Small Ruminant Research 249 (2025) 107517

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Genetic polymorphisms in Agouti signaling protein (*ASIP*) and melanocortin 1 receptor (*MC1R*) genes and their association with coat color in native Bulgarian sheep breeds.

Doytcho Dimov [a,*,1], Milena Kostova [b,*,1], Atanas Vuchkov [a], Ivona Dimitrova [c], Georgi Kalaydzhiev [d], Genoveva Staykova [e], Margarit Iliev [f], Milena Bozhilova [g]

[a] *Agricultural University – Plovdiv, Department of Animal Sciences, Plovdiv 4000, Bulgaria*
[b] *Agricultural University – Plovdiv, Department of Plant physiology, Biochemistry, and Genetics, Plovdiv 4000, Bulgaria*
[c] *University of Forestry, Agronomy Faculty, Sofia 1000, Bulgaria*
[d] *Trakia University Department of Animal husbandry – Ruminants and Dairy Farming, Stara Zagora 6000, Bulgaria*
[e] *Agricultural Institute – Shumen, Agricultural Academy, 3 Simeon Veliki Blvd, Shumen, Sofia 9700, Bulgaria*
[f] *Institute of Agriculture – Karnobat, Agricultural Academy, 1 Industrialna Str., Karnobat, Sofia 8400, Bulgaria*
[g] *Institute of Animal Science, Department of Genetics, Kostinbrod 2230, Bulgaria*

ARTICLE INFO

Keywords:
Polymorphism
ASIP
MC1R
Genes
Native sheep breeds

ABSTRACT

Sheep coat color is an important visual trait for breed differentiation and characterization. In some native sheep breeds, coat color is a patterned phenotype that is the basis of selection. The diversity of coat color provides an important model phenotype for exploring gene roles and studying gene mutations. The present study investigates the genetic polymorphisms in the Agouti signaling protein (*ASIP*) and melanocortin 1 receptor (*MC1R*) genes and their association with coat colour in native Bulgarian sheep breeds. A total of 247 individuals belonging to 7 native sheep breeds from 38 herds were genotyped for the *ASIP* and *MC1R* genes. Five single nucleotide polymorphisms (SNPs) were detected in the *MC1R* gene, and three haplotypes were identified in the studied sheep breeds. The *MC1R* dominant allele (E^D) allele could be the sole cause of fully pigmented sheep (either black or brown coat colour) in the Native Karnobatska population. However, in the population of Karakachanska and Cupper-red Shumenska breeds, the cause of pigmentation can be both E^D and recessive non-agouti (A^a) alleles. In the samples of the Patch-faced Maritza and Dubenska breeds, the E^D allele of the *MC1R* gene is absent, and therefore the fully pigmented and "patch-faced" phenotypes are due to the recessive A^a allele. Genetic mechanisms for white phenotypes exist to some extent in the White Maritza, Native Starazagorska and Dubenska breeds. However, the white phenotype in these breeds cannot be explained by a double copy of the *ASIP* allele alone. The results of this study provide new insights into the genetic causes of coat colour variation in populations of seven native Bulgarian sheep breeds. Further research is needed to elucidate the complex genetic mechanism of coat colour in native Bulgarian sheep breeds, particularly the role of the *Agouti* gene in coat colour expression. Therefore, the results of this study can be considered as a starting point for improving the management and conservation of genetic diversity of coat colour varieties in breeding programmes for native Bulgarian sheep breeds.

1. Introduction

Coat color (hair and fiber cover) is an essential trait in sheep for breed identification, characterization, and morphological selection. Fiber color has important practical applications in wool production from sheep, goats, and other livestock (Allafi et al., 2022; Fontanesi et al., 2010a). Today's consumers have an increasing interest in ecology and the environment. As a result, there is a growing movement for

* Corresponding authors.
E-mail addresses: doytcho.dimov@gmail.com (D. Dimov), mkostova@au-plovdiv.bg (M. Kostova), a_vu@abv.bg (A. Vuchkov), ivonna_dimitrova@yahoo.co.uk (I. Dimitrova), gopo@abv.bg (G. Kalaydzhiev), staikova666@abv.bg (G. Staykova), mar_iliev@abv.bg (M. Iliev), bojilova_milena@abv.bg (M. Bozhilova).
[1] Contributed equally to this work.

https://doi.org/10.1016/j.smallrumres.2025.107517
Received 28 January 2025; Received in revised form 14 April 2025; Accepted 28 April 2025
Available online 10 May 2025

"sustainable fashion" that considers the environmental and social impacts of clothing production. There has also been an increased interest in rare sheep breeds and the preservation of their unique genetic heritage (Daly et al., 2025). Bulgarian native sheep breeds have the potential to produce different types of wool, which is a prerequisite for various applications in the textile, fashion, and art industries. In the immediate future, the market for naturally coloured wool products could be enlarged by increasing public awareness of their consumption and promoting fashion trends that respect environmental protection (Dimov and Vuchkov, 2021). Therefore, the conservation of color varieties within native sheep breeds is a vital breeding task in maintaining genetic diversity within the breed. Description of the breeds' color patterns of Bulgarian native sheep breeds was made by Dimov and Vuchkov (2021).

The diversity of coat color covering the body in sheep is due to the presence, distribution, and biochemical activity in the melanocytes in which two types of melanin (eumelanin and pheomelanin) are synthesized (Barsh et al., 2000). The eumelanin in sheep is either black or brown and phaeomelanin results in a red, tan, or fawn colour (Lundie 2011a) or non-pigmented phenotype. Many genes affect coat color in sheep, but two loci have a significant role in determining coat color - *Agouti* and *Extension* (Barsh et al., 2000; Russell, 1968). The eumelanin in sheep is either black or brown and phaeomelanin results in a red, tan, or fawn colour (Lundie 2011a) or non-pigmented phenotype. Many genes affect coat color in sheep, but two loci have a significant role in determining coat color - *Agouti* and *Extension* (Russell, 1968). These two loci regulate the relative amounts of eumelanin and pheomelanin in the skin and fibers. In addition to *Extension* and *Agouti* loci, in the literature have been reported other genes responsible for coat color in the sheep genome such as the tyrosinase-related protein 1 (*TYRP1*), the v-kit Hardy–Zuckerman 4 feline sarcoma viral onco-gene homolog (*KIT*) (Gurao et al., 2024), and the microphthalmia-associated transcription factor (*MITF*) and also melanocyte-inducing transcription factor (Koseniuk et al., 2018, Zhang et al., 2023).

The *Agouti* locus contains a gene that codes for producing Agouti signaling protein (*ASIP*), and is involved in melanogenesis across various livestock species (Bultman et al., 1992). It is a small paracrine signaling molecule that interacts with the products of the *Extension* locus. The *Extension* locus contains a gene that encodes the melanocortin 1 receptor (*MC1R*), which is present on the surface of the melanocyte membrane. The binding of *MC1R* to α melanocyte-stimulating hormone (αMSH) (Klungland et al., 1995) induces eumelanin synthesis, while alternative binding to *ASIP* induces a switch in pigment synthesis from eumelanin to pheomelanin (Bultman et al., 1992; Yao et al., 2019). The *MC1R* gene in sheep is located on ovine chromosome 14 (OAR14) (Våge et al., 2003). The *ASIP* gene was mapped on a region of chromosome 13 (Bultman et al., 1992; Ollmann et al., 1998). The *Agouti* locus contains a gene that codes for producing Agouti signaling protein (*ASIP*), (Bultman et al., 1992) and is involved in melanogenesis across various livestock species (Apar et al., 2024; Chen et al., 2025). It is a small paracrine signaling molecule that interacts with the products of the *Extension* locus. The *Extension* locus contains a gene that encodes the melanocortin 1 receptor (*MC1R*), which is present on the surface of the melanocyte membrane. The binding of *MC1R* to α melanocyte-stimulating hormone (αMSH) (Klungland et al., 1995) induces eumelanin synthesis, while alternative binding to *ASIP* induces a switch in pigment synthesis from eumelanin to pheomelanin (Bultman et al., 1992; Yao et al., 2019). The *MC1R* gene in sheep is located on ovine chromosome 14 (OAR14) (Våge et al., 2003). The *ASIP* gene was mapped on a region of chromosome 13 (Bultman et al., 1992; Ollmann et al., 1998).

In sheep, classical genetic studies have identified two alleles at the *Extension* locus – dominant black (E^D) and recessive (E^+) (Lu et al., 1994; Våge et al., 2003). Two missense mutations in the *MC1R* gene (p.M73K and p.D121N) were identified by PCR-RFLP. These mutations cause the dominant black color (E^D) allele (Våge et al., 1999). Initially, these mutations in the *MC1R* gene were found in the populations of the Norwegian Dala sheep breed (Lu et al., 1994; Zhang et al., 2023), and the Spanish rare Xalda sheep breed (Royo et al., 2008). The second allele in the *Extension* locus is a wild allele (E^+), widely distributed in most breeds (Russell, 1968). Another *MC1R* missense mutation (p.R67C) was identified (Fontanesi et al., 2010a) in the Italian sheep breed Valle del Belice and the authors suggested that this is the cause of the recessive *e* allele at the ovine extension series. However, it has not yet been well-documented and universally recognized. Examining *MC1R* mutations some authors did not find the putative recessive allele *e* (c.199 C>T), which was previously reported. Stamatis et al., (2017) in their first attempt to assess the genetic polymorphism of the *MC1R* gene in the population of 11 Greek sheep breeds also reported that the putative recessive allele *e* (c.199 C>T) in the *Extension* locus was not found, and they found one non-synonymous mutation that had never been reported (c.789 T > C, p.263 Leu > Pro).

Norris and Whan, 2008 identified a 190-kb tandem duplication encompassing the ovine *ASIP* and *AHCY* coding regions and the *ITCH* promoter region as the genetic cause of the white coat color of the dominant *Agouti* allele (A^{Wt}). Ubiquitous expression of a second copy of the *ASIP* coding sequence regulated by a duplicated copy of the nearby *ITCH* promoter causes the white sheep phenotype. The D_5 deletion and the g.5172 T > A SNP in exon 4 would be predicted to independently cause functional changes to the agouti protein (Norris and Whan, 2008). Despite an in-depth sequence analysis by the Australian authors, they could not conclude that *ASIP* coding regions 5-bp deletion and a g.5172 T > A SNP are the only genetic causes for the expression of recessive black color in Merino sheep.

The relationship between different alleles of *ASIP* and recessive black coat color in the rare Xalda breed in Spain was reported (Royo et al., 2008). The determination in the absence of a E^D allele cannot reject that the homozygous (A^a/A^a) genotype could cause a recessive, black-coated phenotype in sheep (Royo et al., 2008). The genotype (A^a/A^a) cannot be the sole cause of the recessive black colour in the Xalda breed because most black-coated individuals (109 out of 120) were not homozygous for the 5-bp deletion. *ASIP* and *MC1R* gene polymorphisms in two Pramenka sheep populations originated in Bosnia and Herzegovina (Dubian and Privorian sheep) were analyzed (L. Fontanesi et al., 2011b), in which population coat colors and color patterns are not fixed traits. Ninety-five percent of the coat color variation in Brazilian Crioula sheep was explained by epistatic interactions between specific alleles in the *MC1R* and *ASIP* genes (Cavalcanti et al., 2017). Despite their low frequencies, the Brazilian author's evidence suggests an essential role of *TYRP*1 variants in wool color. The marker panel was efficient enough in predicting coat color in the studied animals and, therefore, can be used to implement a marker-assisted selection program in the conservation nucleus of sheep of the Crioula breed. Mutations in *ASIP* and *MC1R* genes and their association with black coat colour were investigated in seven indigenous Swedish sheep breeds in individuals with black, white or grey fleece (Rochus et al., 2019). Previously known mutations in the *ASIP* recessive black allele (g.100_105del and/or g.5172 T > A) were associated with black coat colour in Kleovsjeo and Roslag sheep breeds, and mutations in *MC1R* (dominant black allele): c.218 T > A and/or c.361 G>A) were associated with black coat colour in Swedish Finewool. Swedish sheep breeds have grey individuals in their populations, and grey is thought to result from mutations and allelic copy number variation within the *ASIP* duplication. Finally, a novel missense mutation in MC1R (c.452 G>A) is identified in Gotland, Gute and Vearmland sheep and evidence for a duplication of *MC1R* in Gotland sheep. The four missense mutations included two previously known mutations, c.218 T > A and c.361 G>A, and the two missense mutations, have been found in Norwegian Dala, Damara, Corriedale, and Spanish Merino (Våge, et al., 1999, Royo et al., 2008).

Phenotypic description and characterization of 18 Bulgarian native sheep breeds with colored wool have been made in an extensive review (Dimov and Vuchkov, 2021). Depending on the color of the fleece, the native sheep breeds in Bulgaria have been divided into three groups: breeds with the fully pigmented fleece; breeds with both fully pigmented

and fully white fleece, and breeds with spotted-colored. Some hypotheses about genetic loci and alleles affecting coat color in the populations of Bulgarian native sheep breeds, following the nomenclature of COGNOSAG (Ruvinsky, Sampson, 2001) have been made by (Dimov and Vuchkov, 2021). No report has been made regarding the polymorphism of *MC1R* and *ASIP* genes and the potential association of its mutations with coat colors in Bulgarian native sheep breeds till now.Phenotypic description and characterization of 18 Bulgarian native sheep breeds with colored wool have been made in an extensive review (Dimov and Vuchkov, 2021). Depending on the color of the fleece, the native sheep breeds in Bulgaria have been divided into three groups: breeds with the fully pigmented fleece; breeds with both fully pigmented and fully white fleece, and breeds with spotted-colored. Some hypotheses about genetic loci and alleles affecting coat color in the populations of Bulgarian native sheep breeds, following the nomenclature of COGNOSAG (Ruvinsky, Sampson, 2001) have been made by (Dimov and Vuchkov, 2021). No report has been made regarding the polymorphism of *MC1R* and *ASIP* genes and the potential association of its mutations with coat colors in Bulgarian native sheep breeds till now.

This study aimed to investigate the genetic polymorphisms in *ASIP* and *MC1R* genes and their association with coat color in native Bulgarian sheep breeds.

2. Materials and methods

2.1. Ethical statement

The Animal Research Ethics Committee of the Bulgarian Food Safety Agency (BFSA) (Identification code 154 Art. 381 of the Law on Veterinary Activity) reviewed and approved all the experimental procedures by European Union Directive 86/609.

2.2. Animal sampling

Blood samples were taken from 38 herds in Bulgaria, representing 7 native sheep breeds out of a total of 247 individuals (76 animals from Patch-faced Maritza, 26 animals from White Maritza, 38 animals from Copper-red Shumenska, 22 animals from Dubenska, 31 animals from Karakachanska, 21 animals from Native Starozagorska, 33 animals from Native Karnobatska (Fig. 1, S1). For the White Maritza and Patch-faced Maritza sheep breeds, samples were taken from unrelated animals and verified through a completed pedigree. For the remaining breeds, samples were taken randomly from 3 to 5 samples per herd, and the principle of unrelatedness of the sampled animals was respected by surveying the sheep breeders.

2.3. DNA extraction and PCR conditions

DNA was isolated from 200 μl of liquid blood using the ThermoScientific™ GeneJET Genomic DNA Purification Kit, following the manufacturer's recommendations. Isolated DNA was quality checked on a 1 % agarose gel stained with Green Safe DNA Gel Stain (Canvax) and stored at −20 °C. The quality and quantity of isolated genomic DNA were checked using an Epoch microplate spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

PCR-RFLP was used to genotype the animals for two single nucleotide polymorphisms (SNPs): missense mutations already identified in the ovine *MC1R* gene that determines the black dominant E^D allele

Fig. 1. Pictures of Bulgarian native sheep breeds with different coat color: a – Patch-faced Maritza; b – Piebald phenotype in Patch-faced Maritza breed; c – Piebald phenotype in White Maritza breed; d – Native Starozagorska; e – White Maritza breed; f – Dubenska; g – Native Karnobatska; h – Karakachanska; i – Copper-red Shumenska.

(c.361 G>A or p.D121N) at the *Extension* locus (Våge et al., 1999), one was an SNP (c.–31G>A) located in the 5′-untranslated region (5′-UTR) of the *MC1R* gene (L. Fontanesi et al., 2011). Details of the PCR-RFLP protocols used for the analysis of the *ASIP* and *MC1R* polymorphisms have been already reported (Fontanesi et al., 2011) (Table 1). PCR analyses are performed in a 20 ml volume containing 1 μL My Taq HS red Mix (Bioline). The PCR profile was as follows: 5 min at 95 ^{0}C; 35 amplification cycles from 0.3 min at 95 °C, 0.3 min at 58 °C to 59 °C, 0.3 min at 72 °C, and final extension - 5 min at 72 °C PCR was performed using a TGradient DLAB TC-1000-G (Quanta Biotech, London, UK) (Table 1).

Annealing temperature (^{0}C)/no. of cycles/amplified fragment size (bp).

Prior to the restriction procedure, a pre-purification step was performed. In this step, the distinct DNA fragments were excised from a 1 % agarose gel and then purified using the ISOLATE II PCR and Gel Kit (BIOLINE) as described by the supplier. The restriction reaction is performed with 15 μL of the purified PCR product in a total reaction volume of 20 μL, including 3 U restriction enzyme and 1X reaction buffer, 0.2 μL. BSA. The results are analysed after electrophoresis on a 3 % agarose gel.

2.4. Polymorphism in the ASIP gene

Fragment analysis has also been used to determine whether sampled animals have a duplicate copy of DNA, including *ASIP* (Norris and Whan, 2008). Primers Agt16 and Agt18 spanning the duplication site yielded a unique PCR product of 242 bp, while Agt16 and Agt17 spanning the 5′- point break sequence yielded a fragment of 238 bp. Amplification was performed by asymmetric competitive PCR. If amplification results in these two fragments, the animal carries at least one copy of the duplicated chromosomal region. In contrast, when only 238 bp is amplified, the animal carries no duplicated DNA region.

2.5. Analysis of polymorphisms in the MC1R gene

The polymorphism (c.–31 G>A) located in the 5′-UTR of is genotyped by PCR-RFLP (Fontanesi et al., 2010b). PCR was performed using a TGradient DLAB TC-1000-G (Quanta Biotech, London, UK) in a 20 μl volume containing 1 μl of DNA template, 10 pmol of each primer and PCR profile, as follows: 5 min at 95 °C; 35 amplification cycles of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C; and final extension - 5 min at 72 °C. The reverse primer contains a mismatched nucleotide that introduces an additional artificial restriction site for the *Alu*I endonuclease (Fermentas, Thermo Fisher Scientific, USA) when the c.–31 G allele is amplified. The 202 bp product was subjected to restriction digestion obtained with 5 ml of PCR product in a total of 25 ml of reaction volume including 3 U of restriction enzyme and 1X reaction buffer. The resulting DNA fragments were electrophoresed in 3 % agarose gel. DNA bands were visualized with 1X ROTI®GelStain (Carl Roth, Germany) under UV light. Details of the PCR-RFLP protocols used for the analysis of the three MC1R missense mutations are already reported in Fontanesi et al. (2010a; see also Table 1) The genotype of each animal will be determined as follows GG=c. 31GG (products size of 83 and 96 bp); GA=c.–31GA (products size of 83 and 96 and 119 bp); AA=c.–31AA (products size of 83 and 119 bp).

2.6. MC1R polymorphism analysis in individual samples by PCR-RFLP

PCR-RFLP was used to genotype a polymorphism (c.–31G>A) (Fontanesi et al., 2011),located in the 5-UTR. PCR was performed using a DLab thermal cycler gradient apparatus in a volume of 20 μl containing 10–50 ng DNA template, 10 pmol of each primer (Table 1). The PCR profile was as follows: 5 min at 95 °C; 35 cycles of amplification of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C; final extension - 5 min at 72 °C. The reverse primer contains a mismatched nucleotide that introduces an additional artificial restriction site for the *Alu*I endonuclease when amplifying the c.–31 G allele. The resulting 202 bp PCR product was subjected to restriction with 3 U *Alu*I (Fermentas Thermo Fisher Scientific, USA) and the resulting DNA fragments were separated by electrophoresis on a 3 % agarose gel. Analysis of the c.361 G >A mutation was performed by digesting the 366 bp amplified region of the MC1R gene with the restriction enzyme *Tru*1I (Fermentas; recognition sequence, TTAA, Thermo Fisher Scientific, USA) in a total reaction volume of 25 ml containing 3 U restriction enzyme and 1X reaction buffer, incubated at 65°C for 5 hours. The resulting DNA fragments were electrophoresed in a 3 % agarose gel and visualized with 1X ROTI®-GelStain (Carl Roth, Germany) under UV light (Figure S2).

2.7. SNPs Identification and Genotyping /Sequencing of MC1R gene

SNPs were identified by sequencing amplicons of the whole coding domain sequences (CDS, 954 bp) and parts of the 5'- and 3'-untranslated regions (35 and 125 bp, resp.) of *MC1R* in both directions. DNA from each breed of Patch-faced Maritza, Cooper Red-Shumenska, Dubenska, Karakachanska, Native Starozagorska, and Native Karnobatska were used for identification mutation sites. Primers amplifying the coding region of *MC1R* were used for sequencing (Table 1). All amplifications were performed on TGradient DLAB TC-1000-G (Quanta Biotech, London, UK). The reaction was performed in 25 μL containing 50 ng DNA template, 12 μL My Taq HS red Mix (Bioline), and 10 pM of specific MC1R primers (MF and MR). The PCR condition was as described by (Yang et al., 2013). Macrogen Europe (The Netherlands) directly sequenced the PCR products in both directions. Sequences were analyzed using Geneious software version 4.8.5. (USA) to identify polymorphisms.

Table 1
Primers, PCR conditions, and genotyping analyses for *ASIP* and *MC1R* polymorphisms.

Gene	Primer pair name	Forward and reverse primers (5′ –3′)	PCR[a]	Use
ASIP	ex2_del	CAGCAGGTGGGGTTGAGCACGCTGC CTACCTGACTGCCTTCTCTG	59/25/174	Fragment analysis (genotyping of the exon 2 deletions)
ASIP	Agt17 Agt18 Agt16	GTTTCTGCTGGACCTCTTGTTC GTGCCTTGTGAGGTAGAGATGGTGTT CAGCAATGAGGACGTGAGTTT	58/23/ 238–242	Fragment analysis (duplication breakpoint analysis, asymmetric competitive PCR)
MC1R	MC1R−5'UTR	GCAACTGCACATCCAGAGAA CGCAGGGAGCAGGAAAGGGTGCTAG	58/35/202	PCR-RFLP analysis with *Alu*I (c.-31G>A)
MC1R	3 MC1R	GTGAGCGTCAGCAACGTG ACATAGAGGACGGCCATCAG	61/35/366	PCR-RFLP analysis with *Tru*1I (c.361 G>A; p.D121N)
MF: MR:		GAGAGCAAGCACCCTTTCCT GAGAGTCCTGTGATTCCCCT	60/35/1170	Complete coding region of *MC1R*

3. Results

3.1. Relationship between ASIP polymorphisms and coat colour in sheep

3.1.1. ASIP genotypes

Two hundred and forty-seven sheep were tested for a duplicated allele of the *ASIP* gene. *ASIP* alleles were determined based on their size: 242 bp for the A^{+} allele and 237 bp for the A^{del} allele. For the Patch-faced Maritza breed, seventeen out of 76 animals were found to have duplicated alleles at the *Agouti* locus (Table 2). Duplication was found in six Patch-faced animals, and simultaneous duplication and deletion was found in 11 animals. Duplication and deletion were not found in 16 animals of this breed. Deletion was found in 43 animals (Table 2). The Patch-faced Maritza breed population can be divided into three color varieties: patch-faced, fully pigmented, and piebald. The term "patch-faced" is used to describe a phenotype in which sheep have a white body color, but the animals have typical black patches on the head, ears, and legs. The animals appear piebald when the black spots cover most of the body. Pigmented wool grows on the black spots on the fleece. Fully pigmented sheep are very rare in this breed. There are no duplicates among the pigmented sheep of the Dubenska breed. There are two samples of white phenotypes for this breed in which the duplication was not detected (Table 2). In the Karakachanska breed, where all animals were pigmented, eleven sheep carry a deletion in exon 2. In the Karakachanska breed samples, where all animals were black, there is no duplication at the agouti locus, as expected. Only one animal of the Copper-red Shumenska breed was found to have the deletion in exon 2. The deletion was found at different frequencies in the seven breeds analyzed. In the population of the Dubenska breed, 16 out of 22 animals carry the deletions. In the population of the Karakachanska breed, the deletion was found in only eleven animals. Interestingly, in the native Karnobatska sheep population, duplications were found in 11 animals. However, all animals in the sample group are pigmented, indicating that the dominant E^{D} allele in the extension locus encodes the color of the animals in this sample group.

3.2. MC1R polymorphism analysis in individual samples by PCR-RFLP

3.2.1. Genotyping of the mutation (c.361 G > A; p.D121N)

Fully pigmented animals were selected for restriction analysis outside those tested by *MC1R* gene sequencing. The resulting PCR-RFLP pattern was distinguished by one fragment of 366 bp (allele c.361 G) or two fragments of 265 + 101 bp (allele c.361 A). In black animals, two replacement mutations - Met→Lys at position 73 (M73K) and Asp → Asn at position 121 (*D121N*) - were identified, showing a complete association with black coat color. Both mutations can be easily tested by PCR-RFLP or sequencing. Black animals were found to be predominantly heterozygous at these two positions (73, 121), whereas white animals were homozygous, supporting the assumption of dominant inheritance of black coat color (reddish-brown to black).

PCR-RFLP with *Alu*I restriction was performed on *MC1R* to identify c.–31G>A SNP in different sheep. The genotype of individual animals is indicated as follows: GG=c.–31GG; (83.96 and base pairs) GA=c.–31GA; (83,96 and 119 base pairs) AA=c.–31AA; (83 and 119 base pairs) (Figure S3). The dark pigmentation associated with genotype AA was confirmed in the analyzed animals of the Karakachanska breed, and genotype GG was absent, which was related to white coloration in sheep (Fig. 2).

3.2.2. SNPs identification and genotyping

The present analysis aimed to investigate the expression of *ASIP* gene alleles, and therefore, samples from ewes with fully pigmented phenotype were subjected to sequencing. The idea was to eliminate the influence of the dominant E^{D} allele of the *MC1R* gene, which encodes the pigmented phenotype, from the analysis. A sequence of about 1140 bp including the coding region and parts of the 5- and 3-un-translated regions (UTR) of the

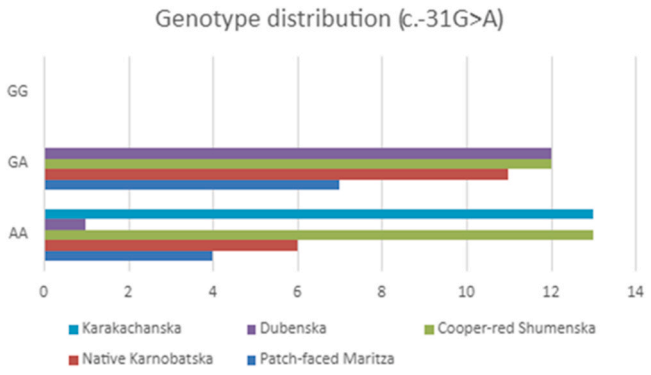

Fig. 2. Genotype distribution among pigmented animals after *Alu*I restriction.

Table 2
Distribution of the analyzed mutations of the *ASIP* locus (duplication, deletion, and the presence simultaneously), in analyzed sheep breeds.

Breeds	Phenotype	n	duplication	deletion	dupl+del	-/-
Patch-faced Maritza		76	6	43	11	16
	Patch-faced*	59	4	35	6	14
	Fully pigmented**	10	1	3	4	2
	Piebald	7	1	5	1	-
White Maritza		26	1	12	10	3
	White	17	-	8	6	1
	Fully pigmented	6	1	4	2	1
	Piebald	3	-	-	2	1
Dubenska		22	-	16	-	6
	White	2	-	1	-	1
	Fully pigmented	20	-	15	-	5
Native Starozagorska		21	4	17	-	-
	White	4	4	-	-	-
	Fully pigmented	17	-	17	-	-
Copper-red Shumenska		38	1	37	-	-
	Fully pigmented	38	-	-	-	-
Native Karnobatska		33	10	23	-	-
	Fully pigmented	33	-	-	-	-
Karakachanska		31				
	Fully pigmented	31	-	11	-	20

* Patch-faced - term used to refer to a phenotype in which sheep have white body color but, on the head, ears and legs, the animals have a typical black spot.
** Fully pigmented – term used to summarize different nuances of reddish-brown to black over the body including head, legs and fleece.

MC1R gene was successfully amplified. Alignment with the sheep reference (GenBank: ANH58923.1,58924–25–26–27–28–29–30, sequences showed 98 % and 99 % sequence homology, respectively (Fig. 3). By analyzing and comparing the electropherogram of the sequences obtained from the DNA pools of 72 sheep, the results showed that five single nucleotide polymorphisms (SNPs), two nonsynonymous mutations previously associated with black coat color (c.218 T > A, p.73 Met>Lys. c.361 G>A, p.121 Asp>Asn) and three synonymous mutations (c.429 C>T, p.143 Tyr>Tyr; c.600 T > G, p.200 Leu>Leu. c.735 C>T, p.245 Ile>Ile) were identified in the CDS of the *MC1R* gene (GenBank accession number: KF198511) (Fontanesi et al., 2010a; L. Fontanesi et al., 2011; Klungland et al., 1995).

3.2.3. Haplotype analysis

All alleles of the mutation (c.218 A, c.361 A, c.429 T, c.600 G, and c.735 T) were found in the Native Karnobatska breed, with two genotypes at each mutation site. Two nonsynonymous mutations (c.218 T > A, p.73 Met>Lys. c.361 G>A, p.121 Asp>Asn) defining the dominant black (E^D) allele (Våge et al., 1999) have not been identified in Patch-faced Maritza and Dubenska sheep. The three synonymous mutations were transmitted with high frequency. The frequency of two non-synonymous mutations is very low or rare in the Native Starozagorska and Karakachanska breeds. The three nonsynonymous mutations in the sheep breeds, except the Native Karnobatska breeds, have three genotypes. Fig. 4 shows the individual haplotype frequencies among the breeds studied. Three haplotypes (haplotype1 [TGCTC], haplotype2 [TGTGT] and haplotype3 [AATGT]) were identified.

Haplotype 3 of all individual mutations were observed as predicted in all animals of the Native Karnobatska sheep breed. Eleven out of sixteen animals of the Cooper -red Shumenska breed and three out of fourteen animals of the Karakachanska breed also showed haplotype 3,

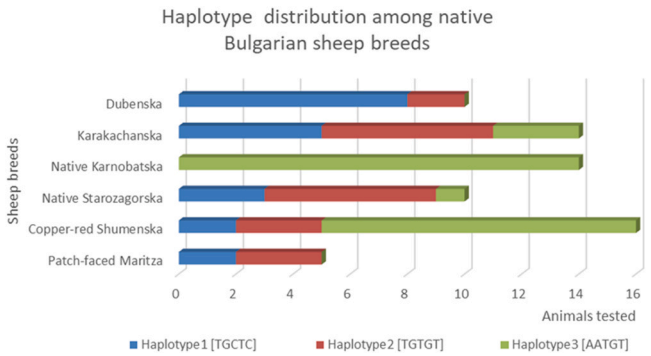

Fig. 4. Distribution of haplotypes detected after the sequencing analyzis of fully pigmented breeds and pigmented animals from non-fully pigmented breeds.

where most animals are pigmented. Only one fully pigmented animal from the Native Starozagorska breed, which is predominantly white, is present here with all five individual mutations. The white coat color breed (Native Starozagorska) was found only in haplotype 1 and haplotype 2. Interestingly, we also observed that the frequency of haplotypes 1 and 2 was significant in animals of the Karakachanska breed.

Analysis of the results showed that five mutations were completely associated with black coat colour in the Native Karnobatska sheep population. At the same time, three haplotypes (haplotype1, haplotype2, and haplotype3) were determined from the SNP mutations in the *MC1R* gene. Interestingly, haplotype 3 is almost fixed in this breed (there are two missense mutations in haplotype 3 causing the E^D allele). Haplotype 3 was not detected in white animals (Fig. 4). We can conclude that haplotype3 alleles could be a possible reason that could explain the

Fig. 3. Multiple alignment for detection of five SNP-s in MC1R gene.

full pigmentation in the Native Karnobatska sheep breed, which has formed the genetic pool of this sheep breed. In the analysis of all pigmented sheep related to haplotype 1, which is associated with light but pigmented coloration, a high level of heterozygosity was also found for the mutations tested (Fig. 5).

Haplotype 3 (E^D) was not found in Dubenska sheep. Combining this with the results of the *ASIP* gene study, where there is no duplication and in 16 out of 22 sheep there is a deletion in exon 2, we can explain the variations in coloration and their assignment to haplotype 1. As expected, the analyzed sheep are from the Native Starozagorska breed, whose population is characterized by a predominantly white phenotype, and here in the pigmented representatives of the breed were found three synonymous mutations in the *MC1R* gene (c.429 C>T, pY143Y; c.600 T > G, pL200L; c.735 C>T, I245I). The alleles of haplotype 3 have not been associated with the Patch-faced phenotype (white body color, but animals have typical black patches on the head, ears, and legs) in the Patch-faced Maritza breed.

4. Discussion

At the end of the 20th and the beginning of the 21st century, the Ministry of Agriculture of the Republic of Bulgaria recognized and legalized the activity of breeding organizations for native sheep breeds. Nowadays, the recognition of breeding organizations is usually connected with the approval of breeding programs for each breed, which necessarily include a description of the breed standard, which includes a description of the morphological characteristics of the breed, including the coat color [Regulation (EU) 2016/1012].

The breed standards of the native Bulgarian breeds, such as the native Karnobatska and the Copper-red Shumenska, were primarily aimed at fully pigmented sheep. For these two native breeds, the standard came into conflict with the biological reality and the mentality of the sheep breeders. For many native breeds, the standard was influenced by the name. For example, for the Copper- red Shumenska breed, the color should be copper red, for the Native Karnobatska breed, the standard defined fully pigmented sheep with a reddish-brown color as typical for the breed, although in the past many authors reported that individuals with white-colored hair and fiber cover also occur in populations of these two fully pigmented breeds, (Dimov and Vuchkov, 2021). In the White Maritza breed, the standard defines white as typical for the breed, although 5–10 % of fully pigmented and less frequently piebald sheep are found in the population (Dimov, 2015).

In the sheep breeding community of the country, opinions and views were formed that the standard defined much more of what sheep should be than they were. As a result of this perception, a white lamb born into a pigmented flock or a black lamb born into a white flock was an undesirable phenotype and often excluded from breeding.

In this case, the flawed thinking is that the standard reflects the reality of the breed, and therefore the genetic mechanism for colored lambs cannot be present in a white breed. Related to this is the misconception that all sheep breeds are ancient and represent ancient, isolated gene pools. For most of these breeds, none of these assumptions are true.

The genetic mechanism for colored lambs persists in many breeds with white-colored coats (Sponenberg and Reed, 2016).

Genetic mechanisms for white phenotypes exist to one degree or another in many native Bulgarian sheep breeds (White Maritza, Native Starazagorska, Dubenska). In this study for the Copper-red Shumenska breed (Fig. 1) 38 pigmented animals were sampled (Table 2). However, a duplication was detected in one of the blood samples, which may encode the white phenotype when homozygous for the *Extension* locus wild allele E^+. The deletion in exon 2 was present in 37 animals in the sampled group in this breed. However, the fact that *MC1R* sequencing identified haplotype 3 (E^D) in 11 of the animals means that pigmentation in the Copperred Shumenska breed may be encoded by deletions when in the homozygous state and if the genotype at the *Extension* locus is E^+E^+ or dominant E^D allele present.

The black spots of different sizes on the face and some parts of the body are crucial for the selection of male and female lambs for breeding purposes in sheep breeds such as the Patch-faced Maritza (Dimov and Vuchkov, 2021). Wool pigmentation is essential for the selection of male and female lambs in native Bulgarian sheep breeds such as Native Karnobatska, Couper-red Shumenska, Karakachanska, and Dubenska. Therefore, it would be essential to identify the genetic causes and genetic variants associated with coat color traits in these breeds. In sheep, at (Najafi, 2025). At least three mutations have been proposed to cause the nonagouti recessive black coat color: a 5 bp deletion in exon 2 (g.100–105delAGGAA, designated D_5), a missense mutation in exon 4 (g.5172 T.A or p.C126S) and a promoter inactivating cis-regulatory mutation have been identified as the genetic cause of the black recessive nonagouti (A^a) allele, which has only been indirectly inferred but not yet well characterized (Norris and Whan, 2008, Royo et al., 2008, Fontanesi et al., 2011b, Gratten et al., 2010). Thus, it would be essential to identify the genetic mechanisms and genetic variants associated with coat colour traits in pigmented sheep breeds. The phenotype of an ewe that is $A^aA^a E^+E^+$ and an ewe that is $A\_A\_ E^DE^+$ or $A\_A\_ E^DE^D$ is identical - there is no visible visual difference. By studying the expression of the *ASIP* gene in Xalda black recessive black sheep, Royo et al. 2008 found that they could not rule out that the A^aA^a genotype could be the cause of recessive black color, but this genotype could not be the only reason, since 91 % of the samples were not homozygous for the A^a allele (Royo et al., 2008). Recently, whole genome sequencing has been widely used to investigate the genetic mechanisms of pigmentation in wild and domestic sheep (Chong et al., 2024; Zhou et al., 2025). Indigenous breeds from South Africa showed high genetic diversity, contrary to fears of inbreeding (Visser et al., 2025).

For this reason, careful breeding and lambing records must be kept if the herder wishes to trace this genetics and preserve genetic diversity.

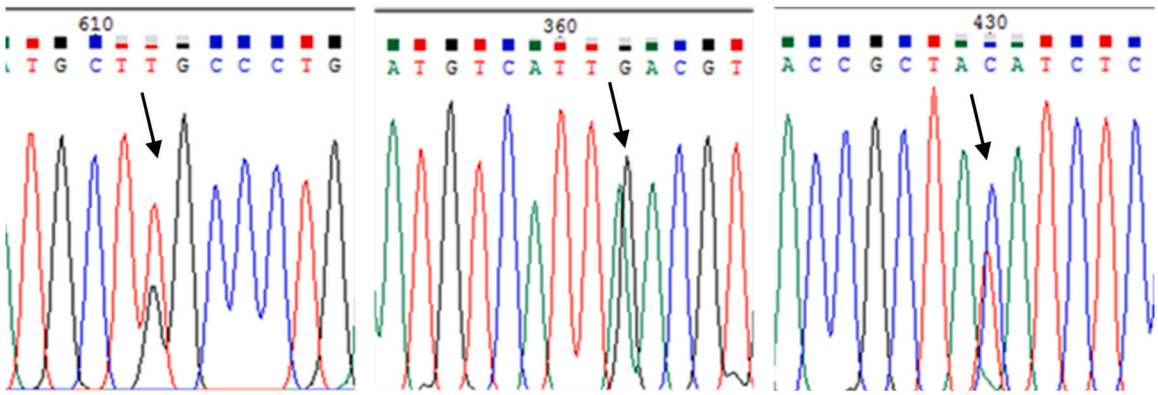

Fig. 5. An electropherogram representing the positions (arrow) of three mutations with heterozygous peaks of pigmented animals assigned to haplotype 1.

We have identified the duplication and deletion at the agouti locus in the native Karnobatska breed, but the E^D allele at the expansion locus is the reason why all animals in the sample are fully pigmented.

In the samples for the Karakachanska breed of sheep, 31 animals were fully pigmented. The *MC1R* sequence results show that haplotype 3 (the dominant E^D allele) was identified in only 3 animals (Fig. 4) and is undoubtedly the determinant of eumelanin synthesis. In the remaining 11 pigmented sheep, the *MC1R* sequence showed the presence of haplotype 1 and haplotype 2, which contain synonymous mutations (c.429 C>T, p.143 Tyr>Tyr; c.600 T > G, p.200 Leu>Leu. c.735 C>T, p.245 Ile>Ile) and in agreement with the studies of (Yang et al., 2013), but the PCR performed on them identified the D_9D_5 deletion, which we believe is the cause of eumelanin synthesis. The analysis of the polymorphisms of the *MC1R* and *ASIP* genes in the Karakachanska breed suggests that the preferred black color in sheep breeding may be due to the E^D allele or the A^a allele in an individual. Selection over the years for individuals with the black coat considered typical of the breed has most likely increased the frequency of the E^D and Aa alleles and decreased the frequency of the duplication in the *ASIP* gene, making the birth of white-coated lambs very rare.

The reddish-brown colour animals in part of the population of the Native Stara Zagora breed is due to the recessive allele A^a (Dimov and Vuchkov, 2021). The PCR performed on the pigmented phenotypes of the Native Starozagorska breed identified the deletion at the *ASIP* locus as the mutation causing the pigmented phenotype. *MC1R* sequencing was performed on 10 samples of pigmented Native Starozagorska sheep. Haplotype 1 and haplotype 2, neutral in coat color, were detected in nine sheep, and haplotype 3 was detected in only one sample. Usually, the typical color for the native Starazagorska sheep breed is white, which, according to classical genetics, is encoded by the dominant allele of the *Agouti* locus (A^{wt}). These studies show that the coloration of the pigmented sheep in this local population is most likely due to the recessive allele of the *Agouti* (A^a) locus. In the native Starazagorska breed, the study should be extended to a more significant number of sheep since as fully pigmented and piebald sheep are rarely found in the population.

Sequencing of the *MC1R* in the Dubenska breed (Fig. 4) showed that the dominant E^D allele was missing in the fully pigmented sheep of this breed tested and that the *Extension* locus contained mainly synonymous mutations encoding haplotype 1 (TGCTC) and haplotype 2 (TGTGT), which are amino acid unchanging and color neutral. The pigmented phenotypes in the Duben sheep are mainly due to the D_5 at the *ASIP* locus. Two of the animals analyzed are white and predictably belong to haplotype 1. In one of the white ewes, the mutations in the *ASIP* locus were not detected, whereas in the other, the deletion D_5 was present. Norris and Whan (2008) identified a 190-kb tandem duplication encompassing the ovine *ASIP* and *AHCY* coding region and the *ITCH* promoter region as the genetic cause of dominant white coat color (A^{wt}) in sheep.

The absence of duplications in *ASIP* has previously been shown to be associated with sheep with darker fleece in Massese (Barsh et al., 2000; Fontanesi et al., 2010b), Dubian and Privorian. Modifications in one or more than two copies of the *ASIP* gene and the additional spontaneous mutations of D_9 and/or D_5 preceding the targeting modification could also involve forming coat color patterns in sheep (Zhang et al., 2017). An uncategorized mutation and removal of 5 bp at exon 2 and non-sense alteration at exon 4 were considered as one of the major causes of the black recessive non-agouti (A^a) allele (Gratten et al., 2010).

Genotyping the Iranian sheep populations via SSCP, led us to discover polymorphisms over the flanking region of DNA. The provided coat colour descriptions suggest that mostly the individuals with light phenotypes as white, light grey and light cream colours in the population show the heterozygous banding pattern (Amin et al., 2018).

The genotype results indicated that these mutations were not associated or not fully associated with the investigated sheep breeds. The above results indicate that the variation in the protein coding region of *ASIP* does not fully explain the variation in coat color phenotypes of the native Bulgarian sheep breeds.

It may also be worthwhile to develop a black line for specific markets to maximize production and maintain the traits associated with black color. These are often called the BASE© of color because this abbreviation includes all four specific gene pairs: the B - black and brown locus, the A - agouti locus, the S - spotted locus, and the E - extension locus. There is no way to identify the agouti pattern alleles of a dominantly colored ewe with one copy of the E^D except by carefully tracking offspring that do not carry the dominant color. Taken together, the *MC1R* and *ASIP* genotypes show that the two genes interact to sincerely modulate the amount of melanin synthesized in alpaca fiber (Pallotti et al., 2020).

In tracing the genetics of sheep color, four specific loci determine much of what is seen as a coat colour when looking at sheep (Lundie 2011a). The selection against black coat color seems to hurt the genetic progress of growth traits in the Menz sheep (Getachew et al., 2020). It may also be worthwhile to develop a black line for specific markets to maximize production and maintain the traits associated with black color.

The fortunes of many sheep breeds have changed in recent decades. Many native sheep breeds have remained as small populations and have reached "endangered" status. On the other hand, breeders of native breeds are holding on to the standard they held during the breed's heyday. Maintaining the historical standard of the breed is not a bad thing. However, at some point, managing the genetic population structure of these "endangered" breeds must take precedence over any historical or economic role of the breed (Sponenberg et al., 2015; Sponenberg et al., 2019).

5. Conclusions

In this study, we found that the color variants in the seven native Bulgarian breeds are in most cases encoded by the alleles of the *ASIP* gene and by the recessive A^a allele. The dominant allele of the Extension locus is the major genetic cause of pigmentation in all individuals of the native Karnobatska breed. E^D may also be a genetic cause of pigmentation in individuals of the Copper-red Shumenska and Karakachanska breeds, but in these two breeds full pigmentation may also be due to the recessive A^a allele of the *ASIP* gene. In the samples studied, the dominant E^D allele of *MC1R* was found to be absent in the Patch-faced Maritza and Dubenska breeds. In the population of the native Starozagorska, our observations and genealogical analysis show that the pigmented variants are due to the recessive non-agouti allele (A^a), but in this population additional studies of a more significant number of samples are required.

In our study, we found that the polymorphism of the *ASIP* gene cannot fully explain the "Patch-faced" phenotype in the Patch-faced Maritza sheep population, and perhaps the expression of this phenotype is influenced by other alleles in the *ASIP* gene and other genes, as shown by the PCR-RFLP analysis of the *ASIP* gene. The PCR-RFLP analysis in the White Maritza sheep breed showed that the white color can be expressed even without the duplication, which also requires additional studies.

Our study provides novel insights into the genetic causes of different color varieties in populations of seven native Bulgarian sheep breeds. The results of this study extend our knowledge on the role of *ASIP* and *MC1R* genes and the different colour patterns in native Bulgarian sheep breeds. In conclusion, further future research is needed to elucidate the complex genetic mechanism of coat colour in native Bulgarian sheep breeds, especially the role of the agouti gene in coat colour expression. Therefore, we consider the results of this study as a starting point for the improvement of the management and the conservation of the color genetic diversity in the breeding programs of the native Bulgarian sheep breeds.

CRediT authorship contribution statement

Kalaydzhiev Georgi: Resources, Investigation, Data curation. **Dimitrova Ivona:** Supervision, Formal analysis. **Vuchkov Atanas:** Validation, Resources, Methodology, Formal analysis. **Dimov Doytcho:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Kostova Milena:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Conceptualization. **Bozhilova Milena:** Supervision, Investigation. **Iliev Margarit:** Supervision, Investigation. **Staykova Genoveva:** Investigation.

Supplementary materials

The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Sample collection, representing 7 native Bulgarian sheep breeds - map.; Figure S2: PCR-RFLP pattern after restriction with *Tru*1 of a product of 366 bp. M- molecular marker 50 bp; Figure S3: PCR-RFLP pattern after *Alu*I restriction PCR-RFLP pattern after *Alu*I restriction of *MC1R*

Ethical statement

All the experimental procedures were reviewed and approved by the Animal Research Ethics Committee of the Bulgarian Food Safety Agency (BFSA) (Identification code 154 Art. 381 of the Law on Veterinary Activity) by European Union Directive 86/609.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

Funding

The research leading to these results received funding from the Ministry of Education and Science of the Republic of Bulgaria under the National Scientific Program Intelligent Animal Husbandry, grant agreement no. Д01–62/18.03.2021.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.smallrumres.2025.107517.

Data availability

Data will be made available on request.

References

Allafi, F., Hossain, M.S., Lalung, J., Shaah, M., Salehabadi, A., Ahmad, M.I., Shadi, A., 2022. Advancements in applications of natural wool fiber: review. J. Nat. Fibers 19, 497–512. https://doi.org/10.1080/15440478.2020.1745128.

Amin, M., Masoudi, A.A., Amirinia, C., Emrani, H., 2018. Molecular study of the extension locus in association with coat colour variation of iranian indigenous sheep breeds. Russ. J. Genet 54, 464–471. https://doi.org/10.1134/S1022795418040026.

Apar, R., Ye, X., Lv, X., 2024. Transcriptome-based screening and validation of key genes for wool color in cashmere goats. Genes Genom. 46, 1239–1252. https://doi.org/10.1007/s13258-024-01562-2.

Barsh, G., Gunn, T., He, L., Schlossman, S., Duke-Cohan, J., 2000. Biochemical and genetic studies of pigment-type switching. Pigment Cell Res. 13, 48–53. https://doi.org/10.1034/j.1600-0749.13.s8.10.x.

Bultman, S.J., Michaud, E.J., Woychik, R.P., 1992. Molecular characterization of the mouse agouti locus. Cell 71, 1195–1204. https://doi.org/10.1016/S0092-8674(05)80067-4.

Cavalcanti, L.C.G., Moraes, J.C.F., Faria, D.A.D., McManus, C.M., Nepomuceno, A.R., Souza, C.J.H.D., Caetano, A.R., Paiva, S.R., 2017. Genetic characterization of coat color genes in Brazilian Crioula sheep from a conservation nucleus. Pesq. agropec. Bras. 52, 615–622. https://doi.org/10.1590/s0100-204x2017000800007.

Chen, Y., Wang, H., Ping, X., Solomon, A.I., Ren, Z., Dong, X., 2025. Two novel SNP variants at *ASIP* and *SNAI2* genes are associated with yellow coat color in rabbits. Anim. Genet. 56, e70006. https://doi.org/10.1111/age.70006.

Chong, Y., Xiong, H., Gao, Z., Lu, Y., Hong, J., Wu, J., He, X., Xi, D., Tu, X., Deng, W., 2024. Genomic and transcriptomic landscape to decipher the genetic basis of hyperpigmentation in Lanping black-boned sheep (Ovis aries). BMC Genom. 25, 845. https://doi.org/10.1186/s12864-024-10772-7.

Daly, K.G., Mullin, V.E., Hare, A.J., Halpin, Á., Mattiangeli, V., Teasdale, M.D., Rossi, C., Geiger, S., Krebs, S., Medugorac, I., Sandoval-Castellanos, E., Özbaşaran, M., Duru, G., Gülcür, S., Pöllath, N., Collins, M., Frantz, L., Vila, E., Zidarov, P., Stoddart, S., Boldgiv, B., Orlando, L., Pearson, M.P., Mullville, J., Askeyev, I.V., Askeyev, A.O., Askeyev, O.V., Shaymuratova, D.N., Van Den Hurk, Y., Zeeb-Lanz, A., Arbogast, R.-M., Hemmer, H., Davoudi, H., Amiri, S., Doost, S.B., Decruyenaere, D., Fathi, H., Khazaeli, R., Hassanzadeh, Y., Sardari, A., Lhuillier, J., Abdolahi, M., Summers, G.D., Marro, C., Bahshaliyev, V., Berthon, R., Çakirlar, C., Benecke, N., Scheu, A., Burger, J., Sauer, E., Horwitz, L.K., Arbuckle, B., Buitenhuis, H., Gourichon, L., Bulatović, J., O'Connor, T., Orton, D., Jalabadze, M., Rhodes, S., Chazan, M., Özkaya, V., Zeder, M., Atıcı, L., Mashkour, M., Peters, J., Bradley, D.G., 2025. Ancient genomics and the origin, dispersal, and development of domestic sheep. Science 387, 492–497. https://doi.org/10.1126/science.adn2094.

Dimov, D., 2015. White Marish sheep - origin, status and development perspectives. Monograph. Publishing house "Intelexpert-94", p. 216. Plovdiv. ISBN 978-619-7220-03-2.

Dimov, D., Vuchkov, A., 2021. Sheep genetic resources in Bulgaria with focus on breeds with coloured wool. GenResJ 2, 11–24. https://doi.org/10.46265/genresj.HXSV9592.

Fontanesi, Luca, Beretti, F., Dall'Olio, S., Portolano, B., Matassino, D., Russo, V., 2011. A melanocortin 1 receptor (*MC1R*) gene polymorphism is useful for authentication of Massese sheep dairy products. J. Dairy Res. 78, 122–128. https://doi.org/10.1017/S0022029910000890.

Fontanesi, L., Beretti, F., Riggio, V., Dall'Olio, S., Calascibetta, D., Russo, V., Portolano, B., 2010b. Sequence characterization of the melanocortin 1 receptor (MC1R) gene in sheep with different coat colours and identification of the putative e allele at the ovine Extension locus. Small Rumin. Res. 91, 200–207. https://doi.org/10.1016/j.smallrumres.2010.03.015.

Fontanesi, L., Beretti, F., Riggio, V., Dall'Olio, S., Calascibetta, D., Russo, V., Portolano, B., 2010a. Sequence characterization of the melanocortin 1 receptor (MC1R) gene in sheep with different coat colours and identification of the putative e allele at the ovine Extension locus. Small Rumin. Res. 91, 200–207. https://doi.org/10.1016/j.smallrumres.2010.03.015.

Fontanesi, L., Dall'Olio, S., Beretti, F., Portolano, B., Russo, V., 2011. Coat colours in the Massese sheep breed are associated with mutations in the agouti signalling protein (ASIP) and melanocortin 1 receptor (MC1R) genes. Animal 5, 8–17. https://doi.org/10.1017/S1751731110001382.

Getachew, T., Abebe, A., Gizaw, S., Rischkowsky, B., Bisrat, A., Haile, A., 2020. Coat color alterations over the years and their association with growth performances in the Menz sheep central nucleus and community-based breeding programs. Trop. Anim. Health Prod. 52, 2977–2985. https://doi.org/10.1007/s11250-020-02315-5.

Gratten, J., Pilkington, J.G., Brown, E.A., Beraldi, D., Pemberton, J.M., Slate, J., 2010. The genetic basis of recessive self-colour pattern in a wild sheep population. Heredity 104, 206–214. https://doi.org/10.1038/hdy.2009.105.

Gurao, A., Vasisth, R., Kumari, N., Chitkara, M., Singh, R., Mukesh, M., Vohra, V., Kumar, S., Dige, M.S., Kataria, R.S., 2024. Identification of *KIT* gene transcript variants in white spotted river buffalo (*Bubalus bubalis*. Anim. Genet. 55, 918–920. https://doi.org/10.1111/age.13484.

Klungland, H., Vage, D.I., Gomez-Raya, L., Adalsteinsson, S., Lien, S., 1995. The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination. Mamm. Genome 6, 636–639. https://doi.org/10.1007/BF00352371.

Koseniuk, A., Ropka-Molik, K., Rubiś, D., Smołucha, G., 2018. Genetic background of coat colour in sheep. Arch. Anim. Breed. 61, 173–178. https://doi.org/10.5194/aab-61-173-2018.

Lu, D., Willard, D., Patel, I.R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R.P., Wilkison, W.O., Cone, R.D., 1994. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature 371, 799–802. https://doi.org/10.1038/371799a0.

Lundie, R.S., 2011a. The genetics of colour in fat-tailed sheep: a review. Trop. Anim. Health Prod. 43, 1245–1265. https://doi.org/10.1007/s11250-011-9850-0.

Najafi, M., 2025. Comparative analysis of gene structure in 5-flanking region of MC1R gene in indigenous sheep breeds. Ecol. Genet. Genom. 34, 100314. https://doi.org/10.1016/j.egg.2024.100314.

Norris, B.J., Whan, V.A., 2008. A gene duplication affecting expression of the ovine *ASIP* gene is responsible for white and black sheep. Genome Res 18, 1282–1293. https://doi.org/10.1101/gr.072090.107.

Ollmann, M.M., Lamoreux, M.L., Wilson, B.D., Barsh, G.S., 1998. Interaction of Agouti protein with the melanocortin 1 receptor in vitro and in vivo. Genes Dev. 12, 316–330. https://doi.org/10.1101/gad.12.3.316.

Pallotti, S., Chandramohan, B., Pediconi, D., Nocelli, C., La Terza, A., Renieri, C., 2020. Interaction between the *melanocortin 1 receptor* (*MC1R*) and *agouti signalling protein genes* (*ASIP*), and their association with black and brown coat colour phenotypes in peruvian alpaca. Ital. J. Anim. Sci. 19, 1518–1522. https://doi.org/10.1080/1828051X.2020.1850216.

Rochus, C.M., Westberg Sunesson, K., Jonas, E., Mikko, S., Johansson, A.M., 2019. Mutations in *ASIP* and *MC1R*: dominant black and recessive black alleles segregate in native Swedish sheep populations. Anim. Genet. 50, 712–717. https://doi.org/10.1111/age.12837.

Royo, L.J., Álvarez, I., Arranz, J.J., Fernández, I., Rodríguez, A., Pérez-Pardal, L., Goyache, F., 2008. Differences in the expression of the *ASIP* gene are involved in the recessive black coat colour pattern in sheep: evidence from the rare Xalda sheep breed. Anim. Genet. 39, 290–293. https://doi.org/10.1111/j.1365-2052.2008.01712.x.

Russell, E.S., 1968. Pigmentation and Inheritance: *Comparative Genetics of Coat Colour in Mammals*. A. G. Searle. Logos Press, London; Academic Press, New York, 1968. xii + 308 pp., illus. $17.50. Science 161, 677–678. https://doi.org/10.1126/science.161.3842.677.

Ruvinsky, A., Sampson, J.C.A.B. International (Eds.), 2001. The genetics of the dog, CABI Books. CABI, Wallingford. https://doi.org/10.1079/9780851995205.0000.

Sponenberg, D.P., Beranger, J., Martin, A., 2015. In: Burns, D., Larkin, A. (Eds.), An introduction to heritage breeds, 155 56. North Adams Published, p. 240. https://doi.org/10.1017/S2078633615000065. ISBN 978-1-61212-125-3 (pbk.: alk. Paper) ISBN 978-1-61212-130-7 (Hardcover: alk. Paper) e-ISBN 978-1-61212-462-9.

Sponenberg, D.P., Martin, A., Couch, C., Beranger, J., 2019. Conservation strategies for local breed biodiversity. Diversity 11, 177. https://doi.org/10.3390/d11100177.

Sponenberg, P., Reed, C., 2016. Effective strategies for local breed definition and conservation.

Stamatis, C., Giannoulis, T., Galliopoulou, E., Billinis, C., Mamuris, Z., 2017. Genetic analysis of melanocortin 1 receptor gene in endangered Greek sheep breeds. Small Ruminant Research 157, 54–56. https://doi.org/10.1016/j.smallrumres.2017.09.018.

Våge, D.I., Fleet, M.R., Ponz, R., Olsen, R.T., Monteagudo, L.V., Tejedor, M.T., Arruga, M. V., Gagliardi, R., Postiglioni, A., Nattrass, G.S., Klungland, H., 2003. Mapping and characterization of the dominant black colour locus in sheep. Pigment Cell Res. 16, 693–697. https://doi.org/10.1046/j.1600-0749.2003.00090.x.

Våge, D.I., Klungland, H., Lu, D., Cone, R.D., 1999. Molecular and pharmacological characterization of dominant black coat color in sheep. Mamm. Genome 10, 39–43. https://doi.org/10.1007/s003359900939.

Visser, C., Retief, A., Molotsi, A.H., 2025. Genetics underlying phenotypic diversity in South African sheep breeds. Small Rumin. Res., 107499 https://doi.org/10.1016/j.smallrumres.2025.107499.

Yang, G.-L., Fu, D.-L., Lang, X., Wang, Y.-T., Cheng, S.-R., Fang, S.-L., Luo, Y.-Z., 2013. Mutations in *MC1R* gene determine black coat color phenotype in chinese sheep. Sci. World J. 2013, 1–8. https://doi.org/10.1155/2013/675382.

Yao, L., Bao, A., Hong, W., Hou, C., Zhang, Z., Liang, X., Aniwashi, J., 2019. Transcriptome profiling analysis reveals key genes of different coat color in sheep skin. PeerJ 7, e8077. https://doi.org/10.7717/peerj.8077.

Zhang, X., Li, W., Liu, C., Peng, X., Lin, J., He, S., Li, X., Han, B., Zhang, N., Wu, Y., Chen, L., Wang, L., MaYila, Huang, J., Liu, M., 2017. Alteration of sheep coat color pattern by disruption of ASIP gene via CRISPR Cas9. Sci. Rep. 7, 8149. https://doi.org/10.1038/s41598-017-08636-0.

Zhang, L., Liang, L., Zhang, X., Li, W.R., Li, H., He, S., 2023. Understanding key genetic make-up of different coat colour in Bayinbuluke sheep through a comparative transcriptome profiling analysis. Small Rumin. Res. 226, 107028. https://doi.org/10.1016/j.smallrumres.2023.107028.

Zhou, W., Li, X., Zhang, X., Zhu, L., Peng, Y., Zhang, C., Han, Z., Yang, R., Bai, X., Wang, Q., Zhao, Y., Liu, S., 2025. Genome-based analysis of the genetic pattern of black sheep in Qira sheep. BMC Genom. 26, 114. https://doi.org/10.1186/s12864-025-11233-5.