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Investigation of polymorphisms on ABCG2 and AA-NAT genes in different sheep breeds in Bulgaria

Milena Bozhilova-Sakova[1], Ivona Dimitrova[2*], Nikolay Petrov[3]

[1]*Institute of Animal Science, Kostinbrod 2232, Bulgaria*
[2]*University of Forestry, Sofia 1756, Bulgaria*
[3]*New Bulgarian University, Sofia 1618, Bulgaria*
**Corresponding author:* ivonna_dimitrova@yahoo.co.uk

Abstract

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The present study was conducted in order to investigate and identify the polymorphism in two sheep genes – ABCG2 (associated with milk production) and AA-NAT (associated with seasonality of reproduction) in 6 breeds, raised in Bulgaria. Blood samples were taken from 42 animals of Karnobat Merino, Caucasian Merino, Northeast Bulgarian Merino, Il de France, Karakachan and Askanian Merino. Genomic DNA was extracted and genotypes were estimated by means of PCR amplification and PCR-RFLP method. After PCR amplification and electrophoretic analysis two alleles and three genotypes for ABCG2 were revealed. The genotypes of AA-NAT gene in all investigated animals were established using RFLP (restriction fragment length polymorphism) analysis with use of SmaI restriction enzyme. There is polymorphism in AA-NAT locus of investigated animals from Askanian Merino, Karnobat Merino, Caucasian Merino and Northeast Bulgarian Merino breeds which could be used in future investigations referring to candidate genes for seasonality of sheep reproduction. AA-NAT locus is monomorphic in investigated animals from Il de France and Karakachan breeds – the allele G and the genotype GG were detected. The ABCG2 locus of Askanian Merino, Karnobat Merino, Northeast Bulgarian Merino and Il de France animals is monomorphic, but in these of Caucasian Merino and Karakachan was found to be polymorphic. This represents the first study of the ABCG2 and AA-NAT genes in sheep breeds reared in Bulgaria.

Key words: sheep; breeds; ABCG2 gene; AA-NAT gene; polymorphism

Introduction

In recent years, genetic progress in livestock breeding has been based on genomic selection – a method of tribal work based on a study of the DNA sequences of the animals. It is based on the use of polymorphic variants in different genes related with commercially useful traits.

Genetic diversity of modern domestic animal populations is crucial for improving breeds, adapting them to different environmental conditions, protection from diseases, parasites and predators and also obtaining high quality animal products. Allelic diversity of candidate genes for productive traits is extremely important and contributes to their differentiation and investigation. Knowing different polymorphic variants of these genes contributes to choosing the best breeding strategy, depending on the productive direction (Tohidi et al., 2013). The selection based on genetic markers for productivity is directed to working with animals with high genetic potential in terms of different productive traits (Deykin et al., 2016). The success of the breeding strategies of different sheep breeds is directly related to the need for a better knowledge of their hereditary characteristics. In Bulgaria they are raised 34 sheep breeds (Executive Agency on Selection and Reproduction in Animal Breeding, 2011).

There is lack of researches on Bulgarian sheep breeds and it is necessary to receive enough information about their genetic diversity for successful breeding and selection.

The membrane-associated protein encoded by ABCG2 gene is included in the superfamily of ATP-binding cassette (ABC) transporters, responsible for transport of different molecules across cell membranes (Higgins, 1992). The ABCG2 gene is located on chromosome 6 from *Ovis aries* genome and expressed in some tissues, including the mammary gland, and the level of expression increases during the lactation period in some farm animals (Al-Mamun et al., 2015). The link between the mutation found in this gene and milk yield, protein and fat percentage and somatic cell count (SCC) is reported in dairy cows (Cohen-Zinder et al., 2005; Mani et al., 2009). The ABCG2 (A) allele decreases milk yield and increases protein and fat concentration of cow milk (Ron et al., 2006). In goat ABCG2 gene expression was a function of lactation stage and parallel to goat lactation curve (Wu et al., 2008). A mutation occurs in the sheep ABCG2 gene, which is a single insertion/deletion of 35 bp (Árnyasi et al., 2013; Oner al., 2014). Studies on sheep ABCG2 gene show presence of polymorphism. This gene is of interest because of its relationship to milk production in sheep, but more research is needed for its impact on different sheep breeds.

Non-seasonal sheep reproduction is a major tool that can be used in breeding schemes. An important step is establishing the genetic base of non-seasonal reproduction. Several genes are associated with this trait. AA-NAT gene is located on chromosome 6 of sheep genome and associated with non-seasonal reproduction in sheep (Carcangiu et al., 2011; Ding-ping et al., 2012; Hristova et al., 2012). The arylalkylamine-N-acetyl-transferase (AA-NAT) is an important enzyme in melatonin biosynthesis regulating the animal seasonal breeding (Klein and Berg, 1970; Mindsing et al., 2013; Oner al., 2014). The melatonin is produced in the pineal gland, which takes part in some physiological and pathological processes (Reiter et al., 1995), and its oscillatory production synchronizes in seasonally oestrus responsible in sheep. In view of its biological role, AA-NAT is a candidate gene for reproductive traits. That is why it could be suggested that genetic mutation of the AA-NAT gene may influence seasonally oestrus responsible in sheep. The researchers reported an A-G transition located at exon 3 and essential difference in genotype frequencies among seasonal and non-seasonal sheep breeds. The frequency of GG genotype was higher in non-seasonal breeds while the frequency of heterozygous genotype GA was higher in seasonal sheep breeds (Ding-ping et al., 2012; Oner et al., 2014).

The purpose of the present work was to identify polymorphic allelic variants of ovine ABCG2 and AA-NAT genes in animals of six breeds, raised in Bulgaria.

Materials and Methods

In this study a total of 42 sheep from 6 breeds were investigated for polymorphisms located in ABCG2 and AA-NAT genes. Blood samples were collected from six animals of each breed – Karnobat Merino, Caucasian Merino, Northeast Bulgarian Merino (rams and ewes), Il de France, Karakachan and Askanian Merino. Blood samples were collected from *v. jugularis* in 3 ml vacuum tubes containing EDTA as anticoagulant and transported to the laboratory. The investigation was carried out in the Laboratory of Genetics of Agronomy Faculty, the University of Forestry.

DNA extraction

The blood samples were stored at –20°C until DNA extraction. Genomic DNA was extracted using manual purification kits Illustra Blood GenomicPrep DNA Purification Kit of GE Healthcare (UK), QIAamp DNA Blood Mini Kit (Qiagen) и GenEluteTM Mammalian Genomic DNA Miniprep Kits (SIGMA) according to the instructions provided in the manual. The DNA concentration of each sample was determined by spectrophotometer Biodrop. The quality of the obtained DNA was about 10–50 ng DNA was tested using gel monitoring on 1% agarose (Healthcare) gel prepared with TBE buffer (Thermo).

PCR amplification

PCR amplifications were carried out in total volumes of 10 µl, containing 4 µl DNA template, 0.2 µl dd H_2O, 0.4 µl of each primer (Bioneer) and 5 µl of 2×(1.5 mM $MgCl_2$) Red Taq DNA Polymerase Master mix (VWR, Int., Belgium).

For genotyping the ABCG2 locus was used only PCR amplification according to Oner et al. (2014). While for the AA-NAT gene was used PCR-RFLP technique according to Ding-ping et al. (2012). The primers sets used for the amplification of two genes are shown in Table 1.

PCR amplifications were carried out in total volumes of 10 µl, containing 4 µl DNA template, 0.2 µl dd H_2O, 0.4 µl of each primer (Bioneer) and 5 µl of 2×(1.5 mM $MgCl_2$) Red Taq DNA Polymerase Master mix (VWR, Int., Belgium).

In Table 2 are presented the specific conditions of the PCR_amplification process for each gene. The sizes of PCR products for ABCG2 gene were determined on 2,5 agarose gel using GeneRuler™ Ladder, 50 bp (Thermo) supplied with 1 ml 6xDNA Loading dye and 10000x RedGel TM Nucleic Acid Stain (Biotium). The obtained PCR products were visualized under UV light.

Table 1. Studied locus, genomic region, primers sequence and length of PCR product

Locus name	Genomic region	Primers (5′→ 3′)	Length of PCR product
ABCG2	Intron 5	GCCTCTTCTCCCATACGTC AAAC CAGTTGTGGGCTCATC	232 bp 267 bp
AA-NAT	Exon 3	AGCGTCCACT GCCTGAAAC GGGATGGAAGCCAAACCTC	1142 bp

Table 2. PCR conditions for each locus

Locus	Primary denaturation	Cycles	Denaturation	Annealing	Elongation	Final elongation
ABCG2	95°C/5 min	30	94°C/30 s	52.6°C/65 s	72°C/1 min	72°C/10 min
AA-NAT	95°C/5 min	35	95°C/40 s	60°C/40 s	72°C/40 s	72°C/10 min

RFLP technique

The genotypes of AA-NAT gene in all investigated animals were established using RFLP (restriction fragment length polymorphism) analysis. All PCR products were digested separately in 10 µl final volume, containing 6 µl PCR product, 2.3-2.5 µl ddH_2O, 0.5-0.7 µl restriction enzyme *SmaI* and 1 µl enzyme buffer. The digestion reactions were carried out at 25°C for 5 h in thermostat. The fragment sizes was determined using GeneRuler™ Ladder, 50 bp (Thermo) supplied with 1 ml 6xDNA Loading dye on 2% agarose (Healthcare) gel and stained by 10000x RedGel TM Nucleic Acid Stain (Biotium). The obtained PCR products and restriction fragments were visualized under UV light.

Results and Discussion

After PCR amplification and electrophoretic analysis two alleles and three genotypes for ABCG2 were revealed. Length of the PCR product with deletion is 232 bp and 267 bp without deletion. Studied animals of Askanian, Karnobat Merino and Northeast Bulgarian Merino breeds and Il de France do not show genetic diversity in this gene. All tested samples were with genotypes -/- which showed presence of deletion in studied region. The animals of the other breeds – Caucasian Merino and Karakachan were found to be polymorphic by this region. Three genotypes were observed: +/+, +/- and -/- (Fig. 1; Table 3). The established frequency of the allele + in the Caucasian merino breed is low (0.08), as is the number of individuals surveyed, so we can assume that when increasing the number of animals studied, it is possible in the other fine flees breeds to detect this allele even though the low frequency.

There are few number of studies related to ABCG2 gene in sheep. Oner et al. (2014) reported the "–" allele as predominant and its frequency differed from 0.50-0.65. On the contrary the "+" allele was found as predominant in other study (Árnyasi et al., 2013). They found relation between the "–" allele and higher SCC.

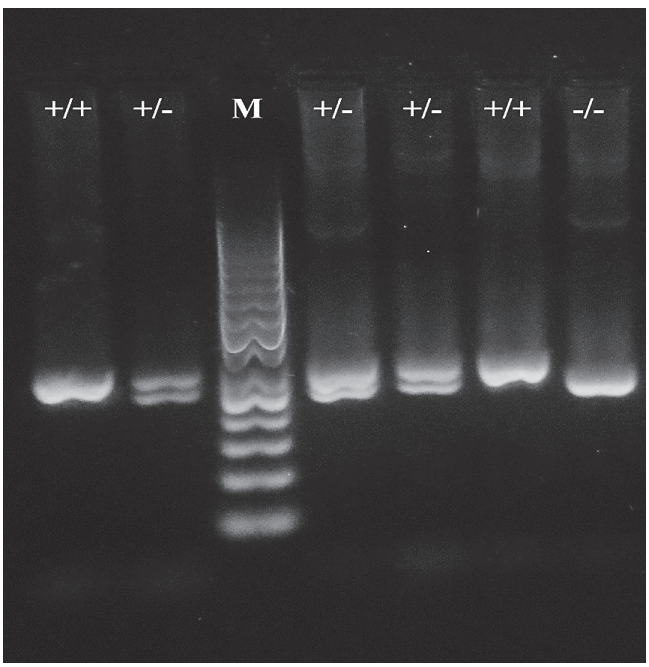

Fig. 1. ABCG2 genotypes, observed in investigated animals

The amplified region of AA-NAT gene showed a PCR product with expected length of 1142 bp. In breeds Askanian, Karnobat Merino, Northeast Bulgarian Merino and Caucasian Merino were observed two genotypes GG and AG. Genotype GG shows the bands 183 bp, 255 bp, 333 bp and 371 bp and genotype AG – bands 183 bp, 255 bp, 333 bp, 371 bp and 516 bp. In the other two breeds included in this study Il de France and Karakachan only genotype GG was observed (Fig. 2; Table 3). In this study the presence of allele G in different breeds was higher than the allele A. These results were in agreement with the results obtained from some Chinese sheep breeds. The study carried out in these Chinese sheep breeds revealed that frequencies of allele G were higher in non-seasonal reproduction breeds while found quite lower

Table 3. Allele and genotype frequencies of ABCG2 and AA-NAT loci

Breed	n	Locus									
		AA-NAT					ABCG2				
		Allele frequency		Genotype frequency			Allele frequency		Genotype frequency		
		A	G	AA	AG	GG	+	–	+/+	+/–	–/–
Askanian Merino	6	0.58	0.42	0.00	0.83	0.17	0.00	1.00	0.00	0.00	1.00
Karnobat Merino	6	0.67	0.33	0.00	0.67	0.33	0.00	1.00	0.00	0.00	1.00
Caucasian Merino	6	0.67	0.33	0.00	0.67	0.33	0.08	0.92	0.00	0.17	0.83
Northeast Bulgarian Merino	12	0.67	0.33	0.00	0.67	0.33	0.00	1.00	0.00	0.00	1.00
Il de France	6	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00
Karakachan	6	0.00	1.00	0.00	0.00	1.00	0.58	0.42	0.33	0.50	0.17

in the seasonal reproduction breeds (Ding-Ping et al.,2012). This study reported a higher frequency of the allele G, similar results are also published by Oner et al. (2016). In our study the frequency of the allele G in breeds Il de France and Karakachan was higher. On the contrary, an investigation of Turkish Awassi sheep raced in Iraq shows higher frequency of the A allele and following distribution of AA-NAT genotypes 56.67% and 33.33% and 10.00% for each of the genotypes AA and GA and GG respectively (Addin et al., 2016). In this study, we received a higher frequency of the A allele in the Merino sheep breeds (Table 3).

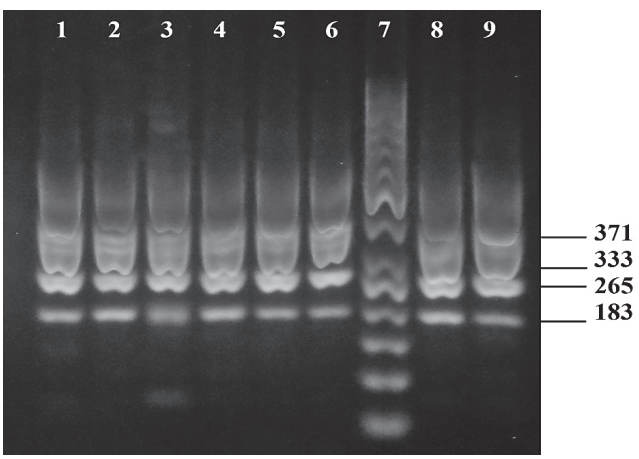

Fig. 2. Electrophoresis of AA-NAT sheep gene after digestion with SmaI restriction enzyme. 1-9 GG genotype; 7 DNA Ladder 5- bp

AA-NAT is a key rhythm-generating enzyme of the melatonin synthesis in the pineal gland (Chattoraj et al., 2009). Polymorphisms in this gene have been reported in studies related to major depression (Soria et al. 2010), melatonin production (Ying et al., 2004), sleep pattern (Wang et al. 2004), delayed sleep phase syndrome (Hohjoh et al., 2003; Pereira et al., 2007), adolescent idiopathic scoliosis (Wang et al., 2008), sheep seasonal and non-seasonal reproduction (Ding-Ping et al., 2012; Oner et al., 2014).

The present results represent the first study of the ABCG2 and AA-NAT genes in sheep breeds reared in Bulgaria and related to the milk productivity and seasonality of reproduction.

Conclusion

✓ In the present study it may be concluded that there is a polymorphism in AA-NAT locus of investigated animals from Askanian Merino, Karnobat Merino, Caucasian Merino and Northeast Bulgarian Merino breeds which could be used in future investigations referring to candidate genes for seasonality of sheep reproduction. AA-NAT locus is monomorphic in investigated animal from Il de France and Karakachan breeds – both the allele G and the genotype GG are detected.

✓ The ABCG2 locus of Askanian Merino, Karnobat Merino, Northeast Bulgarian Merino and Il de France animals is monomorphic, but in these of Caucasian Merino and Karakachan is found to be polymorphic.

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