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IDENTIFICATION OF STAT 5A GENE POLYMORPHISM IN ROMANIAN BUFFALO

IDENTIFICAREA POLIMORFISMULUI GENEI STAT 5A LA BIVOLIȚELE DIN RASA ROMÂNEASCĂ

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ABSTRACT | REZUMAT

The efficiency of the selection process on quantitative and qualitative traits, which are of economic importance, depends on the identification of the candidate genes that are responsible for these traits, as well as on the determination of DNA polymorphism in these genes. The aim of this research was to genetically characterize the structure of some regions in the STAT5A gene, in Romanian buffaloes in order to use possible polymorphisms identified in studies of association with some quantitative and qualitative features of milk production. PCR amplification of the studied regions was done for a 215 bp fragment, located between intron 6 and exon 7 and for a 740 bp fragment located between exons 8 and 9 of the STAT 5A gene. The 215 bp fragment from buffalo has 100% identity with that from the cow, and the study of the C>T polymorphism, from position 178 (c.958), also identified in cows, was performed by PCR-RFLP / Eco88I technique. For the 215 bp fragment, the C > T mutation determines three possible genotypes: CC (178bp, 34 bp); CT (215, 178 and 37 bp) and TT (215 bp), being identified only homozygous genotypes in the analysed sample (N= 20), and the frequency of the two alleles was 0.7 for the C allele and 0.25 for T allele. The 740 bp fragment was sequenced in both directions and a point mutation was detected, type C>T (c.989 + 344 A) in the intronic fragment, located between exons 8 and 9.

Keywords: STAT 5A, SNP, PCR, Romanian buffalo, milk

Eficiența procesului de selecție privind trăsăturile cantitative și calitative, care au importanță din punct de vedere economic, depinde de identificarea genelor candidate care sunt responsabile de aceste trăsături, precum și de determinarea polimorfismul ADN în aceste gene. Scopul acestei cercetări a fost de a caracteriza din punct de vedere genetic structura unor regiuni din gena STAT5A, la bivolițele din rasa românească în vederea utilizării posibilelor polimorfisme identificate în studii de asociere cu unele trăsături cantitative și calitative ale producției de lapte. Amplificarea PCR a regiunilor luate în studiu s-a făcut pentru un fragment de 215pb, situat între intronul 6 și exonul 7 și pentru un fragment de 740pb situat între exonii 8 și 9 ai genei STAT 5A. Fragmentul de 215 pb de la bubaline are identitate 100% cu cel de la vacă, iar studiul polimorfismului C >T, din poziția 178 (c.958), identificat și la vaci, s-a realizat prin tehnica PCR-RFLP/Eco88I. Pentru fragmentul de 215 pb mutația C>T determină trei genotipuri posibile: CC (178bp, 34 pb); CT (215, 178 și 37 pb) și TT (215 pb), fiind identificate doar genotipuri homozigote în proba analizată (N=20), iar frecvența celor două alele a fost de 0,7 pentru alela C și de 0,25 pentru alela T. Fragmentul de 740 pb a fost secvențiat în ambele direcții fiind detectată o mutație punctiformă, de tipul C >T (c.989+344A) în fragmentul intronic, situat între exonii 8 și 9.

Cuvinte cheie: STAT 5A, SNP, PCR, bivolița românească, lapte

Romanian buffaloes have their origin in the Indian wild buffalo (*Bubalus arni*), respectively in the domestic buffalo also called common buffalo (*Bubalus microceros* or *Bubalus bubalis*) (13). In terms of genetics and ecology, buffaloes belong to the river buffalo, respectively to the common Mediterranean riparian buffalo (12,27). The buffalo breed in our country was approved in 1987 (28), under the name of the Romanian buffalo breed, a native breed from the common European river buffalo, which was introgressed with the Bulgarian Murrah breed in 1960 (3,14). The Romanian buffalo population consists of three different types: Carpathian type, Danube type and common type from the Indian buffalo Murrah, a better adapted population (13). The most numerous Romanian buffaloes are of the Carpathian type, with a valuable genetic resource, well adapted to the cold climate (3). The attestation of the buffaloes on the territory of today's Romania was made on the basis of the written information found in

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the locality of Porumbacul de Jos. These documents refer to the buffaloes from Făgăraș County, and the first selection nucleus was made at Șercaia between 1870-1879 (29).

Statistical data from Romania, recorded in 2018, indicate an average production per country of 17189345 liters / year buffalo milk (16). Statistical data recorded by the Romanian Buffalo Breeders Association in 2019 indicate an average production per country of 1876.89 liters / 305 days buffalo milk, milk collected from 49 farms with a total number of over 1197 heads with a total production of 91968 liters / 305 days (23). Buffalo milk has a complex composition and is characterized by a high share of dry matter and fat, superior to cow's milk (8,11). Buffaloes are an important genetic resource for many parts of the world, especially for the production of mozzarella. Taking into account these aspects, we consider that special attention should be paid to the conservation of this species in Romania, where the numbers have decreased greatly or to give greater importance to the process of improving certain productions, in correlation with market needs. Numerous efforts have been made in recent years to detect new loci that affect quantitative traits (QTL). Uninucleotide mutations, due to their numerous presences in the genome, are the subject of many genetic studies in relation to morphoproductive properties. Some SNPs are located in the coding regions and directly affect protein function. These SNPs, but also those in non-coding regions, may be responsible for variations between individuals regarding some important traits. In addition, inherited SNPs are more stable than microsatellites, making them more suitable in the long-term selection process in high-performance genetic analysis, using DNA microarray technology (2). Detection of gene polymorphisms, functional or not, becomes important in the process of identifying QTL regions that could be used to investigate the role of the STAT 5A gene in relation to variations in milk production traits.

The buffalo genome is not fully saturated in mutations and the role of genetic variations and their association with different morphoproductive traits in different breeds has not been sufficiently investigated, therefore establishing associations between genetic polymorphisms and some production traits would be the basis for selection from the earliest stage of life to achieve high yields and at the same time could be integrated into growth and improvement strategies. For this reason, the main purpose of the paper was to identify causal SNP mutations, involved in the acquisition of milk production in the Romanian buffalo.

Genetics of STAT sites

The STAT gene family consists of seven dual-function members, transcription activators and signallers for the translation of genetic information. The seven transcription factors of the STAT family mediate the action of several hormones inside the target cells, being also called latent cytoplasmic factors. When cytokines adhere to cell surface receptors, Janus-associated kinases (JAKs) are activated and cause phosphorylation of STAT factors. Phosphorylated STAT proteins dimerize and enter the nucleus where they bind transcriptional activators by activating target gene expression (26). STAT 5A transcription factors are known to be involved in activating somatotropic axis members. They initiate the growth process in the target cells, mediated by the growth hormone (GH) of the pituitary gland. In addition, STAT 5A factors regulate protein expression in the epithelial tissue of the mammary gland in response to the action of prolactin (30), being involved in mammary gland growth, lactation, etc., but also in reproductive function (7,15, 20). For these reasons, genes encoding transcription and signalling the translation of genetic information, especially STAT factor 5A, have been considered candidate genes for the characteristics of bovine milk production. In cows, the gene encoding STAT 5A was located on chromosome 19q17 between STAT 40KPZ loci that also contain the STAT3 and STAT 5B genes (21,25). In buffaloes (*Bubalus bubalis*) the gene encoding STAT factor 5A is located on chromosome 3, with 97% identity to the *Bos taurus* gene sequence (NC_037346.1). The unique functions of STAT 5A factors occur in distinct and varied systems such as mammary gland cells, hepatocytes and T cells, although recent experimental studies show that they are expressed in many target cells from: colon, heart, skeletal muscle, hypothalamus, etc. (https://www.ebi.ac.uk/gxa/home), and are involved in a wide variety of biological functions. The STAT 5 gene is encoded by two closely related sequences, STAT 5A and STAT 5B (1,22). These proteins have over 90% identity and differ slightly only at the level of the carboxyl (C)-terminus. Both STAT 5 proteins are ubiquitously expressed, but the expression profiles are different. STAT 5A is the predominant form in the mammary gland, while STAT 5B is expressed more prominently in the liver. The 7 STAT proteins in mammals are between 750 and 850 amino acids in structure.

MATERIALS AND METHODS

The biological material used in this research is represented by a number of 25 DNA samples from Romanian buffaloes from the Buffalo Breeding Development Research Station (S.C.D.C.B. Sercaia), Brasov, Romania. Throughout the research, the buffaloes studied were monitored for shelter, hygiene, feeding and watering. Particular attention was paid to the factors that ensure well-being. To identify the polymorphism

of the STAT 5A gene and its influence on productive traits, blood samples were collected on K_2 EDTA. The genomic DNA was extracted from buffalo's blood samples during lactation. All samples were PCR amplified with specific primers, for PCR-RFLP/Eco88I analysis obtaining a fragment of 215 bp and for sequencing a fragment of 740bp. To identify mutations in the 740bp fragment samples were prepared appropriately for sequencing in both directions (Macrogen, The Netherlands). DNA extraction was performed from 200 µl blood using a Quick-DNA-Miniprei Plus Kit, according to the manufacturer's instructions (BioZyme, St Joseph, Missouri, USA). The readings of the purity and the amount of DNA were determined with the Nanodrop ND 1000 spectrophotometer in UV light, the values being between 1.64 and 2 respectively 53 and 199 ng/µl.

Polymorphism studies

Due to the 100% identity of the 215bp sequence from cow to buffalo, in the present study we investigate this polymorphism, associated with the features of milk production and the Romanian buffalo. The C>T polymorphism (c.989 + 344A) located in the ineterexonic region 8-9, was first identified in the Italian buffalo (4) as being associated with a higher percentage of protein in milk. In our study, we aimed to identify all possible polymorphisms of this fragment in the Romanian buffalo.

Detection of point polymorphism (SNP) from the 215bp fragment

Detection of the point polymorphism (SNP) 178/ Eco88I from the 215 bp fragment was performed by PCR amplification with specific primers (Table 1) and enzymatic restriction. The total volume of the mixture was 25 µl, using the 5xFirePol Ready to load (Biozyme, St Joseph, Missouri, USA) kit (5 µl), 1 µl primer (10 pmol, each forward and reverse), 16 µl ultrapure H_2O and 2 µl DNA (with medium concentration of 50 ng/ µl). The samples were amplified with the following program: initial denaturation at 94°C for 5 minutes, followed by 32 cycles at 98°C– 20 seconds, 15 seconds at 65°C and 20 seconds at 72°C. The final extension was 8 minutes at a temperature of 72°C, in an Eppendorf Termocycler. The enzyme restriction protocol included: 15 µl PCR product, 1 µl Eco88I enzyme (Thermo Fischer, Waltham, Massachusetts, United States), 2.5 µl buffer and 6.5 µl ultrapure water, in a final volume of 25 µl. The samples were incubated at 37°C for 5 hours and migrated in agarose gel of 2.5% concentration.

Characterization of SNP polymorphisms from the 740 bp fragment

The 740bp fragment of the STAT 5A gene corresponds to exon sequences 8 and 9 and the corresponding intronic sequence. To identify polymorphism and other possible mutations in the fragment, the sequence was amplified by PCR and sequenced in both directions in five individuals. The identification of the alignment temperatures used can be found in Table 1.

Amplification of the 740bp fragment of the STAT 5A gene for sequencing

PCR amplification was performed in order to sequence the 740bp fragment, located between exons 8 and 9 of the STAT 5A gene with primers specific to each fragment to highlight a point mutation.

The working protocol for amplifying the 740bp fragment contains a final volume (25 µl): 2xKaPaHiFi MasterMix (Zy-moResearch) 12.5µl, 5 pmol of each primer (0.5 µl) 9 µl ultrapure H_2O water, and 2 µl DNA (50 ng/ml). Thermal cycles include: an initial denaturation cycle at 95°C for 5 minutes, followed by 1x 98°C – 20 seconds, 15 seconds at 65°C and 60 seconds at 72°C, repeated 35 times. The final extension was 8 minutes at a temperature of 72°C in an Eppendorf Termocycler.

After amplification, the samples were purified with the MEGAquick-spinTM Plus Total Fragment DNA Purification kit, according to the manufacturer's instructions (iNtRON Biotechnology) and migrated in 1% agarose gel to verify amplification,concentration, DNA integrity and sent for sequencing (Macrogen, The Netherlands). The DNA strands were sequenced in both directions, with the same primers used to obtain PCR products.

RESULTS AND DISCUSSIONS

The result of the PCR-RFLP/Eco88I analysis revealed the presence of two homozygous genotypes in the analysed sample (N = 20): CC (178 and 37 bp bp) and TT (215bp) whose frequency was 0.75 (CC), respectively 0.25 (TT) (Fig. 1).

Table 1

Amplified regions and sequences of primers used

Gene	Amplified region	Sense primer 5'	Antisense primer 3'	PCR product	Annealing temperature
STAT 5A	Intron-Exon 6-7	CTGCAGGGCTGTTCTGAGAG	TGGTACCAGGACTGTAGCACAT	215bp	65C
	Exons 8–9	GTGTGAGAAGTTGGCGGAGA	TTGCGGGTGTTCTCGTTCT	740bp	65C

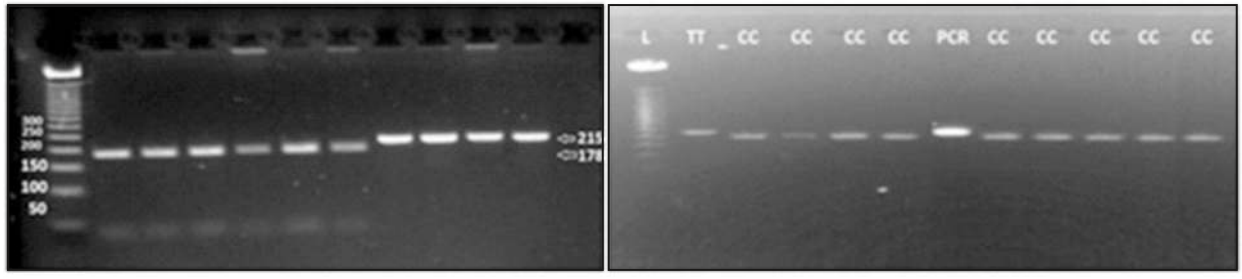

Fig. 1. Electrophoretic profiles of PCR-RFLP/Eco88I analysis (line 1- 50 BP DNA Ladder, lines: 2-11 (left) and 2-6; 8-12 (right) - CC and TT genotypes; line 7 (right- PCR product, 215 bp)

The results of the sequencing analysis

The obtained sequences were processed using Geneious 4.8.5 (Biomatters, New Zealand). First, the forward sequences were compared and assembled with the corresponding antisense sequences, thus obtaining the consensus sequences, used further in the analysis. Consensus sequences were compared with other sequences of the same gene, existing in the Gen Bank® database, using the Basic Local Alignment Search Tool (BLAST) algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The results of the BLAST analysis revealed a similarity of over 99% (99.86%- 99.30 %) with various isolated sequences of the same gene-STAT 5A from buffaloes (References GeneBank: Kj46 1010, KJ461013, KJ461005, KJ461009, KJ461007).

The evaluation of the polymorphisms was performed by aligning all the sequences obtained in this study and the GenBank® reference sequence, with the CLUSTAL Omega function (https://www.ebi.ac.uk/). The 740bp sequence contains the mutation in the intronic region, located between the two exons (8 and 9). The identification of SNPs was performed based on the genomic sequence from *Bubalus bubalis* breed Mediterranean (>NC_037547.1), by aligning the sense sequences of the samples: 8, 11, 19, 24 and 26 (Fig. 2).

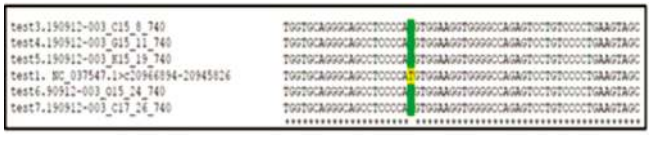

Fig. 2. Alignment of the nucleotide sequences of the 740bp fragment from samples 8,11,19,24 and 26 from the Romanian buffalo and highlighting the C>T mutation

The few international researches in the complete STAT 5A gene sequence reveal an 8-9 interexone mutation in which the C allele of the STAT 5A gene is associated with the properties of milk production, respectively, with the increased percentage of milk protein. In our study, a point mutation was detected, of type C (cytosine) – T (thymine), located in position c.989 + 344A from intron 8-9 of the gene (Fig.3).

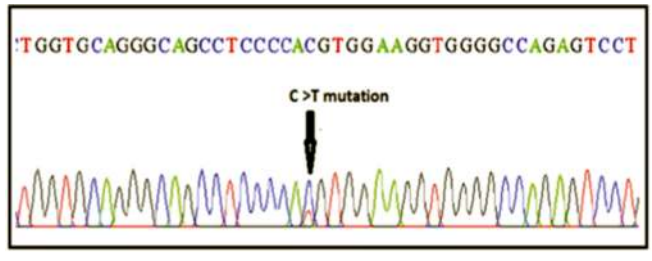

Fig. 3. The C > T mutation, position int 8-9 (c.989+344A) in the STAT5A gene in Romanian buffalo

To identify point mutations, which characterize places of interest and to establish existing polymorphisms that may explain the differences in the lactogenic capacity of the mammary gland in the Romanian buffalo, the sequences of homologous genes were compared comparatively. At the STAT5A locus, the C > T mutation (c.989 + 344A) in the 740bp fragment was associated with a higher amount of milk protein in TT homozygotes (4). In the study performed by us, out of the 5 analyzed samples, only one individual presented the heterozygous CT genotype at the analyzed locus (19), the other four individuals being homozygous CC (8, 11, 24 and 26). For genotyping at the STAT 5A locus, a 215 bp fragment from exon 7 was amplified, a 740 bp fragment located in exon 8-9, where a cytosine / thymine (C / T) substitution was located that characterizes two alleles at this locus C, respectively T. At the locus STAT 5A / fragment 215bp from intron 6-exon 7, the effect of the C allele was determined as favourable, being associated with the characteristics of milk production in cows. The improvement of buffalo populations in order to increase milk production is based on the continuous improvement of favourable allele combinations in the genome of individuals through the selection process. Genetic polymorphisms of the STAT 5A gene in exon 7 have been associated with meat production traits (9), growth performance traits (6) and milk production traits in dairy cows (24).The STAT 5A gene is frequently used as a candidate gene in the study of SNP polymorphisms, so different genomic regions have been identified as active in terms of milk production characteristics in several lactating cattle

breeds (18). Associations between phenotypic traits and different alleles of the STAT 5A gene in animal populations have been the subject of many scientific studies (19). The STAT 5A gene was studied in several European cattle breeds, including Romania but also in the Holstein breed in China where the frequencies of CC, CT and TT genotypes were: 0.79, 0.21, and 0.0 for this gene in Chinese cattle. The frequency of C allele, obtained from the Romanian Simmental breed, was 0.881 (5), slightly lower than that found by Flisikowski et Zwierkowski (2003b) in Red Angus (0.95), Charolaise (0.86), Limousine (0.87) and Simmental breed (0.81), in which no individuals with TT genotype were found either (10). Other studies on STAT 5A genes have shown significant effects on milk fat content and milk production traits in Holstein cattle breeds in Poland and the USA (17), therefore the role of this gene remains one important in studies regarding morpho-productive properties in different species of economic interest.

CONCLUSIONS

Detection of SNP-type gene polymorphisms was performed by PCR-RFLP technique and sequencing to help investigate the role of the STAT 5A gene in relation to variations in milk production traits, which we set out to continue. The results obtained in this research, regarding the existence of polymorphisms within the STAT 5A gene and the sequence differences recorded compared to the genomic reference sequence NC_037547.1 from *Bubalus bubalis* Mediterranean breed, reveal the importance of further studying the STAT 5A locus by establishing associations between the two detected polymorphs (C>T position c.958 and c.989 + 344A) in the STAT 5A gene and milk production properties such as: quantity of milk, percentage of fat and milk protein in the Romanian buffalo breed. The existence of specific SNP mutations, which could be used as genetic markers, remains a desideratum of molecular genetics research in the Romanian buffalo, little characterized from a genetic point of view until now.

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