RESEARCH ARTICLE

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Application of PCR-RFLP method to determine polymorphism in BMP-15 and GDF9 fecundity genes in Northeast Bulgarian Merino sheep breed

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ABSTRACT
The purpose of this study was to determine the presence or absence of mutations in BMP-15 and GDF9 fecundity genes. In this experiment, 60 ewes from the Northeast Bulgarian Merino sheep breed were tested for polymorphisms in the BMP-15 gene and SNP G1 of the GDF9 gene. This is the first investigation of the BMP-15 gene by PCR-RFLP method in the Bulgarian sheep breed. After DNA extraction and PCR amplification, the expected PCR products were received. The digestion with restriction enzyme *HinfI* for the BMP-15 gene and *HhaI* for GDF0 revealed mutations in both genes. In BMP-15, there were found alleles + and *G* with frequencies 0.94 and 0.06, respectively, and genotypes ++ and *G*+ with frequencies 0.80 and 0.12, respectively. In GDF9 were identified alleles *G* and *A* with frequencies 0.94 and 0.06 and genotypes *GG* and *AG* with frequencies 0.88 and 0.12.

Key words: Northeast Bulgarian Merino sheep breed, BMP-15, GDF9, PCR-RFLP analysis, polymorphism

Introduction

In recent years, molecular genetics has been widely used in animal breeding as an alternative method of conventional selection for the faster and more accurate achievement of the desired productive traits. Marker-assisted selection could be successfully managed through candidate genes (Yadav et al., 2019; Khalil, 2020; Saleh et al., 2020).

Polymorphisms in many genes have been reported to be associated with economically important changes of productive traits in sheep. The ovulation rate and litter size have been significantly increased by mutations in a group of genes called fecundity genes. Two of these genes are the Bone morphogenetic protein 15 (BMP15) and Growth differentiation factor 9 (GDF9), which are part of the ovary-derived transforming growth factor-β (TGFβ) superfamily (El Fiky et al., 2017; Mohamed et al., 2020).

Bone morphogenetic protein 15 (BMP15) (FecX) gene is located on chromosome X of the sheep genome and it has two exons separated by one intron. The protein encoded by this gene has 1179 nucleotides (Galloway et al. 2000). BMP15 also called GDF9B or FecX was first found in Romney sheep. The nonsense mutation representing a substitution C<T at position 718 leads to a premature stop codon at amino acid 239 of the unprocessed protein. Several mutations of BMP15 have been identified in different sheep breeds, which could increase the rate of ovulation. Ewes with two inactive copies of the BMP15 gene (homozygous animals) are sterile. Ewes with a single inactive BMP15 gene (heterozygous animals) are fertile and have an increased ovulation rate and a higher incidence of twin or triplet births (Galloway et al., 2000; Hanrahan et al., 2004; Qiuyue et al. 2014).

Growth differentiation factor 9 (GDF9) also called FecG is mapped on chromosome 5 of the genome of *Ovis aries L* (Hanrahan et al., 2004). It covers approximately 2.5 kb and contains two exons and one intron (Ghaffari et al., 2009). Mutations located in the GDF9 gene have been associated with increased ovulation rate and litter size in individuals with heterozygous genotype and sterility in carriers of homozygous mutant genotype (Paz et al., 2015; Barakat et al., 2017).

The purpose of this study was to determine the presence or absence of polymorphisms in BMP-15 and GDF9 fecundity genes in 60 ewes of Northeast Bulgarian Merino sheep breed by PCR-RFLP analysis.

Materials and Methods.

The experiment was carried out in the Laboratory of Genetics part of the Institute of Animal Science, Kostinbrod, Bulgaria.

Animals

In this investigation, 60 ewes from the Northeast Bulgarian Merino sheep breed were tested for polymorphisms in the BMP-15 gene and SNP G1 of the GDF9 gene. Approximately 3 mL of blood from *v. jugularis* were collected from each individual in vacuum tubes containing anticoagulant EDTA. The samples were transported to the laboratory and stored at -20°C till the experimental part.

DNA extraction

DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. After manual DNA extraction, 60 samples were received with a DNA concentration of approximately 10 ng/µl according to the kit specification. The quality of the obtained DNA samples was tested using electrophoresis on 1% agarose (GE Healthcare, UK) gel prepared with 1xTBE buffer (Thermo) and visualized under UV light. Extracted DNA samples were stored at -20°C until use.

PCR amplification

PCR amplification was carried out in total volume of samples 10 µl, containing 40 ng DNA template, 20 pM of each primer and 2× (1.5 mM $MgCl_2$) Red Taq DNA Polymerase Master mix (VWR, Int., Belgium). The polymerase chain reactions were performed using thermal cycler TC-TE (BOECO, Germany) under the condition described in Table 1.

The primer sequences were suggested by Wang et al., (2015) for BMP-15 and by Hanrahan et al. (2004) for GDF9 and they are shown in Table 2.

Restriction analysis

The genotypes of studied animals were established using restriction fragment length polymorphism (RFLP) analysis. The digestion reactions were carried out in a 10 µl final volume, containing a 6 µl PCR product and 10 U/µl specific restriction enzyme. The digestion process for BMP-15 was performed by speed enzyme *HinfI* (Jena Bioscience) for 10-20 min at 37°C in thermo-block. The digestion for GDF9 was performed by the *HhaI* restriction enzyme for 90 min at 37°C in thermo-block. The fragment sizes were determined using GeneRuler™ Ladder, 50 bp (Fermentas) supplied with 1 ml 6xDNA Loading dye (Thermo) on 2,5 – 3 % agarose gel and then visualized under UV light on trans-illuminator Hi-UVTM Duo Capture (HIMEDIA).

Results

RFLP is a technique in molecular biology for the differentiation of small nucleotide sequences in homologous DNA fragments. This analysis is based on the possibility of restriction endonucleases to cut the double-stranded DNA at specific recognition sites. The action of restriction enzymes produces fragments that could be with different lengths depending on the presence or absence of polymorphisms and those fragments could be separated by agarose electrophoresis (Rasmussen, 2012).

Table 1. *Specific PCR conditions for tested loci*

Locus	Primary denaturation	Cycles	Denaturation	Annealing	Elongation	Final elongatiom
BMP-15	94°C/5 min	35	94°C/30 s	63°C/30 s	72°C/25 s	72°C/5 min
GDF9	94°C/5 min	30	94°C/1 min	63°C/45 s	72°C/1 min	72°C/10 min

Table 2. *Primer sequences and length of PCR products of tested loci*

Locus	Primer sequences	Length of PCR product
BMP-15	F:5'-CACTGTCTTCTTGTTACTGTATTTCAATGAGAC-3' R: 5'GATGCAATACTGCCTGCTTG-3'	141 bp
GDF9	F: 5'-GAAGACTGGTATGGGGAAATG-3' R: 5'-CCAATCTGCTCCTACACACCT-3'	462 bp

The PCR product of BMP-15 was 141 bp, as expected (Figure 1). The nonsense mutation C<T which causes the substitution of glutamine with a stop codon (Q239*) was detected by digestion with the *HinfI* (G/ACT) endonuclease enzyme. Two types of restriction patterns were generated – homozygous wild genotype ++ (112bp + 29bp) with frequency 0.88 and heterozygous genotype *G*+ (141bp + 112bp + 29bp) with frequency 0.12. The wild allele + and the wild genotype ++ were more frequent in the population.

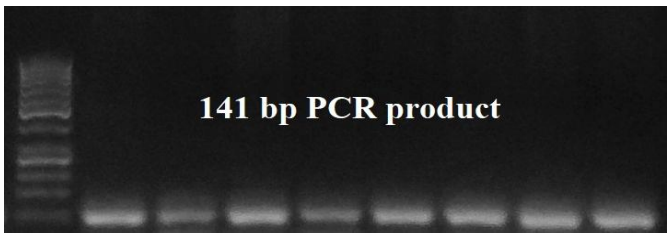

Figure 1. *PCR product of BMP-15 gene*

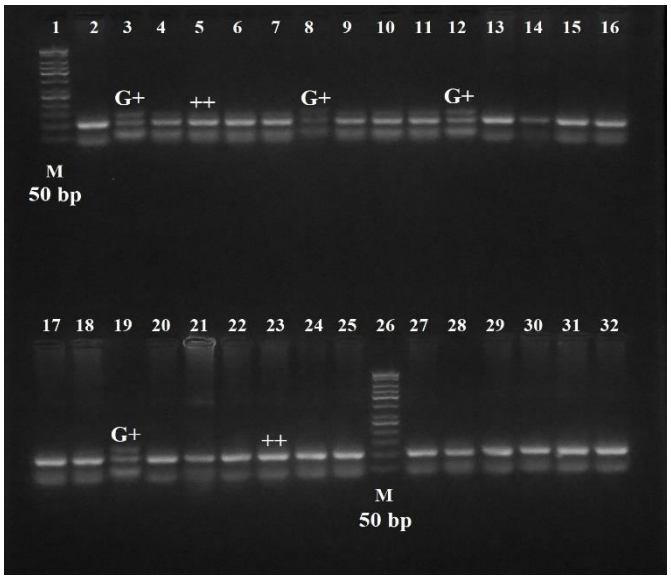

Figure 2. *Image of PCR products of BMP-15 gene digested with enzyme HinfI.*

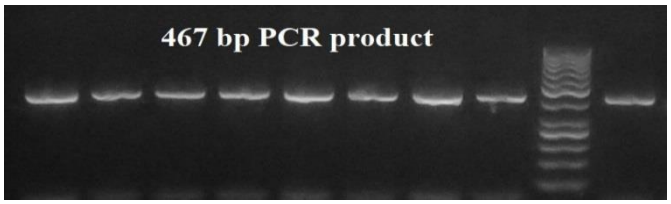

Figure 3. *PCR products of GDF9 gene*

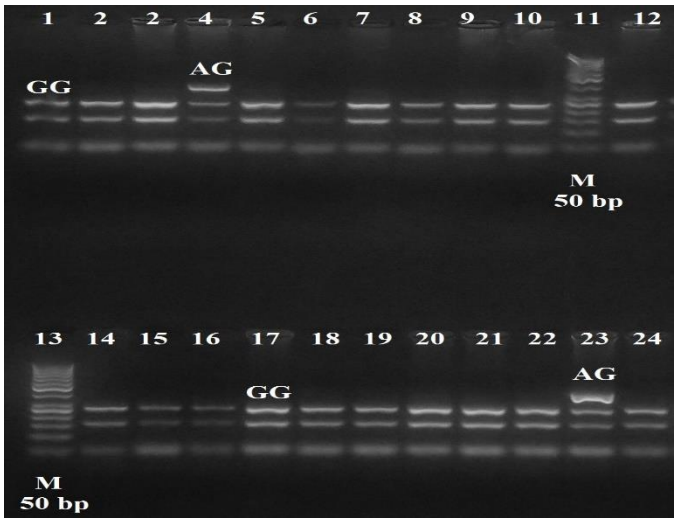

Figure 4. *Image of PCR products of GDF9 gene digested with enzyme HhaI*

None of the animals carried the homozygous mutant genotype *GG* (141bp) (Table 3, Figure 2).

The PCR product of GDF9 was 462bp (Figure 3), located in exon 1 of the GDF9-G1, which included the transition mutation that changes adenine to guanine (G860A) at position 260 in a coding region. The mutation causes a substitution of amino acid arginine. The product was digested with the restriction enzyme *Hha1* (GCG↑C). Digestion of the PCR products revealed two different genotypes – wild genotype *GG* (52, 156, and 254bp) with frequency 0.88 and heterozygous genotype *AG* (52, 156, 254, and 410bp) with frequency 0.12. Similar to results in the BMP-15 the more frequent was the wild allele and the wild genotype (Table 3, Figure 4).

The allele and genotype frequencies were estimated according to Cerit et al. (2004). Expected and observed heterozygosity was received and the statistical significance between them was calculated using standard $\chi 2$ test.

The similarity in the values of the frequencies in both genes was a coincidence. At this stage, there was no established correlation of the presence of mutations in the same individual.

Discussion

Ovine BMP-15 gene plays an essential role in the growth and differentiation of early ovarian follicles. That is why it has been studied in many sheep breeds all over the world (El Fiky et al., 2017).

According to the results in this study Wang et al., (2015) detected the presence of BMP-15 mutation (FecXG) in two sheep breeds (Small Tailed Han and Hu sheep breeds). They also found that the c.718C>T mutation of the BMP-15 gene was significant association with litter size. The homozygous mutant (*GG*) genotype was not detected in Small Tailed Han sheep (n = 869) and Hu sheep (n = 761). Chu et al. (2007) also reported the absence of *GG* genotype in Small Tailed Han sheep, similar to previous studies in the Inverdale and Hanna sheep (Davis et al., 2006). Marzanov et al., (2019) studied reproduction characteristics in Romanov sheep and their association with genetic variation in the BMP-15 gene. The obtained results allowed them to conclude that the genotype frequency at the BMP-15 locus could be useful in estimating the fertility of the ewes.

Opposite to the results in present research Moradband, et al., (2011) studied 152 Baluchi sheep and found only a wild-type allele for BMP15. Another research team conducted an experiment on 32 ewes from Garole sheep intending to explore the DNA polymorphism of exon-2 of BMP-15. They performed PCR-RFLP and sequencing analysis and the result was the absence of polymorphism in the tested locus (Kumar et al., 2016).

Table 3. *Allele frequencies, Genotype frequencies, heterozygosity, df, chi-square and p-value of investigated loci*

Locus	n	Allele Frequencies		Genotype Frequencies			Heterozygosity		df	χ2	p
		+	*G*	++	*G+*	*GG*	*Ho*	*He*			
BMP-15	60	0.94	0.06	0.88	0.12	0.00	0.116	0.112	1	0.000	P<0.05
		G	A	GG	AG	AA	Ho	He			
GDF9		0.94	0.06	0.88	0.12	0.00	0.116	0.112			

GDF9 gene is related to oocyte maturation and folliculogenesis. Polymorphism at G1 point mutation of GDF9 gene was investigated to improve the ovulation rate and litter size in sheep (El Fiky et al., 2017).

In agreement to findings in the present work Hossain et al., (2020) performed an investigation in five distinctly located sheep populations from five regions and a total of 126 individuals. In breeds Tangail and Gaibandha the team announced results like in the current study. They found the presence of two genotypes - homozygous genotype *GG* and heterozygous genotype *AG*. In the Gaibandha sheep breed the highest frequency was reported in homozygous genotype *GG* (71.43%) and lowest in *AG* (28.57%). In contrast in the Tangail sheep breed, the frequency of *AG* (93.33%) was significantly higher than *GG* (6.67%). In the other three breeds, the researchers announced the presence of all three possible genotypes *GG*, *AG*, and *AA* with frequencies - in Noakhali 44.44%, 53.33%, and 2.22%; in Satkhira 82.35%, 14.71%, and 2.94; in Naogaon 44.00%, 48.00 %, and 8.00%, respectively.

In our previous study of the GDF9 gene in Bulgarian indigenous sheep breeds, polymorphism was also detected. Genotypes *GG* and *AG* were detected in the Cooper-Red Shumen sheep breed with frequencies 0.97 and 0.03, respectively. In the Karakachan sheep breed were analyzed all three genotypes *GG*, *AG*, and *GG* with frequency 0.76, 0.17, and 0.07, respectively (Bozhilova-Sakova and Dimitrova, 2021).

In a different experiment of three Merino sheep breeds in Bulgaria, GDF9 was reported as polymorphic. In Ascanian and Caucasian Merino, were determined genotypes *GG* and *AG* with equal numbers of 0.90 and 0.10, respectively. In the Karnobat Merino breed were revealed all three genotypes *GG*, *AG*, and *AA* with frequencies 0.70, 0.27, and 0.03, respectively (Dimitrova et al., 2020).

The observed level of genetic variability in Northeast Bulgarian Merino breed in the two fecundity genes BMP15 and GDF9 studied in this experiment require further and more profound work on their association with fertility to be used as genetic markers in breeding programs.

Conclusions

This is the first study of BMP-15 ever made with Bulgarian sheep breed. After PCR-RFLP analysis polymorphism was detected and two different genotypes were identified in both investigated genes. BMP-15 and GDF9 are extremely important for the reproductive potential of individuals. We strongly recommend research in this direction to be continued with a large number of animals and allele variation of these genes to be associated with phenotype characteristics.

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