Insights into the evolution of extracellular leucine rich repeats in metazoans with special reference to Toll-like receptor 4

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Abstract
The importance of the widely spread leucine-rich repeat (LRR) motif has been studied considering TLRs, the LRR-containing protein involved in animal immune response. The protein connects intracellular signalling with a chain of molecular interaction through the presence of LRRs in the ectodomain and TIR in the endodomain. Domain analyses with human TLR1-9 reported ectodomain with tandem repeats, transmembrane domain and TIR domain. The repeat number varied across members of TLRs and remains characteristic to a particular member. Analysis of gene structure revealed absence of codon interruption with TLR3 and TLR4 as exceptions. Extensive study with TLR4 from metazoans confirmed the presence of 23 LRRs in tandem. Distinct clade formation using coding and amino acid sequence of individual repeats illustrated independent evolution. Although ectodomain and endodomain exhibited differential selection pressure, however, within the ectodomain, the individual repeats displayed positive, negative and neutral selection pressure depending on their structural and functional significance.

Keywords: Leucine rich repeat; LRR, Toll like receptors; TLR, domain architecture, tandem arrays, gene structure, intron phase, codon interruption, evolution, phylogenetics, selection pressure.

1 Introduction

Leucine-rich repeat (LRR) containing domains are abundant in a diverse array of organisms, ranging from bacteria and archaea to eukaryotes. A number of databases such as SMART, PFAM, PROSITE and InterPro reports the presence of huge number of proteins with this highly repeating consensus motif (Sonnhammer et al., 1997; Sigrist et al., 2010; Burge et al., 2012; Letunic et al., 2012). Tandem arrays of characteristic LRR motifs consisting of approximately 20-30 amino acids and the repeat numbers ranging from 2 to 62 have been found in the primary structure of a large number of proteins (Kajava, 1998; Kobe and Kajava, 2001; Matsushima et al., 2010) encompassing a wide range of functions. Each repeat is segmented into a highly conserved segment (HCS) and a variable segment (VS) (Kajava et al., 1995; Ohyanagi and Matsushima, 1997). The HCS usually consists of either the 11-residue sequence LxxLxLxxNxL

or the 12-residue sequence LxxLxLxxCxxL, where L is Leu, Ile, Val, or Phe; N is Asn, Thr, Ser, or Cys; and C is Cys, Ser, or Asn (Kajava, 1998; Kobe and Kajava, 2001). Although these substitutions often preserve hydrophobicity or polarity, it is possible for the first or the last leucine to be replaced by a relatively hydrophilic residue (Matsushima et al., 2007). Based on different length and consensus sequence of the VS, LRRs can be classified into 8 classes: "Typical", "SDS22-like", "Bacterial", "Plant Specific (PS)", "TpLRR (*Treponema pallidum* LRR)", "RI-like (Ribonuclease inhibitor-like)", "IRREKO" and "Cysteine-containing (CC)" (Kajava, 1998; Kobe and Kajava, 2001; Matsushima et al., 2010). LRR containing proteins form a characteristic solenoid to provide a structurally invariant scaffold for protein-protein interaction. The concave side of the solenoid is defined by a series of parallel β-sheet, each constituted by HCS of an LRR motif whereas the convex side is comprised of α-helices, 3_{10} helices, polyproline II helices, β-turns and even short β-strands (Kobe and Deisenhofer, 1994; Kajava et al., 1995; Bella et al., 2008; Botos et al., 2011).

One of the prime functions of LRR involves innate immunity. For example, in organisms like invertebrates (e.g. sea urchin) and Cephalochordates (e.g. amphioxus), which lack adaptive immunity, vast repertoires of LRR proteins are present (Huang et al., 2008). Beyond innate immunity, LRRs being versatile binding motifs exhibit extensive functional significance in a variety of cellular processes like apoptosis, autophagy, cell polarization, neuronal development, nuclear mRNA transport, regulation of gene expression and ubiquitin-related processes (Kobe and Deisenhofer, 1994; Kobe and Kajava, 2001; Matsushima et al., 2005; Matsushima et al., 2005a; Bella et al., 2008; de Wit et al., 2008; Ng and Xavier, 2011). On the basis of domain organization of LRR in a protein, they can be classified into two major types: in tandem repeats (e.g. RI, PGIP and YopM) or in association with other domains like Toll/interleukin-1 receptor (TIR), Gelsolin, and NACHT (e.g. Toll-like receptor (TLR), Protein Flightless-1 homolog, NACHT LRR and PYD domains containing protein 6). In this study, we have focused on the animal protein TLR, involved in innate immune response.

Toll-like receptors, initially discovered as Toll in fruit fly, consists of ten transmembrane glycoproteins in humans (TLR 1-10) which recognizes a wide range of pathogen-associated molecular patterns (PAMPs) from bacteria, virus, fungi as well as some host molecules (Takeda et al., 2003). The domain organization is common across all the TLRs and they have one extracellular domain composed of several LRRs, a helical transmembrane domain and an intracellular TIR domain (Medzhitov, 2007). The extracellular domain recognizes various types of PAMPs. For example, lipopeptides or lipoproteins are recognized by TLR2 forming a heterodimer with TLR1 or TLR6, lipopolysaccharides (LPS) are recognized by TLR4 in association with myeloid differentiation factor 2 (MD-2), bacterial flagellin by TLR5 and viral double stranded and single stranded RNAs are identified by TLR3 and TLR7, TLR8, respectively (Akira, 2003; West et al., 2006). The TIR domains are associated with intracellular signaling pathways that are regulated by various adapters (e.g. MyD88, TIRAP/Mal, TRIF and TRAM). Differential utilization of these TIR domain recruiting adapters provides selection of TLR mediated signaling pathways. Some TLRs are cell surface bound (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) whereas some are sequestered in endosomal compartments (TLR3, TLR7, TLR8 and TLR9). Consequently, the ectodomain portion are either extracellular or face the lumen of an intracellular compartment where they encounter the pathogen derivatives.

The ectodomain part of the TLR varies notably in context to the number of LRR motifs and the

amino acid residues comprising them. These variations help in the recognition of a broad range of ligands thereby activating the signaling cascade through the TIR domain via a defined set of adapter molecules for specific TLRs. It may be mentioned here that the number of signaling pathways and adapter proteins utilized for the same are relatively smaller than the vast spectrum of the recognition machinery of TLRs. The intriguing question that arises is whether the residues (in HCS or VS) of the LRR motif or the number of repeats contribute independently or in combination to play a pivotal role in recognition and initiation of a streamlined immune response. Thus, the ectodomain has to evolve at a higher rate, to protect the host from the repertoire of constantly evolving pathogens, by presumably maintaining co-evolutionary dynamics. There have been numerous views on the evolution of TLRs. Initially, it was thought that evolutionary conservation and strong functional constraint exist on TLRs (Roach et al., 2005). Alternatively, it has been suggested that functional heterogeneity of TLRs arise from occurrence of gene duplication, gene loss and gene conversion (Hughes and Piontkivska, 2008; Roach et al., 2013). Moreover, variation in the ectodomain region of TLRs has been found to be high compared to the TIR domain (Mikami et al., 2012). Thus, we examined evolutionary relationships in TLRs (taking whole protein, ectodomain and endodomain part individually) by generating phylogenetic trees. In addition, we have studied differential selection pressure on distinctive regions of TLRs specifically on the individual LRRs enriching the ectodomain part.

2 Materials and methods

2.1 Sequence Data

The amino acid sequences of human TLR 1-9 were collected from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/protein/) on October, 2017 and the genes for the same were retrieved using the Ensembl Genome Browser v90 (Aken et al., 2017). The following sequences from humans with their respective GI number were considered: TLR1 [49065550], TLR2 [21708105], TLR3 [86161330], TLR4 [6175873], TLR5 [80478954], TLR6 [5006248], TLR7 [74096726], TLR8 [37181712] and TLR9 [74054321]. The sequences were retrieved through extensive keyword match and BLAST (Altschul et al., 1990) searches. Only complete sequences were considered. Each sequence, corresponding to a distinct TLR, was matched for a Uniprot ID and only those having an ID were selected. It may be added that precursor/partial sequences were not considered in the study. Amino acid and gene sequences of TLR4 were obtained for six other metazoans (monkey, rat, mouse, sheep, cow and horse) from NCBI and Ensembl Genome Browser v90 respectively.

2.2 Determination of Domain Architecture of TLRs

The protein sequences were segmented into ectodomain (containing LRR motifs and transmembrane region) and endodomain (consisting of TIR). This division was done based on the results from NCBI Conserved Domain Database (CDD), SMART and UniProt databases. Multiple sequence alignment was performed separately on ectodomain and endodomain of the nine TLRs, using ClustalW (Thompson et al., 1994) and T-Coffee (Notredame et al., 2000; Poirot et al., 2003) with the set of default parameters for MSA. The results were saved in PHYLIP output format, for phylogenetic tree construction.

2.3Study with the gene sequences

The coding sequences and the exon-intron structures of seven organisms for each TLR (TLR1-9) were obtained from the annotations associated with sequences in the Ensembl Genome Browser v90.The gene sequences retrieved from Ensembl were selected in a manner that is provided with RefSeq and Uniprot ID. All the sequences selected belong to transcript level 1 (TSL1).

2.4 LRR identification within TLR4

The protein sequences of TLR4 from nine different organisms were submitted to the SMART and Pfam database in order to calculate the number of LRRs. Following disparities in the results from both the databases, LRRs were counted manually and re-checked to ensure that substitutions or conserved mutations were not overlooked. Multiple sequence alignment was performed on TLR4 protein from nine organisms with the following GI numbers: human [6175873], monkey [194068441], horse [9717253], cow [111414451], goat [334812887], sheep [198281858], buffalo [324121078], mouse [148699131] and Norway rat [149059567]. LRR motifs were marked on the MSA after thorough evaluation based on the usual conserved pattern i.e. L x x L x L x x N/C x L.

2.5 Phylogenetic Tree Analysis

Phylogenetic trees showing evolutionary relationships among the human TLRs were constructed using the protein sequence parsimony method (Protpars) in the PHYLIP (phylogeny inference package) program (Felsenstein, 1989). Three types of trees were made considering the entire TLR protein, ectodomain and endodomain separately. Besides, phylogenetic trees were established using the TLR4 whole protein and 23 individual LRRs of the ectodomain region across seven organisms as the genes for buffalo and goat were unavailable in the Ensembl Genome Browser v90. A total of 1000 bootstrap values were used to generate bootstrap replicates utilizing the seqboot program of the PHYLIP package. On reaching convergence, all the trees were explored and a consense tree was initiated using the consense program of the package. FigTree v1.4.3 (http:// tree.bio.ed.ac.uk/software/figtree/) and iTOL v4 (Letunic and Bork, 2016) were used collectively for both initial visualization and generation of figures of the resulting phylogenetic trees.

Similarly, phylogenetic trees using bootstrapping were constructed for the coding sequences of TLR4 among seven organisms. In addition, a maximum parsimony tree was generated with exon sequences corresponding to 23 LRR regions for each organism using dnapars program in the PHYLIP package.

2.6 Estimation of K_a/K_s ratio

K_a/K_s or dN/dS indicate the ratio of non-synonymous to synonymous substitution rate. Under neutrality, the ratio is not expected to deviate significantly from 1 ($\omega = 1$) whereas notable changes in the ratio may be interpreted in case of positive selection ($\omega >> 1$) or negative selection ($\omega << 1$). The ratio was determined for the coding sequence of 23 LRRs individually taken from TLR4 of seven organisms, using an online available server known as Ka/Ks calculation tool provided by Computational Biology Unit of Bergen Centre for Computational Science (http://services.cbu.uib.no/tools/kaks; Liberles, 2001). Additionally, the ratio was calculated for

TLR1-9 of human and monkey using PAL2NAL (Suyama et al., 2006) considering three cases- ectodomain, endodomain and the whole protein.

3 Results & Discussion

3.1 Domain Organization in TLRs

All mammalian TLR 1-9 are known to consist of unique number of LRRs along with a conserved TIR domain. The SMART and PROSITE databases were consulted to find out the number of LRRs and the position of the TIR domain. The number and position of LRRs differed significantly in these databases. Also, as evident from Figure 1, specific TLRs, namely TLR7, TLR8 and TLR9, are devoid of the TIR domain according to the SMART database, declaring it as an unknown region. We therefore consulted other databases like NCBI and UniProt, the cumulative result of which is tabulated in Table 1. Finally, the amino acid sequences of human TLR1-9 were segmented into two portions, one containing the ectodomain part (comprising the LRRs and TM region) and the other being the conserved endodomain part, bearing the TIR domain based on the above analysis.

3.2 Evaluating the genes

Phases of exons are expressed in pairs corresponding to the phases for the 5' and the 3' end of the exon. For the 5' end, phase zero means that the exon begins with a complete codon, phase one means that the first two bases at the 5' end of the exon constitute the second and third bases of an interrupted codon. Similarly, phase two means that the first base of the exon consists of the last base of an interrupted codon (Sharp, 1981). The phase at the 3' end of the exon is determined by the phase of the following intron. On the other hand, introns that interrupt the reading frame and lie between codons are known as 'phase zero' introns, those that interrupt the codons between the first and second base are known as 'phase one' introns, and those interrupting between the second and third base are known as 'phase two introns'.

We summarized the number, length and phasing pattern of exons of TLR1-9 from seven organisms in Figure 2. The number of exons identified within each TLR ranges from a minimum of 1 to 7. There has been a difference in the number of exons across seven organisms even within a specific TLR except for TLR4 and TLR9. TLR4 and TLR9 consist of 3 and 2 exons, respectively, for all the metazoans. Barring TLR6 and TLR7, the other TLRs (TLR1, 2, 3, 5 and 8) show considerable variation in the number of exons. Interestingly, codon interruption seems to be a rare event in the TLR family with TLR3 and TLR4 as exceptions. Disruption of codon occurs at the same position in all the species. Codon disruption was also noticed in one of the transcript variants of TLR5 and TLR9 for one organism. Another similarity observed between TLR3 and TLR4 is that both of them possess an exon of invariant length for all species, a feature unseen in other members of the TLR family. Unlike TLR3, the position of the exon in TLR4 is restricted owing to the fixed number of exons in all the species.

We concentrated on TLR4 further in the gene study as we observed a high degree of conservation in the number and phasing pattern of exons. Out of the three exons encoding TLR4, the second exon possesses a striking conserved CDS of 167 base pairs across all the seven organisms (Figure 2). Two different multiple sequence alignments were carried out with the second exon and with its corresponding amino acid sequence (Figure 3a and 3b).

From both the multiple sequence alignments, it is confirmed that a conserved pattern prevails in the second exon of TLR4 which is retained after translation. We can possibly infer that there is a selective constraint in maintaining the length as well as the nature of residues in this region. A very pertinent question that arises at this point is that whether the second exon is coding for LRRs, if so then how many of them and which part of the LRR (HCS or VS) is being coded that demands such a level of conservation.

3.3 LRRs within TLR4

As mentioned above, the unique organization of the gene structure of TLR4, exclusively within the metazoans, lead us to work with this member of TLR. Primarily, we identified 23 tandem leucine-rich repeats (LRR1, LRR2, LRR3……LRR23) among nine different organisms by manual annotation in multiple sequence alignment (Figure 4) considering the conserved pattern to be L x x L x L x x N/C x L where L= Leucine (L), Isoleucine (I), Valine (V) and Phenylalanine (F), N= Asparagine (N), Cysteine (C), Serine (S) and Threonine (T). It is to be noted that similar alignment was also obtained from T-Coffee (Supplementary Figure S1). Length of 23 LRR motifs, identified manually in TLR4, ranges from 21 to 31 with their HCS portion fixed to 11 residues and VS portion varying between 10 to 20 residues. Few LRRs such as LRR3, 4, 6, 7, 15, 16, 17, 20 and 23 followed the typical pattern for LRRs without any substitution or conserved mutations in place of 'L' in the 1st, 4th, 6th or 11th position. In case of LRR1, we observed conserved methionine substitutions in the 9th position in place of 'N' whereas the same was evident in the 6th position in place of 'L' for both LRR19 and LRR21. Substitutions of 'L' with 'T' were seen in LRR2 (in all organisms at the 1st position), LRR12 (in 4 organisms at the 1st position), LRR18 (in 5 organisms at the 11th position) and LRR22 (in 2 organisms at the 6th position). In LRR10 and LRR11 substitution of 'N' at the 9th position with 'L' and 'F' were observed, respectively. In LRR1 and LRR22, 'Y' was seen to occupy the 6th position (in all organisms) and 9th position (for 2 organisms). In few LRRs such as in LRR8, 9 and 14, we observed 'N' and 'D' in place of 'L' in many organisms. We also noticed, 'L' changing to 'A' and 'W' in LRR5 and 13 respectively for all organisms. Moreover, substitutions of 'L' with hydrophilic amino acid residues were observed at many positions in the HCS part of LRR motifs. Also, conserved residues in the otherwise variable 'x' positions of the HCS of LRR across nine organisms may imply structural and functional importance.

Trying to map the LRRs of TLR4 with respect to the encoding exons (3), it may be pointed out that the first exon is devoid of any LRR and the second exon consists of LRR1, LRR2 and a portion of LRR3 with the rest of the LRRs, TM and the TIR domain residing entirely in the third exon. Furthermore, we noticed the interacting residues of TLR4, with MD-2 and LPS (ligand) is spread over LRR10, LRR11, 13, 14, 15, 16, 17 and 18 (Park et al., 2009). Despite the fact that the ligand interacting residues lie in LRRs far away from LRR1, LRR2 and portion of LRR3,

what calls for such a level of stringency at the gene and protein level, remains unanswered. A schematic diagram showing the domain architecture of TLR4 has been shown in Figure 5.

3.4Phylogenetic tree analysis

3.4.1 Human TLRs

An unrooted phylogenetic tree was constructed with 1000 bootstrap values using parsimony method for nine TLR protein sequences of human (Figure 6). It may be mentioned that similar tree was obtained from the sequences without TIR domain (ectodomain consisting of LRRs and TM) implying the independence of highly conserved TIR sequence on the tree topology (Figure 7).

TLRs (TLR1, TLR2 and TLR6) with comparatively shorter length formed a cluster while the longer TLRs (TLR7, TLR8 and TLR9) formed another. The topologies of the two trees (whole protein and ectodomain) reflect their functional similarity in terms of ligand recognition. The TLRs that identify bacterial lipopeptides (TLR1, TLR2 and TLR6) constitute one group and those that recognize viral or microbial nucleic acids (TLR7, TLR8 and TLR9) seggregate to another. Although TLR3 share similarity with TLR7, TLR8 and TLR9 with respect to recognition of viral nucleic acids, yet it is relatively of shorter length compared to the other three. In fact, this justifies its distinct position in the phylogeny. Also, TLR4 and TLR5 occupy a distinct position in the tree without clustering in any of the TLR families. Besides, TLR4 and TLR5 are of almost equivalent length and recognize ligands (lipopolysaccharides and flagellin, respectively) that are of completely different nature from the other TLRs. Figure 6 and 7 shows different line of evolution for TLR3, TLR4 and TLR5. It may be suggested that determination of domain architecture and phylogenetic analysis restates LRR-containing TLRs to have evolved through independent evolution of the ectodomain.

A rooted phylogenetic tree was contrived for the TIR domain of nine human TLRs and TIR domain of SARM1 (Sterile alpha and TIR motif-containing protein 1). SARMs are the only known TIR-containing adapters that negatively regulate TLR signalling and have a different line of evolution from other TIR-containing adapters. Therefore, TIR of SARM1 was treated as an outgroup to establish the clustering pattern of TLRs in the presence of a non-TLR TIR domain. Despite the similarities in the length of both TLR and non-TLR TIR domains, SARM1 stood out as an operational taxonomic unit in the tree. Since the TIR domain is evolutionarily conserved, we wanted to see the origin of the nine TIRs, the only part being least susceptible to changes by selection pressure. From this tree (Figure 8), it is clear that TIR domains of TLR3 and TLR5 might have undergone extensive divergence in comparison to the other members. The fact that TIR of TLR4 shares some similar adaptor molecules for signal transduction with TLR2 (Akira and Takeda, 2004; O'Neill and Bowie, 2007), justifies its position in the tree.

3.4.2 TLR4

Figure 9a and 9b showed unrooted phylogenetic trees constructed taking protein and gene sequences of TLR4 for seven organisms. Phylogenetic tree analysis exhibited similar clustering patterns for the two.

Two phylogenetic trees, each with 1000 bootstrap iterations, were crafted considering the amino acid and coding sequences of portions corresponding to the 23 LRR motifs in TLR4 across seven organisms (Figure 10a and Figure 10b). Thus, a total of 161 (23LRRs x 7organisms) sequences were taken into account for constructing each of these unrooted trees. It is worth mentioning that the 23 repeats from seven organisms form distinct clades both at the protein level and also in the coding sequences indicating unique spatial arrangement of the repeats. It may be mentioned that some LRR pairs in the tree with protein sequences seemed very proximate and appeared under a common node. Such LRR pairs are LRR2-LRR16, LRR3-LRR20, LRR4-LRR18, LRR6-LRR19, LRR10-LRR23 and LRR12-LRR22. While in the gene tree, few LRRs are seen to be forming closely related pairs while others are disrupted making separate clades. The following pairs of LRR are closely arranged under a common node: LRR1-LRR17, LRR2-LRR22, LRR6-LRR19, LRR8-LRR16, LRR10-LRR13, LRR11-LRR12 and LRR15-LRR23. Out of these pairs, LRR6-LRR19 was found to be common in both the trees implying a possibility of duplication events (Figure 10a and 10b).

3.5 Selection pressure on individual LRR coding sequences

Selection pressure on genes can be identified from the ratio of non-synonymous (K_a) to synonymous (K_s) nucleotide substitution with pairwise or multiple combinations of genes. We have calculated K_a and K_s of 23 LRR coding sequences from seven organisms using the calculation tool mentioned earlier. Coding sequences corresponding to 23 repeats were translated to protein sequences, multiply aligned on codon boundaries to construct phylogenetic tree with K_a and K_s values. Figure 11 shows the differential selection pressure on branches of gene phylogenetic tree for LRR11, LRR14, LRR6 and LRR21. A ratio significantly greater than one indicates positive selection pressure which was found in almost all LRRs of TLR4 (Supplementary Figure S2). LRR11 and LRR14 needs special mention as they exhibit strong positive selection for most of the organisms considered. The probable reason may be attributed to the fact that the residues responsible for recognition of its ligand and MD-2 (Park et al., 2009), lies in 11th and 14th repeat. Additionally, it may be mentioned that more selection pressure acts on these two LRRs particularly to impart a wide range of recognition ability for diverse array of bacterial LPS. On the other hand, LRR6 and LRR21 are seen to undergo purifying selection.

3.6 Positive selection in the ectodomain of TLR4

According to our previous results, positive selection prevailed on specific LRR motifs in the ectodomain part of TLR4 across seven organisms. The selection pressure was constrained to particular repeats of functional importance instead of being distributed over the entire ectodomain region in other words spanning the repeat motifs. It would thus be intriguing to verify if this specificity in sites for selective pressure is a general rule applicable to other TLRs or not. Consequently, we performed pairwise codon alignment between human and monkey using PAL2NAL considering the ectodomain, endodomain and entire sequence of the nine TLRs (Figure 12). From the results, we observed that the value decreased considerably for the viral or bacterial nucleic acid sensing TLRs (i.e. TLR3, 7, 8 & 9). Our result was shown to be consistent with the findings that evolution in the aforementioned TLRs is constrained thus avoiding response against self-derived nucleic acids (Krieg and Vollmer, 2007; Wlasiuk and Nachman, 2010). In this case, we did not consider the low selection rate in TLR6 ectodomain as it does not

belong to the above mentioned TLR family and also due to its hetero-dimerizing property with TLR2. TLR4 showed highest selection rate among the other TLRs thereby establishing as a special TLR in terms of exon phasing pattern, recruitment of adaptor molecules in alternate basis for signalling and recognition of a myriad of LPS ligands.

Conclusion

Domain architecture analysis of TLRs revealed that each TLR consists of numerous repeat-spanning ectodomain, a transmembrane region and a single TIR domain. On verification of the repeat number using different databases, it was found that the LRR motifs are present in distinct numbers in each TLR remaining invariant across species. Gene structure of TLRs revealed that the number of exons encoding the proteins varied across species for the same TLR and also across different TLRs. TLR4 and TLR9 is an exception to this as both have shown 3 and 2 exons respectively across all species studied. Unlike the coding pattern of ribonuclease inhibitor, an LRR protein, the repeats of TLR are not coded by individual exons. For example, there are cases where few repeats have been found to be coded by a single exon as is a single repeat found to be coded by two exons. Interestingly, only two types of phasing of introns, phase 0 and phase 2 were observed. Phase one introns were missing from all members of the TLRs studied here. Codon interruption has been a rare event in these proteins as introns with phase 2 was observed for only TLR3 and TLR4 thus supporting the early intron hypothesis for this family of proteins. It is worthy of note that the second exon of TLR4 remains conserved both at the CDS and its translated level. On the basis of MSA results, the length and sequence residues of this portion are conserved across all the metazoans studied. Despite being devoid of any ligand or adapter interacting residues in this region, the aforementioned conservation may probably have either some structural implications or functional relevance. Further study of TLR4 ectodomain at the protein level indicated that 23 distinct repeats, following the typical LRR signature sequence, are present in tandem array across seven organisms. Phylogenetic analysis, considering different segments (ectodomain, endodomain and whole protein) of TLR1-9 separately, revealed that TLR3, TLR4 and TLR5 occupy distinct positions between the TLR1 and TLR7 subfamilies. Phylogeny at the CDS and protein level, with individual repeats of the TLR4 ectodomain, reported the presence of each LRR as a separate clade for all the organisms, supporting the fact that individual repeats have undergone distinct evolution. On analysis of selection pressure, we found that the Ka and Ks values vary differentially among organisms for each motif considered. Out of all, two repeats, LRR11 and LRR14, exhibited strong positive selection on most of the organisms while two others, LRR6 and LRR21, exerted negative selection pressure. Both LRR11 and LRR14 harbour ligand and adapter interacting residues thus explaining the cause for positive selection. The cause of negative selection for LRR6 and LRR21 could be for structural maintenance of the scaffold for efficient recognition. Evaluation of selection pressure on different segments of these PRRs (pathogen recognition receptor) revealed a skewed selection pressure along the length of TLR4. The ectodomain consisting of the repeats exhibited positive selection while the endodomain or the TIR domain showed negative selection. The positive selection of the ectodomain is in accordance with its demand of interacting with the ever changing PAMPs to facilitate efficient recognition eventually activating the signalling cascade through the TIR domain conserved across the TLR family.

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Figures and Table

Table 1 Number of LRRs predicted in the nine human TLRs by four databases

	Number of LRRs predicted by			
	SMART	*PROSITE*	*NCBI*	*UNIPROT*
TLR1	4	10	19	19
TLR2	7	11	19	19
TLR3	16	19	22	22
TLR4	11	11	18	18
TLR5	9	12	22	22
TLR6	5	9	19	19
TLR7	12	18	27	27
TLR8	15	20	23	23
TLR9	18	17	26	26

Figure 1 Domain Architecture of human TLR1-9 as per PROSITE and SMART databases

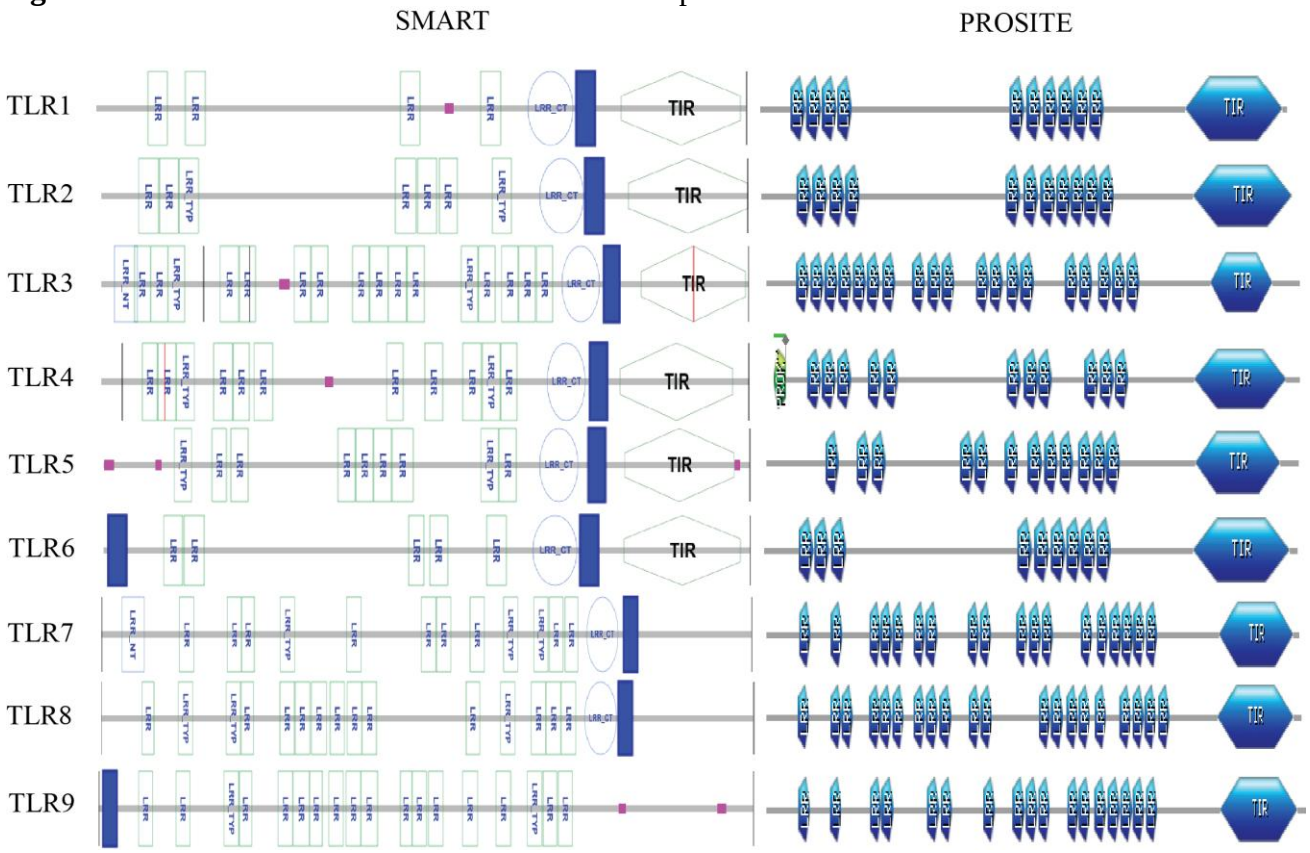


Figure 2 Chart showing base pair length (bp), length of proteins, number, length and phasing of exons in the nine TLRs across seven species

TLRs	*Species*	*No. of base pairs*	*Length of protein (aa)*	*No. of exons*	*Length of exons*							*Phasing of exons*					
TLR1	Human	2779	786	3	71	92	2616					-,-	-,-	-,-			
		2849		4	38	77	92	2642				-,-	-,-	-,-	-,0		
	Mouse	2783	795	3	95	69	2619					-,-	-,-	-,-			
		3025		4	36	300	69	2620				-,-	-,-	-,-	-,-		
	Rat	*	795	*	*							*					
	Monkey	3805	786	4	1030	92	68	2615				-,-	-,-	-,-	-,-		
	Cow	*	792	*	*							*					
	Horse	2498	781	1	2498							-,-					
	Sheep	2391	796	1	2391							0,0					
TLR2	Human	4200	784	1	4200							-,-					
	Mouse	3014	784	3	248	248	2518					-,-	-,-	-,-			
	Rat	2593	784	2	46	2547						-,-	-,-				
	Monkey	3284	784	2	82	3202						-,-	-,-				
	Cow	3613	784	2	120	3493						-,-	-,-				
	Horse	2675	784	2	14	2661						-,-	-,-				
	Sheep	2355	784	1	2355							0,0					
TLR3	Human	6015	904	5	97	448	192	1853	3425			-,-	-,0	0,0	0,2	2,-	
	Mouse	4093	905	6	167	70	453	192	1853	1358		-,-	-,-	-,0	0,0	0,2	2,-
		4096		7	81	183	70	453	192	1853	1264	-,-	-,-	-,-	-,0	0,0	0,2	2,-
		4407		7	167	195	70	453	192	1853	1477	-,-	-,-	-,-	-,0	0,0	0,2	2,-
	Rat	3382	905	7	102	195	69	453	192	1853	518	-,-	-,-	-,-	-,0	0,0	0,2	2,-
		3990	905	5	640	192	1853	279	1026			-,0	0,0	0,2	2,-	-,-	
	Monkey	5516	904	5	604	448	192	1853	2419			-,-	-,0	0,0	0,2	2,-	
	Cow	3025	904	5	106	452	192	1850	425			-,-	-,0	0,0	0,2	2,-	
	Horse	2983	904	5	30	450	192	1853	458			-,-	-,0	0,0	0,2	2,-	
	Sheep	2715	904	4	444	192	1850	229				0,0	0,0	0,2	2,0		
TLR4	Human	4844	839	3	194	167	4483					-,0	0,2	2,-			
	Mouse	4077	835	3	338	167	3572					-,0	0,2	2,-			
	Rat	3341	835	3	331	167	2843					-,0	0,2	2,-			
	Monkey	2824	826	3	436	167	2221					-,0	0,2	2,-			
	Cow	3716	841	3	563	167	2986					-,0	0,2	2,-			
	Horse	3260	843	3	184	167	2909					-,0	0,2	2,-			
	Sheep	2526	841	3	93	167	2266					0,0	0,2	2,0			
TLR5	Human	3368	858	6	87	116	86	183	165	2731		-,-	-,-	-,-	-,-	-,-	-,-
		4088		4	110	183	165	3630				-,-	-,-	-,-	-,-		
	Mouse	3199	873	4	95	128	102	2874				-,-	-,-	-,2	2,-		
		3818	859	1	3818							-,-					
	Rat	4255	871	1	4255							-,-					
	Monkey	3880	858	3	86	166	3628					-,-	-,-	-,-			
	Cow	2577	858	1	2577							0,0					
	Horse	*	859	*	*							*					
	Sheep	2616	871	1	2616							0,0					

Figure 2 (Continued)

TLR6	Human	2938	796	1	2938					-,-				
		5880	796	2	57		5823			-,-		-,-		
	Mouse	3677	806	2	114		3563			-,-		-,-		
	Rat	2700	806	2	211		2489			-,-		-,-		
	Monkey	4930	796	2	1355		3575			-,-		-,-		
	Cow	2382	793	2	2175		207			0,0		0,0		
	Horse	2478	796	1	2478					0,0				
	Sheep	2394	797	1	2394					0,0				
TLR7	Human	5011	1049	3	41	101	4869			-,-	-,0	0,-		
	Mouse	3468	1050	3	97	99	3272			-,-	-,0	0,-		
		3776		3	122	99	3555			-,-	-,0	0,-		
		3897		5	92	99	81	68	3557	-,-	-,-	-,-	-,0	0,-
	Rat	4282	1050	3	117	100	4065			-,-	-,0	0,-		
	Monkey	3402	1049	3	154	101	3147			-,-	-,0	0,-		
		3458		2	311		3147			-,0		0,0		
	Cow	3403	1050	3	152	101	3150			-,-	-,0	0,0		
	Horse	3748	1049	3	30	101	3617			-,-	-,-	-,0		
	Sheep	3376	1050	3	125	101	3150			-,-	-,0	0,-		
TLR8	Human	3367	1059	3	71	137	3159			-,-	-,0	0,-		
		4216	1041	2	90		4126			-,0		0,-		
	Mouse	3158	1032	2	40		3118			-,0		0,-		
		3431		3	135	142	3154			-,-	-,0	0,-		
	Rat	3381	1029	2	135		3246			-,-		-,-		
	Monkey	3120	1040	2	3		3117			0,0		0,0		
		3123		2	3		3120			0,0		0,0		
		3174	1058	2	57		3117			0,0		0,0		
	Cow	*	1024	*	*					*				
			1033											
	Horse	3177	1058	3	27	27	3123			0,0	0,0	0,0		
	Sheep	3108	1035	1	3108					0,0				
TLR9	Human	3870	1032	2	637		3233			-,0		0,-		
	Mouse	3478	1032	2	109		3369			-,0		0,-		
	Rat	3250	1032	2	102		3148			-,0		0,-		
	Monkey	3099	1032	2	3		3096			0,0		0,0		
		3102	1033		20		3082			0,2		2,0		
	Cow	3255	1029	2	126		3129			-,0		0,0		
	Horse	3358	1031	2	101		3257			-,0		0,0		
	Sheep	*	1029	*	*					*				

Note: '*' denotes entry not found in Ensembl Genome Browser. '-' indicates UTR while numbers '0', '1' and '2' correspond to phase 0, phase 1 and phase 2 exons respectively.

Figure 3a Multiple sequence alignment of the coding sequence corresponding to the second exon of TLR4 from seven organisms

```
rat2        GTACTTCCTAATATTACCTACCAATGCATGGATCAGAATCTCAGCAAAATCCCTCATGAC
mouse2      GTAGTTCCTAATATTACCTACCAATGCATGGATCAGAAACTCAGCAAAGTCCCTGATGAC
horse2      GTGGTTCCTAACACTACTTACCAATGCATGGACCTGAATCTCTACAAAATCCCTGAGAAC
cow2        GTTGTTCCTAACATTAGTTACCAGTGCATGGAGCTGAATCTCTACAAAATCCCCGACAAC
sheep2      GTTGTTCCTAACATCAGTTACCAATGCATGGAGCTGAATCTCTACAAAATCCCCGACAAC
human2      GTGGTTCCTAATATTACTTATCAATGCATGGAGCTGAATTTCTACAAAATCCCCGACAAC
monkey2     GTGGTTCCTAACATTACTTATCAATGCATGGAGCTGAAGTTCTACAAAATCCCCGACAAC
            **  ******* *  *  ** ** ******** * ***  **  **** ****  *  **

rat2        ATCCCTTATTCAACCAAGAACCTAGATCTGAGCTTCAACCCCCTGAAGATCTTAAGAAGC
mouse2      ATTCCTTCTTCAACCAAGAACATAGATCTGAGCTTCAACCCCTTGAAGATCTTAAAAAGC
horse2      ATCCCCACATCAACCAAGGAACTGGACCTGAGCTTTAACCCCTTGAAGGAGTTAGGCAGC
cow2        ATCCCCATATCAACCAAGATGCTGGACCTGAGCTTTAACTACCTGAGACATTTAGGCAGC
sheep2      ATCCCCATATCAACCGAGATGCTGGACCTGAGCTTTAACTACCTGAGACATTTAGGCAGC
human2      CTCCCCTTCTCAACCAAGAACCTGGACCTGAGCTTTAATCCCCTGAGGCATTTAGGCAGC
monkey2     ATCCCCTTCTCAACCAAGAACCTGGACCTGAGCTTTAATCCCCTGAGGCATTTAGGCAGC
             * **    ****** **    * ** ******** **   * ***     ***   ***

rat2        TATAGCTTCTCCAATTTCTCACAACTTCAGTGGCTGGATTTATCCAG
mouse2      TATAGCTTCTCCAATTTTTCAGAACTTCAGTGGCTGGATTTATCCAG
horse2      CATAGCTTCTCCAACTTCCCAGAACTGCAGGTGCTGGATTTGTCCAG
cow2        CATAACTTCTCCAGCTTCCCAGAACTGCAAGTGCTGGATTTATCCAG
sheep2      CATAACTTCTCCAGGTTCCCAGAACTGCAAGTGCTGGATTTATCCAG
human2      TATAGCTTCTTCAGTTTCCCAGAACTGCAGGTGCTGGATTTATCCAG
monkey2     TATAGCTTCCTCCGTTTTCCAGAACTGCAGGTGCTGGATTTATCCAG
             *** ****  *   **  ** **** **   ********* *****
```

Figure 3b Multiple sequence alignment of the amino acid residues encoded by the second exon of TLR4 from seven metazoans

```
mouse2      VVPNITYQCMDQKLSKVPDDIPSSTKNIDLSFNPLKILKSYSFSNFSELQWLDLS
rat2        VLPNITYQCMDQNLSKIPHDIPYSTKNLDLSFNPLKILRSYSFSNFSQLQWLDLS
horse2      VVPNTTYQCMDLNLYKIPENIPTSTKELDLSFNPLKELGSHSFSNFPELQVLDLS
human2      VVPNITYQCMELNFYKIPDNLPFSTKNLDLSFNPLRHLGSYSFFSFPELQVLDLS
monkey2     VVPNITYQCMELKFYKIPDNIPFSTKNLDLSFNPLRHLGSYSFLRFPELQVLDLS
cow2        VVPNISYQCMELNLYKIPDNIPISTKMLDLSFNYLRHLGSHNFSSFPELQVLDLS
sheep2      VVPNISYQCMELNLYKIPDNIPISTEMLDLSFNYLRHLGSHNFSRFPELQVLDLS
            *:** :****: :: *:*.::* **: :***** *: * *:.*  * :** ****
```

Figure 4 Multiple Sequence Alignment of the ectodomain part within TLR4 among seven metazoans

```
                                                   LRR1                    LRR2
                                             LxxLxLxxNxL              LxxLx
mTLR4        MMPPWLLARTLIMA-LFFSCLTPGSLNPCIEVVPNITYQCMDQKLSKVPDDIPSSTKNID
rTLR4        MMPLLHLAGTLIMA-LFLSCLRPGSLNPCIEVLPNITYQCMDQNLSKIPHDIPYSTKNLD
hTLR4        MMSASRLAGTLIPAMAFLSCVRPESWEPCVEVVPNITYQCMELNFYKIPDNLPFSTKNLD
moTLR4       MTSALRLAGTLIPAMAFLSCVRPESWEPCVEVVPNITYQCMELKFYKIPDNIPFSTKNLD
hoTLR4       MMPPTRLAGTLIPAMAFLSCLRPESWDPCVQVVPNTTYQCMDLNLYKIPENIPTSTKELD
sTLR4        -MARARRAAALIPAMAILSCLRTESWDPCVQVVPNISYQCMELNLYKIPDNIPMSTEMLD
gTLR4        MMARARRAAALIPAMAILSCLRTESWDPCVQVVPNISYQCMELNLYKIPDNIPVSTEMLD
cTLR4        MMARARLAAALIPATAILSCLRTESWDPCVQVVPNISYQCMELNLYKIPDNIPISTKMLD
bTLR4        MMARARLAAALIPATAILSCLRTESWDPCVQVVPNISYQCMELNLYKIPDNIPISTKMLD
                                            ↑ **   **                    *
                                   LRR3                     LRR4
             LxxNxL              LxxLxLxxNxL              LxxLxLxxNxL
mTLR4        LSFNPLKILKSYSFSNFSELQWLDLSRCEIETIEDKAWHGLHHLSNLILTGNPIQSFSPG
rTLR4        LSFNPLKILRSYSFSNFSQLQWLDLSRCEIETIEDKAWHGLNQLSTLVLTGNPIKSFSPG
hTLR4        LSFNPLRHLGSYSFFSFPELQVLDLSRCEIQTIEDGAYQSLSHLSTLILTGNPIQSLALG
moTLR4       LSFNPLRHLGSYSFLRFPELQVLDLSRCEIQTIEDGAYQSLSHLSTLILTGNPIQSLALG
hoTLR4       LSFNPLKELGSHSFSNFPELQVLDLSRCEIEMIEDDAYQGLNHLSTLILTGNPIRSLALG
sTLR4        LSFNYLRHLGSHNFSRFPELQVLDLSRCEIKIIEDNTFQGLNHLSTLILTGNPIQSLAWG
gTLR4        LSFNYLRHLGSHNFSRFPELQVLDLSRCEIKIIEDNTFQGLNHLSTLILTGNPIQSLAWG
cTLR4        LSFNYLRHLGSHNFSSFPELQVLDLSRCEIKIIEDDTFQGLNHLSTLILTGNPIQSLAWG
bTLR4        LSFNYLRHLGSHNFSSFPELQVLDLSRCEIKIIEDDTFQGLNHLSTLILTGNPIQSLAWG
              **                  *  * *↑ *                *    ** *
                        LRR5                     LRR6                    LRR7
                   LxxLxLxxNxL              LxxLxLxxNxL              LxxL
mTLR4        SFSGLTSLENLVAVETKLASLESFPIGQLITLKKLNVAHNFIHSCKLPAYFSNLTNLVHV
rTLR4        SFSGLTNLENLVAVETKMTSLEGFHIGQLISLKKLNVAHNLIHSFKLPEYFSNLTNLEHV
hTLR4        AFSGLSSLQKLVAVETNLASLENFPIGHLKTLKELNVAHNLIQSFKLPEYFSNLTNLEHL
moTLR4       AFSGLSSLQKLVAVETNLASLENFPIGHLKTLKELNVAHNLIQSFKLPEYFSNLTNLEHL
hoTLR4       AFSGLSSLQTLVAVETKLSSLEKFPIGHLKTLKELNVAHNLIHSFKLPEYFSKMPNLEHL
sTLR4        AFSGLSSLQKLVAVETNLVSLDDFPIGHLKTLKELNVAHNFIHSFKLPEYFSNLPNLEHL
gTLR4        AFSGLSSLQKLVAVETNLVSLDDFPIGHLKTLKELNVAHNFIHSFKLPEYFSNLPNLEHL
cTLr4        AFSGLSSLQKLVAVETNLVSLNDFPIGHLKNLKELNVAHNFIHSFKLPEYFSNLPNLEHL
bTLr4        AFSGLSSLRKLVAVETNLVSLHDFPIGHLRALKELNVAHNFIHSFKLPEYFSNLPNLEHL
                        * **                  *  * **                    *
                                      LRR8                     LRR9
             xLxxNxL                LxxLxLxxNxL              LxxLxLxxNxL
mTLR4        DLSYNYIQTITVNDLQFLRENPQVNLSLDMSLNPIDFIQDQAFQGIKLHELTLRGNFNSS
rTLR4        DLSYNYIQTISVKDLQFLRENPQVNLSLDLSLNPIDSIQAQAFQGIRLHELTLRSNFNSS
hTLR4        DLSSNKIQSIYCTDLRVLHQMPLLNLSLDLSLNPMNFIQPGAFKEIRLHKLTLRNNFDSL
moTLR4       DLSSNKIQNIYCKDLQVLHQMPLSNLSLDLSLNPINFIQPGAFKEIRLHKLTLRSNFDDL
hoTLR4       DLSNNKIQNISHEDLRVLHQMPLLNLSLDLSLNPLEFIQPDAFKEIKLHKLTLRSNFDSI
sTLR4        DLSNNKIQNIYYEDVKVLHQMPLLNLSLDLSLNPLEFIEPGTFKEIKLNGLTLRSNFNSS
gTLR4        DLSNNKIQNIYYEDVKVLHQMPLLNLSLDLSLNPLEFIEPGTFKEIKLNGLTLRSNFNSS
cTLR4        DLSNNKIQNIYYEDVKVLHQMPLLNLSLDLSLNPLDFIEPGTFKEIKLNGLTLRSNFNSS
bTLR4        DLSNNKIQNIYYEDVKVLHQMPLLNLSLDLSLNPLEFIEPGTFKEIKLNGLTLRSNFNSL
             * *                       * * *  *                     * *  *
                        LRR10                    LRR11
                   LxxLxLxxNxL              LxxLxLxxNxL
mTLR4        NIMKTCLQNLAGLHVHRLILGEFKDERNLEIFEPSIMEGLCDVTIDEFRLTYTNDFS--D
rTLR4        NVLKMCLQNMTGLHVHRLILGEFKNERNLESFDRSVMEGLCNVSIDEFRLTYINHFS--D
hTLR4        NVMKTCIQGLAGLEVHRLVLGEFRNEGNLEKFDKSALEGLCNLTIEEFRLAYLDYYL-DD
moTLR4       NVMKTCIQGLAGLEVHRLVLGEFRNERNLEEFDKSSLEGLCNLTIEEFRLTYLDYYL-DN
hoTLR4       DVMKSCIQGLAGLKVNRLVLGEFKNERKLERFDTSALRGLHNLTIEEFRLAYIDNYSSKD
sTLR4        DVMKTCIQGLAGLKINRLVLGEFKNERKLQKFDRSCLEGLCNLTIEQFRIAYLSKFSWN-
gTLR4        YVMKTCIQGLAGLKINRLVLGEFKNERKLQKFDRSCLEGLCNLTIEQFRIAYLNKFSRN-
cTLR4        HVMKTCIQGLAGLKTNRLVLGEFKNERKLQRFDRSFLEGLCNLTIEQFRIAYLDKFSGD-
bTLR4        HVMKTCIQGLAGLKINRLVLGEFKNERKLKIFDRSFLEGLCNLTIEQFRIAYLDEFSGD-
                        *    *                                  *
```

Figure 4 (Continued)

```
                    LRR12                                LRR13                 LRR14
                 LxxLxLxxNxL                          LxxLxLxxNxL           LxxLxLx
mTLR4           DIVKFHCLANVSAMSLAGVSIKYLEDVPKHFKWQSLSIIRCQLKQFPTLDLPFLKSLTLT
rTLR4           DIYNLNCLANISAMSFTGVHIKHIADVPRHFKWQSLSIIRCHLKPFPKLSLPFLKSWTLT
hTLR4           IIDLFNCLTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLKLKSLKRLTFT
moTLR4          IIDLFNCLANVSSFSLVSVSIKRVEDFSYNFRWQHLELVNCKFEQFPTLELESLKRLTFT
hoTLR4          SIDLLNCLADISKISLVSLDLGNLKDFPKGFGWQDFELVNCRIEGFPTLELTSLKRLVFT
sTLR4           DTDLFNCLANVSVISLLSISLGSLQALLKDFRWQHLEMINCDFDKFPALKLRSLKKFVFT
gTLR4           DTDLFNCLANVSMISLLSIPLGSLQALLKDFRWQHLEMINCDFDKFPALELRSLKKFVFT
cTLR4           DTDLFNCLANVSVISLLSISLGSLQALLKDFRWQHLEIINCDFDKFPALKLSSLKKFVFT
bTLR4           DTDLFNCLANVSVISLLSISLGSLQALLKDFRWQHLEIVNCDFEKFPALKLSSLKKFVFT
                       *                                *                  *    *
                                     LRR15                          LRR16
                xNxL            LxxLxLxxNxL                    LxxLxLxxNxL
mTLR4           MNKGSISFKKVALPSLSYLDLSRNALSFSGCCSYSDLGTNSLRHLDLSFNGAIIMSANFM
rTLR4           TNREDISFGQLALPSLRYLDLSRNAMSFRGCCSYSDFGTNNLKYLDLSFNGVILMSANFM
hTLR4           SNKGGNAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNGVITMSSNFL
moTLR4          ANKGGNAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNDVITMSSNFL
hoTLR4          SNKDMKSFNEVKLPSLEFLDLSRNRLSFKSCCSEADLKTTRLKHLDLSFNDVISMSSNFM
sTLR4           DNKDVSTFTKTELPSLQYLDLKRNHLSFKSCCSHTDFGTTNLKHLDLSFNDVITLGSNFM
gTLR4           DNKDVSSFTKTELPSLQYLDLKRNHLSFKTCCSHTDFGTTNLKHLDLSFNDVITLSSNFM
cTLR4           DNKDISTFTEFQLPSLQYLDLKRNHLSFKGCCSHTDFGTTNLKHLDLSFNDVITLGSNFM
bTLR4           DNKDISTFTEFQLPSLQYLDLKRNHLSFKGCCSHTDFGTTNLKHLDLSFNDVITLGSNFV
                                   *  *                          * **
                      LRR17                          LRR18                          LRR19
                   LxxLxLxxNxL                    LxxLxLxxNxL                    LxxLxLx
mTLR4           GLEELQHLDFQHSTLKRVTEFSAFLSLEKLLYLDISYTNTKIDFDGIFLGLTSLNTLKMA
rTLR4           GLEELEYLDFQHSTLKKVTEFSVFLSLEKLLYLDISYTNTKIDFDGIFLGLISLNTLKMA
hTLR4           GLEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSSLEVLKMA
moTLr4          GLEKLEHLDFQHSNLKQMSQFSVFLSLRNLIYLDISHTHTRVAFNGIFDGLLSLKVLKMA
hoTLR4          GLEQLEHLDFQHSTLKQASDFPVFLSLKNLRYLDISYTNTRVVFHGIFDGLVSLQVLKMA
sTLR4           GLEQLEHLDFQHSTLKQINAFSTFLSLRNLRYLDISYTNIRIVFHGIFTGLVSLQTLKMA
gTLR4           GLEQLEHLDFQHSTLKQINAFSTFLSLRNLRYLDISYTNIRIVFHGIFTGLVSLQTLKMA
cTLR4           GLEQLEHLDFQHSTLKQINAFSAFLSLRNLRYLDISYTNIRIVFHGIFTGLVSLQTLKMA
bTLR4           GLEQLEHLDFQHSTLKQINAFSAFLSLRNLRYLDISYTNIRIVFHGIFTGLVSLQTLKMA
                         * **                      * * *                          * *
                                          LRR20                          LRR21
                xNxL                 LxxLxLxxNxL                    LxxLxLxxNxL
mTLR4           GNSFKDNTLSNVFANTTNLTFLDLSKCQLEQISWGVFDTLHRLQLLNMSHNNLLFLDSSH
rTLR4           GNSFKDNTLSNVFTNTTNLTFLDLSKCQLEQISRGVFDTLYRLQLLNMSHNNLLFLDPSH
hTLR4           GNSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSLQVLNMSHNNFFSLDTFP
moTLR4          GNSFQENFLPDIFTDLKNLTFLDLSQCQLEQLSPTAFDTLNKLQVLNMSHNNFFSLDTFP
hoTLr4          GNSFKDNFLPNIFREMTNLTTLDLSKCNLEQVSQEAFCLLPRLRVLNMSHNNLLFLDMLP
sTLR4           GNSFQNNLLPDIFTELTNLTILDLSKCQLEQVSWAAFHSLPSLQVLNMSHNKLLSLDTFL
gTLR4           GNSFQNNLLPDIFTELTNLTILDLSKCQLEQVSWTAFHSLPSLQVLNMSHNKLLSLDTFL
cTLR4           GNSFQNNLLPDIFTELTNLTVLDLSKCQLEQVAQTAFHSLSSLQVLNMSHNKLLSLDTFL
bTLR4           GNSFQNNLLPDIFTELTNLTVLDLSKCQLEQVSQTAFHSLSSLQVLNMSHNKLLSLDTFL
                * *                      *  * *                          * **
                      LRR22                          LRR23
                   LxxLxLxxNxL                    LxxLxLxxNxL
mTLR4           YNQLYSLSTLDCSFNRIETSK-GILQHFPKSLAFFNLTNNSVACICEHQKFLQWVKEQKQ
rTLR4           YKQLYSLRTLDCSFNRIETSK-GILQHFPKSLAVFNLTNNSVACICEYQNFLQWVKDQKM
hTLR4           YKCLNSLQVLDYSLNHIMTSKKQELQHFPSSLAFLNLTQNDFACTCEHQSFLQWIKDQRQ
moTLR4          YKCLPSLQVLDYSLNHIMTSNNQELQHFPSSLAFLNLTQNDFACTCEHQSFLQWIKDQRQ
hoTLR4          YKPLHSLQILDCSFNRIVAFKWQELQHFPSSLASLNLTQNDFACVCEYQSFLQWVKDQRQ
sTLR4           YEPLHSLRILDCSFNRITASKEQELRNLPRNLTWLNLTQNEFACVCEHQSFLQWVKDQRQ
gTLR4           YEPLHSLRILDCSFNRITASKEQELRSLPRNLTWLNLTQNEFACVCEHQSFLQWVKDQRQ
cTLR4           YEPLHSLRILDCSFNRIMASKEQELQNLPRSLTWLNLTQNAFACVCEHQSFLQWVKDQRQ
bTLR4           YEPLHSLRILDCSFNRIMASKEQELQNLPRNLTWLNLTQNAFACVCEHQSFLQWVKDQRQ
                     *   ** *                          * *
```

Note: The number of LRRs in the extracellular domain does not include the LRR-NT and LRR-CT motifs. The organisms used for MSA are abbreviated as follows- mouse (mTLR4), rat (rTLR4), human (hTLR4), monkey (moTLR4), horse (hoTLR4), sheep (sTLR4), goat (gTLR4), cow (cTLR4) and buffalo (bTLR4). The conserved part of the LRR motifs (LxxLxLxxNxL) in the entire MSA had been marked with a user defined color code where positions 1, 4, 6 and 11 containing either leucine (L), isoleucine (I), valine (V) or phenylalanine (F) are colored in red, whereas any other substitution taking place in either of these positions are coded in green. Similarly, position 9 in the conserved segment consisting of asparagine (N), threonine (T), cysteine (C) or serine (S) is marked in blue and any change in this position is coded in purple. Highly conserved residues in the 'x' position of all organisms had been marked with '*'. The position indicating the start of the second and the third exon had been shown with an arrow sign.

Figure 5 Schematic representation of the domain architecture of TLR4

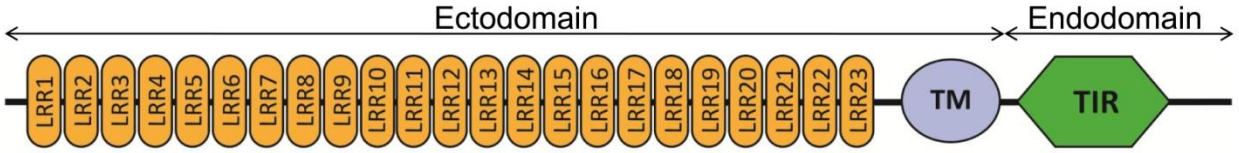


Figure 6 Phylogenetic tree representing the whole protein sequences of nine human TLRs (TLR1-TLR9)

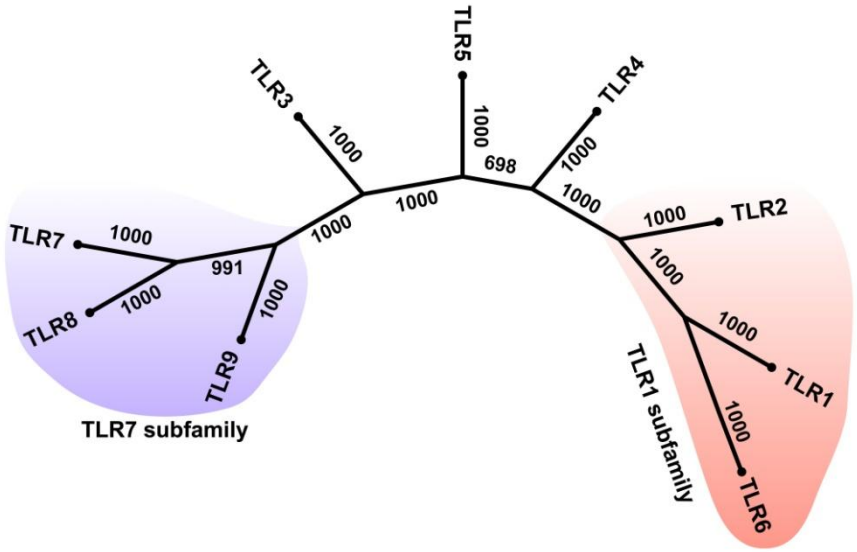


Figure 7 Phylogenetic tree depicting the ectodomain of the nine human TLRs

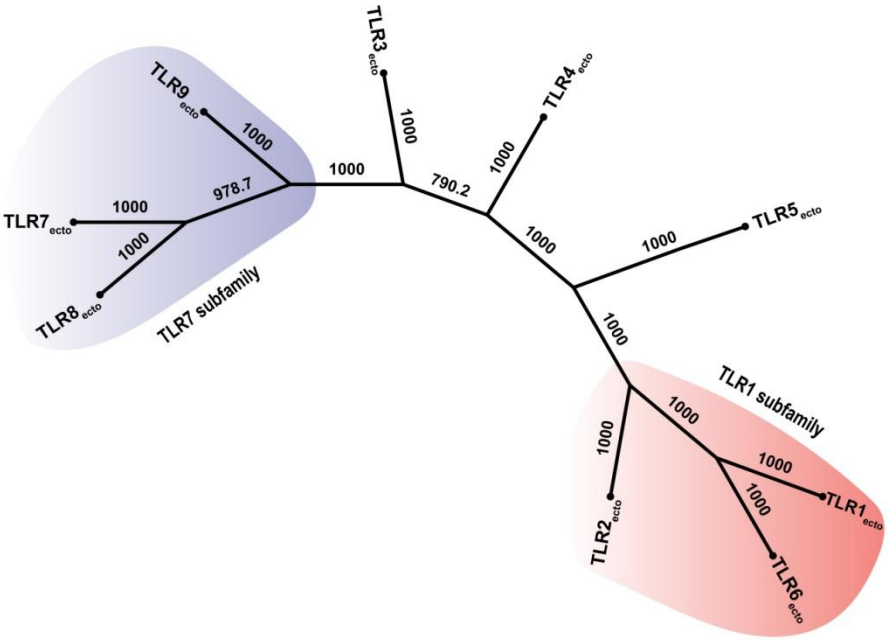


Figure 8 Phylogeny considering the TIR domain part of the nine human TLRs and SARM1, a non-TLR protein

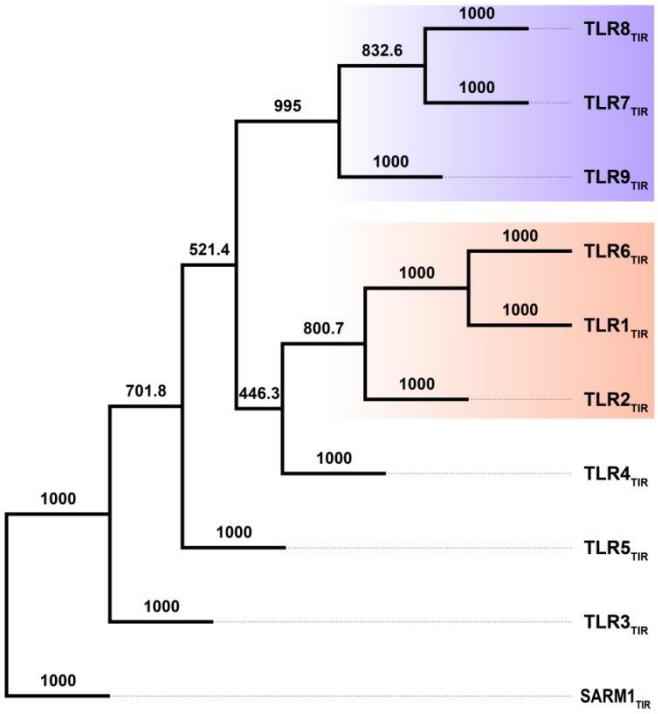


Figure 9a Phylogenetic tree of the TLR4 protein across seven metazoans

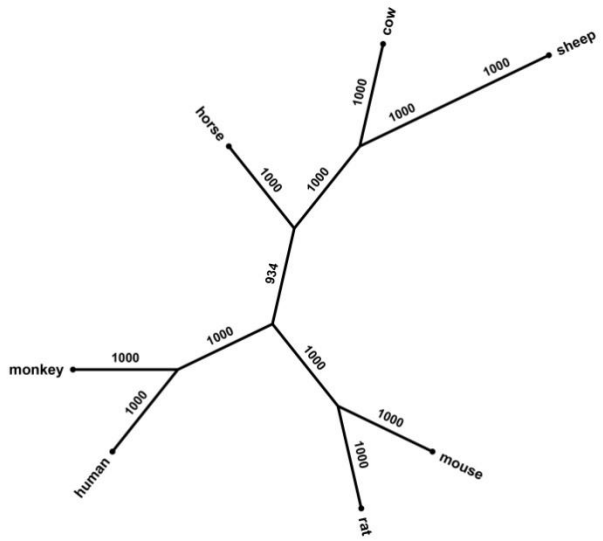


Figure 9b Phylogenetic tree of the TLR4 coding sequence of seven organisms

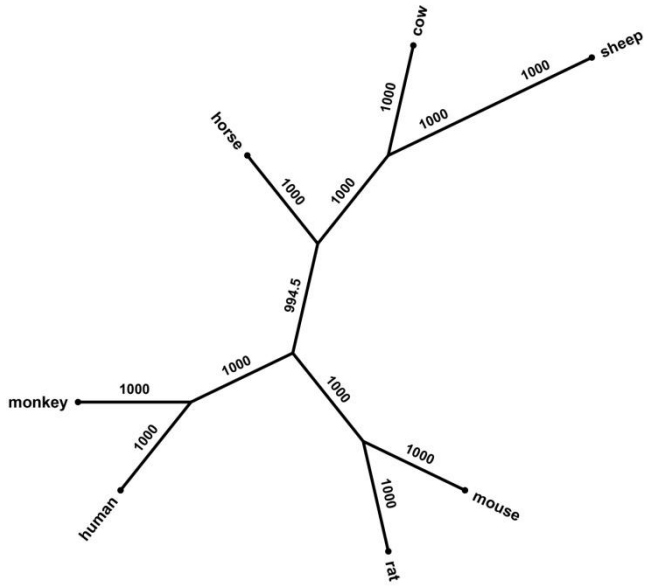


Figure 10a Phylogenetic tree with the protein sequence of 23 individual LRR motifs identified in TLR4 across seven organisms

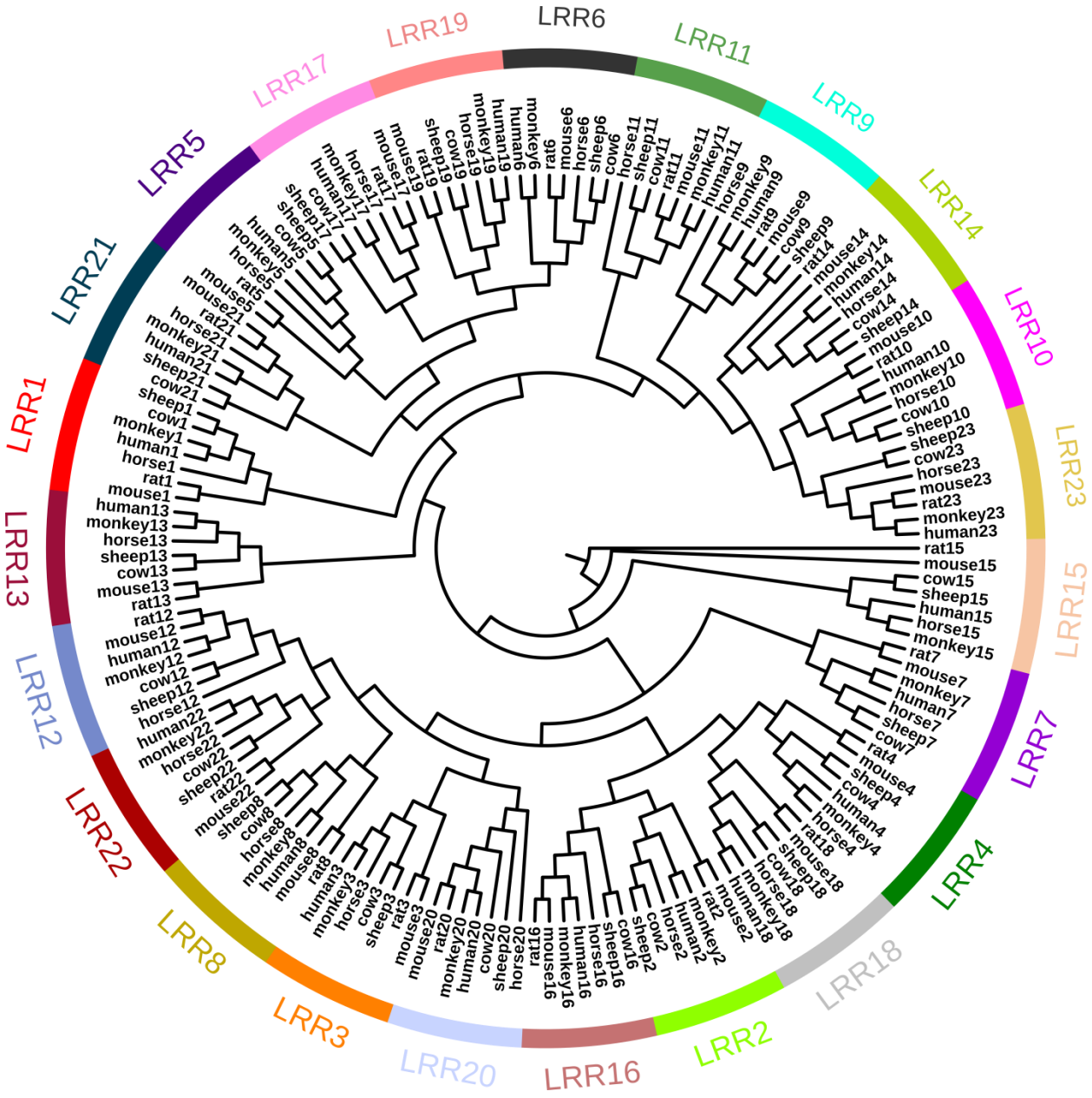
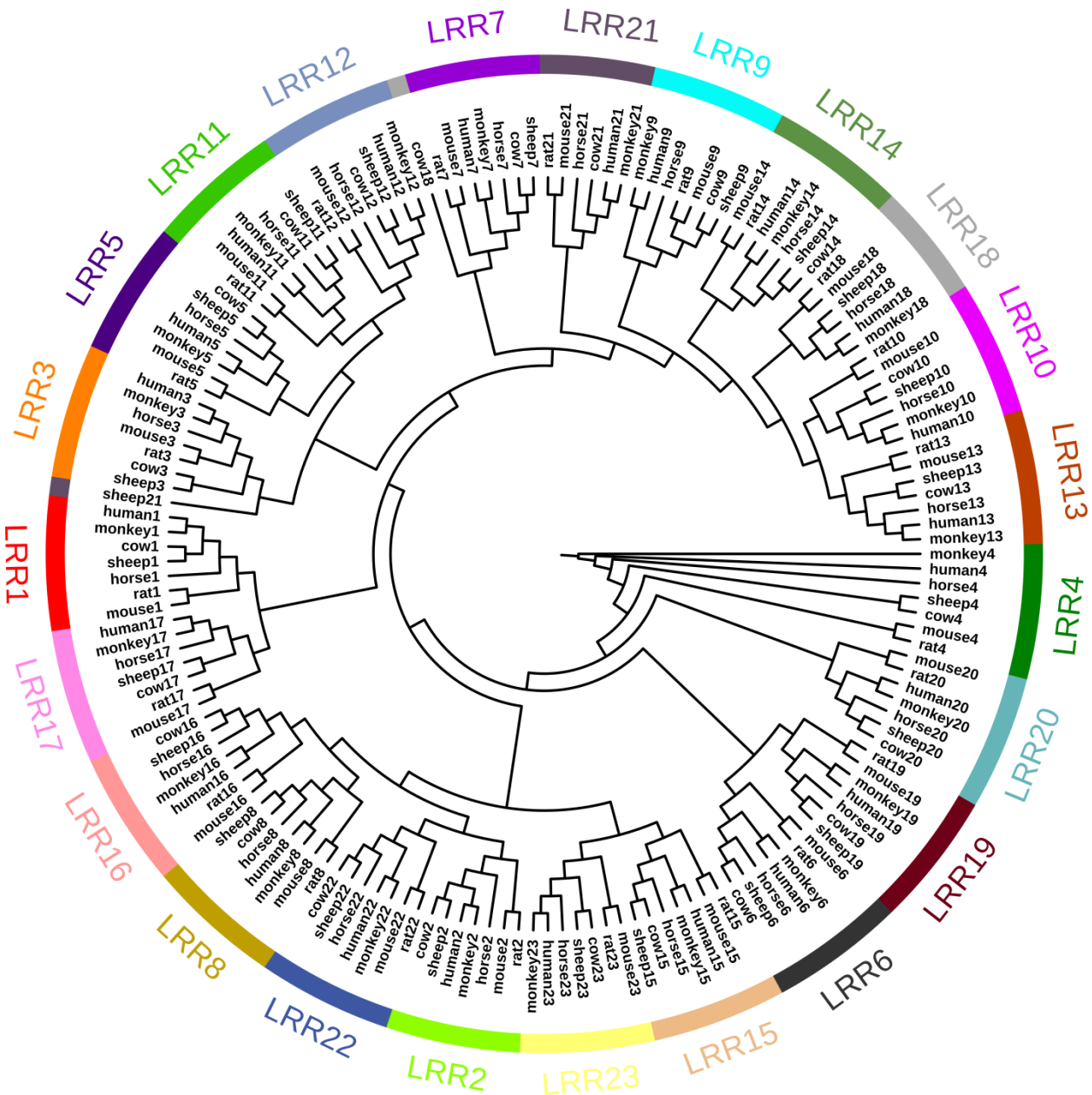
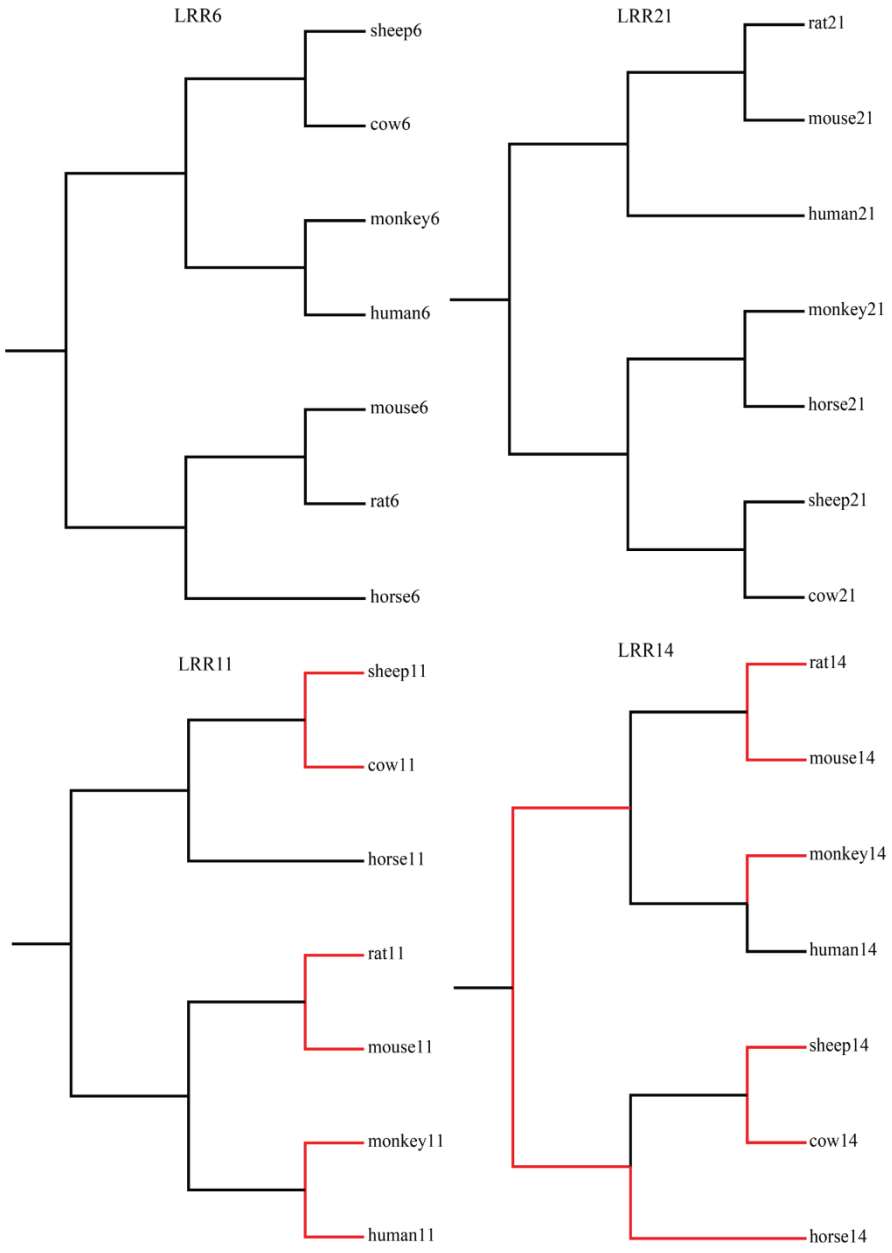


Figure 10b Phylogenetic analysis with the coding sequence of 23 individual LRR motifs across seven organisms

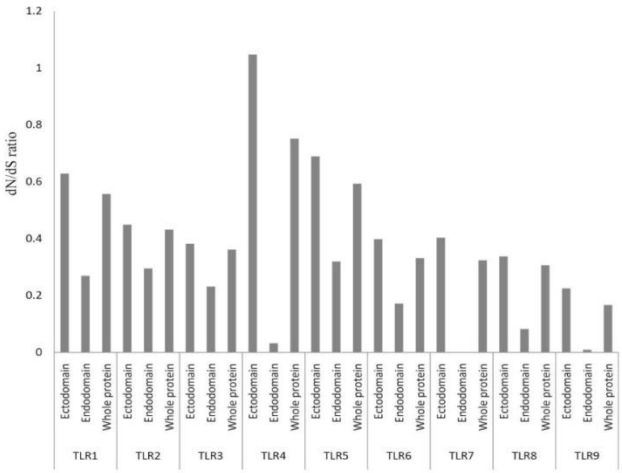


Note: The number beside each organism represents their corresponding LRR number.

Figure 11 K_a/K_s ratio tree showing selection pressure in 4 particular LRRs



Note: The top row shows negative selection (black) in LRR6 and LRR21 whereas the bottom row shows positive selection (red) in LRR11 and LRR14.

Figure 12 Tabular and graphical representation of the dN/dS calculations between human and monkey in nine TLRs

TLR	*Segment of the protein*	*Number of synonymous sites (S)*	*Number of non-synonymous sites (N)*	*Synonymous substitution rate (dS)*	*Non-synonymous substitution rate (dN)*	*dN/dS*
TLR1	Ectodomain	574.6	1327.4	0.0414	0.0260	0.6289
	Endodomain	126.9	308.1	0.0501	0.0135	0.2696
	Whole protein	705.7	1631.3	0.0425	0.0237	0.5575
TLR2	Ectodomain	550.5	1363.5	0.0504	0.0227	0.4495
	Endodomain	147.0	291.0	0.0364	0.0108	0.2953
	Whole protein	692.8	1659.2	0.0475	0.0205	0.4318
TLR3	Ectodomain	657.0	1602.0	0.0647	0.0247	0.3823
	Endodomain	127.7	325.3	0.0525	0.0122	0.2317
	Whole protein	788.2	1923.8	0.0624	0.0226	0.3618
TLR4	Ectodomain	610.4	1402.6	0.0367	0.0384	**1.0474**
	Endodomain	97.6	343.4	0.0908	0.0029	0.0323
	Whole protein	729.7	1724.3	0.0422	0.0317	0.7513
TLR5	Ectodomain	616.9	1453.1	0.0417	0.0287	0.6897
	Endodomain	169.1	334.9	0.0499	0.0160	0.3197
	Whole protein	776.1	1797.9	0.0440	0.0261	0.5932
TLR6	Ectodomain	565.5	1351.5	0.0563	0.0224	0.3982
	Endodomain	134.2	336.8	0.1078	0.0185	0.1718
	Whole protein	704.0	1684.0	0.0653	0.0217	0.3318
TLR7	Ectodomain	834.6	1829.4	0.0332	0.0134	0.4033
	Endodomain	138.2	344.8	0.0455	0.00004	0.0010
	Whole protein	980.3	2166.7	0.0347	0.0112	0.3238
TLR8	Ectodomain	683.0	1948.0	0.0538	0.0182	0.3377
	Endodomain	140.0	349.0	0.0361	0.0030	0.0820
	Whole protein	815.8	2304.2	0.0514	0.0158	0.3064
TLR9	Ectodomain	552.2	2048.8	0.1105	0.0249	0.2253
	Endodomain	100.8	394.2	0.2845	0.0026	0.0093
	Whole protein	657.0	2439.0	0.1276	0.0213	0.1668