GENETIC POLYMORPHISM OF THE CALPASTATIN (CAST) GENE IN ALBANIAN AND IMPORTED SHEEP BREEDS

Anila HODA
Academy of Sciences of Albania
Sulltane AJÇE
Department of Biochemistry and Agrifood, Faculty of Agriculture, University Fan S. Noli, Korça, Albania
Ilia MIKEREZI
Department of Biology, Faculty of Natural Sciences, University of Tirana, Albania
Academy of Sciences of Albania
Xhelil KOLECI
Department of Veterinary Public Health, Faculty of Veterinary Medicine, University of Agriculture, Tirana, Albania
Nazim GRUDA
Department of Horticultural Sciences, University of Bonn, Germany
Academy of Sciences of Albania
Author for correspondence: anila.hoda@akad.gov.al
AH, 0000-0003-0906-2550; SA, 0009-0009-5737-4461;
IM, 0000-0002-5184-513X;
XhK, 0000-0001-8945-7776; NG, 0000-0002-2663-1594

ABSTRACT

Sheep breeding plays a central role in the livestock sector of Albania, contributing significantly to rural livelihoods through the production of meat, milk, and traditional dairy products. However, local sheep breeds often exhibit modest productivity compared with specialized foreign breeds. Advances in molecular genetics have enabled the identification of candidate genes associated with economically important traits, offering opportunities for marker-assisted selection (MAS) in breeding programs. Among these, the calpastatin (CAST) gene is a key regulator of muscle growth and postmortem meat tenderness through its inhibitory effect on calpain proteases. This study investigated the polymorphism of the CAST gene in 112 sheep from two autochthonous Albanian

breeds (Shkodrane and Lara e Polisit) and two imported breeds (Awassi and Île de France) reared at the Centre of Agricultural Technology Transfer in Korça, using PCR–RFLP with the MspI restriction enzyme. A 622 bp fragment encompassing exon 3 was successfully amplified and digested, revealing two alleles (M and N) and two genotypes (MM and MN), while the NN genotype was absent across all breeds. Allele frequencies showed the predominance of M (ranging from 0.865 in Lara e Polisit to 0.981 in Awassi), with breed-specific variations in heterozygosity and fixation indices. The higher genetic diversity detected in the Lara e Polisit breed than in imported populations emphasizes the adaptive potential and conservation value of local Albanian sheep breeds. These findings confirm the polymorphic nature of the CAST gene in Albanian sheep and underline its potential for use in MAS programs aimed at improving growth rate, carcass quality, and meat tenderness, while simultaneously supporting the sustainable conservation of indigenous genetic resources and enhancing competitiveness within the framework of European Union (EU) integration.

Keywords: polymorphism, PCR–RFLP, Albanian sheep breeds, genetic diversity, meat quality, marker-assisted selection (MAS), conservation

1. INTRODUCTION

Sheep breeding plays a vital role in global livestock production, providing meat, milk, and wool as major sources of food and income, particularly in the rural economies of the Western Balkans, especially in Albania. Local breeds in Albania are well adapted to mountainous and semi-arid environments (Hoda *et al*. 2011), however, they often display modest productivity levels compared to specialized foreign breeds. Among the economically important traits in sheep, growth performance and carcass quality are of significant interest to farmers and consumers. These traits are quantitative in nature, influenced by both environmental and genetic factors, and often exhibit low to moderate heritability, which limits the effectiveness of traditional selection methods (Gregula-Kania, 2012). The advent of molecular genetics has enabled the identification of candidate genes and DNA markers associated with production traits, providing opportunities for marker-assisted selection (MAS) to enhance genetic progress in breeding programs.

One of the most widely studied candidate genes in farm animals is calpastatin (CAST), located on chromosome 5 in sheep. CAST encodes calpastatin, an endogenous inhibitor of calcium-dependent proteases, calpains, which regulate muscle protein turnover and play a key role in muscle development and post-mortem meat tenderization (Goll *et al*. 2003;

Yilmaz *et al*. 2024). Increased calpastatin activity reduces protein degradation, which may enhance muscle growth and improve meat quality traits, such as tenderness and carcass composition (Casas *et al*. 2006; Schenkel *et al*. 2006).

Genetic polymorphisms in the CAST gene have been widely documented in sheep populations across different geographical regions. In Turkey (Yilmaz *et al*. 2014; Yilmaz *et al*. 2024), such polymorphisms have been reported in several native breeds, while similar studies in Iran (Azari *et al*. 2012), and Pakistan (Suleman *et al*. 2012), have confirmed variation in local sheep populations. In Europe, genetic diversity at the CAST locus has been identified in breeds such as Polish Merino, Berrichon du Cher, Blackheaded Mutton, and Île de France sheep (Szkudlarek-Kowalczyk *et al*. 2011). Several association studies have shown significant relationships between CAST genotypes and growth or muscling traits (Palmer *et al*. 1999; Byun *et al*. 2008). These findings highlight the global significance of CAST as a candidate gene for small ruminant genetic improvement programs, particularly in relation to growth and meat quality traits.

However, research on genetic diversity and CAST gene polymorphisms in Albanian sheep breeds is absent, despite their socio-economic and cultural importance. Expanding such investigations could provide essential insights for the sustainable genetic improvement and conservation of indigenous Albanian sheep populations, while simultaneously strengthening the competitiveness of the national livestock sector within the framework of European Union (EU) integration.

The study aimed to investigate polymorphisms in the calpastatin (CAST) gene, a potential candidate for future genomic selection, in two autochthonous (Shkodrane and Lara e Polisit) and two imported sheep breeds (Awassi and Île de France) populations raised in Centre of Agricultural Technology Transfer in Korça, using the PCR-RFLP method. Incorporating molecular genetics with performance traits, such as growth and meat quality, can enhance breeding programs for these sheep breeds, while genotyping with CAST markers may allow early classification of carcasses by meat quality before slaughter (Yilmaz *et al.* 2014).

2. MATERIALS AND METHODS

Animals and Sampling

The present study involved two autochthonous sheep breeds (Shkodrane and Lara e Polisit) and two imported breeds (Awassi and Île

de France), all reared at the Centre of Agricultural Technology Transfer in Korça, Albania. In total, 112 blood samples (27–29 individuals per breed) were collected from healthy, unrelated animals of different age categories in the study. For each animal, approximately 5–10 mL of whole blood was obtained via jugular venipuncture and transported on ice to the laboratory for analysis. Genomic DNA was subsequently extracted using the salt-out method described by Gaaib *et al.* (2011).

PCR amplification of CAST gene fragment

A 622-bp fragment of the CAST gene containing the MspI polymorphic site was amplified by polymerase chain reaction (PCR) using primers described by Palmer *et al.* (1998). The forward primer was 5′-TGGGGCCCAATGACGCCATCGATG-3′ and the reverse primer was 5′-GTGGAGCAGCACTTCTGATCACC-3′. PCR amplification was performed in a total reaction volume of 25 µL containing approximately 50 ng of genomic DNA, 1× PCR buffer, 2.0 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer, and 1 U of Taq DNA polymerase. The PCR cycle was performed as follows: an incubation step at 95°C for 12 min, followed by 35 cycles of 95°C for 45 s, annealing at 60°C for 45 s and an extraction step at 72°C for 1 min, and 10 min at 72°C as the final extraction.

Genotyping and polymorphism detection

The amplified 622 bp fragment of the CAST gene was genotyped using the restriction endonuclease MspI. For the digestion reaction, a total volume of 50 µL was prepared, consisting of 10 µL of PCR product, 5 µL of 10× One buffer, 0.5 µL of BSA (100×), 0.3 µL of MspI enzyme, and ultrapure water to reach the final volume. The mixture was incubated at 37°C for 1 hour, followed by enzyme inactivation at 65°C for 20 minutes. The digested fragments were separated by electrophoresis on a 3% agarose gel and visualized under ultraviolet (UV) light after ethidium bromide staining. A 50 bp DNA ladder was used as a molecular size marker to determine the fragment sizes generated by PCR-RFLP analysis.

Statistical analysis

Allelic and genotypic frequencies of the CAST gene were estimated using GenAlEx software (Peakall and Smouse, 2012). The conformity of the populations to Hardy–Weinberg equilibrium (HWE) was tested with the Genepop program (Raymond and Rousset, 1995). To evaluate within-

and between-population genetic variation, genetic diversity parameters, and population differentiation indices, we calculated using FSTAT version 2.9.4 (Goudet, 1995). In addition, Nei's gene diversity (Hs), which represents the expected heterozygosity, was computed for each breed to assess its genetic variability.

3. RESULTS

Genotypic structure and allelic frequencies of the CAST Gene

Polymorphism analysis of the CAST gene was performed using the MspI restriction enzyme. The PCR-RFLP results confirmed the presence of genetic variation in all four sheep breeds (Shkodrane, Lara e Polisit, Île de France, and Awassi). Two genotypes were identified following digestion with MspI: MM (336 and 286 bp fragments) and MN (622, 336, and 286 bp fragments). The NN genotype was not detected in any of the examined populations. This locus is therefore characterized by the presence two alleles, M and N. The allelic frequencies of the CAST gene across the breeds are presented in Table 1, showing that the M allele had the highest frequency in all populations, ranging from 0.865 in Lara e Polisit to 0.981 in Awassi sheep, while the N allele was present at relatively low frequencies.

Table 1. Allelic frequencies of CAST gene for four sheep breeds

Locus	Allele	Shkodrane	Lara e Polisit	in Île de France	Awassi
CAST	M	0.889	0.865	0.964	0.981
	N	0.111	0.135	0.036	0.019

Genetic diversity at the CAST locus in four sheep breeds

The genetic diversity of the CAST locus was evaluated in all four sheep breeds. The results of the population genetic parameters are shown in Table 2. The number of effective alleles (Ne) ranged from 1.038 in Awassi to 1.304 in Lara e Polisit, indicating differences in the level of allelic variation among the breeds. The Shannon's information index (I) showed the highest value in Lara e Polisit (0.395), suggesting relatively greater genetic variability in this population than in other breeds, whereas the lowest value was recorded in Awassi sheep (0.092), reflecting a more homogeneous genetic structure.

The observed heterozygosity (Ho) was highest in Lara e Polisit (0.269), followed by Shkodrane (0.148), whereas markedly lower values were found in Île de France (0.071) and Awassi (0.037). In contrast, the expected heterozygosity (He) was generally higher than Ho in most breeds, indicating a deficiency of heterozygotes. The unbiased expected heterozygosity (uHe) confirmed these trends, with values ranging between 0.037 in Awassi and 0.238 in Lara e Polisit cattle. The fixation index (F) varied from –0.156 in Lara e Polisit, indicating an excess of heterozygotes, possibly due to crossbreeding, to 0.250 in Shkodrane, suggesting a higher degree of inbreeding.

These findings highlight considerable inter-population differences in genetic variability at the CAST loci. The higher diversity observed in Lara e Polisit sheep may be linked to their mixed breeding history and adaptability, whereas the low heterozygosity and near fixation of the M allele in imported breeds, such as Awassi and Île de France, reflect a more uniform genetic base.

Table 2. Genetic diversity of CAST gene in four sheep breeds

Breed	**N**	**Ne**	**I**	**Ho**	**He**	**uHe**	**F**
Shkodrane	27	1.246	0.349	0.148	0.198	0.201	0.250
Lara e Polisit	26	1.304	0.395	0.269	0.233	0.238	-0.156
Île de France	28	1.074	0.154	0.071	0.069	0.070	-0.037
Awassi	27	1.038	0.092	0.037	0.036	0.037	-0.019

4. DISCUSSIONS

Genetic characterization of livestock populations using molecular markers has become an essential tool in modern animal breeding, enabling the identification of allelic variations associated with economically important traits, such as growth performance, carcass composition, and meat quality. In this study, polymorphisms in the calpastatin (CAST) gene were investigated in two autochthonous sheep breeds of Albania (Shkodrane and Lara e Polisit) alongside two imported breeds (Awassi and Île de France).

Genetic polymorphisms of the calpastatin (CAST) gene have been extensively studied in sheep populations across different regions, consistently confirming the role it plays in the growth and meat quality

traits. Gregula-Kania (2012) analysed synthetic lines derived from Berrichon du Cher × Polish Merino and Suffolk × Polish Merino crosses, identifying alleles b and e as favourable for growth rate and genotype ac as associated with muscle mass, although no statistically significant correlations with productivity traits were established. In Pakistan, Suleman *et al*. (2012) investigated the exon 1C/1D region of the CAST gene using PCR–RFLP with the MspI enzyme and reported that the MM genotype has the highest frequency, with all three genotypes detected and the M allele showing the highest frequency across Thalli, Lohi, and Kajli breeds. Likewise, Yilmaz *et al*. (2014) studied Kıvırcık, Sakız, Karacabey Merino, and Gökçeada sheep from Western Anatolia, finding high frequencies of the M allele and variable NN genotype distribution, which was particularly high in Sakız but absent in Gökçeada.

Further evidence of the significance of CAST was provided by Byun *et al*. (2008), who reported associations between CAST variants and growth and carcass traits in Romney sheep. In Iranian Afshari sheep, Nikmard *et al.* (2012) reported two alleles at the CAST locus, though without significant association to carcass or growth traits, while Azari *et al*. (2012) found all three genotypes in Dalagh sheep, with deviations from Hardy–Weinberg equilibrium suggesting selection or genetic drift. In Zandi sheep, Khederzadeh *et al*. (2016) observed polymorphism at the CAST locus within a population in equilibrium, confirming the marker's potential in indigenous sheep breeding programs.

Research from the Middle East and Balkans further supports these findings. Jawasreh *et al*. (2017) reported significant associations between CAST polymorphisms and growth and carcass traits in Awassi lambs, with MN genotypes performing better than MM. In Russian half-bred Poll Dorset × North Caucasian meat-wool sheep, Nikolayevna *et al.* (2022) identified MM and MN genotypes, with MM most frequent, while animals with MN showed favourable carcass characteristics. Similarly, Surov *et al*. (2023) highlighted that the MN genotype of CAST, in combination with AB/BB genotypes of the GH gene, was associated with improved growth rate, slaughter weight, and muscle-to-bone ratio. In Norduz sheep from Turkey, Yilmaz *et al*. (2024) confirmed polymorphism at the CAST locus in populations in Hardy–Weinberg equilibrium. In European breeds Dimitrova *et al*. (2017) reported a high frequency of the M allele and predominance of the MM genotype in Karnobat Merino sheep, whereas Bozhilova *et al*. (2022) found CAST polymorphism only in the Synthetic Population Bulgarian Milk breed, while Cooper-Red Shumen and

Karakachan sheep were monomorphic, reflecting restricted diversity due to small population sizes and historical breeding practices.

The results of our study also confirm that the CAST gene is polymorphic in Albanian sheep breeds, with the presence of the M and N alleles and variable genotypic distributions across both autochthonous (Shkodrane, Lara e Polisit) and imported (Awassi, Île de France) populations. Importantly, the higher level of genetic diversity observed in the Lara e Polisit breed, compared with the more uniform structure of imported breeds such as Awassi and Île de France, underscores the adaptive potential and conservation value of local Albanian sheep breeds. This diversity provides a genetic reservoir that can be strategically utilized in marker-assisted selection (MAS) programs to improve traits, such as lamb growth rate, carcass quality, and meat tenderness, while maintaining breed integrity.

From a broader perspective, the identification of CAST polymorphisms in Albanian sheep contributes to the understanding of small ruminant genetic resources in the country, where studies remain limited compared with other countries. Integrating molecular marker information into national breeding programs can strengthen Albania's capacity to enhance productivity, promote sustainable livestock farming, and support alignment of the livestock sector with European Union standards. Such approaches not only improve economic returns for farmers but also play a vital role in safeguarding biodiversity and traditional sheep breeds that are central to the region's cultural and agricultural heritage.

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