ISSN: 1220-3173; E-ISSN: 2457-7618

THE EFFECT OF GENETIC VARIATIONS OF PITUITARY TRANSCRIPTION FACTOR 1 (*PIT1*) ON MILK PERFORMANCE TRAITS IN ROMANIAN CATTLE

EFECTUL VARIANTELOR GENETICE ALE FACTORULUI DE TRANSCRIPȚIE PITUITARĂ (*PIT1*) ASUPRA PRODUCȚIEI DE LAPTE LA RASELE DE VACI ROMÂNEȘTI

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ABSTRACT | REZUMAT

The aim of this study was to investigate the *HinfI* polymorphism of *PIT1* gene in two cattle breeds, Romanian Spotted and Romanian Brown. Genomic DNA samples were obtained from a total of 137 cattle, of which 82 were Romanian Spotted cattle (RS) and 55 Romanian Brown (RB). All the animals were reared at the Research and Development Station for Bovine, located in Arad, Romania. The cattle involved in the study were milked twice per day and were included in the official dairy control, which consists of milk records at every 28 days. Genotype analyses were performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. We identified three genotypes and two alleles. The frequency of the *B* allele was higher than *A* in both investigated breeds, but more pregnant in the Romanian Spotted population compared to Romanian Brown. The *BB* genotype recorded the highest frequency in Romanian Spotted, while in Romanian Brown, the *AB* genotype was superior in its frequency. However, the *AA* genotype had the lowest frequency in Romanian Brown and it was missing in Romanian Spotted. In our study, the *AA* genotype of *PIT1* had a significant influence on milk fat percentage ($P < 0.05$) when we analysed both breeds together. Cows that carry the *AA* genotype recorded a higher fat percent (4.67±0.49%) compared to *AB* (4.04±0.40%) or *BB* (4.02±0.43%) individuals.

Keywords: *POU1F1*, milk traits, Romanian Brown, Romanian Spotted

Scopul acestui studiu a fost investigarea polimorfismului *HinfI* al genei *PIT1* la două rase de bovine, Bălțată Românească și Brună de Maramureș. S-au obținut probe de ADN genomic de la un total de 137 de bovine, dintre care 82 au fost vaci de rasă Bălțată Românească (RS) și 55 vaci Brună de Maramureș (RB) aparținând Stațiunii de Cercetare Dezvoltare pentru Creșterea Bovinelor din Arad, România. Animalele implicate în studiu au fost mulse de două ori pe zi și au fost incluse în controlul oficial al producției de lapte constând din înregistrarea parametrilor producției de lapte la fiecare 28 de zile. Analizele de genotip au fost efectuate folosind reacția de polimerizare în lanț și polimorfismul de lungime al fragmentelor de restricție (PCR-RFLP). Astfel, s-au identificat trei genotipuri și două alele. Frecvența alelei *B* a fost mai mare față de *A* la ambele rase investigate, dar per total mai mare la vacile de rasă Bălțată Românească comparativ cu cele de Brună de Maramureș. Genotipul *BB* a înregistrat cea mai mare frecvență la rasa Bălțată Românească, în timp ce la rasa Brună de Maramureș, genotipul *AB* a avut o frecvență superioară. Genotipul *AA* a înregistrat cea mai mică frecvență la Brună de Maramureș și a lipsit la rasa Bălțată Românească. În studiul nostru, genotipul *AA* al genei *PIT1* a avut o influență semnificativă asupra procentului de grăsime din lapte ($P<0,05$) atunci când s-au analizat ambele rase împreună. Animalele purtătoare ale genotipului *AA* au înregistrat un procent mai mare de grăsime (4,67 ± 0,49%) comparativ cu indivizii *AB* (4,04 ± 0,40%) sau *BB* (4,02 ± 0,43%).

Cuvinte cheie: *POU1F1*, lapte, Brună de Maramureș, Bălțată Românească

Milk synthesis and secretion, and subsequent milk yield, with interest especially in dairy cattle, is a trait governed by various polymorphic genes and influenced by environmental factors.

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Żukiewicz et al. (2012) reviewed 16 of the milk-yield associated bovine genes and their polymorphism, mentioning here, for example, those for caseins *(CSN1S1, CSN2, CSN1S2, CSN3),* β-lactoglobulin *(LGB),* prolactin and its receptor *(PRL* and *PRLR),* growth hormone and its receptor *(GH* and *GHR),* Leptin *(LEP)* and others (25). The pituitary transcription factor gene *(Pit-1, POUF1* or *POU1F1*, considering its official nomenclature) was also included in this group since its product of synthesis, a protein of about 33

kDa and containing 291 amino acids, includes a DNA binding POU domain and is able, as a transcription factor, to activate the expression of target genes, including here of itself gene and those of *GH, PRL,* somatoliberin or growth hormone-releasing hormone *(GHRH),* β subunit of thyroid-stimulating hormone *(β-TSH),* and the pituitary β2-Thyroid hormone receptor (2,12,14,24,26). Renaville et al. (1997) mentioned that, in this way, *PIT1* intervenes in pituitary cell differentiation and proliferation (15). Besides the role of encoding the proteins involved in animal development (8), Pozovnikova et al. (2020) reviewed the *PIT1* influence on the indices of fertilization and survival rates of embryos (13). The *PIT1* protein mechanism of action is based on its ability to bind the promoters of *GH, PRL*, and *TSH* genes using its domains, the POU-specific and POU-homeo. The POU-homeo includes AA 214-273, while the POU-specific, AA128-198 (4,19). Although both *PIT1* domains have DNA-binding affinity, it seems that the C-terminal half (POU-homeo domain) has a low affinity, whereas the N-terminal half (POU-specific domain) has a high affinity, participating even in protein-protein interactions (12). *PIT1* is a member of POU-family or the family of POU domain containing proteins, together with *OCT-1* (Octamer Transcription Factor) in mammals, and neural *Unc-86* in *Caenorhabditis elegans* (9).

The activity of *GH* and *PRL* genes is controlled by *PIT1* protein since its gene expression precedes the expression of their genes in somatotrophic and lactotrophic cell lines. However, this mechanism of control is alternative, for example the *GH* gene activation in somatotrophic cells is accompanied by its restriction in lactotrophic cells of the anterior pituitary gland. The lack of *PIT1* synthesis is translated into significant decrease in proliferation of *PRL* and *GH* cell lines as a consequence of their genes affected expression (20). The same mechanism and effect are recorded on another adenohypophysis cell type, the thyrotrophic cell lines.

Trakovická et al. (2014) reviewed different ways of action of *PIT1*, considering its three variants resulted from a process of alternative splicing of its mRNA gene, all of them being biologically active (20). Thus, in the case of *PIT1* are known three protein isoforms, *PRL-1α, PRL-1β,* and *PRL-1T*. Compared to the *PRL-1α* major type, the *β* variant loses the ability to activate the *PRL* and *PIT1* promoters, preferentially activating the *GH* gene promoter, considering its modified structure based on a 26 amino acid insertion because of alternative splicing at the end of intron one. On the other hand, *PIT1*T contains an insertion of 14 amino acids because of an alternative splicing of the 3' acceptor site, and is involved only in *TSH-β* expression.

The molecular mass of the final protein is about 31-33 kDa, while the encoding gene is located in the centromeric region of bovine chromosome 1 *(1q21-1q22),* midway between the *TGLA57* and *RM95* loci (4, 5, 8, 9, 11, 20, 21, 23, 26). The *PIT1* gene (ENSBTAT 000000566433) consists of 5 introns and 6 exons, within a total length of 3274 bp (1). In humans, the *PIT1* gene spans more than 14 kb, and its six exons vary in length between 61 bp (exon 5) and 225 bp (exon 3), while the five introns range from 0.7 kb (intron 4) to more than 7.5 kb (intron 2) (4).

As previously discussed, there is a mechanism of *PIT1* gene autoregulation, since the mutation of an enhancer element located upstream of the transcriptional region abolished the positive autoregulation; on the other hand, the mutation of the situs located immediately to the 3' of the cap site increased the *PIT1* promoter expression, demonstrating its usual behaviour as a silencer (3,4,5).

A mutation in *PIT1* was associated with a dwarf phenotype, mainly reviewed in mice and humans (9, 15). There are two dwarf phenotypes known in mice, the Snell and Jackson phenotypes. In 1990, Li et al. (1990) brought evidence of a *G* to *T* mutation in Snell dwarf mice, the *TGG* codon change into *TGT* leading to the tryptophan to cysteine conversion in the POU-homeodomain of the final protein. The authors highlighted this mutation to be conserved in organisms from mammals to yeast (10). Some authors (1,6) reviewed that mutations in human *PIT1* are responsible for a Combined Pituitary Hormone Deficiency *(CPHD),* in which a deficiency of *GH, PRL* or *TSH* is combined with the preservation of adrenocorticotropic hormone *(ACTH),* luteinizing hormone *(LH),* and follicle-stimulating hormone *(FSH)* productions. In fact, the dwarf phenotype is mainly based on the *PIT1* gene expression as one that precedes the expression of *PRL* and *GH* genes, their expression being dependent on the synergistic interactions between *PIT1* and promoters of their genes. Moreover, it seems that the development of distinct cell types within the anterior pituitary, such as somatotrophs and lactotrophs, is related to their cell specific gene activation, and the lack of these factors leads to a subsequent interruption of the normal development of the organ and the loss of *GH, PRL,* and *TSH* synthesis (10). As a consequence, late puberty and hypotyroidism will be also observed (6).

Various polymorphisms involving a single base modification in the structure of the *PIT1* gene were investigated and were reported to be associated with morphological traits of interest in animals, including those related to milk production. Different studies (2, 9) reviewed a mutation located in the *Hinf*I restriction site of the exon 6, which involves a substitution of an adenine with a guanine base (A207G). Another polymorphism located in exon 3 involves a cytosine to adenine change (C577A), which leads to a Pro76His substitution in the mature protein. In addition, one polymorphism located in exon 2 (G545A, *Taq*I restriction

enzyme) (18), two in intron 3, one in intron 4 and one in intron 5 were also reviewed.

Considering the importance of *PIT1*, the purpose of our research was to investigate the *PIT1* gene polymorphism in two Romanian cattle breeds, Romanian Spotted and Romanian Brown, and its corresponding genotype associations with milk production traits.

MATERIALS AND METHODS

DNA samples and phenotypic data collection

Genomic DNA samples were obtained from a total of 137 cattle, of which 82 were Romanian Spotted cattle (RS) and 55 Romanian Brown (RB), reared at the Research and Development Station for Bovine, located in Arad, Romania. DNA samples were extracted from whole blood that was collected from the tail vein in vacutainers containing K3EDTA as an anticoagulant. DNA extraction was carried out using the Wizard Genomic DNA Purification kit (Promega®, USA), following the manufacturer's instructions. The extracted genomic DNA was quantified by spectrophotometry (NanoDrop-2000,Thermo Fisher Scientific®,MA,USA) and assessed for integrity by 1% agarose gel electrophoresis. The cattle involved in the study were milked twice per day and were included in the official dairy control, which consists of milk records collected every 28 days. For the association study between the *PIT1* genotypes and milk traits, only cows with complete lactations were considered.

Milk production data were available for a total of 52 individuals (23 Romanian Spotted and 29 Romanian Brown), normalized for standard lactation length (305 days), mature equivalent. The studied milk phenotypic traits were as following: milk yield (Kg), protein yield (Kg), protein percentage, fat yield (Kg) and fat percentage.

Genotyping

Genotype analyses were performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Amplification of a *PIT1* gene to genotype rs134303957 (g.35419931C > T) polymorphism was performed using the primers presented in Table 1 (22). The PCR amplification was performed in 25 µl reaction mixture containing 50-100 ng of genomic DNA, 20 pM of each primer and Green PCR Master Mix (Rovalab GmbH, Germany).

The reaction conditions were an initial denaturation for 5 min at 95C followed by 35 cycles of 30s at 95C, 30s at 57C, 30s at 72C, and a final extension of 5 min at 72C. After PCR, the amplicons were digested with the *Hinf*I restriction enzyme at 37°C, for two hours. The PCR-RFLP fragments were analysed in 3.5% agarose gel containing Midori Green Advance dye (Nippon Genetics®, Japan) and were visualized by a UV transilluminator. The sequence of primers used for PCR amplification of the *PIT1* gene, annealing temperatures of the primers, expected size of the PCR product, restriction enzyme used and corresponding genotypes are presented in Table 1.

Data analysis and statistics

The genotype and allele frequencies of *PRL* locus were computed using a custom R script. Descriptive statistics were generated using the R package psych v. 1.9.12.31 (16). Scatterplots and correlations were plotted for the whole dataset and for the two breeds, using the pairs.panels function from the R package psych and the ggpairs function from the R package G Gally v.2.0.0 (17), respectively.

To investigate the association of the *PIT1* gene variations with the milk traits of the Romanian Spotted and Romanian Brown cattle, a statistical analysis was carried out using ANOVA and Tukey's test using the corresponding base R methods. Associations with $p < 0.05$ were considered significant.

Ethics statement

The research activities were performed in accordance with the European Union's Directive for animal experimentation (7).

RESULTS AND DISCUSSIONS

The PCR-RFLP technique was used to identity the genotypes for rs134303957 in *PIT1*. Digestion of the 451 bp amplicons using the *Hinf*I enzyme resulted in one restriction fragment of 451 bp for the *AA* genotype; three restriction fragments of 451, 244 and 207 bp for the heterozygotes *AB*; and two restriction fragments of 244 and 207 bp for the *BB* genotype (Fig. 1).

The genotype and allele frequencies of the *PIT1* locus for Romanian Spotted and Romanian Brown cattle breeds are presented in Table 2.

Table 1

Primers used for the PCR amplification of *PIT1*, PCR product size, restriction enzyme and genotypes according to the obtained digestion fragments

Gene	Primers (5'-3')	Annealing temp. (°C)	Amplicon PCR (bp)	Restriction endonuclease	Digestion product size (bp)
PIT1	F: 5' aaa cca tca tct ccc ttc tt 3' R: 5' aat gta caa tgt gcc ttc tga g 3'	57	451	*Hinf*I	(*AA*) 451 (*AB*) 451,144,207 (*BB*) 144,207

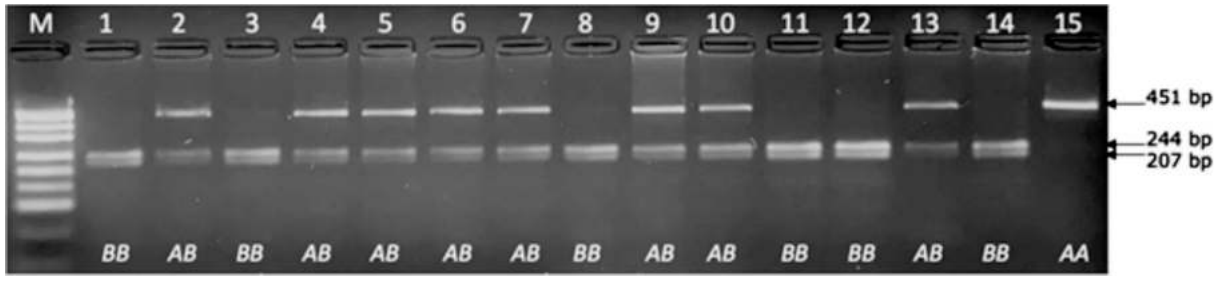

Fig. 1. PCR-RFLP of *PIT1* 451bp/*Hinf*I genotypes on agarose gel electrophoresis. Line M: pUC19/MspI Ladder (Carl Roth); Line 15: genotype *AA* (451 bp); Lines 2, 4-7, 9-10, 13: genotype *AB* (451, 244 and 207 bp); Lines 1, 3, 8, 11-12, 14: genotype *BB* (244 and 207 bp)

The amplified 451 bp DNA fragment and its digestion with *Hinf*I enzyme in order to detect the *A* to *G* point mutation in exon 6 lead to the identification of two alleles, *A* and *B*, and three genotypes, two homozygote *AA* and *BB*, and one heterozygote, *AB*. The frequency of the *B* allele was larger in both investigated breeds, but more pregnant in Romanian Spotted cattle compared to Romanian Brown. The *BB* genotype recorded the highest frequency in Romanian Spotted, while in Romanian Brown, the *AB* genotype was superior in its frequency. However, the *AA* genotype had the lowest frequency in Romanian Brown and it was missing in Romanian Spotted. The predominance of the *B* allele and the *BB* genotype was also reported in various types of Black-and-White or Holstein Friesian cattle (2,5), in hybrid and local Palestinian cattle, but not in the located Friesian (9), in the Podolitica breed (18), or in Italian Holstein, Holstein, Piemontese, Limousine, Angus, and various types of zebu, as Selvaggi and Dario (2011) reviewed (18), or in Chinese Holstein Friesian, as Ebrahimi Hoseinzadeh et al. (2015) reported (6). The same trend of alleles and genotype frequencies as we reported, was also found by Aytekin and Boztepe (2013) for 301 Brown Swiss cows (1).

The results of association testing between the *PIT1* genotypes and milk production traits (milk yield, protein yield, protein percentage, fat yield and fat percentage) are presented in Table 3.

We observed that the *AA* genotype of *PIT1* had a significant influence on milk fat percentage ($P < 0.05$) when we analysed both breeds together. Cows carry-ing the *AA* genotype recorded a higher fat percent (4.67± 0.49%) compared to *AB* (4.04±0.40%) or *BB* (4.02± 0.43%) individuals. However, non-significant differences among *PIT1* genotypes were found for fat percent when the breeds where individually analysed ($P>0.05$). If we compare these results with the literature,although some associations are inconclusive, many other reports, already presented in introduction, also found the *AA* genotype as favourable for milk production, but not on milk components. Therefore, the process of selection is directed according to the farmers' needs, either for superior milk production that will be directed to processing as drinking milk, or to increase the components of milk in order to process it into dairy products.

The *PIT1/Hinf*I polymorphism (A207G) is one of the most studied in cattle populations, particularly on its associations with milk traits. Dybus et al. (2003) found the *A* allele of this *PIT1/Hinf*I polymorphism to be superior for milk and protein yields and inferior for fat percentage in dairy cattle, while in Angus cattle it appeared to affect growth traits. In their study, they found the *B* allele at a higher frequency compared to *A*, and also the *BB* genotype compared to *AB* and *AA* (4). The same trend of allele and genotype frequencies was reported in the Romanian Black-and-White breed and Romanian Grey Steppe (2) and in hybrid and local Palestinian cows but not in Holstein Friesian (9), where *A* allele was dominant; this is an intriguing situation, since Carşai et al. (2012) suggested that the A207G substitution occurred in primitive cattle and afterwards spread in modern cattle breeds. They reported an important effect of the *AA* genotype on milk production, as compared to the intermediate *AB* genotype and the lowest *BB* one (2). The superiority of the *B* and *BB* genotype frequencies was also reported in

Table 2

Distribution of the allele and genotype frequencies for the *PIT1* locus in Romanian Spotted (RS) and Romanian Brown (RB) cattle breeds and total investigated population (RS+RB)

Breed	*n*	Allele frequency		Genotypes frequency (n)		
		A	*B*	*AA*	*AB*	*BB*
RS	82	0.2012	0.7987	0.00 (0)	0.4024 (33)	0.5975 (49)
RB	55	0.3818	0.6181	0.1090 (6)	0.5454 (30)	0.3454 (19)
Total	137	0.2737	0.7262	0.0437 (6)	0.4598 (63)	0.4963 (68)

Table 3

Mean ± SD for milk production and chemical composition according to the *PIT1* locus in Romanian Spotted (RS) and Romanian Brown (RB) cattle breeds

Breed	Genotype (n)	Milk (kg)	Fat (kg)	Fat (%)	Protein (kg)	Protein (%)
RS	*AA*	NA	NA	NA	NA	NA
	AB (7)	6067.00±1765.74[a]	242.57±71.05[a]	4.00±0.47[a]	210.57±70.31[a]	3.45±0.26[a]
	BB (16)	5367.56±1078.12[a]	208.81±47.30[a]	3.90±0.44[a]	174.88±31.71[a]	3.27±0.21[a]
RB	*AA* (4)	4568.00±907.26[a]	209.75±21.856[a]	4.67±0.49[a]	166.75±16.66[a]	3.71±0.49[a]
	AB (16)	5377.06±866.83[a]	217.44±34.07[a]	4.06±0.38[a]	182.31±28.88[a]	3.41±0.34[a]
	BB (9)	5211.44±1373.50[a]	218.67±50.78[a]	4.24±0.31[a]	180.44±40.66[a]	3.51±0.26[a]
Total (RS+RB)	*AA* (4)	4568.00±907.26[a]	209.75±21.856[a]	4.67±0.49[b]	166.75±16.66[a]	3.71±0.49[a]
	AB (23)	5587.04±1211.61[a]	225.09±48.04[a]	4.04±0.40[a]	190.91±45.75[a]	3.42±0.31[a]
	BB (25)	5311.36±1166.68[a]	212.36±47.76[a]	4.02±0.43[a]	176.88±34.45[a]	3.36±0.25[a]
Total RS		5580.43±1323.29[a]	219.09±56.17[a]	3.93±0.44[a]	185.74±48.12[a]	3.33±0.24[a]
Total RB		5214.07±1050.97[a]	216.76±37.66[a]	4.20±0.42[b]	179.59±31.25[a]	3.48±0.34[a]
Total population		5376.12±1181.33	217.79±46.27	4.08±0.45	182.31±39.30	3.41±0.31

Column means with different superscript differ significantly at $P \leq 0.05$, according to the specific variation source (milk production trait)
NA: no available data

900 Black-and-White cows reared in Poland (5), but with no association reported between *PIT1* and milk production traits. Aytekin and Boztepe (2013) found a slight superiority of the *B* allele compared to *A* and of the *AB* genotype compared to *BB*, and the lowest frequency of *AA* genotype in Brown Swiss cattle. They reported the *AA* genotype as associated with higher milk yield, compared to *AB* and *BB*. Considering the milk components, the *AA* and *AB* genotypes were reported with a similar tendency of lower values compared to the *BB* genotype, but without significant associations.

CONCLUSIONS

The marker assisted selection process may be used to consider the *PIT1* gene, since its product of synthesis is able to activate the GH, PRL, and TSH genes, whose hormones are with various metabolic functions and implications in milk production. Our study was centred on the *PIT1/Hinf*I genotyping in two Romanian cattle breeds, Romanian Spotted and Romanian Brown, the obtained results being in most of the cases similar to those reported for other phylogenetically related breeds. We only found a significant influence of the *AA* genotype on milk fat percentage, but this may be attributed to the small number of investigated animals. Considering various reported associations of this locus and milk traits, a selection program may be applied considering the interest for milk processing as drinking milk or for dairy products.

ACKNOWLEDGMENTS

This work was supported by a self-funded grant of SCDCB Arad through the project 4483/2018 and a grant of the Romanian Ministry of Agriculture and Rural Development, through the project ADER 8.1.6/2019.

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