Paralogues From the Expanded Tlr11 Gene Family in Mudskipper (Boleophthalmus pectinirostris) Are Under Positive Selection and Respond Differently to LPS/Poly(I:C) Challenge

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Toll-like receptors (TLRs) are major molecular pattern recognition receptors, which are essential for triggering a series of innate immune responses against invading pathogens by recognizing their evolutionary conserved molecular patterns. The mudskipper, Boleophthalmus pectinirostris is exceptional among fishes due to its amphibious lifestyle and adaptation to living on mudflats. The whole-genome sequencing of B. pectinirostris has revealed that this species possesses an expansion of Tlr11 family [12 Tlr11 family genes (one tlr21, 4 tlr22, and 7 tlr23)] that we focused on in the present study. The full-length cDNA sequences of the 12 tlrs in B. pectinirostris were cloned and their deduced amino acid sequences possessed a typical TLR domain arrangement. Likelihood tests of selection revealed that these 12 Tlr11 family genes are under diversifying selection. A total of 13 sites were found to be positively selected by more than one evolution model, of which 11 were located in the ligand-binding ectodomain. The observed non-synonymous substitutions may have functional implications in antigen and pathogen recognition specificity. These 12 tlrs were highly expressed in immune-related tissues, i.e. spleen and kidney. Tlr21 and tlr22b transcripts were significantly up-regulated by LPS, whereas tlr22a, tlr22d, tlr23b, tlr23e, tlr23g were significantly up-regulated by poly(I:C) in the spleen or/and kidney, which implies that the expanded Tlr11 family genes may play roles in protecting the fish from the invasion of gram-negative bacteria and double-stranded RNA viruses. The results from the present study suggested that the expansion of Tlr11 family genes in B. pectinirostris may recognize ligands from various pathogens found in the intertidal zone.

Keywords: Boleophthalmus pectinirostris, TLR21, TLR22, TLR23, innate immunity, positive selection, LPS, poly(I:C)
INTRODUCTION

The Toll-like receptor (TLR) gene family is a class of pathogen recognition receptors (PRRs) that play crucial roles in the innate immune system by recognizing pathogen-associated molecular patterns (PAMPs) derived from various microbes (1, 2). TLRs interact with PAMPs from pathogens via their clusters of extracellular LRRs (leucine-rich repeats), resulting in conformational changes of TLRs. This further activates cytoplasmic Toll-Interleukin-1 receptor (TIR) domain to recruit cytokines (3). Since the discovery of Toll in fruit fly (Drosophila melanogaster) in 1985 (4, 5), at least 28 TLRs have been identified in vertebrates and can be divided into six major families: TLR1 (TLRs 1, 2, 6, 10, 14, 15, 16, 18, 24, 25, 27 and 28), TLR3 (TLR3), TLR4 (TLR4), TLR5 (TLR5), TLR7 (TLRs 7, 8, 9), and TLR11 (TLRs 11, 12, 13, 19, 20, 21, 22, 23, and 26) (6). Bony fish are thought to have an ancient immune system and there is great scientific interest in comparing their innate and adaptive defense mechanism with mammals (7). So far, at least 21 TLRs have been identified in fishes (8). TLR4 gene has been lost from the genomes of most fishes (9). TLR6 and TLR10 are absent in fishes (10). TLR5s, TLR14, TLR18 to TLR28 are considered to be termed as “teleost-specific TLRs” (8, 11).

Key features of the fish TLRs and the factors involved in their signaling cascade have high structural similarity to the mammalian TLR system. However, fish TLRs also exhibit very distinct features and large diversity which is likely derived from their diverse evolutionary history and the distinct environments that they occupy (7). In particular, some TLR genes in teleosts are known to be shaped by positive (diversifying or adaptive) selection, which enables them to cope with a large number of rapidly evolving pathogens (12–18).

The mudskipper Boleophthalmus pectinirostris (Linnaeus 1758) is a burrow-dwelling fish, widely distributing throughout the intertidal regions of China, Korea and Japan (19). B. pectinirostris is usually found on the soft mudflats of estuaries and coastal waters when they are exposed at ebb tide. Their behaviors, physiological and morphological features have been specialized and adapted for an amphibious lifestyle (20–25). Pathogenic bacteria adhere to and colonize mucosal surfaces of the susceptible host (26), or invade the body mainly through the skin, gill, or gut (27). The peculiar environment of the mudflats, which changes between flood and ebb tides, suggests that B. pectinirostris may have evolved specific immunity genes to adapt to their habitat. Interestingly, genomic study of B. pectinirostris showed that the fish species possesses the largest number (11 copies) of TLR13 in vertebrates sequenced so far (28). However, in the present study, based on the sequences of 11 Tlr13 of B. pectinirostris, we further cloned one more Tlr13 gene from B. pectinirostris. However, the phylogenetic analysis indicated that the 12 Tlr13 from B. pectinirostris should be classified as Tlr21, Tlr22, and Tlr23, which belong to TLR11 family. Similar TLR11 family expansion was also reported in Atlantic cod (Gadus morhua), and 12 tlr22 paralogs of Atlantic cod responded differently to pathogenic challenge, which indicated that they are undergoing neofunctionalization via positive selection and can recognize bacterial pathogen-associated molecular patterns (13).

The aim of this study was to investigate if expansion of Tlr11 family in B. pectinirostris have been retained through adaptive evolution in order to provide special immune defense against pathogens from Vibrio, Klebsiella, Salmonella, etc. in intertidal mudflat (29, 30). After obtaining the full-length cDNA sequence of 12 Tlr11 family genes in B. pectinirostris, we conducted synteny analysis and chromosome localization. In order to assess the adaptive evolution of Tlr11 family genes of B. pectinirostris, positive selection analysis was performed. We further examined the tissue distribution and the expression profiles of these genes in response to lipopolysaccharide (LPS) and polyinosinic-polyribotidic acid [poly(I:C)] challenges.

MATERIALS AND METHODS

Experimental Fish and Sampling

Adult mudskipper B. pectinirostris (body length 105–145 mm, body weight 20–45 g) used in this study were purchased from a seafood market in Xiamen, Fujian province, China. The fish were maintained in plastic tanks with 1.5 cm deep seawater at water temperature of 28–28.5°C, and salinity of 15 ppt. Before sampling, the fish were anesthetized with 0.01% MS222 (Sigma-Aldrich, St. Louis, MO, US). All experiment protocols were approved by the Institute of Animal Care and Use Committee of Xiamen University.

cDNA Cloning, Gene Structures of tlr21, tlr22, and tlr23 Paralouses

The fasta format of whole genome shotgun sequences of B. pectinirostris was downloaded from NCBI, and a local blast database was created with BioEdit (31). The partial fragments of previous published 11 tlr13 genes of B. pectinirostris (28) were obtained from BGI (The Beijing Genomics Institute, Shenzhen), these sequences were used to search for the reference sequences from the database by BioEdit software. We found a new TLR11 family gene from local blast database based on the conserved cDNA sequence of TIR domain of B. pectinirostris. The start and stop codons of these genes were predicted by BLASTP searches (NCBI). Finally, we got sequences of 12 Tlr11 family genes in B. pectinirostris. Specific primers were designed to amplify the open reading frames of these genes (Supplementary Table 1). A cDNA library from kidney tissue was synthesized using the ReverAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

Abbreviations: Bp, Boleophthalmus pectinirostris; CD, cluster of differentiation; CDS, complete coding sequence; Cpg-ODNs, Cpg-oligodeoxynucleotides; CT, C-terminus; MD-2, myeloid differentiation factor 2; eef1, eukaryotic translation elongation factor 1a; hpi, hours post injection; LPS, lipopolysaccharide; LRRs, leucine-rich repeats; Map3k, mitogen-activated protein kinase; miRNA, microRNA; NF-kB, nuclear factor-kappa; NT, N-terminus; PAMPs, pathogen-associated molecular patterns; poly(I:C), polyinosinic-polyribotidic acid; ppme1, protein phosphatase; RIPK2, receptor-interacting serine-threonine kinase 2; SEM, standard error of the mean; Sh3kph1, SH3-domain kinase binding protein 1; Srb, scavenger receptor class B; TIR, Toll-Interleukin-1 receptor; TLR, Toll-like receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β; UTR, untranslated region.
following the manufacturer’s instructions and used to amplify these 12 tlr transcripts. Thirty-five cycles of standard PCR were performed on a Bio-Rad T100 Thermal Cycler, the annealing temperature was 58°C and the elongation time depended on the length of fragments amplified. No more than twenty cycles of nested PCR amplification with an annealing temperature of 58°C were performed when necessary.

The full-length end cDNA sequences of these 12 tlr genes were obtained using a SMART RACE cDNA Amplification kit (BD, Clontech) following the manufacturer’s instructions, and the combined PCR sequences were used to deduce the full-length cDNA sequences of the 12 tlr genes. Briefly, total RNA extracted from the fresh kidney was used to synthesize the RACE Ready first-strand cDNA. The 3′RACE cDNA was synthesized using 3′-CDS Primer A, and the 5′ RACE cDNA was synthesized using 5′-CDS Primer A and SMARTer II A oligo. RACE primers for these 12 genes were designed based on the sequence information of the fragments obtained above (Supplementary Table 2). The PCR products were cloned into pMD19-T simple vector (TaKaRa Dalian, China) and sequenced by Invitrogen Ltd. (Guangzhou, China). Intron-exon boundaries of these 12 tlrS were identified using corresponding genome sequences and gene structure display server (http://gads.cbi.pku.edu.cn/). Furthermore, we searched for the highly conserved tandem repeat sequences in the full-length cDNA sequences of these 12 tlrS from B. pectinirostris using the online software “Tandem Repeats Finder” (32).

Synteny Analysis and Chromosome Location of tlr21, tlr22, and tlr23 Paralogues

Synteny analysis was performed manually based on the genome assemblies of large yellow croaker (Larimichthys crocea) (genome assembly accession no. GCF_000972845.1), green-spotted pufferfish (Tetraodon nigroviridis) (GCA_000180735.1), tiger pufferfish (Takifugu rubripes) (GCF_000180615.1), yellowtail kingfish (Seriola lalandei dorsalis) (GCA_002814215.1), amberjack (Seriola dumerili) (GCF_002260705.1), Asian seabass (Lates calcarifer) (GCF_001640805.1).

A high-quality chromosome map comprising 916.23 Mb (93.2%) of B. pectinirostris entire sequence was constructed as part of our program, and will be published separately (data not shown). The full-length cDNA sequences of these 12 tlr genes were used to determine their locations in the 23 pseudo-chromosomes of B. pectinirostris by BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic Analyses

The deduced amino acid sequences of the expanded Tlr11 family genes were obtained using the ExPaSy Translate Tool (http://www.expasy.ch/tools/dna.html). Protein domains, signal peptide, and transmembrane regions were predicted using SMART (http://smart.embl-heidelberg.de/), SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), respectively. A homology search was performed using the BLAST tool at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic reconstruction was performed using MEGA software 7 (33) by the Neighbor-joining method, and a bootstrap consensus tree was inferred from 1,000 replicates. We also constructed a maximum likelihood phylogenetic tree in MEGA software 7 (33) using the Tamura 3-parameter model and γ distributed rates with invariant sites (G+I) and 5 γ categories, and a bootstrap consensus tree was inferred from 1,000 replicates. GenBank accession numbers of tlr genes for alignment of amino acids and phylogenetic tree construction are as follows: Anser cygnoides TLR21 (AMB20882); Gallus gallus TLR21 (NP_001025729); Epinephelus coioides TLR21 (AAK49148); Takifugu rubripes TLR21 (AAL69371); Oreochromis niloticus TLR21 (AEK49148); Gadus morhua TLR21 (AKF76484.1); Salmo salar TLR21 (CDI93614.1); Danio rerio TLR21 (CAQ13807); Anolis carolinensis TLR21 (XP_009123135.2); Xenopus tropicalis TLR21 (XP_002936443.2); Epinephelus coioides TLR22 (AGA84053.1); Scomphalus maximus TLR22 (AIC75881.1); Takifugu rubripes TLR22 (AAM69372.1); Larimichthys crocea TLR22 (XP_010741403); Tetraodon nigroviridis TLR22 (CAG05452.1); Gadus morhua TLR22b (AKF76486.1); Gadus morhua TLR22d (AKF76488.1); Gadus morhua TLR22g (AKF76491.1); Gadus morhua TLR22i (AKF76493.1); Miichthys miyi TLR23 (ALJ55575.1); Takifugu rubripes TLR23 (AAM70378.1); Gadus morhua TLR23a (AKF76497.1); Gadus morhua TLR23b (AKF76498.1); Labrus bergylta TLR23 (XP_020513361.1); Tetraodon nigroviridis TLR23 (CAF93842.1); Seriola dumerili TLR23a (XP_022616855.1); Seriola dumerili TLR23b (XP_022603128.1); Seriola dumerili TLR23c (XP_022603127.1); Lates calcarifer TLR23a (XP_018537426.1); Lates calcarifer TLR23b (XP_018546010.1); Lates calcarifer TLR23c (XP_01857760.1); Seriola lalandi dorsalis TLR23a (XP_023286622.1); Seriola lalandi dorsalis TLR23b (XP_023252716.1); Seriola lalandi dorsalis TLR23c (XP_023252714.1).

Analyses of Positive Selection

The complete coding sequences (CDS) of the 12 Tlr11 family genes from B. pectinirostris (Table 1) were first aligned with MUSCLE (www.ebi.ac.uk/Tools/msa/muscle) and a codon alignment was obtained using RevTrans 2.0 (www.cbs.dtu.dk/services/RevTrans-2.0/web) followed by Codon Align (www.hiv.lanl.gov). The N- and C- terminal portions (60 and 12 codons, respectively) of the codon aligned TLR11 sequences were too variable and hence not included in the following analysis. Gaps present in more than one sequence were also manually removed. The refined codon alignment used in the selection tests comprised 93% of the total CDS and did not have any stop codons. This alignment was used to construct a maximum likelihood phylogenetic tree in MEGA7 (33) using the Tamura 3-parameter model and γ distributed rates with invariant sites (G+I) and 5 γ categories. A bootstrap consensus tree was inferred from 1,000 replicates.

The average number of synonymous and non-synonymous (amino acid-changing) substitutions, insertions and deletions in the codon alignments were calculated using SNAP (www.hcv.lanl.gov). This algorithm performs pairwise comparisons between all sequences in the alignment using the method
TABLE 1 | The characterization of tlr21, tlr22, and tlr23 paralogues in B. pectinirostris.

| Gene name | Full cDNA (bp) | ORF (bp) | 5’ UTR (bp) | 3’ UTR (bp) | No. of exons | Number of amino acids | Scaffold | Genbank accession no. |
|-----------|----------------|----------|-------------|-------------|--------------|---------------------|----------|---------------------|
| tlr21     | 3520           | 2898     | 256         | 367         | 1            | 965                 | scaffold9 | MH744540            |
| tlr22a    | 3820           | 2853     | 72          | 895         | 4            | 950                 | scaffold291 | MH744541        |
| tlr22b    | 3559           | 2697     | 165         | 697         | 4            | 898                 | scaffold850 | MH744542        |
| tlr22c    | 3219           | 2877     | 74          | 268         | 4            | 958                 | scaffold183 | MH744543        |
| tlr22d    | 4637           | 2856     | 1524        | 257         | 3            | 951                 | scaffold103 | MH744544        |
| tlr23a    | 3694           | 2814     | 194         | 666         | 5            | 937                 | scaffold936 | MH744545        |
| tlr23b    | 2878           | 2748     | 45          | 85          | 4            | 915                 | scaffold1155 | MH744546       |
| tlr23c    | 4825           | 2883     | 37          | 1905        | 5            | 960                 | scaffold294 | MH744547        |
| tlr23d    | 4904           | 2820     | 436         | 1648        | 4            | 939                 | scaffold1045 | MH744548       |
| tlr23e    | 4410           | 2766     | 1410        | 234         | 3            | 921                 | scaffold219 | MH744549        |
| tlr23f    | 3451           | 2805     | 37          | 609         | 5            | 934                 | scaffold294 | MH744550        |
| tlr23g    | 3574           | 2835     | 9           | 730         | 5            | 944                 | scaffold50 | MH744551        |

devolved by Nei and Gojobori (34). In protein coding genes, the ratio (ω) between non-synonymous (dN) and synonymous (dS) substitution rates is related to evolutionary constraints at the protein level (35). A value of ω > 1 indicates positive Darwinian selection, whereas ω < 1 suggests negative or purifying selection. A codon based Z-test of selection was performed to test the hypothesis of positive selection in MEGA7 (33) using the modified Nei-Gojobori method with Jukes-Cantor correction (36). The hypothesis of positive selection was further tested using the likelihood methods implemented in the CODEML program of PAML v4.9 (37) and the Datamonkey adaptive evolution server (38), as detailed elsewhere (39). In PAML, the data set was fitted to 6 models of codon substitution: M0 (one ratio), M1 (two site classes), M2 (positive selection with three site classes, M3 (discrete), M7 (β) and M8 (continuous). Bayesian posterior probabilities were calculated for positively selected sites using naïve empirical Bayes in the case of model M3 or Bayes empirical Bayes for models M2 and M8. Likelihood ratio tests were used to compare the corresponding models with and without selection (i.e., M2 vs. M1, M3 vs. M0, and M8 vs. M7). FEL, SLAC and REL analyses were performed in Datamonkey to calculate dN-dS values for each codon, along with the corresponding probability values. Overall differences in diversifying selection between paralogous genes were determined with GA-branch method implemented in Datamonkey.

The three-dimensional structure of B. pectinirostris Tlr23a was predicted by SWISS-MODEL (40). In brief, structural template searches against the SWISS-MODEL template library were performed with BLAST and HHBlit. The highest quality template was then selected for model building based on the target-template alignment using ProMod3. The global and per-residue model quality has been assessed using the QMEAN scoring function (41). For improved performance, weights of the individual QMEAN terms have been trained specifically for SWISS-MODEL. The local quality plot was shown in Supplementary Figure 1. Positively selected codons were identified in the three-dimensional protein using the web-based viewer iCn3D at NCBI (www.ncbi.nlm.nih.gov/Structure/icn3d/full.html).

Expression of tlr21, tlr22, and tlr23 Paralogues in Different Tissues

To further explore the potential functions of tlr21, tlr22, and tlr23 paralogues in B. pectinirostris, the basal expression levels of these 12 Tlr11 family genes in different tissues were quantified by real-time qPCR. Tissues including brain, heart, spleen, gills, liver, intestine, testis, seminal vesicle, ovary, skin, eye, kidney, blood cells were collected separately from seven B. pectinirostris. All the samples were snap-frozen in liquid nitrogen and stored at −80°C until analyses. Total RNA extraction, cDNA synthesis and real-time qPCR were performed as described in section Real-Time qPCR.

Expression of tlr21, tlr22, and tlr23 Paralogues in Response to LPS and Poly(I:C) Challenges

LPS is the main component of the cell surface of Gram-negative bacteria and poly(I:C) is used here as a model of double-stranded RNA virus infection. To investigate the potential functions of the 12 Tlr11 family genes in B. pectinirostris, the expression levels of these genes in the spleen and kidney were analyzed following intraperitoneal injections of LPS and poly(I:C). Male B. pectinirostris with similar size (body length 119–132 mm, body weight 29.5–33.6 g) were transported live in plastic tanks and acclimated to laboratory conditions (seawater at salinity 15 ppt and temperature 28–28.5°C) for 1 day. For the LPS challenge experiment, fish were randomly divided into two groups and each fish was intraperitoneally injected with LPS (E. coli 0127:B8) dissolved in sterile 100 µL PBS at the dose of 0.1 mg in the treated group or with 100 µL sterile PBS in the control. At 3, 6, 12, 24 h post injection (hpi), the spleen and kidney of five or six individuals from each group at each time point were surgically sampled, frozen immediately in liquid nitrogen and stored at −80°C until analyses. For the poly(I:C) challenge experiment, the fish were prepared as described above. Each fish was intraperitoneally injected with poly(I:C) (Sigma, P0913) dissolved in sterile 100 µL PBS at the dose of 0.1 mg in the treated group or with 100 µL sterile PBS in the control group. The spleen
and kidney of five or six individuals from each group at 3, 6, 12, and 24 hpi were surgically collected. Total RNA extraction and cDNA synthesis of these organs were conducted as described in section Real-Time qPCR. Real-Time qPCR was performed as described in section Real-Time qPCR.

Real-Time qPCR
Total RNA was extracted from tissues using the RNAsiso Plus (TaKaRa Dalian, China) and treated with RNase-free DNase I (Fermentas, USA) to eliminate contaminated genomic DNA. 1.5 μg total RNA was used for the synthesis of the first strand cDNAs using the RevertAid first stand cDNA synthesis kit (Thermo Scientific, USA). Each 20 μL reaction contained 10 μL of PowerUp SYBR Green Real-time PCR Master Mix kit (Thermo Scientific, USA). Each 20 μL reaction contained 10 μL of PowerUp SYBR Green Real-time PCR Master Mix, 2 μL of cDNA template, 1 μL of each primer (10 μM), and 6 μL of water. Sterilized water was substituted for the cDNA in negative control samples. The amplification program was performed as follows: predenaturation at 95 °C for 15 s, 60 °C for 1 min followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Each sample was analyzed in duplicate.

Statistical Analysis
Statistical analysis was performed using Graphpad software and the relative abundance of mRNA for target genes was calculated using 2−ΔΔCt method (42) with the eukaryotic translation elongation factor α1 (eef1α, Genbank accession No. XM_020932525.1) gene as the reference. Data were presented as mean ± standard error of the mean (SEM) and the Student’s t-test was used to assess statistical differences of expression levels between groups. For multiple group comparison, one-way ANOVA followed by Tukey’s test was used for statistical analysis. Differences were considered to be statistical significance when p < 0.05.

RESULTS
cDNA Sequences of tlr21, tlr22, and tlr23 Paralogues
The characterization of tlr21, tlr22, and tlr23 paralogues is summarized in Table 1 and their structures are showed in Figure 1, based on the genome assembly of B. pectinirostris. Although the lengths of these 12 tlr cDNAs varied from 2,878 to 4,904 bp, the deduced protein sizes were conserved, ranging from 898 to 965 amino acids. With 3,520 bp, the tlr21 full cDNA was encoded by a single exon, including 255 bp 5′-UTR, 367 bp 3′-UTR, and the 2,898 bp complete coding region corresponding to a 965 aa protein. Among tlr22 and tlr23 orthologs, only tlr22d and tlr23e comprised 3 exons and had longer 5′-UTRs, while tlr23c and tlr23d contained longer 3′-UTRs.

Several highly conserved tandem repeats were found mainly within 5′-UTR or 3′-UTR of tlr22b, tlr23a, tlr23d (Supplementary Table 3) and tlr22d (Supplementary Figure 2).

| Primer | Sequence (5′ – 3′) | Amplicon length (bp) |
|--------|--------------------|----------------------|
| tr21 f  | AACTCTGTGTCTACATCACAGAGAC | 272 |
| tr21 rR | CAGATAGTGCCTCTTAAGCAGAC | 146 |
| tr22a f | CTGGAGAACGATTCTGAGAACG  | 241 |
| tr22a rR | CACTCGTCTGTGATGATGAC | 241 |
| tr22b f | ATGGAGAACGATTCTGAGAACG | 241 |
| tr22b rR | CACTCGTCTGTGATGATGAC | 241 |

The longest tandem repeats spanning 563 bp were identified in the 5′-UTR of tlr22d, and the shortest tandem repeats spanning 96 bp were found in the 5′-UTR of tlr22b. The copy number of 11 bp tandem repeat within the 3′-UTR of tlr22b was up to 50. The tandem repeats of tlr23a started at the 5′ end of the cDNA. The tandem repeats of tlr23d were completely distributed within 3′-UTR.

Synteny Analysis and Chromosome Location of tlr21, tlr22, and tlr23 Paralogues

Both tlr23c and tlr23f were present in the same scaffold (scaffold294), while the other paralogues were mapped to different scaffolds (Table 1; Figure 2A). Partial synteny analysis based on the current mudskipper genome build revealed conservation between tlr22a in B. pectinirostris and tlr22 coding genes in large yellow croaker, green-spotted pufferfish and tiger pufferfish, within the genomic region containing sh3kbp1, map3k15, and cnksr2 (Figure 2B). Tlr23a in B. pectinirostris and tlr23as in yellowtail kingfish, amberjack and Asian seabass were adjacent with the same gene “ppm1-like” (Figure 2B).

All these 12 tlr genes, except tlr23b, mapped on seven chromosomes of B. pectinirostris (Figure 3, Table 2 | The primers for Real-time qPCR in this study. |
Supplementary Table 4). Tlr21 was located at chr7, tlr22a, tlr22b, tlr22c, and tlr22d were located at chr12, chr8, chr7, and chr17, respectively. Among tlr23 genes, three of them (tlr23c, tlr23d, tlr23f) were present in the same chromosomal region (chr11), while tlr23a and tlr23g were found in chr6 and chr18, respectively. The location of tlr23b needs to be further explored.

Phylogenetic Analysis and Protein Domain Arrangements of tlr21, tlr22, and tlr23i Paralogues in B. pectinirostris
The phylogenetic tree was constructed using the Neighbor-joining method on the basis of deduced amino acid sequences of TLR21, TLR22, and TLR23 of vertebrates (Figure 4).
Phylogenetic analysis showed that TLR21, TLR22, and TLR23 constituted three major groups. In the tree, all TLR21 were grouped under a single clade, while TLR22 and TLR23 formed a separate cluster. The Tlr22 orthologs in *B. pectinirostris* were grouped together. All Tlr23 orthologs except Tlr23a clustered together in *B. pectinirostris*. Similar results were also
FIGURE 4 | Phylogenetic analysis of TLR21, TLR22, and TLR23 using MEGA 7 by the Neighbor-joining method and 1,000 replications of bootstrap. Proteins of *B. pectinirostris* are highlighted within blue box.
FIGURE 5 | Protein domain structures of Tlr21, Tlr22, and Tlr23 proteins in B. pectinirostris. The domain organizations of Tlr21, Tlr22, and Tlr23 in B. pectinirostris were predicted using SMART, SignalP, and TMHMM analyses. LRR, leucine-rich repeat; LRR-TYP, leucine-rich repeat typical subfamily; TIR, Toll/IL-1 receptor; NT, N-(nitrogen) terminal; CT, C-(carboxyl) terminal; ■, signal peptide; ■, transmembrane region; ■, low complexity region.
found in the phylogenetic tree based on Maximum Likelihood method (Supplementary Figure 3).

The protein domain arrangements of tlr21, tlr22, and tlr23 paralogues in B. pectinirostris are presented in Figure 5. All Tlrs amino acid sequences comprised of a signal peptide, several LRRs, a transmembrane domain and a TIR domain. These Tlrs contained various numbers of LRR domains, and the LRR number in each TLR ranged from 10 to 21: 17 (Tlr21), 16 (Tlr22a), 21 (Tlr22b), 15 (Tlr22c), 19 (Tlr22d), 13 (Tlr23a), 10 (Tlr23b), 16 (Tlr23c), 16 (Tlr23d), 11 (Tlr23e), 12 (Tlr23f), 12 (Tlr23g). Most of these Tlrs had C-terminus LRRs (LRR-CT) with the exception of Tlr21 and Tlr23a. Only Tlr23d and Tlr23e contained N-terminus LRRs (LRR-NT). Besides, Tlr22b contained a shorter TIR domain than other Tlrs.

### Molecular Evolution of the Tlr11 Family in B. pectinirostris

A sliding window analysis of the complete coding sequences of tlr21, tlr22, and tlr23 paralogues revealed that the cumulative number of non-synonymous mutations per codon (dN) exceeded the number of synonymous substitutions (dS) and that their occurrence was not uniform throughout the tlr21, tlr22, and tlr23 coding sequences (Figure 6). The average dS of all pairwise comparisons was higher than dN but different between the LRR and TIR domains, with dS/dN ratios of 4.044 and 6.933, respectively. A pairwise codon-based Z-test did not reject the null hypothesis of strict-neutrality (dN = dS) in favor of positive selection (dN > dS) (Supplementary Table 5). Nevertheless, the dN/dS ratios (ω) varied between tlr11 genes and were highest in tlr23 paralogues, particularly in tlr23c-g (Supplementary Figure 4).

A more detailed site-specific analysis was performed using likelihood models to identify codons under diversifying selection. Likelihood ratio tests in PAML showed that models allowing for positive selection fitted the data better than those that did not (M3 vs. M0, 2ΔLnL = 1614, p = 0; M2 vs. M1, 2ΔLnL = 45, p = 0; M8 vs. M7, 2ΔLnL = 40.6, p = 0) (Table 3). Models, M2, M3, and M8 identified 4, 46 and 3 codons under positive selection (Bayesian posterior p < 0.05) and ω values of 5.39, 1.02, and 2.8, respectively. The best model of nucleotide substitution was 012032 with an Akaike information criterion of 63195. FEL and SLAC analyses found 18 and 3 codons under positive selection with p < 0.1 and REL identified 6 positively selected sites with Bayes factor > 50 (Table 3). In total, 13 codons were identified by more than one likelihood model as being under significant positive selection pressure. In particular, codon 68 (F, H, K, N, R, S, T, V, or W) was flagged by all models, except REL (Supplementary Figure 5). Only two out of these 13 codons were present within the TIR domain, whereas 11 were found in the ectodomain. Most positively selected sites (8 out of 13) were found in LRR regions, especially in the coils on the convex surface of this horseshoe-shaped domain. Two sites under diversifying selection (405 and 597) were located in beta sheets within the concave surface (Figure 7, Supplementary Figure 5).

### Tissue Distribution of tlr21, tlr22, and tlr23 Paralogues in B. pectinirostris

Tlr21, tlr22, and tlr23 paralogues showed distinguishable tissue expression patterns (Figure 8). They were all expressed in immune-related organs, i.e., spleen and kidney. In addition, tlr21 was widely distributed and predominantly expressed in the brain, testis and eye (Figure 8A). Among tlr22 paralogues (Figures 8B–E), only tlr22c was detectable in all tissues examined (Figure 8D). Compared with tlr21 and tlr22, tlr23 paralogues were exclusively expressed in the spleen and kidney (Figures 8F–L). The expression of these tlr genes in the seminal vesicle, ovary and skin was weak.

### Expression of the tlr21, tlr22, and tlr23 Paralogues in Response to LPS and Poly(I:C) Challenges

The expression of tlr21, tlr22, and tlr23 paralogues responded differently to LPS (Figures 9, 10) and poly(I:C) (Figures 11, 12) challenges. The expression of tlr21 was significantly up-regulated by LPS in the spleen and kidney at 12 hpi (Figures 9A, 10A). Among tlr22 paralogues, LPS administration significantly stimulated tlr22b in the spleen at 3 hpi (Figure 9C), and down regulated tlr22a in the spleen at 6 hpi (Figure 9B) and tlr22c in the kidney at 6 hpi (Figure 10D). However, the expression profiles of these genes didn’t show time-dependent significant differences. In response to poly(I:C) administration, tlr22a and tlr22d in the spleen showed clear significantly time-dependent increase pattern, and reached the significantly highest levels at 12 hpi (Figures 11B, E). In the kidney, only tlr22a significantly increased at 6 hpi after poly(I:C) stimulation, and...
TABLE 3 | Positively selected sites in mudskipper tlr21, tlr22, and tlr23 paralogues.

| Method   | Model | Parameter estimates | Ln likelihood | Model comparison | Positively selected sites\(^a\) |
|----------|-------|---------------------|---------------|------------------|-------------------------------|
| CODEML\(^b\) | M0: neutral | \(\omega = 0.32\) | -30593.3 | None | |
| CODEML\(^b\) | M1: nearly neutral | \(\omega_0 = 0.15, \omega_1 = 1.00\) | -29907.2 | Not allowed | |
| CODEML\(^b\) | M2: positive selection | \(\omega_0 = 0.15, \omega_1 = 1.00, \omega_2 = 5.39\) | -29884.7 | M2 vs. M1 | 68, 116, 311, 500 |
| CODEML\(^b\) | M3: discrete | \(\omega_0 = 0.04, \omega_1 = 0.33, \omega_2 = 1.02\) | -29726.0 | M3 vs. M0 | 1, 20, 46, 63, 68, 73, 89, 91, 92, 96, 97, 116, 139, 161, 176, 190, 204, 208, 212, 223, 238, 241, 263, 267, 286, 287, 299, 306, 311, 319, 333, 338, 373, 405, 413, 470, 481, 483, 500, 522, 531, 534, 544, 593, 597, 660 |
| M7: \(\beta\) | | \(p = 0.66, q = 1.06\) | -29714.7 | | |
| M8: \(\beta + \omega S > 1\) | | \(p = 0.71, q = 1.24\) | -29694.4 | M8 vs. M7 | 68, 116, 311 |
| Datamonkey\(^c\) | SLAC | | | Not allowed | |
| Datamonkey\(^c\) | FEL | | | | |
| REL | | | | | |

\(^a\)Codons identified by more than one maximum likelihood method are underlined.

\(^b\)Only positively selected sites with Bayesian posterior probabilities equal or >95% are indicated. Sites with a posterior probability > 99% are highlighted in bold.

\(^c\)Only positively selected sites with \(p < 0.01\) (SLAC and FEL) or Bayes factor > 50 (REL) are shown.

followed by significantly dropping down for the rest experimental period (Figure 12B). Among tlr23 paralogues, several genes were significantly down-regulated by LPS stimulation in both spleen and kidney (Figures 9, 10). However, the expression profiles of these genes didn’t show time-dependent significant differences. Similar expression patterns were observed in response profiles of tlr23c and tlr23g after poly(I:C) stimulation in the kidney (Figures 12H,L). In spleen, except tlr23c and tlr23f showed similar down-regulation patterns (Figures 11H,L), tlr23b, tlr23e, and tlr23g were significantly stimulated by poly(I:C) at 3 or 12 hpi (Figures 11G,J,L). However, the expression profile of tlr23b showed significant increasing trend during sampling periods in both control and treated groups (Figure 11G). In contrast to tlr23g (Figure 11L), only tlr23e showed a clear significantly time-dependent increase pattern in the spleen, and reached the highest level at 24 hpi (Figure 11J).

**DISCUSSION**

Several types of gene duplication have been observed in genome to date, including whole genome duplication (43), segmental duplication (44), DNA-mediated duplicative transposition and retrotransposition (45). Paralogous or duplicated Tlr genes in teleosts, probably resulting from the third or fourth round of whole genome duplication event, have been identified in *Danio rerio* (46, 47), *Oncorhynchus mykiss* (48), and *Cyprinus carpio* (49). In the present study, we reported an extensive duplication of tlr23 genes (7 paralogues). Previous study in Atlantic cod has reported an extensive duplication of tlr22 genes (12 paralogues) (13). Such intensive duplication may be not only due to genome duplication but also due to other mechanisms of gene duplication. DNA-mediated duplicative transposition and retrotransposed duplication are ongoing...
FIGURE 8 | Tissue distribution of tlr21 (A), tlr22 (B-E), and tlr23 (F-L) paralogues in B. pectinirostris. The levels of the respective mRNAs were determined using qPCR and normalized to the internal housekeeping gene eef1α. The results were expressed as mean ± SEM (n = 7). Columns with different letters are significantly different from each other (p < 0.05, One-way ANOVA followed by Tukey’s test).
FIGURE 9 | The relative expression of tlr21 (A), tlr22 (B-E), and tlr23 (F-L) paralogues in the spleen after intraperitoneal injection with LPS. Relative expression of these 12 tlr genes in the kidney were examined at different time points (3, 6, 12, 24 h) by Real-time qPCR and all data were expressed as the mean ± SEM (n ≥ 5) and normalized to the expression of eef1α. Significant difference between PBS and LPS treated group was indicated with *(p < 0.05, Student's t-test) or **(p < 0.01, Student's t-test). Columns with different letters are significantly differences with each other (*p < 0.05, One-way ANOVA followed by Tukey's test). Lowercase and uppercase letters indicate control and treated groups, respectively.
FIGURE 10 | The relative expression of tlr21 (A), tlr22 (B-E), and tlr23 (F-L) paralogues in the kidney after intraperitoneal injection with LPS. Relative expression of these 12 tlr genes in the kidney were examined at different time points (3, 6, 12, 24 h) by Real-time qPCR and all data were expressed as the mean ± SEM (n ≥ 5) and normalized to the expression of eef1α. Significant difference between PBS and LPS treated group was indicated with *(p < 0.05, Student’s t-test) or **(p < 0.01, Student’s t-test). Columns with different letters are significantly different from each other (p < 0.05, One-way ANOVA followed by Tukey’s test).
FIGURE 11 | The relative expression of tlr21 (A), tlr22 (B-E), and tlr23 (F-L) paralogues in the spleen after intraperitoneal injection with poly(I:C). Relative expression of these 12 tlr genes in the kidney were examined at different time points (3, 6, 12, 24 h) by Real-time qPCR and all data were expressed as the mean ± SEM (n ≥ 5) and normalized to the expression of eef1α. Significant difference between PBS and poly(I:C) treated group was indicated with *(p < 0.05, Student’s t-test) or **(p < 0.01, Student’s t-test). Columns with different letters are significantly differences with each other (p < 0.05, One-way ANOVA followed by Tukey’s test). Lowercase and uppercase letters indicate control and treated groups, respectively.
FIGURE 12 | The relative expression of *tlr21* (A), *tlr22* (B-E), and *tlr23* (F-L) paralogues in the kidney after intraperitoneal injection with poly(I:C). Relative expression of these 12 *tlr* genes in the kidney were examined at different time points (3, 6, 12, 24 h) by Real-time qPCR and all data were expressed as the mean ± SEM (*n* ≥ 5) and normalized to the expression of *eef1α*. Significant difference between PBS and poly(I:C) treated group was indicated with * (p < 0.05, Student’s t-test). Columns with different letters are significantly differences with each other (p < 0.05, One-way ANOVA followed by Tukey’s test).
process, continually expanding the genetic repertoire of modern organisms (45). A common result of DNA-mediated duplication is a new gene that preserves the intron-exon architecture and the cis-regulatory elements of the parental gene, whereas retrotransposed duplication often generates an intronless gene copy as a result of a process in which a spliced mRNA is reverse-transcribed into cDNA and spontaneously integrated into a random genomic location (45). Besides, it has been suggested that the influence of short tandem repeats may substantially increase the rate of duplication of a DNA segment located between them (50). In *B. pectinirostris*, both tlr22 and tlr23 paralogues showed similar intron-exon architectures. Moreover, short tandem repeat sequences were identified in the UTRs of tlr22b, tlr22d, tlr23a, and tlr23d. Thus, it is possible that tlr22 and tlr23 paralogues resulted from the DNA-mediated duplicative transposition.

Similar to other teleosts (13, 51, 52), there exists only one tlr21 ortholog encoded by a single exon in *B. pectinirostris*. Besides, in *B. pectinirostris* and other fish species, Tlr21 molecules do not have an LRR-CT, in contrast to the chicken and goose TLR21 sequences (53, 54). In the present study, upon stimulation with LPS, the expression of BpTlr21 was significantly up-regulated in the spleen and kidney at 12 hpi in *B. pectinirostris*. In the mammal immune system, the complex of TLR4, CD14, and MD2 has been proved to be the receptor for LPS at the cell surface (55). Unlike in mammals, tlr4 gene has been lost from the genomes of most fishes (9) including *B. pectinirostris*, and Tlr4 in fish does not recognize the stimulation of LPS (56). However, LPS does have multiple biological effects on fish including enhancing the production of immune related cytokines (57, 58). Recently, study in miyu croaker (*Miticthys miyu*) showed that NOD1 can identify LPS and activate the NF-κB signal pathway by recruiting RIPK2 and then promoting the expression of inflammatory cytokines to induce the resistance of organism against bacterial infection (59). Another study further demonstrated that scavenger receptor class B 2a (SRB2a), a novel isoform of the mammalian SRB2 gene, mediates LPS internalization for interaction with NOD1 and NOD2 to initiate NF-κB in teleost macrophages (60). The results from the present study suggest that BpTlr21 may be involved in immune response to bacterial infection in *B. pectinirostris*, and further studies should be focused on whether the stimulatory effects of LPS on BpTlr21 was mediated by SRB2a and NOD in *B. pectinirostris*.

The four tlr22 orthologs cloned from *B. pectinirostris* grouped under a single clade in the phylogenetic tree, separately from the Atlantic cod orthologs of BpTlr22, suggesting that the expansion of tlr22 occurred independently during evolution of these two species. BpTlr22 orthologs are not adjacent in the *B. pectinirostris* genome. The genomic region surrounding tlr22a is conserved in comparison with tlr22 from several fish species, and the region contains the genes *sh3kbp1, map3k15*, and *cnksr2*. It has been proposed that selection favors the organization of gene clusters to facilitate the coordinated control of gene expression and related biological processes (61). In mammals, Sh3kbp1 is involved in the B cell receptor signaling in normal lymphocytes (62). Besides, it has been reported that the B-cell response to CpG S-ODN is mediated through TLR9 (63). Therefore, conserved syntenic localization between Tlr22a and Sh3kbp1 suggests that Tlr22a may participate in biological processes related to B cells in teleosts. Map3k15 (ASK3) is likely a component of ASK1 (Map3k5) signalosome and can interact with ASK1 (64), and ROS-dependent activation of TRAF6-ASK1-p38 pathway is crucial for TLR4-mediated innate immunity (65), which suggest that Map3k15 may take part in Tlr22a signaling pathway. Further study would be interesting to examine these adjacent genes involved in the Tlr22a mediated signaling pathway in *B. pectinirostris*.

In other teleosts, poly(I:C) challenge up-regulated the expression of tlr22 in many tissues (66, 67). It was reported, in tiger pufferfish, that Tlr22 localizes to the cell surface and recognizes long-sized dsRNA or poly(I:C) and links the IFN-inducing pathway via the TRIF adaptor (68). In the present study, the expression of tlr22a and tlr22d in spleen, and tlr22a in kidney showed clear time-dependent up-regulation after poly(I:C) stimulation. These results may suggest that the function of Tlr22a and Tlr22d might play a role in innate immune response to virus. Nevertheless, some Bptlr22 paralogues (including tlr22a) showed a response after LPS stimulation. A study in Atlantic cod indicated that most of tlr22 orthologs transcripts are up-regulated after bacterial bath challenge (13). However, it is worth noting that, after LPS stimulation, the expression profiles of Bptlr22 paralogues didn’t show time-dependent significant differences. Therefore, further studies would be necessary to investigate the possibility that the expansion of Tlr22 likely increases the detectable ligand repertoire, e.g., to recognize dsRNA and PAMPs from pathogen origin.

The function of TLR23 is still largely unknown (13). *B. pectinirostris* encoded 7 copies of tlr23 genes and possessed the largest group of Tlr23 in vertebrates sequenced. It is noteworthy that all BpTlr23 except Tlr23a clustered under a single clade in phylogenetic tree, which suggests that BpTlr23 under the same clade may evolve independently in comparison with BpTlr23a and other teleost Tlr23. We found that Tlr23a genes in several fish species are often adjacent to the *ppme1-like* gene, which suggests that the function of tlr23a may be conserved in different teleost species. Recently, it was found that activation of TLR4 signaling pathway will increase the expression of MFHAS1, which further inhibits expression of inflammatory factors and plays a role in negatively regulating TLR4 signaling pathway (69). During this process, MFHAS1 combines with the B and C subunits of PP2A, which involves up-regulation of PPM1-1 (70). Therefore, it is possible that *ppme1* may be involved in the negative regulation of signaling pathway of tlr23a, and even other tlr23 paralogues.

Tlr23 paralogues in *B. pectinirostris* responded differently to LPS and poly(I:C) challenges. Tlr23e and tlr23g were significantly up-regulated in the spleen upon poly(I:C) stimulation, which
suggest that tlr23e and tlr23g may participate in antiviral immune processes. In contrast to up-regulation, several B. pectinirostris tlr23 paralogues showed down-regulation after LPS and poly(I:C) challenges. Atlantic cod tlr23a is also significantly reduced upon a bath challenge with Gram-negative bacteria V. anguillarum (13). In teleost fish, several studies have identified subsets of microRNAs (miRNAs) that are differentially expressed in organs challenged with DNA or RNA virus, LPS or poly (I:C) (71). Based on the use of bioinformatics approaches and whole transcriptome analysis, increasing studies have discovered that miRNAs negatively regulate the expression of Tlr genes (72). It would be necessary to predict miRNA regulators of tlr23 paralogues and elucidate their roles in the regulation of tlr23 paralogues in B. pectinirostris.

The average number of synonymous changes was higher than the non-synonymous substitutions in all pairwise comparisons between tlr11 genes in B. pectinirostris, indicating overall purifying selection, likely due to functional constraints (73). In fish, the prevalence of purifying selection signatures has been reported not only in tlr genes (13) but also in other immune-related genes, such as antimicrobial peptides (39). Nevertheless, substitution rates were not uniform across tlr11 paralogues in B. pectinirostris and the observed differences contribute to explaining how the TIR domains of Tlr21, Tlr22, and Tlr23 are more conserved than their LRR regions. The TIR domain is generally conserved across species as well as between different TLRs, since it is involved in signal transduction (74).

Homology modeling of B. pectinirostris Tlr23a based on human TLR5 revealed a characteristic horseshoe-shaped structure with a single ectodomain architecture. Most positively selected sites were found in the ectodomain, especially in the convex side of the extracellular solenoid structure, which is most important for ligand binding. Non-synonymous substitutions at the positively selected sites may affect ligand specificity through changes in the amino acids within the beta sheets or in the convex surface of the horseshoe-shaped domain (75). For example, variations in the LRR coil at position 68 between small polar (S and T), positively charged (R, K and H) and large hydrophobic aromatic (F and W) amino acids will likely affect the polarity and structure of the ectodomain, thus affecting ligand specificity. This functional diversification of the B. pectinirostris Tlr11 family through positive selection may be linked to adaptation to evolving pathogens.

In conclusion, we identified and annotated 12 tlr genes (one tlr21, 4 tlr22, and 7 tlr23) representing all members of the high expanded Tlr11 family in the mudskipper B. pectinirostris. The expanded Tlr11 family in B. pectinirostris provides a good model to better understand how and why so many TLR genes have been retained during vertebrate evolution.

**AUTHOR CONTRIBUTIONS**

HQ was involved in entire study. JF analyzed the positive selection. XY, HW, and HY performed the partial synteny and chromosome location analyses. YZ, SH, DL, and QW analyzed results. SC and WH conceived and supervised the project, analyzed results and prepared the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00343/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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