Breed-specific variations in the coding region of toll-like receptor 4 in the domestic cat

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ABSTRACT

Specific point mutations in the human toll-like receptor 4 (TLR4) confer altered risk for diverse diseases including sepsis, aspergillosis and inflammatory bowel disease. Some of these TLR4 polymorphisms are racially specific. We hypothesised that feline TLR4 polymorphisms might underlie an observed increased risk to infections and inflammatory diseases in some cat breeds. The aim of this study was to identify breed-specific variations in the coding region of feline TLR4 and to model the effect of mutations on protein structure and function in silico. The entire coding region of TLR4 was sequenced in 8 groups (7 pure-bred, 1 crossbred) of domestic cats (Felis catus) comprising 158 individuals. Twenty-two single nucleotide polymorphisms (SNPs) were identified in TLR4, with 16 located in the coding region (11 non-synonymous) and four in the 3′UTR. Comparison of breed specific allelic frequencies indicated that Burmese and British shorthairs most commonly differed from other breeds. In silico analyses to predict the impact of the 11 non-synonymous variants indicated a deleterious effect on protein structure for one SNP (c.869 G > A), which was not associated with a specific breed. Overall, findings from this study do not support a role of TLR4 dysfunction in breed-predispositions to infectious diseases in domestic cats in Australia.

1. Introduction

The innate immune response is triggered when pattern recognition receptors (PRRs) on sentinel cells bind conserved molecular motifs on pathogens (pathogen-associated molecular patterns or PAMPs), and damage-associated molecular patterns (DAMPs) released from host cells (Amarante-Mendes et al., 2018). Toll-like receptors (TLRs) are the most extensively studied PRR family to date (Takeuchi and Akira, 2010) (Thompson et al., 2011) with 13 members identified in mammals (Nie et al., 2018). TLRs are characterised by an extracellular, leucine-rich repeat (LRR) domain (Bell et al., 2003) and a conserved intracellular Toll/interleukin-1 receptor (TIR) domain (Janssens and Beyaert, 2003). Binding to the LRR extracellular domain results in recruitment of TIRs and initiation of intracellular signalling leading to inflammatory cytokine production (Akira and Takeda, 2004). TLR4 is a central player in the activation of innate immunity (Zhao et al., 2017) and the gram negative endotoxin lipopolysaccharide (LPS) is its primary ligand (Arbour et al., 2000). Polymorphisms in TLR4 have been associated with hyporesponsiveness to LPS (Arbour et al., 2000) and are proposed to contribute to sepsis (Anderberg et al., 2017; Rosadini and Kagan, 2017). In addition to LPS, TLR4 binds a broad range of structurally unrelated bacterial, viral, fungal and protozoal PAMPs (Mukherjee et al., 2016) so SNPs in the TLR4 gene can potentially alter susceptibility to diverse infectious diseases (Lass-Florl et al., 2013). For example, TLR4 polymorphisms have been associated with an increased risk of invasive aspergillosis in immunosuppressed Caucasian patients (Bochud et al., 2008; Carvalho et al., 2009; de Boer et al., 2011) and with a protective effect against congenital toxoplasmosis (Wujcicka et al., 2015).

Among inflammatory diseases, a meta-analysis of the role of TLR4 polymorphisms in susceptibility to inflammatory bowel disease demonstrated a significant association between the Asp299Gly polymorphism and risk of Crohn’s disease and ulcerative colitis in humans and a possible association between Thr399Ile and inflammatory bowel disease in race specific patient groups (Cheng et al., 2015). Other chronic inflammatory diseases in humans where TLR4 polymorphisms...
are implicated in susceptibility include asthma (Tizaoui et al., 2015; Schurman et al., 2018) and complications of type 2 diabetes (Singh et al., 2013). Among companion animals, two TLR4 polymorphisms have been associated with inflammatory bowel disease in German shepherd dogs (Kathani et al., 2010).

In domestic cats, TLR4 polymorphisms are yet to be investigated although cats suffer many of the infectious and inflammatory conditions that are linked to altered TLR4 expression in humans. Feline TLR4 has been associated with infectious disease pathogenesis in cats; expression of TLR4 is increased in neutrophil exposure to Microsporum canis, and in feline infectious peritonitis (FIP) (Camber et al., 2016; Watanabe et al., 2018) while progesterone-induced TLR4 down-regulation is implicated in the development of pyometra in breeding queens (Jursza et al., 2015). Feline breed-specific differences in susceptibility to viral and fungal infections, including FIP and invasive mycoses, are documented (Pesteau-Somogyi et al., 2006; Worthing et al., 2012; Barrs et al., 2015), but potential underlying immune dysfunction remains under-investigated. A key co-receptor in feline coronavirus infection, the C-type lectin PRR, DC-SIGN, was investigated for polymorphisms and a SNP in the DC-SIGN extracellular domain was associated with FIP susceptibility (Wang et al., 2014).

The objective of this study was to identify polymorphisms in feline TLR4 and to model their effect on protein structure and predicted function, which might influence disease pathogenesis. A secondary aim was to compare variant allele frequency between breeds which might contribute to known breed predisposition to diseases.

2. Materials and methods

2.1. Samples

This study was approved by the Animal Care and Ethics Committee, University of Sydney, Approval numbers N00/7–2013/3/6029 and 2015/902.

A total of 158 Australian domestic cats (Felis catus) from 8 groups (7 purebred, 1 crossbred) were available for study: Persian/Himalayan (n = 30), Burmese (n = 25), British Shorthair/Scottish Shorthair (BSH; n = 30), Siamese (n = 10), Bengali (n = 9), Birman (n = 10), Ragdoll (n = 9) and domestic crossbred cats (n = 35).

The pure breeds included were selected from a range of ancestral groups based on genetic differences using Bayesian clustering of SNPs and short tandem repeats (Kurushima et al., 2013). Of the 158 cats included for study, 25 Persian/Himalayan and 25 BSH cats were from breeding catteries (two catteries for each breed). The remaining cats were client-owned patients of the University Veterinary Teaching Hospital Sydney. Samples used for DNA extraction were either saliva collected using oral swabs (Performagene, DNAgenotek, Ottawa Canada) or residual diagnostic blood samples in EDTA stored at −80 °C.

2.2. DNA extraction, PCR and mutation analysis

Genomic DNA was extracted from EDTA-blood using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden Germany). DNA was extracted from saliva swabs using the manufacturer’s protocol (DNAgenotek, Ottawa Canada) and quantitated using spectrophotometry (Nanodrop™, 2000, ThermoFisher Scientific, Waltham, USA). Primers were designed for the coding region of feline TLR4 using Primer3Plus software (http://www. bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) based on the felCat8 reference genome (GCA_000181335.3) (Table 1). Exon 3 was divided into 6 overlapping segments to facilitate ease of sequencing of the products. Primer sequences are listed in Table1.

Polymerase chain reaction was performed using MyTaq™ DNA Polymerase (Bioline, UK), in a total volume of 25 μL. Each reaction comprised 1.25 units MyTaq™ DNA Polymerase, 5 μL MyTaq™ Reaction Buffer, 1 μL each forward and reverse primers (10 μM), 5–15 ng template DNA and 16.75 μL molecular water. Reactions were heated to 95 °C for 3 min, followed by 35 cycles of 95 °C for 15 s, annealing temperatures (Table 1) for 15 s and 72 °C for 1 min, then a further 72 °C for 7 min. The PCR products obtained were checked for yield and purity on a 1% w/v agarose gel and samples which displayed expected product sizes were submitted to Macrogen Inc (Seoul, South Korea) for purification and Sanger sequencing using the PCR primers. Sequence data was compared with the feline TLR4 cDNA sequence (NM_001009223.1) (Asahina et al., 2003) using Sequencher® 5.4.6 (Gene Codes Corporation, Ann Arbor, USA).

Haplotypes were determined using Haploview (www. broadinstitute.org/haploview/haploview) (Barrett et al., 2005) with all SNPs analysed as a single haplotype block. The effect of each SNP on the protein sequence was predicted using ExPASy (https://web.expasy. org/translate/) and compared to the feline TLR4 mRNA sequence (NP_001009223.1) using Blast Local Alignment Search Tool (BLAST; blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned with reported TLR4 amino acid sequences from humans (NP_612564.1), pigs (NP_001106510.2), cattle (NP_776623.5) and dogs (NP_001002950.2). The functional effects of the non-synonymous SNPs identified were assessed using PolyPhen-2 (http://genetics.bwh.harvard.edu/php2) (Adzhubei et al., 2010).

2.3. Allele frequency analysis

Cats known to be highly related (20 Persian/Himalayans and 20 BSH from two catteries) were excluded from breed analyses. Thus, allele frequency analysis was performed in 118 cats. Variant allele frequencies were calculated for each breed using Microsoft Excel 2010. Differences in allele frequency between breeds were calculated using Fisher’s exact tests. Statistical significance was set at p < 0.05.

3. Results

3.1. Sequence analysis

Sequence analysis of the exonic and exon flanking regions of the feline TLR4 gene in 158 animals representing 8 pure-breed groups and 1 cross-bred group identified 22 polymorphisms (Table 2). Sixteen polymorphisms were identified located in the coding region and four in the 3’UTR of TLR4. Of the 16 polymorphisms in the coding region 11 were non-synonymous.

Evaluation of the predicted consequences of the non-synonymous SNPs revealed that one SNP (c.869 G > A), has a significant functional effect on protein structure. The polymorphism occurred within exon 3 and codes for an amino acid in the extracellular domain in the LRR 10 human homologue (Fig. 1). This variant was present in three of the 158 cats (2 domestic crossbred, 1 Bengal). All three cats were heterozygous at this position. None of the identified polymorphisms resulted in a high effect (STOP codon, frameshift, splicing) on protein function.

The four polymorphisms in the 3’UTR included three SNPs and one single base pair insertion. Two additional variants were identified within the flanking regions of exon 1 when the sequences were compared to the feline reference genome DNA sequence (NC_018735.3). These included a SNP 28bp upstream from exon 1 (g.1–28) and a single base pair deletion in intron 1 (g.154). The latter affected the reverse sequencing of the DNA fragment including exon 1. Due to the high quality of forward sequences, the lack of variations identified within the exon and the non-coding nature of this region, additional primers were not designed to overcome this.

All cats investigated were homozygous for the alternate allele for six of the 22 SNPs identified compared to the feline reference genome (GCF_000181335.3). Three of these were non-synonymous SNPs and none were identified to have a functional effect based on predictive software analysis. Furthermore, when compared to the amino acid sequences of other species, the variant amino acids predicted based on the nucleotide sequences in the present study are the same as for those
Table 1

| Exon | Forward (5'-3') | Reverse (3'-5') | Product length | Annealing temperature |
|------|----------------|----------------|----------------|----------------------|
| 1    | CAGCGTTGCTTTGAAATACAG | ATGAAATTTCGCACTAGCA | 362 | 54°C |
| 2    | GGATGAAAGATGGTTGGATG | GGAGACCTCCTGACATAGAACG | 397 | 58°C |
| 3    | -a TGTCCATGTGTTGAAAGGAC | AGATGCCAGCAGAGAAATGGT | 793 | 54°C |
|      | -b GACTGTGTCCCTAACCTTT | TTGGAAGGAGGTGTCCTG | 844 | 54°C |
|      | -c GTTAAATGGAAAAAGCTTGA | ATACCAGCAAGCAGCCAACA | 852 | 60°C |
|      | -d AACTCTTACTCTCCTCCACG | CATCAGGGTTTTCAGTGC | 829 | 54°C |
|      | -e TTTCAGCCTCTGCTTTCACGT | CATCAAGGCGAAATGATG | 849 | 54°C |
|      | -f CAAGGGCAAGTATTGAGTG | CATCAAGGCGAAATGATG | 679 | 54°C |

3.2. Breed specific allele frequency

118 cats (35 crossbred, 10 Persian/Himalayan, 10 BSH/SHH, 9 Ragdoll, 9 Bengal, 25 Burmese, 10 Birman and 10 Siamese) were included in allele frequency calculations. For six of the identified SNPs (c.564 T > C, c.1057C > T, c.1099 G > A, c.1140C > T, c.352 A > G, c.753 A > G), each breed tested (Cuscó et al., 2014), suggesting the potential for a breed predisposition to diseases where TLR4 function is critical.

3.3. Haplotype frequencies

A total of 13 TLR4 haplotypes were identified in the cats tested with 11 found in crossbred cats and none occurring in all groups (Table 5). The number of breed-specific haplotypes per cat ranged from two, in BSH and Birman cats, to six in Persians. No single haplotype occurred in all breeds. Two haplotypes (TLR4-12 and TLR4-13) were present in one breed each (Bengal and Ragdoll, respectively) and only at very low frequencies. The lowest TLR4 haplotype diversity was in Siamese and Birman cats, with a single haplotype occurring with frequencies of 75% in each breed.

4. Discussion

Polymorphisms in TLR4 have not been previously reported in cats. Here we identified 16 coding and six non-coding variants. One non-synonymous SNP, c.869 G > A, is predicted to have a significant effect on the protein structure. The PolyPhen-2 output of “possibly damaging” for this SNP represents a low-confidence prediction of a deleterious effect rather than a prediction of a minor effect of the substitution (Adzhubei et al., 2010, 2013). An analogous mutation has been identified in humans (rs200276033), although its clinical significance has not been investigated. This polymorphism lies within the region coding for LRR 10 in the human TLR4. Structural deviation of this LRR from the consensus LRR motif is postulated to be crucial in PAMP recognition (Bell et al., 2003). This hypothesis is supported by studies showing that amino acid residues 285–366, a region which includes most of LRR 10, mediate the ability of TLR4 to differentiate between difference types of LPS (Hajjar et al., 2002) and that the Asp299Gly substitution results in LPS hyporesponsiveness (Arbour et al., 2000).

Asp299Gly or Thr399Ile are two of the most commonly occurring amino acid substitutions in TLR4 in humans (ferwerda et al., 2008). The significance of co-segregation and functional consequences of these substitutions continues to be investigated. However, a specific racial distribution of this TLR4 haplotype and its effect on infectious disease susceptibility has been identified (ferwerda et al., 2008; ziakas et al., 2013). No polymorphisms were identified in any of the cats in the present study that corresponded to amino acid substitutions at either locus.

The SNP rate in the current study was 1 SNP/204bp which is higher than the reported feline rate of 1 SNP/5–600, calculated using whole genome sequences (mullikin et al., 2010). This high mutation rate is not unexpected as the TLRs, in particular their extracellular domains, are known to be highly polymorphic (Vaire and Liu, 2014). Seven out of 11 of the point mutations analysed in the coding sequence occurred within the extracellular domain.

The number of variants identified in this study is similar to that found in a canine study of TLR4 (cuscó et al., 2014). Twelve non-synonymous SNPs were identified in the exonic region of TLR4 in 335 dogs comprising 7 breeds compared to 11 non-synonymous SNPs in cats. However, 2/12 of the non-synonymous SNPs identified in canines were predicted to be “probably damaging” and 3/12 “possibly damaging” using PolyPhen-2 software compared to 1/11 identified as “possibly damaging” in cats. When additional predictive software (sift, PROVEAN) was used only 1/5 non-synonymous SNPs in dogs was deemed to be deleterious using all three calculations. Interestingly, the variant allele frequency of this likely damaging SNP was approximately 75% in Boxer dogs, 20% in Shar Peis and less than 5% in the other five breeds tested (cuscó et al., 2014), suggesting the potential for a breed predisposition to diseases where TLR4 function is critical.
Table 2
Variants detected in feline TLR4 by exonic amplification and sequencing.

| Position (gDNA) | Sequence change (cDNA) | Location | Protein | Predicted effect (estimated probability) | Breeds affected |
|----------------|------------------------|----------|---------|----------------------------------------|----------------|
| g.79270717 c.1-28C > A | Intron1 | K57Q | Benign (0.010) | Domestic crossbred, Persian |
| g.79270898 c.96 + 58DelC | Exon 1 | Benign (0.001) | Bengal, Birman, BSH, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79275280 c.564T > C | Exon 2 | Silent | Bengali, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79275290 c.812G > A | Exon 3 | GZV5E | Benign (0.010) | Bengal, Birman, Burmese, Domestic crossbred, Persian |
| g.79279203 c.825A > C | Exon 3 | K277N | Benign (0.001) | Bengal, BSH, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79279213 c.869G > A | Exon 3 | K277N | Benign (0.001) | Bengal, Domestic crossbred |
| g.79279445 c.1087C > T | Exon 3 | P352S | Benign (0.001) | Bengal, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79279468 c.1089C > T | Exon 3 | Silent | Bengali, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79279477 c.1099G > A | Exon 3 | A366T | Benign (0.010) | Bengal, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79279528 c.1140C > T | Exon 3 | Silent | Bengali, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79279552 c.1164G > C | Exon 3 | L387F | Benign (0.002) | Burmese, Domestic crossbred, Persian |
| g.79279770 c.1382A > G | Exon 3 | Q460R | Benign (0.005) | Bengal, BSH, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79280301 c.1913G > T | Exon 3 | G637V | Benign (0.004) | Burmese, Domestic crossbred, Persian |
| g.79280328 c.1940T > C | Exon 3 | P646S | Benign (0.010) | Domestic crossbred, Persian |
| g.79280368 c.1980G > A | Exon 3 | Silent | Domestic crossbred, Persian |
| g.79280599 c.2211C > T | Exon 3 | Silent | Domestic crossbred, Persian |
| g.79280649 c.2261G > A | Exon 3 | G753E | Benign (0.002) | Bengal, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79280934 c.2546C > A | UTR 3' | Not specified | Bengal, Birman, BSH, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79281158 c.2770A > G | UTR 3' | Not specified | Bengal, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79281219 c.2831InA | UTR 3' | Not specified | Bengal, BSH, Domestic crossbred, Persian, Ragdoll, Siamese |
| g.79281452 c.3064C > T | UTR 3' | Not specified | Burmese, Domestic crossbred, Persian |

* Numbering refers to chromosome D4 accession ID NC_018735.3.
† Numbering and reference nucleotide refer to accession number NM_001009223.1.
‡ 100% cats tested were homozygous for alternative allele.
was more similar to that of other species than that based on the feline reference genome assembly (XP_019671055.1). The six SNPs for which all cats in the current study were homozygous for the alternate allele, are also reported as the alternate allele in the reference genomic sequence (GCF_000181335.3), suggesting that these are SNPs within the genome of the cat from which the cDNA reference sequence was created.

Although breed specific differences in allele frequencies were identified for eight of the SNPs, some SNPs were not identified in a number of breeds. All of the variants were present in DSH and most were present in Persians and domestic crossbred cats. Not including the variants found in 100% of cats tested, only one of the coding SNPs was identified in any of the Birmans. Despite these breed differences, only two of these variants were non-synonymous and neither was identified to affect the protein structure of the receptor. As such, TLR4 dysfunction does not appear to be associated with breed differences in disease susceptibility in cats in Australia. Although a single non-synonymous SNP was identified which may affect the protein structure, further investigation is required to determine the clinical effect of this.

Siamese and Birman cats showed the lowest haplotype diversity among the cat breeds investigated, with one haplotype distributed widely in each breed. This low genetic diversity could indicate a genomic region under strong selection, either artificial or natural. Alternatively, this result could demonstrate incomplete sampling of breed representatives or a strong bottleneck event during breed formation, resulting in a reduced genetic pool due to a low population size at the time of breed formation (Nei et al., 1975; Leroy, 2011). Further investigations are required to validate these results.

The effect on the lack of genetic diversity in a single locus in an artificially selected population such as the one in this study has not been investigated. However, an inverse relationship between population heterogeneity and disease-dependent mortality has been previously demonstrated with more heterogeneous populations shown to be less likely to suffer catastrophic epidemics (Springbett et al., 2003). Furthermore, a lack of diversity in innate immune system genes has been proposed as a potential contributing factor in the extinction of a population (Sullivan et al., 2017).

Limitations of this study include that breed identification was based on phenotypic verification by a veterinarian, which could have resulted in misrepresentation of some individuals. However, breed selection in cats has been predominantly based on physical traits, often with basic Mendelian inheritance patterns, making visual identification relatively accurate (Kurushima et al., 2013).

In summary, this study identified 16 previously unreported point mutations for TLR4 in cats, with the majority being identified in DSH and Persians. The lack of diversity in the Birman breed suggests a potential bottleneck event during breed formation.
mutations in feline TLR4. One mutation was predicted to have a deleterious effect on the protein structure and may therefore influence its function. However, this SNP was only identified in only three cats with different genetic backgrounds and breed-specific deleterious TLR4 SNPs that could predispose to infectious and non-infectious diseases in domestic cats were not identified.

**Funding**

This work was supported by the Australian Companion Animal

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**Table 3**

Breed-specific allele frequencies.

| SNP          | Domestic crossbreed variant allele freq. | Persian variant allele freq. | BSH variant allele freq. | Ragdoll variant allele freq. | Bengal variant allele freq. | Burmese variant allele freq. | Birman variant allele freq. | Siamese variant allele freq. |
|--------------|-----------------------------------------|-----------------------------|-------------------------|-------------------------------|-----------------------------|-------------------------------|-------------------------------|-----------------------------|
| g.1-28C > A  | 0.129                                   | 0.05                        | 0.05                    | 0                             | 0.0                         | 0                             | 0                             | 0                           |
| g.96 + 58DelC| 0.514                                   | 0.65                        | 0.5                     | 0.444                         | 0.444                       | 0                             | 0.25                          | 0.1                         |
| c.172A > C   | 0.143                                   | 0.15                        | 0.5                     | 0.056                         | 0.167                       | 0                             | 0                             | 0.1                         |
| c.564T > C   | 1                                       | 1                           | 1                       | 1                             | 1                          | 1                             | 1                             | 1                           |
| c.812G > A   | 0.057                                   | 0.05                        | 0.05                    | 0.12                          | 0.12                       | 0                             | 0                             | 0                           |
| c.825A > C   | 0.129                                   | 0.15                        | 0.5                     | 0.056                         | 0.167                       | 0                             | 0                             | 0.1                         |
| c.869G > A   | 0.029                                   | 0.05                        | 0.05                    | 0.056                         | 0.0                         | 0                             | 0                             | 0                           |
| c.1057C > T  | 1                                       | 1                           | 1                       | 1                             | 1                          | 1                             | 1                             | 1                           |
| c.1098C > T  | 0.257                                   | 0.25                        | 0.5                     | 0.167                         | 0.556                       | 0.56                          | 0.25                          | 0.35                        |
| c.1099G > A  | 1                                       | 1                           | 1                       | 1                             | 1                          | 1                             | 1                             | 1                           |
| c.1140C > T  | 1                                       | 1                           | 1                       | 1                             | 1                          | 1                             | 1                             | 1                           |
| c.1164G > C  | 0.057                                   | 0.05                        | 0.05                    | 0.12                          | 0.12                       | 0                             | 0                             | 0                           |
| c.1382G > G  | 0.129                                   | 0.15                        | 0.5                     | 0.056                         | 0.167                       | 0                             | 0                             | 0.1                         |
| c.1913G > T  | 0.057                                   | 0.05                        | 0.05                    | 0.056                         | 0.167                       | 0                             | 0                             | 0.12                        |
| c.1940T > C  | 0.057                                   | 0.05                        | 0.05                    | 0.056                         | 0.167                       | 0                             | 0                             | 0.12                        |
| c.1980G > A  | 0.014                                   | 0.05                        | 0.05                    | 0.056                         | 0.167                       | 0                             | 0                             | 0.05                        |
| c.2211C > T  | 0.014                                   | 0.05                        | 0.05                    | 0.056                         | 0.167                       | 0                             | 0                             | 0.05                        |
| c.2261G > A  | 1                                       | 1                           | 1                       | 1                             | 1                          | 1                             | 1                             | 1                           |
| c.2546C > A  | 0.329                                   | 0.2                         | 0.5                     | 0.056                         | 0.167                       | 0                             | 0                             | 0.05                        |
| c.2670A > G  | 1                                       | 1                           | 1                       | 1                             | 1                          | 1                             | 1                             | 1                           |
| c.2831aA     | 0.05                                    | 0.2                         | 0.5                     | 0.056                         | 0.167                       | 0                             | 0                             | 0.05                        |
| c.3064C > T  | 0.057                                   | 0.05                        | 0.05                    | 0.056                         | 0.167                       | 0                             | 0                             | 0.12                        |
Table 4
Breed differences in allelic frequency for variants in feline TLR4 (p-values for Fishers exact tests).

|            | Persian | BSH    | Ragdoll | Bengal | Burmese | Birman | Siamese |
|------------|---------|--------|---------|--------|---------|--------|---------|
| c.1-28C > A | 0.4476  | 0.1985 | 0.1943  | 0.1943 | 0.0101  | 0.1985 | 0.5929  |
| Persian     | > 0.9999| > 0.9999| > 0.9999| > 0.9999| 0.2857  | > 0.9999| > 0.9999|
| BSH         | > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999|
| Ragdoll     | > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999|
| Bengal      | > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999|
| Burmese     | > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999|
| Birman      | > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999|
| c.96 + 58DelC| 0.3188  | > 0.9999| 0.7922  | 0.7922  | < 0.0001| 0.0439 | 0.0008  |
| Persian     | 0.5231  | 0.3275  | 0.3275  | < 0.0001| 0.0248  | > 0.9999| > 0.9999|
| BSH         | 0.7568  | 0.7568  | < 0.0001| 0.1908  | 0.0033  | > 0.9999| > 0.9999|
| Ragdoll     | > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999|
| Bengal      | > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999|
| Burmese     | > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999|
| Birman      | > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999|
| c.172A > C  | 0.1078  | 0.0017  | 0.4484  | 0.7243  | 0.005   | 0.1092 | > 0.9999|
| Persian     | 0.0407  | 0.0602  | 0.0945  | 0.0101  | 0.1985  | > 0.9999| > 0.9999|
| BSH         | 0.0037  | 0.0434  | < 0.0001| 0.0004  | 0.1318  | > 0.9999| > 0.9999|
| Ragdoll     | 0.0037  | 0.0434  | < 0.0001| 0.0004  | 0.0138  | > 0.9999| > 0.9999|
| Bengal      | > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999|
| Burmese     | > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999| > 0.9999|
| c.825A > C  | 0.0163  | 0.0163  | 0.0163  | 0.0163  | 0.1985  | > 0.9999| > 0.9999|
| c.1080C > T | 0.0011  | 0.0229  | > 0.9999| 0.0111  | 0.0966  | > 0.9999| > 0.9999|
| c.1382A > G | 0.0027  | 0.0027  | 0.0027  | 0.0027  | 0.0011  | > 0.9999| > 0.9999|
| c.2546C > A | 0.0004  | 0.0004  | 0.0004  | 0.0004  | 0.0011  | > 0.9999| > 0.9999|
| c.2831InA   | 0.0163  | 0.0163  | 0.0163  | 0.0163  | 0.0011  | > 0.9999| > 0.9999|

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Table 5

| TLR4 | Haplotype | Domestic Crossbred | Persian | BSH | Burmese | Bengal | Ragdoll | Siamese | Birman |
|------|-----------|--------------------|--------|-----|---------|--------|---------|---------|-------|
| 1    | CAAAGGCAGTGGCC | 0.514            | 0.65   | 0   | 0.444   | 0.417  | 0.1     | 0.75    |       |
| 2    | CAGAGCTGTGGCC | 0.043            | 0.05   | 0   | 0.444   | 0.389  | 0.111   | 0.75    | 0.25  |
| 3    | CCAGGAGTGGCC | 0.029            | 0.05   | 0   | 0.444   | 0.417  | 0.05    | 0       |       |
| 4    | CGCGAGTGGTGGAC | 0.129           | 0.15   | 0.5  | 0.444   | 0.111  | 0.028   | 0       |       |
| 5    | CCAAGTCTGGTGGAC | 0.043       | 0      | 0   | 0.12    | 0      | 0       | 0       |       |
| 6    | ACCAGGAGTGGCC | 0.086            | 0.05   | 0   | 0       | 0      | 0       | 0       |       |
| 7    | CCAAGGAGTGGCC | 0.071            | 0      | 0   | 0       | 0      | 0       | 0       |       |
| 8    | CAGAAGTGGCC | 0.029            | 0      | 0   | 0       | 0      | 0       | 0       |       |
| 9    | CAGAGGAGTGGCC | 0.029            | 0      | 0   | 0       | 0      | 0       | 0       |       |
| 10   | CCAAGTCTCACTCC | 0.014           | 0      | 0   | 0       | 0      | 0       | 0       |       |
| 11   | ACCAGGAGTGGCC | 0.014            | 0      | 0   | 0       | 0.056  | 0.028   | 0       |       |
| 12   | CCGATAGGTGGTGCAC | 0         | 0      | 0   | 0       | 0      | 0       | 0       |       |
| 13   | CCGGATGGTGGTGCAC | 0            | 0      | 0   | 0       | 0      | 0       | 0       |       |

# individuals
35 10 10 25 9 9 10 10

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Heath Fund and the University of Sydney Calico and Bi-colour Cat Club Feline Research Bequest.

Declarations of interest

None.

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