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Sequence analysis of the 3′UTR region of the myostatin gene in Ukrainian Carpathian Mountain sheep

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Preservation and rational use of the sheep gene pool is a highly urgent issue in the development of sheep breeding. Modern methods of selection and breeding work enable the realisation of genetic potential for increased productivity and breeding value in farm animals. Preservation of the valuable gene pool of the Ukrainian Mountain Carpathian sheep breed should be carried out using scientifically based methods that allow for the protection of the breed's valuable traits, such as high adaptive capacity to the conditions of the mountain region and the dairy and meat direction of productivity. The myostatin (*MSTN*) gene is considered as a marker for improving economically useful traits in livestock. The sequence of 1093 bp of the 3′-untranslated region (3′UTR) of the Ukrainian Mountain Carpathian sheep myostatin gene was analysed to search for potentially beneficial mutations in the gene. Several of eight known SNPs that are polymorphic in Latvian dark-head, Merinolandschaf, Ile de France, Texel, Charollais, Poll Dorset, White Suffolk, Lincoln, Kamieniec, Pomeranian, Colored Polish Merino were found to be monomorphic in the Ukrainian Carpathian Mountain breed. The following genotypes were identified: c.*707TT, c.*709AA, c.*874TT, c.*1098AA, c.*1232GG, c.*1267AA, c.*1316GG, c.*1489GG in the Ukrainian Carpathian Mountain breed. In conclusion, it should be noted that marker-associated selection of the 3′-untranslated region of the myostatin gene (3'UTR) is not suitable for Ukrainian Carpathian Mountain sheep due to the confirmed monomorphism of the studied SNPs in this region. Based on the obtained results, we concluded that it is important to continue studying other regions of the *MSTN* gene, including a larger number of animals and phenotype data. This will help to identify specific mutations within the gene that may serve as potential genetic markers for improving economically important traits in Ukrainian Carpathian Mountain sheep.

Key words: *MSTN*, myostatin, 3′UTR, Ukrainian Carpathian Mountain Sheep-breed, sheep

Introduction

The Ukrainian Carpathian Mountain Sheep (UCM) breed of sheep belongs to the local breeds of sheep in the Western region of Ukraine. This breed is kept in the farms located at the foothill and mountainous regions of the Transcarpathian, Ivano-Frankivsk, Lviv, and Chernivtsi regions. The UCM breed of sheep was bred by the method of reproductive crossing of local coarse-wool ewes of the Tsakel type with rams of the Tsygai breed.

In the mountainous areas of the Carpathians, aboriginal coarse-wool sheep with wool of various colors have been bred for a long period of time and were used for the production of carpet wool. Smooth and pile carpets, artificial fur, coat fabrics, knitwear, and other products were also manufactured [21].

As a breed, the UCM breed began its development in 1993 with the order from the Ministry of Agriculture and Food of Ukraine dated December 31, 1993 no. 363. Despite the unique characteristics of sheep of this breed and their ability to adapt to difficult environmental conditions, the number of livestock has been decreasing uncontrollably over the past 20 years.

According to the database of the Ukrainian Animal Breeding Center, the population of animals of the UCM breed decreased sharply in 2023, from 1,288 sheep in 2019 to 296 in 2023 [1]. Such a rapid decrease is explained by the low profitability of breeding.

Today, 9 sheep breeds have already disappeared without a trace in Ukraine: Walahian, Pirni, Reshetilivska, Chushka, Mazayev Merino, Malich, Hutsulian, Chuntuk [22]. The long-term crisis in sheep breeding and the active war phase in the territory of Ukraine exacerbate this situation even more.

The Carpathian sheep breed played an important role in the historical development of the Carpathian region, preserving cultural crafts, traditions, and the material culture of local communities. Sheep breeding was the main animal husbandry activity of the local Hutsulian population and had a great influence on the entire lifestyle and culture, which over the centuries formed a specific structure of economic activity in the region [4].

The Carpathian Mountain sheep are characterized by a high foraging ability. The selection of these animals was aimed at securing the maximum possible meat productivity when using mainly green pasture fodder. Under the conditions of feeding them with concentrated feed at the rate of 0.4–0.5 kg per head, the young animals reach slaughter conditions (up to 28 kg) by the age of six months. It typically takes 4.1–5.1 feed units to obtain 1 kg of growth in lambs up to 6 months of age, and 7.9 feed units in sheep up to one-year-old [23].

At the same time, the Mountain Carpathian sheep are relatively small — for example, breeding rams have a live weight of 55–65 kg, and ewes have a weight of 36–41 kg. It is important to note that there is a positive relationship between live weight and milk yield of ewes. With an increase in body weight, the milk yield also increases. With a live weight of ewes between 26–30 kg, their milk yield is 71 kg, and with a live weight of 36 kg — 90 kg or higher [3].

A literature analysis over the past 10 years showed that the UCM breed is practically unstudied in terms of genetics. Only in 2018, Chokan and co-authors described the genetic structure of the UCM breed using 11 microsatellite loci. Their analysis indicated significant genetic variability of the studied microsatellite loci. A total of 106 alleles were identified, and the average value of the inbreeding coefficient had a low negative value (0.070), which suggested almost no inbreeding in the studied UCM population [5].

In view of the above context, the urgent issue of developing new breeding methods using molecular genetic markers to increase the meat productivity of sheep is emerging, which will certainly increase the economic potential of the sheep industry in Ukraine. The myostatin gene (*MSTN*) is considered a potential genetic marker for improving economically important traits in livestock due to its well-established role in the negative regulation of muscle growth. Variations in *MSTN*, particularly in regulatory regions such as the 3′UTR, have been associated with enhanced muscle mass, feed efficiency, and overall carcass quality, making it a valuable target for marker-assisted selection in breeding programs [10, 13].

And it is specifically the 3′UTR region of the myostatin gene in the UCM sheep that interests us.

Materials and Methods

The studies were carried out on 14 purebred Ukrainian Carpathian Mountain sheep of both sexes (three rams and eleven ewes), which were bred on the farm “Gafynets” (Pavshino village, Mukachevo district, Transcarpathian region). Blood samples were collected from the sheep’s jugular vein in blood collection tubes with an EDTA anticoagulant.

For genomic DNA extraction, we used a DNA extraction kit (*Zymo Research*, USA) according to the manufacturer’s instructions for extraction from whole blood, serum, and plasma samples. DNA quality and quantity were assessed using agarose gel electrophoresis and spectrophotometry techniques. DNA yields were quantified using a *Nanodrop* spectrophotometer (*Thermo Fisher Scientific Inc.*, USA).

Sequence information for the sheep *MSTN* gene (AY918121, AM992883, and AF393618) available in GenBank was used for the genomic region reconstruction and primer design. *MSTN* gene sequence information for *Oryx dammah* (XM_040227852.1), *Bos taurus* (JQ711180.1), *Cervus elaphus* (OU343110.1), *Capra hircus* (EF591039), *Equus asinus* (XM_014837576.3), and *Sus scrofa* (AY208121.1) was used in the bioinformatic analysis.

A 1.093 kb myostatin gene fragment containing the 3′UTR sequence was amplified by polymerase chain reaction (PCR) from the UCM sheep breed genomic DNA.

The structure of primers: forward: 5′-GCTGAATGGCTGATGTTATC-3′; reverse: 5′-AGCAACTTGACCAGAACCAATGT-3′. The PCR reaction was performed in a 50 µl reaction containing 100 ng of sheep genomic DNA, 1× Taq reaction buffer, 5 nmol dNTPs, 20 pmol of each primer, and 0.25 units of Taq DNA polymerase (*New England Biolabs*).

The PCR program was carried out with an initial 5 min denaturing step at 94°C, followed by 35 cycles (each cycle included 30 s at 94°C, 30 s at 57.5°C, and 1 min at 68°C), and a final 10 min extension at 72°C.

PCR products were then sequenced by the Sanger sequencing method at *Explogen LLC* (Lviv, Ukraine), and the chromatograms were analyzed using *FinchTV* software (*Geospiza Inc.*) for the identification of possible mutations in the 3′UTR of *MSTN*.

Clustal Omega Multiple Sequence Alignment (MSA) was used to generate the sequence multiple alignments [7].

Results and Discussion

The study sequenced of the 3′UTR region of the *MSTN* gene with a length of 1093 bp, localized from 118150011 to 118151103 (fig. 1).

Alignment analysis of the myostatin 1.093 kb sequence of the 3′UTR of myostatin from the NCBI revealed a high degree of its conservation during evolution. The similarity of the 3′UTR region of the myostatin gene between sheep and antelope (*Oryx dammah*, XM_040227852.1), red deer (*Cervus elaphus*, OU343110.1), cattle (*Bos taurus*, JQ711180.1), pig (*Sus scrofa*, AY208121.1), and donkey (*Equus asinus*, XM_014837576.3) was found to be 98.08 %, 97.00 %, 96.80 %, 91.28 %, and 89.34 %, respectively.

The alignment results indicate that this region is quite conserved, which may primarily be associated with its functionality (fig. 2).

For the first time, data on the significant impact of mutations in the 3′UTR of the sheep myostatin gene were published in 2006. The author demonstrated an important role in the regulation of myostatin expression through target sites for microRNAs [6] demonstrated, through target sites. MicroRNAs (miRNAs) are a class of endogenous, short non-coding RNAs about 22 nucleotides in length [9]. There are more than twenty thousand miRNAs identified, and their number is rapidly increasing [18, 20].

The mutations in the Texel's 3′UTR of the *MSTN* (myostatin) gene create a potential target site for miR-1 and miR-206, so its expression is inhibited and the suppression of skeletal muscle growth is removed. Therefore, this sheep breed shows a double-muscled phenotype [6]. It has also been shown that there are regions targeted by miR-27b in the 3′UTR region. According to Zhang et al., miR-27b contributes to the proliferation of sheep skeletal muscle satellite cells, targeting *MSTN* and suppressing its expression. Furthermore, miR-27b controls *MSTN* in ovine satellite cells by suppressing its translation at the initial stage, followed by mRNA degradation [25].

According to the *Ensembl* database [8], 8 polymorphisms are currently known in the studied region (1093 bp). These polymorphisms occur with individual frequencies in different breeds (table). The most studied SNP in the 3′UTR is rs408469734 (c.*1232G>A). It is worth noting that this polymorphism is also found in various publications under other names, in particular: g.9827G>A, g.+6723G-A, c.2360G>A [13].

Polymorphism at this locus occurs in crossbred sheep (Blue-faced Leicester × Scottish Blackface [19]), in Texel and Charollais, in Poll Dorset × White Suffolk rams and AG or GG White Suffolk × (Border Leicester × Merino) dams [14], in Australian Texels, White Suffolk, Poll Dorset, and Lincoln [16], New Zealand Texel-cross sheep [15], and in Polish breeds such as Kamieniec and Pomeranian sheep [17], and in Charollais and Texel sheep bred in Latvia [24].

On the other hand, the Colored Polish Merino sheep were monomorphic for G at the c.*1232 position [10]. No polymorphism was found at this locus in the Latvian dark-head, Merinolandschaf, Ile de France, Dorper [24], NZ Romney, Coopworth, Corriedale, Dorper, Perendale, Suffolk, Merino, Dorset Down, Coopdale, and Poll Dorset [12]. Summarizing the literature data, it can be concluded that this SNP c.*1232G>A is associated with various phenotypic traits related to economic benefits, in particular body weight at birth, average daily gain, dry matter intake, slaughter weight [24], loin and fore shank weights [10], proportion and muscle-to-bone ratio, muscle-to-fat ratio [19], carcass composition, and the proportional weights of the loin and hindquarter muscles [14].

Several of eight known SNPs that are polymorphic in the Latvian dark-head, Merinolandschaf, Ile de France, Texel, Charollais, Poll Dorset, White Suffolk, Lincoln, Kamieniec, Pomeranian, and Colored Polish Merino were monomorphic in the Ukrainian Carpathian Mountain breed. The following genotypes were identified: c.*707TT, c.*709AA, c.*874TT, c.*1098AA, c.*1232GG, c.*1267AA, c.*1316GG, c.*1489GG in the Ukrainian Carpathian Mountain breed.

Thus, the sequencing of the 3′-untranslated region of the myostatin gene in the Ukrainian Carpathian Mountain sheep revealed complete monomorphism across eight known SNP loci, including c.*1232G>A, which has been previously linked to economically important traits in other sheep breeds. While the absence of genetic variation in the studied region suggests that this particular fragment may have limited utility for marker-assisted selection in this population, this conclusion should be interpreted with caution. The small sample size may not fully reflect the genetic diversity of the breed, and the study did not include phenotype-genotype association analysis, which is essential for assessing the functional significance of genetic markers. Moreover, focusing solely on the 3′UTR excludes the possibility that other regions of the *MSTN* gene, such as exons, introns, or promoter regions, may harbor functional polymorphisms with relevance for selection.

GCTGAATGGCTGATGTTATCAGGTTTATCAAGCAAAAAACATTCAGTAAAGTAATAAGTTTCTCCTTTCTTCAGGTGCATTTTTACACTC
CTCCCTATGGGCAATGGATTTTCCATAAAGAAAGAAAAANCMTTTTTCTAGAGGTCTACATTCAATTCTGTAGCATACTTGGAGAAGCTGT
GTTTAAAAGGCAGTCAAAAAGTATTCATTTTTGTCAAAATTTCAAAATTATAGCCTGCCTTTGCAATACTGCAGCTTTTAGGATGAAATAATG
GAAATGACTGATTCTATCAATAKTGTATAAAAAGATTTTGAAAACAGTTGCATTTATATAATATGTATACAATATTGTTTTGTAAATAAATGTCT
CCTTTTTTATTTACTTTGGTATATTTTTACAGTAAGGACATTTCAAATTAAGTATTAAGGCACAAAGACATGTCATGTGGGACATAAAAGCAA
ATGCTTATATTTTGGAGCAAATTAGTTGATTAAATAGTGGTCTTAAAACTCCATATGCRAATGGTTAGATGGTTATATTACAATCATTTTATATT
TTTTTACATTGTTAGCATTCACTTATGGGTTCGTGATGGCTGTATAATGTGAATGTGAAATTTCAATGGTTTACTGTCATTGTATTCAAATCT
CAACRTTCCATTATTTTAATACTTATAAATATTAAGCAARCCAAAATGATTTAACTCTATTATCTGAAATCAGAATAATAAACTGATGRTATCTT
ACAAATTGTTAATTTTATTTTATAATTTGATAATGAATATATTTCTGCATATATTTACTACTATTTTGTAAATTAGGATTTTGTTAATCAAATAAATT
GTACTTATGATTAAGTGAAATTATTTCTTACATCTAATGTGTAGAAACAATGTAAGTTATATTAAAGTRTTTTCACCTTTTTTGAAAGACAACA
GTTTTAGGTTATAATGATTAACTCTAGATTTCTGGCTTCCACTTTATTATAAAAGTTTAAGGACTGAGCACAAAAGTTTGGTTTAGAAATGTT
AGGTCTGCTACTCTAGTTTCTCATGGGTGAAATTCCTGTTACATTGGTTCTGGTCAAGTTGCT

Fig. 1. The work sequence of the DNA. The forward and reverse primers and positions where single nucleotide substitutions could potentially occur are underlined and highlighted: **K** — G or T, **M** — A or C, **N** — A or C or G or T, **R** — A or G. Information about the polymorphisms was obtained from the *Ensembl* database [8]).

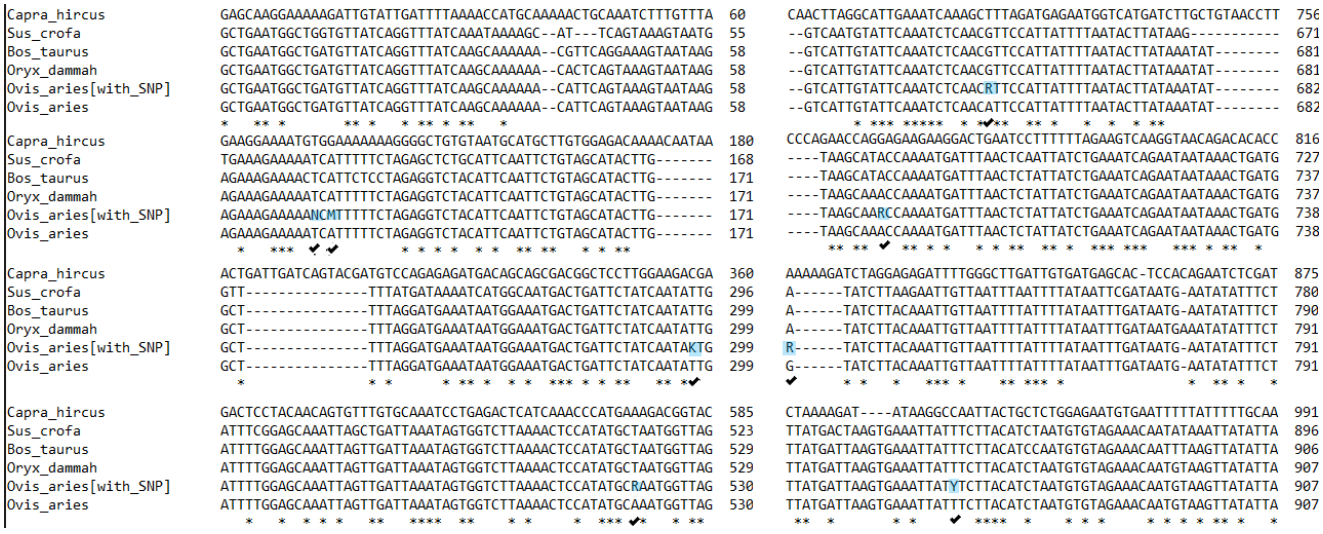

Fig. 2. The *MSTN* gene partial sequence alignment between species. Single-nucleotide polymorphisms (SNPs) in the 3′UTR region are described in sheep using single letter code: **K** — G or T, **M** — A or C, **N** — A or C or G or T, **R** — A or G. GenBank (NCBI) accession numbers of the *MSTN* gene sequences used in the study are indicated in the Materials and Methods section.

Table. Sheep breeds and *MSTN* gene polymorphisms located in the 3′UTR (1093 pb)

ID	Localization	Mutation	Alternative name	Breed in which the mutation was detected	Reference
rs591795591	2:118150140	T/-	c.*707DelT	Latvian dark-head Merinolandschaf, Ile de France and Texel (TEX)	[24]
rs414527527	2:118150142	A/C	c.*709A>C	Charollais	[24]
rs1093989187	2:118150307	T/G	c.*874T>G	—	[8]
rs430092736	2:118150531	A/G	c.*1098A>G	—	[8]
rs408469734	2:118150665	A/G	c.*1232G>A g.9827G>A, g, +6723G>A, c. 2360G>A	Texel, Charollais, Poll Dorset White Suffolk, Lincoln, Kamieniec, Pomeranian, Colored Polish Merino	[10, 11, 12, 14, 16, 17, 19, 24]
rs1088525878	2:118150700	A/G	c.*1267A>G	Romney	[8]
rs419982449	2:118150749	G/A	c.*1316G>A	Charollais	[24]
rs592881811	2:118150922	G/A	c.*1489G>A	Ouled Djellad	[8]

It is also worth noting that while polymorphic (particularly heterozygous) loci are typically used in marker-assisted selection due to their ability to differentiate individuals and associate genotypes with phenotypes, monomorphic loci can also hold value. A fixed (monomorphic) allele may indicate a previously successful selection event and a genetically established trait in the breed. Additionally, such loci can be informative in population comparisons, evolutionary studies, or when evaluating the genetic uniformity of a breed. Therefore, the presence of monomorphic loci should not be interpreted as entirely lacking relevance but rather as part of a broader genetic context. Therefore, it is important to continue studying other regions of the *MSTN* gene, including a larger number of animals and phenotype data. This will help to more accurately assess the potential of *MSTN* as a genetic marker for improving economically important traits in the Ukrainian Carpathian Mountain sheep.

Using sequencing of the 3-untranslated region (3′UTR) of the myostatin gene of the Ukrainian Carpathian Mountain sheep the following genotypes were identified: c.*707TT, c.*709AA, c.*874TT, c.*1098AA, c.*1232GG, c.*1267AA, c.*1316GG, c.*1267AA, c.*1489GG. Based on obtained results, we concluded that it is important to continue studying other regions of the *MSTN* gene, including a larger number of animals and phenotype data. This will help to identify specific mutations within the gene that may serve as potential genetic markers for improving economically important traits in the Ukrainian Carpathian Mountain sheep.

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Аналіз 3′UTR послідовності гену міостатину в овець української гірськокарпатської породи

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Збереження та раціональне використання генофонду овець є дуже актуальною проблемою розвитку вівчарства. Сучасні методи селекційно-племінної роботи забезпечують реалізацію генетичного потенціалу для підвищення продуктивності та племінної цінності сільськогосподарських тварин. Збереження цінного генофонду української гірськокарпатської породи овець має здійснюватися науково обґрунтованими методами, які дозволяють зберегти цінні властивості породи — високу адаптаційну здатність до умов гірського регіону та молочно-м'ясний напрям продуктивності. Ген міостатину (*MSTN*) розглядають як маркер для покращення господарсько корисних ознак у тварин. Було проведено секвенування фрагменту 1093 bp 3′-нетранслюючої ділянки (3′UTR) гена міостатину українських гірськокарпатських овець для пошуку потенційно корисних мутацій у гені. Вісім відомих SNP, кілька з яких є поліморфними у порід латвійська темноголова, мериноландшаф, іль-де-франс, тексель, шаролле, полдорсет, білий суффолк, лінкольн, кам'янська, померанська, кольоровий польський меринос, були мономорфними в української гірськокарпатської породи. У досліджуваній популяції овець виявлено генотипи: c.*707TT, c.*709AA, c.*874TT, c.*1098AA, c.*1232GG, c.*1267AA, c.*1316GG, c.*1267AA, c.*1489GG. Варто зазначити, що у зв'язку з мономорфністю досліджених SNP 3-нетранслюючої ділянки гена міостатину українських гірських карпатських овець маркер-асоційовану селекцію за цим регіоном (3′UTR) гена міостатина проводити не можна. На підставі отриманих результатів ми дійшли висновку про важливість подальшого вивчення інших ділянок гена *MSTN* з охопленням більшої кількості тварин і даних фенотипу. Це допоможе ідентифікувати специфічні мутації в гені, які можуть слугувати потенційними генетичними маркерами для покращення економічно важливих ознак в овець української гірськокарпатської породи.

Ключові слова: *MSTN*, міостатин, 3′UTR, українська гірськокарпатська порода, вівці

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