

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

W. Assavalapsakul<sup>1</sup> and S. Panyim<sup>2,3</sup>

<sup>1</sup>Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand <sup>2</sup>Institute of Molecular Biosciences, Mahidol University, Nakorn Pathom, Thailand <sup>3</sup>Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand

Corresponding author: W. Assavalapsakul E-mail: wanchai.a@chula.ac.th

Genet. Mol. Res. 11 (1): 484-493 (2012) Received July 4, 2011 Accepted December 5, 2011 Published March 6, 2012 DOI http://dx.doi.org/10.4238/2012.March.6.1

**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,

Genetics and Molecular Research 11 (1): 484-493 (2012)

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,

Genetics and Molecular Research 11 (1): 484-493 (2012)

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  $\mu$ g 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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.5 mM MgCl<sub>2</sub>, 2 mM of each dNTP, 2.5 U *Taq* polymerase (Fermentas), 0.2  $\mu$ M of each primer and 2  $\mu$ L 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* DH5 $\alpha$ . Recombinant plasmid from at least five independent clones were extracted using a QIAprep Spin Miniprep kit (QIA-GEN) and sequenced commercially using the BigDye<sup>®</sup> Terminator v3.1 cycle sequencing kit (1st BASE sequencing unit, Malaysia).

Table 1. Primers used to amplify the full-leng	th PmToll sequence of the black tiger shrimp, Penaeus monodon.
Name	Sequence $(5' \rightarrow 3')$
3' RACE primers PRT primer PM1 primer PmT RDW-F	CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTT
First 5' RACE primers PmT GSP1-R PmT Pm-F-1	AGCCTGGGAGTGAGCTGC AGTGTACCTGAAGACCTCTT
Second 5' RACE primers PmT GSP2-R PmT GSP3-R PM1 primer PRC primer	GAGTTCTTCCAAGCTCCTGAGATC GCCTATTTGTGATGTCACTC CCGGAATTCAAGCTTCTAGAGGATCCTT CGGAATTCAAGCTTCTAGAGGATCCTTGGGGGGGGGG
Tissue distribution of PmToll and shrimp β-actin PmToll PmToll PmActin PmActin	F-GTCCAATCAGTTGGAGCTGC R-GAAATCGAGCGTCTTCACATGC F-GACTCGTACGTGGGGCGACGAGG 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-

Genetics and Molecular Research 11 (1): 484-493 (2012)

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  $\mu$ g 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  $\beta$ -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-N-x-Φ-x-x-Φ-x-x-F-x-x-F-x (Bell et al., 2003), where x refers to any amino acid,  $\Phi$  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,

Genetics and Molecular Research 11 (1): 484-493 (2012)

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).

90 180 270 360 450 91 181 361 451 540 58 29 541 59 631 89 721 119 811 149 901 179 991 209 1081 630 88 720 118 810 148 900 178 990 208 1080 238 1170 268 1260 298 1350 269 1261 1440 358 1530 388 1620 418 1710 448 1800 478 1890 508 1980 538 2070 568 1801 479 1891 509 1981 598 599 628 2340 2430 588 2520 718 2610 748 2700 778 2790 689 2521 719 2611 749 2701 L. I. T. V. F. L. L. F. A. V. L. G. T. M. S. F. Y. K. Y. K. Q. G. I. K. V. W. L. F. T. CAT CGT ATG TGT CTT TGG GCC ATA ACA GAG GAC GAA TTA GAT GCT GAC AAG AAA TAT GAT GCC TTC ATC AGC TAT TCT CAC AAG GAT GAA CGC GAC TGG 779 2791 808 2880 V N T V L V P G L E S G D ATC CAA AAC CAG ATC TTG CAG AGT GTA GAG GAC AGC CGT P K Y CGA ACT ATT 809 Е R R 838 2970 2881 AGA ACT AAG CTG CAG AAC 839 K А А Н 0 А D R Ν R 868 2971 TCT ATG AAG ACT TAT 3060 869 D R М К т 898 3150 E К W atg cca cac cca caa gaa ctt ata cag aaa aaa cag caa aag tgc aaa aat M P H P O E L I O K K O O K C K N AAG 899  $\underline{P}$  Q E L I Q K K Q Q K C K N A D K L E L V K S CAG TTT AAG CAA AAC TTT TTT GTG CAT GCG AGT AAC TTG ACT ACA GTC TTC AAC AGT GAT GAC TCA AAG 3240 929 3241 3331 931 3330 3420 3510 3600 3690 3780 3870 3960 4050 4140 CCT CAG ATA GGC TGT GAC AAA TGC CAT AGA ATG GAA TGC AGT GCA GAA ACT AAT GGT ACA AGT CAA TGC TCG CCA AGC AAG

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.

Genetics and Molecular Research 11 (1): 484-493 (2012)

Consensus	XL	XXLXLXXN	χφχχφ	XXXXFXX	LΧ	Position
LRR1	NL	QTLQLVDN	NSASF	PPALLTN	ΤP	135-158
LRR2	KL	EFFRFIGN	RVGSL	PHTMFAS	ТΡ	159-182
LRR3	NL	VMAELGDN	GLTSV	PEDLFAN	LT	183-206
LRR4	KL	LNVSLWNN	QLTDI	QRSLFSD	IΤ	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	ΡK	327-350
LRR10	SL	TYLNLGSNNIS	LPEDYISDS	-GAQFIP	ΥD	352-381
LRR11	ΕL	QHIFLDNN	RINHI	-PSSFNN	LΓ	389-411
LRR12	DL	KTIDLSGN	LISYL	DFPPIHF	IS	413-436
LRR13	GV	-KLNLKNN	LIKAI	SLRQLKFWP	ΙK	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,  $\Phi$  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 Pm-Toll 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

Genetics and Molecular Research 11 (1): 484-493 (2012)

W. Assavalapsakul and S. Panyim

		*	20	*	40	*	60	*	80		
AaToll1		LYDAFTSYSHKDE	EF TEH VPT	PKEPMNEKT	W VRDWMPGE	PT	-OTAKSVEDSR	TVVLSTNF	TESVWGRM		76
AaToll1b		OFDAFISYSHHDE	DEVANHUVPT	<b>POPPMN RT</b>	W VRDWTPGE	LTTE	OMTRSTSESER	TVVLSKGF	LESVWARM		76
AgToll		LYDAEVSYSHKDE	AFTTEHTVPT		WEVRDWTPGE	MISS	OTSSSVEOSRE	TTVLSSF	LESLWGOL		76
AgToll6		LYDAYITYSLODE	HEVSOILTST	PN-DIGERI	THYRDLNANA	YIAD	TIVEAVESSKR	TLVLSKSF	TYNEWTRF	÷	75
AgToll9		HYDVFVSYSNADR	SWVLDHULPN	MBG-VSOINI	THERDFEVGY	GILEN	-IISCMDRSRCI	MLIVSESF	LISHWCOF		75
AmToll		LYDAFTSYSHKDE	DEVVNEL VPK	NGPKP KI	THERDWLAGE	W PT	-OTARSVEESER	TVVLSPNF	LESVWGRM		76
Bm18w	:	LYDAYVCYSPKDE	ESVVOSI VNE	ENGNESTH	THYRDTPHHG		PPDVETAEASKRI	TTVLTRNE	MOTEWSBY	:	80
DmToll		KEDAETSYSHKDO	SFIEDY VPO	FHGPOKTOL	VHERDWLVGG	H PE	NIMESVADSER	TTVLSONF	TKSEWARL	:	76
Dm18w	-	LYDATTLHSEKDY	EFWCRNTAAE	HGRPPERI	TOORDLPPOA	SHLO	-LVEGARASEKI	TLVLTRNL	LATEWNRT		75
DmToll3		REDAELAETHKDE	ALLE-EFVDR	PRGRPRIOL	FYLEDWLAGE	S PD	CTGOSTKDSRRI	TVIMTENE	MNSTWGRL		75
DmToll4	:	KYDAFLSFTHKDE	DLIE-EFVDR	ENGRHKERI (	FYLEDWLVGE	STPD	CINOSVKGSBRI	TTLMTKNE	LKSTWGRL	:	75
DmToll5		TYDAFTSYSHKDE	ELIS-KILPK	SCPHPERL	THORDWLVGD	TPE	OTVETVDDSKRV	TTVLSOHE	TDSVWARM	:	75
DmToll6		PNDAYFAYSLODE	HEVNOTLAOT	EN-DIGYRI	THYRDVNTNA	УТТD	ALTEAAESAKOF	VLVLSKNF	LYNEWSRE	:	75
DmToll7		LYDAVILHSAKDS	EFVCOHLAAO	TGRPPLRV	LOHRDLAHDA	THY0	-TLEATRVSRRV	VILLTRNF	LOTEWARC		75
DmToll8	:	LEDAEVSYSSKDE	LEWNEEL APM	EMGEHRYKI (	THORDERVGG	Y PET		TMVVSENE	TKSEWCRE	:	76
DmToll9	:	WYDTFTSYCONDB	TWYLNELLPN	VEE-TODVSIC	THERDFOLGV	TILDN	-TTSCMDRSYSI	MUTTSSKE	LISHWCOF	:	75
MeToll	:	PYDAFVSFAHEDF	FLUMEO AAR	LESCSRPVRI	THYRDWARCE		-OTAASVRASERI	VAVVSAHV		:	76
TCTOIL	:	TYDAFTSYSHKDE	DEVICIÓN L.PV	I EGGPOPYKI (	THYRNWIPGE	RUTT	-OWTNSVLESER	TWVLSPNF	LESVMCKM	:	76
TtT011	:	TYDAFTSVCSSDS	ETAVNI K-F	TREPUERI	THORDWLACN		NTTYSTONSKDI	TITISKDE	WESAMEHT	:	75
FcToll	:	RADYLLOACARDE	FEWNEVI VDC	INSCORKVET	THYRDWIRGE.	V ON	-OTMOSVEDSER	TVVLSSNE	TESVMCOL	:	76
IuToll	:	KUDAFTSVSUKDE		I SCNDKVPT	THYPOWINGE			TUVISONE		÷	76
MiToll	:	KUDAFISISHKUE	EFWNTVI VBC	SGNERTRIC	T HYPDWI PGA	V 100	OTNOSVEASED	TVVISSNE		:	76
MJTOII MJTOII2	:	KUDAFISISDKVE.	EFWNTVI VPG	I SCOPKVRT	THYPDWIDGE		OTTOSVEDSDD	TVVISSNE	TESVACOL	:	76
PmToll	:	KUDAFISISHKDE	EFWNTVI VPC	I SCOPKVRT	THYPDWIPGE			TVVISSNE	TESVMGQL	÷	76
INTOIT	•	da d		SE C	b P1		QUIQUEDORM	6 663		•	10
		uu u			, 11 1(1		5	0 000	0 11		
		*	100	*	120	*	140	*			
AaToll1		FRTAHLTSMEEK	RARVIVIIVGI		ETKAYTKMN-	TYWKMGI		TRYAMPHP	: 139		
AaToll1b	÷	FRTAHLNSTAER	RSRVTVTLYEI	HEGD-TEOLDA	DIKAYIRTN-				: 139		
Aaroll		FRTAHLOSMAER	RNRTTTTYG	DIGN-TYDIEF	ELBAYTHTN-	-TYWRWGI		TREAMPHP	: 139		
AgToll6		PFKGAIHEVIK-R	RRKLTTTLYG		DMRLYDRTN-	-IICTEWDI		TRIATPHV	: 136		
AgToll9		MHLAOHRLI ETR	RDELTIVILEI	D PRRKCPF	TISYLIKTK-	-TYIKWP1	KSVHEOALEWK	THKTLLTS	: 143		
AmToll		FRVAHCOAL SER	RSKVILILYDI	EIGP-INNLDE	ELKAYMSMN-	T YWK//GI		I RYAL PHA	: 139		
Bm18w		FROGLHEAL KGC	IYKLVLIEEC	SVVA-DAMCDE	DIRPYIKTG-	-SR <mark>II</mark> R//GO	)KGFWE	LRYIMPDS	: 143		
DmToll		FRAAHRSALNEG	RSRIIVIIYSI	DIGD-VEKLDE	ELKAYIKMN-	- TYLKØGI	DPWFWD	IRFAL PHR	: 139		
Dm18w	:	FRNAFHESLRGL	AOKLVIIEET	SVSA-EAEDVA	ELSPYEKSVP	SNRLTCI	DRYEWE	LRYATPIE	: 140		
DmToll3	:	FRLALHATSRDR	CKRLIVVLYPI	NVKN-FDSLDS	ELRTYMAFN-	TYLERSH	IPNEWN	LIYSMPLL	: 138		
DmToll4	:	FRLALHATSRDR	CKRLIVVLYPI	DVEH-FDDLDS	ELRAYMVLN-		JPNEWN	TMYSMPHA	: 138		
DmTol15		FRIAYOATLODK	RKRIIIIILYRI	ELEH-MNGIDS	ELRAYIKLN-		DPLEWS	IYYAM PHN	: 138		
DmTol16		BYKSALHELVK-R	RKRVVEILYGI	DIPORDIDN	IDMRHY RTS-	-IICIEWDI	DKKEWO	IRLALPLP	: 136		
DmToll7		TRRSVHDAT RGR		EVAF-EAESDI	ELLPYTKTSA	VHRIBRSI	DRHEWE	TRYATEVD	: 140		
DmToll8		FKSAHOSVI RDR	RRETTVTVLGI	EVPOKELDE	DIRLYTKTN-			TREATEDV	: 138		
DmToll9		MYLAOHRIFEVS	KEHLTLVELEI	DIPRRKRPF	TLOYLMDVK-		-AKEDRKLEWK	TKRSDEVT	: 142		
MsToll		DIREATAASLOEG	MPRETTYLLD	TORLMIDIDE	ELHAYVENN-	TYVRWHI	)PWFWE	TKOATPPP	: 140		
TcToll		<b>PERTAHTOAMTEG</b>	RARVITVIYGI		ELKTYLKTN-	-TYWKMGI	)PYEWN	TRYATPHT	: 138		
TtToll		PHAAHYOTI EDK	VNRLIVVVTNI	NTPP-KDSTDP	DIOYLISTK-	- TYLLWKF		LRYAMPHR	: 138		
FcToll	÷	FKAAHSOALODR	TNRTTVTVYG	OVPP-ESELDE	KURLYUSMK-	TYWKMGI	)AKPWE	TRYIMPHP	: 139		
LvToll		FKAAHSOALODR	TNRTTVTVYG	OVPP-ESELDE	KURLYUSMK-		)AKPWE	TRYIMPHP	: 139		
MiToll		FKTAHYOAT KDR	HNRTTVTVLG	EVPP-ENELDE	ELKLYLSTR-		)PKPWE	TRYAMPHP	: 139		
	_		the second se	and the second sec		and the second sec		the second se			
MITOLIZ		FKAAHSOALODR	INRIIVIVYG	OVPP-ESELDE	KURLYUSMK-	-TYWKMGI	)AKEWE	IRYIMPHP	: 139		
MJTOII2 PmToll	:	DFKAAHSQALQDR DFKAAHSOALODR	INRIIVIVYG INRIIVIVYG	QVPP-ESELDE	KIRLYISMK-	-TY <mark>V</mark> KWGI -TYVKWGI	DAKEWE	(LRYIMPHP (LRYIMPHP	: 139 : 139		

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.

Genetics and Molecular Research 11 (1): 484-493 (2012)



**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.

Genetics and Molecular Research 11 (1): 484-493 (2012)

<sup>©</sup>FUNPEC-RP www.funpecrp.com.br

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.

## ACKNOWLEDGMENTS

Research supported by the Thailand Research Fund and Commission on Higher Education, the National Research University Project of CHE and Ratchadaphiseksomphot Endowment Fund (FW643A), and the Thai Government Stimulus Package 2 (TKK2555), under the Project for Establishment of Comprehensive Center for Innovative Food, Health Products and Agriculture. We would like to extend our gratitude to Prof. Anchalee Tassanakajon for suggestions, and also to Mr. Wanlop Chinnirunvong at the Institute of Molecular Biosciences for technical assistance. W. Assavalapsakul is supported by TRF-CHE (grant #MRG5180160). S. Panyim is a TRF Senior Research Scholar. The funding agencies had no role in the study design, in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.

### REFERENCES

Arts JA, Cornelissen FH, Cijsouw T, Hermsen T, et al. (2007). Molecular cloning and expression of a Toll receptor in the giant tiger shrimp, *Penaeus monodon. Fish Shellfish Immunol.* 23: 504-513.

Genetics and Molecular Research 11 (1): 484-493 (2012)

©FUNPEC-RP www.funpecrp.com.br

Bell JK, Mullen GE, Leifer CA, Mazzoni A, et al. (2003). Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol.* 24: 528-533.

Bendtsen JD, Nielsen H, von Heijne G and Brunak S (2004). Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* 340: 783-795.

- Boonyaratpalin S, Supamattaya K, Kasornchandra J, Direkbusaracom S, et al. (1993). Non-occluded baculo-like virus, the causative agent of yellow head disease in the black tiger shrimp (*Penaeus monodon*). Fish Pathol. 28: 103-109.
- Bowie A and O'Neill LA (2000). The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for proinflammatory interleukins and microbial products. J. Leukoc. Biol. 67: 508-514.
- Cerenius L and Soderhall K (2004). The prophenoloxidase-activating system in invertebrates. *Immunol. Rev.* 198: 116-126.
- Flegel TW (1997). Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. *World J. Microbiol. Biotech.* 13: 433-442.
- Han-Ching WK, Tseng CW, Lin HY, Chen IT, et al. (2010). RNAi knock-down of the *Litopenaeus vannamei* Toll gene (LvToll) significantly increases mortality and reduces bacterial clearance after challenge with *Vibrio harveyi. Dev. Comp. Immunol.* 34: 49-58.
- Jiravanichpaisal P, Lee BL and Soderhall K (2006). Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology* 211: 213-236.
- Lee SY and Soderhall K (2002). Early events in crustacean innate immunity. Fish Shellfish Immunol. 12: 421-437.
- Lemaitre B and Hoffmann J (2007). The host defense of Drosophila melanogaster. Annu. Rev. Immunol. 25: 697-743.
- Letunic I, Doerks T and Bork P (2009). SMART 6: recent updates and new developments. *Nucleic Acids Res.* 37: D229-D232.
- Mekata T, Kono T, Yoshida T, Sakai M, et al. (2008). Identification of cDNA encoding Toll receptor, MjToll gene from kuruma shrimp, Marsupenaeus japonicus. Fish Shellfish Immunol. 24: 122-133.
- Tamura K, Dudley J, Nei M and Kumar S (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596-1599.
- Yang C, Zhang J, Li F, Ma H, et al. (2008). A Toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to *Vibrio anguillarum* infection. *Fish Shellfish Immunol*. 24: 564-574.
- Yang LS, Yin ZX, Liao JX, Huang XD, et al. (2007). A Toll receptor in shrimp. Mol. Immunol. 44: 1999-2008.

Genetics and Molecular Research 11 (1): 484-493 (2012)