SNP Marker Discovery in Koala TLR Genes

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Abstract

Toll-like receptors (TLRs) play a crucial role in the early defence against invading pathogens, yet our understanding of TLRs in marsupial immunity is limited. Here, we describe the characterisation of nine TLRs from a koala immune tissue transcriptome and one TLR from a draft sequence of the koala genome and the subsequent development of an assay to study genetic diversity in these genes. We surveyed genetic diversity in 20 koalas from New South Wales, Australia and showed that one gene, TLR10 is monomorphic, while the other nine TLR genes have between two and 12 alleles. 40 SNPs (16 non-synonymous) were identified across the ten TLR genes. These markers provide a springboard to future studies on innate immunity in the koala, a species under threat from two major infectious diseases.

Introduction

The koala (Phascolarctos cinereus) is an arboreal herbivorous marsupial which was widespread across eastern Australia until the end of the 19th century, when populations have suffered declines due to the fur trade, habitat degradation and disease [1]. The koala is distributed from Queensland, New South Wales (NSW) and Victoria and translocated to islands off the south coast of Australia and to South Australia [2]. The Queensland, NSW and Australian Capital Territory populations are listed as “vulnerable” under the federal government Environmental Protection and Biodiversity Conservation Act 1999. Genetic diversity in koalas is extremely low on the introduced islands and in Southern Australia due to founder effects [3]. Levels of diversity are higher in NSW and Queensland [3]. North-eastern koalas have twice as much diversity as south-eastern koalas (A = 11.5+/−1.4 vs A = 5.3+/−1), and microsatellite variability is comparable to that seen in other wild species [3,4]. Thirty-one mitochondrial haplotypes from the hypervariable Control Region (D-loop), have been characterized across the Australian east coast distribution [5,6], including 9 novel haplotypes recently characterized from NSW (unpublished data, Australian Museum). Mitochondrial DNA haplotype diversity within south-eastern Queensland and north-eastern NSW populations was much higher than that in Victoria and South Australian island populations, which contained only a single mitochondrial haplotype [5]. Diversity in key immune genes belonging to the Major Histocompatibility
Complex (MHC) in NSW and Queensland koalas is also robust [7], and is on par with that of New Zealand brushtail possums (*Trichosurus vulpecula*) [7,8].

Many koala populations are currently threatened by habitat loss and vehicular injuries [1] as well as infections by koala retrovirus (KoRV), which is associated with immune suppression and lymphoma and leukemia [9] and by *Chlamydia pecorum and C. pneumoniae* [10], obligate intracellular bacterial pathogens that cause debilitating ocular and reproductive tract disease [11]. In wild populations, chlamydial infection rates range from 20% to 100% [11]. However, depending on the populations, the level of clinical disease in association with infection has been noted to vary. This anecdotal evidence supported by studies monitoring *C. pecorum* shedding in individual koalas with and without clinical disease [12], would suggest that infection alone is not the primary determinant of chlamydial disease development in this host. Indeed, while studies continue to emerge that key genetic differences may exist in the infecting chlamydial strain in koalas [13] studies in other hosts suggest that the host’s immune response is key to the eventual outcome of the infection [14]. KoRV, on the other hand, is in the process of endogenising into the koala’s genome. While the direct relationship of KoRV to diseases such as leukemia and lymphoma are yet to be proven, it is widely assumed that the integration of KoRV sequences into the koala genome has affected the koala’s immune responsiveness to KoRV and potentially chlamydial infections [15].

Toll-like receptors (TLRs), encoded by a range of TLR genes, are key components of the innate immune response. TLRs are the first receptors to interact with invading microorganisms by recognising pathogen-associated molecular patterns (PAMPs) on a wide spectrum of pathogens [16]. TLRs are encoded by a large gene family, with 10 TLR homologs (TLR1–10) characterised in human (*Homo sapiens*; TLR1–10), cow (*Bos taurus*) and pig (*Sus domesticus*), 12 in house mouse (*Mus musculus*) [16–18] and 10 in gray short-tailed opossum (*Monodelphis domestica*) [19] and Tasmanian devil (*Sarcophilus harrisii*) [20]. The TLR molecules contain three domains; a large extracellular domain consisting of 18–30 Leucine-Rich Repeats (LRRs), a transmembrane domain and an intracellular Toll/interleukin I (TIR) domain. The extracellular domain forms a horseshoe shape, and it can recognize bacteria, fungi, parasites and viruses. TLRs can be sub-divided based on their functional roles into viral and non-viral. Viral TLRs are expressed in the cell, and include TLR3, TLR7, TLR8 and TLR9. They can recognize dsRNA and DNA viruses (TLR3) [21], ssRNA (TLR7 and TLR8) [22] and unmethylated Cpg-containing DNA, which is commonly found in the genomes of DNA viruses (TLR9) [23]. Non-viral TLRs are expressed on cell surface and can respond to lipopeptide from bacteria and parasites (TLR1, TLR2, TLR6 and TLR10) [24,25], lipopolysaccharides (LPS) from Gram-negative bacteria (TLR4) [26], flagellins (TLR5) [25] and bacterial 23S ribosomal RNA (TLR13) [27]. Although most immunological studies have been limited to mouse models, there is strong evidence to suggest that TLR2 and/or TLR4 activation and signaling has a strong influence on the clearance of the development of immunopathological sequelae as a result of chlamydial infection [28]. Interestingly, recent studies in humans have revealed that genetic Variants in the TLR1 and TLR4 genes may increase inflammation and are associated with risk of chlamydial infection and development of pelvic inflammatory disease [29], raising questions over whether genetic variation at these loci may serve as a biomarker of chlamydial infection and disease in other species as well.

Genomics technologies have facilitated rapid elucidation of the basic architecture of the koala’s immune system. Recent studies have described the characterisation of MHC class I and II [7,8,30], interleukins [31–33], interferon gamma, T cell markers and other immune genes [34]. In an effort to provide more tools to understand the role of TLRs in the koala response to infectious diseases, in the current study, we describe nine TLR-encoding genes in the koala and the development of a series of molecular markers that may be applied to study TLR genetic diversity in koala populations with different chlamydial infection outcomes.
Material and Methods

Koala transcriptome dataset

The koala transcriptome dataset consists of four cDNA libraries which were established from immune related tissues including liver, lymph node, spleen and bone marrow [35]. All tissues are from a single male koala obtained from the Australia Zoo Wildlife Hospital. All cDNA libraries were deposited in the Sequence Read Archive at NCBI [31] with the accession number SRR1106690, SRR1106707, SRR1121764, SRR1122141 for bone marrow, lymph node, liver and spleen libraries, respectively. The koala genome is published as a marker paper [36] and was made available to us for this project.

Searching strategy

Nine koala TLR genes were identified by searching all 4 koala cDNA libraries [35] using BLAST with human (Homo sapiens) and mouse (Mus musculus) TLR coding sequences. Koala TLR13 was obtained by searching the draft koala genome using BLAST using a Tasmanian devil (Sarcophilus harrisii) TLR13 nucleotide coding sequence [20]. Phylogenetic relationships between koala TLR genes and their homologs in eight other species, including three eutherians, human, cow and mouse, and two marsupial species, gray short-tailed opossum and Tasmanian devil [20], one bird species (chicken, Gallus gallus), one amphibian (western clawed frog, Xenopus tropicalis) and one fish species (zebrafish, Danio rerio) were analysed in MEGA 5 [37] using the Neighbour joining method [38] with 1000 bootstrap replicates to infer the level of confidence on the phylogeny. GenBank and Ensembl accession numbers of sequences are listed in S1 Table.

Analysis of TLR diversity in 20 wild koalas

Genetically diverse koalas were selected in an attempt to maximize the TLR gene diversity discovered in this study. Control Region mitochondrial diversity was used as a proxy measure to choose 20 genetically diverse animals, representing six koala mitochondrial DNA (mtDNA) Control Region haplotypes (Australian Museum, unpublished data). These koalas were all from New South Wales ranging from Narrandera (southern NSW) to Kyogle (northern NSW). Koala samples were either collected by the Port Macquarie Koala Hospital when animals were brought in for veterinary care or from the Australian Museum Tissue Collection. Australian Museum registration numbers are provided in S2 Table. For mtDNA PCR protocols and conditions see Frankham et al 2014 [39].

Gene specific primers (Table 1) were designed using Oligo6 [40]. Primers for TLR2, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR13 were designed at both ends of the coding region, amplifying the LRRs, transmembrane and cytoplasmic domains since the coding sequences of these genes are encoded within a single exon. The target fragments of TLR3, TLR4 and TLR10 contain partial LRR region and partial cytoplasmic region and include the peptide-binding region for each gene. The coding sequences of these three genes have multiple exons. PCR amplifications were carried out in a Bio-Rad MJ Mini Personal Thermal Cycler in 25μl reactions containing 1× high-fidelity buffer (Invitrogen, Mulgrave, Australia) that consists of 60 mM Tris-HCl (pH8.9) and 18 mM (NH₄)₂SO₄, 0.2 mM each dNTP, 2.0 mM MgSO₄, 0.5 uM each forward and reverse primers, 1.5U of Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Mulgrave, Australia) and approximately 30 ng template DNA. The general cycle conditions were initial denaturation at 94°C for 2 min, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 55–66°C (Table 1), and 1–3 min extension at 68°C and a final extension at 68°C for 10 min. A negative control without DNA was included in all PCR reactions. PCR
products were isolated on a 1.2% agarose gel, using EasyLadder I (Bioline, Alexandria, Australia) as a size marker, and purified with QIAEX II Gel Extraction Kit (QIAGEN, Chadstone Centre, Australia). PCR products were inserted into pGEM-T Easy Vector System I (Promega, Alexandria, Australia), and then transformed into JM109 High-Efficiency Competent Cells (Promega, Alexandria, Australia). Eight positive clones were picked for each PCR sample to make sure we captured both alleles from each locus within each individual. The plasmids were extracted using DirectPrep 96 MiniPrep Kit (QIAGEN, Chadstone Centre, Australia) and sequenced with T7 forward and SP6 reverse primers at Australian Genome Research Facility (AGRF, Westmead, Australia).

Sequences were quality-checked with Sequencher 4.9 and aligned in BioEdit v7.2.3 [41]. To minimise sequence artefacts from PCR, cloning and sequencing, a sequence variant was considered a real TLR allele when identified in multiple PCR amplifications from each individual. The plasmids were extracted using DirectPrep 96 MiniPrep Kit (QIAGEN, Chadstone Centre, Australia) and sequenced with T7 forward and SP6 reverse primers at Australian Genome Research Facility (AGRF, Westmead, Australia).

The overall rate of synonymous substitution per synonymous site ($d_S$) and non-synonymous substitutions per non-synonymous sites ($d_N$) in each gene were conducted using the Nei-Gojobori method with Jukes-Cantor adjustment in MEGA 5 [37]. Codon-based Z-tests of selection were performed with 5000 bootstrap replications to generate the standard error.

### Result and Discussion

We identified nine koala TLRs with clear eutherian orthologs (TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, TLR10 and TLR13). The evolutionary relationships of these genes are depicted in Fig. 1, and resemble those previously described by Roach et al. 2005. TLRs are present across a wide range of taxonomic groups, including insects [42,43], fish [44,45], amphibian [46], birds [47,48] and mammals [16,49]. TLRs from each family have similar functions across species [50,51]. The koala TLR genes shared on average 64% amino acid identify to their
human counterparts, with TLR3 and TLR7 the highest at 73%, and TLR4 the lowest at 53%.
The transcripts described here contained complete coding sequences, and ranged in length
from 2350 (TLR2) to 3141bp (TLR7), similar to the human TLRs, which ranged from 2352
(TLR2) to 3147bp (TLR7). The primers we designed to study diversity amplified between 1502
and 3065bp (details provided in Table 1).

Based on the well characterized human TLR sequences, the koala TLR genes contained all
of the key functionally conserved residues in the extracellular domain [25] S2–S11 Figs. The
leucine-rich motif in each LRR was conserved. Residues 1–10 in every LRR motif were present
in all LRRs and are predicted to form a β-strand within each LRR. The residues which follow
residue 10 are variable among TLRs, and this variability is usually associated with pathogen
binding S12–S21 Figs. [25,52–56].

The phylogenetic tree shown in Fig. 1 highlights that vertebrate TLRs can be divided into
six subfamilies which include the TLR1 family (including TLR1, TLR6, TLR10 and TLR2),
TLR3, TLR4, TLR5, the TLR7 family (including TLR7, TLR8 and TLR9) and the TLR11 family
(including TLR11–22). All vertebrate species appear to have at least one copy of a gene from
each subfamily. As expected, the koala TLR2, TLR3, TLR4, TLR5, TLR7, TLR8 TLR9 TLR10
and TLR13 each had a single clear ortholog in the other marsupial and eutherian species, so we
were able to annotate koala TLRs confidently. The koala sequences have been deposited to
GenBank and have been assigned the GenBank accession numbers. S3 Table. A single gene,
which we designate TLR1/6-like due to its location at the base of the eutherian TLR1 and TLR6
clades (Fig. 1) was also found in the transcriptome assembly. This gene, while not reported in
humans or other eutherians, has been found in opossum [19] and the Tasmanian devil [20]
and its emergence appears to predate the duplication of TLR1 and TLR6 in eutherians [19]. It
shares 58.5% amino acid identity with human TLR1 and 60.2% amino acid identify with
human TLR6.

The phylogenetic tree allows us to speculate about the evolutionary history of the TLR1
gene family. A single TLR1-like gene and a single TLR2 gene are seen in zebrafish and Xenopus
[19]. The chicken genome contains two copies of TLR1, which duplicated relatively recently, as
depicted by the short branch lengths, as well as two copies of TLR2 genes. It does not contain
TLR10. It appears that the ancestral avian TLR1, duplicated to give rise to TLR10 and TLR1/
6-like in mammals. TLR1/6-like is located adjacent to TLR10 in the koala, Tasmanian devil
and opossum genomes and is found in present day marsupials. TLR1/6-like then went on to
duplicate to give rise to the TLR1 and TLR6 families, which are seen in present day eutherians.
The mammals all retained TLR10 and TLR2.

The TLR genes evolve independently in different lineages in response to species-specific
pathogens [57]. Genes in the TLR1 subfamily form homo or heterodimers with each other and
with TLR2, indicating redundancy in pathogen recognition. For instance, the human TLR2/
TLR1 heterodimer responds to microbial triacyl lipoproteins [58], and TLR2/TLR6 responds
to diacyl lipopeptides [59], however, mutation of the F343 and F365 residues in TLR6 allows
the TLR2/TLR6 heterodimer to respond to triacyl lipopeptides [59]. Similarly, heterodimers of
human TLR1/TLR2 and TLR2/TLR10 can recognize the same pathogens [60]. On the other
hand, in chickens, heterodimers of any TLR1/TLR2 combination can respond to diacyl and
triacyl lipopeptides [61]. We therefore predict that marsupial TLR1/6-like will also be able to

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Fig 1. Neighbour-joining phylogenetic analysis of TLRs. Ten TLR amino acid sequences of the koala
compared to TLRs in eight species, including three eutherians (human, cattle and house mouse), two
marsupials (Tasmanian devil and gray short-tailed opossum), chicken, Xenopus and zebrafish. The
bootstrap values are displayed at each branch point.
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form homo and heterodimers with other members of the TLR1 family and respond to diacyl and triacyl lipopeptides.

Genetic polymorphisms were detected in all koala TLR genes, except TLR10. In the nine polymorphic genes, the number of alleles per gene ranged from 2–12, and the number of SNPs per gene between 1–8 (Table 2). In this study, TLR4 showed the highest level of genetic variability, with 12 alleles containing 8 SNPs. It is also the most polymorphic TLR in humans,

Table 2. Polymorphisms in koala TLRs.

| Gene      | Observe haplotypes | syn:nsyn | SNPs | Amino acid |
|-----------|--------------------|----------|------|------------|
| TLR2      | 4                  | 3:0      | 213: C/T | Asp        |
|           |                    |          | 834: G/A | Leu        |
|           |                    |          | 1665: A/C | Thr        |
| TLR3      | 4                  | 3:1      | 471: T/C | Thy        |
|           |                    |          | 1059: T/C | Leu        |
|           |                    |          | 1236: C/T | Val        |
| TLR4      | 12                 | 5:3      | 474: C/T | Ile        |
|           |                    |          | 576: T/C | Asn        |
|           |                    |          | 867: A/G | Thr        |
|           |                    |          | 959: C/A | Ser/Tyr    |
|           |                    |          | 1458: A/G | Gln        |
|           |                    |          | 1543 C/G | Leu/Val    |
|           |                    |          | 1660: A/G | Met/Val    |
| TLR5      | 8                  | 3:4      | 509: A/G | Gln/Arg    |
|           |                    |          | 528: A/G | Val        |
|           |                    |          | 589: A/G | Asn/Asp    |
|           |                    |          | 864: C/T | His        |
|           |                    |          | 1452: A/G | Ser        |
|           |                    |          | 1723: C/G | Gln/Glu    |
|           |                    |          | 2117: A/G | His/Arg    |
| TLR1/6like| 2                  | 1:0      | 384: T  | Thr        |
| TLR7      | 4                  | 3:2      | 102: T/C | Ser        |
|           |                    |          | 385: G/A | Glu/Lys    |
|           |                    |          | 807: C/A | Ile        |
|           |                    |          | 1970: A/G | Glu/Gly    |
|           |                    |          | 2439: C/T | Ser        |
| TLR8      | 5                  | 2:2      | 909: T/C | Phe        |
|           |                    |          | 1319: C/A | Thr/Lys    |
|           |                    |          | 1352: T/C | Met/Thr    |
|           |                    |          | 1935: A/G | Pro        |
| TLR9      | 10                 | 4:3      | 136: A/G | Thr/Ala    |
|           |                    |          | 701: C/T | Ala/Val    |
|           |                    |          | 1158: C/T | Leu        |
|           |                    |          | 1917: T/C | Pro        |
|           |                    |          | 2033: A/G | His/Arg    |
|           |                    |          | 2043: G/A | Thr        |
|           |                    |          | 2709: G/A | Arg        |
| TLR13     | 2                  | 0:1      | 2122: C/A | Arg/Ser   |

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cattle, pigs and robins [62–65]. A total of 40 SNPs were identified across all loci, all of which were biallelic and 16 were non-synonymous (Table 2). 13 of the non-synonymous SNPs were located in the extracellular domain, while the others in the intracellular domain. For each individual we have provided a summary of TLR genotypes and mitochondrial genotypes in S4 Table.

The level of TLR diversity that was observed in koalas in this study is comparable to that observed in other species (Table 3). For instance, 73 alleles have been identified at TLR5 in 158 humans from Africa, Europe and East-Asia [66]. A study on 259 pigs from six populations identified 16 SNPs within the TLR4 exon 3. To make our comparison more relevant, we have compared the koala results with those 56 outbred pigs, rather than incorporating inbred populations [67]. Grueber et al. 2012 studied a bottlenecked population of 24 New Zealand Robins from an isolated island population, and observed a range of 1–5 alleles within all TLRs coding sequences [65] (Table 3). The koala samples used here were selected to maximize genetic diversity, but it is important to note that additional alleles are likely to be found if more samples were analysed, particularly if samples from additional geographic regions were analysed.

By comparing rates of synonymous (dS) and non-synonymous (dN) substitutions [68] we found that dS is higher than dN in all nine koala TLR genes characterized here. No non-synonymous substitutions were observed in TLR2 and TLR1/6-like (Table 2). The absence of non-

| Species, Genus and Species Name | Genes | Samples | SNPs | Alleles | References |
|--------------------------------|-------|---------|------|---------|------------|
| Koala, Phascolarctos cinereus | TLR2  | 20      | 3    | 4       | This study |
|                                | TLR3  |         | 4    | 4       |            |
|                                | TLR4  |         | 8    | 12      |            |
|                                | TLR5  |         | 7    | 8       |            |
|                                | TLR1/6like | 1 | 2 |        |            |
|                                | TLR7  |         | 5    | 4       |            |
|                                | TLR8  |         | 4    | 5       |            |
|                                | TLR9  |         | 7    | 10      |            |
|                                | TLR10 |         | 0    | 1       |            |
|                                | TLR13 |         | 1    | 2       |            |
| New Zealand Robin, Petroica australis rakiura | TLR2A | 17–24 | 1 | 2 | [65] |
| Pig, Sus scrofa | TLR4 | 56 | 16 | 23 | [67] |
| Human, Homo sapiens | TLR1 | 158 | 59 | 52 | [66] |
|                                | TLR2  |         | 24   | 23      |            |
|                                | TLR3  |         | 41   | 55      |            |
|                                | TLR4  |         | 66   | 59      |            |
|                                | TLR5  |         | 73   | 61      |            |
|                                | TLR6  |         | 44   | 36      |            |
|                                | TLR7  |         | 26   | 31      |            |
|                                | TLR8  |         | 33   | 46      |            |
|                                | TLR9  |         | 27   | 30      |            |
|                                | TLR10 |         | 64   | 55      |            |

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synonymous mutations may be the result of selective pressures [69]. Purifying selection appears to be acting on TLR2 and TLR4 (Table 4) and it is tempting to speculate that mutations in the pathogen-binding region of extracellular domain in these genes may adversely affect fitness. In future it will be interesting to investigate whether there is an association between these genes and response to disease. Polymorphisms at human TLR2 and TLR4 have been found to be associated with the inflammation and increased disease susceptibility. For instance, SNPs Arg753Gln and Arg677Trp at TLR2 contribute to the course of sepsis [70] and D299G and T399I at TLR4 are associated with infections caused by *Legionella pneumophila* [71].

Given the locations that these koalas were sampled from, it is likely that all of the koalas tested in this study were KoRV positive based on previous observations that koalas in north-eastern NSW were 100% KoRV-positive [72]. Nothing is known about the chlamydial infection status of the animals sampled in this preliminary study, limiting any further commentary on the association between TLR variants and koala disease. While this is an unfortunate limitation, future studies utilising the TLR molecular markers described here alongside the general availability of tissue samples from *Chlamydia*-free, natural *Chlamydia*-infected but clinically healthy koalas and koalas that have developed severe *Chlamydia*-related ocular or reproductive tract pathology means that koala researchers will now be in a strong position to investigate whether TLRs have an impact on koala chlamydial disease pathogenesis. To this end, work in naturally infected women has already provided the first suggestions that TLR sequence variation may be associated with increased risk of infection and inflammatory disease [29], but such studies have been generally limited by sample size. With the recent availability of genetic resources for the koala [73], expanded koala studies such as those proposed may provide new insights into host genetic susceptibility more generally while also providing stakeholders with important management information that can be used to deploy conservation tools such as a prototype koala chlamydial vaccine [74].

### Supporting Information

**S1 Fig. Mitochondrial Haplotypes.** Haplotype network showing stepwise sequence divergence, for koala samples from NSW used in this study. Each step depicts a nucleotide difference between haplotypes. H12, 18 and H5 previously reported by [5], and Q1 previously reported

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**Table 4. Selection test at nine koala TLRs.**

| Gene        | $d_N$ | $d_S$ | $d_N-d_S$ | $Z$ test of neutral selection $(d_N=d_S)$ | $Z$ test of purifying selection $(d_N<d_S)$ |
|-------------|-------|-------|-----------|------------------------------------------|-------------------------------------------|
|             | Statistic | P     | Statistic | P       |
| TLR2        | $0$   | $0.003$ | $-0.003$  | $-1.823$ | $0.071$ | $1.801$ | $0.037^*$ |
| TLR3        | $0$   | $0.004$ | $-0.004$  | $-1.565$ | $0.12$  | $1.552$ | $0.062$   |
| TLR4        | $0.001$ | $0.004$ | $-0.003$  | $-1.634$ | $0.105$ | $1.803$ | $0.036^*$ |
| TLR5        | $0.001$ | $0.002$ | $-0.001$  | $-0.938$ | $0.35$  | $0.926$ | $0.178$   |
| TLR1/6like  | $0$   | $0.002$ | $-0.002$  | $-1.038$ | $0.301$ | $1.031$ | $0.152$   |
| TLR7        | $0.001$ | $0.002$ | $-0.001$  | $-1.362$ | $0.176$ | $1.371$ | $0.086$   |
| TLR8        | $0$   | $0.001$ | $-0.001$  | $-0.969$ | $0.334$ | $0.971$ | $0.167$   |
| TLR9        | $0.001$ | $0.002$ | $-0.002$  | $-1.574$ | $0.118$ | $1.571$ | $0.059$   |
| TLR13       | $0.001$ | $0$   | $0.001$   | $1.059$  | $0.292$ | $-1.078$ | $1.000$   |

*Significant purifying selection $p < 0.05$
by [6]. 1, 2, 3, 6, 8 all novel haplotypes previously unreported.

S2 Fig. Koala TLR2 Nucleotide and Amino acid sequences. Boxes and arrows means the locations of primers (→: forward primer, ←: reverse primer). Vertical lines show the boundaries of Leucine-Rich Repeats, transmembrane and cytoplasmic region.

S3 Fig. Koala TLR3 Nucleotide and Amino acid sequences.

S4 Fig. Koala TLR4 Nucleotide and Amino acid sequences.

S5 Fig. Koala TLR5 Nucleotide and Amino acid sequences.

S6 Fig. Koala TLR1/6like Nucleotide and Amino acid sequences.

S7 Fig. Koala TLR7 Nucleotide and Amino acid sequences.

S8 Fig. Koala TLR8 Nucleotide and Amino acid sequences.

S9 Fig. Koala TLR9 Nucleotide and Amino acid sequences.

S10 Fig. Koala TLR10 Nucleotide and Amino acid sequences.

S11 Fig. Koala TLR13 Nucleotide and Amino acid sequences.

S12 Fig. Amino acid alignment at TLR2. Amino acid alignment of coding sequences of TLR2 in koala, Tasmanian devil, gray short-tailed opossum, house mouse, cattle and human. Dashes in the sequences represent gaps. Dots represent conservation of amino acids with the koala sequence. The ruler has been adjusted according to the koala TLR sequence. The LRR motifs are in green and are marked above with the consensus pattern: LxxLxLxxNxL according to human TLRs (“L” is Leu, Ile, Val, or Phe. “N” is Asn, Thr, Ser, or Cys. “x” represent residue.) [54]. The positions of the β-strand are shown as arrows above the sequences [58]. The stars above the residues indicate predicted pathogen binding positions in human [58].

S13 Fig. Amino acid alignment at TLR3. The stars above the residues are predicted pathogen binding positions in human TLR3 [53]. The predicted pathogen binding positions are in box according to human TLR3 [54]. The “^” above the residues indicate sites of non-synonymous substitutions.

S14 Fig. Amino acid alignment at TLR4. The predicted pathogen binding positions are in box according to human TLR4 [55].
S15 Fig. Amino acid alignment at TLR5. The predicted pathogen binding positions are in box according to human TLR5 [56]. (TIF)

S16 Fig. Amino acid alignment at TLR1/6like. The predicted pathogen binding positions are in box according to human TLR6 [25]. (TIF)

S17 Fig. Amino acid alignment at TLR7. The predicted pathogen binding positions are in box according to human TLR7 [25]. (TIF)

S18 Fig. Amino acid alignment at TLR8. The predicted pathogen binding positions are in box according to human TLR8 [25]. (TIF)

S19 Fig. Amino acid alignment at TLR9. The predicted pathogen binding positions are in box according to human TLR9 [25]. (TIF)

S20 Fig. Amino acid alignment at TLR10. The predicted pathogen binding positions are in box according to human TLR10 [25]. (TIF)

S21 Fig. Amino acid alignment at TLR13. (TIF)

S1 Table. Accession numbers of Toll-like receptor sequences used in this study. Amino acid sequence accession numbers were all obtained from GenBank, except Xenopus tropicalis TLR9 that was from Ensemble. (XLS)

S2 Table. Australian Museum registration numbers of investigated koalas. (XLS)

S3 Table. GenBank accession number of koala TLRs. (XLS)

S4 Table. Genotype of TLRs and mitochondrial DNA. (XLS)

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Author Contributions
Conceived and designed the experiments: KB. Performed the experiments: JC GF. Analyzed the data: JC GF DOM YC. Contributed reagents/materials/analysis tools: GF RJ AP PT. Wrote the paper: JC GF RJ AP PT DOM YC KB.
References

1. Melzer A, Carrick F, Menkhorst P, Lunney D, John BS. Overview, critical assessment, and conservation implications of koala distribution and abundance. Conserv Biol. 2000; 14: 619–628.

2. Lee AK, Martin RW. The koala: a natural history: New South Wales University Press. Kensington. 1988

3. Houlden B, England P, Taylor A, Greville W, Sherwin W. Low genetic variability of the koala Phascolarctos cinereus in south-eastern Australia following a severe population bottleneck. Mol Ecol. 1996; 5: 269–281. PMID: 8673272

4. Lee KE, Seddon JM, Corley SW, Ellis WA, Johnston SD, de Villiers DL, et al. Genetic variation and structuring in the threatened koala populations of Southeast Queensland. Conserv Genet. 2010; 11: 2019–2103.

5. Houlden BA, Costello BH, Sharkey D, Fowler EV, Melzer A, Ellis W, et al. Phylogeographic differentiation in the mitochondrial control region in the koala, Phascolarctos cinereus (Goldfuss 1817). Mol Ecol. 1999; 8: 999–1011. PMID: 10434420

6. Fowler E, Houlden B, Hoeben P, Timms P. Genetic diversity and gene flow among southeastern Queensland koalas (Phascolarctos cinereus). Mol Ecol. 2000; 9: 155–164. PMID: 10672159

7. Jobbins SE, Sanderson CE, Griffith JE, Krockenberger MB, Belov K, Higgins DP. Diversity of MHC class II DAB1 in the koala (Phascolarctos cinereus). Aust J Zool. 2012; 60: 1–9.

8. Lau Q, Jobbins SE, Belov K, Higgins DP. Characterisation of four major histocompatibility complex class II genes of the koala (Phascolarctos cinereus). Immunogenetics. 2013; 65: 37–46. doi: 10.1007/s00251-012-0658-5 PMID: 23089959

9. Tarlinton RE, Meers J, Young PR. Retroviral invasion of the koala genome. Nature. 2006; 442: 79–81. PMID: 16823453

10. Mehta SD, Moses S, Agot K, Parker C, Ndinya-Achola JO, Maclean I, et al. Adult male circumcision does not reduce the risk of incident Neisseria gonorrhoeae, Chlamydia trachomatis, or Trichomonas vaginalis infection: results from a randomized, controlled trial in Kenya. J Infect Dis. 2009; 200: 370–378. doi: 10.1086/600074 PMID: 19545209

11. Polkinghorne A, Hanger J, Timms P. Recent advances in understanding the biology, epidemiology and control of chlamydial infections in koalas. Vet Microbiol. 2013; 165: 214–223. doi: 10.1016/j.vetmic.2013.02.026 PMID: 23523170

12. Wan C, Loader J, Hanger J, Beagley K, Timms P, Polkinghorne A. Using quantitative polymerase chain reaction to correlate Chlamydia pecorum infectious load with ocular, urinary and reproductive tract disease in the koala (Phascolarctos cinereus). Aust Vet J. 2011; 89: 409–412. doi: 10.1111/j.1751-0813.2011.00827.x PMID: 21933169

13. Bachmann NL, Polkinghorne A, Timms P. Chlamydia genomics: providing novel insights into chlamydial biology. Trends Microbiol. 2014.

14. Darville T, Hiltke TJ. Pathogenesis of genital tract disease due to Chlamydia trachomatis. J Infect Dis. 2010; 201: S114–S125. PMID: 20524234

15. Denner J, Young PR. Koala retroviruses: characterization and impact on the life of koalas. Retrovirology. 2013; 10: 108.101–108.107.

16. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol. 2001; 2: 675–680. PMID: 11477402

17. McGuire K, Jones M, Werling D, Williams J, Glass E, Jann O. Radiation hybrid mapping of all 10 characterized bovine Toll-like receptors. Anim Genet. 2006; 37: 47–50. PMID: 16441295

18. Werling D, Coffey TJ. Pattern recognition receptors in companion and farm animals—The key to unlocking the door to animal disease? Vet J. 2007; 174: 240–251. PMID: 17137812

19. Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, et al. The evolution of vertebrate Toll-like receptors. P Natl Acad Sci USA. 2005; 102: 9577–9582. PMID: 15976025

20. Cui J, Cheng Y, Belov K. Diversity in the Toll-like receptor genes of the Tasmanian devil (Sarcophilus harrisii). Immunogenetics. 2015; 1–7.

21. Alexeopoulos L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kB by Toll-like receptor 3. Nature. 2001; 413: 732–738. PMID: 11607032

22. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science. 2004; 303: 1526–1529. PMID: 14976262

23. McCartney SA, Colonna M. Viral sensors: diversity in pathogen recognition. Immunol Rev. 2009; 227: 87–94. doi: 10.1111/j.1600-065X.2008.00726.x PMID: 19120478
Campos MA, Almeida IC, Takeuchi O, Akira S, Valente EP, Procópio DO, et al. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol. 2001; 167: 416–423. PMID: 11418678

Bell JK, Mullen GE, Leifer CA, Davies DR, Segal DM. Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol. 2003; 24: 528–533. PMID: 14552836

Poltorak A, He X, Smirnova I, Liu M-Y, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57Bl/10ScCr mice: mutations in Tlr4 gene. Science. 1998; 282: 2085–2088. PMID: 9851930

Oldenburg M, Krüger A, Ferstl R, Kaufmann A, Nees G, Sigmund A, et al. TLR13 Recognizes Bacterial 23S rRNA Devoid of Erythromycin Resistance–Forming Modification. Science. 2012; 337: 1111–1115. doi:10.1126/science.1220363 PMID: 22821982

Yang X, Joyee A. Role of toll-like receptors in immune responses to chlamydial infections. Curr Pharm Design. 2008; 14: 593–600. PMID: 18336303

Taylor BD, Danville T, Ferrell RE, Kammerer CM, Ness RB, Haggerty CL. Variants in toll-like receptor 1 and 4 genes are associated with Chlamydia trachomatis among women with pelvic inflammatory disease. J Infect Dis. 2012; 205: 603–609. doi: 10.1093/infdis/jir822 PMID: 22238472

Houlden BA, Greville WD, Sherwin WB. Evolution of MHC class I loci in marsupials: characterization of sequences from koala (Phascolarctos cinereus). Mol Biol Evol. 1996; 13: 1119–1127. PMID: 8656665

Morris K, Prentis PJ, O’Meally D, Pavalasovic A, Brown AT, Timms P, et al. The koala immunological toolkit: sequence identification and comparison of key markers of the koala (Phascolarctos cinereus) immune response. Aust J Zool. 2014.

Mathew M, Beagley KW, Timms P, Polkinghorne A. Preliminary characterisation of tumour necrosis factor alpha and interleukin-10 responses to Chlamydia pecorum infection in the koala (Phascolarctos cinereus). Plos one. 2013; 8: e59958. doi:10.1371/journal.pone.0059958 PMID: 23527290

Mathew M, Waugh C, Beagley KW, Timms P, Polkinghorne A. Interleukin 17A is an immune marker for chlamydial disease severity and pathogenesis in the koala (Phascolarctos cinereus). Dev Comp Immunol. 2014; 46: 423–429. doi: 10.1016/j.devimm.2014.05.015 PMID: 24915607

Maher IE, Griffith JE, Lau Q, Reeves T, Higgins DP. Expression profiles of the immune genes CD4, CD8β, IFNγ, IL-4, IL-6 and IL-10 in mitogen-stimulated koala lymphocytes (Phascolarctos cinereus) by qRT-PCR. PeerJ. 2014; 2: e280. doi: 10.7717/peerj.280 PMID: 24688858

Mathew M, Pavalasovic A, Prentis PJ, Beagley KW, Timms P, Polkinghorne A. Molecular characterisation and expression analysis of Interferon gamma in response to natural Chlamydia infection in the koala, Phascolarctos cinereus. Gene. 2013; 527: 570–577. doi: 10.1016/j.gene.2013.06.019 PMID: 23792018

Johnson RN, Hobbs M, Eldridge MDB, King AG, Colgan DJ, Wilkins MR, et al. The Koala Genome Consortium. Tech. Rep. Aust. Mus., Online. 2014: No. 24, pp.91–98.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011; 28: 2731–2739. doi: 10.1093/molbev/msr121 PMID: 21546353

Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987; 4: 406–425. PMID: 3447015

Frankham GJ, Handasyde KA, Norton M, Murray A, Eldridge MD. Molecular detection of intra-population structure in a threatened potoroid, Potorous tridactylus: conservation management and sampling implications. Conserv Genet. 2014; 15: 547–560.

Zhang X-y, Gao Y-n. To design PCR primers with Oligo 6 and Primer Premier 5 [J]. Bioinformatics. 2004; 4: 003.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. 1999. pp. 95–98.

Medzhhitov R, Janeway C Jr. The Toll receptor family and microbial recognition. Trends Microbiol. 2000; 6: 452–456. PMID: 11044679

Kopp EB, Medzhhitov R. The Toll-receptor family and control of innate immunity. Curr Opin Immunol. 1999; 11: 13–18. PMID: 10047546

Parcell MK, Smith KD, Aderem A, Hood L, Winton JR, Roach JC. Conservation of Toll-like receptor signaling pathways in teleost fish. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics. 2006; 1: 77–88. PMID: 17330145

Meijer AH, Gabby Krens S, Medina Rodriguez IA, He S, Bitter W, Ewa Snaar-Jagalska B, et al. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. Mol Immunol. 2004; 40: 773–783. PMID: 14687934
46. Ishii A, Kawasaki M, Matsumoto M, Tochinai S, Seya T. Phylogenetic and expression analysis of amphibian Xenopus Toll-like receptors. Immunogenetics. 2007; 59: 281–293. PMID: 17265063
47. Temperley ND, Berlin S, Paton IR, Griffin DK, Burt DW. Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. BMC genomics. 2008; 9: 62. doi: 10.1186/1471-2164-9-62 PMID: 18241342
48. Iqbal M, Philbin VJ, Smith AL. Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. Vet Immunol Immunop. 2005; 104: 117–127. PMID: 15661337
49. Akira S. Mammalian Toll-like receptors. Curr Opin Immunol. 2003; 15: 5–11. PMID: 12495726
50. Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immun. 2006; 117: 979–987. PMID: 16675322
51. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004; 4: 499–511. PMID: 15229469
52. Matsushima N, Tanaka T, Enkibayar P, Mikami T, Taga M, Yamada K, et al. Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. BMC genomics. 2007; 8: 124. PMID: 17517123
53. Bell JK, Askins J, Hall PR, Davies DR, Segal DM. The dsRNA binding site of human Toll-like receptor 4. P Natl Acad Sci USA. 2005; 102: 10976–10980. PMID: 16043704
54. Hajjar AM, Ernst RK, Tsai JH, Wilson CB, Miller SI. Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nat Immunol. 2002; 3: 354–359. PMID: 11912479
55. Mizel SB, West AP, Hantgan RR. Identification of a sequence in human toll-like receptor 5 required for the binding of Gram-negative flagellin. J Biol Chem. 2003; 278: 23624–23629. PMID: 12711596
56. Huang Y, Temperley ND, Ren L, Smith J, Li N, Burt DW. Molecular evolution of the vertebrate TLR1 gene family-a complex history of gene duplication, gene conversion, positive selection and co-evolution. BMC Evol Biol. 2011; 11: 149. doi: 10.1186/1471-2148-11-149 PMID: 21619680
57. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik S-G, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell. 2007; 130: 1071–1082. PMID: 17889651
58. Jiang Y, Ranoa DRE, Jiang S, Mutha SK, Li X, Baudry J, et al. Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. J Immunol. 2010; 184: 5094–5103. doi: 10.4049/jimmunol.0901888 PMID: 20348427
59. Hijichi M, Matsuo A, Shingai M, Shida K, Ishii A, Funami K, et al. Combinational recognition of bacterial lipoproteins and peptidoglycan by chicken Toll-like receptor 2 subfamily. Dev Comp Immunol. 2008; 32: 147–155. PMID: 17614130
60. Smirnova I, Poltorak A, Chan E, McBride C, Beutler B. Phylogenetic variation and polymorphism at the toll-like receptor 4 locus (TLR4). Genome Biol. 2000; 1: 002.010. PMID: 11178226
61. Miller SI, Ernst RK, Bader MW. LPS, TLR4 and infectious disease diversity. Nat Rev Microbiol. 2005; 3: 36–46. PMID: 15608698
62. Seabury CM, Seabury PM, Decker JE, Schnabel RD, Taylor JF, Womack JE. Diversity and evolution of 11 innate immune genes in Bos taurus taurus and Bos taurus indicus cattle. P Natl A Sci. 2010; 107: 151–156.
63. Grueber CE, Wallis GP, King TM, Jamieson IG. Variation at innate immunity Toll-like receptor genes in a bottlenecked population of a New Zealand robin. Plos one. 2012; 7: e45011. PMID: 23024782
64. Barreiro LB, Ben-Ali M, Quach H, Laval G, Patin E, Pickrell JK, et al. Evolutionary dynamics of human Toll-like receptors and their different contributions to host defense. Plos Genet. 2008; 5: e1000562. doi: 10.1371/journal.pgen.1000562 PMID: 19609346
65. Palermo S, Capra E, Torremorell M, Dolzan M, Davoli R, Haley C, et al. Toll-like receptor 4 genetic diversity among pig populations. Anim Genet. 2009; 40: 389–393. doi: 10.1111/j.1365-2052.2008.01833.x PMID: 19290993
66. Yang Z. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. Mol Biol Evol. 1998; 15: 568–573. PMID: 9580986
67. Zhang DX, Hewitt GM. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. Mol Ecol. 2003; 12: 563–564. PMID: 12675814
68. Woeirie T, Du W, Goetz A, Hsu H-Y, Joos TO, Weiss M, et al. Pathogen specific cytokine release reveals an effect of TLR2 Arg753Gln during Candida sepsis in humans. Cytokine. 2008; 41: 322–329. doi: 10.1016/j.cyto.2007.12.006 PMID: 18249133
71. Hawn TR, Verbon A, Janer M, Zhao LP, Beutler B, Aderem A. Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires’ disease. P Natl Acad Sci USA. 2005; 102: 2487–2489. PMID: 15699327

72. Simmons G, Young P, Hanger J, Jones K, Clarke D, McKee J, et al. Prevalence of koala retrovirus in geographically diverse populations in Australia. Aust Vet J. 2012; 90: 404–409. doi: 10.1111/j.1751-0813.2012.00964.x PMID: 23004234

73. Hobbs M, Pavasovic A, King AG, Prentis PJ, Eldridge MD, Chen Z, et al. A transcriptome resource for the koala (Phascolarctos cinereus): insights into koala retrovirus transcription and sequence diversity. BMC Genomics. 2014; 15: 786. doi: 10.1186/1471-2164-15-786 PMID: 25214207

74. Kollipara A, George C, Hanger J, Loader J, Polkinghorne A, Beagley K, et al. Vaccination of healthy and diseased koalas (Phascolarctos cinereus) with a Chlamydia pecorum multi-subunit vaccine: Evaluation of immunity and pathology. Vaccine. 2012; 30: 1875–1885. doi: 10.1016/j.vaccine.2011.12.125 PMID: 2230583