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Original article

ASSOCIATIONS BETWEEN NEWLY DISCOVERED POLYMORPHISMS OF THE *MyoD1* GENE AND BODY PARAMETERS IN STAVROPOL BREED RAMS

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Summary

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The aim of this study was to investigate the influence of the *MyoD1* gene polymorphisms on some body parameters in sheep. *MyoD1* polymorphisms were detected using NimbleGen sequencing technology (Roche, USA). The effect of polymorphisms identified was investigated in Stavropol sheep breed rams (n=30). In coding and regulatory parts of gene 26, single nucleotide polymorphisms (SNP) were detected. These were the substitutions: c.-2112C>G, c.-1806A>G, c.-1687T>C, c.-1608C>T, c.-1603G>T, c.-1578G>A, c.-1235G>A, c.-910G>T, c.-909G>T, c.-880G>A, c.-637C>T, c.-412G>T, c.244C>T, c.246G>T, c.253G>T, c.259G>C, c.261C>T, c.269C>G, c.274C>A, c.276C>G, c.277C>A, c.279C>T, c.281C>A, c.287C>A, c.325T>C and c.483C>T. Sixteen of these SNPs have been identified for the first time. The complex of the four SNPs c.-1578, c.-880, c.-637, c.325 and c.-1235 occurred in Stavropol sheep breed only together. The study of the influence of these SNPs on the body parameters of rams showed the absence of significant correlation between their presence and the body size of the animals. The SNPs c.-910 and c.-909 were associated with improved body parameters, but they were found in a very small numbers of animals, which does not allow making definitive conclusions.

Key words: *MyoD1*, sequencing, sheep, SNP, Stavropol breed

INTRODUCTION

A modern and promising method for prediction of breeding value and productive potential of animals is marker-assisted selection. It can be used in association with traditional selection methods to accelerate selection of animals with best production traits and reduce costs (Hagen *et al.,* 2005).

The myostatin gene has a leading position among several genes related to meat quality of farm animals (Gan *et al*., 2008). However, often an increase in muscle mass is not associated with changes in the

coding region of myostatin gene. Therefore, candidate genes that affect the myostatin gene function and muscle development in general have attracted more attention (Muroya *et al.,* 2002; Hagen *et al*., 2005).

One of these genes is a member of the myogenic regulatory factors (MRF) genes family – *MyoD1* gene (Busanello *et al.,* 2012). Members of the MRF family are basic helix-loop-helix (bHLH) transcription factors. This family of genes also comprises *Myf5*, myogenin, and *MRF4*. (Buckingham, 1992). They play a key role in the differentiation and structure of skeletal muscle in vertebrates. The protein MyoD1 is capable to convert non-muscle cells, such as fibroblasts, into myoblasts that have the ability to fuse into myotubes (Davis *et al*., 1987). The MRF proteins contain several functionally distinct domains responsible for transcriptional activation, chromatin remodelling, DNA binding, nuclear localisation, and heterodimerisation (Tapscott *et al*., 1988; Weintraub *et al*., 1991; Vandromme *et al*., 1995; Gerber *et al*., 1997). The bHLH domain is highly conserved in all members of the family and allows for the formation of heterodimers that are bound to the E-box (CANNTG) locus. This locus is found in most of the regulatory regions of muscle-specific genes, including myostatin gene (Murre *et al.,* 1989; Zhang *et al.,* 2006).

It was previously shown that the *MyoD1* gene interacts with the promoter of the muscle specific creatine kinase gene (Huynen *et al.,* 1992), and the myostatin gene (Du *et al.,* 2007; Deng *et al.,* 2012). In the regulation of postnatal myogenic programming of satellite muscle cells, the *MyoD1* gene plays a critical role. Thus, mice with a knockout mutation in *MyoD1* gene have a severely reduced regenerative capacity after injury (Megeney *et al.,* 1996). High levels of the MyoD protein inhibit the proliferation of satellite muscle cells and lead to either myogenic differentiation or apoptosis (Asakura *et al.,* 2007; Pan *et al.,* 2015).

A positive correlation between the level of expression of *MyoD1* and the cold carcass yield was found in sheeps (Lobo *et al*., 2012). A polymorphism of *MyoD1* gene in pigs is associated with meat quality (Klosowska *et al.*, 2004; Kapelanski *et al.*, 2005; Liu *et al.*, 2008), including fatness (Kuryl *et al.*, 2002) and meat marbling (Verner *et al.,* 2007). In chickens, a mutation in both *MyoD1* and *Mrf4* genes was associated with an increase in the diameter of muscle fibres. These genes are recommended as molecular markers for chicken marker-assisted selection (Yang *et al.,* 2015). The effect of single nucleotide polymorphisms in *MyoD1* gene on the growth was examined in the Hanwoo breed of Korean cattle (Bhuiyan *et al.,* 2009). In Chinese cattle breed, an influence of *MyoD1* gene polymorphism on meat quality traits was detected (Ujan *et al.*, 2011). The relationship between mutations in *MyoD1* gene and indicators of meat quality in sheep has not been studied.

The Stavropol breed of sheep was bred in the period from 1921 to 1950. The base for the creation of the Stavropol breed was Novokavkazskaya fine-fleeced sheep breed. During the breeding, American Rambouillet and Australian Merino rams were used. Among the wool breeds of sheep, the Stavropol breed is the biggest in size and has good meat productivity; it is well adapted to the climate of Stavropol territory. Nowadays, Stavropol breed is one of the largest fine-wool sheep breeds in Russia (Aboneev *et al.,* 2011). It is also characterised by some good meat quali-

ties, inherited from the original parental forms (Dmytryk & Ovchinnikova, 2013).

The aim of research was to examine the structure of the gene *MyoD1* in the Stavropol breed of sheep and to identify polymorphisms associated with vital body conformation traits.

MATERIALS AND METHODS

Sample collection

The investigation was carried out on randomly selected one-year-old Stavropol breed rams (n=30) from a livestock-breeding farm in Stavropol Krai, Russian Federation. All animals were healthy, kept in optimal conditions and fed with a total mixed ration. Body measurement parameters were analyzed in order to describe meat production.

DNA isolation

Genomic DNA was extracted from blood samples obtained from the jugular vein under aseptic conditions. Blood samples were collected in Vacutainer® vials with stabiliser EDTA (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and were transported to the laboratory at +4 °C within 6 hours. DNA was extracted from 0.2 mL of blood using the PureLink Genomic DNA MiniKit (Invitrogen Life Technologies, Grand Island, NY, USA).

Targeted enrichment and next generation sequencing

In order to detect mutations in the genes, targeted enrichment was done and the investigated DNA fragments were sequenced. For enrichment of target regions, we used NimbleGen technology (Roche NimbleGen, Inc., Madison, WI, USA). Probes for target regions were developed in cooperation with Roche NimbleGen (USA). Libraries of DNA fragments were prepared in accordance with the protocol in the Rapid Library Preparation Method Manual undergoing the procedure of enrichment using NimbleGen SeqCap EZ Developer Libraries (Roche NimbleGen, Inc., Madison, WI, USA).

Monoclonal amplification of the enriched target regions of DNA was carried out according to a standard protocol in the emPCR Amplification Method Manual, Lib-L (Roche NimbleGen, Inc., USA).

Sequencing was performed using a GS Junior genomic sequencer (Roche NimbleGen, Inc., Madison, USA). The resulting sequences were mapped to the reference genome assembly *Ovis aries* oviAri3 (The National Center for Biotechnology Information. Genome, 2012, *Ovis aries* [sheep], 2015) by GS Reference Mapper v2.9 software (Roche NimbleGen, Inc., Madison, USA).

To describe a single nucleotide polymorphism (SNP) the HGVS nomenclature (www.hgvs.org) based on transcript ENSOART00000027076 (Anonymous, 2016) was used.

Statistical analysis

Phylogenetic analysis was performed using Unipro UGENE 1.15.1 software (Unipro, Russia). For statistical analysis, we used Student's t-test in Excel for Windows statistical plug-in. Significant difference was detected at $P<0.05$.

RESULTS

As a result of our work we found 26 SNPs in the coding and regulatory regions of the *MyoD1* gene. Sixteen SNPs were detected for the first time; 10 were previously added to dbSNP NCBI database (Table 1).

Replacement identified only in a heterozygous variant accounted for 34% of all

Table 1. The frequency of the *MyoD1* gene polymorphic alleles in Stavropol sheep breed

No	SNP name, HGVS nomenclature	Identifier in the NCBI database	Position in contig	Amino acid	Allele		Genotype		
1	c.-2112C>G	rs 404884444	34373234	–	G 0.97	C 0.03	GG 0.93	GC 0.07	CC 0.00
2	c.-1806A>G	rs 424553252	34372928	–	T 0.2	C 0.8	TT 0.13	TC 0.13	CC 0.73
3	c.-1687T>C	rs 406278149	34372809	–	A 0.3	G 0.7	AA 0.26	AG 0.07	GG 0.67
4	c.-1608C>T	Not in database	34372730	–	G 0.07	A 0.93	GG 0.00	GA 0.13	AA 0.87
5	c.-1603G>T	Not in database	34372725	–	C 0.07	A 0.93	CC 0.00	CA 0.13	AA 0.87
6	c.-1578G>A	Not in database	34372700	–	C 0.83	T 0.17	CC 0.67	CT 0.33	TT 0.00
7	c.-1235G>A	rs 412308724	34372357	-	C 0.6	T 0.4	CC 0.2	CT 0.8	TT 0.0
8	c.-910G>T	rs 591152513	34372032	–	C 0.9	A 0.1	CC 0.8	CA 0.2	AA 0.0
9	c.-909G>T	rs 601707240	34372031	–	C 0.9	A 0.1	CC 0.8	CA 0.2	AA 0.0
10	c.-880G>A	rs 412662330	34372002	–	C 0.83	T 0.17	CC 0.67	CT 0.33	TT 0.00
11	c.-637C>T	rs 409662616	34371759	–	G 0.83	A 0.17	GG 0.67	GA 0.33	AA 0.00
12	c.-412G>T	rs 420129038	34371534	–	C 0.933	A 0.067	CC 0.87	CA 0.13	AA 0.0
13	c.244C>T	Not in database	34370878	R\C	G 0.00	A 1.00	GG 0.00	GA 0.00	AA 1.00
14	c.246G>T	Not in database	34370876		C 0.00	A 1.00	CC 0.00	CA 0.00	AA 1.00
15	c.253G>T	Not in database	34370869	G\C	C 0.03	A 0.97	CC 0.00	CA 0.07	AA 0.93
16	c.259G>C	Not in database	34370863	G\R	C 0.00	G 1.00	CC 0.00	CG 0.00	GG 1.00
17	c.261C>T	Not in database	34370861		G 0.00	A 1.00	GG 0.00	GA 0.00	AA 1.00
18	c.269C>G	Not in database	34370853	P\R	G 0.00	C 1.00	GG 0.00	GC 0.00	CC 1.00
19	c.274C>A	Not in database	34370848	P\T	G 0.00	T 1.00	GG 0.00	GT 0.00	TT 1.00
20	c.276C>G	Not in database	34370846		G 0.00	C 1.00	GG 0.00	GC 0.00	CC 1.00
21	c.277C>A	Not in database	34370845	P\T	G 0.00	T 1.00	GG 0.00	GT 0.00	TT 1.00
22	c.279C>T	Not in database	34370843		G 0.00	A 1.00	GG 0.00	GA 0.00	AA 1.00
23	c.281C>A	Not in database	34370841	T\N	G 0.00	T 1.00	GG 0.00	GT 0.00	TT 1.00
24	c.287C>A	Not in database	34370835	A\D	G 0.00	T 1.00	GG 0.00	GT 0.00	TT 1.00
25	c.325T>C	rs 599663516	34370797	L	A 0.83	G 0.17	AA 0.67	AG 0.33	GG 0.00
26	c.483C>T	Not in database	34370639	A	G 0.00	A 1.00	GG 0.00	GA 0.00	AA 1.00

Table 2. The *MyoD1* gene haplotypes identified in Stavropol sheep breed. Homozygous mutant genotype is highlighted in black, the heterozygous – in gray, and the homozygous wild-type genotype – in white

No	Genotype	SNP								
		Upstream gene								
		c.-2112	c.-1806	c.-1687	c.-1608	c.-1603	c.-1578	c.-1235	c.-910	c.-909
1	A									
2	B									
3	C									
4	D									
5	E									
6	F									
7	G									
8	H 1									
	H 2									

	Genotype	SNP								
		Upstream gene			Exon II					
		c.-880	c.-637	c.-412	c.244	c.246	c.253	c.259	c.261	c.269
9	A									
10	B									
11	C									
12	D									
13	E									
14	F									
15	G									
16	H 1									
	H 2									

	Genotype	SNP							
		Exon II							
		c.274	c.276	c.277	c.279	c.281	c.287	c.325	c.483
17	A								
18	B								
19	C								
20	D								
21	E								
22	F								
23	G								
24	H 1								
	H 2								

found SNPs and they are mainly located in the 5` flanking region, with the exception of SNP c.325T>C located in exon 2.

The SNPs having both the heterozygous and mutant homozygous forms are found in 19% of cases and they are also located in the non-coding region of the gene. Substitutions detected in second exon are found in all tested animals only in homozygous form and constitute 46% of the total number of identified SNPs. From all detected SNPs, 42% led to a change of the encoded amino acids.

Among the detected mutations in the Stavropol breed transversions prevailed (54%). The highest frequency of mutant alleles among animals examined were detected for SNPs c.244C>T, c.246G>T,

c.253G>T, c.259G>C, c.261C>T, c.269C>G, c.274C>A, c.276C>G, c.277C>A, c.279C>T, c.281C>A, c.287C>A, c.483C>T, c.325T>C. All of them were located in the second exon. They were presented in the homozygous mutant variants, except for c.325T>C replacement. It was found in the heterozygous form. Frequencies of mutant alleles of SNPs c.-2112C>G, c.-910G>T, c.-909G>T were rare. They were found in less than 2% of rams. Replacements c.-1578G>A, c.-880G>A, c.-637C>T and c.325T>C were identified in the Stavropol breed only together, and only in the heterozygous form.

Nine out of the ten previously described mutations were in 5' flanking region and one (c.325T>C) was located in the second exon. The SNPs c.-1608C>T, c.-1603G>T, c.-1578G>A, detected for the first time, were located in the 5' flanking region. In the second exon, c.244C>T, c.246G>T, c.253G>T, c.259G>C, c.261C>T, c.269C>G, c.274C>A, c.276C>G, c.277C>A, c.279C>T, c.281C>A, c.287C>A and c.483C>T, also detected the first time in sheep, were found out.

The SNPs c.325T>A and c.483C>T are synonymous. Twelve SNPs led to amino acid replacements. Some of non-synonymous SNP's were located within one triplet. In the *MyoD1* gene four modified triplets were found. Inside one of the triplet the following pairs of replacements have occurred: c.244C>T and c.246G>T; c.259G>C and c.261C>T; c.274C>A and c.276C>G; c.277C>A and c.279C>T.

From the combination of mutations, the animals were divided into eight main groups (A-H) and two subgroups within the group H (Table 2). The phylogenetic tree of detected genotypes is shown on Fig. 1. Haplotypes A and B had the least replacements and were found in 7% of the animals. The greatest numbers of SNPs had haplotypes found in 27% of animals in groups H and E, identified in 7% of the animals. Haplotypes C, D, G had from 16 to 19 SNPs. The most common haplotype was F, detected in 33% of the rams.

Sequencing of *MyoD1* gene in Stavropol breed allowed to reconstruct the amino acid sequence of the encoded protein. Twelve out of twenty six found replacements lead to changes in the amino acid composition of protein. Structure of encoded MyoD1 peptide in Stavropol breed differed from reference MyoD1 sequence by presence of cysteine in positions 82 and 85, arginine in positions 87

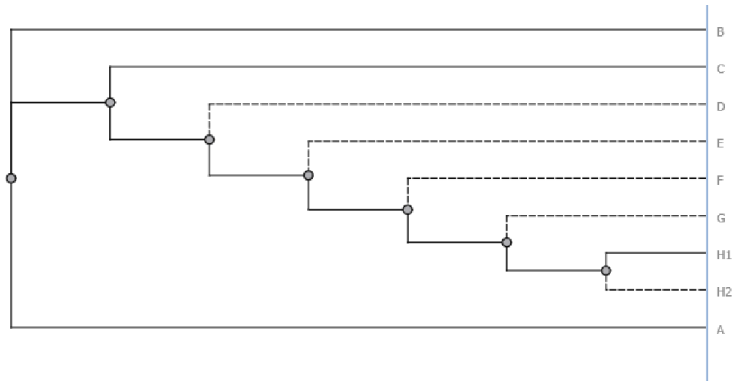

Fig. 1. Phylogenetic tree of detected haplotypes in Stavropol sheep.

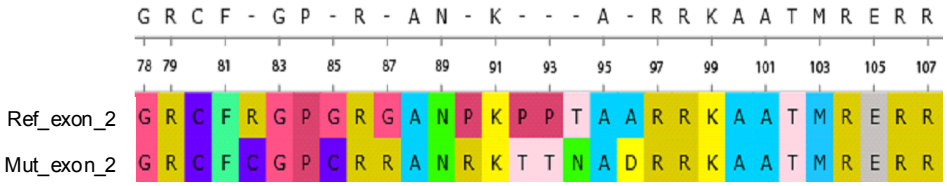

Fig. 2. Amino acids chains of the Myod1 protein in Stavropol sheep breed and reference (www. uniprot.org, 2016).

and 90, threonine at positions 92 and 93, asparagine in position 94, an aspartic acid in position 96 (Fig. 2).

The impact of replacements c.-1578, c.-880, c.-637, c.325 and c.-1235 on the ram's body parameters was studied to assess the possibility of using them as genetic markers in selection (Table 3). The complex of the four first SNPs are of interest to research because these replacements occurred only together in Stavropol sheep breed, indicating their linked inheritance. In addition, one of the replacements (c.325) was in the coding region of exon II. SNP c.-1235 occurred in a larger number of animals in the group than the previous four replacements. Heterozygous rams did not exhibit any significant difference in live weight with wild-type homozygotes. However, the chest girth, metacarpal girth and loin width in heterozygous rams was higher than in homozygotes. The study of described SNPs influence on the lifetime body parameters of rams showed almost no significant correlation between their presence and the size of the animals. Therefore, the use of SNP's c.-1578, c.-880, c.-637, c.325 and c.-1235 as a genetic marker in the selection of Stavropol breed of sheep is not allowed.

Unfortunately, we couldn't conduct a study on correlation between SNP's, accompanied by the replacement of amino acids in the peptide chain and body parameters. All these mutations in Stavropol were present only in the homozygous mutant variant. Therefore, within-breed comparison with individuals carrying the wild haplotype was not possible.

Perhaps SNPs c.-910 and c.-909 were associated with greater body parameter values, as they were found in animals with higher weight.

DISCUSSION

In our study, *MyoD1* gene structure was investigated for the first time and assessment of the identified polymorphisms in the body parameters in rams of Stavropol sheep breed was done. Genotyping of Russian sheep breeds has not been done earlier and there is no information on *MyoD1* gene structure. There are also no data of the effect of *MyoD1* gene structure on sheep body parameters in other countries.

The study of *MyoD1* gene structure identified regions of the DNA chain, which differed in the number of single nucleotide replacements. The greatest number of SNPs contained Exon II, the least variable was 5' flanking region.

Most SNPs found in the second exon are in the homozygous mutant variant. The exception was SNP c.325T>C, which was in the heterozygous form. Apparently, the carriers of mutant alleles differed by best qualities in the selection of pairs for

Table 3. Body measurements of rams with different *MyoD1* genotypes. W –the wild type allele; Mu –the mutant allele. Data are presented as mean ± SEM

Trait	Genotype					
	c.-1578, c.-880, c.-637, c.325			c.-1235		
	W/W n=20	W/Mu n=10	p value	W/W n=6	W/Mu n=24	P value
Live weight (kg)	44.99±2.07	48.16±1.76	0.23	42.57±1.44	46.92±1.76	0.06
Height at wither (cm)	49.34±2.33	52.42±1.90	0.29	46.73±1.81	51.28±1.96	0.09
Height at croup (cm)	47.94±2.26	50.92±1.85	0.29	45.40±1.77	49.82±1.91	0.09
Width at croup (cm)	68.00±0.89	67.80±0.74	0.86	67.67±2.04	67.83±0.81	0.93
Length of croup (cm)	70.20±0.98	70.04±0.94	0.88	69.67±2.04	70.25±0.78	0.77
Carcass length (cm)	16.60±0.28	16.80±0.65	0.77	16.33±0.41	16.75±0.32	0.39
Chest width (cm)	22.70±0.79	24.00±0.61	0.18	22.00±1.41	23.42±0.64	0.35
Chest depth (cm)	79.90±1.47	81.40±1.92	0.51	80.00±1.22	80.50±1.39	0.77
Chest girth (cm)	22.70±0.63	23.80±0.82	0.27	21.67±0.41	23.42±0.57	0.02
Metacarpal girth (cm)	31.30±0.57	32.20±0.42	0.19	30.02±0.71	32.08±0.41	0.04
Metacarpal length (cm)	88.80±1.02	89.80±2.68	0.71	86.67±2.04	89.75±1.15	0.20
Metatarsus length (cm)	9.50±0.24	9.00±0.35	0.23	9.67±1.08	9.33±0.20	0.74
Loin width (cm)	15.70±0.42	15.40±0.27	0.53	14.33±0.41	15.92±0.27	0.01
Width of back (cm)	17.01±0.44	16.40±0.22	0.21	16.02±0.71	17.10±0.34	0.22
Half girth of back (cm)	13.20±0.34	13.60±0.25	0.35	13.00±0.69	13.42±0.27	0.56

crossing. That led to the consolidation of mutations in Stavropol breed in homozygous form.

Four SNPs: c.-1578G>A, c.-880G>A, c.-637C>T and c.325T>C in Stavropol breed of sheep were found in the heterozygous form and only together, which may indicate their linked inheritance. Three of them (c.-1578G>A, c.-880G>A, c.-637C>T) were identified for the first time.

In Ensembl database we found information about the frequency of occurrence of single nucleotide replacements in *MyoD1* gene in Iranian and Moroccan breeds of sheep, which were close to Stavropol breed of sheep. In Stavropol rams the SNP c.-2112C>G in the flanking region occurs with a frequency of 3%, which is lower than the Iranian (10%) and Moroccan (19%). SNPs c.-910G>T, c.-909G>T in Stavropol and Iranian breeds are found with the same frequency (10%), which is by 5% higher than that in the Moroccan. The frequency of replacement c.637C>T in Moroccan sheep is by 12% lower than in Stavropol and Iranian breeds (17%). c.-1235G>C replacement in Moroccan breed occurs with a frequency of 32%, which is lower than that of the Stavropol (40%) and Iranian (37%) breeds. The replacement c.-880G>A with a frequency of 17% is higher than in Iranian (8%) and Moroccan (3%) breeds. The SNP c.325T>C located in exon II of Stavropol breed has a same frequency of occurrence as those of Moroccan and Iranian breeds (Anonymous, 2016).

This indicates the need for further research of encoded peptide features. The linked pairs of SNP's inside coding nucleotide triplets are c.-244C T and c.-246G>T; c.-259G>C and c.-261C>T; c.-274C>A and c.-276C>G; c.-277C>A and c.-279C>T, which are accompanied with non-synonymous amino acid changes in MyoD1 protein. As these SNP have been detected for the first time, this provides evidence for the unique structure of the MyoD1 protein in the Stavropol breed. Replacements c.325T>C and c.483C>T in exon II are associated with synonymous amino acid replacements, but may be related to functional characteristics of the encoded peptide. Previously, some cases of SNP's present in coding regions of proteins with identical amino acid sequence, which differ in functional and structural parameters of proteins, were described (Komar, 2007).

In all investigated animals, we found a unique structure of the *MyoD1* gene. It is accompanied by changes in the amino acid composition of the encoded protein in Stavropol sheep breed, which had not been detected in any other sheep breeds. However, in our study we were unable to study its influence on meat production. In this case one way to detect the influence of gene structure on meat quality is a breeding hybrid with another breed, which will carry heterozygous haplotype of these SNP's. Study of mass indexes and body size of the obtained heterozygous offspring compared with the native wild haplotype allows detecting the effect of the unique structure of *MyoD1* gene in Stavropol sheep breed.

In our study, SNPs c.-910 and c.-909 occurred in a very small number of animals, which did not allow making definitive conclusions. In the future, their influence on the lifetime and slaughter yield indicators of sheep productivity will be further investigated in detail.

CONCLUSION

The study shows a significant difference of *MyoD1* gene in Stavropol breed of

sheep from the reference gene variant, the changes relating to the coding regions. During our work, we found 26 SNPs, 16 of these were discovered for the first time and were not entered into dbSNP NCBI. The study of the effect of described SNPs on the body parameters of rams showed the absence of correlation between their presence and the size of the animals. It is not possible to recommend the use of SNP c.-1578, c.-880, c.-637, c.325 and c.-1235 as a genetic marker in the selection of Stavropol breed of sheep.

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