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Investigation into the genetic diversity in toll-like receptors 2 and 4 in the European badger *Meles meles*

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ABSTRACT

The Toll-like receptor (TLR) genes are a conserved family of genes central to the innate immune response to pathogen infection. They encode receptor proteins, recognise pathogen associated molecular patterns (PAMPs) and trigger initial immune responses. In some host-pathogen systems, it is reported that genetic differences, such as single nucleotide polymorphisms (SNPs), associate with disease resistance or susceptibility. Little is known about TLR gene diversity in the European badger (*Meles meles*). We collected DNA from UK badgers, carried out PCR amplification of the badger TLR2 gene and exon 3 of TLR4 and determined DNA sequences for individual badgers for TLR2 (n = 61) and TLR4 exon 3 (n = 59). No polymorphism was observed in TLR4. Three TLR2 amino acid haplotype variants were found. Ninety five percent of badgers were homozygous for one common haplotype (H1), the remaining three badgers had genotypes H1/H3, H1/H2 and H2/H2. By broad comparison with other species, diversity in TLR genes in badgers seems low. This could be due to a relatively localised sampling or inherent low genetic diversity. Further studies are required to assess the generality of the low observed diversity and the relevance to the immunological status of badgers.

Toll-like receptors (TLRs) are a family of proteins that target highly conserved molecules essential for parasite and pathogen survival (Takeda and Akira, 2005). Single nucleotide polymorphisms (SNP) in TLR genes have been linked to pathogen susceptibility in some host species. For example, there is a relationship between TLR variation and *Borrelia afzelii* susceptibility (Tschirren et al. (2013)) and TLR polymorphism and susceptibility to bovine tuberculosis (bTB) in both Chinese Holstein cattle (Sun et al., 2012) and water buffalo (Alfano et al., 2014). Other studies have shown links between TLR variation and risks of cancer (Gomaz et al., 2012), diabetes (Liu et al., 2012), asthma (Schwartz and Cook, 2005) and TB (Zhang et al., 2013) in humans.

TLR genes are conserved throughout evolution (Lu et al., 2008) and homologues are found across a wide range of species (Vasselon and Detmers, 2002). The proteins that they encode have two broad domains; an extracellular Leucine Rich Repeat (LRR) domain which recognises and binds certain pathogen associated molecular patterns (PAMPs) and an intracellular Toll-Interleukin receptor homology domain (TIR). The LRR displays variability which is thought to be driven by an evolutionary arms race with invading parasites and pathogens (Roach et al., 2005). The TIR domain is highly conserved and functions to deliver intracellular signals triggering an innate immune response. Studies on TLR variation have found a higher degree of variation within the LRR domains, some of which appears to be generated by positive selection on amino acid diversity (Jann et al., 2008; Werling et al., 2009). Polymorphisms in the LRR domain can be associated with enhanced susceptibility to disease. For example, the change from arginine to glutamine at position 753 in the human TLR2 gene increases susceptibility to staphylococcal infection (Lorenz et al., 2000), tuberculosis (Ogus et al., 2004), rheumatic fever (Berdeli et al., 2005) and urinary tract infection (Tabel et al., 2007).

TLR population studies are of increasing interest for analysis of broad host responses to disease. This is particularly the case in humans

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but relevant equivalent data is sparse in wildlife species. To the best of our knowledge, there are no reported studies of TLR variation in European badgers (*Meles meles*) and there are no reported DNA sequences of TLRs from this species. An investigation into badger immune molecules is pertinent in the context of the role of the badger in the perpetuation of bTB in cattle in the UK and Republic of Ireland (Krebs et al., 1997; Bhuachalla et al., 2015). TLR 2 and TLR 4 recognise glycosyl-phosphatidyl-inositol (GPI) anchors, and thus may be relevant to both the pathogenesis of bTB infection and to other pathogens of the badger such as *Toxoplasma gondii* (Anwar et al., 2006; Hide et al., 2009), *Eimeria melis, Isospora melis*, (Newman et al., 2001; Cottrell, 2011), and *Trypanosoma pestanai*, (Lizundia et al., 2011; Ideozu et al., 2015). In the present study, we set out to investigate TLR2 and TLR4 genetic diversity in the European badger using a sample of animals from a well-studied population at Woodchester Park, in south-west England, alongside geographically distinct badgers from other UK locations.

For DNA extraction, blood samples were collected from 54 badgers captured as part of a long-term capture-mark-release study of badgers at Woodchester Park (e.g. Rogers et al., 1998; Delahay and Frolich, 2000), Gloucestershire, England. Ethical approval, ethical practice and appropriate licensing of badger capture and examination were described previously (Ideozu et al., 2015). Additionally, a further 14 DNA extractions were performed on other blood samples provided by Secret World Wildlife Rescue, which were collected from badgers originating from eight geographically distinct locations in a 150 km radius of the Woodchester Park site. DNA was extracted from badger blood samples using a modification of a phenol-chloroform protocol (Morley et al., 2005, 2008; Ideozu et al., 2015). Appropriate measures were taken to prevent cross contamination in DNA extractions and subsequent PCR reactions (Williams et al., 2005; Bajnok et al., 2015). Briefly, 1.0 ml of badger blood was centrifuged at 2500 RPM for 10 min and the recovered pellet was then incubated, by the addition of 400 µl lysis buffer (100 mM NaCl, 25 mM EDTA, 0.5% SDS, 20 mM Tris pH 8.0) and 100 µl proteinase K (20 mg/ml), at 56 °C overnight. Then 500 µl Tris buffered phenol-chloroform (pH 8.0) was added, mixed for 10 min before separation of phases by centrifugation for 10 min at 13,000 rpm. The phenol chloroform extraction was repeated twice more. Then 90 µl sodium acetate (3 M pH 5.2) and 900 µl of 100% ethanol were added to the final supernatant and incubated overnight at −20 °C. Following centrifugation for 20 min at 13000 *g*, the pellet was washed in 70% ethanol, centrifuged again and re-dissolved in 100 µl TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). To design PCR primers, TLR nucleotide sequences were retrieved from PubMed-NCBI and ENSEMBLE, and TLR amino acid sequences from UniProt using database searches. As no badger TLR gene information was available, primers suitable for TLR2 and TLR4 amplification from badgers were predicted from evolutionarily related species (ferret, *Mustela putorius furo*, cat *Felis catus,* dog *Canis lupus familiaris*, giant panda *Ailuropoda melanoleuca* and Pacific walrus *Odobenus rosmaus divergens*). Badger TLR sequences were run through predictive modelling software using the PubMed position-specific iterated basic local alignment search tool (PSI-BLAST) to identify possible protein domains from known sequences.

Two overlapping primer sets were designed and optimised to amplify 1909 bp of the 2238 bp third exon of TLR4 TLR44.1F 5′CTGTATCTCTTTTCCCTRTAGGTGTGA3′, 5′GTTTAGGAAGCGAGCCAAATG3′ nucleotide 1 to 1110 (see accession number KR780356), annealing 56 °C; TLR44.2F 5′TTACCTTGATATTTCTTATAC3′, TLR44.2F 5′ GAAN CCTTCCTGGATGAT3′, nucleotide 1071 to 1909 (see accession number KR780356), annealing 50 °C. This fragment covers all of the leucine rich repeat regions of TLR4. Three overlapping primer sets were designed and optimised to amplify the single 2358 bp exon of TLR2 in its entirety TLR22SF, 5′ATGTCACGTGTTYTGTGGACA3′, TLR22SR 5′GTCRCTGAGATCCAAATATTCTA3′, nucleotide 1 to 1110, annealing 60 °C; TLR22XF 5′GATGGAAGTTTTAGCGAACTTGTG3′, TLR22XR 5′ TCCCGAGTGAAAGACAGGAAT3′, nucleotide 789 to 1643, annealing 56 °C; TLR22F 5′GCTCCTGTGAATTCCTGTCTTTC3′, TLR22R 5′GCCGT GTCAGAATAAGCTACCAC3′, nucleotide 1613 to 2435 (77 bases beyond the stop codon), annealing 64 °C. Each PCR Reaction contained 0.1 volume 10× NH4 buffer (Bioline, UK), 2 mM $MgCl_2$, 1 mM dNTP (Bioline UK), 0.5 µM forward primer and 0.5 µM reverse primer and 2.5 units BioTaq Polymerase (Bioline, UK). PCR amplification was carried out using a Stratagene Robocycler, denaturing for 40 s at 94 °C, annealing as specified in Table 1, depending on the primer combination, and extension at 72 °C for 1 min 30 s. The number of PCR cycles also varied for each primer set as shown in Table 1. All PCRs were run with 10 min denaturing at 94 °C before cycling and 5 min extension at 72 °C at the end of the final cycle. Amplified DNA was examined by gel electrophoresis using 1% agarose then checked for purity and quantity using a nanodrop spectrophotometer (Thermo Fisher Scientific, UK). PCR products were adjusted to 10 ng/µl and sequenced by Sanger sequencing (SourceBioscience, Cambridge) using both forward and reverse primers and a consensus sequence was generated. Sequence graphics were examined by eye, using the program FinchTV (https://digitalworldbiology.com/FinchTV), to check correct base calling and identify heterozygous bases (identified as two overlapping peaks of, usually, a smaller amplitude at the same point on the electropherogram). All polymorphisms and heterozygotes were rechecked by resequencing at least three times. Investigation of predicted structures of TLRs were carried out by sequence alignment with example mammalian species (Program, Clustal Omega www.ebi.ac.uk) and Leucine Rich Repeats (LRRs) were predicted using LRR Finder (http://www.lrrfinder.com/lrrfinder.php).

Table 1
DNA sequence polymorphisms in Badger TLR2.

Haplotype	Nucleotide variation	SNP type	Amino acid variation	Mutation
H1 (Wild type)	Position 1007 ACA Position 1722 CAG			
H2	Position 1007 A**C**A > A**T**A	Transition	Position 336; Threonine (T) > Isoleucine (I)	Missense
H3	Position 1722 CA**G** > CA**C**	Transversion	Position 574; Glutamine. (Q) > Histidine (H)	Missense

From Woodchester Park and other locations, 59 badgers were sequenced (accession number KR780356) across exon 3 of TLR4 (1909 bp of the 2238 bp third exon) which covers the LRR region of TLR4. No polymorphisms were found in the TLR4 gene. Analysis of the predicted protein sequence shows good identity to TLR4 from other species (ferret, 92%; human, 73%; bovine 76%; mouse 62%). Eighteen LRRs are predicted compared to 20 for the other species. The badger sequence is missing LRRs 7,8,19 (relative to the human sequence) but has an additional predicted LRR before LRR1 (human).

Sixty-one badgers, from Woodchester Park and other locations, were sequenced across the entire 2355 bp of the single exon TLR2 gene. Low genetic diversity was found. In addition to the wild type sequence, Haplotype 1 (H1), two single nucleotide polymorphisms were found in TLR2 (H2 and H3) and both were non-synonymous missense mutations (Table 1) (Accession numbers KR780353 (TLR2-H1), KR780354 (TLR2-H2), KR780355 (TLR2-H3)). The three haplotypes generated two homozygous genetic types, one of which (the wild type H1/H1 n = 58) predominated over the other (H2/H2 n = 1). Two heterozygote haplotype combinations were seen (H1/H2, n = 1; H1/H3, n = 1). One out of 52 (2%) of the badgers from the Woodchester Park study showed variation from the majority consensus TLR2 sequence (heterozygous H1/H3), while 2/9 (22%) badgers from the other regions showed TLR2 sequence variation (H1/H2 or H2/H2).

Analysis of the predicted protein sequences showed good identity to