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Molecular characterisation of Toll-like receptors in the black flying fox *Pteropus alecto*

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ABSTRACT

Bats are believed to be reservoir hosts for a number of emerging and re-emerging viruses, many of which are responsible for illness and mortality in humans, livestock and other animals. In other vertebrates, early responses to viral infection involve engagement of Toll-like receptors (TLRs), which induce changes in gene expression collectively leading to an “antiviral state”. In this study we report the cloning and bioinformatic analysis of a complete set of TLRs from the black flying fox *Pteropus alecto*, and perform quantitative tissue expression analysis of the nucleic acid-sensing TLRs 3, 7, 8 and 9. Full-length mRNA transcripts from TLRs homologous to human TLRs 1–10 were sequenced, as well as a nearly intact TLR13 pseudogene that was spliced and polyadenylated. This prototype data can now be used to design functional studies of the bat innate immune system.

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1. Introduction

Bats belong to the order Chiroptera which is divided into two suborders: Megachiroptera (megabats, also known as fruit bats or flying foxes), and Microchiroptera (microbats). They are highly diverse (>1000 species) and possess many unusual anatomical and physiological adaptations including flight, longevity, diverse thermoregulation, echolocation and constrained genome size (Calisher et al., 2006; Sulkin and Allen, 1974; Smith and Gregory, 2009). Bats are also known to harbour a huge range of viruses and have been increasingly implicated as carriers and/or natural reservoir hosts of numerous zoonotic viruses (Calisher et al., 2006; Wong et al., 2007). Fruit bats are known reservoir hosts of Hendra virus (Murray et al., 1995; Halpin et al., 2000), Nipah virus (Chua et al., 2000), Ebola virus (Leroy et al., 2005), Marburg virus (Towner et al., 2009), Melaka virus (Chua et al., 2007), and Australian bat lyssavirus (van der Poel et al., 2006; Speare et al., 1997), whereas microbats are known reservoir hosts of Rabies, and likely reservoir hosts of Severe acute respiratory syndrome coronavirus (SARS-CoV) (Lau et al., 2005; Li et al., 2005).

Although many of the viruses carried by bats are highly pathogenic in other mammals, experimental infections and virus

isolation studies have demonstrated that infected bats rarely display clinical symptoms associated with disease (Williamson et al., 1998; Calisher et al., 2006; Middleton et al., 2007; Towner et al., 2009). One unique aspect of bat biology that may be related to the role of bats as viral reservoirs was revealed in a recent study of bat DNA transposons. In contrast to other mammalian lineages, in which it was widely believed that DNA transposon activity had ceased at least 40 million years ago, multiple waves of extensive and recent DNA transposon activity were identified in the genomes of two microbat species. Speculating upon a possible connection with the ability of bats to withstand viral infections, the authors proposed either a general tolerance of bats for “genomic parasitism”, or that the extensive acquisition of DNA transposons may in fact be a consequence of bats’ tolerance of viruses (Ray et al., 2007, 2008). In contrast to bats’ seeming ability to withstand viruses, some hibernating species are highly vulnerable to fungal infections, as seen by the emergence of white nose syndrome in North America (Blehert et al., 2009). As a result of these observations, the question has been raised of whether bat immune systems may be qualitatively different to other mammals, particularly in response to viral infections. However, few studies have examined the innate or adaptive mechanisms involved in resistance to viral infection in any species of bat.

In vertebrates, viruses are first recognised by the innate immune system via pattern recognition receptors, including Toll-like receptors (TLRs), which can induce expression of type-I interferons

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leading to an “antiviral state” (Uematsu and Akira, 2007; Xiao, 2009). TLRs are considered by many to be the first line of defence, as they are located either extracellularly or in endosomal compartments, and thus are the first receptors to come into contact with invading microorganisms. TLRs are type-I integral membrane proteins characterised by a ligand binding, extracellular domain containing multiple leucine-rich repeat (LRR) regions, a trans-membrane (TM) domain and a highly conserved Toll/interleukin-1 receptor (TIR) domain which transduces signals and induces expression of innate immune response genes (Uematsu and Akira, 2007).

Ten different TLRs have been described in humans, sheep, cows and pigs (TLRs 1–10), while additional TLRs have been described in other mammals such as mice (TLRs 11, 12 and 13), and other vertebrates such as birds (TLRs 15 and 21) amphibians (TLRs 14, 16, 21 and 22) and fish (TLRs 14, 21, 22 and 23) (Roach et al., 2005). In numerous cases vestigial TLRs are present as pseudogenes, such as TLR10 in mice (Hasan et al., 2005) and TLRs 11 and 12 in humans (Roach et al., 2005). TLRs 3, 7, 8 and 9 are located in endosomes, and are important for innate immune recognition of microbial nucleic acids (Xiao, 2009). TLRs 3, 7, 8 recognise viral RNA, whereas TLR9 recognises viral, bacterial and protozoan DNA (Xiao, 2009; Uematsu and Akira, 2006). TLRs 1, 2, 4, 5, 6, and 11 are cell surface expressed and bind various protein, lipid and carbohydrate moieties in bacteria, protozoa and fungi (Uematsu and Akira, 2006; Yarovinsky and Sher, 2006; Roeder et al., 2004). TLRs 2 and 4 have also been demonstrated to bind viral proteins (Kurt-Jones et al., 2000; Bieback et al., 2002). Ligands for TLRs 10, 12 and 13 have not been identified, however TLR13 (as well as TLRs 3, 7 and 9), has been shown to interact with Unc93B1, a molecule involved in transport of proteins from the endoplasmic reticulum to endosomal compartments, thus indicating that it may be a virus-sensing TLR (Tabeta et al., 2006; Brinkmann et al., 2007).

In this report we hypothesised that fruit bats could have a unique arrangement of TLRs or differences in the structure of one or more TLRs that could be indicative of an atypical innate antiviral immune system. To address this, we cloned and analysed the complete set of TLR mRNAs from the fruit bat *P. alecto*, and performed extended analysis of the nucleic acid-sensing TLRs, including tissue expression analysis. Three TLR sequences from the fruit bat *Rousettus leschenaultii* were known prior to this study (Iha et al., 2010); however our report contains the first TLR sequences from a member of the genus *Pteropus*, and the first complete set of TLR sequences reported for any bat species to date. This information can now be used to study bat innate immunity to viruses and other pathogens.

2. Materials and methods

2.1. Tissue collection and RNA extraction

P. alecto bats were trapped in Southern Queensland, Australia, and transported alive by air to the Australian Animal Health Laboratory (AAHL) in Victoria, where they were euthanized for dissection using methods approved by the AAHL animal ethics committee. Tissues were stored at -80°C in RNAlater (Ambion). Total RNA was extracted from frozen *P. alecto* tissues using a Precellys 24 tissue homogeniser (Bertin technologies) and an RNeasy mini kit (Qiagen) with on-column DNase-I treatment (Qiagen) to remove traces of genomic DNA. Total RNA was extracted from peripheral blood mononuclear cells (PBMC) harvested from *P. alecto* blood by density centrifugation, using lymphoprep (Axis-Shield) and an RNeasy mini kit with on-column DNase-I treatment, but using Qias shredders (Qiagen) for homogenisation. Quantification of nucleic acids was carried out by absorption spectrometry.

2.2. PCR, rapid amplification of cDNA ends (RACE), cloning and DNA sequencing

PCR and RACE primers were designed using Clone Manager 9.0 (Sci-Ed Software) and are listed in Table 1. Total spleen RNA was reverse transcribed into cDNA using Omniscript reverse transcriptase (Qiagen) and full-length amplification was performed using Long-amp DNA polymerase (New England Biosystems). 5' and 3' RACE were performed using a GeneRACER kit (Invitrogen). PCR and RACE products were cloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit for sequencing (Invitrogen). M13 forward and M13 reverse primers were employed for DNA sequencing using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems) and an Applied Biosystems 3130 XL Genetic Analyser.

2.3. Genome analyses

TLR sequences were identified in the genome of the Malaysian flying fox *Pteropus vampyrus* located in the Ensembl database (assembly pteVam1, 2.63X coverage, July 2008) and the GenBank trace file archive using BLAST (Altschul et al., 1990). Sequence assemblies were performed using Seqman PRO (Lasergene) and Clone Manager 9.0 (Sci-Ed Software). Intron–exon maps were drawn using Fancy Gene v1.4 (<http://host13.bioinfo3.ifom-iec-campus.it/fancygene/>).

2.4. Phylogenetic and pair-wise analysis

Accession numbers of sequences used for comparative analysis are listed in Table 2. Sequence alignments and phylogenetic trees were generated using MEGA 4.1 (Tamura et al., 2007), using Clustal W (Thompson et al., 1994) and the Minimum Evolution method (Rzhetsky and Nei, 1992) with 500 bootstrap replicates. Pair-wise sequence analysis was conducted using Clone manager and Ka/Ks ratios were determined using K-Estimator 6.1 (Comeron, 1999).

2.5. Analysis of domain architecture and modelling

Protein domains were determined using the Simple Modular Architecture Research Tool (SMART) (Letunic et al., 2009). TM domains were identified using TMHMM 2.0 (Krogh et al., 2001), TMPRED (Hofmann and Stoffel, 1993), HMMTOP (Tusnady and Simon, 2001), and Clone Manager 9.0 (hydrophobicity). Protein structures were modelled using CPHmodels 3.0 (Lund et al., 2002) and PyMOL 1.2.8 (DeLano scientific).

2.6. qPCR

Quantitative PCR (qPCR) was performed using tissues collected from three apparently healthy wild-caught *P. alecto* bats. Total RNA was prepared from peripheral blood mononuclear cells (PBMC), lymph node, spleen, liver, lung, heart, kidney, small intestine, brain and salivary gland, following the procedure described in Section 2.1. cDNA was generated using a Quantitect reverse transcription kit for RT-PCR (Qiagen). For each sample, 500 ng total RNA was used as template in a 20 μl cDNA synthesis reaction. qPCR primers were designed using Primer Express 3.0 (Applied Biosystems) with default parameter settings, and are listed in Table 1. Reactions were carried out using EXPRESS SYBR[®] GreenER[™] qPCR Supermix Universal (Invitrogen) and an Applied Biosystems 7500 Fast Real-Time qPCR instrument. Final qPCR reaction volumes were 10 μl and carried out in 96-well plates. Each reaction contained a final concentration of 200 nmol each primer and 2 μl of 1:5 diluted cDNA, equivalent to 10 ng total starting material. Thermocycling consisted of 90 $^{\circ}\text{C}/1$ min, 40 cycles of 90 $^{\circ}\text{C}/15$ s \rightarrow 60 $^{\circ}\text{C}/1$ min, and was followed by melt curve analysis. Copy numbers of target sequences

Table 1
RACE, full-length amplification, and qPCR primers used in this study.

Gene	Primer	Nucleotide sequence (5'–3')	Application
TLR1	5'	TGATAGGATGTCAGAAGTCCGAAGCTCAG	RACE
	3'	GTGAAACTTGGCAAGGTGAAGACAC	RACE
	Forwards	CAGATGGCAAGTTGTTTCG	Full-length amplification
	Reverse	CCATTATGTGGCCAAGCAG	Full-length amplification
TLR2	5'	CCCTTCTCCCAACAGGCTGATTGC	RACE
	3'	ACGGAAGATGATGAACACCAAGACTACC	RACE
	Forwards	TCTTCCCAGCCAGCGACTC	Full-length amplification
	Reverse	GATCACCAGCAGACCAGAC	Full-length amplification
TLR3	5'	TGGCACAATTCTGGCTCCAGTTTTGA	RACE
	3'	CTGGCCACTTCAGAAAGAAC	RACE
	Forwards	TGGAGAAGCTACTCTGATTA	Full-length amplification
	Reverse	CCCTCCCAATTTAGTCTTTA	Full-length amplification
	RT-PCR forwards	TGGGACTGTGCCCTTTTG	qPCR
	RT-PCR reverse	CTACTTCATGCCTCACAGCACAT	qPCR
TLR4	5'	AAGGGTCCCAGCTCTCAGGTCTC	RACE
	3'	GCGGCACATCTCTGGAGACGAC	RACE
	Forwards	CACCGAGCAAGGAAAGAAG	Full-length amplification
	Reverse	GAATCCAGCTGCACAAGAAG	Full-length amplification
TLR5	5'	TAAGGCCATCCGACCATCAGAGGAG	RACE
	3'	ACGTCCGGTGGTTTCTCGATAAACTCTC	RACE
	Forwards	TGACCACCATCATGTCTGAG	Full-length amplification
	Reverse	TGTCCGCATCTGTAAGTTC	Full-length amplification
TLR6	5'	CAGAGAAGTGGAAATGTCGTTCCAACACTAC	RACE
	3'	ACAGAACTTGCACTTGGGTTGAGAG	RACE
	Forwards	AAGATCAGCCACAACCTTTT	Full-length amplification
	Reverse	GATTTCTTAAGTTGGATTTT	Full-length amplification
TLR7	5'	GACATCACAGGGCAGAGTTT	RACE
	3'	AAGAGGCTCTGTGGAGTTC	RACE
	Forwards	CCCATCTTTGGCTGACCTTG	Full-length amplification
	Reverse	CAGAGTCCCACCTCTTCTAC	Full-length amplification
	RT-PCR forwards	CCTGGATGGAACCAGCTTCT	qPCR
	RT-PCR reverse	GCTCAGCAGCCGTAAGCTAGA	qPCR
TLR8	5'	TGGGGAACCTCTTTCAGC	RACE
	3'	CCTCCAGTGGCCTGACAATC	RACE
	Forwards	CCTGGCCCTTCACCTGTACCTC	Full-length amplification
	Reverse	GGGTCACTTCTTTGATTTG	Full-length amplification
	RT-PCR forwards	CGGGAACGTCCAAGGTGTT	qPCR
	RT-PCR reverse	TTCTGCGCAAGGCGTACA	qPCR
TLR9	5'	GCATGGCGGCCTGCACCAGGAGAG	RACE
	3'	TGACCAGGGACAACCCTACTTCTATAAC	RACE
	Forwards	ACCAAGACCCTCAGGAGAAG	Full-length amplification
	Reverse	CCGGTCGCATTATTTAGTGTG	Full-length amplification
	RT-PCR forwards	TGCAAGAACTTGACCTTCACCTT	qPCR
	RT-PCR reverse	AGAGCCCAGGCAACATCT	qPCR
TLR10	5'	GTCTGAGACCTGCCAGTGAATACCAAG	RACE
	3'	TCGACAGAGGTGAATGAAGAGTCTCAAG	RACE
	Forwards	ACAAGGGAACAGTGTCTAAA	Full-length amplification
	Reverse	GGCAACTCCAACATTATAG	Full-length amplification
TLR13	5'	TCTTCCATGTGACTTGGCTGCAAGG	RACE
	3'	GTGCCATGAGAGGCCACTCTTCTG	RACE
	Forwards	TGCCGTTGACGGTGATTTT	Full-length amplification
	Reverse	CCTGCATGGTGTCTCTGTTG	Full-length amplification
18s	RT-PCR forwards	CACGGCGACTACCATCGAA	qPCR
	RT-PCR reverse	CGGCGACGACCATTTC	qPCR

were calculated using standard curves and normalised relative to 18s ribosomal RNA.

3. Results

3.1. Identification of Toll-like receptors in pteropid bats from existing sequence data

Although no genome sequence is yet available for *P. alecto*, a low-coverage genome sequence is publicly available from the closely related pteropid bat *P. vampyrus*. This genome has been automat-

ically assembled and annotated based on homology to the human genome. As a result, low-coverage assemblies of TLRs 1, 3, 4, 7 and 8 were available through Ensembl by keyword searching. In addition; TLRs 3; 7 and 9 from the Fulvous fruit bat; *R. leschenaultii* have been sequenced previously (AB472355–AB472357). Draft sequences of additional TLR-like genes were identified by BLAST searches of the *P. vampyrus* genome; and were subsequently reassembled from *P. vampyrus* trace file data obtained from the GenBank trace file archive. Near-complete draft sequences corresponding to mammalian TLRs 1–10 and TLR13 were identified in the *P. vampyrus* genome; as well as three additional short fragments resembling

Table 2
Accession numbers of TLR protein sequences used in phylogenetic analysis.

Species	TLR3	TLR7	TLR8	TLR9
Mammals				
Bats				
<i>Rousettus leschenaultii</i> (Leschenault's rousette)	BAH02555.1	BAH02556.1		BAH02557.1
Carnivores				
<i>Canis lupus familiaris</i> (dog)	XP_540020.2	ABC69204.1		NP_001002998.1
<i>Felis catus</i> (domestic cat)	NP_001073298.1	NP_001073602.1	ABS28967	NP_001009285.1
Even-toed ungulates				
<i>Bos taurus</i> (cattle)	NP_001008664.1	ABC69205.1	ABN71683.1	NP_898904.1
<i>Ovis aries</i> (sheep)	NP_001129400.1	NP_001128531.1	ACQ57805.1	NP_001011555.1
<i>Sus scrofa</i> (pig)	NP_001090913.1	BAG68214.1	ABQ52585.1	NP_999123.1
Odd-toed ungulates				
<i>Equus caballus</i> (horse)	NP_001075267.1	NP_001075240.1	NP_001104771	NP_001075259.1
Placentals				
<i>Loxodonta africana</i> (African savanna elephant)	ABC95781.1	ABC95782.1	ABS28968	
Primates				
<i>Aotus nancymae</i> (Ma's night monkey)				AAX14714.1
<i>Callithrix jacchus</i> (white-tufted-ear marmoset)	ACC68086.1			
<i>Cercocebus torquatus atys</i> (sooty mangabey)	ABY64989.1	ABY64994.1	ABY64997.1	ABY64998.1
<i>Gorilla gorilla</i> (Western Gorilla)	BAG55031.1	BAG55059.1	BAG55066.1	BAG55073.1
<i>Homo sapiens</i> (human)	NP_003256.1	NP_057646.1	AAI01076.1	NP_059138.1
<i>Hylobates lar</i> (common gibbon)	ACC68077.1	ACC68081.1	ACC68082.1	
<i>Macaca fascicularis</i> (crab-eating macaque)	BAG55033.1		BAG55068.1	
<i>Macaca mulatta</i> (rhesus monkey)	BAG55034.1	NP_001123898.1	NP_001123899.1	NP_001123903.1
<i>Pan paniscus</i> (pygmy chimpanzee)	BAG55030.1	BAG55058.1	BAG55065.1	BAG55072.1
<i>Pan troglodytes</i> (chimpanzee)	NP_001123942.1	NP_001123605.1	NP_001123944.1	NP_001138338.1
<i>Pongo pygmaeus</i> (Bornean orangutan)	BAG55032.1	BAG55060.1	BAG55067.1	BAG55074.1
Rabbits & hares				
<i>Oryctolagus cuniculus</i> (rabbit)	NP_001075688.1			
Rodents				
<i>Cavia porcellus</i> (domestic guinea pig)	ABD77101.1			
<i>Mus musculus</i> (house mouse)	NP_569054.2	NP_573474	NP_573475.2	NP_112455.2
<i>Rattus norvegicus</i> (Norway rat)	NP_942086.1	NP_001091051.1	NP_001094479.1	NP_937764.1
Marsupials				
<i>Monodelphis domestica</i> (gray short-tailed opossum)	XP_001368540.1		XP_001381093.1	XP_001380386.1
Monotremes				
<i>Ornithorhynchus anatinus</i> (platypus)		XP_001515229.1	XP_001515241.1	XP_001506154
Birds				
<i>Anas platyrhynchos</i> (mallard)		ABK51522.1		
<i>Gallus gallus</i> (chicken)	ABL74502.1	CAG15146.1		
<i>Taeniopygia guttata</i> (zebra finch)	XP_002190888.1	XP_002194932.1		
Amphibians				
<i>Xenopus (Silurana) tropicalis</i> (western clawed frog)		NP_001120883.1		
Fish				
<i>Carassius auratus</i> (goldfish)	ABC86865.1			
<i>Ctenopharyngodon idella</i> (grass carp)	ABI93941.1			
<i>Cyprinus carpio</i> (common carp)	ABL11473.1			
<i>Danio rerio</i> (zebrafish)	AAT37633.1	XP_701101.3	XP_001340186.2	NP_001124066.1
<i>Dentex tumifrons</i> (yellowback seabream)				ABY79218.1
<i>Epinephelus coioides</i> (orange-spotted grouper)				ACV04893.1
<i>Gobiocypris rarus</i>	ABL11471.1			
<i>Ictalurus punctatus</i> (channel catfish)	ABD93872.1			
<i>Megalobrama amblycephala</i> (Wuchang bream)	ABI83673.1			
<i>Oncorhynchus mykiss</i> (rainbow trout)	AAX68425.1	ACV41797.1	ACV41799.1	
<i>Pagrus major</i> (red seabream)				ABY79217.1
<i>Salmo salar</i> (Atlantic salmon)			NP_001155165.1	NP_001117125.1
<i>Takifugu rubripes</i> (torafugu)	AAW69373.1	AAW69375.1	AAW69376.1	
<i>Tetraodon nigroviridis</i> (spotted green pufferfish)	CAF96592.1			

TLRs 10, 11 and 12. The three truncated fragments were not examined in further detail as they do not appear to be transcribed and are unlikely to encode proteins.

3.2. Sequencing of *P. alecto* TLR mRNAs

Full-length mRNA transcripts of TLRs 1–10 and TLR13 were sequenced from cDNA made from poly A⁺ RNA isolated from the spleen of an apparently healthy, wild-caught *P. alecto* fruit bat. Initially, RACE primers were designed from the draft sequences of *P. vampyrus* TLRs for end-sequencing of *P. alecto* TLR mRNA. RACE products were obtained for all 11 putative genes and featured splicing as determined by alignment with the *P. vampyrus* genome, demonstrating that they were transcribed *in vivo*. PCR primers

designed from the RACE products were then used to amplify the full-length mRNAs for TLRs 1–10 and TLR13. For each gene we chose one allele and selected the longest transcript to record as the prototype. Sequences of *P. alecto* mRNA for TLRs 1–10 and TLR13 were deposited into GenBank under the accession numbers GU045600–GU045610.

To minimise sequencing and assembly errors, a long-range, proof-reading DNA polymerase was used to amplify the entire predicted coding region of each gene as a single fragment, thus eliminating the possibility of chimeric sequence assemblies. Preliminary alignments of nucleotide sequence data from *P. alecto* and *P. vampyrus* confirmed that the two bats were very closely related at the genetic level, and suggested that TLRs 1 and 6 had approximately 1000 bp of nearly identical sequence at the 3' end. To resolve

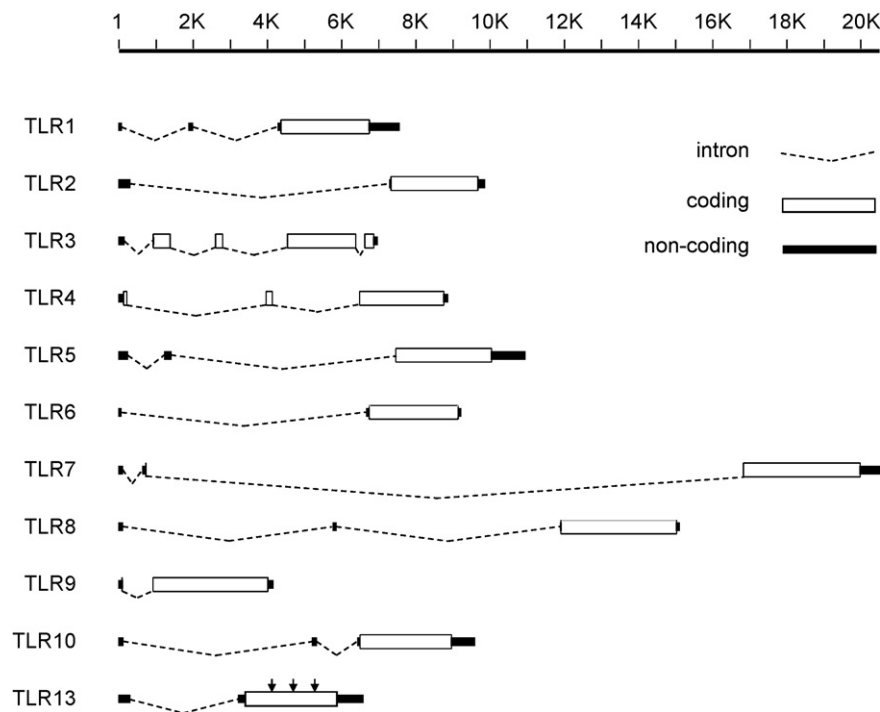


Fig. 1. Predicted intron–exon organisation of pteropid bat TLRs 1–10 and TLR13. The intron–exon arrangements of pteropid bat TLRs were predicted by nucleotide alignment of *P. alecto* mRNA and *P. vampyrus* genomic DNA. Putative coding and non-coding sequences are drawn as light and dark rectangles respectively, while introns are indicated by dotted lines. *P. alecto* TLR13 is predicted to be a pseudogene due to the presence of three in-frame stop codons, as indicated by arrows. The figure is drawn to scale and distances are given in bp, however introns in TLRs 2, 4, 6, 7, 8 and 10 in the *P. vampyrus* genome assembly contained gaps so the actual sizes of these introns are estimates. The first exon of TLR5 was not represented in the *P. vampyrus* genome assembly and is therefore only assumed to be contiguous. Correspondingly, the first intron of TLR5 has been arbitrarily drawn at 1000 bp. In all other cases, intron–exon boundaries were clearly identified by homology in other species, and by the presence of splice site consensus sequences. In TLRs 7 and 9, the first coding exon contains only three coding nucleotides (ATG) that are hard to see in the diagram due to the scale.

this, 3' RACE primers for these genes were positioned upstream of the homologous region, and for all of the TLRs, the entire coding region was amplified by PCR as a single large product for sequence confirmation. As a result, it was shown that *P. vampyrus* TLR1 and TLR6 genes were miss-assembled in Ensembl, where they are represented as a single gene rather than two loci. The *P. vampyrus* gene identified in Ensembl as TLR1 is a chimera consisting of ~1300 bp from the 5' end of TLR1 followed by ~1000 bp of TLR6. As predicted, the two genes have approximately 1000 bp of nearly identical sequence at the 3' end; however they diverge considerably in the final 160 bp of the coding region.

3.3. Gene organisation of Pteropid bat TLRs

P. alecto mRNAs for TLRs 1–10 each had one long open reading frame (ORF) with start and stop codons comparable to those in the TLRs of other mammalian species. In contrast, *P. alecto* TLR13 lacked a suitable start codon and contained three in-frame stop codons, suggestive of a recently inactivated pseudogene. Based on the confirmed *P. alecto* mRNA sequences, it was possible to reassemble genomic DNA sequences for *P. vampyrus* TLRs using shotgun sequence data available in the GenBank trace file archive, enabling a more accurate comparison of *P. alecto* mRNA with *P. vampyrus* genomic DNA. Alignments of *P. alecto* mRNA and *P. vampyrus* genomic DNA were performed for each gene, to determine the intron–exon organisation and splice sites of TLRs of the two pteropid bat species (Fig. 1). Due to gaps in some of the introns within the *P. vampyrus* genome, some of the intron sizes had to be estimated, however in all cases splice site consensus sequences were used as the basis for assigning intron–exon junctions. TLRs 2, 6, 9 and 13 were each found to consist of two exons, while TLRs 1, 4, 5, 7, 8 and 10 each consisted of three exons. TLR3 consisted of

five exons. The coding regions of TLRs 1, 2, 5, 6, 8 and 10 were each located within a single exon, while the coding regions of TLRs 7 and 9 were spread across two coding exons, the first of which contains just the first three nucleotides corresponding to the start codon. The coding region of TLR3 was spread out across four exons, while the coding region of TLR4 was spread across three exons.

P. alecto TLR13 was transcribed, spliced and polyadenylated, despite the fact that it appears to be an inactivated pseudogene. *In vivo* transcription was demonstrated by the fact that it could be amplified from cDNA generated from DNase-I treated *P. alecto* RNA. The identification of a large intron with GT–AG splice sites provided evidence of mRNA splicing, and a putative polyadenylation signal (AATAAA) located a few bases upstream of a poly A+ tail provided evidence of polyadenylation (although the location of the poly A+ tail corresponded to a run of A's in the *P. vampyrus* genome, so it was unclear whether this was a genuine example of polyadenylation). In addition, the 5' end of exon 1 featured a 170 bp sequence widely represented in the *P. vampyrus* genome, and the intron contained a sequence resembling a LINE-1 transposable element. A frame-shift mutation relative to mouse TLR13 was also observed close to the 3' end of *P. alecto* TLR13, consisting of a 2 bp deletion that was present in both *P. alecto* and *P. vampyrus*.

3.4. Phylogenetic and pair-wise analysis of *P. alecto* TLRs

Due to the importance of TLRs 3, 7, 8 and 9 in viral recognition, we performed phylogenetic analyses on these from *P. alecto* with a variety of other mammals and non-mammalian vertebrates, including TLRs 3, 7 and 9 from *R. leschenaultii*, that were obtained from GenBank (Table 2, Fig. 2a–d). *P. alecto* and *R. leschenaultii* TLRs 3 and 7 clustered with other members of Laurasiatheria (horse, dog, cat). In contrast, *P. alecto* TLR8 was an outlier in the Laurasiatheria

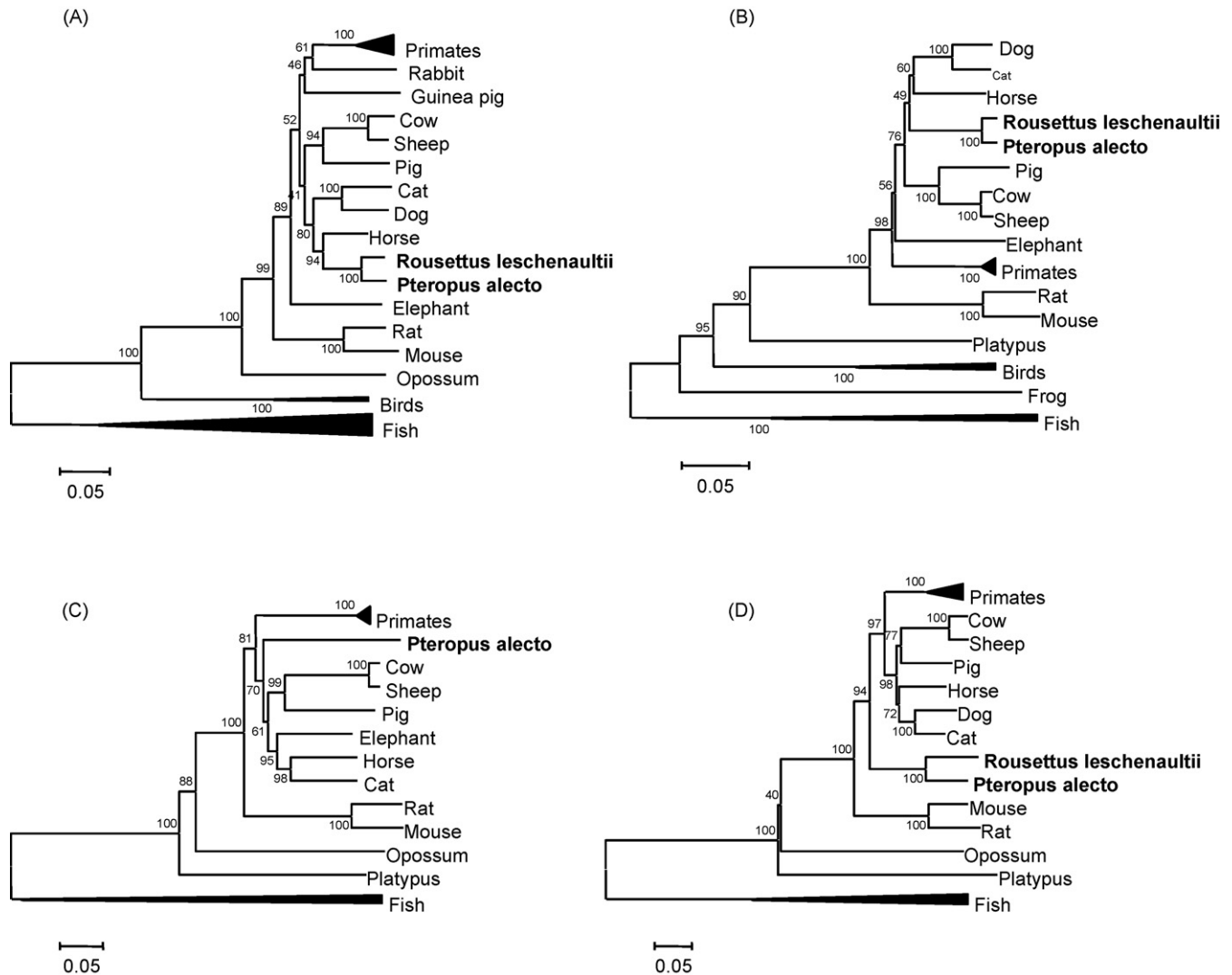


Fig. 2. Phylogenetic analysis of the virus-sensing TLRs 3, 7, 8 and 9 in *P. alecto* and other vertebrates. Phylogenetic trees generated from amino acid sequence alignments of (A) TLR3, (B) TLR7, (C) TLR8 and (D) TLR9 from *P. alecto* and other vertebrates. Refer to Table 2 for a list of accession numbers of protein sequences.

group and *P. alecto* and *R. leschenaultii* TLR9 sequences did not cluster within Laurasiatheria at all. Laurasiatherian and primate TLR9 genes appeared to be more closely related to each other than they were to bat TLR9; however bat TLR9 was more similar to both of these groups than it was to rodent TLR9.

P. alecto TLRs were also subjected to pair-wise analysis with human, bovine and mouse TLRs (Table 3). In each case the RNA-sensing TLR7 was found to be the most conserved TLR at the protein level, whereas TLRs 8, 2 and 4 were found to be the least conserved relative to human, bovine and mouse, respectively. The TLR13 pseudogene in *P. alecto* was approximately 78% identical to mouse TLR13 at the DNA level, and if the frame-shift mutation in *P. alecto* TLR13 was corrected, approximately 74% identical at the protein level. A sequence resembling TLR13 identified in the bovine genome (which also appears to be a pseudogene) had even greater similarity to *P. alecto* TLR13, with 83% identity at the DNA level and 76% identity at the protein level after frame-shift corrections were applied (Table 3). Pair-wise analysis was also performed on the *P. alecto* TLRs with sequences from the reassembled draft sequences from *P. vampyrus* and TLRs 3, 7 and 9 from *R. leschenaultii* (Iha et al., 2010). All *P. alecto* and *P. vampyrus* TLRs were approximately 98% identical at the DNA level and approximately 97% identical at the protein level (not shown). *P. alecto* and *R. leschenaultii* TLRs 3, 7 and 9 were

96, 97 and 92% identical at the DNA level, and 95, 97 and 87% identical at the protein level, respectively (not shown). *P. alecto* TLR9 also had a 6 bp insertion relative to *R. leschenaultii* TLR9.

When *P. alecto* TLR genes were aligned with TLRs in other species, the percent sequence identities were almost invariably higher at the DNA level than at the protein level (Table 3). This prompted us to examine the ratio of non-synonymous to synonymous nucleotide substitutions (the Ka/Ks ratio), which can be informative about the type of selection pressure acting on genes (Hurst, 2002). Ka/Ks ratios were calculated for pair-wise alignments of the coding regions of *P. alecto* TLRs with those in human, bovine and mouse, however, none were found to be >1 (Table 3).

3.5. Domain architecture and modelling of the predicted *P. alecto* TLR proteins

TLRs 1–10 typically encode transmembrane receptors. To verify this, the domain architecture of the *P. alecto* TLRs were determined by SMART analysis, which combines homology searches with predictive algorithms to identify putative protein domains. Signal peptides, LRR domains and TIR domains were identified in *P. alecto* TLRs 1–10 (Fig. 3). TM domains were identified by SMART in all *P. alecto* TLRs with the exception of TLR9. SMART utilises a hid-

Table 3
Similarity of *P. alecto* TLRs to human, bovine and mouse genes.

Gene	Accession	% identity								
		Human			Bovine			Mouse		
		nt	aa	Ka/Ks	nt	aa	Ka/Ks	nt	aa	Ka/Ks
TLR1	GU045600	84	78	0.43	86	79	0.45	76	70	0.27
TLR2	GU045601	83	76	0.43	83	75	0.51	73	67	0.29
TLR3	GU045602	86	83	0.29	85	82	0.29	79	76	0.23
TLR4	GU045603	83	74	0.52	82	76	0.38	72	65	0.39
TLR5	GU045604	82	78	0.30	80	77	0.30	73	70	0.26
TLR6	GU045605	85	80	0.37	85	80	0.39	75	69	0.28
TLR7	GU045606	88	86	0.32	88	86	0.31	81	79	0.25
TLR8	GU045607	80	71	0.57	78	72	0.42	75	70	0.34
TLR9	GU045608	80	75	0.41	82	78	0.43	75	71	0.34
TLR10	GU045609	85	79	0.33	85	81	0.30	–	–	–
TLR13	GU045610	–	–	–	83 ^a	76 ^a	–	78 ^a	74 ^a	–

^a *P. alecto* TLR13 is a pseudogene with no counterpart in humans. It aligns with high homology to a 2800bp region of mouse TLR13 and a bovine sequence which also appears to be a pseudogene. The amino acid sequence similarities of TLR13 are derived from a conceptual translation in which the bat ($n=1$) and bovine ($n=6$) frame-shift mutations have been corrected.

den Markov model for prediction of TM domains (TMHMM) and also fails to identify TM domains in TLR9 in other species, including human (not shown). An alternative method, HMMTOP, also failed to predict a TM domain, however TMPRED and hydrophobicity modelling enabled identification of the TM domain of *P. alecto* TLR9 (Fig. 4a). When *P. alecto* TLR5 was examined using the above meth-

ods, two putative TM domains were predicted with high probability scores using all three predictive models as well as hydrophobicity modelling (Fig. 4b). The second predicted TM domain was located within the TIR domain, explaining why it was not observed by SMART. Although the structure of TLR5 has not been solved, structures of human TLR1 and 2 TIR domains are available (Xu et al.,

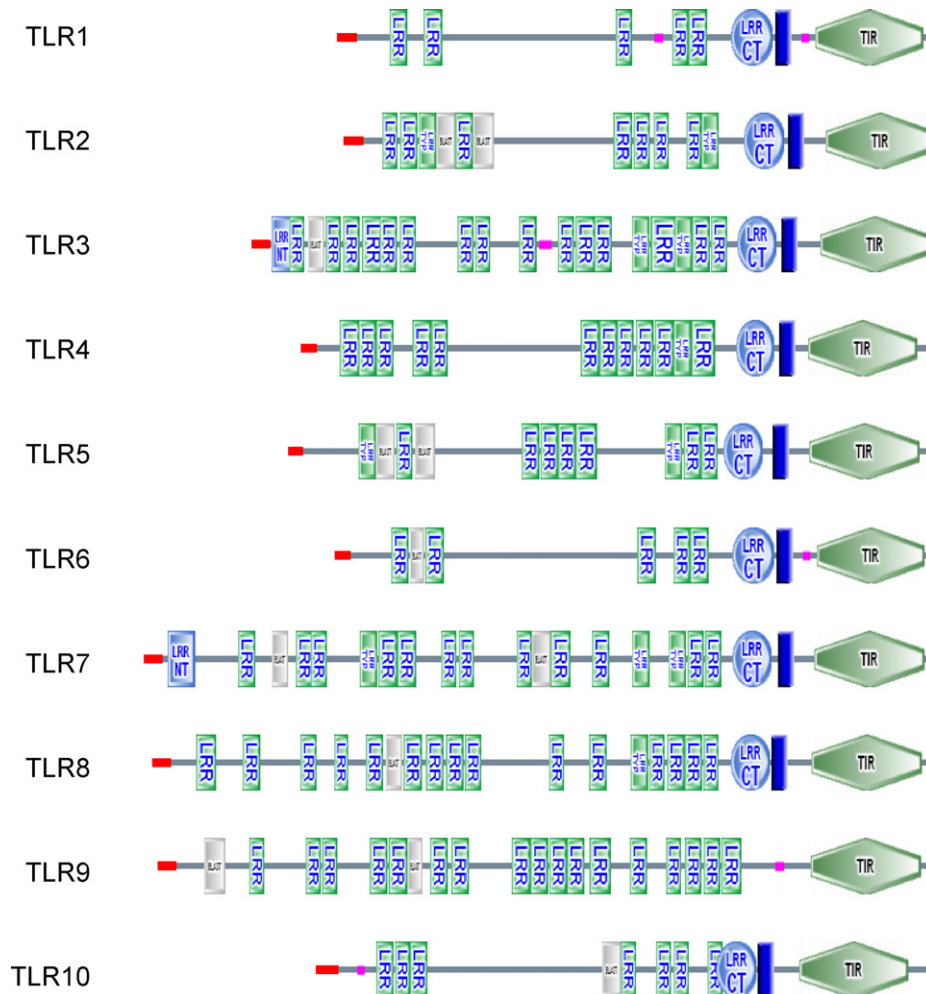


Fig. 3. Predicted protein domain architecture of *P. alecto* TLRs 1–10. Protein domain architectures of *P. alecto* TLRs 1–10 were determined by SMART analysis. Signal peptides are shown in red, TM domains in dark blue and low complexity regions in pink. Other domains including LRR domains and TIR domains are labelled accordingly. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

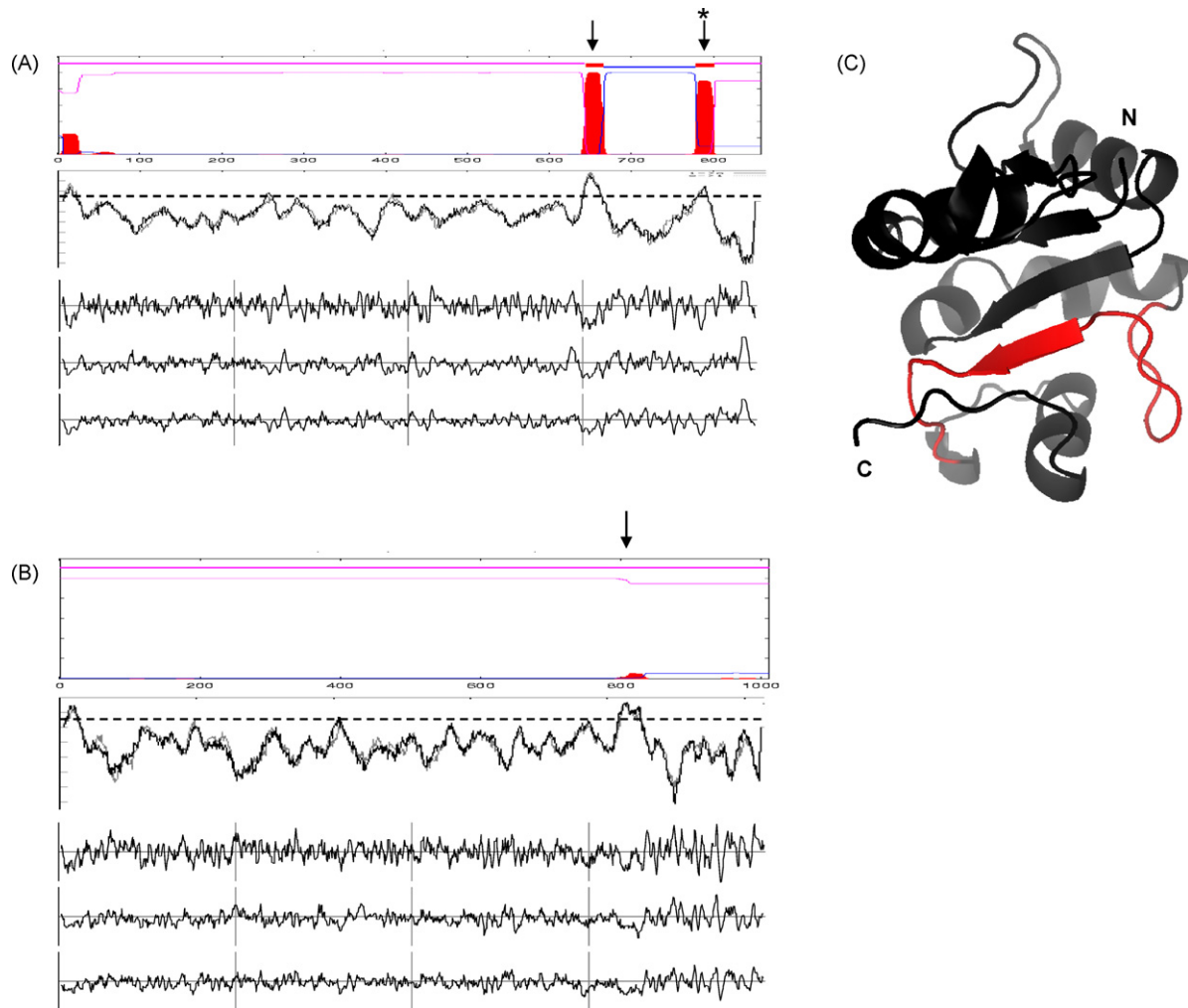


Fig. 4. Transmembrane domain prediction in *P. alecto* TLRs 5 and 9. TM domain predictions were performed for (A) *P. alecto* TLR5 and (B) *P. alecto* TLR9. The output from two predictive algorithms (TMHMM, TMPRED) and three models of hydrophobicity (Kyte and Doolittle, Hopp and Woods, Surface exposure) are shown top to bottom for each protein. In the top plots (TMHMM), red peaks indicate probability of a TM domain, while the pink and blue lines indicate the best model (pink indicates outside the membrane while blue is inside the membrane). In the second plots, the dotted line is shown to indicate the predictive threshold—above the line is predicted to be a TM domain. The hydrophobicity plots are shown inverted—below the line represents hydrophobicity while above the line represents hydrophilicity. Putative TM domains are indicated by arrows. Outputs from the third predictive algorithm described in the text (HMMPOT) are not shown as they were text-based. *A second putative TM domain that was predicted in *P. alecto* TLR5 by all of the models tested. (C) Structural model of *P. alecto* TLR5 TIR domain based on human TLR2. The region in which a second putative TM domain was predicted by TMHMM and other methods is highlighted in red, and appears to form one of the central parallel β sheets. The C terminal helix of the TIR domain has been masked for clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

2000). Structural modelling of the TIR domain of *P. alecto* TLR5 based on the crystal structure of human TLR2 showed that the putative second TM domain corresponds to one of the central parallel β sheets that form the hydrophobic core of the TIR domain (Fig. 4c).

Due to the highly diverse nature of LRR domains, SMART could only identify a subset of them in each TLR. Each TLR is expected to contain one N terminal LRR domain and one C terminal LRR domain, flanking a variable number of regularly spaced LRR domains that form the ligand-binding region of these receptors. SMART was able to identify N terminal LRRs in TLRs 3 and 7 only and C terminal LRRs in TLRs 1–10 but not in TLR9. SMART also failed to detect a substantial proportion of the remaining LRRs, so structural modelling was performed to determine whether the number of LRRs could be predicted with greater accuracy (Fig. 5a–d). Structural models of the ectodomains of *P. alecto* TLRs 3, 7, 8 and 9 were generated, based on the crystal structure of human TLR3 (Choe et al., 2005). This enabled the identification of additional LRR domains that were not detected by SMART. Mammalian TLR3 proteins typically have 25 LRR domains, whereas mammalian TLRs 7, 8 and 9 proteins typically have 27 LRRs (Matsushima et al., 2007). While

SMART identified 20, 19, 18 and 19 LRR domains in *P. alecto* TLRs 3, 7, 8 and 9, respectively, structural modelling enabled identification of 25 LRR domains in each case. In TLRs 7, 8 and 9, loops extending away from the main structure may contain additional LRR domains.

3.6. qPCR analysis of mRNA expression levels of the nucleic acid-binding TLRs 3, 7, 8 and 9 in *P. alecto* tissues

Expression levels of mRNA encoding TLRs 3, 7, 8 and 9 were measured using SYBR Green qPCR in ten tissues collected from three individual wild-caught *P. alecto* fruit bats (Fig. 6a–d). TLR3 was strongly expressed in liver and moderately expressed in lymph node, PBMC, spleen, small intestine, lung and salivary gland. TLR7 was strongly expressed in PBMC and moderately expressed in lymph node, spleen, lung and possibly small intestine. TLR8 was strongly expressed in PBMC and spleen, and moderately expressed in lymph node and lung. TLR9 was strongly expressed in PBMC, spleen and lymph node, and moderately expressed in lung. Brain, heart and kidney expressed low levels of all four mRNAs.

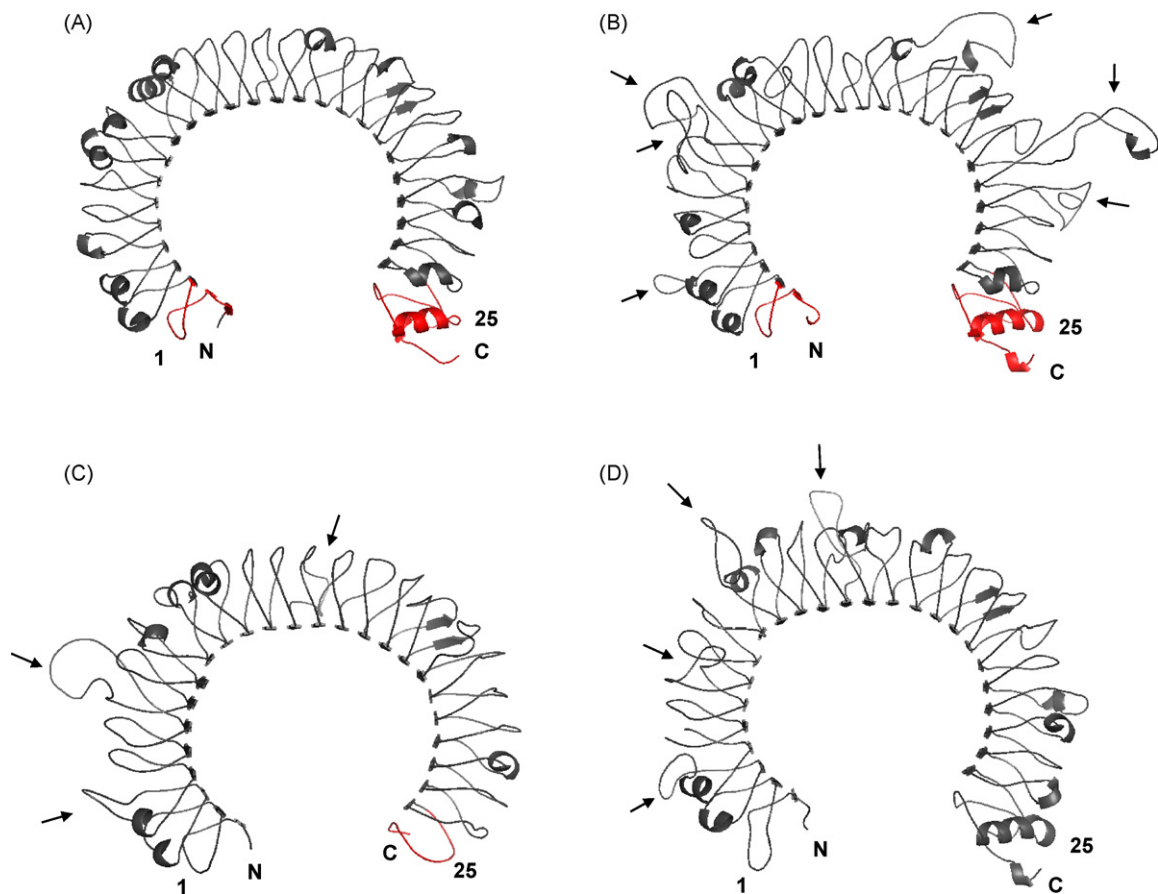


Fig. 5. Structural modelling of *P. alecto* TLR ectodomains. Structural models of *P. alecto* TIR ectodomains (minus signal peptides) based on human TLR3: (A) TLR3, (B) TLR7, (C) TLR8, and (D) TLR9. The atypical N and C terminal LRRs identified by SMART are highlighted in red. The first and last LRR domains are numbered in each figure. All TLRs were slightly truncated at the ends by the modelling program. N and C terminals are indicated by capital letters, and arrows indicate regions that loop out from the main structures and may contain additional LRR domains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

Bats are believed to harbour many zoonotic viruses and are among the least studied of all mammals. The association of bats and viruses has been long recognised (Calisher et al., 2006), however very little basic information has been obtained on the immune system of any bat species, and the majority of existing bat immunological studies were undertaken prior to the discovery of fundamental components of vertebrate immune systems, including the mechanisms of innate pathogen recognition. *P. alecto* has been used as a model bat species in a number of studies, including experimental infection studies (e.g. van den Hurk et al., 2009), establishment of bat cell lines (Crameri et al., 2009), and immunological studies (Baker et al., 2010). In this report, we describe the molecular cloning and analysis of the expressed TLR repertoire of the black flying fox, *P. alecto*, with comparison to the germline repertoire of the large flying fox *P. vampyrus*. These two closely related pteropid bat species are natural hosts of the important paramyxoviruses, Hendra and Nipah, respectively (Field et al., 2007), and *P. alecto* is also a known carrier of Australian bat lyssavirus (Speare et al., 1997).

4.1. Pteropid bat TLRs are highly conserved with those of other mammals

TLRs 1–10 were identified in *P. alecto*, as well as a number of pseudogenes including a nearly-intact TLR13 that was transcribed, spliced and polyadenylated. In common with TLRs in other species,

P. alecto TLRs feature signal peptides, LRR-rich ectodomains for ligand-binding, TM domains and cytoplasmic TIR domains for signal transduction. Structural modelling enabled the identification of more LRR domains in *P. alecto* TLRs than homology searching using SMART, and the highest accuracy of LRR domain prediction appeared to be for *P. alecto* TLR3, due to the availability of the human TLR3 ectodomain structure, combined with the fact that *P. alecto* TLR3 is highly conserved. The predicted intron–exon organisation of pteropid TLRs was found to be similar to that observed in other mammals, however varying numbers of 5′ non-coding exons have been reported on occasion in some species. For example, bovine TLR1 is reported to have four non-coding 5′ exons (Opsal et al., 2006) whereas only two were detected in *P. alecto* TLR1. The inability for automated gene assembly to detect many TLRs in the *P. vampyrus* genome, as well as significant miss-assemblies highlighted the need for laboratory confirmation of gene sequences mined from low-coverage genome assemblies. Overall however, TLRs in *P. alecto* were shown to be highly similar to TLRs in human and other mammalian species.

The phylogenetic relationship of Chiroptera with other eutherian mammals has long been a subject of controversy, however it is now widely accepted that bats belong within the clade Laurasiatheria (Cetartiodactyla, Perissodactyla, Carnivora, Pholidota, Chiroptera and Eulipotyphla) (Madsen et al., 2001; Murphy et al., 2001). Attempts to resolve the phylogenetic relationship of bats within this group in greater detail have produced varying results. Analysis of mitochondrial DNA placed bats within an existing subgroup of Laurasiatheria termed Fereuungulata

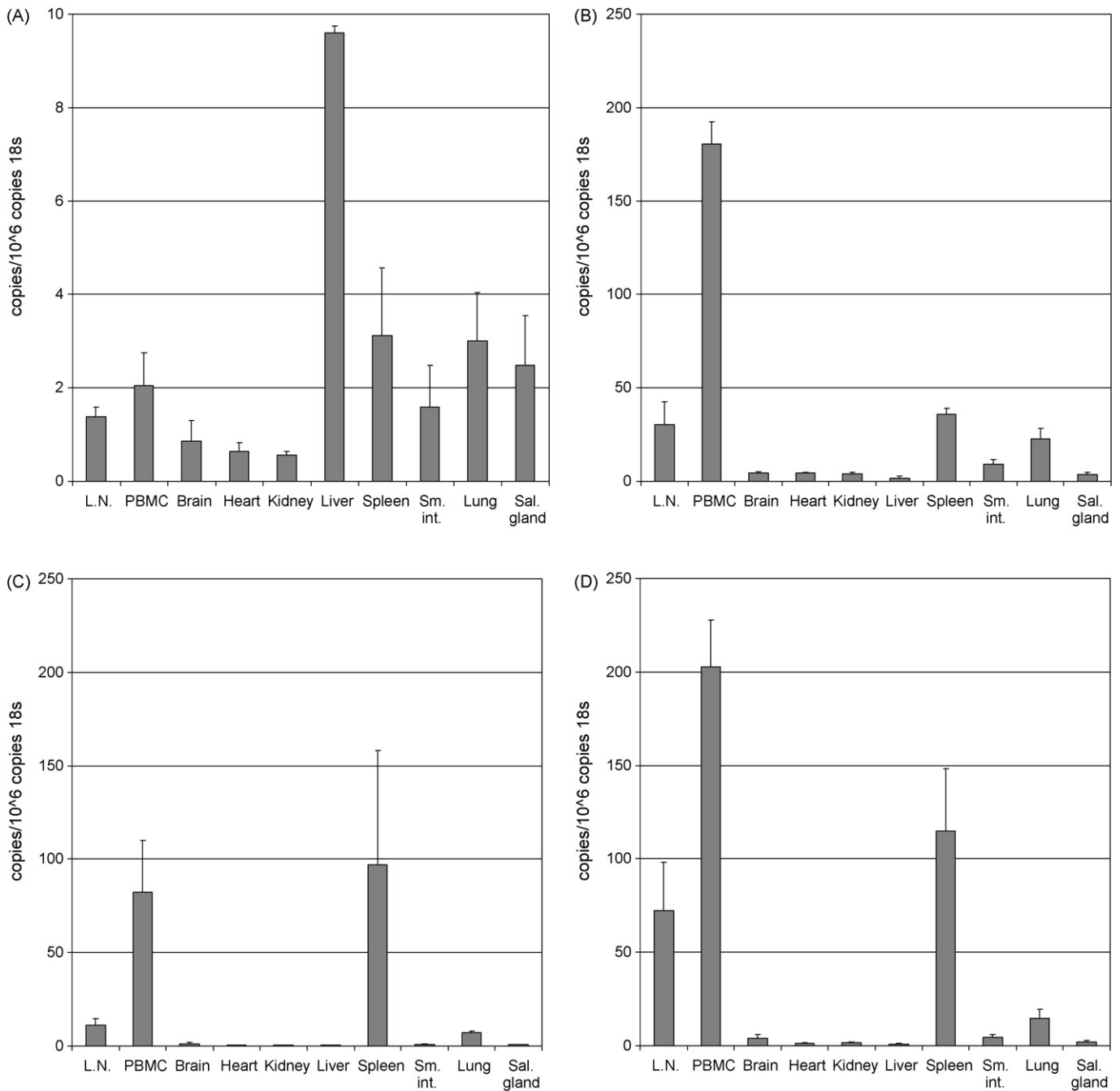


Fig. 6. Quantitative mRNA expression of TLRs 3, 7, 8 and 9 in *P. alecto* tissues. Tissue mRNA expression levels of: (A) TLR3, (B) TLR7, (C) TLR8, (D) TLR9. $n=3$ individual apparently healthy wild-caught bats. Error bars represent standard deviation. *Abbreviations:* lymph node (L.N.), small intestine (Sm. int.), salivary gland (Sal. gland).

(Carnivora + Perissodactyla + Cetartiodactyla) (Nikaido et al., 2000), whereas analysis of LINE-1 retrotransposon insertions argued against the existence of *Fereungulata* as a natural grouping and instead suggested the creation of a new group termed *Pegasoferae* (Chiroptera + Perissodactyla + Carnivora + Pholidota) (Nishihara et al., 2006). Phylogenetic analysis of pteropid bat CD4, interferon- α and interferon- β genes showed that they clustered within Laurasiatheria and were most closely related to sequences from pig, horse, cat and dog (Omatsu et al., 2006, 2008). The results of our phylogenetic analysis of *P. alecto* TLRs found that while bat TLRs 3 and 7 clustered with horse, cat and dog sequences, bat TLR8 was the least conserved sequence in Laurasiatheria, and bat TLR9 fell outside Laurasiatheria altogether. Despite this, the nucleic acid-sensing TLRs 3, 7, 8 and 9 can be considered highly conserved in

bats, consistent with them being capable of viral recognition similar to other species.

The observation that percent sequence identities of bat and other mammalian TLRs were almost invariably higher at the DNA level than at the protein level prompted us to examine the ratio of non-synonymous to synonymous nucleotide substitution, which can be informative about the nature of selection pressure acting on a gene (Hurst, 2002). K_a/K_s ratios <1 are interpreted as evidence of negative (purifying) selection pressure, meaning that evolution is selecting against mutations in that gene. In contrast, K_a/K_s ratios >1 (which are encountered much less frequently) are interpreted as evidence of positive selection, whereby mutations are favoured over conservative replication. Viruses and other parasites are under constant positive selection pressure to mutate in order to escape

the immune responses of their host, and correspondingly some vertebrate immune genes are also under positive selection pressure in order to keep up in a so-called “molecular arms race” (Nielsen et al., 2005). Analysis of the Ka/Ks ratio of *P. alecto* TLRs with other mammals suggested weak negative selection, supporting the findings of previous studies and consistent with selection to conserve important binding specificities (Roach et al., 2005; Nakajima et al., 2008; Barreiro et al., 2009).

The tissue mRNA analysis agreed with, and significantly extended the findings of Iha et al. (2010). We increased the sensitivity of measurement to a true quantitative level, examined additional tissues, and measured TLR8 levels for the first time. TLRs 7, 8 and 9 had very similar expression patterns, with the highest levels observed in PBMC, lymph node, spleen, and to a lesser extent in lung. These observations suggest that these genes are predominantly expressed on professional immune cells, as they are in other mammals.

4.2. Atypical observations of *P. alecto* TLRs

Although the *P. alecto* TLRs appeared to be highly conserved with those of other mammalian species, some atypical characteristics were observed among the *P. alecto* TLR dataset. Analysis of *P. alecto* TLR5 using three predictive algorithms and three models of hydrophobicity predicted the presence of a second putative TM domain within the C terminal TIR domain. Structural modelling suggested that this region correlated with one of the parallel β sheets located in the core of the TIR domain, however the model was based on the structure of human TLR2 (the TLR5 TIR domain structure has not been solved). The presence of this hydrophobic sequence may affect TLR5 signal transduction, and may suggest a functional region worthy of closer examination. The TM domain of *P. alecto* TLR9 was unable to be predicted by TMHMM, which is surprising as this method is reported to have a very low false negative prediction rate (Krogh et al., 2001), however using alternative methods we were able to identify a putative TM domain in *P. alecto* TLR9.

The qPCR data confirmed the finding of Iha et al. (2010) that TLR3 is highly expressed in bat liver, something that is not observed in humans and mice. TLR3 is predominantly expressed in dendritic cells in other mammals, so it will be interesting to see if this is also the case in bats once reagents become available to distinguish cell types. It was also observed that transcription levels of TLRs 3, 7, 8 and 9 in *P. alecto* tissues were very tightly regulated, as demonstrated by the surprisingly low variation observed between three individual wild-caught bats.

The minor mutations in the fruit bat TLR13 suggesting a recent loss of function are intriguing, particularly since it appeared to be transcribed, spliced and polyadenylated. While the function of TLR13 in other species remains unknown, recent studies have raised the possibility of it being a virus-sensing TLR (Brinkmann et al., 2007; Kim et al., 2008). In light of this it will be interesting to ascertain whether other bat species carry an intact copy of TLR13.

Although these findings do not shed light on how bats can remain asymptomatic to viral infections, they at least indicate that *P. alecto* TLRs 1–10 are likely functional genes, and a starting point for further studies to understand whether they are more effective at ligand recognition and signalling through downstream pathways. Many questions remain about the functional significance of the TLR system in bats in relation to their role as carriers of zoonotic viruses. Unravelling the events that occur downstream of TLR signalling, such as induction of inflammatory cytokines and the potential for viruses to block TLR signalling pathways, depends firstly upon knowledge of which TLRs are present in the bat. It is hoped that this study fills that requirement and provides a prototype dataset that can be used to design functional assays. In summary, the fruit

bat *P. alecto* appears to have a complement of TLRs resembling those present in humans and other mammals. It now remains to be determined whether the fruit bat TLR system functions the same or differently from other species during viral infections.

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