RESEARCH ARTICLE

Domain combination of the vertebrate-like TLR gene family: implications for their origin and evolution

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Abstract

Domain shuffling, which is an important mechanism in the evolution of multi-domain proteins, has shaped the evolutionary development of the immune system in animals. Toll and Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate and adaptive immune systems. Draft genome sequences provide the opportunity to compare the Toll/TLR gene repertoire among representative metazoans. In this study, we investigated the combination of Toll/interleukin-1 receptor (TIR) and leucine-rich repeat (LRR) domains of metazoan Toll/TLRs. Before Toll with both domains occurred in Cnidaria (sea anemone, *Nematostella vectensis*), through domain combinations, TIR-only and LRR-only proteins had already appeared in sponges (*Amphimedon queenslandica*). Although vertebrate-like TIR (V-TIR) domain already appeared in Cnidaria, the vertebrate-like TLR (V-TLR) with both domains appeared much later. The first combination between V-TIR domain and vertebrate-like LRR (V-LRR) domain for V-TLR may have occurred after the divergence of Cnidaria and bilateria. Then, another combination for V-TLR, a recombination of both domains, possibly occurred before or during the evolution of primitive vertebrates. Taken together, two rounds of domain combinations may thus have co-shaped the vertebrate TLRs.

[Wu B., Huan T., Gong J., Zhou P. and Bai Z. 2011 Domain combination of the vertebrate-like TLR gene family: implications for their origin and evolution. *J. Genet*. **90,** 401–408]

Introduction

Innate immune responses protect organisms from harm caused by invading pathogens, and can also collaborate with the acquired immune responses (Pasare and Medzhitov 2004). A Toll gene that plays a key role in development and immunity was first discovered in the fruit fly (*Drosophila melanogaster*) (Lemaitre *et al.* 1996). One year later, the first mammalian Toll homologue, Toll-like receptor 4 (TLR4), was cloned from humans (Medzhitov *et al.* 1997). After which, a series of TLR genes (TLR1-TLR23) were identified from jawed vertebrates (Oshiumi *et al.* 2003; Ishii *et al.* 2007a,b; Yilmaz *et al.* 2005; Guo *et al.* 2009). From an evolutionary point of view, these TLRs are collected into six families: TLR2 family (including TLR1, 2, 6, 10, 14 and 15), TLR7 family (TLR7, 8 and 9), TLR11 family (TLR11, 12, 13, 16, 21, 22 and 23), TLR3 family (TLR3), TLR4 family (TLR4) and TLR5 family (TLR5) (Roach *et al.* 2005). Most of them play a significant role in immunity by recognizing pathogen associated molecular patterns (PAMPs), which are characteristics of microbial component structures (Takeda and Akira 2005). A typical TLR consists of a leucine-rich repeat (LRR) ectodomain for ligand recognition and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain for downstream signal transduction. Nevertheless, several kinds of soluble LRR-only TLRs were found in some animals (human, *Homo sapiens*; amphibian, *Xenopus tropicalis*; fugu fish, *Takifugu rubripes*; rainbow trout, *Onchorhynchus mikiss* and lamprey, *Petromyzon marinus*), which may modulate TLR-mediated signalling (LeBouder *et al.* 2003; Tsujita *et al.* 2004; Roach *et al.* 2005; Guo *et al.* 2009). As lamprey is one of the oldest extant primitive vertebrates (Chang *et al.* 2006; Gess *et al.* 2006), its TLRs may be taken to represent the primitive vertebrate TLRs. Sequence and RT-PCR analyses suggested that eight kinds of TLRs are present in the lamprey genome (TLR2/TLR14, TLR3, TLR5S, TLR7/8, TLR21/22) (Kasamatsu *et al.* 2010).

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Keywords. Toll-like receptors; TIR domain; LRR domain; domain combination.

There are two structural types of TLRs: the vertebrate-like TLRs (V-TLRs) and the protostome-like TLRs (P-Tolls). P-Tolls are mainly present in protostomes and possess an extra LRRCT-LRRNT motif in the LRR domains compared with V-TLRs (Hibino *et al.* 2006). V-TLRs refer to TLRs from some invertebrate and all vertebrate TLRs. The V-TLR/P-Toll genes have so far been intensively studied in vertebrates and some lower metazoans. However, the evolutionary pathways involved in the domain shuffling of TLR genes have not been thoroughly analysed or reported. The draft genome sequences of representative metazoans provide the opportunity to explore the issue. In this paper, we concentrated on the domain combinations that shaped the P-Tolls, V-TLRs and vertebrate TLRs. Our analyses suggest that at least two rounds of domain combinations contributed to the formation of vertebrate TLRs.

Materials and methods

Data extraction

The V-TLR/P-Toll protein sequences from Cnidaria, sponge (*Amphimedon queenslandica*), lamprey (*Petromyzon marinus*) and fugu fish (*Takifugu rubripes*) were taken from the previous reports (Oshiumi *et al.* 2003; Miller *et al.* 2007; Wiens *et al.* 2007; Guo *et al.* 2009; Sasaki *et al.* 2009; Kasamatsu *et al.* 2010). Insect Toll proteins were obtained from Entrez (http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi) using gene names as search keys. The Ambulacraria (sea urchin, *Strongylocentrotus purpuratus* and acorn worm, *Saccoglossus kowalevskii*) V-TLR/P-Toll proteins were obtained from the Baylor College of Medicine Human Genome Sequencing Center (BCM-HGSC) (http://www.spbase.org/SpBase/search) or via Entrez. V-TLR/P-Toll protein models of amphioxus (*Branchiostoma floridae*) were obtained from the JGI genome assembly (http://genome.jgi-psf.org/cgi-bin/searchGM?db=Brafl1) or via Entrez. In addition, we searched for V-TLR/P-Toll related protein models using domain and species names against the SUPERFAMILY database (http://supfam.cs.bris.ac.uk/SUPERFAMILY). The species searched in SUPERFAMILY included choanoflagellate *Monosiga brevicollis* (probable sister group of animals), placozoan (*Trichoplax adhaerens*), Cnidarians (sea anemone, *Nematostella vectensis* and hydra, *Hydra magnipapillata*), fruit fly (*Drosophila melanogaster*), nematode (*Caenorhabditis elegans*), mosquito (*Anopheles gambiae*), silk moth (*Bombyx mori*), gastropod snail (*Lottia gigantean*), sea urchin (*S. purpuratus*) and amphioxus (*Branchiostoma floridae*). The evolutionary positions of all species involved in this paper are illustrated in figure 1.

Phylogenetic analysis

Gene structures were extracted from databases (GenBank, Ensemble and JGI) or predicted by Genscan (http://genes.mit.edu/GENSCAN.html). Protein domains used for constructing phylogenetic trees were obtained from the SMART predictions (http://smart.embl-heidelberg.de) or

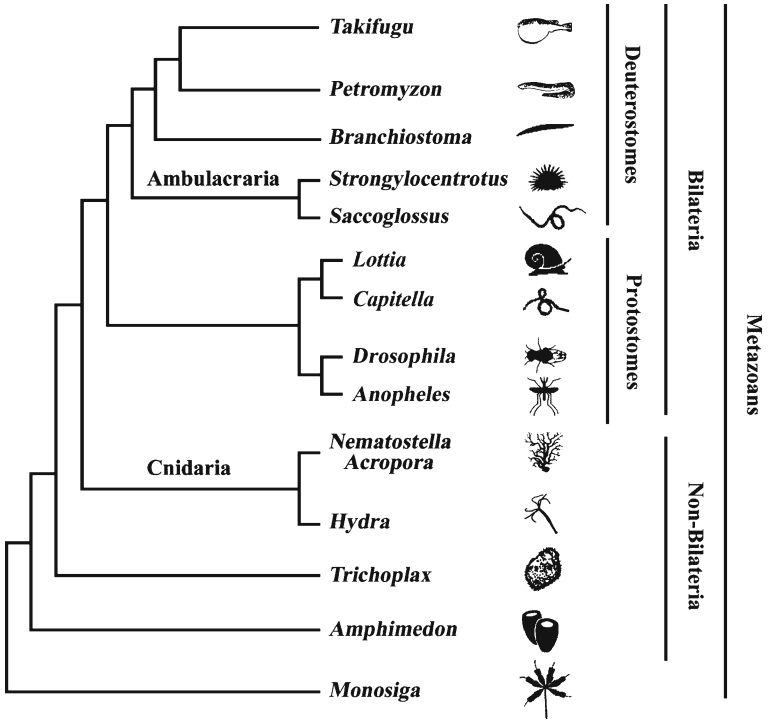

Figure 1. A reduced evolutionary tree of metazoans. The known genome sequences of the model organisms in this tree are used in this work.

Pfam predictions (http://pfam.sanger.ac.uk/search). Amino acid sequences of these proteins were aligned using ClustalX v 2.0 program with default parameters (Larkin *et al.* 2007). MEGA4.0 was used for phylogenetic analysis with the neighbour-joining (NJ) method with 1000 bootstrap values (Tamura *et al.* 2007). In addition, to further verify the reliability of the NJ trees, the maximum-likelihood (ML) analysis was performed using ProtTest v 2.4 (Abascal *et al.* 2005). The best-fit model considers the relative rates of amino acid replacement and the evolutionary constraints imposed by conservation of protein structure and function. The Akaike information criterion (AIC) was implemented in ProtTest to estimate the most appropriate model of amino acid substitution for tree building analysis. Then, according to the best-fit model predicted by ProtTest, unrooted ML trees were constructed with the PhyML v 3.0 online program (Guindon *et al.* 2005), and the reliability of interior branches was assessed with 100 bootstrap resamplings.

Nomenclature

Typical P-Toll or V-TLR consists of an extracellular LRR domain and an intracellular TIR domain. TIR-only and LRR-only proteins (including complete coding sequences) exist in invertebrates and are similar to the TIR and LRR domains of vertebrate TLRs. V-TIR*n* or V-LRR*n* indicates a protein with a TIR or an LRR domain that is similar to the TIR or LRR domain of vertebrate TLR*n* (*n* indicates the family number). Also, we used this denomination for TIR or LRR domains of canonical V-TLRs.

Results and discussion

TLR contains two separated domains, LRR domain and TIR domain, which are connected by a 20 amino acids long transmembrane helical stretch. Thus, to decipher the origin of V-TLRs, three questions about the evolutionary histories of both domains are to be addressed. First, when did V-TIR and V-LRR domains first appear? Second, when were V-TIR and V-LRR domains assembled together, which shaped the V-TLRs? Third, how is the appearance of vertebrate TLRs described by the evolutionary pathway?

P-Toll and V-TIR domains appeared in nonbilateria

Search results from SUPERFAMILY show that TIR-only proteins appeared as early as in *Monosiga brevicollis* (the closest known relative of metazoans) and *Trichoplax adhaerens* (lower metazoan). However, their sequences are not similar to the TIR domains of vertebrate TLRs because a BLASTP search for them against the NCBI protein database did not return a vertebrate TLR as hit. There is a TLR-like protein in *Amphimedon queenslandica* that possesses a single TIR domain (Wiens *et al.* 2007). However, we found that this TIR domain is distantly related to that of vertebrate TLRs. We further analysed the TIR-only proteins in Cnidaria, which are less primitive than *M. brevicollis*, *A. queenslandica* and *T. adhaerens*. Cnidarian TLRs provide two important evolutionary clues towards the domain combination of TLRs. First, there are several TIR-only proteins in Cnidaria, which are similar to the TIR domains of vertebrate TLRs. Our phylogenetic analyses of Cnidaria TIR-only proteins and vertebrate TLRs reveals that these TIR-only proteins belong to the vertebrate TLR family (figure 2). Here, we name these TIR-only proteins V-TIR. Although V-TIRs have already appeared in Cnidaria, the V-TLR with both domains has not been found. Second, P-Toll appears in the Cnidarian *N. vectensis* (Hemmrich *et al.* 2007; Miller *et al.* 2007), which suggests that a combination between protostome-like TIR (P-TIR) and protostome-like LRR (P-LRR) may have preceded the split between Cnidaria and bilateria. The phylogenetic tree of P-Tolls from 10 species

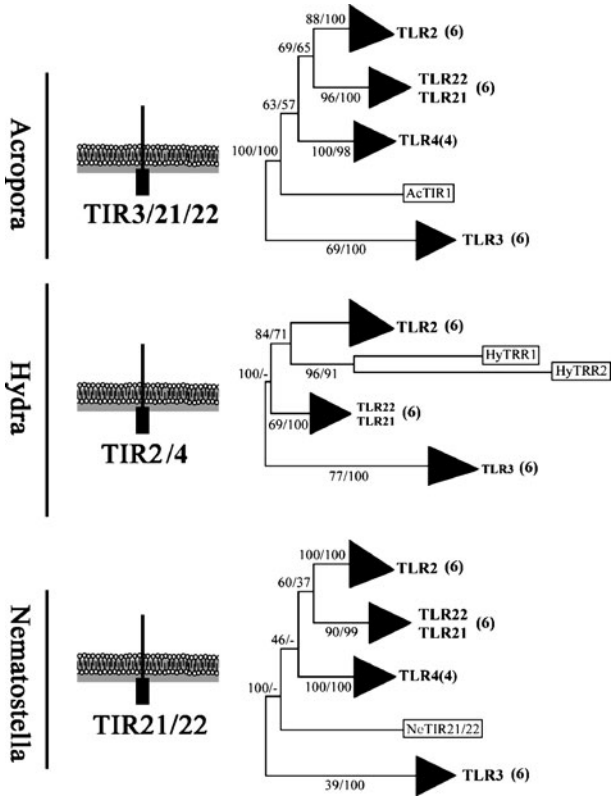

Figure 2. Homology and evolutionary analyses of Cnidaria TIR-only proteins. The black arrowheads stand for omitted sub-branches (sequences) that we do not concern, and the number in the parenthesis following the arrowhead indicates the number of sequences in this sub-branch. The bootstrap values of ML (former) and NJ (latter) analyses are both labelled. Ho, *Homo sapiens*; Mu, *Mus musculus*; Ga, *Gallus gallus*; Xe, *Xenopus tropicalis*; Da, *Danio rerio*; Ta, *Takifugu rubripes*; Ac, *Acropora millepora*; Hy, *Hydra magnipapillata*; Nv, *Nematostella vectensis*. The database accession IDs of these sequences are listed in table 2 of electronic supplementary material.

show that *N. vectensis* Toll shares a common ancestor with other Tolls (see figure 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). On the other hand, a number of LRR-only proteins from *M. brevicollis* and nonbilateria were also reported by SUPERFAMILY. However, due to a high diversity of LRR domains, we failed to recognize a V-LRR from them.

V-TLR appeared after the divergence of bilateria and nonbilateria

To address when V-TIR domain was combined with V-LRR domain and explore the origin of vertebrate TLRs, we investigated V-TLR protein sequences from the known genomes of three basal deuterostome invertebrates and five protostome mammals (species indicated in figure 1).

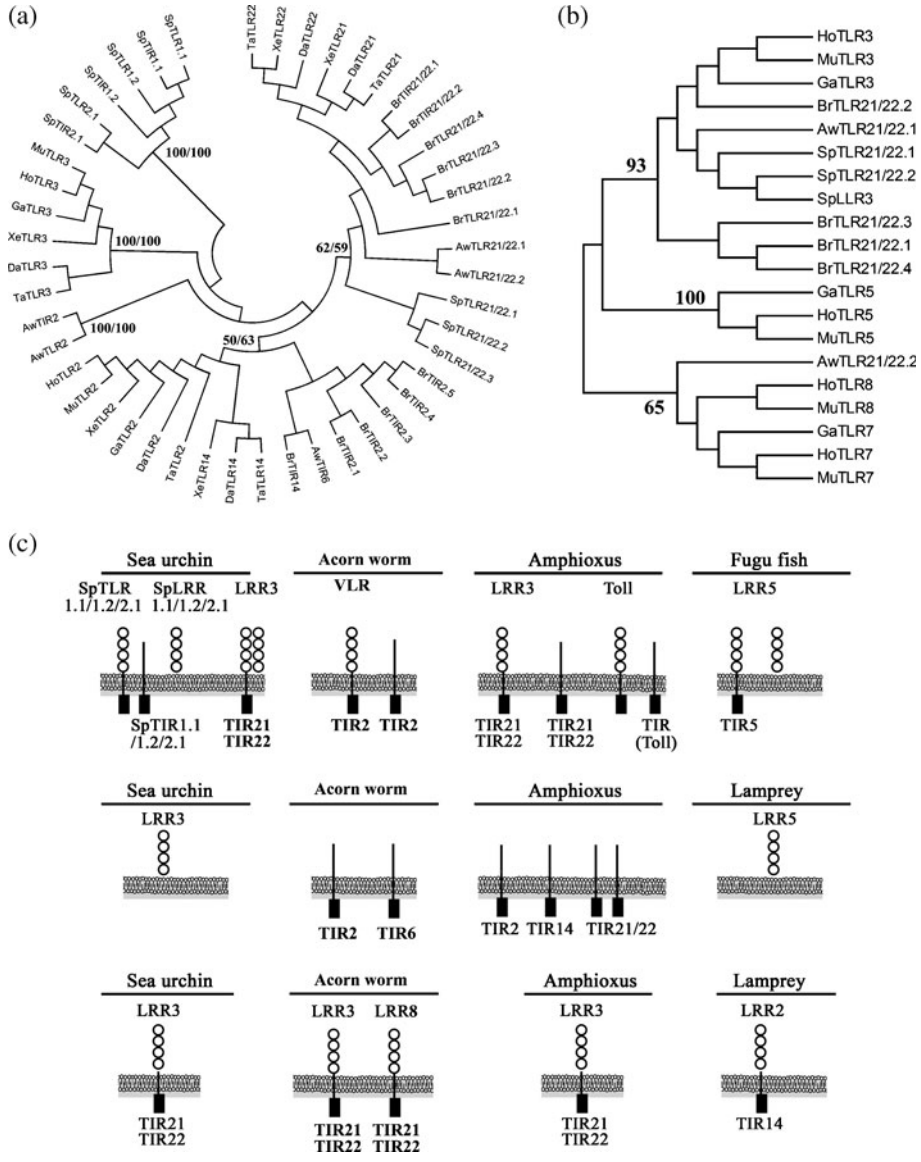

Figure 3. (a) Unrooted phylogenetic tree of deuterostome TLRs and a selected set of TIR*n*s. The evolutionary relationships were calculated based on amino acid sequences of cytoplasmic TIR domains. The bootstrap values of ML (former) and NJ (latter) analyses are both labelled. (b) Unrooted phylogenetic tree of deuterostome TLRs and a selected set of TIR*n*s. The relationships were calculated based on amino acid sequences of extracellular LRR domains. The bootstrap value of NJ (latter) analyses is labelled. All *Branchiostoma floridae* TLRs and TIR*n*s are labelled with Br; *Strongylocentrotus purpuratus* TLRs and TIR*n*s with Sp; *Saccoglossus kowalevskii* TLRs and TIR*n*s with Aw. Abbreviations are detailed in figure legend of figure 2. The database accession IDs of these sequences are listed in table 2 of electronic supplementary material. (c) Structural summary of V-TLRs, V-TIRs and V-LRRs from five representative deuterostomes. TIR*n*, LRR*n* and TLR*n* co-exist in specific species (upper row). Appearances of TIR2/14/21/22 and LRR3/5 were prior to typical vertebrate TLRs (middle row). In lower deuterostome animals, some TLRs resemble a recombination of vertebrate TIR and LRR domains. The possible recombination is denoted (lower row).

Recently, about 23 V-TLRs were found in protostomes *Capitella capitata* and *Helobdella robusta* (Davidson *et al.* 2008). Here, we also found two V-TLRs in *Lottia gigantean* (see figure 2 in electronic supplementary material). These observations suggest that V-TLRs widely exist in lophotrochozoa, a branch of protostomes.

Most *S. purpuratus* V-TLRs (SpTLRs) can be divided into two groups: SpTLR1 (SpTLR1.1 and SpTLR1.2) and SpTLR2 (SpTLR2.1) (Hibino *et al.* 2006). In the phylogenetic tree of deuterostome TLRs (figure 3a), the SpTLR1 and SpTLR2 constitute a sister group adjacent to vertebrate TLRs. Here, we found another three SpTLRs from BCM-HGSC. In contrast to SpTLR1 and SpTLR2 proteins, these three SpTLRs occur deeply in the vertebrate branch of the tree, suggesting that they are more closely related to the vertebrate TLR family than to SpTLR1 or SpTLR2 (figure 3a and figure 3a in electronic supplementary material). In the genomic trace archive of the hemichordate *Saccoglossus kowalevskii*, we found either P-Tolls or V-TLRs (table 1 and see table 1 in electronic supplementary material). A BLASTP search within *S. kowalevskii* genome against the NCBI protein database returned four potential V-TLRs (table 1).

The published *Branchiostoma floridae* genome shows that although it has 36 V-TLRs, no ortholog to vertebrate TLR genes have been identified (Huang *et al.* 2008). We obtained 20 V-TLRs from SUPERFAMILY, and then constructed a phylogenetic tree for them together with a selected set of vertebrate TLR3 and TLR11 family members (figure 3b in electronic supplementary material). The tree reveals that several *B. floridae* V-TLRs are more similar to vertebrate TLRs than to the other *B. floridae* V-TLRs.

The above investigations of TIR domains and LRR domains in basal deuterostome and protostome genomes provide two important clues: i) the combination between V-TIR and V-LRR domains for V-TLR took place after the divergence of bilateria and nonbilateria; ii) V-TLR and P-Toll widely spread in basal deuterostomes and protostomes, while V-TLRs were lost in some deuterostome animals.

Origin of vertebrate TLRs

Phylogenetic analyses on TIR-only and LRR-only proteins from deuterostomes: There are a large number of *S. purpuratus* TIR-only proteins and LRR-only proteins (TIR1.1/LRR1.1, TIR1.2/LRR1.2 and TIR2.1/LRR2.1) present in SUPERFAMILY, which are similar to TIR and LRR domains of SpTLR1.1/1.2/2.1 (see table 1 in electronic supplementary material). In the phylogenetic tree, these TIR-only proteins are clustered with TIR domains of SpTLR1.1/1.2/2.1 (figure 3a). TIR-only proteins were also found in *Saccoglossus kowalevski*, which are similar to TIR domains of the vertebrate TLR2 and TLR6 (see table 1 in electronic supplementary material). The phylogenetic tree shows that these TIR-only proteins are grouped with vertebrate TLR2 family (figure 3a and see table 1 in electronic supplementary material).

Further, evolutionary analyses were performed on *B. floridae* TIR-only proteins. Unexpectedly, we found V-TIR2, V-TIR14 and V-TIR21/22 in the amphioxus genome (see table 1 in electronic supplementary material). From our phylogenetic tree based on TIR domains, we found that *B. floridae* TIR2/14 and 22 form a stable paraphyletic relationship with the vertebrate TLR2 family and the TLR21/22 lineage, respectively (figure 3a).

Due to the irregularity of LRR domain sequences, it is difficult to determine LRR*n* from basal deuterostome

Table 1. The overall sequence information of V-TLRs from three representative invertebrate deuterostomes.

Species	V-TLR genes	Most similar vertebrate TLR (LRR domain)	Most similar vertebrate TLR (TIR domain)	Number of exons
Strongylocentrotus purpuratus NCBI/SP base	TLR1.1	GaTLR3	PoTLR3	1
	TLR1.2	TgTLR3	DaTLR20	1
	SPU_018838	GaTLR3	DaTLR22	3
	SPU_005830	GaTLR3	DaTLR21	2
	SPU_005832	GaTLR3	DaTLR22	2
Saccoglossus kowalevskii NCBI	XP_002733943.1	LaVLR	XeTLR2	2
	XP_002741806.1	LaVLR	XeTLR2	2
	XP_002732314.1	GaTLR3	FuTLR21	1
	XP_002732817.1	SsTLR8	FuTLR21	1
Branchiostoma floridae JGI database	68489	GaTLR3	DaTLR22	14
	82252	GaTLR3	DaTLR21	6
	82677	DaTLR3	DaTLR22	4
	88496	GaTLR3	DaTLR22	1
	88657	GaTLR3	DaTLR22	9
	97448	GaTLR3	DaTLR21	5

Da, *Danio rerio*; Ga, *Gallus gallus*; La, *Petromyzon marinus*; Po, *Paralichthys olivaceus*; Ss, *Salmo salar*; Fu, *Takifugu rubripes*; Tg, *Taeniopygia guttata*; Xe, *Xenopus laevis*; VLR, variable lymphocyte receptor.

animals. The only evident LRR*n*, an LRR3, which is highly similar to the LRR domain of vertebrate TLR3 (e.g., E-value 8e-77 to *Gallus gallus* TLR3), was found in *S. purpuratus* (SpLRR3 in figure 3b). Most LRR*n*s annotated by NCBI or JGI in *S. purpuratus* and *B. floridae* genomes show weak similarities to LRR domains of vertebrate TLRs (see table 1 in electronic supplementary material). However, *Petromyzon marinus* TLR5s and *Takifugu rubripes* TLR5s are typical LRR*n* proteins (Guo *et al.* 2009; Kasamatsu *et al.* 2010; Tsukada *et al.* 2005), which confirmed the existence of V-LRR and LRR-only proteins.

Taken together, two assumptions can be drawn from the analyses of basal deuterostome TIR-only and LRR-only proteins: i) V-TIR-only proteins in basal deuterostome animals coexisted with their corresponding V-TLRs (figure 3c, upper row); ii) V-TIR-only proteins or V-LRR-only proteins in basal deuterostome appeared earlier than the typical vertebrate TLRs (figure 3c, middle row), and they were 'awaiting' their partners. These data strongly support that the process of domains combination has been working from Ambulacraria to Agnatha.

Phylogenetic analysis of V-TLR genes: Phylogenetic analyses were also performed on basal deuterostome V-TLRs based on their TIR domains and LRR domains, respectively (figure 3, a&b). TIR domains of basal deuterostome V-TLRs form a stable paraphyletic relationship with corresponding TIR domains of vertebrate TLRs (figure 3a). Due to the irregularity of LRR domains, the topology of the NJ tree and ML tree of LRR domains is slightly different. Several LRR domains of basal deuterostome V-TLRs could not be clustered with the corresponding LRR domains of vertebrate TLRs in the ML tree (see figure 4 in electronic supplementary material); whereas in NJ tree these LRR domains were clustered with the corresponding vertebrate LRR domains (figure 3b). Compared with the primitive vertebrate *Petromyzon marinus* TLRs (TLR2, 3, 7/8, 14, 21 and 22), whose TIR and LRR domains are in proper corresponding relationship (e.g., TIR3 with LRR3), V-TLRs from basal deuterostome animals were composed through 'mismatch' of their V-TIR and V-LRR domains (figure 3c), because there were no TIR and LRR pairs in proper corresponding relationship. After the corresponding TIR and LRR pairs had appeared, the vertebrate TLRs would be constructed. On the other hand, the 'mismatched' V-TLRs might be lost by vertebrates or disassembled (fission) to recombine vertebrate TLRs.

Few introns are present in *S. purpuratus* V-TLR and TLR genes of higher vertebrates (Hibino *et al.* 2006; Ishii *et al.* 2007a). Nevertheless, the three SpTLRs located in the vertebrate branch of the tree possess multiple exons (figure 3a; table 1). Most *B. floridae* V-TLRs, that are clustered with the vertebrate TLR11 family (figure 3a), also contain varying numbers of introns in their coding regions (table 1). The multiple exons might have provided opportunities for fission and fusion of V-TIR and V-LRR domains.

Conclusion and perspective

Based on our results, we propose a hypothesis that describes the potential evolutionary pathways of P-Toll and V-TLR (figure 4). The combination between P-TIR and P-LRR domains occurred in nonbilateria, while the early combination between V-TIR and V-LRR domains for V-TLR took place after the divergence of bilateria and nonbilateria. Moreover, another combination for V-TLR, possibly a 'recombination' of both domains, occurred before or during the

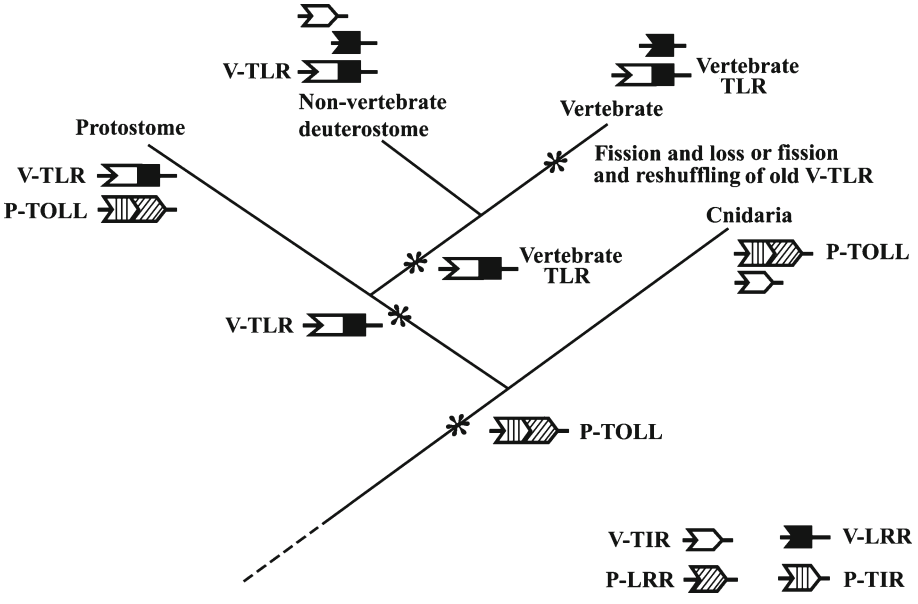

Figure 4. Hypothetical evolutionary pathways of P-Toll and V-TLR.

evolution of primitive vertebrates. These two rounds of domain combinations coshaped the vertebrate TLRs (figure 4).

Previous research suggested that complicated domain rearrangements appeared in the innate immune network during the evolution of the cephalochordate, amphioxus (Zhang *et al.* 2008). Yuan *et al.* (2009) constructed a chimeric TLR, which is a hybrid of amphioxus LRR1 and human TIR2 (Yuan *et al.* 2009). Although the chimeric TLR could significantly activate an NF-κB response, it could not directly respond to various PAMPs. This lack of response may be due to two reasons. First, the hybrid of amphioxus LRR1 and human TIR2 was heterologously expressed in human culture cells instead of the native environment. Second, a TLR molecule whose LRR and TIR domains are from different TLR families inherently could not cooperate in function (the amphioxus LRR1 does not belong to the TLR2 family). By contrast, a combination from the same TLR family or from TLR families with similar functions may retrieve the activity, e.g., the V-LRR3-TIR22 of amphioxus (JGI 68489) and the V-LRR2-TIR14 of lamprey (contig 7641.4). If the latter explanation is true, we speculate that the combination of TIR and LRR domains is not random but follows certain rules, that might be selective pressure exerted by pathogens in specific environment.

Acknowledgements

We thank the JGI, BCM-HGSC and the SUPERFAMILY databases for genome or protein data. We also thank Hong Yu and Dr Tiandi Wei for discussion and art work. This work was supported by the Chinese National '863' Project under grant no. 2008AA092603.

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Received 21 December 2010, in final revised form 17 May 2011; accepted 20 May 2011
Published on the Web: 9 November 2011