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Molecular cloning and tissue distribution of the Toll receptor in the black tiger shrimp, *Penaeus monodon*

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ABSTRACT. The black tiger shrimp (*Penaeus monodon*) is economically important in many parts of the world, including Thailand. Shrimp immunity is similar to that of other invertebrate organisms; it consists of an innate immunity system. Toll or Toll-like receptors (TLRs) play an essential role in recognizing the cleaved form of the cytokine Spätzle, which is processed by a series of proteolytic cascades activated by secreted recognition molecules. We isolated a full-length Toll receptor from *P. monodon*. The cloned full-length sequence of the PmToll cDNA consists of 4144 nucleotides, containing a 5'-UTR with 366 nucleotides, a 3'-terminal UTR with 985 nucleotides, with a classical polyadenylation signal sequence AATAAA, a poly A-tail with 27 nucleotides, and an open reading frame coding for 931 amino acids. The deduced amino acid sequence of PmToll is a typical type I membrane domain protein, characteristic of TLR functional domains. It includes a putative signal peptide, an extracellular domain consisting of leucine-rich repeats, flanked by cysteine-rich motifs, a single-pass transmembrane portion,

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and a cytoplasmic TLR domain. PmToll was expressed in all tissues tested, including gill, hemocytes, heart, hepatopancreas, lymphoid organs, muscle, nerve, pleopod, stomach, testis, and ovary. The deduced amino acid of PmToll is closely related to that of other shrimp Tolls, especially FcToll. Further studies elucidating the mechanism of action of Tolls will be of benefit for understanding the defense mechanisms of this economically important aquatic species.

Key words: Molecular cloning; Toll receptor; *Penaeus monodon*

INTRODUCTION

The black tiger shrimp (*Penaeus monodon*) is an economically important aquatic organism in many parts of the world, including Thailand. However, farming of this species has significantly decreased in recent years, primarily because of the susceptibility of this species to shrimp pathogens such as the yellow head (Boonyaratpalin et al., 1993) and white spot syndrome (Flegel, 1997) viruses. Shrimp immunity is similar to that in other invertebrate organisms, and consists of an innate immunity, which can be divided into humoral and cellular defenses (Lee and Soderhall, 2002; Cerenius and Soderhall, 2004; Jiravanichpaisal et al., 2006; Han-Ching et al., 2010). Toll or Toll-like receptors (TLRs) play an essential role in recognizing the cleaved form of the cytokine Spätzle, which is processed by a series of proteolytic cascades activated by secreted recognition molecules (Lemaitre and Hoffmann, 2007). Toll or TLRs are evolutionarily conserved transmembrane glycoproteins characterized by an extracellular domain containing various numbers of leucine-rich repeat (LRR) motifs and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (Bowie and O'Neill, 2000). Recently, a truncated PmToll from *Penaeus monodon* (Arts et al., 2007), a full-length LvToll from *Litopenaeus vannamei* (Yang et al., 2007), a full-length MjToll from *Marsupenaeus japonicus* (Mekata et al., 2008), and a full-length FcToll from *Fenneropenaeus chinensis* (Yang et al., 2008) have been cloned, and some of their functions in shrimp innate immunity against foreign molecules have been determined. The objective of the present study was to clone the full-length Toll receptor from *P. monodon*, PmToll, to compare its sequence with other invertebrate organisms, and to study its tissue distribution.

MATERIAL AND METHODS

Shrimp culture

Healthy juvenile *P. monodon* (weighing 8-10 g) were purchased from a commercial farm in the domestic area and reared in a sea water tank system with a salinity of 10 parts per thousand (ppt) at 25-28°C for 7 days before the experiments.

RNA extraction

Total RNA was extracted from various tissues using TRIzol-Reagent (Invitrogen,

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USA) following the manufacturer protocol. The extracted RNA was then treated with RQI RNase-free DNase (Promega, USA) to remove contaminating DNA. The RNA concentration and its quality were determined (A_{260}) and monitored (A_{260}/A_{280} ratio >1.8) using a NanoDrop 1000 spectrophotometer.

cDNA cloning of PmToll

To synthesize the first-strand cDNAs, 5 µg total RNA was subjected to reverse transcription using the M-MLV Reverse Transcription System (Fermentas, USA) according to the supplied procedure with PRT primer (Table 1). PCR was performed using the cDNA prepared as above, which amplifies the initial sequence using the Toll RDW and PM1 primers (Table 1). Having isolated this partial PmToll sequence, the entire sequence was obtained using 5'-RACE-PCR with the gene-specific primers shown in Table 1. PCR was performed using 1X *Taq* buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 2 mM of each dNTP, 2.5 U *Taq* polymerase (Fermentas), 0.2 µM of each primer and 2 µL template cDNA. The PCR conditions consisted of 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 50-55°C for 30 s and 72°C for 1.5 min, followed by an extension of 72°C for 10 min. The products were cloned into the pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5α. Recombinant plasmid from at least five independent clones were extracted using a QIAprep Spin Miniprep kit (QIAGEN) and sequenced commercially using the BigDye® Terminator v3.1 cycle sequencing kit (1st BASE sequencing unit, Malaysia).

Table 1. Primers used to amplify the full-length PmToll sequence of the black tiger shrimp, *Penaeus monodon.*

Name	Sequence (5'→3')
3' RACE primers	
PRT primer	CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTTTTTT
PM1 primer	CCGGAATTCAAGCTTCTAGAGGATCCTT
PmT RDW-F	TGCCTTCACTACCGCGACTGG
First 5' RACE primers	
PmT GSP1-R	AGCCTGGGAGTGAGCTGC
PmT Pm-F-1	AGTGTACCTGAAGACCTCTT
Second 5' RACE primers	
PmT GSP2-R	GAGTTCTTCCAAGCTCCTGAGATC
PmT GSP3-R	GCCTATTTGTGATGTCACTC
PM1 primer	CCGGAATTCAAGCTTCTAGAGGATCCTT
PRC primer	CGGAATTCAAGCTTCTAGAGGATCCTTGGGGGGGGGGGGGGG
Tissue distribution of PmToll and shrimp β-actin	
PmToll	F-GTCCAATCAGTTGGAGCTGC
PmToll	R-GAAATCGAGCGTCTTCACATGC
PmActin	F-GACTCGTACGTGGGCGACGAGG
PmActin	R-AGCAGCGGTGGTCATCTCCTGCTC

Sequence analysis and phylogenetic tree

Nucleotide sequence and deduced amino acid sequence comparisons were carried out using the BLAST algorithm at the NCBI GenBank database (NCBI, http://ncbi.nlm.nih.gov/BLAST/). Sequence alignments were performed using AlignX (Part of the Vector NTI Version 10). The signal peptide was predicted using the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen et al., 2004). Potential N-linked glycosylation sites were pre-

dicted by the NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc/). The simple modular architecture research tool (SMART, http://smart.emblheidelberg.de) (Letunic et al., 2009) was used to analyze the deduced amino acid sequence. Phylogenetic and molecular evolutionary analyses of the predicted amino acid sequences of different Tolls were conducted using the neighbor-joining method and were drawn using *MEGA* version 4 (Tamura et al., 2007). The nucleotide sequence and deduced amino acid sequence of PmToll were submitted to GenBank (GenBank ID: GU014556 and ADK55066).

Tissue distribution of the PmToll gene

To investigate the tissue distribution of PmToll, total RNA was extracted from the gills, hemocytes, heart, hepatopancreas, lymphoid organ, muscle, nerve, pleopod, stomach, testis, and ovary, and 1 μg RNA from each tissue was used to produce first-strand cDNA with an oligo dT primer. The first-strand cDNA was used directly as a template in subsequent multiplex-PCR to amplify the PmToll gene and shrimp β-actin (internal control), using PmToll-F and PmToll-R primers and actin-F and actin-R primers, respectively (Table 1). The temperature profile for PCR conditions was as follows: 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. After 30 cycles, the reaction was held at 72°C for another 10 min.

RESULTS AND DISCUSSION

In this study, we report the first isolation of a full-length Toll receptor from *P. monodon* (Figure 1). The cloned full-length sequence of the PmToll cDNA consists of 4144 nucleotides containing a 5'-UTR of 366 nucleotides, a 3'-terminal UTR of 985 nucleotides with a classical polyadenylation signal sequence (AATAAA) and a poly A-tail of 27 nucleotides, and an open reading frame coding for 931 amino acids. The deduced amino acid sequence of PmToll is a typical type I membrane domain protein, characteristic of TLRs' functional domains. It includes a putative signal peptide (residues 1-19), an extracellular domain (residues 133-706) consisting of LRRs flanked by cysteine-rich motifs, a single-pass transmembrane portion (residues 713-735), and a cytoplasmic TIR domain (residues 766-904). Twelve potential N-linked glycosylation sites, which were predicted by NetNGlyc 1.0, are located in the ectodomain. Finally, many structural features are conserved in the regions that flank the LRRs, including all 18 cysteine residues in the LRR-CT and LRR-NT regions, and the sequence NPXXC(N/D)C in the two LRR-CT regions of PmToll. The prevailing LRR consensus sequence in TLRs is the 24-residue motif of x-L-x-x-L-x-L-x-x-N-x-Φ-x-x-Φ-x-x-x-x-F-x-x-L-x (Bell et al., 2003), where x refers to any amino acid, Φ is any hydrophobic residue, L and F are frequently replaced by other hydrophobic residues. Alignment of LRRs in PmToll (Figure 2) revealed that 14 tandem LRR repeats exist in PmToll, whereas only 8 LRRs were predicted by the SMART program (Figure 3). All LRRs contained the conserved asparagine residue at position 10, while highly conserved leucine residues were found at positions 2, 5 and 7 of each LRR. In addition, an insertion of seven residues was identified in LRR-10. Moreover, alignment of the TIR domain of the PmToll protein with other shrimp and arthropod Toll proteins showed a similar structure (Figure 4). The expression of PmToll was investigated in several tissues including gills, hemocytes, heart, hepatopancreas, lymphoid organs, muscle, nerve, pleopod,

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stomach, testis, and ovary, and expression was detected in all tissues using multiplex RT-PCR, although an apparently low level of expression was observed in the hepatopancreas (Figure 5).

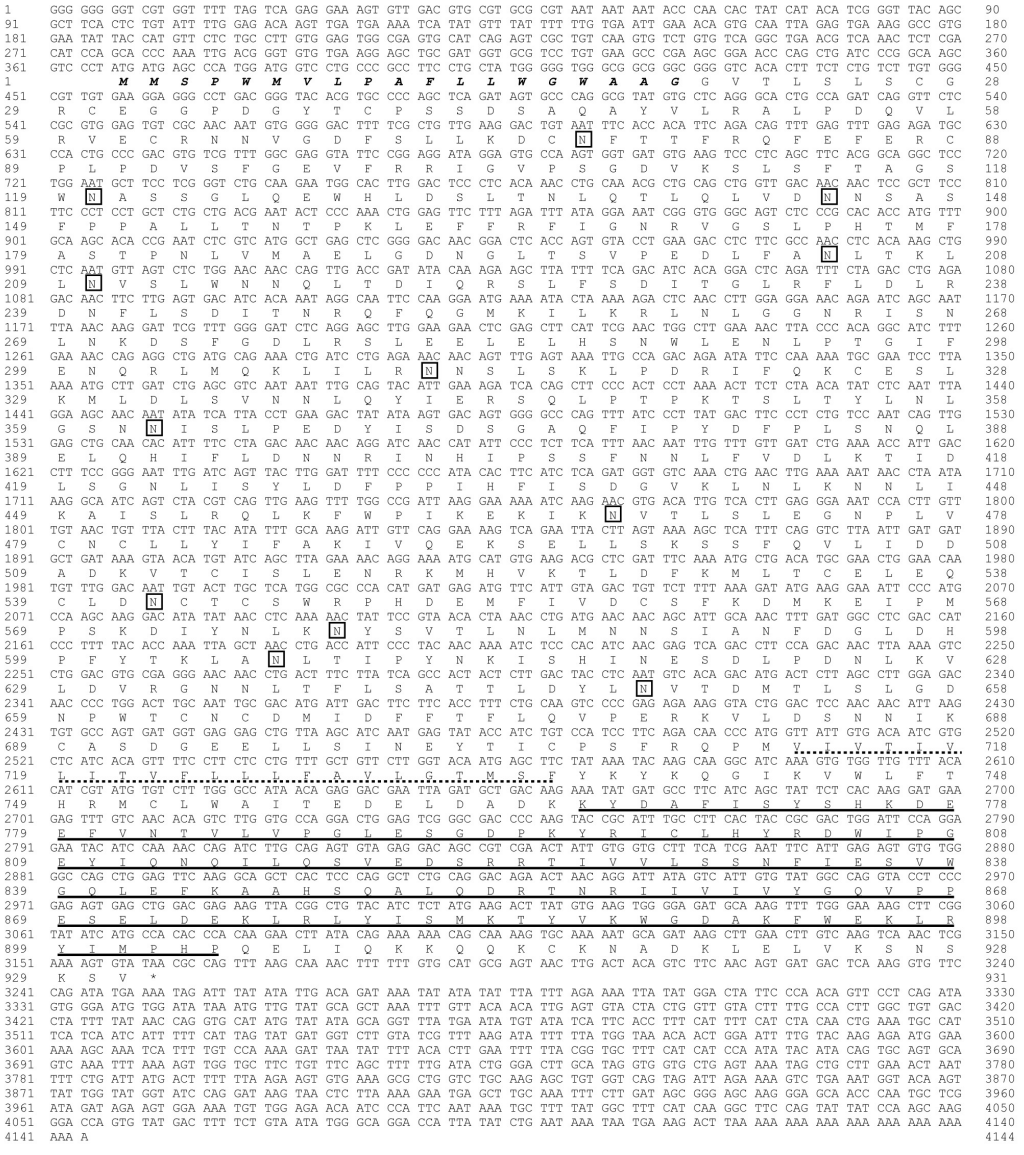

Figure 1. The full-length cDNA sequence and deduced amino acid sequence of PmToll from *Penaeus monodon.* The result of the amino acid sequence is coded with one-letter underneath the nucleotide sequence. The predicted signal peptide is italicized. The potential N-liked glycosylation sites in the extracellular domain are shown in boxes. The transmembrane region is underlined with a dotted line, while the TIR domain is underlined with a solid line.

Consensus	XL	XXLXLXXN	XΦXXΦ	XXXXFXX LX	Position
LRR1	NL	QTLQLVDN	NSASF	PPALLTN TP	135-158
LRR2	KL	EFFRFIGN	RVGSL	PHTMFAS TP	159-182
LRR3	NL	VMAELGDN	GLTSV	PEDLFAN LT	183-206
LRR4	KL	LNVSLWNN	QLTDI	QRSLFSD IT	207-230
LRR5	GL	RFLDLRDN	FLSDI	TNRQFQG MK	231-254
LRR6	IL	KRLNLGGN	RISNL	NKDSFGD LR	255-278
LRR7	SL	EELELHSN	WLENL	PTGIFEN QR	279-302
LRR8	LM	QKLILRNN	SLSKL	PDRIFQK CE	303-326
LRR9	SL	KMLDLSVN	NLQYI	ERSQLPT PK	327-350
LRR10	SL	TYLNLGSNNISLPEDYISDS		-GAQFIP YD	352-381
LRR11	EL	QHIFLDNN	RINHI	-PSSFNN LF	389-411
LRR12	DL	KTIDLSGN	LISYL	DFPPIHF IS	413-436
LRR13	GV	-KLNLKNN	LIKAI	SLRQLKFWP IK	438-462
LRR14	NL	KVLDVRGN	NLTFL	SATTLDY LN	625-648

Figure 2. Alignment of leucine-rich repeats (LRRs) in PmToll. LRRs of PmToll are aligned with the 24-residue prevailing LRR consensus sequence of TLRs (Bell et al., 2003). X refers to any amino acid, Φ is any hydrophobic residue, and L and F are frequently replaced by other hydrophobic residues. Residues that are conserved with the consensus sequence are shaded in gray.

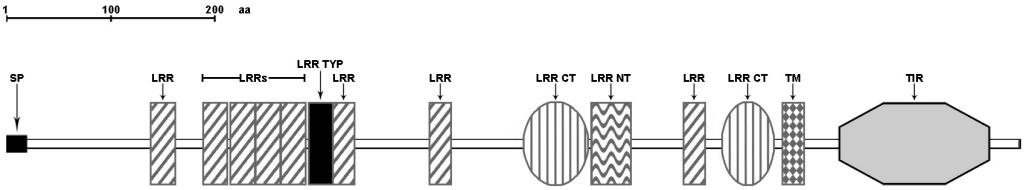

Figure 3. Schematic diagram of the PmToll protein predicted by the SMART program. The ectodomain of PmTolls consists of SP, LRR, LRR-CT and LRR-NT. TM is the transmembrane region. The cytosolic domain consists of the TIR/IL-1 domain. SP = signal peptide; LRR = leucine-rich reperat; LRR-CT = LRR C-terminal domain; LRR-NT = LRR N-terminal domain; TIR = Toll/interleukin-1R domain.

The phylogenetic relationship between the deduced amino acid sequence of PmToll and other arthropod Tolls is shown in Figure 6 and Table 2, and the analysis suggests that PmToll is closely related to other shrimp Tolls, especially FcToll. Moreover, shrimp Toll proteins are more closely related to DmToll5, DmToll3 and DmToll4 than to other DmTolls. In *F. chinensis*, the expression of FcToll in the lymphoid organ has been characterized after bacterial or WSSV challenge, and was shown to have distinct expression profiles. After bacterial challenge, FcToll expression was upregulated, whereas FcToll expression after WSSV stimulation was downregulated (Yang et al., 2008). More recently, the function of LvToll was studied using an RNAi silencing approach to downregulate expression of LvToll, followed by WSSV or *V. harveyi* challenge. While there was a significant increase in mortality and bacterial CFU counts in LvToll-silenced shrimp following *V. harveyi* challenge, there was no difference in mortality rates following WSSV challenge, suggesting that LvToll is an important factor in the shrimp innate immune response to acute *V. harveyi* infection, but not to WSSV (Han-Ching

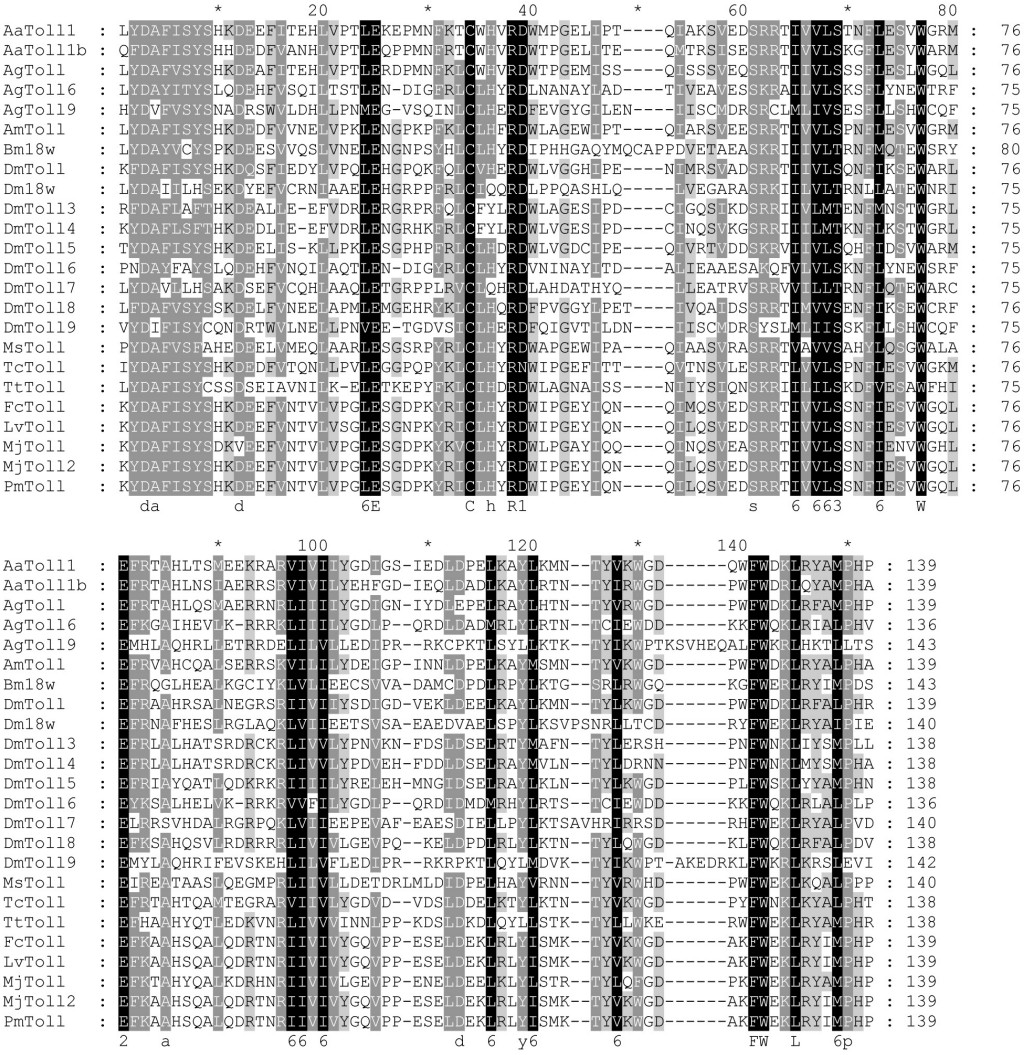

Figure 4. Alignment of TIR domains of Arthropoda Tolls.

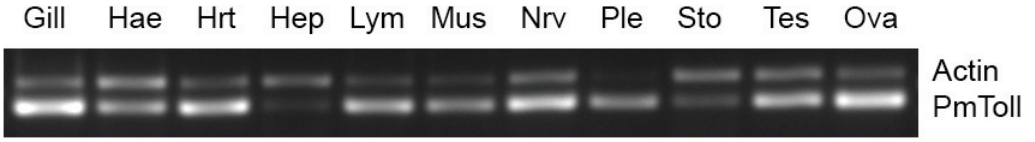

Figure 5. Tissue expression profile of PmToll using multiplex RT-PCR detection. Hae = hemocytes; Hrt = heart; Hep = hepatopancreas; Lym = lymphoid organ; Mus = muscle; Nrv = nerve; Ple = pleopod; Sto = stomach; Tes = testis; Ova = ovary.

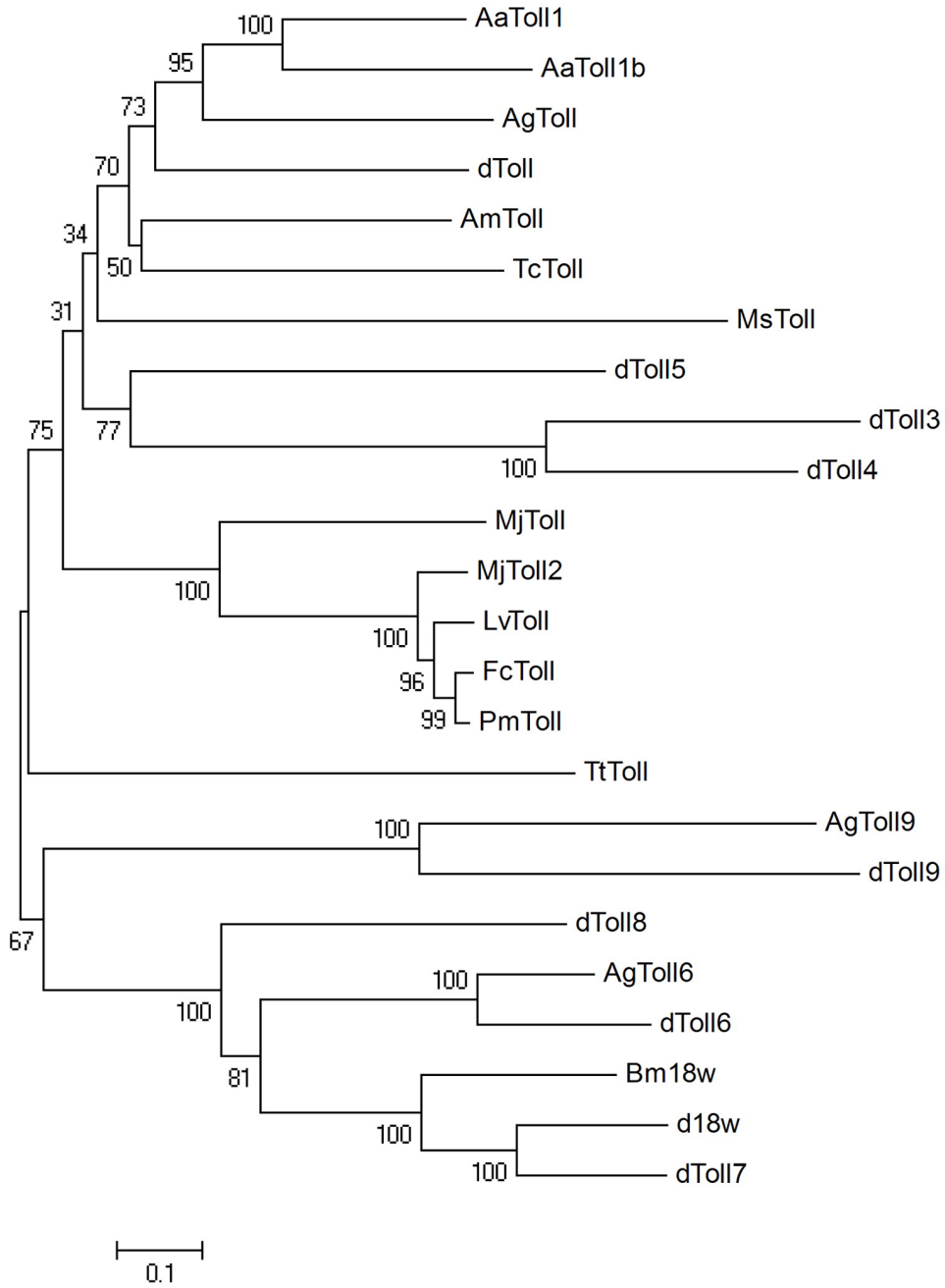

Figure 6. Phylogenetic tree of arthropod TLRs. All full-length amino acid sequences of TLR from arthropod sequences (Table 2) were aligned and the phylogenetic tree was constructed using the bootstrap NJ method with the MEGA4.02 program. The reliability of each branch was tested by 1000 bootstrap replications. Numbers at the branch nodes indicate bootstrap values. The scale bar indicates a branch length of 0.1 amino acid sequence.

Table 2. Details of genes used for PmToll analysis.

Species	Name	Accession number
Aedes aegypti	AaToll1	AAM97775
	AaToll1b	AAM97776
Anopheles gambiae	AgToll	AAL37901
	AgToll6	AAL37902
	AgToll9	AAL37903
Apis mellifera	AmToll	XP_396158
Bombyx mori	Bm18w	BAB85498
Drosophila melanogaster	DmToll	AAQ64935
	Dm18w	AAF57509
	DmToll3	AAF54021
	DmToll4	AAF52747
	DmToll5	AAF86227
	DmToll6	AAF49645
	DmToll7	AAF57514
	DmToll8	AAF49650
	DmToll9	AAF51581
Fenneropenaeus chinensis	FcToll	ABQ59330
Litopenaeus vannamei	LvToll	ABK58729
Manduca sexta	MsToll	ABO21763
Marsupenaeus japonicus	MjToll	BAF99007
	MjToll2	BAG68890
Tribolium castaneum	TcToll	XP_967796
Tachypleus tridentatus	TtToll	BAD12073

et al., 2010). Similarly, PmToll was not found to be regulated during WSSV challenge of *P. monodon* (Arts et al., 2007). Collectively, these results suggest that shrimp Tolls are involved in the innate immune response to bacterial rather than viral infection, and as such, further studies elucidating the mechanism of action of Tolls will be of benefit in understanding the mechanism of bacterial pathogenesis in economically important aquatic species.

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