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*Article*

# Polymorphisms within the Toll-Like Receptor (TLR)-2, -4, and -6 Genes in Cattle

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**Abstract:** In mammals, members of the TLR gene family play a primary role in the recognition of pathogen-associated molecular patterns from bacteria, viruses, protozoa and fungi. Recently, cattle TLR genes have been mapped to chromosomes using a radiation hybrid panel. Nucleotide sequences of bovine TLR2, TLR4 and TLR6 genes were screened to identify novel SNPs that can be used in studies of cattle resistance to diseases. In total, 8 SNPs were identified and were submitted to the NCBI dbSNP database. The frequencies of the SNPs were assessed in 16 different bovine European cattle breeds and a phylogenetic analysis carried out to describe the relationships between the breeds. Even if from our analysis the SNPs do not appear located in loci under selection, a deviation of three SNPs from Hardy Weinberg equilibrium was observed, and we hypothesize that some of the polymorphisms may be fixated since many generations. The described variations in immune function related genes will contribute to research on disease response in cattle. In fact, the SNPs can be used in association studies between polymorphisms and cattle resistance to diseases.

**Keywords:** toll-like receptors; cattle; SNPs; diversity; genetic distances

## 1. Introduction

The immune system in mammals consists of innate and adaptive immune responses. Adaptive immunity is mediated by antigen specific T and B cells responses, and is observed only in vertebrates. Innate immunity, however, is conserved between invertebrates and vertebrates [1]. Toll-like Receptors (TLRs) play an important role in the recognition of components of pathogens and subsequent activation of the innate immune response, which then leads to development of adaptive immune responses [2,3]. The TLRs are an ancient gene group which is found both in invertebrates and vertebrates; related genes are found also in plants [4]. In mammals, members of the TLR gene family play a primary role in the recognition of pathogen-associated molecular patterns (PAMPs) in proteins from bacteria, viruses, protozoa and fungi [5,6]. Mammalian TLRs derive their name from the Drosophila Toll protein, with which they share sequence similarity. The drosophila Toll protein was shown to be involved in dorsal-ventral pattern formation in fly embryos and was also implicated as a key component of host immunity against fungal infection [7-9].

The TLRs consist of a large extracellular domain responsible for PAMP binding, a transmembrane domain and an intracellular Toll/interleukin-1 receptor (TIR) domain which binds molecules and initiates cellular immune responses [10]. The extracellular domains are composed of about 20 leucine-rich repeats (LRRs) motifs of 20–30 amino acids (AA) and form a solenoid shape with the potential to bind the TLR specific PAMP [11].

Ten TLRs, which recognize molecular patterns from all major classes of pathogens, have been identified in mammals, eleven in mice [12,13]. TLRs operate with diverse variety of ligands ranging from hydrophilic nucleic acid to LPS, furthermore the heterodimerization expands the ligand spectrum [14]. TLR2 and TLR4 recognize bacterial cell components, and are critical in the immune response against Gram positive and negative bacteria [15]. TLR6 in association with TLR2 recognizes a wide variety of bacterial cell wall components including lipopolysaccharides, teichoic acid and lipoproteins [16,17] and induce NFkB signalling pathway [18].

Recently, all 10 TLR genes have been mapped in cattle using a radiation hybrid panel: TLR2 and TLR4 have been previously mapped to the proximal end of *Bos taurus* chromosome (BTA) 17 and the distal end of BTA 8, respectively [19]. TLR6, TLR1 and TLR10 cluster on BTA 6 [20], as observed on human chromosome 4; this organization is most likely the result of gene duplication [21].

Several studies have shown that mutations in the TLR may reduce the ability of the protein to recognise PAMP and hence interfere with innate immune activation. Describing genetic variation in these loci in relation to resistance against specific diseases in livestock may be useful in guiding genetic selection for disease resistance. Single nucleotide polymorphisms (SNPs) within TLR genes in humans seem to be associated with susceptibility to infection by specific diseases [22]. Among cattle genes TLR1, TLR5 and TLR10, 98 polymorphisms have been identified, 14 of which are non synonymous SNPs positioned in domains considered to be functionally significant [23]. Eighty three polymorphisms have been also identified for bovine TLR2 and TLR6 [24]. The initiation of the innate response to bovine respiratory syncytial virus (BRSV) requires the interaction of the viral F protein with TLR4, which leads to activation of NFkB via the Myd88-dependent pathway [25]. A recent study showed an association between TLR mutations and increased susceptibility to MAP (*Mycobacterium avium*

*paratubercolosis*) infection in cattle, exactly two missense mutations in TLR4 (LRR domain) were associated with MAP infection [26].

In this study, we screened nucleotide sequences of bovine TLR2, TLR4 and TLR6 genes to identify SNPs that can be used in disease resistance studies in cattle. Eight new SNPs were identified and their frequency assessed in 16 different European cattle breeds.

## *Materials and Methods*

### *Samples:*

A total of 951 individuals belonging to the following European breeds were analysed: Jersey (50), South Devon (43), Aberdeen Angus (45) and Highlands (48), from Great Britain; Holstein (60), Danish Red (59) and Simmental (30), from Denmark; Asturiana de los Valles (66), Casina (66), Avilena (65) and Pirenaica (73), from Spain; Piemontese (67), Marchigiana (36) and Maremmana (91), from Italy; Limousin (72) and Charolais (80), from France. Genomic DNA was isolated from blood using conventional methods and concentration and quality were evaluated by agarose gel electrophoresis.

### *Polymerase Chain Reaction (PCR) Conditions:*

PCR primers for TLR2, 4 and 6 were designed using *PolyPrimers* [27] from the sequences available in Genebank (TLR2: AY634629, TLR4: DQ839567, TLR6: AJ618974) to amplify genomic fragments of approximately 1 kb (Table 1, Figure S1) covering most of the gene sequence. Each polymerase chain reaction (PCR) was performed in a total volume of 30 μL containing 30 ng of genomic DNA, 1.6 pMol of each primer (Sigma-Aldrich), 200 μM dNTPs, 1X PCR buffer and 0.2 units of *Taq* DNA polymerase (Promega) on a PCR Express cycler (Hybaid), using the annealing temperatures reported in Table 1. A 5 minutes denaturation step was followed by 14 cycles of denaturation at 94 °C (30 sec), annealing starting from T.A. + 7 °C and decreasing 0.5 °C per cycle (45 sec) and extension at 72 °C (40 sec), then by 20 cycles of denaturation at 94 °C (30 sec), annealing at T.A. (45 sec) and extension at 72 °C (40 sec); the final extension step was carried out at 72 °C for 5 minutes.

### *Sequence analysis:*

PCR products were purified through ExoSap-IT (USB Corporation) to remove residual primers and dNTPs and used as templates for forward and reverse sequencing reactions. Sequencing was performed by means of a ceq 8,800 sequencer using DTCS QuickStart Kit and purifying with Agencourt CleanSEQ 96 (Beckman Coulter), according to manufacturer instructions. To identify SNPs, sequences of at least one individual each of six different breeds (Maremmana, Charolais, Jersey, Holstein, Pirenaica and Piemontese) were analysed and aligned with *Bioedit* software [28]. The putative SNPs identified by sequencing were confirmed and allele frequencies estimated by genotyping 951 individuals. SNP genotyping was performed by Kbiosciences using the patented technology KASPar (www.Kbioscience.com).

*Data analysis:*

Allelic frequencies, Gene Diversity, Heterozygosity and PIC were calculated using *Powermarker* software [29]. Genotypes were analysed using *Fdist2* software to verify whether any of the loci were under selection [30]. Hardy-Weinberg equilibrium and *Nei* genetic distances [31] between populations pairs were calculated using *Powermarker*. The *Neighborjoining algorithm* was used to calculate the phylogeny relationship which was visualised using *Treeview* [32].

**Table 1.** Sequence of Forward (Fw) and Reverse (Rw) primers, annealing temperature (T.A.), amplicon size and amplicon position relatively to Genbank sequences.

| ***Locus*** | **Sequence (5'→3')** | **T.A. (℃)** | **Amplicon size (bp)** | **Amplicon position** | **Genbank Accession #** |
|---|---|---|---|---|---|
| *TLR2* | Fw: CTGTCCAACAATGAGATCACCT<br>Rw: AATTCTGTCCAAACTCAGTGCT | 49 | 735 | 311-1045 | AY634629 |
| *TLR2* | Fw: GTTCAGGTCCCTTTATGTCTTG<br>Rw: ATGGGTACAGTCATCAAACTCT | 47 | 509 | 493-1003 | AY634629 |
| *TLR2* | Fw: ACTACCGCTGTGACTCTCCCTC<br>Rw: GACCACCACCAGACCAAGACT | 55 | 711 | 1818-2530 | AY634629 |
| *TLR2* | Fw: CTCCCTTTCTGAATGCCACA<br>Rw: AAAGTATTGGAGCTTCAGCA | 47 | 754 | 1876-2631 | AY634629 |
| *TLR4* | Fw: GTGTGGAGACCTAGATGACTGG<br>Rw: GTACGCTATCCGGAATTGTTCA | 50 | 705 | 7938-8644 | DQ839567 |
| *TLR4* | Fw: CTACCAAGCCTTCAGTATCTAG<br>Rw: GGCATGTCCTCCATATCTAAAG | 47 | 741 | 8880-9623 | DQ839567 |
| *TLR4* | Fw: TCAGGAACGCCACTTGTCAGCT<br>Rw: TGAACACGCCCTGCATCCATCT | 55 | 710 | 9635-10346 | DQ839567 |
| *TLR6* | Fw: AAAGAATCTCCCATCAGAAGCT<br>Rw: GAAGGATACAACTTAGGTGCAA | 46 | 515 | 228-745 | AJ618974 |
| *TLR6* | Fw: CTGCCCATCTGTAAGGAATTTG<br>Rw: GATAAGTGTCTCCAATCTAGCT | 47 | 739 | 624-1382 | AJ618974 |
| *TLR6* | Fw: TTGGAAACACTGGATGTTAGCT<br>Rw: ACTGGAGAGTTCTTTGGAGTTC | 49 | 710 | 1428-2138 | AJ618974 |
| *TLR6* | Fw: CTGCCTGGGTGAAGAATGAATT<br>Rw: TGTAGTTGCACTTCCGGGCT | 50 | 715 | 2173-2888 | AJ618974 |

## 2. Results and Discussion

To discover SNPs in the three TLR genes, 12 PCR fragments were amplified and sequenced, five for TLR2, three for TLR4 and four for TLR6. One of the TLR2 primer pairs was soon discharged because of BLASTing problems. We then choose nine of 12 fragments, giving better results in terms of amplification and sequencing. In total eight SNPs were identified, three in TLR2, three in TLR4 and two in TLR6 [33] and were deposited in NCBI dbSNP (the ss# identities are listed in Table 2). These three genes are very important because they could be involved in immune response against various bovine diseases. In fact, TLR2 and TLR6 are critical in the immune response against Gram positive bacteria, TLR4 against Gram negative bacteria and virus. The polymorphisms in TLRs may reduce the ability of the protein to recognise ligands.

**Table 2.** Characterization of the detected SNPs.*: also described by Seabury and Womack [24].

| SNPs | Position in GeneBank sequence[1] | aa change | Position in the protein[2] | SNP ID number |
|---|---|---|---|---|
| *TLR2_591G>A* | 591 | non - coding | | ss107911951 |
| *TLR2_738A>G* | 738 | non - coding | | ss107911952 |
| *TLR2_767G>A** | 767 | non - coding | | ss107911953 |
| *TLR4_254G>A* | 254 | non - coding | | ss107911954 |
| *TLR4_1678C>T* | 1678 | Synonymous (Ser) | 552: LRR domain | ss107911955 |
| *TLR4_2043T>C* | 2043 | non - coding | | ss107911956 |
| *TLR6_855G>A** | 855 | Asp/Asn | 214 | ss107911957 |
| *TLR6_2315T>C** | 2315 | Synonymous (Phe) | 315: TIR domain | ss107911958 |

[1] Nucleotide positions are numbered relatively to the first base of the sequence in GeneBank.
[2] Aminoacid position are numbered according to protein sequence in GeneBank (TLR4: NP776623, TLR6: NP001001159).

The allele frequencies are reported in Table 3 and major allele frequencies ranged from 0.557 (locus TLR2_767) to 0.969 (locus TLR2_738). Except for the latter, in all SNPs the frequency of the minor allele is greater than 5%. Observed heterozygosity (Ho) and Expected heterozygosity (He) of the loci determined from SNP frequencies ranged from 0.051 to 0.466 and from 0.060 to 0.493, respectively. Polymorphism Information Content (PIC) ranged from 0.058 to 0.372 (Table 3).

Eight of the breeds analysed were polymorphic at all the SNPs (Holstein, Asturiana de los Valles, Casina, Avilena, Pirenaica, Piemontese, Charolais ) and five SNPs were polymorphic in all the breeds (*TLR2_767*, *TLR4_254*, *TLR4_1678*, *TLR6_855*, *TLR6_2315*). Both SNPs identified in TLR6 gene were polymorphic in all the breeds.

**Table 3.** Frequencies of the major allele (M.A.F.), expected heterozygosity (He), observed heterozygosity (Ho), Polymorphism Information Content (PIC) of the 8 characterized SNPs.

| SNP | M.A.F. | He | Ho | PIC |
|---|---|---|---|---|
| *TLR2_591* | 0.866 | 0..33 | 0.205 | 0.206 |
| *TLR2_738* | 0.969 | 0.060 | 0.051 | 0.058 |
| *TLR2_767* | 0.557 | 0.493 | 0.461 | 0.372 |
| *TLR4_254* | 0.595 | 0.482 | 0.466 | 0.366 |
| *TLR4_1678* | 0.655 | 0.452 | 0.421 | 0.350 |
| *TLR4_2043* | 0.843 | 0.264 | 0.234 | 0.230 |
| *TLR6_855* | 0.608 | 0.477 | 0.444 | 0.363 |
| *TLR6_2315* | 0.688 | 0.429 | 0.425 | 0.337 |

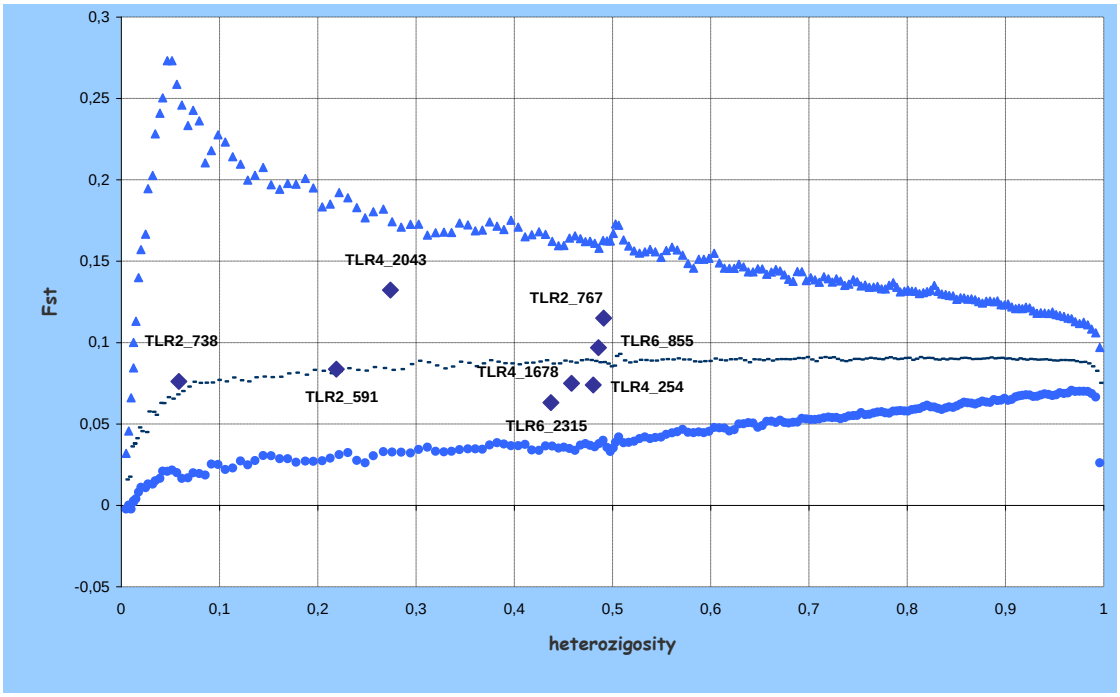
Some breeds were fixed at a number of SNPs, particularly *TLR2_738* is fixed in seven breeds (Highlands, Jersey, Limousine, Marchigiana, Maremmana, Simmenthal and South Devon), as shown in Table 4. *TLR2_591* and *TLR4_2043* are fixed in one breed (South Devon and Highlands, respectively). Interestingly, the SNP *TLR2_73*8 is fixed in three breeds from Great Britain: Highlands, Jersey and South Devon. Indeed, the only two breeds with two fixed alleles are South Devon and Highlands. Moreover, its rare allele frequency is lower than 0.05 in seven other breeds (Aberdeen Angus, Asturiana de los Valles, Avilena, Charolais, Danish Red, Piemontese and Pirenaica), being higher than 0.05 in Holstein and Casina breeds only (Table 4). This suggests an involvement of the gene in some important roles which prevents its polymorphism.

**Table 4.** Allelic frequencies in the 16 European cattle breeds.

| **SNP** | ***TLR2_591*** | | ***TLR2_738*** | | ***TLR2_767*** | | ***TLR4_254*** | | ***TLR4_1678*** | | ***TLR4_2043*** | | ***TLR6_855*** | | ***TLR6_2315*** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ALLELE** | **A** | **G** | **A** | **G** | **A** | **G** | **A** | **G** | **C** | **T** | **C** | **T** | **A** | **G** | **C** | **T** |
| *A. Angus* | 0.011 | 0.989 | 0.012 | 0.988 | 0.693 | 0.307 | 0.837 | 0.163 | 0.683 | 0.317 | 0.989 | 0.011 | 0.581 | 0.419 | 0.349 | 0.651 |
| *A. Valles* | 0.086 | 0.914 | 0.016 | 0.984 | 0.548 | 0.452 | 0.581 | 0.419 | 0.635 | 0.365 | 0.898 | 0.102 | 0.373 | 0.627 | 0.234 | 0.766 |
| *Avilena* | 0.250 | 0.750 | 0.031 | 0.969 | 0.317 | 0.683 | 0.712 | 0.288 | 0.533 | 0.467 | 0.913 | 0.087 | 0.437 | 0.563 | 0.353 | 0.647 |
| *Casina* | 0.327 | 0.673 | 0.152 | 0.848 | 0.432 | 0.568 | 0.538 | 0.462 | 0.649 | 0.351 | 0.848 | 0.152 | 0.302 | 0.698 | 0.196 | 0.804 |
| *Charolais* | 0.114 | 0.886 | 0.044 | 0.956 | 0.487 | 0.513 | 0.545 | 0.455 | 0.671 | 0.329 | 0.840 | 0.160 | 0.353 | 0.647 | 0.336 | 0.664 |
| *Danish Red* | 0.035 | 0.965 | 0.017 | 0.983 | 0.595 | 0.405 | 0.632 | 0.368 | 0.582 | 0.418 | 0.796 | 0.204 | 0.500 | 0.500 | 0.324 | 0.676 |
| *Highlands* | 0.011 | 0.989 | **0** | **1** | 0.932 | 0.068 | 0.415 | 0.585 | 0.564 | 0.436 | **1** | **0** | 0.260 | 0.740 | 0.239 | 0.761 |
| *Holstein* | 0.147 | 0.853 | 0.154 | 0.846 | 0.759 | 0.241 | 0.417 | 0.583 | 0.740 | 0.260 | 0.855 | 0.145 | 0.366 | 0.634 | 0.364 | 0.636 |
| *Jersey* | 0.170 | 0.830 | **0** | **1** | 0.372 | 0.628 | 0.707 | 0.293 | 0.888 | 0.112 | 0.413 | 0.587 | 0.776 | 0.224 | 0.582 | 0.418 |
| *Limousine* | 0.271 | 0.729 | **0** | **1** | 0.479 | 0.521 | 0.583 | 0.417 | 0.616 | 0.384 | 0.819 | 0.181 | 0.340 | 0.660 | 0.326 | 0.674 |
| *Marchigiana* | 0.030 | 0.970 | **0** | **1** | 0.529 | 0.471 | 0.894 | 0.106 | 0.338 | 0.662 | 0.833 | 0.167 | 0.557 | 0.443 | 0.559 | 0.441 |
| *Maremmana* | 0.093 | 0.907 | **0** | **1** | 0.517 | 0.483 | 0.572 | 0.428 | 0.742 | 0.258 | 0.884 | 0.116 | 0.330 | 0.670 | 0.331 | 0.669 |
| *Piemontese* | 0.215 | 0.785 | 0.038 | 0.962 | 0.405 | 0.595 | 0.675 | 0.325 | 0.538 | 0.462 | 0.817 | 0.183 | 0.500 | 0.500 | 0.281 | 0.719 |
| *Pirenaica* | 0.132 | 0.868 | 0.014 | 0.986 | 0.650 | 0.350 | 0.418 | 0.582 | 0.690 | 0.310 | 0.963 | 0.037 | 0.215 | 0.785 | 0.174 | 0.826 |
| *Simmenthal* | 0.096 | 0.904 | **0** | **1** | 0.717 | 0.283 | 0.522 | 0.478 | 0.591 | 0.409 | 0.875 | 0.125 | 0.519 | 0.481 | 0.395 | 0.605 |
| *S. Devon* | **0** | **1** | **0** | **1** | 0.838 | 0.163 | 0.638 | 0.363 | 0.907 | 0.093 | 0.643 | 0.357 | 0.116 | 0.884 | 0.085 | 0.915 |

Selection can leave, in the genes under its influence, a set of signatures that can be analyzed to identify genes or chromosomal regions which are likely targets of positive selection. We used *FST* statistic to assess if the variation of SNP allele frequencies among populations leads to signatures of selection. For each *locus*, the allele frequencies are used to compute *FST* values conditional on heterozygosity and to calculate P-values for each *locus*. This method provides evidence for divergent selection by looking for outliers with *FST* values higher than expected, controlling for heterozygosity. The analysis performed using *FDist2* software to identify outlier loci revealed that none of the SNPs lied outside the 95% confidence limits assumed for conditional joint distribution of *FST* vs. mean heterozygosity. Analysis was performed by bootstrapping 200,000 replications on real data using a coalescent model (Figure 1).

**Figure 1.** Upper (▲) and lower (●) confidence limits of 95% quantiles; median (-) of 200,000 replications of expected Fst and heterozygosity using the coalescent model.




None of the identified SNPs is located in loci under selection according to the model of Beaumont and Nichols [30]. Anyway, significant deviations from Hardy-Weinberg equilibrium over all populations (p-value < 0.01) were observed in three SNPs at two loci: *TLR2_591*, *TLR2_738* and *TLR4_2043* (Table 5). We hypothesize that polymorphisms are fixed in the analysed breeds since many generations, and that the coalescent model employed is not powerful enough to identify selection events happened too far in the past.

Distance based phylogenetic analysis was used to describe the relationships between breeds regarding the investigated TLRs. Table 6 presents the Nei genetic distances relating the 16 breeds studied. The lowest distance values are observed between Charolais and Asturiana de los Valles (0.002), while the highest distance is observed between Highlands and Jersey (0.117). Furthermore, the Jersey breed results very distant from all the other breeds of Great Britain, confirming the results obtained by AFLP and suggesting isolation within the Jersey island as the major cause of distinctiveness [34]. Indeed, Nei distances show that the highest genetic diversity is

observed in the geographically isolated breeds: it is suggestive (Figure 2) that the breeds of Great Britain (Aberdeen Angus, Highlands, South Devon and Jersey), using the analysed polymorphisms, are distributed accordingly to their geographic provenience.

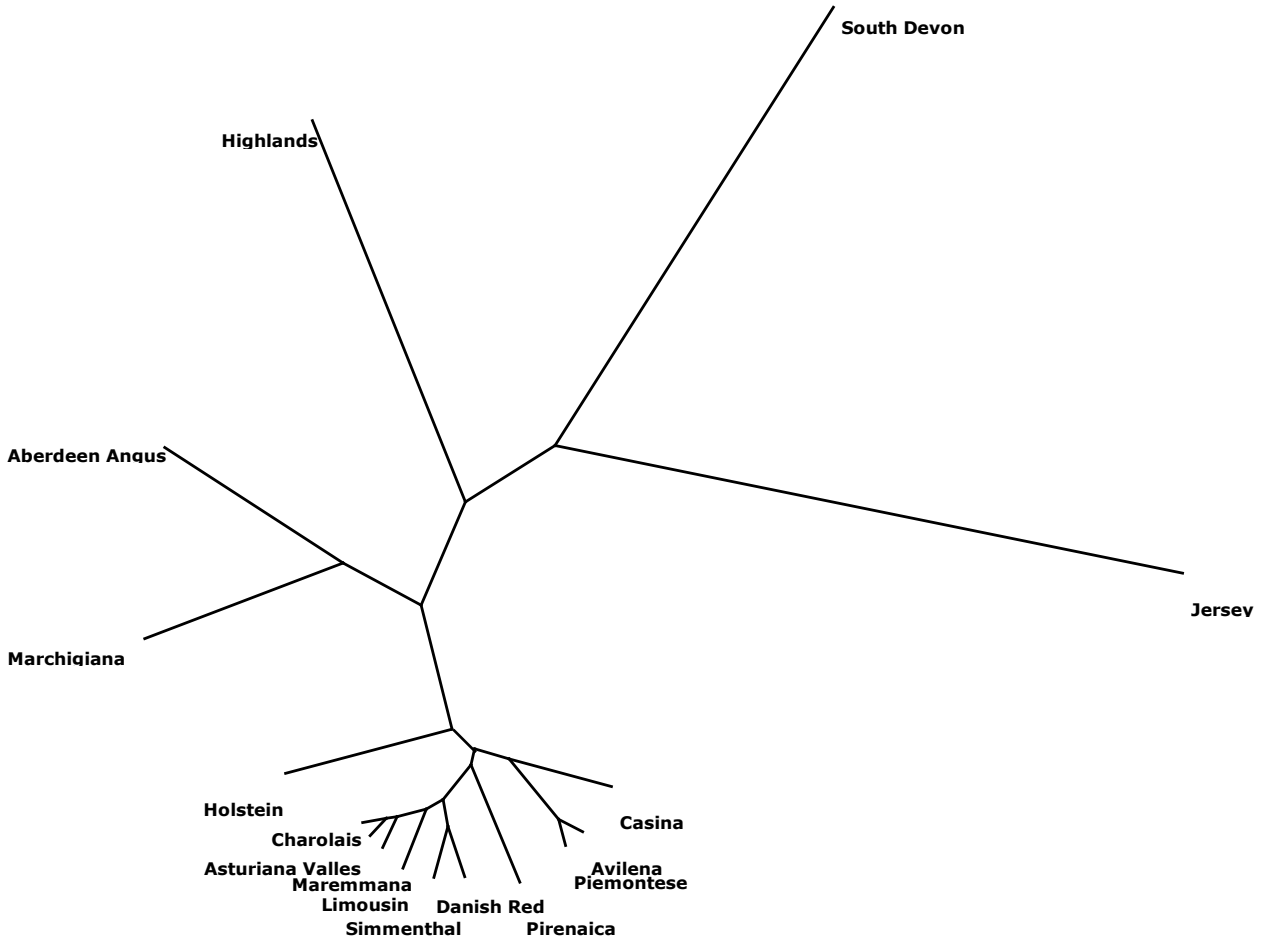
**Table 5**. Hardy-Weinberg equilibrium Test.

| ***Locus*** | ***Hw Test*** |
|---|---|
| *TLR2_591* | 0.0007 |
| *TLR2_738* | 0.0011 |
| *TLR2_767* | 0.0542 |
| *TLR4_254* | 0.2693 |
| *TLR4_1678* | 0.0345 |
| *TLR4_2043* | 0.0012 |
| *TLR6_855* | 0.0303 |
| *TLR6_2315* | 0.7460 |

**Table 6**. Nei genetic distances. GB: Great Britain; ES: Spain; FR: France; DK: Denmark; IT: Italy.

| | **Geographic location of the breed** | **Aberdeen Angus** | **Asturiana de los Valles** | **Avilena** | **Casina** | **Charolais** | **Danish Red** | **Highlands** | **Holstein** | **Jersey** | **Limousin** | **Marchigiana** | **Maremmana** | **Piemontese** | **Pirenaica** | **Simmenthal** | **South Devon** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Aberdeen Angus** | GB | 0 | | | | | | | | | | | | | | | |
| **Asturiana de los Valles** | ES | 0.016 | 0 | | | | | | | | | | | | | | |
| **Avilena** | ES | 0.027 | 0.010 | 0 | | | | | | | | | | | | | |
| **Casina** | ES | 0.044 | 0.012 | 0.011 | 0 | | | | | | | | | | | | |
| **Charolais** | FR | 0.023 | **0.002** | 0.008 | 0.009 | 0 | | | | | | | | | | | |
| **Danish Red** | DK | 0.014 | 0.004 | 0.015 | 0.022 | 0.005 | 0 | | | | | | | | | | |
| **Highlands** | GB | 0.029 | 0.027 | 0.057 | 0.058 | 0.037 | 0.034 | 0 | | | | | | | | | |
| **Holstein** | DK | 0.032 | 0.013 | 0.027 | 0.014 | 0.009 | 0.015 | 0.033 | 0 | | | | | | | | |
| **Jersey** | GB | 0.061 | 0.048 | 0.044 | 0.058 | 0.039 | 0.036 | **0.117** | 0.056 | 0 | | | | | | | |
| **Limousin** | FR | 0.032 | 0.007 | 0.007 | 0.012 | 0.006 | 0.012 | 0.045 | 0.020 | 0.038 | 0 | | | | | | |
| **Marchigiana** | IT | 0.020 | 0.026 | 0.023 | 0.052 | 0.028 | 0.015 | 0.060 | 0.050 | 0.048 | 0.029 | 0 | | | | | |
| **Maremmana** | IT | 0.019 | 0.003 | 0.013 | 0.018 | 0.004 | 0.007 | 0.030 | 0.016 | 0.040 | 0.005 | 0.028 | 0 | | | | |
| **Piemontese** | IT | 0.026 | 0.007 | 0.003 | 0.009 | 0.006 | 0.008 | 0.055 | 0.020 | 0.035 | 0.006 | 0.022 | 0.011 | 0 | | | |
| **Pirenaica** | ES | 0.029 | 0.006 | 0.023 | 0.016 | 0.010 | 0.018 | 0.018 | 0.013 | 0.076 | 0.014 | 0.052 | 0.009 | 0.021 | 0 | | |
| **Simmenthal** | DK | 0.016 | 0.007 | 0.019 | 0.027 | 0.009 | 0.005 | 0.023 | 0.014 | 0.041 | 0.010 | 0.021 | 0.007 | 0.014 | 0.015 | 0 | |
| **South Devon** | GB | 0.053 | 0.035 | 0.073 | 0.060 | 0.040 | 0.037 | 0.046 | 0.044 | 0.082 | 0.049 | 0.076 | 0.033 | 0.060 | 0.035 | 0.044 | 0 |

**Figure 2.** Phylogenetic relationship among the 16 breeds studied. The genetic distances were calculated from allelic frequencies by using Nei distances. The reconstruction was done with UPGMA (Sneath and Sokal, 1973).




0.01 (meaning 0.01 nucleotide substitutions per site)

## 3. Conclusions

We could identify eight SNPs in genes of great interest in cattle management by screening the nucleotide sequences of bovine TLR2, TLR4, and TLR6 genes. These variations in immune function related genes will contribute to research on disease response in cattle. In fact, the newly identified SNPs can be used in association studies between polymorphisms and cattle resistance to diseases.

The SNPs characterization was performed by analysing a conspicuous number of individuals from 16 European breeds, and the main statistics were calculated. Even if from our analysis the SNPs do not appear located in loci under selection, a deviation of three SNPs from Hardy Weinberg equilibrium was observed. We hypothesize that some of the polymorphisms were fixated many generations ago within breed and the coalescent model could not be powerful enough to reveal selection events so far in the past. It would be interesting to apply a more powerful model to confirm the absence of selection in the SNPs and their suitableness as neutral markers.

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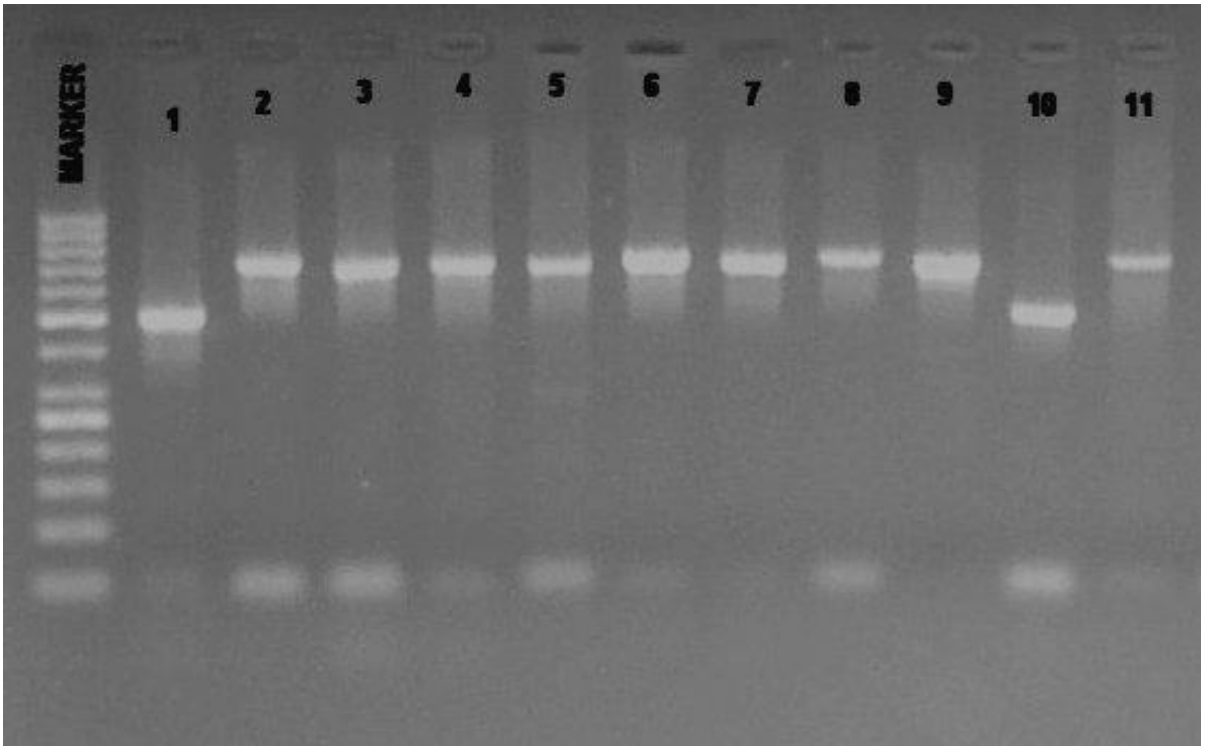
**Electronic Supplementary Information**

**Figure S1.** PCR amplified fragments: 1. TLR6_1; 2. TLR6_2; 3. TLR6_3; 4. TLR6_4; 5. TLR4_1; 6. TLR4_2; 7. TLR4_3; 8. TLR2_5; 9. TLR2_4; 10. TLR2_3; 11. TLR2_2.

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