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BRIEF COMMUNICATION

Diversity in the Toll-like receptor genes of the Tasmanian devil (*Sarcophilus harrisii*)

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Abstract The Tasmanian devil is an endangered marsupial species that has survived several historical bottlenecks and now has low genetic diversity. Here we characterize the Toll-like receptor (TLR) genes and their diversity in the Tasmanian devil. TLRs are a key innate immune gene family found in all animals. Ten TLR genes were identified in the Tasmanian devil genome. Unusually low levels of diversity were found in 25 devils from across Tasmania. We found two alleles at TLR2, TLR3 and TLR6. The other seven genes were monomorphic. The insurance population, which safeguards the species from extinction, has successfully managed to capture all of these TLR alleles, but concerns remain for the long-term survival of this species.

Keywords Toll-like receptors · Genetic diversity · Tasmanian devil · Population bottleneck

The Tasmanian devil (*Sarcophilus harrisii*) is the world's largest remaining carnivorous marsupial. It is now restricted to the island state of Tasmania, Australia, but was once distributed across the Australian mainland, and went extinct there approximately 3000 to 4000 years ago, probably due to hunting and competition from dingoes (Brown 2006). The devil is currently under threat of extinction due to a fatal contagious cancer called Devil Facial Tumour Disease (DFTD). DFTD was first observed in 1996 and has led to a decline of devil population by up to 84 % (Hawkins et al. 2006). The disease continues to spread and extinction of the species in the wild has been predicted (McCallum et al. 2007). To prevent this, the Save the Tasmanian Devil Program has established an insurance population which currently has 622 devils in captivity (Department of Primary Industries, Parks, Water and Environment, 2012–2013 Annual Program Report). The aim of this programme is to capture and retain the entire genetic diversity of the species in captivity until it is safe to release devils back into the wild (http://www.tassiedevil.com.au/tasdevil.nsf).

Previous studies have revealed that devils have low genetic diversity at microsatellite loci (Jones et al. 2004), in the mitochondrial and nuclear genome (Miller et al. 2011), and particularly at the major histocompatibility complex (MHC), a gene family involved in immune surveillance and disease resistance (Cheng and Belov 2014; Cheng et al. 2012; Siddle et al. 2007). However, to date, diversity has not been studied in the Toll-like receptor genes, an important innate immune gene family.

Toll-like receptors (TLRs) are a gene family found in both vertebrate and invertebrate species involved in recognizing and binding conserved patterns on pathogens. TLR molecules can be expressed either on the cell surface or membrane compartment of immune and non-immune cells (Takeda et al. 2003). They are the first line of defence against pathogens since they are the first receptors to interact with invading microorganisms, including viral, bacterial, fungal and parasitic pathogens (Jin and Lee 2008). TLRs encode type I transmembrane glycoproteins that consist of an extracellular domain, containing 20–30 leucine-rich repeats (LRRs), a transmembrane domain and a cytoplasmic Toll/interleukin I receptor (TIR) domain (Uematsu and Akira 2007). The extracellular domain (ECD) forms a horseshoe shape and is responsible for binding conserved regions on microbes known as

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'pathogen associated molecular patterns' (PAMPs). Residues within LRR9 and LRR15 at the convex surface of the extracellular domain are involved in PAMP binding (Bell et al. 2003; Kobe and Kajava 2001). Once the pathogen is bound, the TIR domain triggers a cascade of signal transduction that activates both the innate and the adaptive immune responses (Pasare and Medzhitov 2005).

The vertebrate TLRs can be grouped into six subfamilies according to phylogenetic analysis (Roach et al. 2005). Most TLR subfamilies consist of a single ortholog, a copy of which is found in each species, but the exception is the TLR1 family, which contains four paralogues (TLR1, TLR2, TLR6 and TLR10) in mammals and two paralogues (TLR1-like genes TLR1A and TLR1B and TLR2-like genes TLR2A and TLR2B) in birds. TLRs can also be classified into non-viral (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) and viral (TLR3, TLR7-9) TLRs by their functions (Iwasaki and Medzhitov 2004).

It has been hypothesized that populations should maintain genetic diversity in order to maintain population fitness, as low genetic diversity increases inbreeding rate and decreases species survival (Fredrickson et al. 2007; Gutierrez Espeleta et al. 2001; Mattila and Seeley 2007; Reed and Frankham 2003). In order to focus on immunological fitness, conservation geneticists have begun to study diversity at immune genes (Lazzaro et al. 2004; Sommer 2005). Although some populations have survived with low MHC diversity, including cheetahs (Castro-Prieto et al. 2011), some amphibians (Babik et al. 2009) and Eurasian beavers (Ellegren et al. 1993), populations with high diversity are more likely to harbour gene variants that provide resistance to emerging diseases (Bradley and Altizer 2007; Keesing et al. 2006; Reed and Frankham 2003). Specific alleles at the major histocompatibility complex (MHC) have been linked to reduced resistance to infectious diseases caused by bacteria, viruses and parasites, including mycobacteria in humans (Casanova and Abel 2002), canine parvovirus in Mexican wolves (Hedrick et al. 2003) and nematode infections in the yellow-necked mouse (Meyer-Lucht and Sommer 2005). However, little is known about the relationship between TLR diversity and species fitness. Low levels of TLR diversity have been found in the bottlenecked and endangered New Zealand robin (Grueber et al. 2012). Here we measured TLR diversity in the endangered Tasmanian devil in order to increase our understanding of innate immunity in this species and to provide new measures of fitness for management of the species.

We used the Tasmanian devil genome (Murchison et al. 2010) to identify TLR sequences and designed primers to study diversity within the genes (Table 1). Ten TLR genes (accession numbers are listed in Table 2) were predicted by Ensembl. All 10 genes are likely functional, as full-length transcripts with an open reading frame encoding intact extracellular, transmembrane and intracellular domains have been identified in the transcriptome datasets (Murchison et al. 2010, 2012). The annotated TLR sequences in the devil genome were aligned with TLRs from other species in BioEdit7 v7.2.3 (Hall 1999). Phylogenetic analysis was conducted using the neighbour-joining method with 5000 bootstrap replicates (Saitou and Nei 1987) in MEGA5 (Tamura et al. 2011) to verify their classification (Fig. 1). GenBank accession numbers of sequences used for comparative analyses are listed in Table 2. Gene-specific polymerase chain reaction (PCR) primers (Table 1) were designed using Oligo 6 (Zhang and Gao 2004) within a single exon (primer binding sites shown in supplementary material 1). The coding sequences of TLR2, TLR5, TLR6, TLR7, TLR8, TLR10 and TLR13 are encoded within a single exon. Primers for each of these genes were designed at both ends of the coding region, amplifying the LRRs, transmembrane and cytoplasmic domains. In order to confirm the intron and exon boundaries, devil TLR full-length transcripts were blasted against the devil genome. The coding sequences of TLR3, TLR4 and TLR9 have multiple exons (supplementary material 1). The target fragments include the peptide-binding region for each gene. TLR3, TLR4 and TLR9 contain partial LRR region and partial cytoplasmic region. Sequence alignments of TLR2-10 (in the devil, opossum and human) and TLR13 (in the devil, opossum and mouse) have been provided in the supplementary data.

Genomic DNA was extracted from ear biopsies or from frozen blood stored at −20 °C using DNeasy Blood and Tissue Kit (QIAGEN, Chadstone Centre, Australia). Twenty-five animals were used to study genetic diversity, 14 of which belong to the subpopulation from eastern Tasmania and 11 from northwestern Tasmania. Previous studies have found genetic differentiations between eastern and northwestern subpopulations (Jones et al. 2008; Miller et al. 2011; Siddle et al. 2010). PCRs were carried out in a total volume of 25 μl for each reaction. PCR products were separated in a 1.2 % agarose 1× TBE gel stained with SYBR Safe DNA gel stain (Invitrogen, Mulgrave, Australia). Target bands were extracted and purified with QIAEX II Gel Extraction Kit (QIAGEN, Chadstone Centre, Australia). The TLR fragments were then cloned in a pGEM-T Easy Vector (Promega, Alexandria, Australia) cloning system, and eight positive clones from each sample were sequenced in both directions at the Australian Genome Research Facility (AGRF, Westmead, Australia). To minimize sequence artefacts from PCR, cloning and sequencing, putative novel alleles were considered to be real if amplified independently in two separate PCRs. Plasmid products were sequenced at the Australian Genome Research Facility (AGRF, Westmead, Australia). Raw sequences were quality checked and edited with Sequencher 4.9 and aligned with BioEdit v7.2.3 (Hall 1999). Selection test was performed in MEGA5 (Tamura et al. 2011) and allele frequency was calculated in DnaSP 5.10 (Librado and Rozas 2009).

Table 1 Primers used in this study

Gene	Full length (bp)	Fragment length	Forward primer		Reverse primer	
TLR2	2352	2300	TLR2F	5′-ATGAGACATGTCAGGTGGACAGT-3′	TLR2R	5′-TGACTTTATAGCAGCTCTCAAATTGA-3′
TLR3	2847	1756	TLR3F	5′-TTTTCTCCAGGATGCTTTCAGTC-3′	TLR3R	5′-CTTCTTGCTCCTTTTTATGCTGTG-3′
TLR4	2486	2202	TLR4F	5′ TTCCTCAATGCAAACAATATCT 3′	TLR4R	5′ CTTCTTTCTGCCTCTTTGTTCT 3′
TLR5	2577	2526	TLR5F	5′-ATGGGCCATCTTGCATTTCTCT-3′	TLR5R	5′-TAGGATACAGTTGCTACGGTTTGTAAGG-3′
TLR1-6	2418	2366	TLR1-6 F	5′-ATGACAGTGACCCTTTGGGAT-3′	TLR1-6R	5′-AATTTCATTGTCTTCTGTAACTAATGCT-3′
TLR7	3264	2035	TLR7F	5′-TACCGTGCTTATTTGAATTTGTC-3′	TLR7R	5′-TAGAGAGTCTCTTTAAACACCTTACTATA-3′
TLR8	3455	1370	TLR8F	5′ ACTGTCCACGGTGTTTCAATAAT 3′	TLR8R	5′ TGTTTTTCCCCAGCAATAGTATC 3′
TLR9	3183	2553	TLR9F	5′ ACCTCACCGTGTTGGACCTCAG 3′	TLR9R	5′ AGCAGGTTCCCAGACAGGTCAA 3′
TLR10	2550	1959	TLR10F	5′ GACTTTGACAACATTCCCATCT 3′	TLR10R	5′ CTCTGAAATCGTCTGTGAACTGT 3′
TLR13	2859	1500	TLR13F	5′-CGTTTGCAGTCTCTCTCTCTTTC-3′	TLR13R	5′-TTCTCAGGCCAGGTAATAAATGTT-3′

The marsupial TLR repertoire contains 10 genes, nine of which have eutherian orthologs (*TLR2*, *TLR*3, *TLR4*, *TLR5*, *TLR7*, *TLR8*, *TLR9*, *TLR10* and *TLR13*). Our phylogenetic tree shows that a single TLR gene, which we designate TLR1-6-like, is found in the two marsupial species and is basal to the eutherian TLR1 and TLR6 gene clusters. We propose that a gene duplication of TLR1-6-like gave rise to the TLR1 and TLR6 gene families in eutherians. TLR1-6-like is located adjacent to TLR10 in both the Tasmanian devil and opossum genomes, and it appears that TLR10 and TLR1-6-like arose through gene duplication. TLR1-6-like then duplicated again in the eutherian lineage. Clustering of these genes within the genome provides a blueprint of the evolutionary history of this gene family.

TLR13 is found in the Tasmanian devil, opossum and some eutherians. It is not found in birds, fish or reptiles. We propose that it arose prior to the divergence of marsupials and eutherians but may not be critical for all lineages and hence has been lost in some mammalian lineages. In rodents, this gene is an RNA-sensing receptor and recognizes bacterial RNA (Hidmark et al. 2012; Li and Chen 2012; Manzoor and Koh 2012). Its function in marsupials is not yet known.

In the 25 wild devils, no single nucleotide polymorphisms (SNPs) were found at TLR 4, TLR5, TLR7, TLR8, TLR9, TLR10 and TLR13, while one SNP was found at TLR2 and TLR1-6-like, and five SNPs at TLR3 (Table 3). The SNPs in TLR2 and TLR1-6-like are non-synonymous substitutions, resulting in two alleles differing at one amino acid residue. The five SNPs in TLR3 include four synonymous and one non-synonymous substitution. The locations of the non-synonymous substitution in TLR2, TLR3 and TLR6 are shown in the supplementary material 1. TLR diversity in devils is low compared with that observed in other species (Table 3). The lack of genetic diversity at the TLRs in devils is likely to be the result of their evolutionary history with low level of diversity maintained since the last glacial maximum (Brüniche-Olsen et al. 2014; Morris et al. 2013). It is possible that purifying selection has acted on TLR3 ($d_N/d_S=0.25$, p value=0.022); however, this could also be the result of genetic drift.

Table 2 GenBank accession numbers of sequences used in this study

Gene	Devil, *Sarcophilus harrisii*	Opossum, *Monodelphis domestica*	Human, *Homo sapiens*	Mouse, *Mus musculus*	Cattle, *Bos taurus*
TLR2	XM_003773130.1	XM_001375753.1	NM_011905.3	NM_003264.3	NM_174197.2
TLR3	XM_003773017.1	XM_001368503.1	NM_126166.4	NM_003265.2	NM_001008664.1
TLR4	XM_003757076.1	XM_001368732.1	NM_021297.2	NM_003266	NM_174198.6
TLR5	XM_003767727.1	XM_001376152.1	NM_016928.2	NM_003268.5	NM_001040501.1
TLR6 (TLR1-6-like)	XM_003773313.1	XM_003341433.1	NM_011604.3	NM_006068.4	NM_001001159.1
TLR7	XM_003765580.1	XM_003341792.1	NM_133211.3	NM_016562.3	NM_001033761.1
TLR8	XM_003765579.1	XM_001381056.2	NM_133212.2	NM_138636.5	NM_001033937.1
TLR9	XM_003762588.1	XM_001380349.1	NM_031178.2	NM_017442.3	NM_183081.1
TLR10	XM_003773322.1	XM_00749659.1		NM_001017388	NM_001076918.2
TLR13	XM_003774898.1	XM_001372402.1		NM_205820.1	

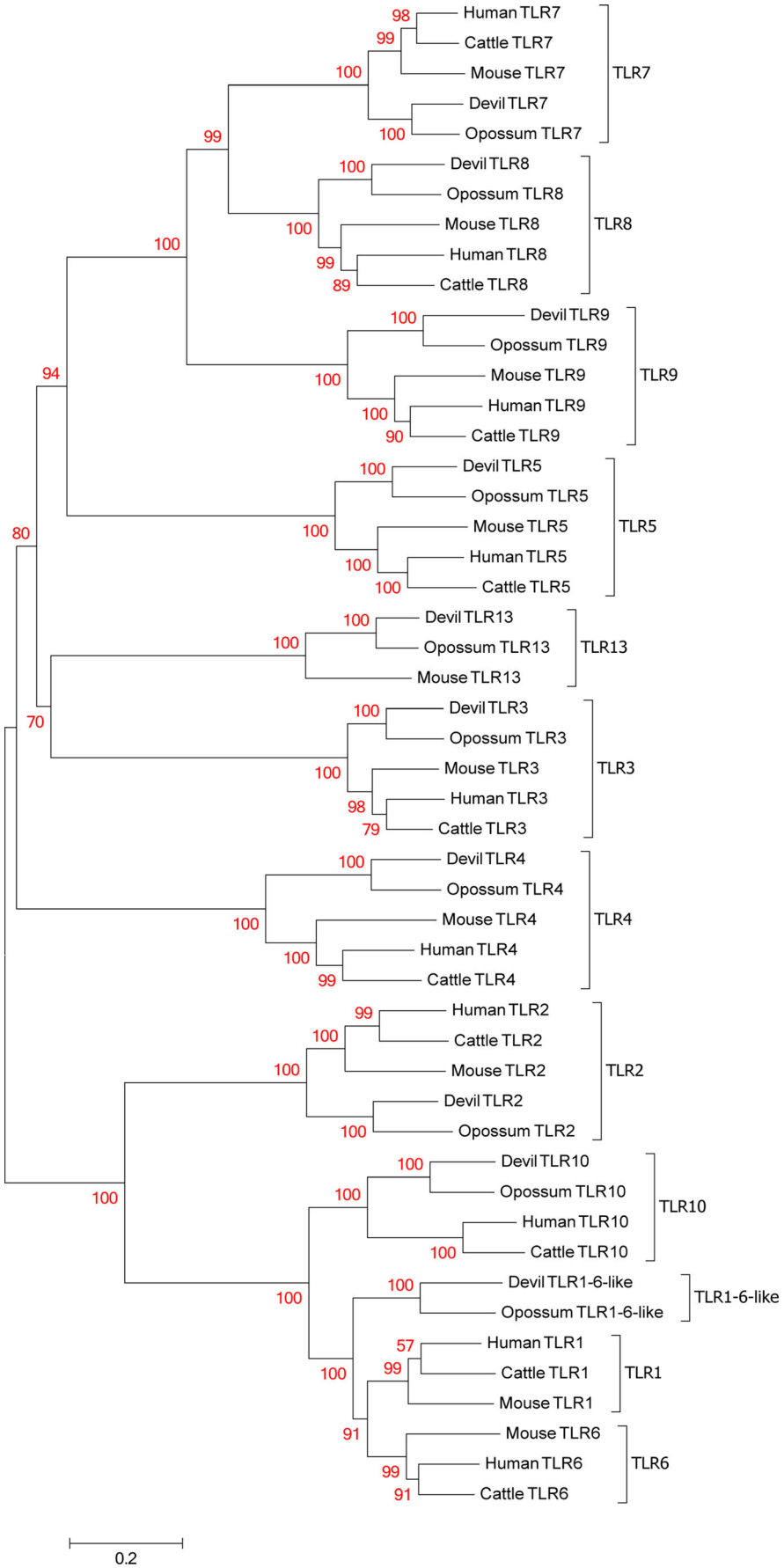

Fig. 1 Neighbour-joining phylogenetic tree of TLR gene families. The bootstrap values are displayed at each branch point

Table 3 Variants identified in TLR genes

Species	Genes	Samples	SNPs	Non-syn	Alleles	References
Tasmanian devil	TLR2	75	1	1	2	This study
	TLR3	75	5	1	2	
	TLR4	25	0	0	1	
	TLR5	25	0	0	1	
	TLR1-6-like	75	1	1	2	
	TLR7	25	0	0	1	
	TLR8	25	0	0	1	
	TLR9	25	0	0	1	
	TLR10	25	0	0	1	
	TLR13	25	0	0	1	
New Zealand robin	TLR2A	17–24	1	0	2	(Grueber et al. 2012)
	TLR2B		5	1	3	
	TLR3		0	0	1	
	TLR4		4	4	5	
	TLR5		2	2	3	
	TLR7		2	3	2	
Human	TLR1	100			11	(Georgel et al. 2009)
	TLR2				7	
	TLR4				4	
	TLR5				14	
	TLR6				10	
	TLR7				6	
	TLR8				9	
	TLR9				8	
	TLR10				15	

Non-syn non-synonymous

Polymorphisms in TLR genes are associated with differing host immunity against particular pathogens. For instance, specific SNPs in TLR4 (Sackesen et al. 2005), TLR6 (Tantisira et al. 2004) and TLR10 (Lazarus et al. 2004) have been associated with increased asthma risk in humans. SNPs at TLR9 are associated with rapid progression of HIV-1 infection (Bochud et al. 2007). Polymorphisms at TLR3 in grass carp are associated with resistance to grass carp reovirus (Heng et al. 2011). SNPs at human TLR4 are associated with decreased susceptibility to rheumatoid arthritis (Reismann et al. 2004) and malaria (Mockenhaupt et al. 2006). SNPs at TLR4 and TLR5 are associated with resistance/susceptibility to Legionnaires' disease (Hawn et al. 2003, 2005). Polymorphisms at TLR2, TLR4 and TLR9 enhance the risk of tuberculosis and leprosy (Bharti et al. 2014; Xue et al. 2010). Based on these observations, we postulate that diversity of TLRs in wild species may be beneficial for their long-term fitness and survival, providing populations with a greater capacity to respond when new disease threats emerge. And therefore, it is important to ensure that all existing TLR diversity in wild devils is captured in the insurance population. To do this, we sequenced the three polymorphic genes, TLR2, TLR1-6-like and TLR3, in 50 devils from the insurance population. All alleles found in wild devils were identified and no novel alleles were observed.

In conclusion, we found that the genetic diversity at TLRs is extremely low in the Tasmanian devil. Some loci are monomorphic (TLR4, TLR5, TLR7-10 and TLR13); others have only two alleles (TLR2, TLR3 and TLR1-6-like; Table 4). The low TLR diversity in devils is probably a consequence of previous historical bottlenecks, but by successfully capturing

Table 4 Allele frequencies of TLR2, TLR3 and TLR1-6-like in wild and captive population

	Wild devils		Captive devils	
	Allele frequency		Allele frequency	
Locus	A1	A2	A1	A2
TLR2	0.66	0.34	0.82	0.18
TLR3	0.77	0.23	0.71	0.23
TLR1-6-like	0.87	0.13	0.89	0.11

A allele

all of these TLR alleles in the insurance population, we are helping to ensure that the species does not go through an additional bottleneck within the captive breeding programme.

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