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Research Concerning the Polymorphic Expression of Pit-1 and STAT5A Genes in Cattle

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Abstract. Recent advances in molecular genetic techniques led us to make possible the accession of a large number of polymorphisms at DNA level in the international database. Some of them are molecular markers and genotyping many individuals for this loci makes possible the association with important economical traits, which can lead to faster genetic gain when information is used in molecular assisted selection. The somatotropic axis plays an important role in the control of the regulation of metabolism and physiological process through growth hormone (GH) and insulin-like growth factors (IGF-I and IGF-II) and their associated receptors. The pituitary transcription factor – Pit1 is the cellular specific transcription factor for activating expression of the prolactin (PRL) and GH genes in the anterior pituitary gland. The STAT5 transcription factors initiate the growth process in the target cells, a process mediated by the pituitary growth hormone. This evidence makes possible to test association between polymorphisms of these genes and quantitative traits in animal population. Two PCR-RFLP polymorphisms (Pit1/*Hinf*1 and STAT5A/*Eco*88I) were tested in order to establish the association with milk performance traits (milk yield, fat and protein content) in Romanian Simmental cattle. The average of milk yield, fat and protein content was in favor of A allele ($p<0.001$) for Pit-1 gene and only the difference in average of fat content was statistically significant ($p<0.05$) in favor of C allele in STAT5A gene.

Keywords: Pit-1, STAT5A, RFLP, milk yield, fat, protein content

INTRODUCTION

One of the obstacles to progress in dairy cattle selection is that milk production traits are only expressed after the first calving. However, the use of the quantitative trait loci (QTL) technology will improve the efficiency of dairy industry with a positive image for the consumers. QTL are part of the genome showing a preponderant action and explaining the major part of variation of the trait production (Parmentier *et al.*, 1999). Pit-1 and STAT5A genes are candidate genes for milk production and characteristics improvement. The candidate gene approach is the best tool for detecting polymorphic loci in an unstructured population (Rothschild and Soller, 1997).

The pituitary is well known as one of the most important glands in the endocrine system, and secretes hormones that orchestrate many physiological processes such as growth, sexual development, metabolism and the stress response. The anterior pituitary gland consists of five cell types: somatotrophs (producing growth hormone-GH), lactotrophs (producing prolactin-PRL), gonadotrophs (producing luteinizing hormone-LH and follicle stimulating hormone-FSH), thyrotrophs (producing thyroid stimulating hormone-TSH), and corticotrophs (producing

adrenocorticotropic hormone-ACTH, proteolytically digested from proopiomelanocortin-POMC) (Ma *et al.*, 2009). The somatotropic axis includes growth hormone (GH), upstream hypothalamic hormones, the insulin-like growth factors (IGFs) and downstream signaling molecules (Brown-Borg, 2009). POU1F1 gene (Pit-1) encodes a member of the POU family of transcription factors that regulate mammalian development. The pituitary transcription factor (Pit-1) is the cellular specific transcription factor for activating expression of the prolactin (PRL), thyrotropin (TYR), and GH genes in the anterior pituitary gland (Ben-Batalla *et al.*, 2010; Tuggle and Trenkle, 1996). Growth hormone (GH) is known to be responsible for galactopoiesis and persistency of lactation (Bell 1995; Svennersten-Sjaunja *et al.*, 2005), and the uncoupled somatotropic axis (GH-insulin-like growth factor I axis, IGF-I) mediates nutrient partitioning for lactogenesis in high producing dairy cows (Lucy *et al.*, 2009; Renaville *et al.*, 2002; Ruprechter *et al.*, 2011). In addition, some genes in the JAK/STAT signaling pathway downstream of POU1F1 have been shown to be associated with different production traits in dairy cattle (Hunag *et al.*, 2008).

Transcription factors from the family of Signal Transducers and Activator of Transcription (STAT) include 7 members, which become known as latent cytoplasmatic factors. The STAT5 transcription factors are also known as members of the somatotropic axis. They initiate the growth process in the target cells, a process mediated by the pituitary growth hormone. In addition, the STAT5A factors regulate the protein expression in the tissue of mammary gland in response to prolactine action. For these reasons, the genes, which encode the transcription and signaling factors of the STAT5A transducers are candidate genes for the quantitative traits in cattle.

GH activates a number of transcription factors including STATs 1, 3, 5a, and 5b. Upon GH binding, STATs are recruited to the GHR complex and become both tyrosine phosphorylated by JAK kinases (resulting in STAT dimerization and nuclear translocation) and serine phosphorylated (which is important for activation of transcription (Pilecka *et al.*, 2006). The factors STAT1 and STAT2 are involved in IFNα/β- and IFNγ-response, the factors STAT3 are involved in response to several cytokines including IL6 (Meton, 1999). They activate several genes involved in acute phase response, they are important in growth regulation, embryonic development and organogenesis. Activation of STAT3 members is correlated with cell growth, linked to cancer in human (Molenaar, 2000). The STAT4 and STAT6 members are involved in IL4 and IL12 response. The STAT5A and STAT5B are involved in response to several cytokines such as prolactin, growth hormone, erythropoietin and interleukins. All members of the STAT family share a highly conserved molecular structure (Hennighaussen *et al.*, 2008). The two forms of STAT5 (STAT5A and STAT5B), encoded by two different genes, have been identified in sheep, mouse, human, rat and cattle cells (Hou *et al.*, 1995; Goldammer *et al.*, 1997; Liu *et al.*, 1995; Rippenger *et al.*, 1995). The genes encoding STAT5A and STAT5B are highly homologous, being ~ 90% identical in coding sequence (Dario *et al.*, 2009).

The aim of this study was to test two RFLP polymorphisms (Pit1/*Hinf*I and STAT5A/*Eco*88I) in 21 Romanian Simmental breed cattle and to make preliminary associations between detected polymorphisms and milk production parameters (milk yield, fat and protein content).

Pit1 and STAT5A genes polymorphisms in cattle. The bovine Pit1 gene was located in centromeric region of chromosome 1 (position 1q-21-q22), between TGLA57 and RM95 loci. This location creates a chain transmission of the following group of genes: TGLA49-RM95-PIT1-TGLA57 (Moody *et al.*, 1995). The Pit-1 gene, named also transcription factor 1, growth

factor 1, POU1F1 has the accession numbers in NCBI database: gene ID 282315, mRNA-NM_174579.3 and protein-NP_777004.1 or ENSBTAG00000009128 in Ensembl database. It consists in 6 exons and 5 introns. The RefSeq of the gene present a 1307 bp mRNA product length, which codifies a protein of 291 AA (Source: RefSeq peptide; Acc:NP_777004). In the transcript of Pit-1 gene, 5 variations have been identified. All these are presented in Table 1. The other 74 variants, consisting in SNPs or insertions, were identified in the intronic regions of the gene as they are present in the Ensmbl database.

Tab. 1

The genetic variations of the pituitary- specific positive transcription factor 1
(Protein ID: ENSEMBTAP00000012033, Source: Ensembl database)

Residue	Variation ID	Alleles	Ambiguity code	Residues	Codons
91	rs110357819	C/T	Y	S	TC**G**/TC**A**
117	rs109135550	T/G	Y	P	CC**A**/CC**G**
143	rs135305350	A/C	R	L	**T**TG/**C**TG
156	rs133796768	T/C	Y	S	TC**A**/TC**G**
333	rs134303957	C/T	Y	L	CT**G**/CT**A**

Underling nucleotide represents the SNP mutation in the specific codon

Many studies regarding polymorphism of Pit1 gene and their association with economically important traits were performed. In cattle, Pit-1 was found to be associated with body weight, average daily gains and milk composition (Renaville *et al.*, 1997a, b), and growth performances and carcass traits Angus cattle (Zhao *et al.*, 2004). *Hinf*I polymorphism has been reported in exon 6 of the bovine Pit-1 gene by PCR-RFLP technique (Woollard *et al.*, 1994). Moody et al. (1995) using also PCR/RFLP technique for amplification of a 1355 bp fragment, corresponding to an intron of 1.1 kb flanked by exons 5 and 6, has studied the same polymorphic site. This single nucleotide polymorphism (SNP) founded in the coding region, namely 6th exon, of the bovine Pit-1 gene consists in the substitution A→G. All studies conducted in this polymorphic site reveal a favorable A allele in selection regarding to variable quantitative traits.

Moreover, four intronic polymorphisms were also reported: two located in intron 3, one in intron 4 and the last in intron 5 (Zhao *et al.*, 2004). Recently, a new silent mutation in exon 2 was discovered by Pan *et al.* (2008) and also reported by Huang *et al.* (2008): this SNP is a substitution G→A at position 545, recognized by the *Taq*I restriction enzyme.

In cattle, the STAT5A gene has been assigned to chromosome 19q17 (Seyfert *et al.*, 2000; Molenar *et al.*, 2000), it contains 4 conserved domains of a total length of 44.39 kb.STAT5A gene, also known as signal transducer and activator of transcription 5A or MGF (Mammary gland factor)(Wakao *et al.*, 1997). It has the ID 282375 in NCBI database and respectively ENSBTAG00000009496 in Ensemble database. The transcript length of the gene consists in 2470 bp (19 exons), which codifies 794 AA of the proteins (http://www.ensembl.org/Bos_taurus/Gene/Sequence; Transcript ID: ENSBTAT00000034831, Ensemble database).

Many SNP polymorphisms have been assigned to this gene, some of them being in the coding or noncoding region of the gene (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref). The research for SNP detecting in 15,291 bp of genomic *STAT5A*, conducted by Khatib *et al.*

(2008) revealed a total of 12 SNP, of which 11 SNP were identified in introns and 1 SNP (SNP12195) was identified in exon 8. Three SNP (SNP3117, SNP3419, and SNP3470) were identified in intron 4; and SNP12885, SNP12924, SNP13244, SNP13319, SNP13516, SNP13654, and SNP14217 were identified in intron 9. Only SNP15541 was identified in intron 12. Association testing of SNP12195 (exon 8) and SNP14217 (intron 9) with milk composition in Holstein cows revealed that allele G of SNP12195 was associated with a decrease in both protein and fat percentages and with low survival rate. However, SNP14217 in intron 9 showed no significant association with milk production or health traits in Holstein cows.

Several studies have been initiated to investigate polymorphism in the STAT5A encoding gene. So far, only a few associations have been established with product characteristics. Through the comparison of restriction maps in the 215 bp fragment of the 7 exon, the substitution C→T occurs in position 6853 of the gene (|AJ237937|) which is also the site of the *Ava*I(Eco88I) restriction enzyme (Dario *et al.*, 2008, Flisikowski *et al.*, 2003a and b; Selvaggi *et al.*, 2009).

MATERIALS AND METHODS

The polymorphism studies were carried out on 21 unrelated Romanian Simmental cattle from SCDB Targu Mures, randomly selected from population. The DNA from 200 µl fresh blood, collected on K-EDTA tubes, was extracted using Wizard®DNA Purification kit (Promega). DNA quantification was performed on spectrophotometer Nanodrop ND1000. The polymorphism at Pit-1 locus was studied according to Moody *et al.* (1995) protocol by PCR-RFLP/*Hinf*I in an 1355 bp fragment, corresponding to 1.1 kb intron flanked by exons 5 and 6.The polymorphism in the STAT5A gene has been studied according to the Flisikovski *et al.* (2003, a) protocol, using the PCR-RFLP/*Eco*88I technique for the amplification of a 215 bp fragment of the gene. The polymorphic site is located in the 7 exon. Primers structure, PCR conditions and restriction protocols were previously described (Cosier *et al.*, 2008; Cosier and Vlaic, 2009).

The sequences of primers used for amplification of the 1355 bp fragment of Pit-1 gene and for the 215 bp of STAT5A gene are presented in Table 2.

Tab. 2

Sequence of the Pit-1 and STAT5A primers, product size expected in PCR reaction, amplified region and references

Primer sequence	Product size	Amplified region	References
Pit-1_F 5'-CAA TGA GAA AGT TGG TGC-3'	1355bp	Intron 5 and exon 6	Moody *et al.*, 1995
Pit_1_R 5'-TCT GCA TTC GAG ATG CTC-3'			
ST5A_F 5'-CTGCAGGGCTGTTCTGAGAG-3'	215 bp	Exon 7	Flisikovski *et al.*, 2003a
ST5A_R5'-GGTACCAGGACTGTAGCACAT-3'			

The genetic structure of investigated population for both RFLP polymorphisms, Pit-1 and STST5A, were calculated according to co-dominat manner of the allele's interaction, by simple counting. In addition, the combined analysis of the two considered loci was performed. A chi-square test was applied to verify if the distribution of the genotypes, both Pit-1 and STAT5A, was in Hardy–Weinberg equilibrium.

The data for a 305-day milk production (normal lactation) including milk yield (MY), protein and fat yield (PY and FY) were obtained from the UARZ Targu Mures. The mean and standard error of milk yield and milk parameters were calculated and the differences were statistically analyzed by Student test (t).

RESULTS AND DISCUSSIONS

Polymorphism's description and genetic structures determinations.

Pit 1 polymorphism: Digestion of PCR products of Pit-1 gene with *Hinf*I revealed two alleles corresponding to the following genotypes: AA (660, 425 and 270 bp), AB (660, 425, 385 and 270 bp) and BB (660, 385 and 270 bp). The Pit-1 mutation is a substitution (A→G) (NM_174579) which modifies the *Hinf*I restriction enzyme site (Fig.1-left).

STAT5A polymorphism: Digestion of the 215 bp PCR products with *Eco*88I restriction nuclease revealed two alleles: C and T. Variants of STAT5A gene are the result of point mutation, from CCC to CCT which both encode proline. Allele C is digested at position 181 by the *Eco*88*I* enzyme, while the T allele remains uncut, resulting in three possible genotypes: CC, CT and TT (Fig. 1-right).

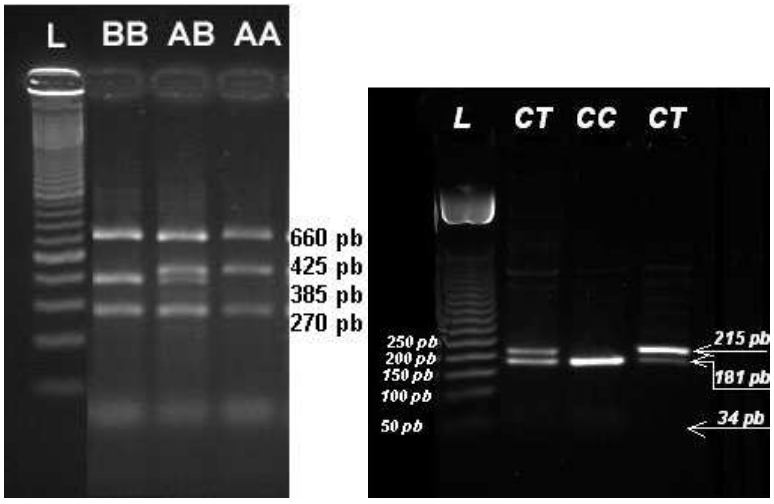

Fig. 1. Partial genomic structure of *Hinf*I polymorphism between exon 5 and 6 of Pit-1 gene, 100bp DNA step ladder -Promega (left) and for 215 bp fragment of STAT5A gene, 7th exon, 50 bp DNA step ladder (Fermentas) (right) on 3% agarose gel

Genetic structure at Pit-1 and STAT5A loci is presented in Table 3. The genotypes AA, AB and BB corresponding to Pit-1 locus were detected as a result of the *Hinf*I restriction enzyme action. Also, three genotypic patterns are produced as a result of *Eco*88I restriction enzyme in 215 bp fragment of STAT5A gene, namely CC, CT and TT.

The allele's frequency for Pit-1/*Hinf*I locus was recorded at the value 0.31 and 0.69 for A and B allele respectively. These alleles generated three patterns, and the calculated genotype frequencies were: 0.096, 0.428 and 0.476 for AA, AB and BB genotypes, respectively. The recorded values for STAT5A/*Eco*88I locus were 0.835 for C allele and 0.165 for T allele; the CC

genotype recording 67%, CT–33% while the TT genotype was not found in the analyzed population. The combined analysis of genotypes showed a superior value of frequencies for ABCC, BBCT and BBCC as compared to AACC and BBCT.

Tab. 3

Frequencies of genotypes, alleles, and combined genotypes in the sample of Romanian Simmental breed for both considered SNPs

Genotype	Genotype frequency	Allele	Allele frequency	Combined genotype	Combined genotype frequency (%)
AA	0.096	A	0.31	AACC	0.095
AB	0.428	B	0.69	AACT	0
BB	0.476			ABCC	0.381
CC	0.67	C	0.835	ABCT	0.048
CT	0.33	T	0.165	BBCC	0.19
TT	0			BBCT	0.286

The expected values of frequencies of the three genotypes calculated according to Hardy-Weinberg equilibrium were 2.018 (AA), 8.98 (AB) and 9,998 (BB). The calculated χ^2 value (χ^2_{calc} 0.2132< χ^2_{tab} (5.99)) indicates the Hardy-Weinberg equilibrium in the population. For STAT5A gene the expected values of genotype frequencies were 14.637 (CC), 5.796 (CT) and 0.567 (TT) and χ^2_{calc} was 0.85, indicating the same significance.

The data included in Table 4 showed the effects of the Pit-1/*Hinf*I polymorphism on milk performance traits on the normal lactation. The average of milk yield on the AB genotypes was higher (4866.67±89.23 kg), by 877.97 kg compared to those of BB genotypes (3988.7±153.14). For the other two parameters tested by the mean differences, the AB genotype was superior to BB genotype for both characteristics: fat and protein content. Very significant differences were found in all three cases for milk yield and for milk parameters ($p<0.001$), and always in the favor of genotype with A allele (AB).

The data recorded for milk production characteristics on the normal lactation related with the genetic structure of STAT5A/*Eco*88I locus are presented in Table 5. As they are presented in Table 5, average of milk yield, fat and protein content show superiority for CC genotype, but the difference was statistically significant (+) only for fat content.

Tab. 4

Mean and standard error of milk production traits in Romanian Simmental population, with different Pit-1 genotype

Genotype	n	Milk (kg)	Fat content (kg)	Protein content (kg)
		$\overline{X} \pm s_e$	$\overline{X} \pm s_e$	$\overline{X} \pm s_e$
AA	2	5203.5±179.16*	200.33±21.4*	243.39±0.59*
AB	9	4866.67±89.23^A	193.89±5.46^A	155.78±4,27^A
BB	10	3988.7±153.14^A	152.70±5.47^A	129.5±4,81^A

Note: The means ^{A}within rows bearing the same superscript differ very significantly at $p<0.001$ (Student test); ^{B}-distinct significant differences $p<0.01$; and ^{C}-insignificant ($p>0.05$).

* Small number of individuals with AA genotype/differences not tested

Tab. 5

Mean and standard error of milk production traits in Romanian Simmental population, with different STAT5A genotype

Genotype	n	Milk (kg)	Fat content (kg)	Protein content (kg)
		$\overline{X} \pm s_e$	$\overline{X} \pm s_e$	$\overline{X} \pm s_e$
CC	14	4723.60 ± 166.24^{C}	188.24 ± 8.17^{B}	151.07 ± 5.67^{C}
CT	7	4122.29 ± 248.10^{C}	157.71 ± 9.17^{B}	133.43 ± 6.87^{C}
TT	0	-	-	-

Note: The means ^{A}within rows bearing the same superscript differ very significantly at $p<0.001$ (Student test); ^{B}-distinct significant differences $p<0.01$; and ^{c}-insignificant ($p>0.05$)

The polymorphism within 6th exon of Pit-1 gene was studied in several cattle breeds (Angus, Holstein-Friesian, Brown Swiss, Hereford, Limousine, Belgian-Blue, Indian zebuine, Polish Black and White, Chinese Holstein etc) and in other Romanian cattle breeds (Maramures Brown, Romanian Black and White and Romanian Grey Steppe breeds) and association among different genotypes and milk yield/quality, and also with different conformation traits, were performed. Regarding gene frequencies Renaville *et al.* (1997a) founded A allele in Belgian Blue breed at 0.53 value and the B allele at 0.47 while in Italian Holstein –Friesian breed the A allele frequency was 0.19 (Renaville *et al.*, 1997b). Woolard *et al.* (1994), Zhao *et al.* (2004), Dybuse *et al.* (2003, 2004) Selvaggi *et al.* (2011) founded the A allele with the frequencies 0.15 in Holstein breed, 0.33 in Angus breed, 0.27 in Limousine and 0.30 in Podolica breed respectively.

The results regarding the frequencies of A allele in other Romanian cattle breeds are 0.16 for Maramures Brown breed (Cosier *et al.*, 2008), 0.1-0.091 for Romanian Black & White breed and 0.25 for Romanian Grey Steppe breed (Carsai *et al.*, 2012).

The results of CC and CT frequencies are in accordance with the data provided by the other authors. Several researchers have recorded such associations between C allele and milk yield. Kmiec *et al.* (2008) have demonstrated the superiority of C allele in increased fat content in milk in a research conducted on 723 Holstein cattle in Poland. The same result was demonstrated by a study conducted by Flisikowski and Zwierzchowski in 2003 on 72 cattle of 5 breeds (Charolaise, Limousine, Red Angus, Hereford and Simmental) and 49 Friesian cattle.

CONCLUSION

In conclusion, in the present work two SNP polymorphisms (Pit1/*Hinf*1 and STAT5A/*Eco*88I) were tested in order to establish the association with milk performance traits (milk yield, fat and protein content) in Romanian Simmental cattle. Both polymorphisms are in relation with important economical traits so the results presented in this paper confirm that these loci are candidate genes that may produce differences in milk characteristics and can be used in marker assisted selection in Romanian Simmental cattle.

The distribution of the genotypes was within Hardy-Weinberg equilibrium in the tested population ($P>0.05$). The superiority of AB genotype animals (Pit-1 locus) was proven in what concerns milk yield, protein and fat content as compared to homozygotes BB in Pit-1 genotypes but only statistically significant (+) and in favor of C allele, only for fat content in STAT5A

locus. The nucleotide substitution C→T at position 6583 of the STAT5A gene, located in exon 7, was identified in Romanian Simmental breed but no TT genotype individual was found in the analyzed sample. For this point of view and to test combined effects associations between genotypes further studies on larger sample should be carried out concerning analyzed loci.

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