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Author(s)	Kasamatsu, Jun; Oshiumi, Hiroyuki; Matsumoto, Misako; Kasahara, Masanori; Seya, Tsukasa
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Phylogenetic and expression analysis of Lamprey Toll-like receptors

Jun Kasamatsu, Hiroyuki Oshiumi, Misako Matsumoto, Masanori Kasahara, Tsukasa Seya*

Jun Kasamatsu, Hiroyuki Oshiumi, Misako Matsumoto, Tsukasa Seya*

Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8638 Japan. e-mail: seya-tu@pop.med.hokudai.ac.jp, Tel.:+81-11-7065073, FAX.: +81-11-7067866,

Masanori Kasahara

Department of Molecular Pathology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8638 Japan.

Running title: **Lamprey TLRs**

Abstract

Toll-like receptors (TLRs) have been identified as pivotal sensors recognizing microbial pattern molecules in vertebrates. Whole genome analysis of the teleost *Takifugu rubripes* supports the existence of a fundamental family of *TLR* genes in fish. However, the role of the innate immune system in the context of raising acquired immunity in jawless fish remains unclear. In this study, we annotated 16 lamprey *TLR* genes predicted from the latest genome assembly of lamprey on the basis of homology, and identified their cDNAs from Japanese lamprey, *Lethenteron japonicum*. Phylogenetic analyses indicated that the repertoire of lamprey TLRs consisted of both fish (F)- and mammalian (M)-type TLRs, and it was also demonstrated that lamprey TLRs are constitutively expressed in various organs. Our results suggest that lampreys protect against microorganisms using the innate system consisting of a similar set of M- and F-type TLRs, despite possessing a unique acquired immune system. In addition, *type I interferon (IFN), interferon-regulatory factor (IRF)-3,* and *IRF7* were not identified in the lamprey genome although TLR adaptor and signal transduction genes were highly conserved upstream of (IRF)-3/7 and type I IFN in most vertebrates. This is the first report to describe the TLR repertoire and IFN system in one of the most primitive vertebrates, the lamprey.

Key Words

Toll-like receptor, lamprey, Evolution, Phylogeny, Interferon, Innate immunity

Introduction

Many pathogens possess their own genomes that can change the nature of the host, while vertebrates defend against microbial invasion using sophisticated microbial recognition systems. The representative recognition systems for signaling the presence of invading pathogens are known as innate and acquired immunity. In jawed vertebrates, including mammals, these systems have been characterized at the molecular level, with details of the acquired immune system having first emerged from ancient cartilaginous fish [1-2]. The acquired immune system is characterized by the actions of highly variable peptide-antigen receptors, such as immunoglobulins and T-cell receptors. Recent progress on the study of the innate immune system, involving dendritic cells (DCs) and pattern-recognition receptors (PRRs), has revealed that its activation precedes the acquired immune system. PRRs are now a focus of study due to recent elucidation of the ligand properties of Toll-like receptors (TLRs) and TLR-mediated DC maturation. Accumulating evidence regarding TLR-mediated DC maturation has helped us solidify the current understanding that TLRs facilitate driving effector cells by maturation of antigen (Ag)-presenting DCs. Accordingly, Ags determine the object toward which immune cells are proliferated whereas microbial patterns determine which effectors are selected for immunological output [3].

Recent findings have indicated that lamprey possesses a unique acquired immune system involving variable lymphocyte receptors (VLRs) and the lymphoid system [4]. In agnathans, which includes lamprey and hagfish, clonally diversed receptors are generated by the assembly of genes for VLRs, which are comprised of leucine-rich repeat (LRR) subunits similar to TLRs. The variable VLR gene products consist of soluble forms and GPI-anchored membrane forms similar to antibodies and antigen-receptors [4]. However, no relationship between lamprey TLRs and VLR-based acquired system has yet been reported.

TLRs with similar host-defense functions to drosophila Toll protein [5] have been identified in mammals [6]. TLR is a type-1 membrane protein consisting of an LRR extracellular domain, a transmembrane domain, and a C-terminal Toll/IL-1 receptor homology domain (TIR).

TLRs recognize pathogen-associated molecular patterns (PAMPs), which are characteristic of microbial structures, and induce anti-microbial responses [7]. In the human genome, 10 TLR members have been indentified and their functions determined from analyses of TLR-deficient mice [7]: the TLR2 subfamily recognizes bacterial cell wall peptidoglycan and acylated lipopeptides, TLR4 recognizes Gram-negative bacterial lipopolysaccharides, TLR5 recognizes bacterial flagellin, while TLR3, 7, 8, and 9 recognize microbial nucleic acids.

Mouse TLR stimulation results in signal through one of five adaptor proteins (MyD88, TIRAP/MAL, TICAM-1, TICAM-2 and SARM) [8]. All TLRs with the exception of TLR3, can signal through the adaptor molecule MyD88. MyD88 recruits members of the interleukin-1 receptor associated kinase (IRAK) family which in turn activate the key ubiquitin E3 ligase, tumour necrosis factor receptor-associated factors (TRAFs) and transforming growth factor-β (TGF-β)-activating kinase (TAK1), leading to the activation of the transcription factor NF-κB [7,8]. The activated NF-κB is translocated to the nucleus and induces expression of inflammatory cytokine genes such as IL-1β and IL-6. In contrast, TLR3 recruits the adaptor molecule TICAM-1, leading to activation of interferon (IFN) regulatory factor-3 (IRF-3) and induces an anti-viral response through expression of type I IFN and IFN-inducible genes [8,9]. Although the MyD88 pathway is conserved in a wide range of vertebrates, whether or not the TICAM-1 pathway is conserved in lower vertebrates is still controversial [9].

Advances in whole genome sequencing and annotation have enabled the identification of TLRs from several vertebrates, including osteichthyes fish (fugu) [10], amphibian, (frog) [11], bird (chicken) [12] and mammals (human and mouse). Comparison of the TLR families across the vertebrate species has revealed that water-living vertebrates possess TLR14, 21 and 22, in addition to the mammalian-type TLRs [13,14]. Our earlier studies speculate that the TLR repertoire was established during evolution in a species-specific manner in jawed vertebrates depending upon their living environment, such as water or land [10,11,15]. On the other hand, some invertebrates, the sea urchin and amphioxus, possess a large number of TLRs [16-19], while *Ciona intestinalis* has only 2 functional TLRs [32] from their genome projects. Thus, what

happens in the TLR system lamprey is of interest in tems of the evolution.

Our previous study identified two lamprey TLRs, named laTLR14a and 14b, by PCR-based cloning using sequences of TLR2 from various animals [21]. However, no conclusive identification of TLRs in the lamprey genome had been made until recently. Here, we surveyed the amino acid sequences of predicted TIR-containing proteins from the latest *Petromyzon marinus* genome database (Pre-Ensemble Lamprey Genome Browser) and NCBI trace archive, and the predicted protein sequences of the lamprey TLRs and their respective TIR domains were subjected to comparative analyses using the NCBI non-redundant protein database and BLASTP search. Ultimately, we determined the repertoire of predicted lamprey TLRs in this report.

Materials and methods

Identification of TLR and innate immune genes in the *Petromyzon marinus* genome.

Sea lamprey (*P. marinus*) EST sequences were retrieved from the NCBI trace archive (http://www.ncbi.nlm.nih.gov/Traces/trace.cgi) and a private database was constructed using the GENETYX-PDB program package (version 5, GENETYX Corporation). This database, and the EST database from NCBI, was searched with the TBLASTN program using putative amino acid sequences of lamprey TLRs and innate immunity genes from previously annotated human and fugu TLRs [10]. The genes that were not found by these analyses were searched with the TBLASTN program using the Pre Ensembl genome browser (http://pre.ensembl.org/Petromyzon_marinus/Info/Index). Domain structures of the *P. marinus* TLR (pmTLR) proteins were analyzed by the SMART program (http://smart.embl-heidelberg.de). An unrooted phylogenetic tree based on the amino acid sequences was constructed by the Neighbor-joining (NJ) method in the ClustalX version 2 program [22] and the MEGA version 4 program [23]. The distance matrix was obtained by calculating p-distances for all pairs of sequences. Sites containing gaps were excluded from the analysis using the pairwise deletion option. The reliability of branching patterns was assessed by

bootstrap analysis (1000 replications). The accession numbers of the sequences used for gene-searching and phylogenetic analysis are listed in Table 1.

Expression analysis of TLRs and their adaptor genes in *Lethenteron japonicum*

An adult Japanese lamprey (*L. japonicum*) was used to analyze the differential expression of *L. japonicum* TLR (LjTLR) mRNAs in various tissues. This is because 1. we have no sea lamprey (*P. marinus*) in Japan, and 2. *L. japonicum* belongs to Petromyzoninae, the same family as *P. marinus*. Although we have no genome database of *L. japonicum*, it is expected that the genome of *L. japonicum* has highly homologous to that of *P. marinus*. For the analysis, 100 mg of each frozen tissue was homogenized and total RNA was extracted using an RNeasy mini kit (Qiagen). One μg of total RNA was treated with RQ1 RNase-free DNase (Promega) and reverse-transcribed with M-MLV RTase (Promega) using random primers. For amplification of TLR and innate immunity gene cDNA fragments, PCR reactions were typically performed by denaturation at 94°C for 2 min followed by 30-45 cycles of 94°C for 30 sec, 50-60°C for 30 sec, and extension at 72°C for 30 sec using Ex-Taq polymerase (Takara) (each primer set and PCR condition are listed in Table 3). The size of the cDNA fragments were confirmed using electrophoresis with 3% agarose gels. All amplicons were cloned into vectors using the pGEM®-T Easy Vector System (Promega) or TOPO TA Cloning System (Invitrogen) and their sequences were determined using an automated sequencer.

Lamprey blood cell stimulation

Blood cells were separately collected from each individual of lampreys (*L. japonicum*) one day after arrival. Peripheral blood leukocytes (PBLs) were drawn from the severed tails of fish into PBS containing 30 mM EDTA. Buffy-coat leukocytes were collected by centrifugation for 30 min at 1500 rpm. The cells were incubated with polyI:C and heat killed *Escherichia coli* (*E. coli*) as indicated in Fig. 5 and allowed to stand for 3h or 6 h. Then, total RNA was extracted using an RNeasy mini kit (Qiagen). RT-PCR was performed as described above.

Results

Identification of TLRs in the Lamprey genome

A typical TLR consists of multiple LRRs at the N-terminus, a transmembrane domain, and a TIR domain in the C-terminus. The TIR domain is important for signal transduction and recruits adaptor molecules which also contain a TIR domain [7]. We attempted to identify genes encoding TLRs and their adaptors from the EST and genome databases of sea lamprey (Pre-Ensemble Lamprey Genome Browser) using human TLR1-10, TICAM-1, MyD88, TICAM-2, TIRAP, and SARM [8], mouse TLR12 and 13, and Fugu TLR21 and 22 [11] as query sequences. Ultimately, 20 genes predicted to encode typical TIR domains were identified (Table 2), of which 16 harbored multiple LRRs and were therefore defined as lamprey TLR proteins (pmTLRs) (Fig. 1). The other 4 proteins were defined as TLR adaptor-like proteins since they are similar to MyD88, TICAM or SARM.

Based on the Genscan ID and EST accession numbers, the contig positions and E-values for the 16 pmTLR proteins were determined, and each protein was annotated based on their most likely TLR homolog (Table 2). Sea lamprey pmTLR14a and pmTLR14b were most homologous to Japanese lamprey laTLR14a (ljTLR14a) and laTLR14b (ljTLR14b), respectively, which we previously identified [21]. An additional two genes, pmTLR14c and pmTLR14d, encoded paralogs of ljTLR14a and b, and were more homologous to zebrafish TLR18 than any other TLR family member. Additionally, we found four TLR2-like genes formed a unique cluster independent of the clade of the mammalian TLR2 (Fig. 2). Therefore, we designated these pmTLR genes TLR24a-d, which represent a novel TLR2 subfamily. TLR2c and d both localized to contig 1344, suggesting a cause of gene duplication, however, the presence of an ambiguous gap between the two genes deterred us from concluding that they originated from a tandem duplication. It is notable that the TLR7/8, TLR14, and TLR21 genes were mapped to distinct contigs. Although pmTLR2c appeared to be an ortholog of *Canis lupus* TLR6, its functional similarity to mammalian TLR6 could not be determined. Lamprey possessed

orthologous genes for TLR3, TLR5, TLR7, and TLR8 but not for TLR4 or TLR9. In addition to the M-type TLRs, three TLR21 and a single TLR22 orthologs classified as F-type TLRs were identified in the lamprey genome database. Taken together, the phylogenetic analyses revealed that sea lamprey has a TLR system comprised of an incomplete set of M-type and full F-type TLRs, as in fish (Fig. 1).

Although many animal TLR genes are predicted to be intronless [13], *Takifugu rubripes* TLR genes has been reported to contain introns and be dispersed over wide regions on a variety of chromosomes [10]. In the Pre-Ensemble Lamprey Genome database, lamprey TLR2d, 3, 14c, 22, and 24d contain several introns in coding regions, while TLR2a, 2b, 14a, 14b, 21a, 21b, 21c, 24a and 24b are intronless. As only partial sequences for TLR2c, 5, 7, 8a, 8b, 14d, and 24c were obtained from the genome database, it was impossible to conclusively determine if introns were present. Nevertheless, the results infer that lamprey TLRs are encoded by both intron-containing and intronless genes, as is the case in teleosts [10].

Using the SMART program, typical TLR structures of pmTLR proteins were predicted (Fig. 1). Almost all proteins consisted of multiple LRRs in the N-terminal region and a single TIR domain in the C-terminus, split by a transmembrane domain. Although we failed to detect the N- or C-terminal regions in several pmTLRs, their mRNAs, except for TLR24c, were detected by RT-PCR, suggesting that the mRNAs of these pmTLRs are present as complete forms but their signal peptides and N-terminal LRRs could not be detected in the SMART browser. The size and number of the LRRs in each pmTLR were similar to the TLR counterparts of *T. rubripes* (Fig. 1).

Phylogenetic analysis of TLRs

To examine the relationships between vertebrate (human, mouse, chicken, xenopus, zebrafish, fugu, and lamprey) TLRs, a phylogenetic tree was constructed based upon the TLR sequences using the Clustal X and MEGA programs (Fig. 2). Phylogenetic analyses, which included the cytoplasmic TIR and extracellular LRR-regions, were performed as described

previously [21]. The constructed tree revealed the presence of several TLR subfamilies in pmTLRs: single clades were formed with TLR2a-b, TLR14a-c, TLR7/8a-b, TLR21a-c, and TLR24a-b. It is possible that each subfamily diverged through gene duplications that occurred after lampreys separated from the ancestor of common vertebrates.

There appeared to be a distinct cluster of pmTLR24, which formed a distinct clade from TLR2 and TLR1, 6, 10 in jawed vertebrates and the TLR14 subfamily. It is likely that the lamprey TLR2 subfamily expanded in a lamprey-specific manner. TLR14d was identified as an ortholog of TLR14 of jawed vertebrates with a high bootstrap value, forming a clade with the teleost TLR14 subfamily. In contrast, pmTLR14a-c showed a high similarity to ljTLR14a/b as indicated by their bootstrap values, suggesting that lamprey has two types of TLR14 with different primary sequences.

Although both pmTLR7/8a and 7/8b were mapped to the TLR7/8 cluster of jawed vertebrates, they separated in lamprey species independently of the divergence of TLR7 and TLR8 in jawed vertebrates. Whether pmTLR7/8a and 7/8b recognize nucleic acid structures with different properties is presently unknown. The pmTLR21a-c genes were in the same cluster as the teleost TLR21 clade, while pmTLR22 was closest to the teleost TLR22 gene. There are only a single orthologs of TLR3 and TLR5 in lamprey (Fig. 2), which is consistent with a previous report on vertebrate TLR phylogenetic analysis [24]. An examination of the tree suggests a rationale that lamprey TLRs correspond to the jawed vertebrate TLR orthologs (Fig. 2). The loci of these genes did not suggest that gene duplications simply occurred in the region resulting in two tandem sets of pmTLR14 and pmTLR24; the splitting of tandem genes may have occurred during the long history of the lamprey. The phylogenetic analysis based on their amino acid sequences reinforced the differential clustering of these TLR clades, which was likely rooted in the ‘TLR big-bomb’ which occurred ~600 million years ago [11].

Phylogenetic analyses of lamprey TLR adaptors

Human and mouse TLRs recruit the TIR-containing adaptors, MyD88 and TICAM-1 [8].

For example, TLR2 recruits the complex adaptor TIRAP-MyD88, while TLR4 recruits two complex adaptors: TIRAP-MyD88 and TICAM-2-TICAM-1 [7,8]. The MyD88 pathway is dominant in mammals given that all TLRs, except for TLR3, bind MyD88. Only TLR3 and TLR4 link to the TICAM-1 pathway [25]. The present lamprey TLR adaptor analyses allowed us to identify two TICAM-1 homologs, pmTICAM-1a and pmTICAM-1b (Fig. 3A), which resemble zebrafish TICAM-1, lacking the TRAF6-binding motif [26]. The RHIM-like domain was conserved in pmTICAM-1b, as in zebrafish TICAM-1, but not in TICAM-1a. In the phylogenetic tree, pmTICAM-1a and b formed a clade with jawed vertebrates as well as mammalian TICAM-1 and 2 with high bootstrap values (Fig. 3B). In contrast, pmMyD88 and pmSARM belongs to their respective clades to form a single cluster with MyD88 and SARM of other jawed species with a high bootstrap support (Fig. 3B). From the observed amino acid identities between lamprey and jawed vertebrate TICAM genes, pmTICAM-1b was more similar to jawed vertebrate TICAM-1 than to TICAM-2, although pmTICAM-1a was nearly equidistant from all TICAM genes (Table 4). Therefore, these analyses indicate that pmTICAM-1b is the ortholog of jawed vertebrate TICAM-1 while pmTICAM-1a is either an ancestral or lamprey-specific TICAM gene.

Expression analysis of lamprey TLRs

RNA expression of pmTLRs in several lamprey tissues were analyzed by RT-PCR. cDNA libraries were constructed from the eye, brain, gill, intestine, kidney, liver, muscle, skin, heart, and peripheral blood leukocytes (PBLs) from adult *L. japonicum* tissues. Each TLR primer set, except for pmTLR14a and b whose sequences were reported earlier [21], was derived from the nucleotide sequences of pmTLRs (Table 3). Almost all *L. japonicum* TLR cDNAs were successfully amplified using the sea lamprey primers, demonstrating that the sea lamprey and Japanese lamprey share similar TLR sets with very high homologies (Fig. 4). However, we could not amplify the TLR24c gene using any of the generated primer sets, nor could pmTLR24c be amplified using genomic DNA as a template. Further TBLASTN analysis using

pmTLR24a as a query revealed that the N-terminal sequence of the TLR24c gene contained stop codon (data not shown), suggesting that ljTLR24c may in fact be a pseudogene, formed during the speciation of *P. marinus* and *L. japonicum*.

The tissue distribution analysis indicated that every TLR mRNA was detected in each organ subjected to RT-PCR analyses (Fig 4), although the level of expression differed among individual organs. All amplicons were sequenced and compared with sea lamprey TLR sequences by BLASTN analysis which revealed their partial nucleotide sequences were reasonably aligned with those predicted from the sea lamprey TLR genome (data not shown). Most lamprey TLR genes tended to be highly expressed in the gill, kidney, and PBLs. Since these organs were rich in phagocytes and lymphocyte-like cells, TLRs may be dominantly expressed in the myeloid cells. Similarly, pmTICAM-1 adaptors were also expressed in the gill, kidney, and PBLs. Interestingly, pmTICAM-1a was predominantly expressed in the gill and PBLs, whereas pmTICAM-1b with the RHIM domain, was predominant in the kidney.

The ljTLR mRNA levels in PBLs were up- or down-regulated in response to polyI:C and heat-killed *E. coli*, which contain PAMPs (Fig. 5). ljTLR24a appeared to be transiently down-regulated while other ljTLRs were up-regulated 6 h after polyI:C stimulation. In contrast, *E. coli* stimulation tended to down-regulate mRNA levels of ljTLR3, 7/8b, 14b, and 21a. Thus, not only ljTLR3 but also ljTLR7/8a/b, 21a, 24b/c may be polyI:C-inducible genes and gene expression of most ljTLRs are controlled by exogenous microbial stimuli in lamprey.

Discussion

In this study, we annotated TLRs in the *P. marinus* genome using the latest assembled version of the Ensemble Lamprey Genome Browser database (released on Aug. 2008), and determined their expression profiles by RT-PCR analyses within various organs in adult lampreys of *L. japonicum*. The overall features of the lamprey TLR system appear to resemble those of teleosts living in water in that they commonly express incomplete M-type TLRs and have more sophisticated F-type TLRs than land animals. The levels of ljTLRs in

lamprey PBLs are regulated by PAMP stimuli as observed in mouse macrophages.

The prominent characteristics of the lamprey TLR system as compared to the teleost TLR system, are outlined below. We found three types of TLRs from the TLR2 subfamily in the lamprey, which correspond to TLR24 (pmTLR2a-d), TLR14 (pmTLR14a-c), and the ortholog of jawed vertebrate TLR14 (TLR14d), forming clearly distinct clusters in the phylogenetic tree. The TLR2 subfamily consists of multiple members displaying wide variability across animal species [27]. Our past studies have shown that members of the chicken and amphibian TLR2 subfamily arose by lineage-specific duplication events [11,15]. In combination with TLR2, human and mouse TLR1 and TLR6 facilitate the discrimination between triacylated and diacylated bacterial lipoproteins, respectively[7]. In contrast, chicken TLR2 proteins (chTLR1-1,2 and chTLR2-1,2) recognize bacterial lipoproteins and peptidoglycan in a different manner than human and mice [15]. These studies suggest that divergence of the TLR2 subfamily seems to have developed by gene duplication events and have allowed animals to specifically cope with pathogens sharing living environment with them.

Similarly, we also identified two TLR7/8 and three TLR21 genes in the lamprey genome. TLR3, 7, and 8, and teleost TLR22 recognize foreign RNA, while TLR9 and chicken TLR21 recognize unmethylated CpG DNAs [28,29]. Therefore, our analysis indicates that lamprey has developed not only the TLR2 subfamily, but also other TLR proteins which recognize nucleic acids. This then begs the question: what was the original vertebrate TLR system? Table 5 shows a summary of the TLR repertoire present in deuterostomes. The TLR2 subfamily, TLR3, TLR5, TLR7/8, and TLR21/22 are essentially conserved in the lamprey and teleosts, suggesting that lampreys and jawed vertebrates conform to the same TLR family with hybrid M- and F-type TLRs, which may represent the origin of the TLR repertoire in vertebrates.

The vertebrate TLR phylogenetic tree in Fig. 2 appears as a star-like tree [13], and such a phylogram shape suggests that each TLR family was generated at the same time,

which we termed the "TLR big-bomb". While the sea urchin and amphioxus possess approximately 200 [16,17] and 50 TLRs [18,19] , respectively, *Ciona intestinalis* has only 2 functional TLRs [20] identified from their genomes. In phylogenetic analyses of these studies, invertebrate TLRs did not clearly belong to vertebrate M- and F-type TLRs [16,18-20]. Therefore, M- and F-type TLRs may have arisen together with the origin of vertebrates. Interestingly, despite having only two TLRs, *C. intestinalis* can recognize a broad spectrum of PAMPs [20], indicating these unique invertebrate TLRs may have been an alternative origin of mammalian TLRs. In other words, vertebrate TLR proteins did not always recognize just one specific PAMP, but were able to recognize several PAMPs using a single type of M-type TLR [20]. The gain or loss of these TLRs may have occurred during the evolution of vertebrates, although it is also possible that differential environmental factors promoted TLR divergence for animals to survive against microbial milieu, which although interesting to speculate, is beyond the scope of this analysis.

Our database analyses failed to identify orthologs of TLR4, 9 and 15, nor the TLR4-related genes, CD14, MD-2, and TIRAP in the Pre-Ensemble Lamprey Genome Browser database (Table 2). Absence of orthologs of CD14, MD-2 and TIRAP in lamprey may reflect the lack of a TLR4 system in lamprey as well as in teleosts and amphibia [11]. It has long been established that teleosts are resistant to toxic effect of LPS. Recent study shows that zebrafish TLR4 acts as a negative regulator for NF-κB-signaling pathway, not for recognition of LPS [30]. Hence, these observations indicate that first the TLR4 gene arose in jawed vertebrates as a negative regulator of NF-κB-signaling pathway, and then it may have gained the function of LPS recognition with CD14 and MD-2 in an ancestor of amniota.

Additionally, we identified two TICAM genes, named TICAM-1a and b, in the lamprey genome. A previous study indicated that mammalian TICAM-1 and 2 genes resulted from two rounds of genome duplication, termed the 2R hypothesis, in early vertebrate evolution [26]. In this hypothesis, one of the ancestral TICAM genes was lost after the first round of genome duplication, while after the second genome duplication, the TICAM-1 and 2 genes

appeared. Although the timing of the genome duplications is still unclear [24,31], preliminary analyses of *HOX* gene clusters indicates the first round of genome duplication occurred in a common ancestor of jawed and jawless vertebrates [32]. In our analysis, pmTICAM-1b was identified as the ortholog of jawed vertebrate TICAM-1, whereas pmTICAM-1a did not show clear similarity to either TICAM-1 or TICAM-2. It is possible that pmTICAM-1a represents an ancestral gene of TICAM-2 that occurred during the first genome duplication event without gene loss. There are many TLRs binding to TICAM-1 in teleosts [28,33], and TICAM-1a and b might have diverged due to the necessity for differential signaling of TICAM-associated TLRs in the lamprey.

Although the orthologs of nucleic acid-recognizing TLRs were predicted in the genome and confirmed by RT-PCR, no genes related to type I IFN induction except for the antiviral Mx gene were identified in the lamprey. While RIG-I/IPS-1, TBK1/IKKε and IRF-2, 4, 5, and 8 are present, genes corresponding to IRF-1, IRF-3, IRF-7, and type I IFN are not conserved in the lamprey genome (Table 2). The presence of the IFN-inducing pathway in lamprey is controversial as whole genome sequencing projects in ectoderms and primitive chordates, including sea urchin, amphioxus, and ascidian, have revealed the lack of type I IFN and its essential transcription factors, IRF-3 and IRF-7 (Table 5) [16,18-20]. Therefore, IFN-inducing pathway would be completed in a common ancestor of jawed vertebrates. Type I IFN plays a critical role in facilitating TCR-MHC-mediated lymphocyte activation of the acquired immune response in mammals. Although lamprey has many orthologs of jawed vertebrate TLRs and TLR-associated signaling molecules, it does not have MHC or authentic TCR/BCR.

Recent findings have indicated that lamprey possesses a unique acquired immune system involving variable lymphocyte receptors (VLRs) and the lymphoid system [4,34-36]. In agnathans, which includes lamprey and hagfish, clonally diversed receptors are generated by the assembly of genes for VLRA and VLRB, which are comprised of leucine-rich repeat (LRR) subunits and an invariant membrane-proximal stalk region [4,37]. Interestingly, the

variable VLRA and VLRB products consist of a number of LRR motifs representing soluble forms and membrane-bound forms, respectively [38]. Hence, the VLR-expressing lymphocytes are similar to authentic B and T cells in jawed vertebrates. Our study indicates that VLR-based acquired immunity may be regulated by TLRs with IFN-independent systems in jawless vertebrates. Functional analyses of lamprey TLRs need to be conducted in the future.

We can speculate from the current results that both water and land vertebrates possess common TLR proteins that may participate in the recognition of common PAMPs [39]. Indeed, lamprey also expresses a set of TLRs as in fish despite their evolutionary separation approximately 500 million years ago. The present results allow us to surmise that the TLR orthologs in lamprey and mammals recognize common PAMPs for host defense. The TLR system may serve to protect lampreys from infections despite the fact that their acquired immune system is modally different.

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Figure legends

Fig. 1. Structures of the predicted *Petromyzon marinus* TLR proteins. Domains in the protein were predicted by the SMART program. LRRNTLRR, LRRCT transmembrane region and TIR domains are indicated in the picture. Left; lamprey TLRs. Right; fugu (*T. rubripes*) TLRs.

Fig. 2. Unrooted phylogenetic tree of vertebrate TLRs. The relationships were calculated on the basis of amino acid sequences of cytoplasmic TIR and extracellular LRR-regions. Bootstrap values (>60) are indicated. Hs; human (*Homo sapiens*), Mm; mouse (*Mus musculus*), Gg; chicken (*Gallus gallus*), Xt; frog (*Xenopus tropicalis*), Dr; zebrafish (*Danio rerio*), Tr; fugu (*Takifugu rubripes*), Pm; Sea lamprey (*Petromyzon marinus*), Lj; Japanese lamprey (*Lethenteron japonicum*).

Fig. 3. (A) Alignment of vertebrate TICAM-1 sequences. An asterisk indicates the TRAF6 binding motif. Black shaded area, 100% identity; gray shaded area, 80~99% identity; light gray shaded area, 60~79% identity. (B) Unrooted phylogenetic tree of TIR-containing adaptors in vertebrates. The relationships were calculated on the basis of amino acid sequences of TIR domains. Bootstrap values (>60) are indicated. Hs; human (*Homo sapiens*), Mm; mouse (*Mus musculus*), Gg; chicken (*Gallus gallus*), Dr; zebrafish (*Danio rerio*), Tr; fugu (*Takifugu rubripes*), Pm; lamprey (*Petromyzon marinus*).

Fig. 4. Tissue expression profiles of *Lethenteron japonicum* TLR genes. All amplifications of the TLR cDNAs were performed by an identical PCR procedure. EF1 alpha was used as a positive control. No DNA was amplified by PCR regarding the EF1 alpha template from the non-reverse transcribed sample. Typical results were obtained using 30~45 PCR cycles.

Fig. 5. Expression of blood cell ljTLRs in response to PAMP stimulation. Peripheral blood

leukocytes were harvested from individuals of *L. japonicum*. Cells were separated into three groups: first group with no stimulation (Unstimulation), second group with 3 h stimulation (3h) and third group with 6 h stimulation. PolyI:C (10μg/ml) and Heat killed *E. coli* ($3x10^7$ cell/ml) were used as stimulators as indicated. EF1 was used as a positive control.

Table 1 The accession numbers of TLRs and innate immunity genes used for BLAST and phylogenetic analyses. The accession numbers of NCBI are listed. Only *Xenopus tropicalis* TLRs are indicated by JGI ID.

Species	Gene name			
Homo sapiens	TLR1 (U88540)	TLR2 (U88878)	TLR3 (U88879)	TLR4 (U88880)
	TLR5 (NM_003268)	TLR6 (NM_006068)	TLR7 (NM_016562)	TLR8 (NM_016610)
	TLR9 (AF259262)	TLR10 (AF296673)	MyD88 (NP_002459.1)	TICAM-1 (AB086380)
	TICAM-2 (NP_067681.1)	TIRAP (NP_001034750.1)	SARM (NP_055892.2)	LBP (NP_004130.2)
	CD14 (NP_001035110.1)	MD2 (NP_056179.2)	RIG-I (AF038963)	MDA5 (AF095844)
	LGP2 (NP_077024.2)	IPS-1 (NP_065797.2)	IRAK1 (NM_001025242)	IRAK2 (NM_001025242)
	IRAK3 (NP_001135995.1)	IRAK4 (NM_001114182)	TRAF3 (NM_003300)	TRAF6 (NM_004620)
	TAB2 (NM_015093)	TAB3 (NM_152787)	TBK1 (NP_037386.1)	RIP1 (NP_003795)
	CASP8 (NP_001219.2)	FADD (NP_003815.1)	TANK (NP_004171.2)	SINTBAD (NP_055541.1)
	IKKα (NP_001269.3)	IKKβ (NP_001547.1)	IKKγ (NP_001093327.1)	IKKε (NP_054721.1)
	NAP1 (NP_071906.1)	MKK6 (NP_002749.2)	MKK3 (NP_002747.2)	JNK (NP_002741)
	TAB2 (NM_015093)	IRF3 (NP_001562.1)	IRF7 (NP_001563.2)	RELA (NP_001138610.1)
	NFκB1 (NP_001158884.1)	ATF2 (NP_001871.2)	AP-1 (NP_034721.1)	Mx (NM_001144925)
	PKR (NM_001135651)	OAS1 (NM_001032409)	IFNα (NM_024013)	IFNβ (NM_002176)
	TNFα (NP_000585)	IL-6 (NM_000600)	IL-12p40 (NP_002178.2)	
Mus musculus	TLR1 (NM_030682)	TLR2 (NM_011905.3)	TLR3 (NM_126166)	TLR4 (NM_021297)
	TLR5 (NM_016928)	TLR6 (NM_011604)	TLR7 (NM_133211)	TLR8 (NM_133212)
	TLR9 (NM_031178)	TLR11 (NM_205819)	TLR12 (NM_205823)	TLR13 (NM_205820)
	MyD88 (NP_034981.1)	TICAM-1 (NP_778154.1)	TICAM-2 (NP_775570.1)	TIRAP (NP_473437.1)
	SARM (NP_766383.2)			
Gallus gallus	TLR1 (NM_001007488)	TLR2 (NM_204278)	TLR3 (NM_001011691)	TLR4 (NM_001030693)
	TLR5 (NM_001024586)	TLR6 (NM_001007488)	TLR7 (NM_001011688)	TLR15 (NM_001037835)
	TLR21 (NM_001030558)	MyD88 (NP_001026133.1)	TICAM-1 (NM_001081506)	TIRAP (NP_001020000.1)
	SARM (XP_415814.2)			
Xenopus tropicalis	TLR1 (jgi371271)	TLR2.1 (jgi320872)	TLR2.2 (jgi320954)	TLR3 (jgi271893)
	TLR5 (jgi459490)	TLR6.1 (jgi281677)	TLR6.2 (jgi371307)	TLR7 (jgi323633)
	TLR8.1 (jgi161716)	TLR8.2 (jgi323721)	TLR9 (jgi350411)	TLR12 (jgi187046)
	TLR14.1 (jgi190020)	TLR14.2 (jgi30694)	TLR14.3 (jgi421728)	TLR14.4 (jgi421736)
	TLR21 (jgi349648)	TLR22 (jgi414791)	MyD88 (DR867184)	TICAM-1 (CX999107.1)
	TIRAP (NM_001044460)			
Danio rerio	TLR1 (XM_692439)	TLR2 (NM_212812.1)	TLR3 (AY616582)	TLR4a (NM_001131051)
	TLR4b (NM_212813)	TLR5a (XP_001919052)	TLR5b (XM_001343113)	TLR7 (XP_701101.3)
	TLR8a (XP_001920594)	TLR8b (XM_001340150)	TLR9 (XM_685911)	TLR12 (XM_685162)

	TLR14 (NM_001089350)	TLR18(BC162732)	TLR21 (XP_001923227.1)	TLR22 (XM_692565)
	MyD88 (NP_997979.2)	TICAM-1 (NP_001038224.1)	TIRAP (XP_001922965.1)	SARM (XP_001344407.1)
Takifugu rubripes	TLR1 (AAW69368)	TLR2 (AAW69370)	TLR3 (AC156436)	TLR5 (AC156437)
	TLR5S (AC156440)	TLR7 (AC156438)	TLR8 (AC156438)	TLR9 (AC156439)
	TLR14 (AC156431)	TLR21 (NM_001032579)	TLR22 (NM_001113193)	TLR23 (AC156435)
	MyD88 (NM_001113195)	TICAM-1(NM_001113194)	TIRAP (NM_001113196)	
Lethentron japonicum	TLR14a (AB109402)	TLR14b (AB109403)		

Table 2 Annotation of TLR and other innate immunity genes in the *Petromyzon marinus* genome. Each TLR and other innate immune gene was annotated using a BLASTP search and described as a TLR after meeting specific criteria. The encoded contig numbers and E-value are shown.

Gene categories	Gene name (*P. marinus*)	Genscan prediction ID or EST accession no.	Contig no.	Most similar gene [species] (accession no.)	E-value
TLR and their adaptor genes	TLR2a	GENSCAN00000027249	7641	Toll-like receptor 2 [*Sus scrofa*] (BAD90590)	2e-108
	TLR2b	GENSCAN00000120185	1179	TLR2 type2 [*Gallus gallus*] (BAB16842)	3e-121
	TLR2c	GENSCAN00000113984	1344	Toll-like receptor 6 [*Canis lupus familiaris*] (ACB41375)	2e-83
	TLR2d	GENSCAN00000096816	1344	Toll-like receptor b [*Lethenteron japonicum*] (BAE47506)	4e-50
	TLR14a	GENSCAN00000120396	35588	Toll-like receptor a [*Lethenteron japonicum*] (BAE47505)	0.0
	TLR14b	EB082826	4136	Toll-like receptor b [*Lethenteron japonicum*] (BAE47506)	0.0
	TLR14c	GENSCAN00000075432	9099	Toll-like receptor a [*Lethenteron japonicum*] (BAE47505)	7e-170
	TLR14d	GENSCAN00000133414	47492	Toll-like receptor 18 [*Danio rerio*] (AAI62732)	2e-135
	TLR3	GENSCAN00000116058	18297	Toll-like receptor 3 [*Taeniopygia guttata*] (XP_002190888)	3e-134
	TLR5	GENSCAN00000136769	38231	TLRS5 [*Takifugu rubripes*] (AAW69378)	4e-87
	TLR7/8a	GENSCAN00000159232	7539	Toll-like receptor 7 [*Taeniopygia guttata*] (XP_002194911)	0.0
	TLR7/8b	Not predicted	30480	Similar to TLR8 [*Ornithorhynchus anatinus*] (XP_001515241)	2e-122
	TLR21a	GENSCAN00000145839	1528	Toll-like receptor 21 [*Ictalurus punctatus*] (ABF74622)	8e-175
	TLR21b	GENSCAN00000112026	15848	Toll-like receptor 21 [*Ictalurus punctatus*] (ABF74622)	2e-154
	TLR21c	GENSCAN00000006564	16741	Toll-like receptor 21 [*Danio rerio*] (CAQ13807)	8e-146
	TLR22	GENSCAN00000052970	44660	Toll-like-receptor [*Oncorhynchus mykiss*] (CAF31506)	5e-92
	MyD88	gnl|ti|1309469868	8673	Similar to MyD88 [*Canis familiaris*] (XP_534223)	5e-47
	TICAM-1.1	GENSCAN00000029725	3393	Similar to TICAM-1 [*Monodelphis domestica*] (XP_001375102)	1e-27
	TICAM-1.2	GENSCAN00000011455	28649	TIR-containing adaptor molecule [*Ictalurus punctatus*] (ABD93874)	4e-22
	SARM	EC383618	12108	Sterile alpha and TIR motif containing 1 [*Danio rerio*] (AAI63770)	2e-95
RLR and adaptor genes	RIG-I	CO546225	50584	RIG-I isoform 1 [*Pan troglodytes*] (XP_001156442)	5e-36
	LGP2	GENSCAN00000019608	2627	DHX58 [*Salmo salar*] (NP_001133649.1)	2e-13
	IPS-1	FD727562	427	Zgc:158392 [*Danio rerio*] (AAI29222)	6e-11
TLR/RLR associated genes	LBP	DW024367	9431	MGC108117 protein [*Xenopus tropicalis*] (NP_001015694.1)	2e-42
	IRAK1	GENSCAN00000011724	14150	IRAK1 protein [*Bos taurus*] (AAI08133.1)	5e-25
	IRAK3	GENSCAN00000072175	17420	novel protein similar to vertebrate IRAK3 [*Danio rerio*] (CAQ13227.1)	1e-15
	IRAK4	FD717813	6289	IRAK4 [*Plecoglossus altivelis altivelis*] (BAH58736.1)	1e-22
	TRAF3	FD703125	45178	TRAF3 [*Oncorhynchus mykiss*] (NP_001118087.1)	1e-50
	TRAF6	GENSCAN00000012099	4693	TNF receptor-associated factor 6 [*Sus scrofa*] (NP_001098756.1)	3e-134

	TAK1	FD720776	2510	MAP kinase 7 [*Xenopus tropicalis*] (NP_001093731.1)	2e-84
	TAB2	GENSCAN00000012731	19927	Similar to KIAA0733 [*Monodelphis domestica*] (XP_001370832.1)	7e-26
	TBK1	EB718759	13265	TANK-binding kinase 1 [*Xenopus tropicalis*] (NP_001135652.1)	2e-21
	RIP1	gnl|ti|1229574012	1789	RIP1 [*Rattus norvegicus*] (NP_001100820.1)	5e-33
	CASP8	EG023282	11270	Caspase 8 [*Gallus gallus*] (NP_989923)	4e-14
	IKKα	GENSCAN00000034882	5202	IKKα [*Taeniopygia guttata*] (XP_002186613.1)	3e-60
	IKKβ	GENSCAN00000073839	4708	Unnamed protein product [*Tetraodon nigroviridis*] (CAG09394.1)	3e-12
	IKKγ	CO548529	49136	Similar to IKKγ [*Ornithorhynchus anatinus*] (XP_001505706.1)	5e-40
	IKKε	GENSCAN00000078948	48353	IKKε [*Bos taurus*] (NP_001039810.1)	8e-16
	NAP1	GENSCAN00000070143	780	5-azacytidine induced 2 [*Bos taurus*] (NP_001070473.1)	6e-13
	MKK6	GENSCAN00000036211	17299	Unnamed protein product [*Tetraodon nigroviridis*] (CAG03047.1)	2e-14
Transcription factor genes	IRF2	GENSCAN00000117845	17476	Similar to IRF2 isoform 1 [*Canis familiaris*] (XP_532847.2)	8e-47
	IRF4	GENSCAN00000134889	77775	Interferon regulatory factor 4 [*Equus caballus*] (XP_001487915.1)	6e-23
	IRF5	GENSCAN00000119547	65651	unnamed protein product [*Tetraodon nigroviridis*] (CAF90666.1)	2e-12
	IRF8	gnl|ti|1220653061	74379	Interferon regulatory factor 8 [*Danio rerio*] (NP_001002622.1)	1e-11
	RELA	GENSCAN00000045445	69770	RELA isoform 1 [*Homo sapiens*] (NP_068810.3)	3e-15
	NFκB1	CO548333	23299	Similar to NFκB1 p105 subunit [*Danio rerio*] (XP_001339487.2)	1e-49
	ATF2	gnl|ti|1229751035	1160	Similar to ATF2 [*Monodelphis domestica*] (XP_001376719.1)	6e-09
	AP-1	GENSCAN00000027406	61254	unnamed protein product [*Coturnix coturnix*] (CAA33553.1)	2e-32
Antiviral genes	Mx	EE737404	23777	Mx protein [*Ctenopharyngodon idella*] (Q6TKS7)	2e-11
Not found	TLR4, TLR9, TLR13, TLR15, CD14, MD2, TIRAP, MDA5 IRAK2, TAB3, FADD, TANK, SINTBAD, MKK3, PKR, OAS1, Type I IFN (IFNα, β), TNF-α, IL-6, IL-12p40				

Table 3 Primers used in this study. The primers sets, excluding TLR14a and TLR14b, were constructed based on nucleotide sequences obtained from *Petromyzon marinus*.

Gene name	Forward primers (5'-3')	Reverse primers (5'-3')	PCR conditions (Cycle and annealing temperature)
TLR2a	TGACTACCAATGCTCAAATCCAGAG	CCAGCTCAGGCAGGAGTTTC	35 cycle, 50°C

TLR2b	CAACACACTACTGGGGATGAAACTAA	GTACCACAGCGCATCCAGGT	40 cycle, 50°C
TLR2d	TCAATGCTCCAATCCAGAGAA	ATGATGTGGTGGCTGGGAAC	40 cycle, 50°C
TLR14a	TCCTTGAGAGAGCTGTATCTGACG	AGTCCGAGTCCATGTGGCTGTAGG	40 cycle, 60°C
TLR14b	TACATTGCACCCGAGTTGTACTCC	GTGGGCACCAGGGTGTTCTCCACC	45 cycle, 60°C
TLR14c	TGGTCCCGACACTTGAGCAT	CGAGCGAGTCTTGGTTCTCC	40 cycle, 50°C
TLR14d	CTACCGCTTCCACGCCTTC	GCTCGATGCTGTCGATGATG	35 cycle, 50°C
TLR3	CGCTGTTCGTCCACTTTCA	GCTCCAGGTGTCTGCTCGTC	35 cycle, 50°C
TLR5	CAGCATTGACCTCAGCCACA	GGCTATTGTTTGGGCTCCAC	40 cycle, 55°C
TLR7/8a	TGCTACAATGCCCCTTACCC	GCCCTCAGCCAGTGCTTTT	35 cycle, 50°C
TLR7/8b	GCTTCGACTGGTAGGGAATGG	CATCCCAAGGAATACGTGTCAG	35 cycle, 50°C
TLR21a	GCGGTGTGCCAATCTTTCTC	TGGTTTCCACCCAGATCCAA	35 cycle, 50°C
TLR21b	CCACGAGTTCCATGTGTCGT	CTTGAGGGTGATGAGGTTGCT	40 cycle, 50°C
TLR21c	CCCCCAGTTGGAGAAAGAGG	ATGCGGTAGTAGGGCGACAG	35 cycle, 50°C
TLR22	GTCTGCACCACCGCGACT	GCAGGTAGCTCCGCGTCA	35 cycle, 50°C
MyD88	CACGTCCCGTAACAACAGCA	TGTCGGCGTAGCAGTAGCAG	33 cycle, 50°C
TICAM1.1	GAGGGGCAGAATGATGAAGA	TTGTTCGGGGTTAGGATGGA	40 cycle, 50°C
TICAM1.2	CTGGGCAGTACAGGGGTGTC	CGTCTCGTGCTGAATGCTGT	40 cycle, 50°C
SARM	GCGATGAAGGAGAGCGTCA	CGCAGCAGTCCGTAGGTGT	35 cycle, 50°C
EF1 alpha	CCATCGACATCTCTCTGTGGA	TAGTGCCGACGATGAGCTGCT	30 cycle, 60°C

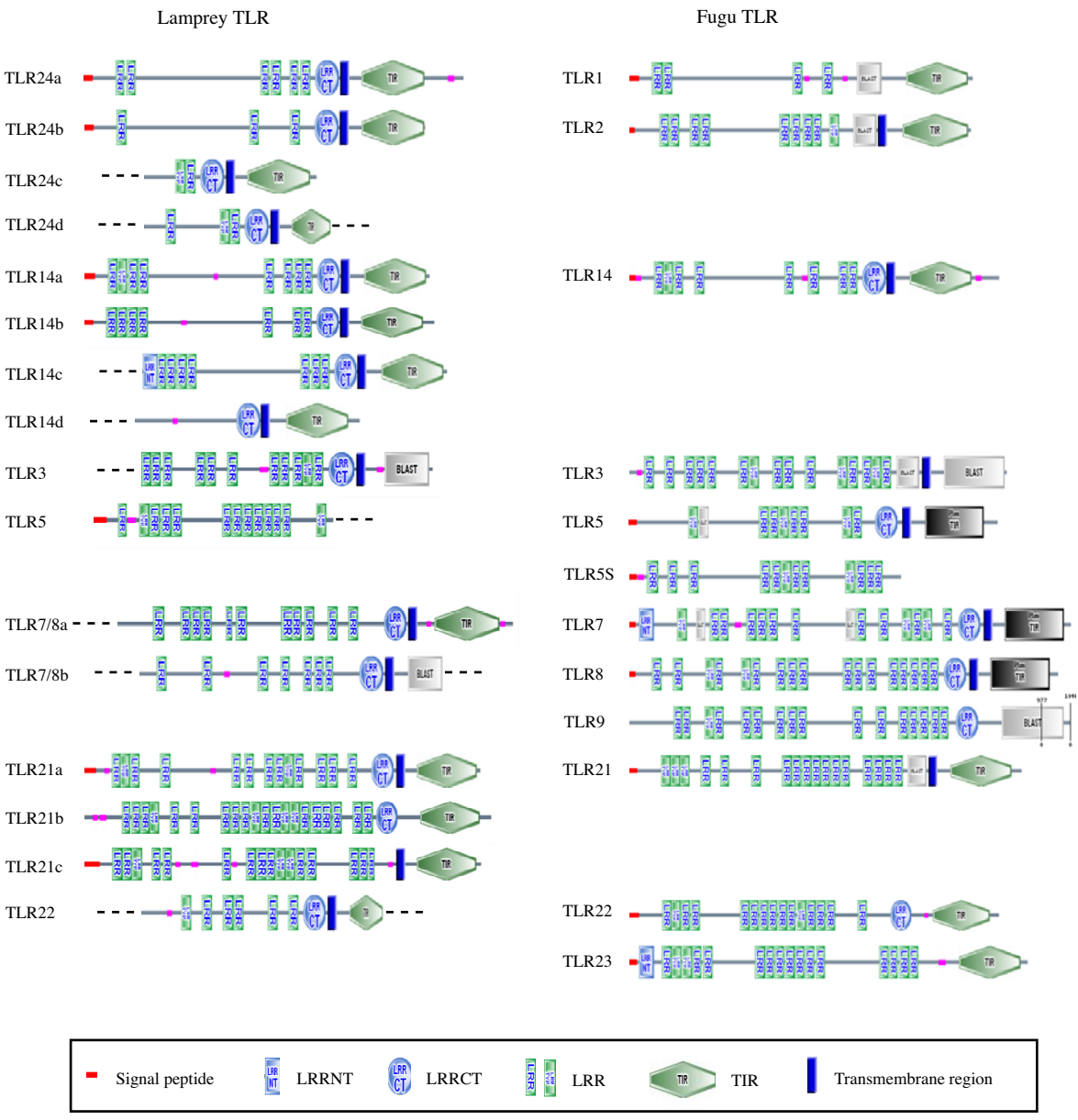
Table 4 Amino acid identities between human, mouse, zebrafish, and lamprey TICAM genes. Hs; human (*Homo sapiens*), Mm; mouse (*Mus musculus*), Dr; zebrafish (*Danio rerio*), Pm; lamprey (*Petromyzon marinus*).

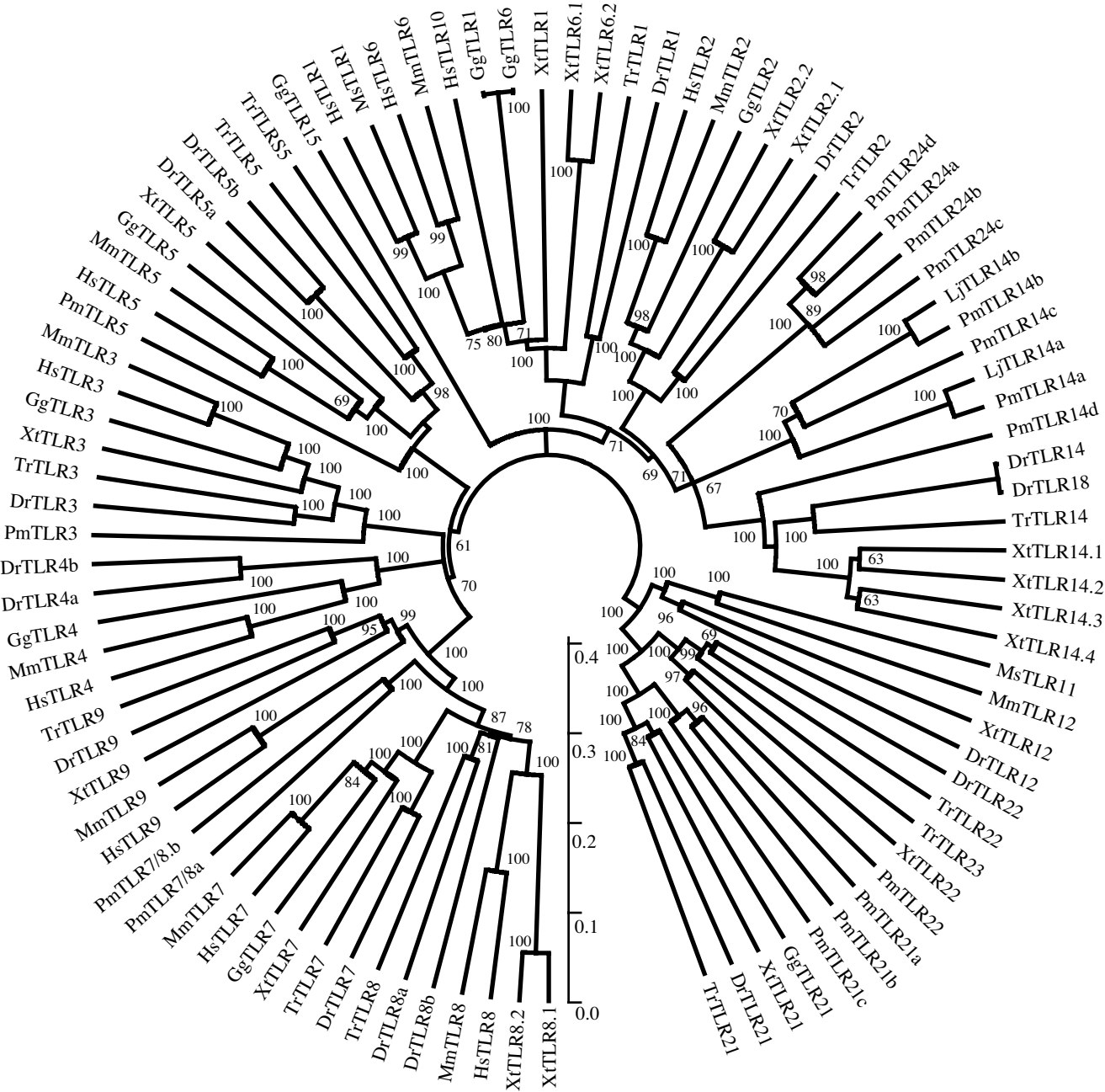
	HsTICAM-1	MmTICAM-1	DrTICAM-1	HsTICAM-2	MmTICAM-2	PmTICAM-1b
PmTICAM-1a	15.6%	14.0%	18.8%	18.2%	15.5%	18.9%
PmTICAM-1b	19.8%	19.3%	18.5%	9.4%	9.1%	-

Table 5 TLR repertoires in deuterostomes.

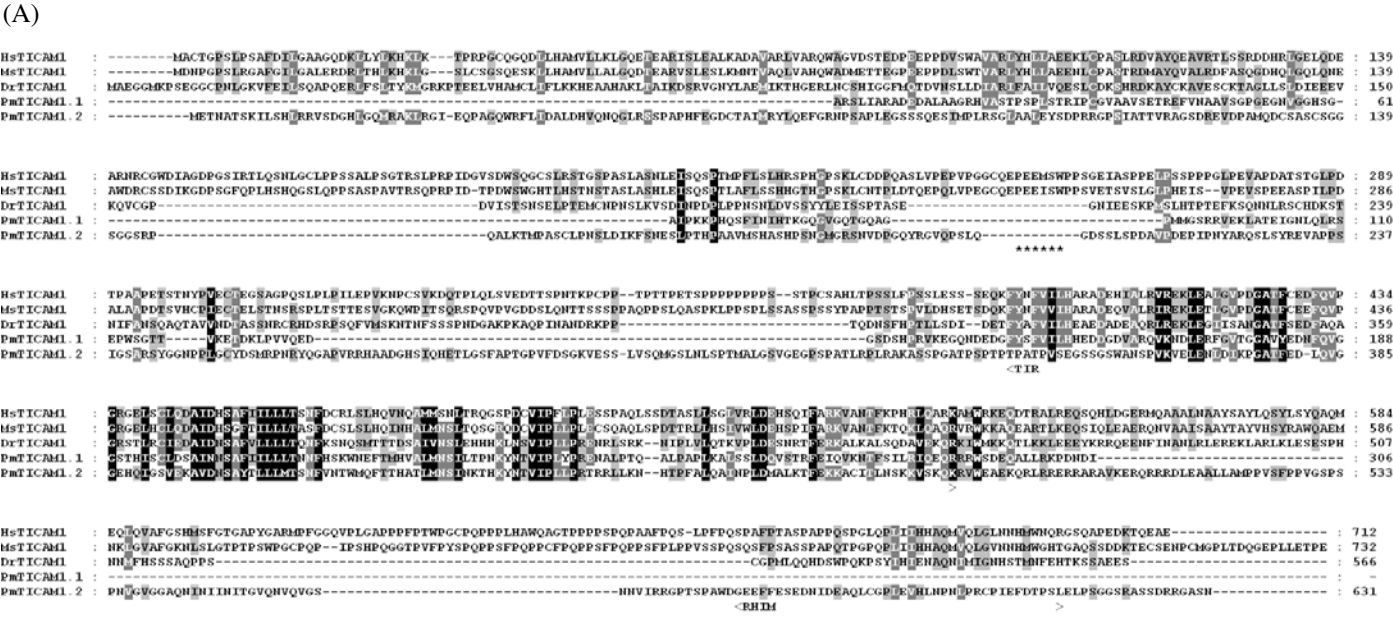
	Human	Mouse	Chicken	Frog	Zebrafish	Fugu	Lamprey	Ascidian	Amphixus	Sea urchin
TLR1	+	+	+	+	+	+	-			
TLR2	+	+	+	+	+	+	-			
TLR3	+	+	+	+	+	+	+			
TLR4	+	+	+	+	+	-	-			
TLR5	+	+	+	+	+	+	+			
TLR6	+	+	*psd*	-	-	-	-			
TLR7	+	+	+	+	+	+	+			
TLR8	+	+	+	+	+	+	+	**~2?**	**~36?**	**~300?**
TLR9	+	+	-	+	+	+	-			
TLR10	+	*psd*	-	-	-	-	-			
TLR12 (TLR11)	-	+	-	+	-	+	-			
TLR13	-	+	-	+	-	-	-			
TLR14 (TLR15)	-	-	+	+	+	+	+			
TLR21	-	-	-	+	+	+	+			
TLR22 (TLR23)	-	-	-	+	+	+	+			
TLR24	-	-	-	-	-	-	+			
MyD88	+	+	+	+	+	+	+	+	+	+
TICAM	+	+	+	+	+	+	+	-	+	-
RIG-I	+	+	+	+	+	+	+	-	+	-
MDA5	+	+	+	+	+	+	-	-	-	+
IPS-1	+	+	+	+	+	+	+	-	-	+
IRF3	+	+	-	+	+	+	-	-	-	-
IRF7	+	+	+	+	+	+	-	-	-	-
IFN	+	+	+	+	+	+	-	-	-	-

+; exists, -; does not exist, *psd*; pseudogene.

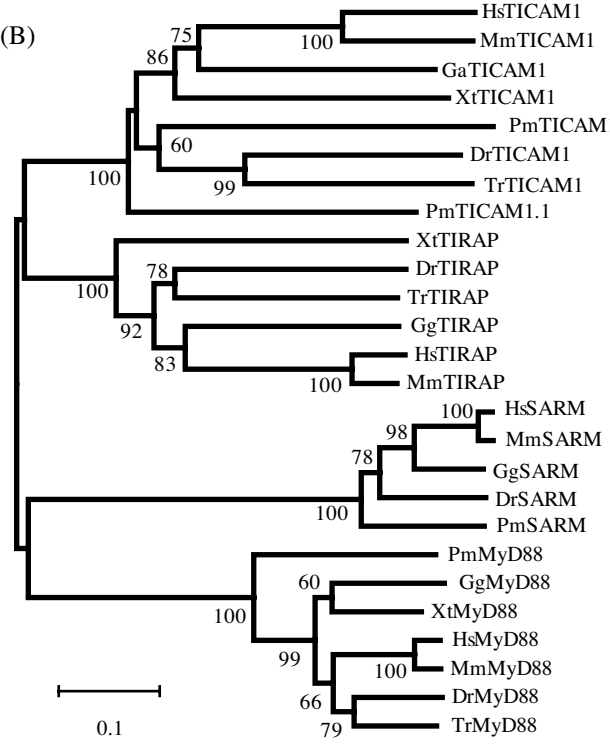

Lamprey TLR
Fugu TLR
TLR24a
TLR24b
TLR24c
TLR24d
TLR14a
TLR14b
TLR14c
TLR14d
TLR3
TLR5
TLR7/8a
TLR7/8b
TLR21a
TLR21b
TLR21c
TLR22
TLR1
TLR2
TLR14
TLR3
TLR5
TLR5S
TLR7
TLR8
TLR9
TLR21
TLR22
TLR23
Signal peptide
LRRNT
LRRCT
LRR
TIR
Transmembrane region

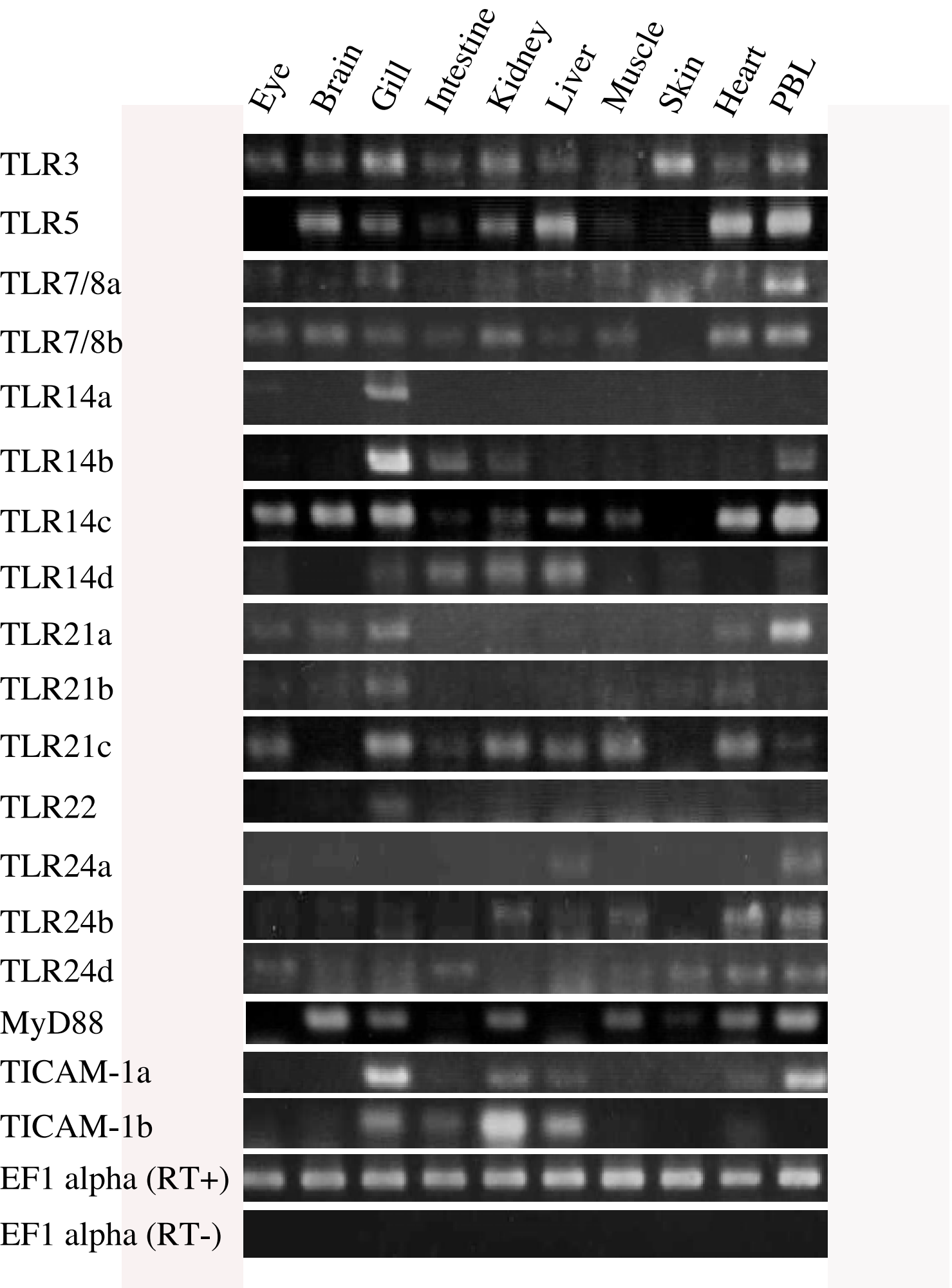

GgTLR6
XtTLR1
XtTLR6.1
XtTLR6.2
TrTLR1
DrTLR1
HsTLR2
MmTLR2
GgTLR2
XtTLR2.2
XtTLR2.1
DrTLR2
TrTLR2
PmTLR24d
PmTLR24a
PmTLR24b
PmTLR24c
LjTLR14b
PmTLR14b
PmTLR14c
LjTLR14a
PmTLR14a
PmTLR14d
DrTLR14
DrTLR18
TrTLR14
XtTLR14.1
XtTLR14.2
XtTLR14.3
XtTLR14.4
MsTLR11
MmTLR12
XtTLR12
DrTLR12
DrTLR22
TrTLR22
TrTLR23
XtTLR22
PmTLR22
PmTLR21a
PmTLR21b
PmTLR21c
GgTLR21
XtTLR21
DrTLR21
TrTLR21
XtTLR8.1
XtTLR8.2
HsTLR8
MmTLR8
DrTLR8b
DrTLR8a
TrTLR8
DrTLR7
TrTLR7
XtTLR7
GgTLR7
HsTLR7
MmTLR7
PmTLR7/8a
PmTLR7/8.b
HsTLR9
MmTLR9
XtTLR9
DrTLR9
TrTLR9
HsTLR4
MmTLR4
GgTLR4
DrTLR4a
DrTLR4b
PmTLR3
DrTLR3
TrTLR3
XtTLR3
GgTLR3
HsTLR3
MmTLR3
PmTLR5
HsTLR5
MmTLR5
GgTLR5
XtTLR5
DrTLR5a
DrTLR5b
TrTLR5
TrTLRS5
GgTLR15
HsTLR1
MsTLR1
HsTLR6
MmTLR6
HsTLR10
GgTLR1
0.4
0.3
0.2
0.1
0.0

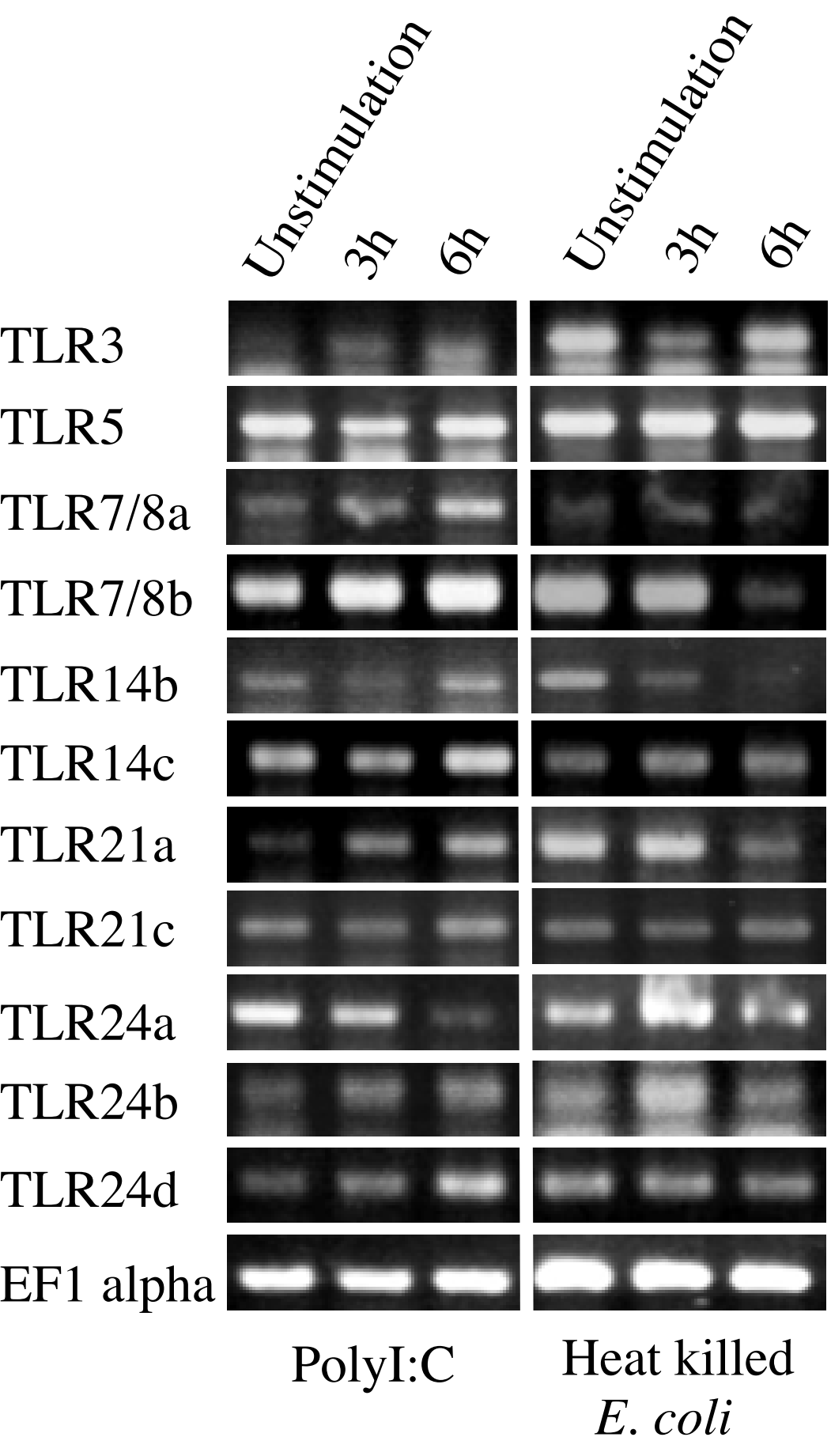
(A)


HsTICAM1
MsTICAM1
DrTICAM1
PmTICAM1.1
PmTICAM1.2
TIR
RHIM

(B)


HsTICAM1
MmTICAM1
GaTICAM1
XtTICAM1
PmTICAM1.2
DrTICAM1
TrTICAM1
PmTICAM1.1
XtTIRAP
DrTIRAP
TrTIRAP
GgTIRAP
HsTIRAP
MmTIRAP
HsSARM
MmSARM
GgSARM
DrSARM
PmSARM
PmMyD88
GgMyD88
XtMyD88
HsMyD88
MmMyD88
DrMyD88
TrMyD88
0.1


Eye
Brain
Gill
Intestine
Kidney
Liver
Muscle
Skin
Heart
PBL
TLR3
TLR5
TLR7/8a
TLR7/8b
TLR14a
TLR14b
TLR14c
TLR14d
TLR21a
TLR21b
TLR21c
TLR22
TLR24a
TLR24b
TLR24d
MyD88
TICAM-1a
TICAM-1b
EF1 alpha (RT+)
EF1 alpha (RT-)


Unstimulation
3h
6h
Unstimulation
3h
6h
TLR3
TLR5
TLR7/8a
TLR7/8b
TLR14b
TLR14c
TLR21a
TLR21c
TLR24a
TLR24b
TLR24d
EF1 alpha
PolyI:C
Heat killed
E. coli