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POLYMORPHISM IN SNP G1 OF THE GDF9 GENE IN RAMS FROM TWO BULGARIAN SHEEP BREEDS

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Abstract

Fertility is of great importance for the profitability of sheep farming as its traits are under the genetic control of several key genes known as fertility genes (Fec) among which is GDF9 (Growth Differentiation Factor 9). The aim of this study is to determine the presence or absence of polymorphism in SNP G1 of the GDF9 gene in rams of two Bulgarian breeds with different productive direction. 43 rams from the Bulgarian dairy synthetic population (breed for milk, with good fertility) and 44 rams from the North-East Bulgarian Merino breed (for wool and meat, with lower fertility) were included in the study. Using the PCR-RFLP method, 462 bp fragments of exon 1 of the gene were amplified and with subsequent treatment with the restriction enzyme HhaI, two alleles were identified in both breeds - wild G and mutant A. All three possible genotypes - GG, AG and AA - were found in rams from the more fertile BDSP breed, while only GG and AG were found in rams from the less fertile NEBM breed.

Key words*: Bulgarian Dairy Synthetic Population (BDSP), GDF9 gene, North-East Bulgarian Merino breed (NEBM), rams, SNP G1.*

INTRODUCTION

In sheep farming, fertility is a key reproductive trait with fundamental importance for the profitability of livestock production. For this reason, various strategies are applied to increase it, such as the ram effect and the use of exogenous hormones, but the former is not effective enough, and the latter, although easy and practical, has recently raised concerns about their impact on human health and the environmental footprint (Symeon et al., 2024). While such interventions offer short-term solutions, genetic selection offers a more sustainable and long-term strategy for improving fertility. In this regard, it is safest and most expedient to select and distribute appropriate hereditary variants that will ensure a long-term effect on this indicator.

Fertility traits have high economic value (Notter, 2008) and are under the genetic control of several major genes known as fecundity genes (Fec) (Abdoli et al., 2019) which exhibit moderate level of heritability (Alkass et al., 2021; Al-Thuwaini & Al-Hadi, 2022; Sahib et al., 2024). Their genetic improvement within existing animals can be achieved either by selection within the breed (which is a very slow process) or by crossing local breeds with exotic breeds that grow quickly and have a higher production capacity in the next generation (Shenkute et al., 2020). The authors report differences in fertility between rams within a breed and between rams of the same age between breeds. Overall fertility levels in rams are a culmination of both genetic and non-genetic factors (Petrovic et al., 2012; Ajafar et al., 2022).

The main genes influencing fertility in sheep are BMP-15, BMPR-1B, GDF9, B4GALNT2 and others. The first three of them are part of the transforming growth factor β (TGF-β) gene superfamily, which affect fertility in both ewes and rams. Genetic variations in members of the transforming growth factor-beta (TGF-beta) superfamily of proteins have been widely

documented in sheep, with significant differences between breeds and extensive variations within breeds (Mohammadi, 2016; Tong et al., 2020), which significantly affect, for example, the rate of ovulation in sheep.

The GDF9 gene, which is considered one of the main candidate genes for ovine fertility, influences the growth and differentiation of follicles and germ cells, as well as the secretion of reproductive hormones (Belli et al., 2018). The ovine GDF9 gene is located on chromosome 5 and contains two exons and one intron, spanning 1126 base pairs (bp), and the mature peptide of the gene consists of 135 amino acids (Hanranhan et al., 2004). Initially, the action of GDF9 was only associated with female fertility, but Fitzpatrick et al. (1998) reported its expression in tissues outside the ovaries, including the testes, brain, pituitary gland and bone marrow. Growth differentiation factor 9 (GDF9) is strongly associated with the cell cycle transition from G_0/G_1 to S and G_2/M phases and regulates the growth and development of testicular germ and somatic cells. GDF9 was detected in 8 tissues of rams, with the highest expression of GDF9 found in the epididymis, confirming that the gene is related to epididymal function, playing a supporting role in spermatogenesis, with the high fertility of a given ram likely due to high levels of GDF9 gene expression in it (Chen et al., 2020). Studies in Tibetan rams have shown the presence of GDF9 gene expression in spermatogonia and Leydig cells in the testes (Wang et al., 2022).

El Fiky et al. (2017) investigated the presence of a polymorphism in exon 1 of the GDF9 gene using DNA sequencing and PCR-restriction fragment length polymorphism (RFLP) methods in Egyptian sheep breeds, which can serve as a marker for crossbreeding and are useful in breeding to design selection programs for genetic improvement. Eight different point mutations (G1–G8) were identified in the GDF9 gene by Hanranhan et al. (2002; 2004) that increase the rate of ovulation in heterozygous individuals, but in two of the four mutations identified, follicle development is impaired in homozygous individuals and as a result, carriers are infertile. In the study of ewes in China, the distribution of genotypes in the GDF9 gene showed a significant difference between a sheep breed with low fertility (Tan - with seasonal estrus and single birth) and three other sheep breeds with high productivity (Small-tailed Han sheep, Hu sheep and Wadi sheep are excellent native breeds in China for their significant characteristics of year-round estrus and hyper-prolificacy) (Wang et al., 2024).

To date, we have studied the allele frequencies in SNP G1 of the GDF9 gene in ewes from different herds of Bulgarian sheep breeds (Bozhilova-Sakova & Dimitrova, 2021; Dimitrova et al., 2024), but it is also of interest to establish the frequencies of allelic variants in rams, which, considering the application of artificial insemination, affect the genetic diversity in the next generation even more significantly.

Bulgarian Dairy Synthetic Population and North-East Bulgarian Merino breed are two Bulgarian breeds of sheep (Ovis aries) from different productive directions – the first for milk, and the second for meat and wool, both with a seasonal estrus regime. A difference is observed between the two breeds of sheep in their fertility, with the first one being within 150% (Stancheva et al., 2014), and the second one – around 115% (Stancheva et al., 2020).

The aim of this study was to determine the level of polymorphism in SNP G1 of the GDF9 gene in rams from two Bulgarian breeds with different productive direction and fertility: the Bulgarian dairy synthetic population (breed for milk, with good fertility) and the North-East Bulgarian Merino breed (for wool and meat, with lower fertility).

MATERIALS AND METHODS

Animals

The study included 43 rams from the Bulgarian Dairy Synthetic Population (BDSP) (a milk breed with good fertility) from the herd of the Agricultural Institute - Shumen (Figure 1), and 44 rams from the North-East Bulgarian Merino breed (NEBM) (a wool and meat breed with lower fertility) from the herd of the Scientific Agriculture Center - Targovishte (Figure 2).

Figure 1. Rams from BDSP of the herd of the Agricultural Institute - Shumen (own source)

Blood samples were collected from *v. jugularis* in 3 ml vacuum tubes with added EDTA as an anticoagulant and transported to the laboratory. The survey was fulfilled in the Laboratory of Genetics of Agronomy Faculty, the University of Forestry - Sofia.

Figure 2. Rams from NEBM of the herd of the Scientific Agriculture Center - Targovishte (own source)

Extraction of genomic DNA

The blood samples were stored at –20°C until DNA extraction. DNA was isolated from stored whole blood samples using manual commercial kit for DNA purification according to the manufacturer's instruction (Illustra Blood GenomicPrep DNA Purification Kit, *GE Healthcare*). The concentration and purity of each DNA sample was measured with a Biodrop spectrophotometer. The quality of about 10-50 ng DNA obtained was tested using a 1% agarose (Bioline) gel prepared with TBE buffer (Jena Bioscience).

Polymerase chain reaction (PCR) condition

DNA analysis was performed according to the methodology for identifying polymorphisms in SNP G1 of the GDF9 gene proposed by Hanrahan et al. (2004) in which a pair of primers was used for PCR amplification as follows:
F:5'-GAAGACTGGTATGGGGAAATG-3' and
R: 5'-CCAATCTGCTCCTACACCCT-3'.
PCR reactions consist of 1 cycle of pre-denaturation at 94°C for 5 minutes, followed by 30 cycles: denaturation at 94°C for 1 minute, annealing at 63°C for 45 s, extension at 72°C for 1 minute. Finally, the process is terminated by waiting at 72°C for 10 min and storage at 10°C.

Restriction fragment length polymorphism (RFLP) and gel electrophoresis

Subsequent processing was with the restriction enzyme *Hhal* (Thermo Fisher) recognizing the site 5'GCG↓C3'
3'C↑GCG5' in the amplified fragments of the GDF9 gene. The resulting restriction products were run through electrophoresis in 2.5 % agarose gel stained by fluorescent dye GelRed (Biotium) and visualized under UV light.

RESULTS AND DISCUSSIONS

PCR amplified fragments of 462 bp from exon 1 of the GDF9 gene. Two alleles were identified in both breeds - the wild G (with fragments of 254 bp, 156 bp and 52 bp) and the mutant A (with fragments of 410 bp and 52 bp) with frequencies of 0.8 and 0.2 in rams of the BDSP breed, respectively, and with frequencies of 0.94 and 0.06 in those of the NEBM breed, respectively. In rams of the more fertile BDSP breed, all three possible genotypes were identified - GG, AG and AA with frequencies of 0.65, 0.30 and 0.05, respectively. However, in rams of the lower fertile NEBM breed, only GG and AG were identified with frequencies of 0.89 and 0.11, respectively (Table 1).

Table 1. Allele and genotype frequencies, average heterozygosity (observed Ho, expected He) and coefficient of inbreeding (Fis) of SNP G1 of GDF9 gene

Breed	n	Allele frequency		Genotype frequency			Heterozygousity		Fis	χ2	P
		G	A	GG	AG	AA	Ho	He			
BDSP	43	0.80	0.20	0.65	0.30	0.05	0.302	0.320	0.056	1.07	NS*
NEBM	44	0.94	0.06	0.89	0.11	0.00	0.114	0.113	-0.009	0.10	NS*

*statistically non-significant difference

As our previous studies have shown, the GDF9 gene (growth differentiating factor 9) is of primary importance for the fertility of Bulgarian sheep breeds, unlike the BMP-15 and BMPR-1B genes (Bozhilova-Sakova et al., 2020; Dimitrova et al., 2020; Bozhilova-Sakova, & Dimitrova, 2021; Bozhilova-Sakova et al., 2023; Dimitrova et al., 2024), although contradictory results have been reported by other authors regarding the significance of the G1 mutation on sheep fertility (Eghbalsaied et al., 2017; Talebi et al., 2018).

In our previous study of ewes from the same herd of the Bulgarian Dairy Synthetic Population breed, similar frequencies were found, slightly higher for allele A, which is probably due to the fact that animals with genotype AA and AG predominate among those born as twins, triplets and more, and they are given preference in the selection of replacement animals. Genotype frequencies show a lower value for homozygous GG (0.59), higher for heterozygous AG – 0.35 and approximate for homozygous mutant – 0.6 (Dimitrova et al., 2024).

When studying the ewes of the North-East Bulgarian Merino breed from the same flock, the same allele frequencies and the presence of two genotypes were found – GG and AG with frequencies of 0.88 and 0.12, respectively (Bozhilova-Sakova & Dimitrova, 2021).

In the present study, the inbreeding coefficient for rams from both breeds was calculated based on the values for Ho and He, and in both breeds the values of the expected and established heterozygosity were very close. In animals from SPBM, a low positive value of the indicator (Fis = 0.056) was found, indicating a low level of inbreeding, while in SIBT a low, negative value (Fis = –0.009) was reported for this parameter (Table 1).

Similar results to ours on the predominant influence of FecG1 on biological fertility were obtained in a study of indigenous sheep from Bangladesh revealing the frequency of the genotypes in the general population GG, AG, AA respectively 51.59%, 45.24% and 3.17%, and the frequencies of the G and A alleles are respectively 74.21% and 25.79%. The authors found that the homozygous genotype GG had the smallest litter size (1.59) and the homozygous genotype AA had the highest litter size (2.00), but the number of the ewes with genotype AA was very low (Hossain et al., 2020).

In a study of 40 rams of the Prikatun type of Altai Mountains breed, the authors reported the A allele as desirable in breeding to improve the reproductive qualities and productivity of sheep, with rams carrying the AA genotype outperforming their peers of the AG and GG genotypes in terms of pre-slaughter weight, slaughter weight and yield, as well as carcass meat content (Selionova & Podkorytov, 2021).

Better reproductive abilities of carriers of the mutant A allele were also reported by Younis et al. (2017) in a study of Awassi ewes, in which heterozygous GA individuals on G1 of the GDF9 gene were associated with year-round reproduction, while frequencies of the wild GG genotype were higher in seasonal Awassi ewes.

The frequency of allelic diversity in the GDF9 gene in rams selected for breeding, in addition to influencing the allelic frequencies in the next generation of ewes, is also important for the fertility of these male individuals, taking into account the clarification of the role of the GDF9 gene in the development of Leydig cells and germ cells, and from there for the molecular mechanisms of the gene in spermatogenesis of male animals (Wang et al., 2022).

In this regard, the study is to be expanded in order to clarify the influence of the different allelic variants of the gene on the fertility of rams from the two studied breeds.

CONCLUSIONS

As a result of the study, a genetic polymorphism in SNP G1 of the GDF9 gene was identified in the studied rams, with a higher level in animals from the Bulgarian Dairy Synthetic Population. Both allelic variants, A and G, were detected in the two groups, with the A allele showing a higher frequency in rams from the Bulgarian Dairy Synthetic Population.

All three possible genotypes - GG, AG, and AA were observed in rams from the more fertile Bulgarian Dairy Synthetic Population breed. In contrast, only the GG and AG genotypes were present in rams from the less fertile North-East Bulgarian Merino breed.

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