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# INVESTIGATING C.260G>A MUTATION IN THE GROWTH DIFFERENTIATION FACTOR 9 GENE IN BREZNIK, BLACK-HEADED PLEVEN AND BULGARIAN DAIRY SYNTHETIC POPULATION SHEEP BREEDS

**Milena BOZHILOVA-SAKOVA[1], Ivona DIMITROVA[2], Nevyana STANCHEVA[3]**

[1]Agricultural Academy, Institute of Animal Science, Spirka "Pochivka", 2232, Kostinbrod, Bulgaria
[2]University of Forestry, Faculty of Agronomy, 10 "Kliment Ohridski" Blvd, 1756, Sofia, Bulgaria
[3]Agricultural Academy, Agricultural Institute, Department of Animal Science, 3 "Simeon Veliki" Blvd, 9700, Shumen, Bulgaria

Corresponding author email: ivonna_dimitrova@yahoo.co.uk

***Abstract***

*Growth differentiation factor 9 (GDF9) contains multiple mutations related to the fecundity in sheep. In the present experiment was studied the genetic variation in exon 1 (G1) and investigating of mutation c.260G>A, related to litter size in three sheep breeds raised in Bulgaria (Breznik, Bulgarian Dairy Synthetic Population and Black-headed Pleven). A total of 99 ewes were genotyped trough PCR-RFLP method. Results proved polymorphism in all of the three breeds. The highest genetic diversity was calculated in the BDSP population (0.385), where all three possible genotypes GG, AG and AA were identified with frequencies of 0.54, 0.41 and 0.05, respectively. The other two breeds are local breeds and as expected the genetic variation in them was lower. Genotypes GG and AG were found with very closed frequencies in both breeds. The statistical analysis manifested that all tested population were consisted with Hardy-Weinberg equilibrium.*

***Key words****: fecundity, GDF9, genetic diversity, polymorphism.*

## INTRODUCTION

According to a report of the Food and Agriculture Organization of the United Nations (FAO), published in June 2024, world production of sheep meat is about 17.3 million which represents an increase of almost 1% compared to the amount produced in 2023. Sheep meat production takes around 5% of world meat production this year. 82% of the world's sheep meat production comes from Asian and African countries. The largest producer of sheep meat is China (5.3 million tons), followed by India (833 thousand tons), Pakistan (816 thousand tons), Turkey (762 thousand tons), Nigeria (425 thousand tons) and Algeria (363 thousand tons). Australian farmers sent more sheep and lambs to slaughterhouses in 2024 than the previous year, so Australia's sheep meat production was increased by 3.6 % (915,000 tonnes). A slight increase was expected in New Zealand (+0.7%). In 2024, a growth of 3.5% was expected in both exports and imports in the world trade in sheep meat. In 2023, Bulgaria officially produced 9361 tons of sheep and goat meat or 0.054% of the world production of lamb meat. The world consumption of lamb is increasing in parallel with the increase in population. That is why it is extremely important to find fast and cost-effective methods to develop the sheep breeding sector worldwide and more specifically to increase the number of lambs born per ewe. In 2029, the average consumption of sheep meat is expected to reach 4.2 kg per person (FAO, 2024).

The only way to ensure permanently high production of sheep meat is by maintaining high level of lambing (twins, triplets etc.). Marker assisted selection gives a fast pathway through improving process. Different QTLs have been pointed out as genetic markers associated with economically important traits. Regarding the prolificacy there are several major genes reported as eventual enhancers of litter size in sheep. One of those genes is the growth differentiation factor 9 (GDF9) (Sae-Foo et al., 2024). This gene is a member of the transforming growth factor (TGF-B) family and is located on chromosome 5. The ovine GDF9

gene spans approximately 2.5 kb and contains two exons separated by one intron. Exon 1 encodes for 134 amino acids and exon 2 for 135-456 amino acids, while the intron spans about 1126 bp (Hanrahan et al., 2004). Eight different mutations have been discovered in GDF9 (G1 to G8). They could affect the ovulation rate and even could cause infertility. Mutations G2 and G3 are located in the intron. Mutation G5 is located in exon 2 and do not result in amino acid changes. The other five mutations (G1, G4, G6, G7, and G8) lead to amino acid substitutions. Different studies announced that in G1 mutation the heterozygous genotype AG is expressed by the higher litter size per ewe (Abdelgadir et al., 2021).

The aim of the current trial is to determine the allelic variants in exon 1 (G1) c.260G>A, related to the litter size in 99 ewes from three sheep breeds raised in Bulgaria (Breznik, Bulgarian Dairy Synthetic Population and Black-headed Pleven).

## MATERIALS AND METHODS

### Blood sample's collection and DNA isolation

Blood samples were collected using vacuumed tubes with $K_2$-EDTA from 30 sheep of Breznik (Br) sheep breed, 30 sheep of Black-headed Pleven (BHP) and 39 sheep of Bulgarian Dairy Synthetic Population from 3 different distinct flocks raised in Bulgaria. DNA was obtained from the whole blood taken from *vena jugularis* using a DNA purification kit QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

The blood collection was in accordance with the requirements for the humane treatment of animals listed in the regulatory documents of the Republic of Bulgaria and the European Union. All DNA analysis procedures were performed in the Laboratory of Genetics of the Faculty of Agronomy at the University of Forestry, Sofia.

### DNA analysis

The extracted DNA was further processed by PCR analysis. A 462 bp fragment of the GDF9 gene exon 1 was amplified by polymerase chain reaction (PCR) in all tested samples using the following primers (Hanrahan et al., 2004)
Forward:
5′GAAGACTGGTATGGGGAAATG3′ and
Reverse:
5′CCAATCTGCTCCTACACACCT3′.

PCR reactions were performed in a final volume of 25 µl, including 12.5 µl Red Taq DNA polymerase master mix (2x) (VWR), 1 µl of each primer (10 pmol/µl) (Bioneer), 10 µl DNA template and distilled water to final volume. PCR conditions were performed as described: primary denaturation at 94°C for 5 minutes, followed by 30 cycles a denaturation at 94°C for 1 minute, annealing at 63°C for 45 s, extension at 72°C for 1 minutes, and final extension at 72°C for 10 minutes. The PCR procedure was performed in a VerityPro 96-Well Thermal Cycler (Applied Biosystems by Thermo Fisher Scientific).

### Genotyping of the GDF9 gene

All PCR products were subjected to restriction with *HhaI* enzyme. The reactions were incubated at 37°C for 20 minutes and then inactivated at 65°C for 10 minutes. The restriction mixture was prepared in a final volume of 20 µL, containing 1 µL (5 U) of restriction enzyme *HhaI* (cat. no.ER1851, Thermo Fisher Scientific), 5 µL $ddH_2O$ and 2 µL Tango Buffer). After digestion, samples were run in 2.5% agarose gel electrophoresis for 60 min in 120 V and 80 mA. The genotypes were determined under UV transilluminator (Hi-UVTM Duo Capture, HIMEDIA).

### Statistical analysis

Statistical software was used to determine genotype and allele frequencies, observed and expected heterozygosity, coefficient of inbreeding for the studied locus (Yeh et al., 1997). The chi-square test was used to analyze whether the studied population were in Hardy-Weinberg equilibrium.

## RESULTS AND DISCUSSIONS

A total of 99 ewes were genotyped trough PCR-RFLP method. Results proved polymorphism in all of the three breeds (Table 1). In all samples were determined the PCR products with expected length of 462 bp (Figure 1). The highest genetic diversity was calculated in the Bulgarian Dairy Synthetic Population (0.385), where all three possible genotypes GG, AG and AA were identified with frequencies of 0.54,

0.41 and 0.05, respectively (Figure 2). In ewes from Breznik and Black-headed Pleven sheep breeds the genetic variation was lower as expected. This could be explained with the fact that those two breeds are local Bulgarian breeds and they are more or less stable and conservative. Genotypes GG and AG were found with very closed frequencies in both breeds. Homozygous genotype AA was not observed in those two local Bulgarian sheep breeds.

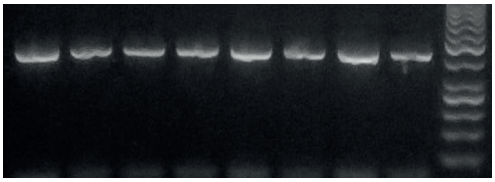

Figure 1. Electropherogram of PCR products of GDF9 with length of 462 bp

In Table 1 was clearly demonstrated that the genetic diversity (Ho) was higher in the herd of Bulgarian Dairy Synthetic population sheep breed (0.410) The statistical analysis manifested that all tested population were consisted in Hardy-Weinberg equilibrium. The coefficient of inbreeding (Fis) is the proportion of the variance in the population contained in an individual. A high Fis implies a considerable degree of inbreeding. In the present study all breeds had a negative degree of Fis which meant that despite the lower genetic diversity for the tested locus in the two local breeds the implemented selection aimed to avoid inbreeding.

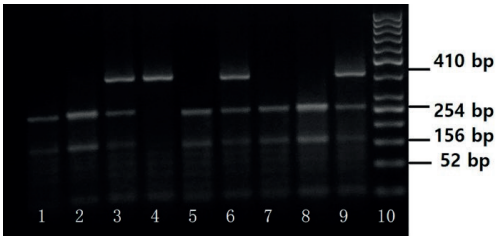


Figure 2. Separated fragments and different genotypes pattern in ewes from Bulgarian Diary Synthetic Population

On Figure 2 were showed the results from fragment separation in Bulgarian Dairy Synthetic Population where all three genotypes could be seen. The homozygous genotype GG was represented by 3 bands with lengths of 254 bp, 156 bp and 52 bg. The heterozygous genotype AG revealed 4 fragments with lengths 410 bp, 245 bp, 156 bp and 52 bp. The homozygous genotype AA demonstrated 2 bands with lengths of 410 bp and 52 bp.

Sheep (*Ovis aries*) are the most widely farmed animal species in many parts of the world. There are over 1300 sheep breeds worldwide. Sheep are multipurpose animals that are raised for their meat, milk, fleece, skin, fur and fertilizer (Koyun et al., 2021).

The crucial factor in sheep breeding is the litter sized. The reproduction potential is determined by breed, environment and most of all by genetics (Hassooni and Zamit, 2024).

The growth differentiation factor 9 is one of the most studied genes worldwide associated with the prolificacy in sheep. PCR-RFLP technique appears to be easy and fast method for genotyping and identification of allelic variants of this gene. Kırıkçı (2022) studied 50 Akkaraman sheep in order to establish the genetic variability in G1 of GDF9 gene. The author reported very similar result to these in Black-headed Pleven and Breznik sheep breeds in the present study. In Akkaraman animals were determined genotypes GG and AG with frequencies 0.87 and 0.13 respectively, and genotype AA was not found.

A study on indigenous sheep in Bangladesh Results suggested that the genotypes had a significant effect on litter size. The study also showed that the homozygous genotype GG had the lowest litter size (1.59±0.09) and the homozygous genotype AA had the highest litter size (2.00±0.41) (Hossain et al., 2020), which was in agreement to a previous our work regarding G1 mutation in GDF9 in ewes from Bulgarian Dairy Synthetic population breed where a proven higher fertility was observed in ewes born as twins with genotype AA (1.97) compared to twins born as carriers of heterozygous genotype AG (1.65) ($p \leq 0.05$) (Dimitrova et al., 2024).

Conversely - in a study by Kasiriyan et al. (2011) in 150 Sangsari sheep, it was found that sheep with a homozygous mutant genotype AA were fertile, but had the smallest litter size compared to those homozygous for the wild allele GG and heterozygous AG ewes.

Our research team has conducted a massive study on different sheep breeds raised in

Bulgaria regarding productive traits in sheep including fecundity. In a previous study of the GDF9 gene in Bulgarian indigenous sheep breeds, a genetic variation was detected. Homozygous genotype GG and heterozygous genotype AG were identified in the Cooper-Red Shumen sheep breed with frequencies 0.97 and 0.03, respectively. In the Karakachan sheep breed were determined all three genotypes GG, AG, and GG with frequency 0.76, 0.17, and 0.07, respectively (Bozhilova-Sakova and Dimitrova, 2021).

Other investigation of some Merino sheep breeds in Bulgaria, showed also that GDF9 locus was polymorphic. In Ascanian and Caucasian Merino, were detected genotypes GG and AG with similar frequencies of 0.90 and 0.10, respectively. In the Karnobat Merino breed were revealed the three genotypes GG, AG, and AA with frequencies 0.70, 0.27, and 0.03, respectively (Dimitrova et al., 2020). In 60 ewes from Northeast Bulgarian Merino sheep breed the GDF9 gene was identified as polymorphic with presence of two alleles G and A with frequencies 0.94 and 0.06 and genotypes GG and AG with frequencies 0.88 and 0.12, respectively (Bozhilova-Sakova and Dimitrova, 2020).

In disagreement to the present investigation Al-Mutar and Younis (2020) tested the effect of point mutation in the Growth Differentiation Factor 9 Gene of oocytes on the sterility and fertility of Awassi sheep. They announced that the mutant allele A in G(199)A locus was demonstrated in sterile samples, while the wild G allele was detected in fertile ewes. The ovarian tissues taken from the non-fertile carriers of the mutant allele A showed severe hypoplastic changes. Hypoplasia was characterized by diminished developmental follicles mostly replaced with stromal connective tissue.

In an experiment conducted by Aboelhassan et al. (2021), the authors studied the different variants of GDF9 in Egyptian sheep, using the T-ARMS-PCR technique to calculate the effect of genetic variability on reproductive traits, the average litter size, and twining in particular. According to the results, the GDF9 was identified as the most important genetic marker for improving fertility in Egyptian sheep.

All stated so far prove that growth differentiation factor 9 gene was polymorphic in the investigated sheep breeds worldwide and hence it is suitable for conducting in marker assisted selection aimed to improving reproduction in sheep.

Table 1. Effective allele number, allele and genotype frequencies, heterozygosity, coefficient of inbreeding, chi-square and p-value of the tested breeds

| Breed | n | Ae | Allele frequencies | | Genotype frequencies | | | Heterozygosity | | Fis | X2 | p |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | G | A | GG | AG | AA | Ho | He | | | |
| BDSP | 39 | 1.63 | 0.74 | 0.26 | 0.54 | 0.41 | 0.05 | 0.410 | 0.385 | -0.064 | 0.4 | NS* |
| BR | 30 | 1.10 | 0.95 | 0.05 | 0.90 | 0.10 | 0.00 | 0.100 | 0.095 | -0.053 | 0.0 | NS* |
| BHP | 30 | 1.15 | 0.93 | 0.07 | 0.87 | 0.13 | 0.00 | 0.133 | 0.130 | -0.023 | 0.0 | NS* |

*statistically non-significant difference

## CONCLUSIONS

The presence of polymorphism was found in all three studied breeds. The highest genetic diversity was observed in sheep from Bulgarian Dairy Synthetic Population, where all three possible genotypes were found.

The two local breeds (Breznik and Black-headed Pleven) are characterized by lower genetic diversity and the presence of two genotypes (GG and AG).

The results found are probably due to the different breeding systems, as the local breeds are bred extensively, with continuous application of "maintaining" selection in order to preserve the typical characteristics of the breed, and on the other hand the number of animals studied is limited.

Additional studies are needed, including larger sample sizes, to confirm the current results.

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