Fish and Shellfish Immunology 90 (2019) 134–140

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short sequence report

Two toll-like receptors identified in the mantle of *Mytilus coruscus* are abundant in haemocytes

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ARTICLE INFO

Keywords:
Mytilus coruscus
Toll-like receptors
Haemocytes
Mantle

ABSTRACT

Toll-like receptors (TLRs) are a large family of pattern recognition receptors (PRRs) that play a critical role in innate immunity. TLRs are activated when they recognize microbial associated molecular patterns (MAMPs) of bacteria, viruses, or fungus. In the present study, two TLRs were isolated from the mantle of the hard-shelled mussel (*Mytilus coruscus*) and designated McTLR2 and McTLR3 based on their sequence similarity and phylogenetic clustering with *Crassostrea gigas*, CgiTLR2 and CgiTLR3, respectively. Quantitative RT-PCR analysis demonstrated that McTLR2 and McTLR3 were constitutively expressed in many tissues but at low abundance.

1. Introduction

The Toll and Toll-like receptors (TLRs) are an important component of innate immunity in metazoans [1,2]. TLRs are single pass transmembrane receptors that share a conserved structure. Two main classes of TLRs have been identified, and they are grouped according to their CF motifs (cysteine cluster in the domain of leucine rich repeat; LRR) into single and multiple cysteine cluster TLRs (sccTLR, mccTLR) [3]. Most invertebrate TLRs are classified as mccTLR, whereas mammalian TLRs, of which 10 to 12 members have been identified, are of the sccTLR type [4,5]. The LRR domain in TLRs recognize, pathogen/microbial associated molecular patterns (PAMPS/MAMPs) present on the surface of, or associated with bacteria, viruses, or fungus [6]. In mammals TLR4 is activated by lipopolysaccharide (LPS), TLR5 senses bacterial flagellin, TLR11 interacts with uropathogenic bacteria [7] and there is evidence that TLR9, TLR7 and TLR8 detect host nucleic acids [8–11].

The interaction of TLRs with PAMPs such as LPS and Peptidoglycans (PGN) [12,13], causes receptor dimerization and the cytoplasmic Toll–IL-1-resistence (TIR) domain initiates signalling [14,15]. The TLR TIR dimer, recruits adaptor molecules such as, Myeloid-differentiation-factor-88 (MyD88) [16], Myd88-adaptor-like protein (MAL), TIR domain-containing adaptor protein inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM), sterile-α- and armadillo-motif-containing protein 1 (SARM1) [17]. The interaction of TLR TIR and adaptor proteins creates the signalling complex that activates diverse downstream kinases and ultimately transcription factors (NF-kB, AP-I and IRF). Transcription of immune effectors such as NF-κB, p38 MAPK and JNK leads to the clearance of invading microorganisms [17,18].

Invertebrates lack an adaptive immune system and are solely dependent on innate immunity for protection from pathogens [12]. This probably explains the expansion of innate immune gene families and due to selective pressure, their functional divergence in some invertebrates [19,20]. In the bivalves, results from transcriptome and genomic analysis clearly indicate that expansion of the TLR gene family has occurred. This may be linked to the adaptation of these filter-feeding animals to the pathogen-rich coastal waters they inhabit and the complex abiotic and biotic stresses to which they are exposed, including microorganisms, such as *Vibrio* [21,22]. In the Pacific oyster (*Crassostrea gigas*) 83 TLRs were reported, and exposure of oysters to bacteria and viruses caused up-regulation of TLR expression [19]. In the Zhikong scallop (*Chlamys farreri*), CfToll-1 transcription was stimulated

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https://doi.org/10.1016/j.fsi.2019.05.001
Received 19 September 2018; Received in revised form 28 April 2019; Accepted 1 May 2019
Available online 02 May 2019
1050-4648/

Table 1
Oligonucleotide primers used to amplify McTLR2 and McTLR3.

Primer	Sequence (5′ – 3′)	Application	Tm	Annealing temperature
McTLR2-3′F	GCTCGCCATGAGAGTAGCCGACCCT	3′ RACE	67.7	68
McTLR2-5′R	CTTGCACCCTTTAAGTCCACCAA	5′ RACE	64.0	68
McTLR2-RT-F	GTGGCTCGAAGCAAACTTACTA	RT-qPCR	54.7	55
McTLR2-RT-R	CTTCGCTCCAAGCTGCATAA	RT-qPCR	55.5	55
McTLR3-3′F	GAAATCAGTTGGTTGGGCTTGGCG	3′ RACE	70.6	68
McTLR3-5′R	GTGTCTTCAAAGGCATCTGGTTCT	5′ RACE	66.0	68
McTLR3-RT-F	TACATGGCGAAAGAGCGATAC	RT-qPCR	54.7	55
McTLR3-RT-R	AGTCGTGCCTTTCCATCTTAC	RT-qPCR	54.6	55
EF-1α-F	CACCACGAGTCTCTCCCTGA	RT-qPCR	59.0	60
EF-1α-R	GCTGTCACCACAGACCATTCC	RT-qPCR	58.9	60

by exposure to LPS [23]. However, in the Mediterranean mussel, *Mytilus galloprovincialis*, injection of LPS or PGN was relatively ineffective in stimulating transcription of any of the 23 TLRs identified to date [21].

The hard-shelled mussel (*M. coruscus*) is an economically important species in China [24]. Recently, pathogen-associated mortality of the hard-shelled mussel has had a negative impact on the sustainability of their aquaculture. Under the current scenario of rising sea water temperatures, increased anthropogenic inputs into marine ecosystems and the associated increase in bacteria and other microorganisms it is important to better understand immunity in marine bivalves. So far, only one TLR (McTLR-a) gene has been reported in *M. coruscus* [25]. Here we aimed to extend understanding of the TLRs in this species, since insight into the function of TLRs in bivalves will be the key to developing effective disease control approaches. The purpose of the present study therefore was to: (1) clone two TLR transcripts (McTLR2 and McTLR3) previously identified in a preliminary study of *M. coruscus* mantle tissue; and (2) examine the expression pattern of the identified TLRs in different mussel tissues.

2. Materials and methods

2.1. Mussel samples

M. coruscus (shell length/height: 10.1 ± 0.4/4.9 ± 0.3 cm) were obtained from Gouqi Island, (30°72′N; 122°77′E) (Zhoushan, China) and maintained in 10 L polycarbonate tanks (10 tanks, 20 mussels/tank) at 21 ± 1 °C for one week before the experiments. The seawater (salinity: 30 ppt) in the tanks was renewed daily. The mussels were fed daily with *Platymonas helgolandica* var. *tsingtaoensis* (2×10^4 cells·ml^{-1}) and *Isochrysis zhanjiangensis* (8×10^5 cells·ml^{-1}) during acclimation to the experimental facility.

To assess the tissue distribution of TLRs some of the acclimated mussels were sacrificed by placing them in iced water and the tissues of interest dissected out. The tissues collected included the mantle, gill and digestive gland (directly in contact with the environment) and the adductor muscle, foot, gonad and the haemolymph. Tissues were stored at −80 °C until analysis. Haemolymph was collected by introducing a fine needle into the adductor muscle and gently withdrawing the fluid. Haemocytes were harvested by centrifugation of the haemolymph (800 × *g*, 10 min, 4 °C) and retaining the cell pellet.

The experimental and the mussel handling procedures were approved by the institutional animal care and use committee (IACUC) of Shanghai Ocean University, China (Review number: SHOU-DW-2018-055).

2.2. RNA isolation and synthesis of first-strand cDNA

Total RNA was isolated from the mantle and haemocytes using an RNAiso Plus kit (TaKaRa, Japan). Contaminating genomic DNA was removed from the total RNA using an Ambion Turbo DNase kit (Ambion, USA) and following the manufacturer's instructions. The RNA quality and concentration were evaluated using a Nanodrop 2000 and by analyzing the extracted RNA by 1% agarose gel electrophoresis.

First strand cDNA synthesis for quantitative PCR was conducted using 500 ng of extracted RNA and a Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). RACE PCR was used for amplification of full–length cDNA of two forms of TLR (designated TLR2 and TLR3, see the results section) using a SMARTer™ RACE 5′/3′ Kit (Clontech, Japan).

2.3. Cloning of full-length of McTLR2 and McTLR3

Specific primers for TLR2 (McTLR2-3′F, McTLR2-5′R) and for TLR3 (McTLR3-3′F, McTLR3-5′R) were designed using Primer Premier 6.0 software (Table 1) and the available transcriptome data of the hard-shelled mussel (unpublished). RACE amplification procedures were performed following the protocol provided by the manufacturer (Clontech, Japan).

The final reaction volume of the PCR was 20 μl. Touchdown PCR was conducted following the thermocycle proposed by the company (Clontech, Japan), which consisted of: 94 °C for 4 min, 5 cycles of 94 °C for 30 s, 72 °C for 3 min, and another 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min, followed by another 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. The final elongation step was conducted for 10 min at 72 °C. The presence of a single PCR reaction product and the amplicon size was confirmed by 1% agarose gel electrophoresis before it was purified using a Gel Extraction Mini Kit (Takara, Germany). The purified PCR product was cloned into a pMD 19-T vector (Takara, Dalian) and transformed into competent DH5α cells. The cDNA sequences of the cloned amplicons were confirmed by sequencing (Sangon biotech, China).

2.4. Sequence analysis

Sequence homology of the McTLR2 and McTLR3 cDNA with other TLRs was established using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The ORF of McTLR2 and McTLR3 were identified via ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder). The signal peptide and signature domains of the deduced proteins were identified using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) [26] and SMART Tool (http://smart.embl-heidelberg.de/) [27], respectively. The molecular weight and isoelectric point of the proteins was predicted via Compute pI/Mw (https://www.expasy.org/). Potential *N*-glycosylation sites were predicted using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) [28].

The amino acid sequence of the TIR domain of the 2 *M. coruscus* (Mc) TLRs and McTLRa [25], the TIR domains of the 23 TLR sequences from the mussel, *M. galloprovincialis* (Mg) [12], the 10 TLRs from the carpet shell clam *Ruditapes decussatus* (Rd) [29], the 9 TLRs from the oyster *Crassostrea gigas* (Cg) [3,30–32] and the single TLR from *Chlamys farreri* (Cf) [23] were aligned using the MUSCLE algorithm [33] in the

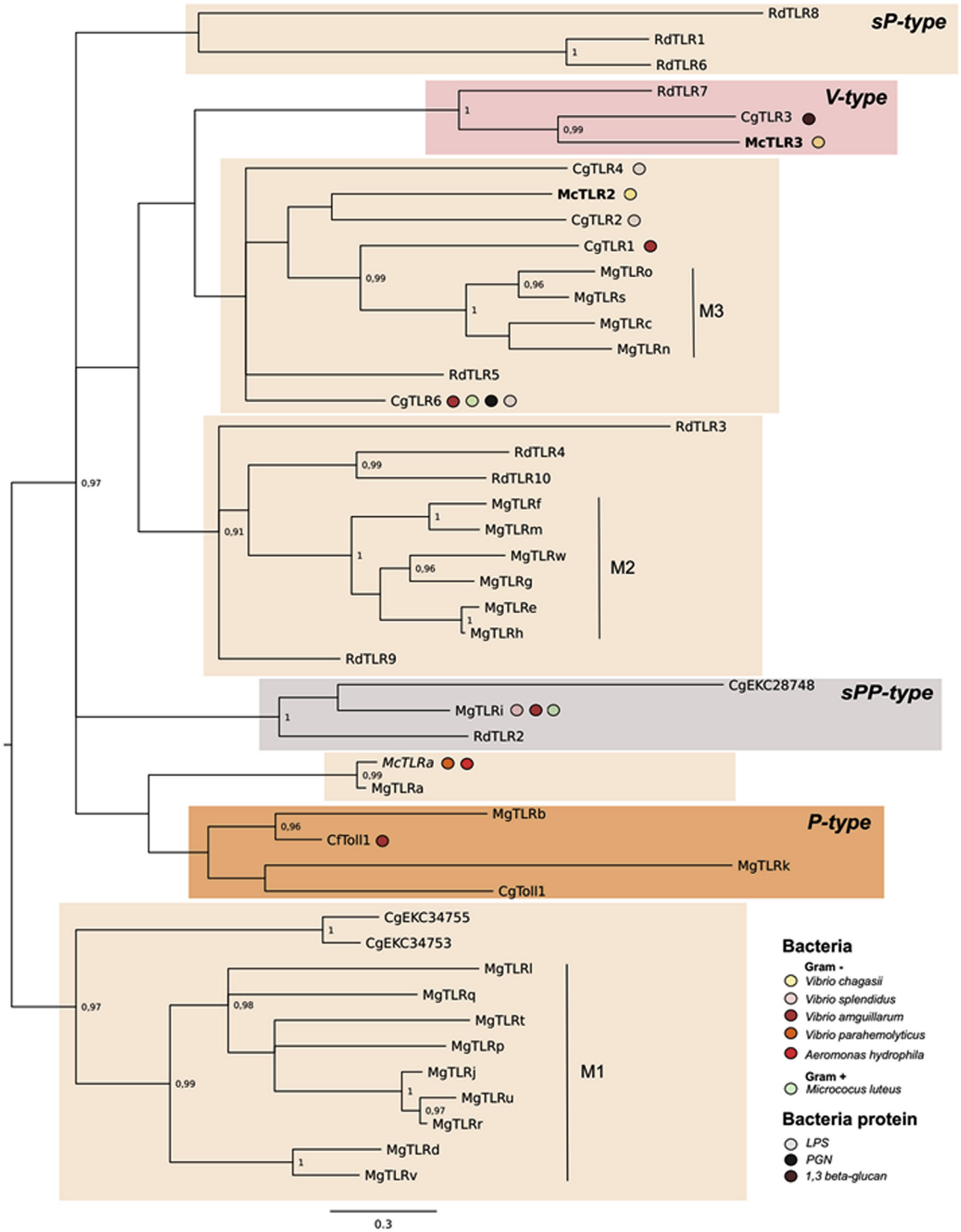

Fig. 1. Phylogenetic tree built using the TIR domain of the bivalve toll-like receptors (TLRs) constructed using the BI method. The unrooted tree was built using the sequence alignment available as Supplementary Fig. 2. Only posterior probability values > than 0.90 are represented. A similar tree was obtained using the ML method and 100 bootstrap replicates (Fig. S3). The bivalve TLRs were grouped according to the classification proposed by Ref. [19] for the oyster TLRs: V-type (vertebrate-type, pink), P-type (protostome-like type, orange), sP-type (short protostome-like type, salmon) and sPP-type (short protostome-like, grey). Coloured circles next to the TLRs indicate the differentially expressed TLR transcripts identified in other studies after challenge with gram-negative bacteria: *V. chagasii* (this study, yellow) and *V. anguillarum* (bordeaux) and *V. splendidus* (pink), *Vibrio parahemolyticus* (orange) and *Aeromonas hydrophila* (red); gram-positive bacteria, *Micrococcus luteus* (green), lipopolysaccharide (LPS, light grey), peptidoglycan (PGN, black), 1, 3-beta-glucan (grey). The changes in TLR transcription in response to a challenge was obtained from Refs. [12,23,25,29,31]. *M. coruscus* TLR2 and TLR3 sequences are highlighted in bold and the recently described TLR (TLRa) in this species is represented in italics. M1, M2 and M3 indicate the mytilid clusters described in Ref. [28]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Percentage of TIR domain identity (upper right)/similarity (lower left) of the *M. coruscus* TLRs with other TLRs.

Similarity (%)	McTLR2	McTLR3	CgTLR1	CgTLR2	CgTLR3	CgTLR4	CgTLR6
McTLR2		22/48	33/61	35/62	21/50	23/59	31/61
McTLR3	22/48		15/42	21/47	44/70	16/46	23/46
CgTLR1	33/61	15/42		27/54	19/45	30/53	33/57
CgTLR2	35/62	21/47	27/54		21/55	27/56	35/60
CgTLR3	27/50	44/70	19/45	21/55		20/45	22/47
CgTLR4	23/59	16/46	30/53	27/56	20/45		33/61
CgTLR6	31/60	23/46	33/57	35/60	22/47	33/61	

Aliview platform 1.18 [34]. To further consolidate the assignment of the isolated Mc TLRs, phylogenetic analysis was also performed with the full-length amino acid sequence of the isolated bivalve TLRs. The phylogenetic analysis was conducted using maximum likelihood (ML) and Bayesian inference (BI) methods.

ML trees were established using the PhyML 3.0 [35] algorithm and the ATGC bioinformatics platform with the SMS automatic model selection [36] for the best model of protein evolutionary analysis according to the AIC (Akaike Information Criterion). The TIR domain ML tree was constructed using an LG substitution model [37] and the full-length TLR tree was constructed with a VT substitution model [38]. For both trees reliability of internal branching was assessed using 100 bootstrap replicates. The BI tree was performed using MrBayes 3.2 [39] and an LG (for TIR) or VT (for full-length TLRs) substitution model (Aamodel = LG or VT) and 1.000.000 generations sampling for probability values supporting tree branching. ML and BI trees were displayed in FigTree 1.4.2 and were edited in Inkscape. Both phylogenetic trees of the TLRs gave similar topologies. The percent sequence similarity and identity between TLR isoforms were calculated for the TIR domain using GeneDoc (2.7.000) [40].

2.5. Quantitative real-time PCR analysis

Expression of McTLR2 and McTLR3 transcripts in *M. coruscus* tissues (haemocytes, gill, mantle, digestive gland, gonad, adductor muscle and foot) was determined by RT-qPCR. Table 1 indicates the specific primers designed for McTLR2 and McTLR3 using Primer Premier 6.0 software. Quantitative real-time qPCR (RT-qPCR) analysis of McTLR2 and McTLR3 was performed using a LightCycler960 in a 96 multiwell plate (Roche) with five biological replicates for each tissue.

The RT-qPCR reactions were performed in duplicate and each reaction contained 1 μl template cDNA (around 200 ng), 0.3 μl of each of the primers (0.3 μM), 5 μl of 2 × FastStart Essential DNA Green Master (Roche) and sterile MilliQ water to give a final reaction volume of 10 μl. The RT-qPCR amplification protocol was as follows: an initial denaturation step at 95 °C for 10 min followed by 45 cycles of 10 s at 95 °C and 10 s at the optimal annealing temperature for primer pairs. A melting curve analysis was conducted to confirm a single reaction peak was obtained.

Elongation factor 1α (EF-1α) was used as the reference gene. An absolute quantification approach was conducted to determine the relative mRNA expression. Amplicons of each gene were isolated after 1% agarose gel electrophoresis and their sequence confirmed as previously described. The template of the standards used for absolute quantification consisted of the PCR reaction amplicon isolated from the reaction mix using a NucleoSpin Gel kit and PCR Clean-Up Kit (Clontech, Japan) and quantified by spectrophotometry. A standard curve was included in each RT-qPCR reaction and ranged from 10^7 – 10^1 DNA molecules. The Ct value of each sample was used in conjunction with the standard curve to determine the copy number of the target genes.

2.6. Statistical analysis

For statistical analysis of RT-qPCR data, a Kruskal-Wallis test followed by a Steel-Dwass All Pairs test were used, as assumptions of normality and homogeneity of variance for the parametric tests were not met. Data analysis was conducted using JMP™ software (SAS Institute, China). Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Characterization of McTLR2 and McTLR3

Full-length cDNA sequences of McTLR2 (Fig. S1A) and McTLR3 (Fig. S1B) were obtained using RACE (Genbank accession number: MH591769 for McTLR2; MH591768 for McTLR3) (Fig. S1). The McTLR2 cDNA encoded a 696 amino acid protein with a predicted molecular mass of 80.90 kDa and a theoretical isoelectric point (pI) of 9.35. The McTLR3 cDNA encoded an 872 amino acid protein with a predicted molecular mass of 98.97 kDa and theoretical isoelectric point (pI) of 9.20.

3.2. Phylogenetic analysis and multiple sequence alignments of McTLR2 and McTLR3

A multiple sequence alignment of the TIR domain of TLRs from *C. gigas, M. galloprovincialis, R. decussatus*, the single sequence from *C. farreri* and the 3 isolated *M. coruscus* TLRs including the *M. coruscus* TLRa (see Fig. S2) were used to build the phylogenetic trees. The sequence similarity and clustering of deduced proteins of the cDNAs isolated from *M. coruscus* in the BI phylogenetic tree, indicated that they belong to members of a TLR gene family (Fig. 1). The ML (Fig. S3) and BI tree for the bivalve TLRs had a similar topology. Expansion of the TLR gene family occurred in the mytilids (clusters M1, M2 and M3) (Fig. 1). The naming of the isolated McTLRs was based on their amino acid sequence similarity with other TLRs and their clustering in the phylogenetic tree. The McTLRs were designated McTLR2 and McTLR3 since they clustered in the BI tree with CgiTLR2 and CgiTLR3, respectively (Fig. 1). Furthermore, McTLR2 shared 35% identity and 62% similarity with CgiTLR2, and McTLR3 shared 44% identity and 70% similarity with CgiTLR3 (Table 2). The expression of the bivalve TLRs previously reported to respond to bacteria or bacterial molecules were mapped onto the tree (Fig. 1). A phylogenetic tree constructed using BI and the full-length deduced protein sequences of the TLRs concurred with the tree topology obtained when only the TIR domain was used in the analysis (see Fig. S4).

To identify the main functional domains of the TLRs and further confirm the identity of the TLRs isolated from *M. coruscus* a multiple sequence alignment (MSA) was performed. McTLR2 was aligned with CgiTLR1, CgiTLR2, MgTLRc, MgTLRn, MgTLRo and MgTLRs (Fig. 2A) and McTLR3 was aligned with CgiTLR3 and RdTLR7 (Fig. 2B). The deduced protein sequence of McTLR2 contained 1 signal peptide, 6 LRRs, 1 LRR-CT, 1 transmembrane domain and 1 TIR domain (Fig. 2A). The deduced protein sequence of McTLR3 contained 1 signal peptide, 13 LRRs, 1 LRR-NT, 4 LRR-TYP, 1 LRR-CT, 1 transmembrane domain and 1 TIR domain (Fig. 2B).

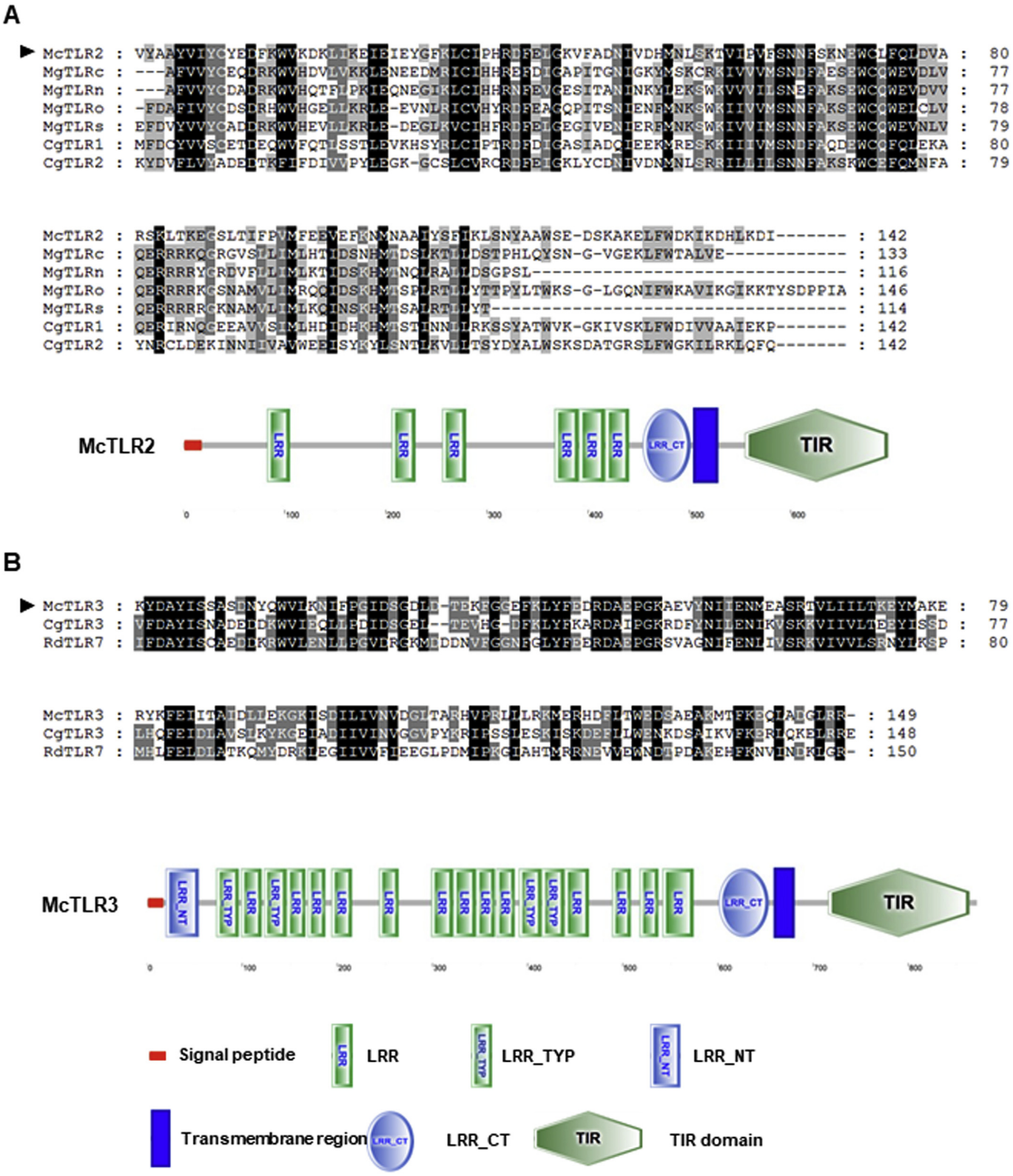

Fig. 2. Multiple sequence alignment (MSA) of the TIR domain from *M. coruscus*, *M. galloprovincialis*, *C. gigas* and *Ruditapes decussatus*. A) indicates the MSA generated for McTLR2 and below a schematic representations of the full length McTLR2 is provided. The main domains identified that are characteristic of TLRs are indicated. B) indicates the MSA generated for McTLR3 and below it is a schematic representations of the full length McTLR3. In the MSA conserved amino acids are shaded, and identical (black) and similar (grey) residues are also indicated.

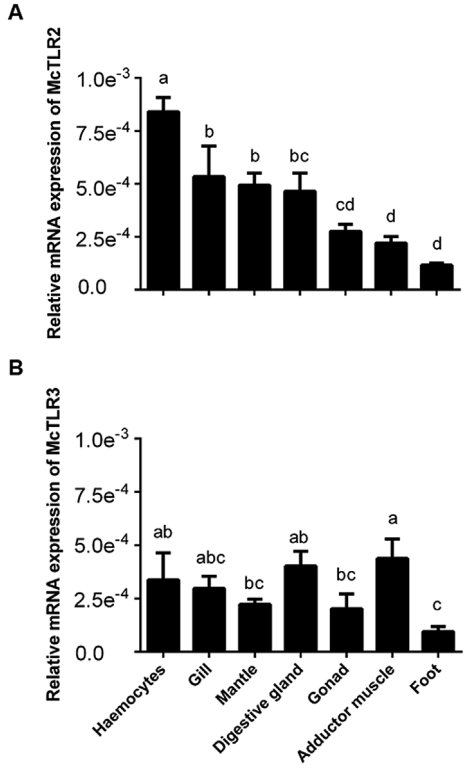

Fig. 3. McTLR2 (A) and McTLR3 (B) mRNA transcript abundance in seven tissues (haemocytes, gill, mantle, digestive gland, gonad, adductor muscle and foot). The vertical bars represent the mean ± S.E (n = 5 samples/tissue). Different letters above the bars indicate significant different ($P < 0.05$).

3.3. McTLR2 and McTLR3 mRNA tissue localization

The McTLR2 and McTLR3 mRNA expression levels in the haemocytes, gill, mantle, digestive gland, gonad, adductor muscle and foot were examined by RT-qPCR (Fig. 3). McTLR2 and McTLR3 mRNA were expressed in all of the tissues analyzed. The haemocytes had a significantly higher abundance ($P < 0.05$) of McTLR2 transcripts relative to other tissues (Fig. 3A). The mRNA expression of McTLR3 was significantly more abundant ($P < 0.05$) in the haemocytes, digestive gland and adductor muscle relative to the foot (Fig. 3B).

4. Discussion

The molluscs are one of the most successful and diverse groups of extant animals and the basis of their success are probably due to their capacity to adapt to a wide diversity of habitats [31]. As filter-feeding organisms, bivalves are susceptible to stress from biotic and abiotic factors. Characterization of the TLRs required for protection from pathogens through PAMP recognition, highlights the importance of this components of innate immunity in bivalves. In the present study, two full-length cDNAs, belonging to the TLR gene family, were isolated from *M. coruscus* (McTLR2 and McTLR3). The two TLRs had a widespread tissue distribution and McTLR2 was highly abundant in haemocytes.

Gene family expansions of TLRs are commonly found in bivalves. In the genome of the pacific oyster *C. gigas* [19,31] and transcriptomes of the Mediterranean mussel *M. galloprovincialis* (23 TLRs) [12], Venus Clam *Cyclina sinensis* (32 TLRs) [41] and carpet shell clam *R. decussatus* (10 TLRs) [29], there is a higher number of TLRs relative to the vertebrates and other invertebrates such as *Drosophila*. For the naming of the identified TLRs in the hard-shelled mussel we adopted that of the pacific oyster, a bivalve in which TLRs have been extensively investigated [3,19,31]. The clustering in the phylogenetic tree of McTLR2 with TLR2 from the pacific oyster (Cgi_TLR2) and the conserved domains in the deduced protein revealed it was an sP form (short protostome-like without LRRCT-LRRNT ectodomain). In contrast, McTLR3 clustered tightly with the pacific oyster TLR3 and the deduced protein contained the characteristic motifs of vertebrate type (V-type) TLRs. Interestingly, the recently identified novel McTLR-a in the hard-shelled mussel clustered in a sister clade to McTLR2 and functional conservation was evident as it was also responsive to a challenge with gram negative bacteria [25]. The TLR gene family expansion and the maintenance of an increased number in the genome of bivalves are associated with rapid evolution as a consequence of lineage and species-specific gene expansions and sequence divergence. Overall, there is low sequence homology between TLRs in different species and this makes orthologous relationships difficult to establish. Nonetheless, the identified TLRs in the present study contained the typical domains of the TLR family such as the signal peptide, LRR, LRR-CT and TIR domain and a transmembrane domain.

TLRs are classified as type I transmembrane receptors and possess an extracellular LRR domain and a cytoplasmic TIR domain [42]. McTLR2 and McTLR3 are single cysteine cluster TLRs (sccTLR), a feature they share with the previously identified, McTLR-a, in *M. coruscus* [25]. In fact, in *M. galloprovincialis* 19 of the 23 TLRs identified were classified as sccTLR and only four belonged to the TLPs with multiple cysteine clusters (mccTLR) [12]. The LRR domain in the TLRs is responsible for their highly variable structure and it has a key role in ligand recognition and signal transduction [43,44]. The McTLR2 and McTLR3 identified in the present study contained a variable number of LRR domains (6 and 13, respectively) and a large variable extracellular region, suggesting each may recognize a different spectrum of PAMPs. Preliminary studies revealed the transcriptional response of McTLR2 and McTLR3 to *V. chagasii* differed (data not shown) and supports the notion they recognize different PAMPs. A similar variable number of LRR (from 3 to 17) repeats was also identified in the different forms of TLRs in *M. galloprovincialis* [12]. The TLR gene family expansion in mussels and other bivalves may be linked to the acquisition of increased versatility in pathogen recognition during their evolution. So far relatively few studies have screened for PAMP recognition using mollusc TLRs displayed in cell lines, but this will be an essential step if the full functional scope of these molecules is to be deciphered.

McTLR2 and McTLR3 were constitutively expressed in several different tissues, although the high expression of McTLR2 transcripts in haemocytes, key cells in the bivalve immune repertoire, suggests a role in the internal system of host defense [45]. The presence of appreciable levels of both McTLR2 and McTLR3 transcripts in mucosal tissue suggests they participate in the immune protection to external immune barriers. A previous study has shown that injection of bacteria induced TLR upregulation in haemocytes of the ark shell *Scapharca broughtonii* [46].

In conclusion, two TLRs (McTLR2 and McTLR3) of the sP- and V-type were identified in the mantle of the mussel *M. coruscus*. Phylogenetic analysis of the TLR family in mytilids revealed that gene family expansion occurred. McTLR2 transcripts were significantly higher ($P < 0.05$) in the haemocytes compared to the gill, mantle, digestive gland, gonad, adductor muscle and foot. In contrast, McTLR3 was significantly more abundant ($P < 0.05$) in the haemocytes, digestive gland and adductor muscle compared to the foot. It will be important to establish PAMP recognition of McTLR2 and McTLR3 to establish how they participate in immune recognition.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This study was supported by Key Program for International Science and Technology Cooperation Projects of Ministry of Science and

Technology of China (No. 2016YFE0131900), the National Natural Science Foundation of China (No. 41476131), the Peak Discipline Program for Fisheries from the Shanghai Municipal Government to JLY. DMP, JRC and FMB acknowledge support from the Fundação para a Ciência e a Tecnologia, Portugal (UID/Multi/04326/2013) and an FCT/China bilateral cooperation project (No. 441).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.001.

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