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The polymorphisms of *MyoD1* gene in Manych Merino sheep and its influence on body conformation traits

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ABSTRACT. Candidate genes associated with meat productivity often affect the performance of myostatin and muscle development in general. The *MyoD1* gene is a member of the myogenic regulatory factor (MRF) family and plays a key role in the differentiation of skeletal muscle cells in vertebrates. We investigated the structure of the *MyoD1* gene. The effect of polymorphisms on meat production was studied in the Russian sheep breed Manych Merino. To detect alleles, we used NimbleGen sequencing technology (Roche, USA). In the Manych Merino breed, we found 14 single nucleotide polymorphisms (SNPs) associated with substitutions, all in the second exon, namely, 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. All of these SNPs, except from c.325T>C, were detected here for the first time. The Manych Merino breed had 13 substitutions that were present as homozygous type. Only SNP c.325T>C occurred as the wild type of homozygotes and heterozygotes in the ratio 2:1. We found that SNP c.325T>C was associated with some vital body parameters, including parameters of height and croup measurement. Thus, the determination of allelic variants of the *MyoD1* gene may be used in marker assisted genetic selection schemes.

Keywords: *MyoD1* gene, myoblast determination protein, Manych Merino, SNP, meat and carcass traits

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INTRODUCTION

Marker assisted selection is a modern and promising method for accurate assessment and prediction of breeding value and productive potential of animals. It can be used to accelerate selection of animals with desired traits and reduce costs associated with traditional selection methods (Hagen et al. 2005).

Among several genes responsible for the meat quality of farm animals, the myostatin gene takes a leading position (Gan et al. 2008). However, an increase in muscle mass is often unlinked to changes in the coding region of myostatin. Therefore, interest has turned towards candidate genes affecting the performance of myostatin and muscle development in general (Megeney et al. 1996; Hagen et al. 2005).

One of these genes is *MyoD1,* which is a member of the myogenic regulatory factor (*MRF*) gene family (Busanello et al.2012). This family of genes also includes *Myf5*, myogenin, and *MRF4*. Members of the *MRF* family are basic helix-loop-helix (bHLH) transcription factors (Buckingham 1992). They play a key role in the differentiation and structure of skeletal muscle in vertebrates. *MyoD1* has the potentiality 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 remodeling, DNA binding, nuclear localization, and heterodimerization (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 bind to the E-box (CANNTG) locus. This locus is found in most of the regulatory regions of muscle-specific genes, including myostatin (Murre et al. 1989; Zhang et al. 2006).

It has already been shown that the *MyoD* gene interacts with the promoter of the myostatin gene (Du et al. 2007; Deng et al. 2012), a muscle specific creatine kinase gene (Murre et al. 1989). The *MyoD* gene plays a critical role in the regulation of postnatal myogenic programming of satellite muscle cells. Thus, mice with a knockout mutation in *MyoD1* have severely reduced regenerative capacity after injury (Megeney et al. 1996). High levels of MyoD protein inhibit proliferation of satellite muscle cells and leads to either myogenic differentiation or apoptosis (Asakura et al. 2007; Pan et al. 2015).

In pigs, a positive correlation between the level of expression of *MyoD1* and the cold carcass yield was found (Lobo et al. 2012). In chickens, a mutation in both *MyoD* and *Mrf4* genes was associated with an increase in the diameter of muscle fibers. These genes are recommended as molecular markers for marker-assisted selection (Yang et al. 2015). The relationship between mutations in *MyoD1* and indicators of meat quality in sheep has not been studied.

One of the sheep breeds raised in Russia is the Manych Merino breed, which was first bred in 1983. This breed is characterized by high levels of wool production and good meat quality (Babichev and Moroz 1992). Manych Merino sheep are widely used in different climates under different farming systems. Their distinguishing feature is their stable breeding quality and high productivity in the arid steppe zone of the North Caucasus (Surov and Aboneev 2009).

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

MATERIALS AND METHODS

Animals

The investigation was carried out on 30 randomly selected Manych Merino rams at the age 376±3 days 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 analysed in order to describe meat production. Live weight was measured by using electronic scales. Height at wither and croup, length of croup and carcass, girth

of chest and metacarpal, metacarpal and metatarsus length, half girth of back was measured by using a measuring band. Width at croup, chest width and depth, loin width and width of back were measured by metal caliper.

Sample collection-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 stabilizer 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, target 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 undergo 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., Madison, WI, USA).

Sequencing was performed using a GS Junior genomic sequencer (Roche NimbleGen, Inc., Madison, WI, 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, WI, USA).

To describe a single nucleotide polymorphism (SNP) we used HGVS nomenclature (www.hgvs.org). We used this nomenclature based on transcript ENSOART00000027076 (www.ensembl.org).

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 detected if $p<0.05$.

RESULTS

During sequencing, 14 SNPs in the *MyoD1* gene of Manych Merino sheep were found (Table 1). All of the SNPs were located in the coding region in exon 2. Eight of the detected SNPs caused substitutions in amino acid coding triplets: six did not alter the coding triplets and two lead to the substitution of amino acids to the alternative.

Thirteen of the identified SNP were not registered into the dbSNP database. In Manych Merino sheep, most of these single nucleotide substitutions are only present as homozygous for the mutant variant. For SNP c.325T>C, the population has both homozygotes and heterozygotes, with twice as many homozygotes as heterozygotes.

Researching variants of genotypes of sheep depending on combinations of presented SNP in the *MyoD1* gene allowed to allocate only one substitution that defines the differences in allele - c.325T>C. Despite the fact that it is synonymous and does not change the encoded amino acid, it makes sense to consider its use as a marker for certain genotypes. In regards to this SNP, the examined animals can be divided into two genotypes—homozygous for the wild type allele (n = 20) and heterozygotes (n = 10).

The comparison of vital parameters of meat productivity of rams with different alleles of the *MyoD1* gene showed that the presence of SNP c.325T>C is not associated with any significant change in val-

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

	Name of SNP in HGVS nomenclature	Amino-acid	Identifier in the NCBI/ Ensemble database	Position in contig	Allele		Genotype		
1	c.244C>T	R/W	Not in database	34370878	G	A	GG	GA	AA
					0	100	0	0	100
2	c.246G>T	R	Not in database	34370876	C	A	CC	CA	AA
					0	100	0	0	100
3	c.253G>T	G/C	Not in database	34370869	C	A	CC	CA	AA
					0	100	0	0	100
4	c.259G>C	G/R	Not in database	34370863	C	G	CC	CG	GG
					0	100	0	0	100
5	c.261C>T	G	Not in database	34370861	G	A	GG	GA	AA
					0	100	0	0	100
6	c.269C>G	P/R	Not in database	34370853	G	C	GG	GC	CC
					0	100	0	0	100
7	c.274C>A	P/T	Not in database	34370848	G	T	GG	GT	TT
					0	100	0	0	100
8	c.276C>G	P	Not in database	34370846	G	C	GG	GC	CC
					0	100	0	0	100
9	c.277C>A	P/T	Not in database	34370845	G	T	GG	GT	TT
					0	100	0	0	100
10	c.279C>T	P	Not in database	34370843	G	A	GG	GA	AA
					0	100	0	0	100
11	c.281C>A	T/N	Not in database	34370841	G	T	GG	GT	TT
					0	100	0	0	100
12	c.287C>A	A/D	Not in database	34370835	G	T	GG	GT	TT
					0	100	0	0	100
13	c.325T>C	L	rs599663516/ ss1139613360	34370797	A	G	AA	AG	GG
					83.3	16.7	66.6	33.3	0
14	c.483C>T	A	Not in database	34370639	G	A	GG	GA	AA
					0	100	0	0	100

Table 2. Body measurements of rams with different *MyoD1* genotypes (n represents the number of animals; W represents the wild type allele; Mu represents the mutant allele; significantly different if p<0.05).

	Trait	SNP c.325 genotype		P Value
		W/W, M±m (n=20)	W/Mu, M±m (n=10)	
1.	Live weight (kg)	58.86±1.20	55.82±1.19	0.075
2.	Height at wither (cm)	74.20±0.70	69.02±1.80	0.027
3.	Height at croup (cm)	76.20±0.86	72.60±0.76	0.005
4.	Width at croup (cm)	20.50±0.67	18.80±0.42	0.039
5.	Length of croup (cm)	24.70±0.32	23.04±0.35	0.003
6.	Carcass length (cm)	86.30±0.99	84.06±0.61	0.055
7.	Chest width (cm)	27.40±0.61	26.60±0.76	0.392
8.	Chest depth (cm)	31.80±0.26	31.04±0.52	0.165
9.	Chest girth (cm)	102.00±1.04	103.80±1.64	0.339
10.	Metacarpal girth (cm)	9.80±0.14	10.40±0.84	0.472
11.	Metacarpal length (cm)	14.90±0.11	15.80±1.08	0.407
12.	Metatarsus length (cm)	17.20±13.40	17.06±1.02	0.837
13.	Loin width (cm)	13.40±0.17	13.62±0.27	0.516
14.	Width of back (cm)	23.70±0.39	22.81±0.55	0.177
15.	Half girth of back (cm)	70.30±0.99	73.40±1.52	0.098

M – mean; m – standard error of mean.

ues of external measurements and indicators of live weight (Table 2). However, different alleles were associated with significant difference in parameters such as height at wither, height at croup, width at croup and length of croup.

The presence of the heterozygous c.325T>C SNP was associated with a significant reduction of height at wither by 6.7 %, compared with the wild type homozygotes. For heterozygotes, height at croup was significantly decreased by 4.5 % compared to wild type homozygotes. Width at croup was significantly increased by 7.9 % for wild type homozygotes compared to heterozygotes. Length of croup was also reduced by 7.7 % in heterozygotes compared to wild type homozygotes.

Thus, we have found a relationship between the presence of an allele of the gene in *MyoD1* with SNP c.325T>C and the parameters of height and croup measurement in Manych Merino rams.

DISCUSSION

Investigation of the *MyoD1* gene structure revealed a number of variable spaced single nucleotide polymorphisms. Most of the revealed SNPs are in the homozygous form. An exception is the substitution of c.325T>C, which is either in a homozygous form with a base analogous to reference genome OAR 3.1, or in a heterozygous form.

Unfortunately, making a comparative analysis of the influence of certain mutations to the parameters of meat productivity in Manych Merino sheep is not possible. This is because the animal sample contains only those with the presence of the SNP, so there is no opportunity to compare with the heterozygous and homozygous

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        10         20         30         40         50
MELLSPPLRD VDLTGPDGSL CNFATADDFY DDPCFDSPDL RFFEDLDPRL uniprot.org
MELLSPPLRD VDLTGPDGSL CNFATADDFY DDPCFDSPDL RFFEDLDPRL MM

        60         70         80         90        100
VHVGALLKPE EHSHFPAAAH PAPGXXXGRC FRGPGRGANP KPPTAARRKA uniprot.org
VHVGALLKPE EHSHFPAAAH PAPGXXXGRC FWGPCRRANR KTTNADRRKA MM
       110        120        130        140        150
ATMRERRRLS KVNEAFETLK RCTSSNPNQR LPKVEILRNA IRYIEGLQAL uniprot.org
ATMRERRRLS KVNEAFETLK RCTSSNPNQR LPKVEILRNA IRYIEGLQAL MM
       160        170        180        190
LRDQDAAPPG AAAAFYAPGP LPPGRSGEHY SGDSDASSPR SNCSDGMV   uniprot.org
LRDQDAAPPG AAAAFYAPGP LPPGRSGEHY SGDSDASSPR SNCSDGMV   MM
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Figure 1. Comparison of the reference amino acid sequence of the peptide MyoD1 (uniprot.org, 2014) with the peptide at the Manych Merino (MM) sheep breed

wild type animals. Therefore, estimation of the influence of *MyoD1* variants on sheep meat productivity was conducted with only one SNP (c.325T>C).

It is worth noting that only the SNP c.325T>C among the revealed SNPs in sheep has been registered into the dbSNP NCBI database. In Manych Merino sheep, the percentage of nucleotides A/G (83.3/16.7) was close to that of Iranian sheep (80/20) and Moroccan sheep (85/15) (Ensembl project. NextGen Project populations, www.ensembl.org, 2014).

SNP c.325T>C is synonymous and does not alter the encoded amino acid. However, it has been shown that certain SNPs with amino acid sequence identity may differ in structural and functional parameters, and alter the structure of substrate and inhibitor interaction sites (Yang et al. 2015).

The rest of the 13 SNPs found here are novel and unique in the Manych Merino breed. All of them are located in the coding region of the gene, and lead to changes in the amino acid sequence of the MyoD1 protein. Figure 1 shows the differences in the structure of the encoded peptide in Manych Merino sheep compared to the reference sequence of the MyoD1 peptide (*Ovis aries* (Sheep), www.uniprot.org, 2014).

Such a gene structure in Manych Merino sheep indicates that 13 of the SNPs we have identified in the genome have been present for a long time and during breeding, the wild form of *MyoD1* was actually eliminated from the population. Apparently, carriers of mutant alleles have advantages in terms of mate choice, which led to the prevalence of mutations in the homozygous form.

Investigating the effect of the c.325T>C SNP on vital parameters of meat productivity in sheep gave mixed results. Despite the fact that most of the measurements, including weight of rams, do not depend on the presence of the substitution in the genome, animals with heterozygous genotype were significantly shorter in height and had narrower croups. As we investigated only vital body conformation traits, an indirect indicators of meat productivity of sheep, it is possible that the presence of the c.325T>C SNP has a more pronounced effect on the quality of the meat. The same live weight may have different combinations of bone skeleton, muscles and internal

organs, which calls for more in-depth study of the impact of this mutation on slaughter indicators of meat productivity.

CONCLUDING REMARKS

The investigation shows a significant difference in the *MyoD1* gene in Manych Merino sheep compared to the reference gene variant, with the changes located in the coding region. During this study, we found 14 SNPs, 13 of which were detected for the first time and not present in the dbSNP database. We found a correlation between certain vital body conformation traits of sheep with the c.325T>C mutation, which calls for further study of the impact of this SNP on meat quality.

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CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest to declare.

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