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Diversity of the bovine genes *IRAK1* and *IRAK4* in the Toll-like receptor signaling pathway

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Abstract: Innate immunity is the organism's first line of defence, and the receptors of immune cells play a key role. Interleukin 1 receptor-associated kinase 1 and 4 (IRAK1 and IRAK4) are necessary for signal transmission from Toll-like receptors, which are the most important receptors of immune cells. The main objective of this study was to investigate the gene polymorphisms of *IRAK1* and *IRAK4* in Czech Fleckvieh cattle (CF) and to compare their diversity with other cattle breeds as there is still little information about these genes. PacBio Technology of next-generation sequencing (NGS) was used to detect genetic variability in 164 CF bulls. The results revealed 17 single nucleotide variants (SNVs) in the *IRAK1* gene and 12 SNVs and three deletions in the *IRAK4* gene. Three methods were then used to determine allelic variability: Sanger sequencing, the primer extension method and Illumina DNA BeadChip microarray in cooperation with the Czech Moravian Breeder's Corporation. Sanger sequencing enabled analysis of the population variability in single nucleotide polymorphisms (SNPs) rs210710958 and rs48726521 of *IRAK1* in 164 CF bulls. SNaPshot genotyping was employed to determine the allelic frequencies of SNP rs211379365 and a novel polymorphism in *IRAK1*, and rs380202447 in the *IRAK4* gene. DNA microarray method, which was applied to 74 CF dairy cows, revealed the allelic frequency of *IRAK1* rs110533802 including the frequency 0.07 of mutant allele *T*. Additional microarray data allowed the comparison of the allelic frequencies among the Czech and Hungarian populations of Holstein dairy cows and beef breed populations. The results show that the functionally significant diversity in the *IRAK1* and *IRAK4* genes in the studied CF population is rather limited. The higher frequency 0.4 of the *T* mutant allele of SNP rs110533802 of *IRAK1* in Holstein cattle could be the consequence of breeding for milk performance.

Keywords: NGS sequencing; innate immunity; cattle; DNA microarray

There are two fundamentally different types of reactions when foreign substances or microorganisms invade animals – innate (nonspecific) immunity and acquired (specific; adaptive) immunity. The innate immune system responds using the same mechanism every time it encounters an antigen, while

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acquired immunity is highly specific for a particular antigen, including the development of immunologic memory. The layout of innate immunity is transmitted as genetic information from generation to generation and remains unchanged by external factors, while acquired immunity improves upon repeated exposure to a given infection.

Cow health is crucial to the operation of farms. Improving the health of dairy cattle can be achieved through changes in external environmental conditions, breeding management, and genetic background (Zavadilova et al. 2021). Variability in immune genes is often associated with different responses to bacterial and viral infection in cattle (Goldammer et al. 2004; Chen et al. 2017; Dusza et al. 2018). Recent developments in molecular genetics and genomics methods brought significant advantages to accelerating genetic progress in cattle breeding. Functional gene annotation supports the detection of important genetic markers in genetic improvement programs in the field of cattle health (Kyselova et al. 2021).

The innate immune system recognises microorganisms via a limited number of pattern recognition receptors (PRRs). The form of these receptors is germline-encoded, and they are located in the cells of the innate immune system. PRRs can recognise microbial components known as pathogen-associated molecular patterns (PAMPs). The stability of the target structures is guaranteed by the fact that they perform a key role in the survival of microorganisms (Medzhitov and Janeway 1997).

PRRs also include 12 members of the family of Toll-like receptors in mammalian species. These are type I integral membrane glycoproteins and are expressed on the surface of various immune cells, including macrophages, B cells, specific types of T cells, dendritic cells, and non-immune cells (fibroblasts and epithelial cells). Toll-like receptors can recognise several classes of PAMPs. For example, TLR1, TLR2, and TLR6 recognise bacterial lipopeptides, TLR4 bacterial lipopolysaccharides, and TLR5 bacterial protein flagellin, whereas TLR7, TLR8, and TLR9 recognise different categories of nucleic acids of pathogens (Akira et al. 2006).

TLRs activate several types of signalling pathways. Signal transfer is mediated by the combination of four adaptor molecules containing the TIR (toll-interleukin receptor) domain. The group of adaptors includes Toll-interleukin receptor domain-containing adaptor protein (TIRAP), Toll-like receptor domain-containing adaptor inducing interferon B (TRIF/TICAM1), myeloid differentiation primary response 88 (Myd88), and TRIF related adaptor molecule 2 (TRAM/TICAM2) (Horng et al. 2001; Kawai and Akira 2007). The importance of signalling molecules on the phenotype level was demonstrated in the study by Capparelli et al. (2013), which described the association between genotype *AC* in the single nucleotide polymorphism 625A>C of *MyD88* and a 5-fold reduced risk of active pulmonary tuberculosis. Interleukin-1 receptor-associated kinases (IRAKs), which include the members IRAK1, IRAK2, IRAK3 and IRAK4, belong to the family of death domain containing protein kinases and play an essential role in the signaling cascade of Toll-like receptors and interleukin-1 receptors (Gottipati et al. 2008).

The molecules IRAK1 and IRAK4, which are the subject of this study, are serine/threonine kinases (specifically, interleukin-1 receptor-associated kinases 1 and 4) that play a critical role in initiating the innate immune response. The activation of the intracellular signalling pathway culminates in the induction of inflammatory cytokines, chemokines, interferons (IFNs), and upregulation of costimulatory molecules (Kawai and Akira 2007). The *IRAK1* gene is located on chromosome X from position 37 220 194 to 37 212 624 with a length of 7 571 bp and contains 14 exons according to the ARS-UCD1.2 RefSeq NC_037357.1. The *IRAK4* gene is located on chromosome 5 from position 36 708 802 to 36 680 671 with a length of 28 132 bp and contains 12 exons according to the ARS-UCD1.2 RefSeq NC_037332.1.

In humans, a polymorphism in the *IRAK1* gene is associated with autoimmune diseases. A study by Kucinskas et al. (2020) and Zhao et al. (2020) associated the rs1059703 and rs3027898 polymorphisms with a higher risk of developing the autoimmune diseases rheumatoid arthritis and psoriasis. A study by Sperry et al. (2014) reports the *IRAK1* rs1059703 gene polymorphism in humans as a predictor of multiple organ failure and mortality postinjury and demonstrates the importance of TLR receptor signaling pathways after injury. Polymorphisms in *IRAK1* (rs1059701 and rs1059702) and *IRAK4* (rs4251513 and rs1461567) genes are associated with the occurrence of severe invasive pneumococcal disease (SIPD). These poly-

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morphisms are related to a higher risk of developing SIPD (Carrasco-Colom et al. 2015). Likewise, Song et al. (2022) described the relationship of the rs4251545 polymorphism in the *IRAK4* gene with susceptibility and severity of enterovirus-71 infection in children.

There are few reports on the *IRAK1* and *IRAK4* genes in cattle, so the main objective of this study was to investigate their polymorphism in Czech Fleckvieh (CF) cattle and compare their diversity with several other cattle breeds.

MATERIAL AND METHODS

Ethical statement

Sample collections (insemination sperm doses and hair bulb samples) were performed using non-invasive method and carried out in accordance with the Act of the Czech National Council for the Protection of Animals Against Cruelty NO246/199.

Sample collection and DNA isolation

A total of 164 Czech Fleckvieh bulls were included in this study for primary screening for SNPs in *IRAK1* and *IRAK4*. Samples were obtained from the breeding company Impuls (Bohdalec, Czech Republic). Genomic DNA was isolated from insemination sperm doses using the MagSep Tissue kit (Eppendorf, Hamburg, Germany). A total of 72 Czech Fleckvieh dairy cows were used for DNA microarray technology. Genomic DNA was isolated from hair bulbs using GeneAll SV mini kit (Exgene, Seoul, South Korea). Hair bulb samples were collected at the experimental farm of the Institute of Animal Sciences (Prague, Czech Republic). The DNA concentration was determined spectrophotometrically by NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Additional genotyping data from Illumina (San Diego, CA, USA) Bovine SNP_chip_V3 were included in cooperation with the Czech Moravian Breeders' Corporation for the populations of Czech Holstein cattle (4 954 dairy cows), Hungarian Holstein (4 268 dairy cows) and beef breeds of 1 196 animals, namely Limousine (365), Charolais (261), Simmental (281), and Aberdeen Angus (289).

Primer design and PCR amplification

Amplification primers for cattle *IRAK1* and *IRAK4* genes were designed according to the NCBI database reference sequences (UMD_3.1.1 – RefSeq: AC_000161.1, AC_000187.1). The resulting five primer pairs for the *IRAK1* gene and 20 primer pairs for *IRAK4* covered the entire regions of the *IRAK1* and *IRAK4* genes (Table S1 in electronic supplementary material (ESM), for ESM see the electronic version; Table S2 in ESM). Primers were synthesised at Eurofins Genomics (Konstanz, Germany). According to the supplier's instructions, the lyophilised primers were diluted to the resulting concentration of 100 µM with deionised water without nucleases and stored at –20 °C. In the second step, primers were diluted to a 10 µM working concentration in 96-well microplates.

PCR amplification was performed in a 10 µl mixture containing 5 µl of gb Basic PCR Master Mix (Generi Biotech, Hradec Králové, Czech Republic), 3.9 µl of PCR grade H_2O, 0.3 µl of genomic DNA (50 ng/µl) and 0.4 µl of each primer at 10 pmol/l. Two types of PCR thermocyclers were used for DNA amplification: Biometra Thermocycler T Gradient Thermoblock (Biometra, Göttingen, Germany) and Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany). In total, 43 PCR cycles included an initial denaturation step at 95 °C for 60 s, then five cycles of a denaturation step at 95 °C for 30 s, an annealing step at 62 °C for 30 s, and elongation at 72 °C for 120 s, followed by 38 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 120 s. The final step involved incubation at an elongation temperature of 72 °C for 360 seconds. Amplified fragments were checked using 1.6% agarose gel electrophoresis with GelRed dye (Biotium, Fremont, USA) and prepared for the subsequent next-generation sequencing (NGS) methodology.

Next generation sequencing

A mixture of *IRAK1* or *IRAK4* amplicons prepared from gDNA of the entire studied population of the CF bulls pooled in equimolar concentrations was prepared for NGS. Subsequently, a mixture of PCR fragments was purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany). Pooled mixed samples were sequenced using the sequenc-

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ing machine PacBio RSII (Pacific Biosciences, Menlo Park, CA, USA) in the service laboratory Eurofins Genomics (Konstanz, Germany).

The obtained results were further processed using the bioinformatics software Geneious Prime (Biomatters, Auckland, New Zealand) and mapped to the reference sequence (UMD_3.1.1 – RefSeq: AC_000161.1, AC_000187.1). The "Find Variations" function identified single nucleotide polymorphism (SNP) variants that had a probability of random origin of less than 0.01. Variants that were caused by other types of errors in NGS technology, such as fluctuations in the number of repeated nucleotides, often G(4)–G(5) variations, were excluded. The obtained SNPs were compared with polymorphisms registered in the European Bioinformatics Institute (EBI) database (https://www.ebi.ac.uk/eva/). A list of eligible variants was exported as a CSV file from the Geneious program (Table 1, Table 2). The localisation of the particular variants was based on inspecting the bovine reference.

Sanger sequencing

Sanger sequencing technology was applied to resequence regions of the *IRAK1* gene that failed to be sequenced by NGS technology and to independently confirm the NGS results. The primer sequences were designed using the module Design of New Primers for Sequences by the Geneious Prime program (Table 3) and synthesised at the Generi Biotech company (Hradec Králové, Czech Republic). Amplification was performed in a 10 µl mixture containing 5 µl of gb Basic PCR Master Mix, 3.9 µl of PCR grade H_2O, 0.3 µl of 50 ng/µl genomic DNA and 0.4 µl of each primer (10 pmol/l). The PCR program was as follows: initial denaturation at 94 °C for 120 s, denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 50 s, and final elongation at 72 °C for 300 seconds. Sanger sequencing applied to all 164 bull DNA samples was provided by Biogen (Prague, Czech Republic).

SNaPshot genotyping

The primer extension method (Hoogendoorn et al. 1999), specifically its commercial version SNaPshot (Thermo Fisher Scientific, Waltham, MA, USA), was used to determine population variability in the *IRAK1* and *IRAK4* genes. Amplicons of 67 bull DNA samples were obtained using primers IK1-4F, IK1-4R, IK4-18F and IK4-18R (Table S1 in ESM, Table S2

Table 1. *IRAK1* gene variants found in the Czech Fleckvieh population using next-generation sequencing

Polymorphism name	Position	Change	Type	Predicted impact	Exon number of total	Intron number of total	Amino acid change	Reference SNP
Poly_1	37 216 403	T>G	intron variant	modifier	–	10_13	–	rs134381860
Poly_2	37 216 559	T>C	intron variant	modifier	–	10_13	–	rs209170931
Poly_3	37 216 571	C>T	intron variant	modifier	–	10_13	–	–
Poly_4	37 216 696	A>G	intron variant	modifier	–	10_13	–	–
Poly_5	37 217 154	G>A	synonymous variant	low	9_14	–	Tyr395Tyr	–
Poly_6	37 217 426	C>T	intron variant	modifier	–	8_13	–	rs110533802
Poly_7	37 217 653	G>T	intron variant	modifier	–	8_13	–	–
Poly_8	37 218 228	C>T	intron variant	modifier	–	7_13	–	–
Poly_9	37 218 523	C>G	intron variant	modifier	–	6_13	–	rs210710958
Poly_10	37 218 627	A>G	missense variant	moderate	6_14	–	Val252Ala	–
Poly_11	37 218 688	G>A	intron variant	modifier	–	5_13	–	–
Poly_12	37 218 694	G>A	intron variant	modifier	–	5_13	–	–
Poly_13	37 218 927	G>A	synonymous variant	low	5_14	–	Thr234Thr	rs211379365
Poly_14	37 218 948	G>A	synonymous variant	low	5_14	–	Tyr227Tyr	–
Poly_15	37 219 120	C>T	intron variant	modifier	–	4_13	–	–
Poly_16	37 219 187	G>T	intron variant	modifier	–	4_13	–	–
Poly_17	37 219 214	A>G	intron variant	modifier	–	4_13	–	–

SNP = single nucleotide polymorphism

Chromosome X position according to the reference genome ARS-UCD1.2 RefSeq NC_037357.1

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Table 2. The *IRAK4* gene variants found in the Czech Fleckvieh population using next-generation sequencing

Polymorphism name	Position	Change	Consequence type (VEP)	Predicted impact	Exon number of total	Intron number of total	Reference SNP
Polym_1	36 681 209	A>G	3'UTR variant	modifier	12_12	–	–
Polym_2	36 681 523	C>T	3'UTR variant	modifier	12_12	–	rs379546685
Polym_3	36 681 596	G>A	3'UTR variant	modifier	12_12	–	–
Polym_4	36 681 659	A>G	3'UTR variant	modifier	12_12	–	–
Polym_5	36 682 518	A>C	intron variant	modifier	–	10_11	rs382209396
Polym_6	36 682 935	A>G	intron variant	modifier	–	10_11	rs385218628
Junction_3	36 683 060	deletion size – 3	intron variant	modifier	–	10_11	–
Junction_4	36 683 075	deletion size – 3	intron variant	modifier	–	10_11	–
Polym_7	36 683 073	T>G	intron variant	modifier	–	10_11	rs209992951
Polym_8	36 683 123	G>A	intron variant	modifier	–	10_11	–
Polym_9	36 683 143	(AC)7>(AC)6	intron variant	modifier	–	10_11	–
Polym_10	36 689 302	G>A	intron variant	modifier	–	8_11	–
Polym_11	36 704 337	T>C	intron variant	modifier	–	1_11	rs132981883
Junction_26	36 704 358	deletion size – 4	intron variant	modifier	–	1_11	rs380202447
Polym_12	36 704 466	G>A	intron variant	modifier	–	1_11	rs385762758

VEP = variant effect predictor (Ensembl); UTR = untranslated region; SNP = single nucleotide polymorphism
Position on chromosome 5 according to the reference genome ARS-UCD1.2 RefSeq NC_037332.1

in ESM) under the same PCR conditions and were subjected to subsequent genotyping. Six microlitres of PCR product were purified with 1.8 µl of thermosensitive alkaline phosphatase FastAP (1 IU/µl, Fermentas, Vilnius, Lithuania) and 0.8 µl of exonuclease I (20 IU/µl, Fermentas, Vilnius, Lithuania) by incubation for one hour at 37 °C. Subsequently, the enzymes were inactivated by heating at 85 °C for 15 minutes. Extension primers were designed by means of the Geneious program to adhere to the analysed variable nucleotides in the direct or reverse orientation. Their length has been optimised in the range of 20 to 30 nucleotides, and their GC content is approximately 60%. Poly(T) sequences in five nucleotide intervals were added to the 5' end of the primers to simultaneously analyse more PCR amplicons in the reaction mixture. The substituted nucleotide was determined based on the primer extension with specific fluorescently marked ddNTPs in the SNaPshot Ready Reaction Mix (Thermo Fisher Scientific, Waltham, MA, USA). Three extension primers were designed as follows: GACCTCCTTGAGCCTCTTCACAGCATAGAC for polymorphism rs211379365, (T6)GCATAGACGGTGTTCCTCATCACCGCCCG for substitution G>A on chromosome X at position 37 218 948 (ARS-UCD1.2 RefSeq NC_037357.1) and (T24)TATAAGTCACCTAGCATGGGC for

Table 3. Primers used for validation of the revealed SNPs by re-sequencing of the *IRAK1* gene

Primer name	Primer Sequence 5'→3'	Starts	Ends
IK1PRIM1F	CAAGGGGTTGAGGGCCATG	37 213 463	37 213 481
IKPRIM1R	TAGGCTGGGGATGGGGAAG	37 214 097	37 214 115
IK1PRIM2F	GTCCTGGATCTCTCCTCCCA	37 214 360	37 214 379
IK1PRIM2R	TCTCTTCTCTGGCCCTGACC	37 214 938	37 214 957
IK1PRIM3F	GTAGATGAGGGGGCTGGAGA	37 219 248	37 219 267
IK1PRIM3R	CCTGACGCCCCCTGACCC	37 219 909	37 219 926
IR1F1INT	TGCCAAGCGTGATACAGTGT	37 218 221	37 218 240
IR1R1INT	CAGGAGTCTCACACTGCCAG	37 218 686	37 218 705

Chromosome start and end position of amplicons according to the reference sequence ARS-UCD1.2 RefSeq NC_037357.1

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deletion rs380202447. Primers were also designed for more single nucleotide variants (SNVs) but did not pass validation for unknown technological reasons. The genotyping reaction was performed in a 10 µl mixture containing 3 µl of the PCR product, 5 µl of SNaPShot Ready Reaction mix, and 2 µl of a 0.1 µM mixture of extension primers. The genotyping program included 25 cycles consisting of three steps: the first step was 10 s at 96 °C, the second was 5 s at 50 °C, and the third step was 30 s at 60 °C. The mixture was purified by adding 1 µl of the FastAP enzyme per reaction, followed by incubation at 37 °C for one hour and by inactivation of the enzyme by heating at 85 °C for 15 seconds. Two microlitres of the purified product were transferred to 8 µl formamide with 0.25 µl of GeneScan 120 LIZ size standard (Thermo Fisher Scientific, Waltham, MA, USA). Then the mixture was denatured at 95 °C for 5 minutes. Genotyping of the variants was performed by means of an Applied Biosystems 3130 Genetic Analyser (Waltham, MA, USA) in the fragment analysis mode. The results were evaluated in Peak Scanner Software v1.0 (Thermo Fisher Scientific, Waltham, MA, USA) and GeneMapper v4.0 (Thermo Fisher Scientific, Waltham, MA, USA).

Microarray technology

The microarray SNP detection method was realised in cooperation with the iGenetics Laboratory of the Czech Moravian Breeders' Corporation – CMSCH (Hradištko, Czech Republic). A standard methodology using commercial kits and chips was used in the service lab of CMSCH. DNA samples with concentrations of approximately 50 ng/µl from 74 dairy cows were hybridised with the BovineSNP50 V3 BeadChip microarray containing 53 218 informative SNPs distributed across the entire genome. Moreover, the Illumina Infinium HTS Assay technology directly determined the population variability of SNP rs110533802 of the *IRAK1* gene.

RESULTS

NGS and Sanger sequencing

The targeted NGS analysis revealed the presence of 17 single nucleotide polymorphisms located in the *IRAK1* gene (Table 1). Variants *Poly_1*, *Poly_2*, *Poly_6*, *Poly_9* and *Poly_13* were confirmed in the SNP database of the EBI. Four of them were identified as exon-synonymous mutations.

The obtained NGS results of *IRAK4* revealed 12 single-nucleotide polymorphisms and three deletions (Table 2). No polymorphism that could cause functional amino acid changes was found. Polymorphisms *Polym_1* to *Polym_4* are located in exons, but in 3'UTR regions, thus, no significant functional changes can be expected. According to the Variant Effect Predictor (VEP, European Bioinformatics Institute, Cambridgeshire, UK) program, even relatively more significant interventions in the *IRAK4* sequence, such as deletions, are not associated with the altered function of the protein due to their localisation inside introns.

The results of the *IRAK1* Sanger sequencing, using primers IR1F1INT and IR1R1INT, further specified population variability of polymorphism rs210710958 and detected an additional polymorphism rs48726521. Mutant allele *G* of the rs210710958 polymorphism was present at a frequency of 0.08, and the mutant allele *G* of rs48726521 was present at a frequency of 0.13 in the CF cattle. Sanger sequencing using primers IK1PRIM1F, IK1PRIM1R, IK1PRIM2F, IK1PRIM2R, IK1PRIM3F and IK1PRIM3R did not reveal any additional polymorphisms.

SNaPshot genotyping

SNaPshot genotyping determined the allelic frequencies of the three polymorphisms: rs211379365 G>A with a frequency of 0.04 for mutant allele *A*, rs380202447 T>C with a frequency of 0.1 for mutant allele *C*, and G>A at the position 37 218 627 with a mutant allele *A* frequency of 0.08. Even the last polymorphism, primarily detected using the NGS method, was successfully validated in this way (Figure 1).

Genotyping by DNA microarray

In this part of the study, 74 dairy cows from the population of Czech Fleckvieh cattle were analysed using the DNA microarray method. Probe ARS-BFGL-NGS-107270 on the Illumina Bovine SNP_chip_V3 targets the site of the polymorphism C>T *Poly_6* in *IRAK1* (rs110533802). The mutant

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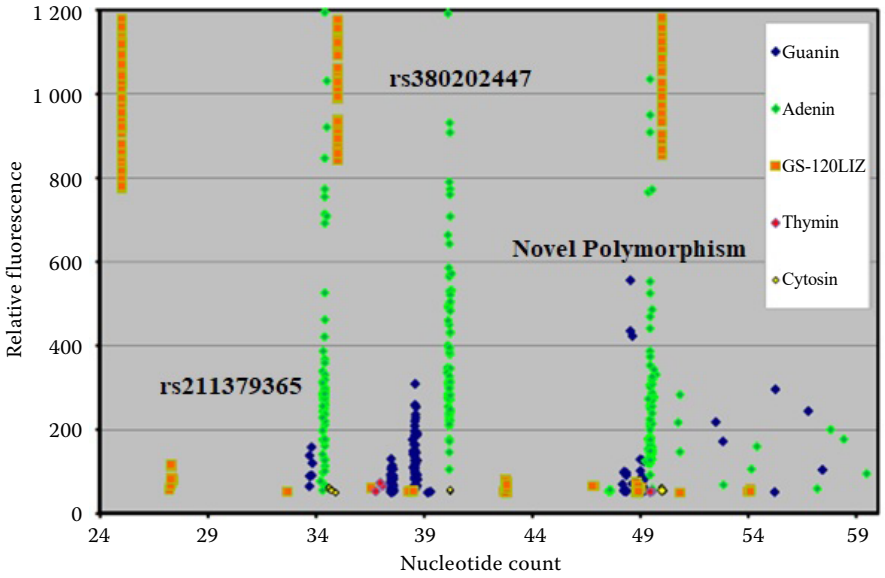

Figure 1. Reproducibility and frequency of the SNPs *Poly_13* (rs211379365) in *IRAK1*, *junction_26* (rs380202447) in *IRAK4* and the newly detected polymorphism *Poly_14* in *IRAK1* visualised in the GeneMapper software
GeneScan-120LIZ is used as the size standard

allele *T* was represented with an allele frequency of 0.07 in this population (Table 4).

Data provided by the Czech Moravian Breeders' Corporation allowed the comparison of the Czech and Hungarian populations of 9 222 dairy cows of the Holstein breed. Both populations showed the same frequency (0.4) of mutant allele *T* (Table 4).

The chip genotyping data also allowed a comparison of the rs110533802 polymorphism frequency among beef breeds (Table 4). Beef breeds showed a lower frequency (0–0.1) of the mutant allele *T* than the Holstein breed. Moreover, the *T* allele was absent in the Aberdeen Angus breed (289 animals).

DISCUSSION

The study combined several methods to discover variability in the genes *IRAK1* and *IRAK4*. Despite the comprehensive use of the sequencing pipeline, the detected polymorphisms were additionally confirmed in the EBI database or by independent genotyping. Out of 32 variants found in total in both genes, 11 polymorphisms and one deletion were also found in EBI. The other variants must be considered putative and confirmed independently, e.g., by genotyping. Since research on the bovine genes *IRAK1* and *IRAK4* is still sparse, the results are only partially comparable with other published studies.

Table 4. Comparison of variability (rs110533802) of the breeds using DNA microarray

Breed	Dairy cows (*n*)	Wild-type allele frequency *C*	Mutant allele frequency *T*
Czech Fleckvieh	74	0.93	0.07
Holstein (HU)	4 268	0.60	0.40
Holstein (CZ)	4 954	0.60	0.40
Limousine	365	0.95	0.05
Charolais	261	0.94	0.06
Simmental	281	0.90	0.10
Aberdeen Angus	289	1	0

Our results might be compared with variability studies of another class of bovine proteins with a related function from the same signalling pathway, the Toll-like receptors and their corresponding genes. A comprehensive study by Bilgen et al. (2016) revealed variability in *TLR2*, *TLR4* and *TLR6* genes in Turkish Holstein cows, along with historical cattle breeds. In this gene screening, 45 variants for the *TLR2* gene, 155 for *TLR6*, and 74 for *TLR4* were detected. A subsequent study by Novak et al. (2019) in populations of Czech Fleckvieh and Czech Red cattle using PacBio technology resequencing reported 34 variants in the *TLR2* gene, 21 variants in *TLR4* and 23 variants in *TLR6*. Sequencing of the *TLR3*, *TLR7*, *TLR8* and *TLR9* genes in nine breeds representing *Bos taurus* and *Bos indicus* revealed the presence of 130 single nucleotide polymorphisms and nine insertion-deletions (Cargill and Womack 2007).

The importance of functional variability in immune genes of the Toll signalling pathway was demonstrated by several association studies. The *TLR2* and *TLR4* genes have been reported to be associated

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with resistance to mastitis in cattle caused by bacterial agents (Goldammer et al. 2004; Zhou et al. 2017). Polymorphisms rs8193946, rs8193060 and rs29017188 in *TLR4* are associated with an increase in the number of somatic cells in fresh cow's milk (Sharma et al. 2006). Moreover, *TLR4* mutations 349G>C and 355C>A are connected with susceptibility to digital dermatitis (El-Shafaey et al. 2017).

The functional impact of other genes of the TLR signaling pathway has been demonstrated in studies in other mammalian species. In the case of the human *IRAK4* gene, a crucial role in developing the normal inflammatory response induced by bacterial and nonbacterial factors has been ascertained (Medvedev et al. 2003). Variability in the *IRAK1* gene can lead to autoimmune diseases, as documented in studies by Zhang et al. (2013) and Hosseini et al. (2020), who described SNPs significantly associated with the risk of human rheumatoid arthritis. Lack of IRAK1-mediated signaling in IRAK1-deficient mice downregulated the early phase of the cytokine cascade during peritonitis-initiated sepsis. Paradoxically, the attenuated response increased the chance of the infected animals surviving induced sepsis (Chandra et al. 2013).

In this study, substantial efforts were devoted to confirming the non-synonymous polymorphism G>A at position 37 218 627 in the *IRAK1* gene (ARS-UCD1.2 RefSeq NC_037357.1). The primers IR1F1INT and IR1R1INT were used for targeted amplification and verification by Sanger sequencing. However, the results did not confirm the initial detection of this variant by NGS. Nevertheless, DNA microarray technology enabled further comparison of the variability in the *IRAK1* gene between both Holstein populations. Both Holstein cattle populations harboured the mutant allele *T* of the polymorphism rs110533802 with a frequency of 0.4. This fact could be caused by similar breeding and the potential relationship of the polymorphism rs110533802 to milk traits of dairy cattle, as this mutation is maintained at high frequencies in both populations. This probably corresponds to the fact that Toll signaling pathway molecules play a significant role not only in immunity (Capparelli et al. 2013) but also in metabolic processes. The results of the study by Eberlein et al. (2011) raised awareness of the functional importance of *IRAK1* in several tissues, including the bovine liver, skeletal muscle, mammary gland, and subcutaneous fat, which are involved in energy and fat metabolism in cattle. Consistently, different expression of the *IRAK1* gene was found in animals with different intramuscular fat contents.

Our work demonstrated that the *IRAK1* mutant allele *T* of polymorphism rs110533802 occurred significantly less in the populations of beef cattle breeds (between 0–0.1) than in the Holstein breed. Among 289 Aberdeen-Angus animals, the mutant allele did not occur at all. Our results obtained for the population of Czech Fleckvieh cattle were more similar to the *IRAK1* allelic frequencies obtained in beef than in Holstein cattle. This finding could be related to the traditional selection for dual-purpose efficiency, as it is still defined in the current CF breeding goal.

CONCLUSION

This study estimated the variability of the *IRAK1* and *IRAK4* genes, considered important members of the innate immune system in cattle. The NGS method revealed 17 SNVs in the *IRAK1* gene and 12 SNVs, and three deletions in the *IRAK4* gene. Comparison of variability (rs110533802) of the breeds using DNA microarray places CF breed more in the group of beef breeds. The significantly higher allelic frequency of the polymorphism rs110533802 in Holstein cattle populations indicates this polymorphism as a potential genetic marker associated with milk yield traits, which brings a new perspective on this unexplored polymorphism. The molecular methods designed and validated for screening the variability of these genes in Czech Fleckvieh cattle serve as proven methodological procedures for further research on these genes in other breeds and herds. The relatively low gene diversity found in the CF contrasts sharply with the high diversity registered in the European Variation Archive. Investigation of variations may also be useful for subsequent association studies and breeding for health traits and resistance against pathogens. Even if they were not predicted as causal mutations affecting immunity and health, they may act as genetic markers if they are in absolute linkage with mutations that have a more pronounced effect. The study provides new insights into the variability of native immunity genes, which can affect resilience and performance in cattle. These findings are useful for scientific and practical breeding implications. Research of this nature can aid in developing an improved method

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for DNA microarray design. Collaboration between researchers and breeding organisations is a practical approach to promptly convey new knowledge to farmers and enhance the competitiveness of the entire agricultural industry.

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Conflict of interest

The authors declare no conflict of interest.

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