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Research paper

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 infectious 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

Abbreviations: BSH, British shorthair; LPS, lipopolysaccharide; LRR, leucine-rich repeat; PRR, pattern recognition receptor; SNP, single nucleotide polymorphism; SSH, Scottish shorthair; TLR, toll-like receptor; TLR4, toll-like receptor 4

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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 (Kathrani 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) (Cambier 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 (Pesteanu-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), Bengal (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 templated 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/pph2) (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 affect 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
Primers designed for amplification of exonic DNA of feline TLR4 based on Feline Genome Assembly v.8.

	Forward (5'-3')	Reverse (5'-3')	Product length	Annealing temperature
Exon 1	CAGCGTTGCTTTGAATACAG	ATGAAATTCGGCAACTAGCA	362	54°C
Exon 2	GGATGAAAGATGGTTGGATG	GGAGACTCCTGACATAAGAACG	397	58°C
Exon 3				
- a	TGTCACATCTGTGTGAAGAGC	AGATGCACCAGAGAAATGGT	793	54°C
- b	GACTTGTCCCTCAACCCTTT	TTTGGAAGGAAGTTGTCCTG	844	54°C
- c	GGTTAAGTTGGAAAGCCTTGA	ATACACCAGAACCACCACCA	852	60°C
- d	AACCTCTTCACTCCCTCCAG	CAGCAAGGCTTTTCTGAGTC	829	54°C
- e	TTTTCAGCTCTGCCTTCACT	GCATTCAATAGAAAAGGGAAAA	849	54°C
- f	CAAGGGCAGATGATTCAGTG	CATCAAGCGACAAAGTGATG	679	54°C

reported in *Canis familiaris* (Asahina et al., 2003) and *Bos taurus* (GenBank accession number UAB056444) in all three positions and in *Homo sapien* (GenBank accession number U88880) and *Equus caballus* (GenBank accession number AY005808) in two positions (amino acid positions 352 and 753).

3.2. Breed specific allele frequency

118 cats (35 crossbred, 10 Persian/Himalayan, 10 BSH/SSH, 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.2261 G > A, c.2770 A > G), 100% of cats tested were homozygous for the alternative allele (Table 3).

Allelic frequencies differed between at least two breeds for eight of the SNPs (2 synonymous, 2 non-synonymous, 4 in the flanking regions). Within these, Burmese and BSHs most commonly differed from the other breeds (Table 4). No significant differences in allelic frequency of any SNP were noted between domestic and Persian cats.

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 with a high frequency among all breeds. TLR4-1 was the most common haplotype in domestic cats, Persians, BSH, Bengals, Ragdolls and Birmans but occurred at a relatively low frequency in Siamese cats and was not identified in Burmese cats. 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 Birmans, 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 (Vaure 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. Interesting, 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.

Previous studies of the bovine *TLR4* have identified over 800 SNPs, 160 of which are located within the coding sequence. 19/110 non-synonymous SNPs have been identified as damaging using multiple predictive software programs (Mishra et al., 2017). The higher number of non-synonymous SNPs identified in bovines compared to dogs and cats may reflect the higher number of individuals investigated or greater overall genetic diversity in this species. Notwithstanding, the rate of deleterious SNPs is similar between the three species. This is similar also in humans, where despite the high number of SNPs identified in the *TLR4* gene, most have minimal detrimental effects on phenotype (Vaure and Liu, 2014).

Due to the limited number of available feline *TLR4* genomic sequences the "wild-type" is yet to be determined. In our study the previously reported feline cDNA sequence (Asahina et al., 2003) was selected as the reference sequence since its predicted amino acid sequence

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				Domestic crossbred, Persian
g.79270898	c.96 + 58DelC	Intron1			Bengal, Birman, BSH, Domestic crossbred, Persian, Ragdoll, Siamese
g.79275280	c.172 A > C	Exon2	K57Q	Benign (0.010)	Bengal, BSH, DLH, DSH, Persian, Ragdoll, Siamese
g.79278952	c.564 T > C#	Exon 3	Silent		Bengal, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese
g.79279200	c.812 G > A	Exon 3	G270E	Benign (0.001)	Birman, Burmese, Domestic crossbred, Persian
g.79279213	c.825 A > C	Exon 3	K274N	Benign (0.001)	Bengal, BSH, Domestic crossbred, Persian, Ragdoll, Siamese
g.79279257	c.869 G > A	Exon 3	R289Q	Possibly damaging (0.895)	Bengal, Domestic crossbred
g.79279445	c.1057C > T#	Exon 3	P352S	Benign (0.001)	Bengal, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese
g.79279468	c.1080C > T	Exon 3	Silent		Bengal, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese
g.79279487	c.1099 G > A#	Exon 3	A366T	Benign (0.010)	Bengal, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese
g.79279528	c.1140C > T#	Exon 3	Silent		Bengal, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese
g.79279552	c.1164 G > C	Exon 3	L387F	Benign (0.002)	Burmese, Domestic crossbred, Persian
g.79279770	c.1382 A > G	Exon 3	Q460R	Benign (0.005)	Bengal, BSH, Domestic crossbred, Persian, Ragdoll, Siamese
g.79280301	c.1913 G > T	Exon 3	G637V	Benign (0.004)	Burmese, Domestic crossbred, Persian
g.79280328	c.1940 T > C	Exon 3	F646S	Benign (0.119)	Burmese, Domestic crossbred, Persian
g.79280368	c.1980 G > A	Exon 3	Silent		Domestic crossbred, Persian
g.79280599	c.2211C > T	Exon 3	Silent		Domestic crossbred, Persian
g.79280649	c.2261 G > A#	Exon 3	G753E	Benign (0.002)	Bengal, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese
g.79280934	c.2546C > A	UTR 3'			Bengal, BSH, Domestic crossbred, Persian, Ragdoll, Siamese
g.79281158	c.2770 A > G#	UTR 3'			Bengal, Birman, BSH, Burmese, Domestic crossbred, Persian, Ragdoll, Siamese
g.79281219	c.2831InA	UTR 3'			Bengal, BSH, Domestic crossbred, Persian, Ragdoll, Siamese
g.79281452	c.3064C > T	UTR 3'			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.

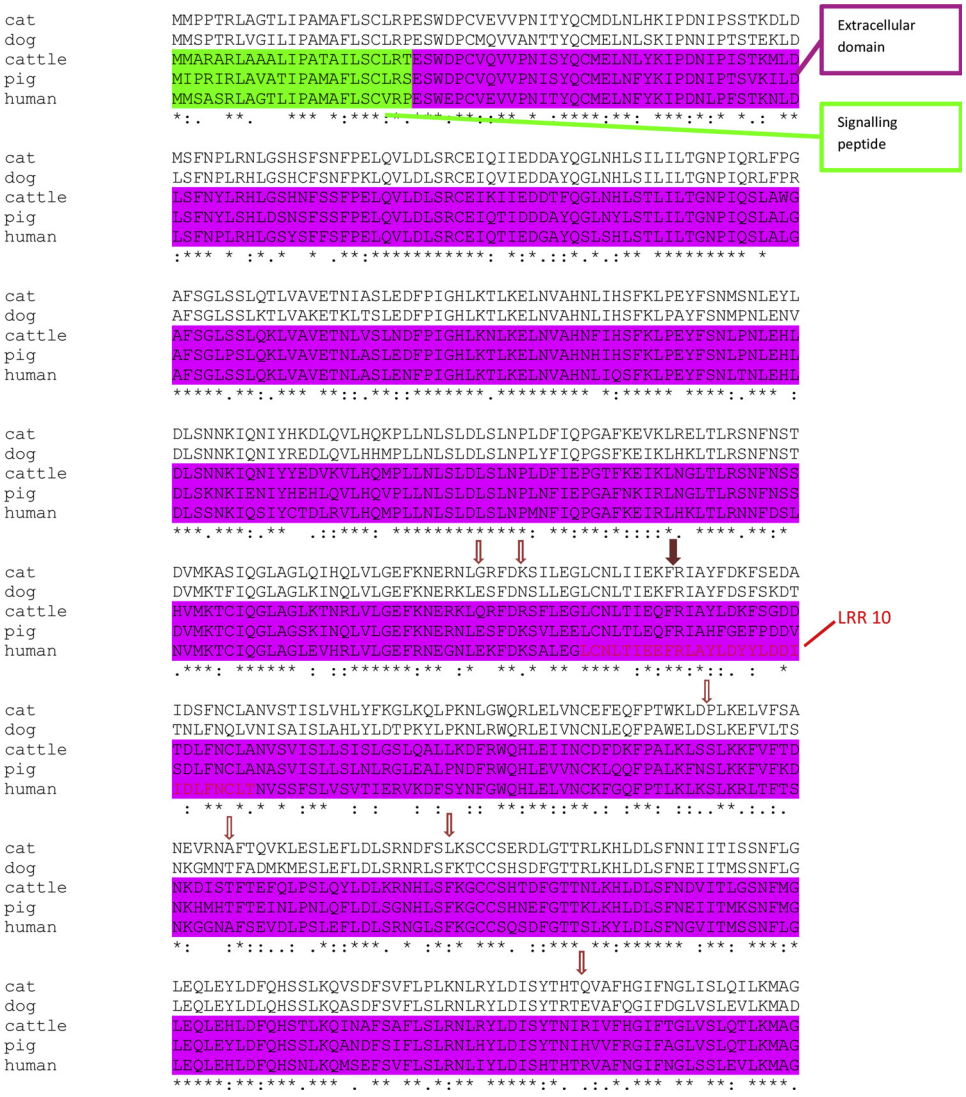

Fig. 1. Multiple alignment of cat, dog, cattle, pig and human TLR4 protein. The sequences were derived from Genbank accessions NP_001009223.1 (cat), NP_001002950.2 (dog), NP_776623.5 (cattle), NP_001106510.2 (pig) and NP_612564.1 (human). Protein domains are indicated by coloured shading. The position of identified non-synonymous SNPs with (closed arrow) and without (open arrow) predicted effects of protein structure are shown.

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 amongst 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

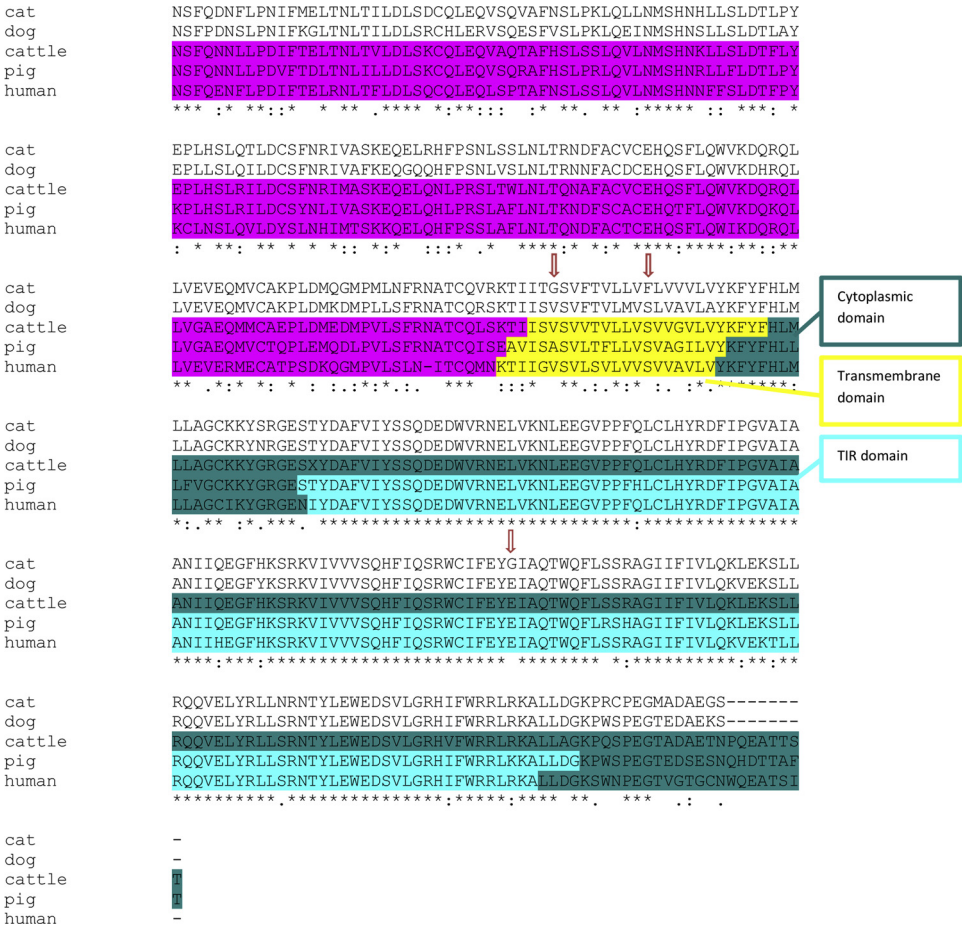

Fig. 1. (*continued*)

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.

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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	0	0	0	0	0
g.96 + 58DelC	0.514	0.65	0.5	0.444	0.444	0	0.25	0.1
c.172 A > C	0.143	0.15	0.5	0.056	0.167	0	0	0.1
c.564 T > C	1	1	1	1	1	1	1	1
c.812 G > A	0.057	0.05	0	0	0	0.12	0	0
c.825 A > C	0.129	0.15	0.5	0.056	0.167	0	0	0.1
c.869 G > A	0.029	0	0	0	0.056	0	0	0
c.1057C > T	1	1	1	1	1	1	1	1
c.1080C > T	0.257	0.25	0.5	0.167	0.556	0.56	0.25	0.35
c.1099 G > A	1	1	1	1	1	1	1	1
c.1140C > T	1	1	1	1	1	1	1	1
c.1164 G > C	0.057	0.05	0	0	0	0.12	0	0
c.1382 A > G	0.129	0.15	0.5	0.056	0.167	0	0	0.1
c.1913 G > T	0.057	0.05	0	0	0	0.12	0	0
c.1940 T > C	0.057	0.05	0	0	0	0.12	0	0
c.1980 G > A	0.014	0.05	0	0	0	0	0	0
c.2211C > T	0.014	0.05	0	0	0	0	0	0
c.2261 G > 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.2770 A > G	1	1	1	1	1	1	1	1
c.2831InA	0.05	0.2	0.5	0.056	0.167	0	0	0.05
c.3064C > T	0.057	0.05	0	0	0	0.12	0	0

Table 4
Breed differences in allelic frequency for variants in feline TLR4 (p-values for Fishers exact tests).

c.1-28C > A	Persian	BSH	Ragdoll	Bengal	Burmese	Birman	Siamese
Crossbred	0.4476	0.1985	0.1943	0.1943	***0.0101***	0.1985	0.5929
Persian		> 0.9999	> 0.9999	> 0.9999	0.2857	> 0.9999	> 0.9999
BSH			> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
Ragdoll				> 0.9999	> 0.9999	> 0.9999	> 0.9999
Bengal					> 0.9999	> 0.9999	> 0.9999
Burmese						> 0.9999	> 0.9999
Birman							> 0.9999
c.96 + 58DelC	Persian	BSH	Ragdoll	Bengal	Burmese	Birman	Siamese
Crossbred	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***	***0.0008***
BSH			0.7568	0.7568	< ***0.0001***	0.1908	***0.0138***
Ragdoll				> 0.9999	< ***0.0001***	0.3071	***0.0265***
Bengal					< ***0.0001***	0.3071	***0.0265***
Burmese						***0.0013***	0.0787
Birman							0.4075
c.172 A > C	Persian	BSH	Ragdoll	Bengal	Burmese	Birman	Siamese
Crossbred	> 0.9999	***0.0017***	0.4484	0.7243	***0.005***	0.1092	> 0.9999
Persian		***0.0407***	0.6062	> 0.9999	***0.0208***	0.2308	> 0.9999
BSH			***0.0037***	***0.0434***	< ***0.0001***	***0.0004***	0.0138
Ragdoll				0.6026	0.2647	0.4737	> 0.9999
Bengal					***0.0163***	0.0967	0.6525
Burmese						> 0.9999	0.0787
Birman							0.4872
c.825 A > C	Persian	BSH	Ragdoll	Bengal	Burmese	Birman	Siamese
Crossbred	0.7248	***0.001***	0.68	0.7045	***0.0101***	0.1985	> 0.9999
Persian		***0.0407***	0.6062	> 0.9999	***0.0208***	0.2308	> 0.9999
BSH			***0.0037***	***0.0434***	< ***0.0001***	***0.0004***	***0.0138***
Ragdoll				0.6026	0.2647	0.4737	> 0.9999
Bengal					***0.0163***	0.0967	0.6525
Burmese						> 0.9999	0.0787
Birman							0.4872
c.1080C > T	Persian	BSH	Ragdoll	Bengal	Burmese	Birman	Siamese
Crossbred	> 0.9999	0.0549	0.5444	***0.0229***	***0.0011***	> 0.9999	0.4412
Persian		0.1908	0.6968	0.096	***0.0327***	> 0.9999	0.7311
BSH			0.0434	0.7568	0.7915	0.1908	0.5231
Ragdoll				***0.0354***	***0.0054***	0.6968	0.2778
Bengal					> 0.9999	0.096	0.3275
Burmese						***0.0327***	0.1852
Birman							0.7311
c.1382 A > G	Persian	BSH	Ragdoll	Bengal	Burmese	Birman	Siamese
Crossbred	0.7248	***0.001***	0.68	0.7045	***0.0101***	0.1985	> 0.9999
Persian		***0.0407***	0.6062	> 0.9999	0.0208	0.2308	> 0.9999
BSH			***0.0037***	***0.0434***	< ***0.0001***	***0.0004***	***0.0138***
Ragdoll				0.6026	0.2647	0.4737	> 0.9999
Bengal					***0.0163***	0.0967	0.6525
Burmese						> 0.9999	0.0787
Birman							0.4872
c.2546C > A	Persian	BSH	Ragdoll	Bengal	Burmese	Birman	Siamese
Crossbred	0.4072	0.1928	***0.0195***	0.2504	< ***0.0001***	***0.0024***	***0.0192***
Persian		0.0958	0.3436	> 0.9999	0.0053	0.106	0.3416
BSH			***0.0037***	***0.0434***	< ***0.0001***	***0.0004***	***0.0033***
Ragdoll				0.6026	0.2647	0.4737	> 0.9999
Bengal					***0.0163***	0.0967	0.3282
Burmese						> 0.9999	0.2857
Birman							> 0.9999
c.2831InA	Persian	BSH	Ragdoll	Bengal	Burmese	Birman	Siamese
Crossbred	> 0.9999	***0.0258***	0.1772	0.7522	**0.0002**	**0.0182**	0.1051
Persian		0.0958	0.3436	> 0.9999	***0.0053***	0.106	0.3416
BSH			***0.0037***	***0.0434***	< ***0.0001***	***0.0004***	***0.0033***
Ragdoll				0.6026	0.2647	0.4737	> 0.9999
Bengal					***0.0163***	0.0967	0.3282
Burmese						> 0.9999	0.2857
Birman							> 0.9999

Table 5
TLR4 haplotypes and their frequency in various cat populations.

TLR4	Haplotype	Domestic Crossbred	Persian	BSH	Burmese	Bengal	Ragdoll	Siamese	Birman
1	CAAGAGCGAGTGCC:C	0.514	0.65	0.5	0	0.444	0.417	0.1	0.75
2	C:AGAGTGAGTGCC:C	0.043	0.05	0	0.44	0.389	0.111	0.75	0.25
3	CCAGAGCGAGTGCC:C	0.029	0.05	0	0.44	0	0.417	0.05	0
4	CCCGCGTGGGTGCAAC	0.129	0.15	0.5	0	0.111	0.028	0.1	0
5	CCAAAGTCATCGCC:T	0.043	0	0	0.12	0	0	0	0
6	ACAGAGCGAGTGCAAC	0.086	0.05	0	0	0	0	0	0
7	CCAGAGCGAGTGCA:C	0.071	0	0	0	0	0	0	0
8	CCAGAATGAGTGCC:C	0.029	0	0	0	0	0	0	0
9	ACAGAGCGAGTGCA:C	0.029	0	0	0	0	0	0	0
10	CCAAAGTCATCATC:T	0.014	0.05	0	0	0	0	0	0
11	ACCGAGCGAGTGCAAC	0.014	0	0	0	0	0	0	0
12	CCCGCATGGGTGCAAC	0	0	0	0	0.056	0	0	0
13	C:CGCGTGGGTGCAAC	0	0	0	0	0	0.028	0	0
	# individuals	35	10	10	25	9	9	10	10

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Declarations of interest

None.

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