

SUMMARY

Title of the PhD thesis: Research on the genetic determinism of the growth process in some sheep breeds

Keywords: *Ovis aries*; Myostatin; Single Nucleotide Polymorphisms; intron 1; 3'UTR; haplotypes; genetic diversity; growth traits; morphometric traits; Teleorman Black Head; Suffolk; Île de France; MAS (Marker-Assisted Selection).

Sheep farming represents an important sector of animal husbandry, contributing significantly to valuable productions, some of which have high biological value. In the context of increasing demand for sheep meat, the improvement of growth performance has become a priority, and the use of specialized breeds in crossbreeding with local breeds has led to the production of individuals with superior body development. However, the absence of systematic genetic selection has limited the progress achieved, making it necessary to adopt a modern approach based on molecular markers.

The present research focuses on the myostatin gene (MSTN/GDF-8), a marker involved in the negative regulation of muscle growth. Its mutations can lead to muscular hyperplasia and hypertrophy (McPherron & Lee, 1997), resulting in significant variations in growth and muscle development traits. Previous studies (Clop et al., 2006; Gan et al., 2008; Johnson et al., 2009; Han, 2012; Hickford et al., 2010; Kolenda, 2016; Grochowska et al., 2019; Osman et al., 2021; Trapina et al., 2024; Zhao et al., 2024) have identified numerous SNP (Single Nucleotide Polymorphism) variants in the coding regions (exons) as well as in non-coding regions such as intron 1 and the 3'UTR of the MSTN gene, with implications for several traits related to growth, body development, and muscle development in sheep.

The biological material used in the study consisted of individuals from the Teleorman Black Head, Suffolk, and Île-de-France sheep breeds. The first is a robust local breed characterized by large body size, resilience, and good aptitude for meat production, while Suffolk and Île-de-France are imported improver breeds, recognized for their marked muscular development and high slaughter yield. The selection of these breeds was justified by their relevance in improving meat production in Romanian sheep and by their presence in the northeastern area of the country, where the research was conducted.

The general objective of the thesis is to identify and characterize genetic variations in the intron 1 and 3'UTR regions of the MSTN gene and to evaluate their influence on growth and body development traits in the three breeds.

The first part of the thesis was developed based on the review of the scientific literature and is structured into three chapters that address theoretical aspects regarding the origin of the species *Ovis aries*, its economic importance, and the main sheep breeds raised for meat production in Romania, with emphasis on those included in the study. Morphoproductive traits related to body development, meat production, and carcass quality are also analyzed, as well as the genetic parameters associated with these traits.

The third chapter is dedicated to the role of genetic markers in the selection and improvement of meat production in sheep, presenting the most relevant candidate genes involved in growth and muscle development, with a focus on the characterization of the myostatin (MSTN) gene, described in detail from the perspective of its role as a genetic marker, the mutations identified in its sequence, and their impact on growth traits and body conformation, according to studies reported by numerous authors in other sheep breeds.

The second part of the thesis integrates the experimental research carried out and complements the information available in the literature regarding the genetic diversity of sheep breeds and the relationship between nucleotide variation and production traits. This section comprises four chapters structured as follows: the methodology used in the research, results regarding the phenotypic and genetic characterization of the analyzed populations, identification of polymorphisms in the intron 1 and 3'UTR regions of the MSTN gene, and the association of the identified haplotypes and mutations with production traits.

The aim of this research is to investigate the genetic determinism of growth and body development traits in sheep from the Teleorman Black Head, Suffolk, and Île-de-France breeds by identifying and analyzing SNP polymorphisms in two specific regions of the MSTN (GDF-8) gene—intron 1 and 3'UTR. The choice of these regions is justified by numerous studies describing them as hypervariable areas involved in modulating MSTN gene expression, particularly intron 1, where polymorphisms with effects on muscle growth have frequently been reported (Clöp et al., 2006; Gan et al., 2008; Johnson et al., 2009; Han, 2012; Hickford et al., 2010; Kolenda, 2016; Grochowska et al., 2019; Osman et al., 2021; Trapina et al., 2024; Zhao et al., 2024). In addition, the c.1232G>A mutation in the 3'UTR region has been consistently associated with superior muscular development in sheep, being considered a valuable genetic marker for selection aimed at meat production.

The research was oriented along two main directions:

a) Phenotypic evaluation of growth traits (birth weight, weaning weight, average daily gain) and main morphometric traits (body weight, height at withers, height at croup, trunk length, chest width, croup width, chest girth, and cannon bone circumference);

b) Molecular analysis of variability in the intron 1 and 3'UTR regions of the MSTN gene by identifying SNPs and evaluating their association with the phenotypic traits analyzed.

Based on these directions, the following specific objectives were formulated:

- ✓ Phenotypic characterization of individuals based on body measurements and growth data;
- ✓ Collection and processing of biological samples for DNA isolation and quality assessment;
- ✓ Amplification of target fragments from the intron 1 and 3'UTR regions and their sequencing using the Sanger method;

- ✓ Identification and description of nucleotide polymorphisms (SNPs) by aligning the obtained sequences with reference sequences from the NCBI database;
- ✓ Identification of mutations with potential as genetic markers and evaluation of their importance in improving the studied breeds;
- ✓ Statistical analysis of the influence of identified haplotypes and SNPs on growth and body development traits.

The achievement of the proposed objectives was made possible by conducting experimental stages both in the field and in specialized laboratories in Romania and abroad. Field activities were carried out in the farms of origin of the sheep populations, where blood samples were collected, and body measurements necessary for evaluating growth and body development traits were taken for the individuals included in the study. The stages of documentation, theoretical foundation, methodology development, and data systematization were carried out at the Faculty of Food and Animal Sciences of the University of Life Sciences “Ion Ionescu de la Brad” of Iași.

Molecular analyses were performed in the Molecular Genetics Laboratory of the Faculty of Biology, “Alexandru Ioan Cuza” University of Iași, where DNA isolation and purification from blood, amplification of target fragments via PCR, and preparation of samples for sequencing were carried out. In total, 35 blood samples were collected, of which 34 were validated for genetic analyses.

DNA sequencing for the intron 1 and 3'UTR regions of the MSTN gene was performed in collaboration with the international laboratory Macrogen-Europe (Amsterdam, The Netherlands), specialized in next-generation sequencing technologies. The method used was Sanger sequencing (Chain Termination). The results were delivered electronically as FASTA files and chromatograms corresponding to each analyzed sample.

To analyze the molecular variability of the MSTN (GDF-8) gene, the obtained sequences were compared with reference sequences in NCBI-GenBank (*Ovis aries* ARS-UI_Ramb_v3.0, Accession GCF_016772045.2; chromosome 2, Accession NC_056055.1). The raw sequences, exported in FASTA format, were edited, aligned, and analyzed in MEGA 11 (Tamura et al., 2021), using the ClustalW algorithm (Thompson et al., 1994). Based on these alignments, SNPs were identified and then grouped into distinct haplotypes used in comparative analyses.

Genetic diversity, haplotype frequencies, genetic distances, and linkage disequilibrium (LD) were calculated in DnaSP v.6 (Rozas et al., 2017), and haplotype networks were reconstructed in Network 10 (Fluxus Technology Ltd.). To highlight phylogenetic relationships, MSTN sequences from the analyzed regions for other sheep breeds downloaded from GenBank were integrated into the analysis, and trees were constructed in MEGA 11 using the Neighbor-Joining method (Saitou & Nei, 1987).

Statistical analysis of phenotypic and genetic data was performed in IBM SPSS Statistics (v.26) and supplemented with Real Statistics Resource Pack (Zaiontz, 2013–2024). The main estimators (mean, standard deviation, coefficient of variation) were calculated according to classical methodology (Ivancia, 2007; Dronca & Ivancia, 2023).

The effects of haplotypes, sex, and breed on growth and body development traits were evaluated using a general linear model (GLM) with three factors:

$$y_{ijk} = \mu + H_i + S_j + R_k + \varepsilon_{ijk}, \text{ where:}$$

- y_{ijk} = trait studied;
- μ = overall mean;
- H_i = effect of haplotype (H_1, H_2, H_3);
- S_j = effect of sex (female, male);
- R_k = effect of breed (Teleorman Black Head, Île de France, Suffolk);
- ε_{ijk} = residual error.

Significant differences between groups were tested using Tukey HSD, at a significance threshold of $P < 0.05$.

Phenotypic analysis

The results for growth traits showed, in all three breeds (Teleorman Black Head, Île-de-France, Suffolk), higher values in males for all traits analyzed. Birth weight ranged from 3.5 to 5.8 kg, weaning weight from 24 to 52 kg, and weight at evaluation between 51 and 78 kg. Average daily gain was higher in the first 90 days, with values between 230 and 520 g/day. Coefficients of variation were generally below 15%, indicating that the populations were homogeneous.

Body measurements revealed a pronounced sexual dimorphism, males showing body weights between 80 and 115 kg, and females between 70 and 85 kg, with marked differences for height at withers (63–79 cm) and chest girth (100–120 cm).

The comparison between breeds highlighted clear differences in the level of growth and body development performance: Suffolk individuals showed the highest values for weights and average daily gains; Île-de-France recorded intermediate values but with lower variability; Teleorman Black Head had lower values for growth traits, but balanced body proportions, characteristic of its morphological type.

Analysis of gene sequences

Genetic analyses performed for the 34 samples from Teleorman Black Head, Île-de-France, and Suffolk breeds targeted the sequences from the intron 1 and 3'UTR regions of the MSTN (GDF-8) gene, in order to identify nucleotide variations relative to the *Ovis aries* reference sequence (GenBank: NC_056055.1). Spectrophotometric quantification of DNA using a Nanodrop ASP-3700 indicated concentrations between 10.4 and 86.7 ng/μl (mean 34.7 ng/μl) and A260/A280 ratios between 1.7 and 2.0, confirming adequate purity for amplification and sequencing.

Identification of SNPs and construction of the haplotype network

Analysis of the intron 1 and 3'UTR regions of the MSTN gene revealed SNPs with different distributions among Teleorman Black Head, Île-de-France, and Suffolk breeds. In intron 1, ten nucleotide substitutions were identified, three of which (g.824G>A, g.827T>C, and g.840G>T) showed high frequencies and were present in all three breeds. The g.840G>T polymorphism was the most frequent, present in 71.4% of Teleorman Black Head individuals, 40% of Île-de-France, and 100% of Suffolk. The

g.824G>A and g.827T>C variants showed similar distributions but with higher proportions in Teleorman Black Head, where many individuals presented all three substitutions simultaneously, defining a haplotype specific to this population. In contrast, rare mutations (g.607A>T, g.672A>G, g.822T>C, etc.) were also identified in Suffolk, suggesting higher internal genetic diversity in this breed.

Compared with the literature, eight of the polymorphisms identified here coincide with those previously reported in other sheep breeds (Clop et al., 2006; Hickford et al., 2010; Kolenda et al., 2016; Han et al., 2013).

In the 3'UTR region, eight variants were identified, most of them found in Teleorman Black Head individuals, where two animals exhibited six mutations (g.6363T>A, g.6405T>C, g.6414T>G, g.6432T>G, g.6468C>G, g.6438T>G, g.6507A>G). In Île-de-France, only a single substitution (g.6412A>C) was detected, and no variation was observed in Suffolk, confirming the high conservation of this region. Moreover, the c.*1232G>A polymorphism, frequently reported in other sheep populations, was not detected in any of the sequences analyzed.

Genetic diversity analysis based on the average number of substitutions per site (Maximum Composite Likelihood model, MEGA11) indicated low variability, unevenly distributed between breeds. Within breeds, mean distances were 0.0046 for Teleorman Black Head, 0.0050 for Île-de-France, and 0.0062 for Suffolk, reflecting the highest internal genetic heterogeneity in Suffolk and the lowest in Teleorman Black Head. Between breeds, distances ranged from 0.00491 to 0.00639 substitutions/site, with the smallest difference between Teleorman Black Head and Île-de-France (0.00491) and the largest between Île-de-France and Suffolk (0.00639).

Based on intron 1 sequences, ten haplotypes (H₁–H₁₀) were identified, with a haplotype diversity $H_d = 0.7701$, indicating a moderate–high level of variability in this region. H₁ is the most frequent haplotype (13 individuals from all three breeds), predominating in Teleorman Black Head (57.1%) and occurring in Suffolk (40%) and Île-de-France (10%). H₂ (10 individuals) appears only in Teleorman Black Head and Île-de-France and corresponds to the NCBI reference sequence, and can be considered an ancestral haplotype. H₃ (4 individuals) occurs mainly in Île-de-France, while the other haplotypes (H₄–H₁₀) are rare, each represented by a single individual; these unique haplotypes are much more frequent in Suffolk, where 60% of individuals carry their own specific variants.

Analysis of the 3'UTR region of the MSTN gene confirmed a high level of genetic conservation in all three breeds, with almost no variability. Nucleotide diversity was very low: 0 in Suffolk, 0.0009 in Île-de-France, and 0.0055 in Teleorman Black Head, where a few rare haplotypes were identified. Genetic distances between breeds ranged from 0.00044 to 0.00329 substitutions per site, the smallest between Île-de-France and Suffolk and the largest between Teleorman Black Head and Île-de-France.

The haplotype structure in 3'UTR is simple and unbalanced, with four haplotypes identified ($H_d \approx 0.17$). H₁ dominates the population (91.2% of all individuals), being present in all Suffolk animals, 90% of Île-de-France individuals, and

85.7% of Teleorman Black Head individuals. Haplotypes H_2, H_3, and H_4 are rare, each found in a single individual.

The haplotype network shows H_1 as the central haplotype, from which the others derive through single substitutions, while the very low linkage disequilibrium values ($D \approx 0-0.06$) confirm the absence of stable haplotype blocks and the independent segregation of rare variants. The phylogenetic tree, characterized by short branches and low bootstrap support, reflects the limited variability of the region, with tight clustering of H_1 carriers and isolation of individuals with rare haplotypes on terminal branches.

The phylogenetic trees constructed for the intron 1 and 3'UTR regions of the MSTN gene, based on comparisons between the obtained sequences and those available in NCBI, provided a clear picture of the genetic position of the individuals analyzed.

For intron 1, the tree topology reveals a high degree of conservation, with compact clusters grouping individuals from all three breeds (Teleorman Black Head, Île-de-France, and Suffolk). Most of the sequences obtained cluster alongside international records of breeds such as Najdi, Barki, Romney, and Latvian Darkhead.

In the 3'UTR region, the tree structure is more branched and indicates greater genetic variability. Most sequences from the sample are close to those of the Malpura breed, while others cluster with sequences from European breeds such as Texel and Romney. This topology reflects both a common genetic background among breeds and specific point differentiations in some individuals that appear on terminal branches, defining unique haplotypes.

Association of the identified haplotypes with phenotypic traits

Chapter 7 evaluated associations between MSTN variability and growth and body development performance. For intron 1, statistical analysis showed that haplotypes significantly influenced early growth traits. Individuals with haplotype H_1 had the highest values for birth weight (4.58 kg compared with 3.86 kg in H_2 and 3.45 kg in H_3), with clearly significant differences compared with H_2 and H_3. A similar pattern was observed for weaning weight (33.9 kg in H_1, 26.21 kg in H_2, 30.85 kg in H_3) and average daily gain from 0–90 days (325.81 g in H_1, 248.33 g in H_2, 304.45 g in H_3), where pairwise comparisons revealed highly significant differences between H_1–H_2 and significant differences between H_2–H_3, while H_1–H_3 differences were not significant. Overall, haplotypes including intron 1 mutations (especially H_1 and partially H_3) are associated with better early growth compared with H_2, which corresponds to the reference-like sequence without polymorphisms, supporting the idea that intron 1 variations may indirectly modulate MSTN expression and, consequently, growth performance.

Regarding morphometric traits, the same intron 1 haplotypes did not generate statistically significant differences, although a consistent trend was outlined: individuals with H_3 had, on average, higher values for body weight (95.65 kg), chest girth (114.75 cm), chest width and croup width (≈ 29.25 cm), and cannon bone circumference (8.58 cm) than H_1 and H_2 individuals, suggesting a potentially more robust body format that, however, cannot be confirmed under the conditions of the current small sample size.

For the 3'UTR region, the SNPs identified were rare and concentrated in very few individuals (3FCN, 4MCN, 5MI); although these carriers often had above-average values for body weight and average daily gain, the analyses did not reveal consistent or reproducible effects on either growth traits or morphometric traits, with the observed variability being better explained by other genetic and environmental factors. Overall, the results support a clearer role of intron 1 polymorphisms of the MSTN gene in the determinism of early growth traits and a limited impact of 3'UTR variation on body conformation in the breeds analyzed.

The thesis contributes to the enrichment of the database on MSTN polymorphisms in sheep, having scientific importance—by highlighting intra- and inter-breed genetic diversity—and practical relevance—through the potential use of these markers in the selection of individuals with superior muscular growth potential. Overall, the research supports the integration of molecular genetics into breeding programs for meat production improvement in Romanian sheep.