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POLYMORPHISM IDENTIFICATION OF *FABP3* GENE IN SHEEP OF BULGARIAN DAIRY SYNTHETIC POPULATION

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Abstract

This experiment was conducted in order to be identified the allelic and genotypic polymorphisms of FABP3 (heart-type fatty acid binding protein) gene in 30 ewes from Bulgarian Dairy Synthetic Population breed reared in Experimental base - Tzarev Brod - part of the Agricultural Institute - Shumen. FABP3 gene is a candidate marker that influences milk fat content and marbling of meat. Thirty blood samples were collected from v. jugularis in vacuum tubes with EDTA. Genomic DNA was extracted manually with commercial kit. By means of PCR-RFLP technique with endonuclease BseDI in exon 2 of FABP3 gene (SNP3) were determined the allele and genotype variants of the investigated animals. In this population were observed two alleles - wild allele A with frequency 0.15 and mutant allele G - with 0.85. Two different genotypes were identified - homozygous GG with frequency 0.67 and heterozygous genotype AG with frequency 0.33. Ho (observed heterozygosity) was 0.330 and He (expected heterozygosity) was 0.255. This herd was found to be in Hardy-Weinberg equilibrium (p>0.05).

Key words*: FABP3 gene, PCR-RFLP method, polymorphism, sheep.*

INTRODUCTION

In recent years, consumers have paid increasing attention to healthy eating and food quality. In this regard, due to the increased demands of consumers of animal products, the efforts of breeders are aimed at improving the quality of sheep meat and milk through the rapid development of breeding programs in this direction.

The tender and marbled meat is preferable due to its better taste, and the high fat content of milk is a suitable raw material for cheese production. Therefore, researchers seek to identify the main genes affecting economically important traits as initial and decisive steps to develop genetic markers associated with different productive characteristics that will allow the selection of animals producing products of better quality - more tender marble meat, milk with higher fat and protein content, etc. (Deykin et al., 2016; Clark et al., 2017; Xu et al, 2017; Mohammadi et al., 2020).

Many genes affect the milk and meat productivity of sheep. Nowadays, diverse molecular genetic markers are increasingly used in sheep breeding programs in order to improve the quality of milk and meat (Selvaggi et al., 2014; Eer et al., 2020).

Fatty acid binding proteins (FABPs) form a small family of low molecular weight cytoplasmic proteins that have a high binding capacity for long chain fatty acids. FABPs play a crucial role in hormone action and cellular functions in adipocytes and other cells. They are essential mainly in the storage, transport and metabolism of lipids in the cell and are therefore the subject of research. These small proteins coordinate the transport of fatty acids from the plasma membrane to the sites of oxidation, triacylglycerol and phospholipid synthesis (Chmurzynska, 2006; Jurie et al., 2007; Furuhashi & Hotamisligil, 2008; Eer et al., 2020). They accelerate the absorption of long-chain fatty acids and delivering fatty acids to intracellular organelles (Lanier & Corl, 2015). Fatty acid-binding proteins are small intracellular proteins having a molecular size of 14 to 16 kDa with 126 to 134 amino acids (Lang et al., 2017). They are found in all animal

species and are involved in their active lipid metabolism (Damcott et al., 2004; Michal et al., 2006; Kulig et al., 2010; Cho et al., 2011; Wang et al., 2015; Wang et al., 2016; El-Mansy et al., 2019).

In sheep, one reliable candidate gene for meat quality and milk fat content, member of this family is FABP3, also known as H-FABP or HFABP, which is associated with cardiac activity and whose molecular weight is 15 kDa (Calvo et al., 2002). According to studies of Lang et al. (2017) in Oula sheep, the H-FABP gene and its expression in muscle tissue are associated with the content of intramuscular fat (IMF) in meat. FABP3 is present in many tissues with a high need for fatty acids such as the heart muscle, skeletal muscle and mammary gland during lactation (Calvo et al., 2002; Jurie et al., 2007). Some studies on the effect of the fatty acid-binding protein 3 gene on different productive traits in sheep have shown the influence of different genotypes on fatty acid metabolism in both muscle and milk and is linked to muscle development, milk fat content and meat marbling (Calvo et al., 2004; Aurora et al., 2014).

The *FABP3* gene was mapped in the distal part of chromosome 2 of *Ovis aries* genome and it consists of four exons according to Calvo et al. (2002) or five according NCBI (2021) which are separated by introns. Thirteen single-nucleotide polymorphisms (SNPs) were identified on sheep *FABP3* gene from Calvo and co-authors (2002) in samples of animals from Manchega breed. Two of SNPs were located in exon 2 and intron 3, respectively named SNP3 (G/A) and SNP13 (G/A) have codominant segregation of the polymorphisms and are suitable to be studied in connection with certain productive qualities. The expression of the *FABP3* gene had a positive effect on the intramuscular fat content in different muscles of sheep (Xu et al., 2020).

In Bulgaria, sheep breeding has a thousand-year tradition and despite the fact that the number of sheep has decreased significantly in the last two decades both in sheep population and the total number of sheep farms, sheep production is essential for the country's economy (Boykovski et al., 2008; Sabkov et al., 2017; Annual Agricultural Reports, 2021). In Bulgaria are bred around 34 breeds of sheep, as 88.4% of the available ewes are dairy, and 70% of them are from the Bulgarian Dairy Synthetic Population. In the case of dairy breeds, the income is generated from the sale of lambs and milk, that is why the breed is a very valuable source for both meat and dairy industries (Slavova et al., 2015; Krastanov et al., 2018; Annual Agricultural Reports, 2021). In this regard, it is interestingly to establish the level of genetic polymorphism in certain productivity-related genes including *FABP3* in farmed sheep breeds. The objective of our study was to determine allele and genotype frequencies of SNP3 of *FABP3* gene in 30 ewes from Bulgarian Dairy Synthetic Population sheep breed.

MATERIALS AND METHODS

Animals

In present investigation were tested 30 animals from Bulgarian Dairy Synthetic Population sheep breed reared in Experimental base in Tzarev Brod - part of the Agricultural Institute - Shumen. The samples of peripheral blood of each individual were collected from the jugular vein into tubes containing EDTA as an anticoagulant factor. The probes were stored at -20°C until DNA extraction process.

DNA extraction

The experimental work was carried out in the Laboratory of Genetics, University of Forestry, Sofia, Bulgaria. Genomic DNA was extracted from whole blood using a manual commercial kit for DNA purification according to the manufacturer's instruction (Illustra Blood GenomicPrep DNA Purification Kit, GE Healthcare, US). The DNA concentration of each sample was determined by spectrophotometer Biodrop. The criteria of DNA quality control were DNA concentration must be between 10 to 50 ng/µL. The quality of the obtained DNA was tested also on 1% agarose (Bioline, UK) gel prepared with TAE buffer (Jena Bioscience, Germany).

PCR amplification

The polymerase chain reaction amplifications were carried out in total volumes of 10 µl, containing 4 µl DNA template, 0.2 µl sterile water, 0.4 µl of each primer and 5 µl of 2 × (1.5 mM $MgCl_2$) MyTaq TM HS Red Mix 2x (Bioline, UK). The primer set used in this experiment was suggested by Calvo et al., (2004):

F: 5′-GGTTTTGCTACCAGGCAGGT-3′ and
R: 5′-TTCCCTATTCCCCTTCAGGG-3’.
Amplification reactions were accomplished by thermal cycler QB-96 (Quanta Biotech) under the following conditions: primary denaturation at 94ºC for 2 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 56ºC for 30 s, elongation at 72ºC for 1 min. The reaction was completed by final extension at 72ºC for 10 min.

RFLP analysis
The genotypes of all tested animals from Bulgarian Dairy Synthetic Population were determined using restriction fragment length polymorphism analysis (RFLP). All amplify-cation products of the *FABP3* gene fragment (exon 2 - 222 bp) were digested separately in 10 µl final volume, containing 6 µl PCR product, 2.5 µl dd H_2O, 10 U/µl restriction enzyme *BseDI* (Thermo, US) and 1 µl enzyme buffer. The digestion reactions were carried out at 60˚C for 3 h in thermal block. The fragment sizes were identified using Ready-to-Use DNA Ladder, 50 bp (Thermo, US) on 2.5 % agarose (Bioline) gel and stained by RedGel Nucleic Acid Stain (Bioline, UK). The obtained PCR products and restriction fragments were visualized under UV light.

Statistical Analysis
In the present study, the allelic and the genotypic frequencies of *FABP3* gene were estimated using simple gene counting method (Falconer and Mackay, 1996). The expected and the observed genotypic frequencies were compared using χ^2 test. The population was found to be consistent with the Hardy-Weinberg equilibrium so the value of p was > 0.05.

RESULTS AND DISCUSSIONS

After DNA extraction of received blood samples were obtained 30 specimens of genomic DNA with concentration from 10 ng/µl to 50 ng/µl. All samples were equalized to concentration of 10 mg/µl with TE buffer and tested through 1% agarose gel electrophoresis (Figure 1).
By means of PCR technique were amplified fragments with expected length of 222 bp from exon 2 of *FABP3* locus, included SNP3. The received from amplification products from Bulgarian Dairy Synthetic Population ewes were tested on 2% agarose gel electrophoresis and visualized under UV light (Figure 2).

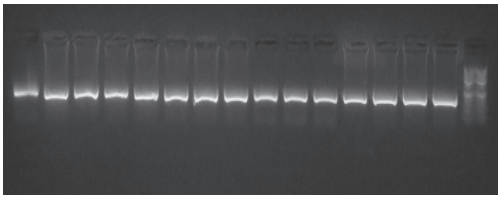

Figure 1. Extracted DNA samples tested on 1% agarose gel electrophoresis

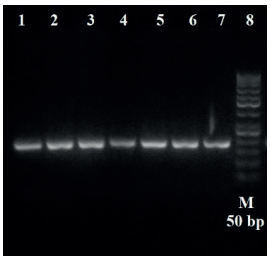

Figure 2. PCR products of *FABP3* gene tested on 2% agarose gel electrophoresis

All samples were digested with *BseDI* restriction enzyme. Two alleles *G* and *A* were detected. The digestion of the PCR products of three different fragments (143, 43 and 36 bp) produced mutant allele *G* and the digestion of the amplification products of two fragments (186 and 36 bp) produced wild allele *A* (Figure 3).

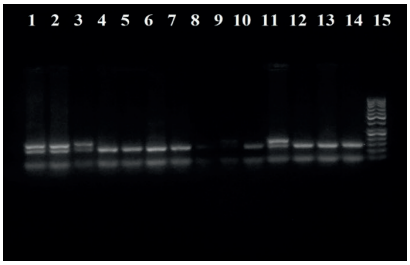

Figure 3. Restriction fragments of *FABP3* gene visualized on 2.5 % agarose gel under UV light. Lane 1, 2, 3, 9, 11 heterozygous genotype *AG*; lane 4, 5, 6, 7, 8, 10, 12, 13, 14 - homozygous genotype *GG*; lane 15 - DNA marker 50 bp

In the investigated Bulgarian Dairy Synthetic Population ewes from herd of the Agricultural Institute - Shumen were identified both possible alleles mutant *G* and wild *A* with frequency 0.85 and 0.15, respectively. Two different genotypes

were identified in SNP3 of fatty acid-binding protein 3 gene - homozygous genotype *GG* with frequency 0.67 and heterozygous genotype *AG* with frequency 0.33. Observed heterozygosity (H_o) was 0.330 and expected heterozygosity (H_e) was 0.255. The variations of alleles and genotypes of *SNP3* of *FABP3* gene were presented in Table 1. This herd was found to be in Hardy-Weinberg equilibrium.

In previous our study of animals from the same breed Bulgarian Dairy Synthetic Population but from herd of Institute of Animal Science - Kostinbrod the allelic frequency was the same. It was obtained also two genotypes - *GG* - with frequency 0.70 and *AG* – with 0.30.

The presence in SNP3 of the fatty acid binding protein 3 gene of the *AG* genotype was associated with increased litter size, while the presence of the *GG* genotype showed a tendency with increased lactation productivity (Dimitrova et al., 2021).

Table 1. Allele and genotype frequencies of *SNP3* of *FABP3* gene

Breed	n	Allele frequencies		Genotype frequencies			Ho	He	Fis	p
		G	A	GG	AG	AA				
SPBM	30	0.85	0.15	0.67	0.33	0.00	0.330	0.255	0.870	ns

The results reported in this experiment were in agreement with other study of the SNP3 of the Fatty acid-binding protein 3 gene in different breeds. It was study five Bulgarian breeds - three fine fleece (Ascanian, Caucasian and Karnobat) and two local (Cooper-Red Shumen and Karakachanian) breeds. The frequency of mutant allele G and homozygous genotype GG was also higher in all breeds, but in all these five breeds all three genotypes have been identified. Research team established that of allele *G* varied between 0.77 to 0.87 and genotype frequency for genotype *GG* - differed from 0.57 to 0.80. The frequency of allele *A* ranges from 0.13 to 0.23, with the lowest frequency observed in the homozygous genotype *AA* - from 0.03 to 0.07, and the heterozygous genotype is in the range from 0.13 to 0.40 (Dimitrova et al., 2020).

The research of Calvo et al. (2002) show that in the predecessor of European sheep breeds - mouflon, only allele A was found in *SNP3* of the fatty acid binding protein 3 gene. The *G* allele was also found in domestic sheep, and it is predominant in the majority of the studied breeds. The frequency of allele *A* in domestic sheep breeds varies between 0.26 in Raza Aragonesa to 0.33 in Awasi, 0.38 in Assaf, 0.39-0.46 in Manchega (Calvo et al., 2002; Calvo et al., 2004).

The results in this study essential differ from the results reported in 100 investigated animals from three populations of Turkish sheep breed Kıvırcık (Oner et al., 2014). It was established genetic diversity in the same region of the *FABP3* gene - SNP3, with frequency of allele *A* was 0.42 and of the allele *G* - 0.58. In contrast to the present study in this Turkish breed was observed homozygous genotype *AA* with frequency 0.30, while the other two genotypes - *GG* (0.46) and *AG* (0.24) were lower in frequency than we found.

Other research team studied 10 different SNPs in *FABP3* gene in 250 Tan sheep and 174 Hu sheep. All of them are located in sequences related to the qualities of meat as H-FABP is considered one of the main genes that affect the content of intramuscular fat and is an important gene that affects slaughter traits and controls meat quality (Huang et al., 2006; Eer et al., 2020).

PCR-RFLP method was used also to detected polymorphism in two regions of fatty acid binding protein 3 gene - *SNP3* and *SNP13* in 50 sheep of the Slovak breed Zošľachtená Valaška (Kowalewska-Łuczak et al., 2017). They found only heterozygous genotypes in *SNP3*, while polymorphism was found in *SNP13*. Study was aimed to determine the prevalence of alleles and genotypes in relation to the SNP polymorphisms in *FABP3* locus and to determine possible relationships between genotypes and qualitative characteristics of sheep's milk. The scientific team reported that animals with the homozygous *AA* genotype had the highest content of fat, protein and solids in the milk of tested sheep. The sheep with the heterozygous genotype *AG* demonstrated the highest content of solids and urea in milk.

The establishment and inclusion of molecular genetic markers in breeding programs in order to improve the quality of meat and milk, as well as their composition is gradually becoming a

future goal in sheep breeding (Selvaggi et al. 2014; Kowalewska-Łuczak et al., 2017). The established polymorphism in *SNP3* of the fatty acid-binding protein 3 gene indicated that the study of this locus in Bulgarian Dairy Synthetic Population sheep should be studied more detailed. It is necessary to extend both the number of the tested animals and the studied area of the gene to determine the relationship to specific productive characteristics before its implementation in sheep breeding programs.

CONCLUSIONS

The results obtained in this study show that sheep fatty acid-binding protein 3 is a polymorphic gene. Genetic diversity was detected with presence of two alleles (wild *A* and mutant *G*) and only two genotypes (*GG* and *AG*). Higher frequency was observed in the homozygous genotype *GG*.

There is no difference in allelic and genotypic frequencies between the animals of the two herds of the breed Bulgarian Dairy Synthetic Population in *SNP3* of the *FABP3* gene.

The established genetic diversity in *SNP3* of *FABP3* gene in 30 animals from Bulgarian Dairy Synthetic Population sheep breed indicated that after additional research this gene could be suitable for implementing in breeding programs for improving milk productivity in sheep.

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