

PCR-RFLP ANALYSIS AND GENETIC DIVERSITY OF GDF9 IN TWO INDIGENOUS BULGARIAN SHEEP BREEDS

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Abstract

GDF9 (growth differentiating factor 9) is a genetic marker affecting prolificacy. The aim of present study was to determine the allelic variability and genetic diversity in locus SNP G1 of the GDF9 gene in two indigenous Bulgarian sheep breeds – Cooper-Red Shumen and Karakachan. 60 ewes (30 of each breed) were tested for polymorphisms in SNP G1 of the GDF9 gene. A 462 bp fragment from GDF9 gene was amplified using the PCR method. The genotypes of all animals were determined using PCR-RFLP strategy with restriction enzyme HhaI. In Karakachan sheep breed were identified all possible genotypes - GG, AG and AA with frequencies 0.76, 0.17 and 0.07, respectively. In Cooper-Red Shumen breed was found only one animal with heterozygous genotype AG (0.03), all others were with wild genotype GG (0.97). The frequencies of the wild allele G were significantly higher than mutant allele A in both tested breeds.

Key words: *Cooper-Red Shumen, Karakachan, GDF9, PCR-RFLP, genetic diversity*

Introduction

Sheep farming as an agricultural sector aims to provide high quality meat and milk to the population, as well as to develop ways to make more efficient use of the gene pool of available sheep breeds. Sheep are bred in extensive or intensive production systems and under different climat conditions. They face challenges such as various diseases and environment conditions (Kijas et al., 2012). Their adaptation to highly different geographic and climat conditions and the specialization of different productivity lead to the creation of breeds with significant phenotypic diversity (Gutiérrez-Gil et al., 2014). For preventing breeds extinction, animal breeding programs are necessary to identify effective candidate genes for the economical important traits, especially genes with effect on litter size and ovulation rate (Paz et al., 2014; Khodabakhshzadeh et al., 2016). A major gene found to be associated with prolific phenotype is GDF9, which is responsible for increasing of ovulation rate in heterozygous individuals and sterility in homozygous individuals (Silva et al., 2010). Growth differentiation factor 9 (GDF9) also called Fec G (Elvin et al., 1999; Hanrahan et al., 2004) is mapped on chromosome 5 of the genome of *Ovis aries* L., it covers approximately 2.5 kb and contains two exons and one intron (Sadighi et al., 2002).

Mutations located in the GDF9 gene have been associated with increased ovulation rate and litter size ((Paz et al., 2014). The genotypes of the GDF9 gene have been described in several sheep breeds with effects on ovulation rate and litter size. Hanrahan et al (2004) 8 different SNPs in

sheep, the so-called G1, G2, G3, G4, G5, G6, G7 and G8, perhaps the most intensively studied is G1.

The aim of present study was to determine the allelic variability and genetic diversity in locus SNP G1 of the GDF9 gene in two indigenous Bulgarian sheep breeds – Cooper-Red Shumen and Karakachan.

Materials and methods

Animals

In this investigation 60 ewes from two indigenous Bulgarian sheep breeds – Cooper-Red Shumen and Karakachan (30 of each breed) were studied for polymorphisms in SNP G1 of the GDF9 gene. About 3 mL blood from *v. jugularis* was collected from each individual in vacuum collection tubes containing EDTA as anticoagulant and transported to the laboratory. The examination was carried out in the Laboratory of Genetics of Agronomy Faculty, the University of Forestry.

DNA extraction

DNA was extracted following the standard protocol from the whole blood using Illustra Blood GenomicPrep DNA Purification Kit, (GE, Healthcare) according to the manufacturer's instruction manual. Extracted DNA was stored in -20°C until use.

DNA quantification and detection

After extraction, the concentration of DNA was measured by spectrophotometer Biodrop Agarose gel electrophoresis for visualizing/detecting of the obtained DNA was performed. The mean concentration and purity for Karakachan sheep breed were 21,4 ng/µl and 260/280 - 1,696. For Cooper-Red Shumen breed were 22,1 ng/µl and 260/280 - 1,786.

PCR amplification

DNA samples were amplified in QB-96 thermocycler (Quanta Biotech) using following PCR protocol - primary denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 45 s, elongation at 72°C for 1 min. The process was completed by final extension at 72°C for 10 min and storage at 10°C forever. PCR was carried out in 10 µl reaction mixture containing 4 µl DNA template, 0.2 µl ddH₂O, 20 pM of each primer and 5 µl of 2×(1.5 mM MgCl₂) MyTaq™ HS Red Mix 2x (Bioline). The primer set was suggested by Hanrahan et al. (2004):

forward primer: F: 5'-GAAGACTGGTATGGGGAAATG-3'

reversed primer: R: 5'-CCAATCTGCTCCTACACACCT-3',.

Digestion of PCR products with endonuclease enzyme

After confirmation of PCR amplification of target DNA sequence by electrophoresis, digestion of the PCR products was done with *HhaI* restriction enzyme (Hanrahan et al., 2004) using a digital thermoblock. All samples were prepared in 10 µl final volume containing a mixture of 6 µl of PCR product, 0.5 µl of *HhaI* enzyme, 1 µl of buffer and 2,5 µl of deionized water were kept incubated for 90 min at 37°C. The digested products were checked in 2.5% agarose gel. The gel was observed

in documentation system (Canon UVDI, Major Science) and pictures were captured for further analysis.

Results and discussion

A 462 bp fragment from exon 1 of GDF9 locus was amplified in all 60 ewes. The obtained PCR products were cut with restriction enzyme *HhaI* (GCG↓C to C↑GCG). Two alleles were detected - wild G with three fragments with length 254, 156 and 52 bp and mutant A with 2 fragments 410 and 52 bp.

Genotype GG was presented by the bands 254, 156 and 52 bp, genotype AG – by the bands 410, 254, 156 and 52 bp and the genotype AA – with 410 and 52 bp (Figure 1).

The allele frequencies and genotype frequencies were presented in Table 1. The three possible genotypes (GG, AG and AA) were observed in Karakachan breed. In Cooper-Red Shumen breed were identified only two genotypes (GG and AG). In general, the frequencies of allele G and the homozygous genotype GG were significantly higher than those of allele A and other genotypes in both breeds.

Table 1. Allele and genotype frequencies, average heterozygosity (observed Ho, expected He) and coefficient of inbreeding

Breeds	n	Allele frequency		Genotype frequency			Ho	He	Coefficient of inbreeding	p
		A	G	GG	AG	AA				
Karakachan	30	0.15	0.85	0.76	0.17	0.07	0.166	0.255	0.349	>0.05
Cooper-Red Shumen	30	0.02	0.98	0.97	0.03	0.00	0.033	0.039	0.153	

^a p - non significant

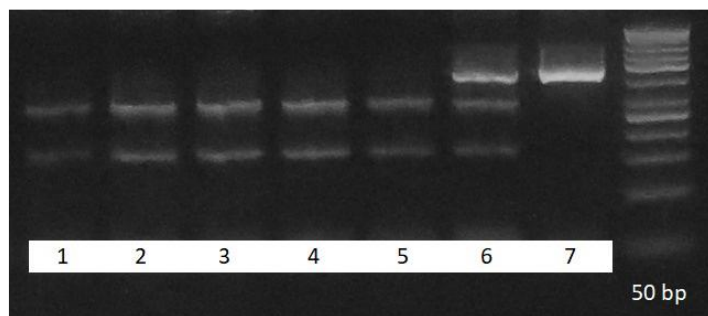


Figure 1. PCR-RFLP analysis and band patterns obtained by digesting 462 bp PCR fragment from exon 1 of ovine GDF9 gene with *HhaI* restriction enzyme. Lines 1 - 5 represented genotype GG, line 6 – genotype AG, line 7 – AA genotype, line 8 – GeneRuler 50 bp DNA Ladder

Higher frequency of allele A (0.15) was observed in Karakachan breed. Cooper-Red Shumen breed showed lower rate of allele A (0.02) and higher frequency of allele G (0.98), respectively. The frequency of homozygous mutant genotype AA identified only in Karakachan breed was 0.07. Higher frequency of heterozygous genotype AG (0.17) was also observed in Karakachan breed and lower (0.03) in the other breed. The homozygous wild genotype GG was with higher frequency (0.97) in Cooper-Red Shumen breed and with lower (0.76) in Karakachan breed. The observed heterozygosity was almost equal to the expected one for Cooper-Red Shumen breed. The coefficient of inbreeding was 0.349 in Karakachan breed and 0.153 in Cooper-Red Shumen breed.

GDF9 gene is related to oocyte maturation and folliculogenesis. Polymorphism at G1 point mutation of GDF9 gene was investigated in order to improve the ovulation rate and litter size in sheep. Liandris et al., (2012) studied two Greece sheep breed. The allele frequency in Karagouniki breed were almost equal to those in Cooper-Red Shumen breeds in our study - for the allele A - 0.03 and for the wild type allele G - 0.97. Similar results were reported by Getmantseva et al., (2019) in their research on GDF9 in Volgograd sheep breed. The authors identified two alleles - A and G with frequencies of 0.04 and 0.96, respectively, and two genotypes - AG and GG with frequencies of 0.09 and 0.91, respectively. The homozygous genotype AA was not observed, similar to Cooper-Red Shumen in our study.

The results of studies of Salskaya and Romanov sheep indicated polymorphism of the GDF9 gene by points G1 with the presence of two genotypes - GG and AG (Kolosov et al., 2015). The allelic frequencies in Karakachan were close to Romanov breed and in Cooper-Red Shumen to Salskaya sheep.

Our investigation for other Bulgarian breeds showed that Ascanian and Caucasian merino breeds were close to those obtained for Cooper-Red Shumen breed, both in allele frequency and due to the presence of two genotypes - GG and AG. Results for Karakachan breed were similar to Karnobat breed – allele frequency was near and also were found the three possible genotypes (GG, AG and AA) (Dimitrova et al., 2020).

Conclusions

This is the first study of GDF9 in indigenous Bulgarian sheep breeds. Karakachan breed showed satisfactory diversity with presence of all three possible genotypes. In Cooper-Red Shumen breed was identified only one individual with heterozygous genotype AG, all others were homozygous for wild allele GG. In general, the results showed higher frequency of allele G in both tested breeds.

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