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Polymorphisms in Melanocortin Receptor 1 Gene in Goat Breeds: A Window for Coat Color Controling Mechanism

Research Article

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ABSTRACT

The broad goal of this research was to examine the nature of the Melanocortin receptor 1 (MC1R) locus on the coat color phenotype of seven goat breeds with different color coat. Blood samples were collected from five Iranian indigenous (Khalkhal, Markhor, Naeini, Najdi and Tali) and two exotic (Cashmere and Saanen) goat breeds. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique and DNA sequencing were used to detect polymorphism of the MC1R gene. Digestion of polymerase chain reaction products with *EarI* revealed two alleles of A and B and three genotypes of AA, AB and BB. The observed allele size was similar to previous reports. The genotype and gene frequencies for each breed were determined and shown to be variable among the breeds. In the goat breeds with black / brown coat color, AA genotype showed a higher frequency than white color goat breeds. In this aspect, Tali had only AA and AB genotypes and Cashmere had AB and BB genotypes. Comparison of genotype frequencies using χ^2 test showed significant ($P<0.01$) differences between colored and white coat phenotypes. The Lys226Arg (A676G) mutation of the MC1R was verified by sequencing distinguished the B allele from the A allele. The phylogenetic tree also separated these breeds into two clusters, including exotic breeds with white / yellow coat color and indigenous breeds with black/brown phenotype. A replacement mutation was verified in Lys to Arg at position 676 bp. In conclusion, the results of this study confirmed the previous findings on MC1R gene as a useable tool for breed identification and white mohair marketing and production.

KEY WORDS coat color Melanocortin receptor 1, goat breed, PCR-RFLP, specific phenotype.

INTRODUCTION

Coat color variation in domestic animals is of considerable interest and it can be traced back to at least 5000 years ago. It is a well-documented fact that in farm animal breeds, coat color is one of the most important traits in distinguishing individuals as large number of phenotypes has been described in different species. The use of qualitative traits such as coat color to measure livestock performance is gaining wider application and might offer a cheap and indirect method of improving production performance of indigenous livestock (Zeder, 1994). The genetic basis of coat color inheritance has been unraveled in different farm animals, inclucing: Pigs (Fang *et al.* 2009; Jinlong *et al.* 2012), cattle (Russo *et al.* 2007; Xi *et al.* 2012), sheep (Fontanesi *et al.* 2010; Bemji *et al.* 2012; Hepp *et al.* 2012) and goats (Zhao-Long *et al.* 2006; Fontanesi *et al.* 2009). Melatonine is responsible for coat color variation in mammals. There are many different methods of measuring the coat color variation in mammals. In the melanocyte cells, which are the sites of melanin production, the Extension (E) and Agouti (A) loci control the relative amount of the two basic

types of tyrosine-derived melanin, eumelanin (black/brown) and phaeomelanin (yellow/red). Numerous studies have provided a detailed description of MC1R locus with the aim to elucidate the biology of coat color. Previous molecular studies have revealed that Extension and Agouti encodes MC1R and a 131-amino-acid peptide antagonist to MC1R, respectively.

MC1R, also denoted melanocyte stimulating hormone receptor, is a G-protein-coupled receptor (Robbins *et al.* 1993), which is expressed on the surface of melanocytes and has been mapped to cattle chromosome 18 (Carruthers, 2009), human chromosome 16 (Makova and Norton, 2005), chicken chromosome 11 (Guo *et al.* 2010) and sheep chromosome 14 (Fontanesi *et al*. 2010). This gene is included complete coding region (CDS, 954 bp) and parts of the 5'- and 3'-untranslated regions (38 and 284 bp, respectively) with accession No FM212940 (Fontanesi *et al.* 2009). The majority of previous studies proposed the polymorphisms of this gene as a useable tool for breed identification (Jinlong *et al.* 2012). The goat population of Iran is about 25 million heads and is kept mainly for meat and milk production. The main breeds of goats in Iran are Naeini, Nadoushan, Birjandi, Abadeh, Markhor, Tali, Khalkhal and Najdi. The Najdi goats are gray, dark brown, brown with a black back line colored. Markhor goat mostly are black, white and chocolate brown colored and are raised for colored mohair production. The Tali goats are medium-sized and their coat color is mostly brown or light brown. The phenotypes of the Naeini and Khalkh goat breeds are black Saanen and white or cream, which is mostly reared in the north and east of Iran. Cashmere goat breed produces cashmere hair with different coat color but white coat color is more frequent than others (Saadat-Noori and Siah-Mansoor, 1987). The general purpose of the current study was to examine the relationship between variations in MC1R gene and coat colors among seven goat breeds including five indigenous breeds (Khalkhal, Markhor, Naeini, Najdi and Tali) and two exotic (Cashmere and Saanen).

MATERIALS AND METHODS

Animals and DNA extraction

In the present study, 210 individuals of seven goat breeds (30 individuals of each breed) were investigated. Blood samples were collected from the jugular vein and stored at –20 ˚C for a few weeks. DNA extraction was conducted in accordance with Quigen commercial Kit. The DNA was washed once with 70% ethanol, dried and suspended in 20 µL of distilled H_2O. A 416 bp fragment of MC1R gene was amplified by PCR. Primer sequences are considered as described by Zhao-Long *et al*. (2006) with the accession number (Y13965). Sequence of forward and reverse primers were as follows:

Forward: 5'GTGGACCGCTACATCTCCAT-3';
Reverse: 5' TTGAAGATGCAGCCACAGG-3'.

Figure 1 illustrates the target sequence and position of annealing of primers in the candidate gene.

PCR program

PCR was performed using a T Gradient Biometra model (Germany) thermal cycle in a volume of 30 µL containing 50-100 ng of genomic DNA, 2.25 m*M* $MgCl_2$, 200 M dNTP, 0.1 µ*M* of each primer and 2 units of Taq DNA polymerase (Cinagen, Iran).

The PCR reactions were performed as follows: the first cycles of 3 min at 94 ˚C, 1 min at 65 ˚C and 2 min at 72 ˚C followed by 34 cycles of 45 Sec at 94 ˚C, 1 min at 63 ˚C, 1 min at 72 ˚C and ending with a 10 min extension phase in 72 ˚C. The size of the alleles was determined based on a 100 bp DNA size standard (Fermentas Company) using the Computer software BIO $1D^{++}$.

PCR-RFLP and sequencing

The PCR products (30 µL) for each sample were digested with 10 units of *EarI* at 37 ˚C for at least 2 h. Digestion products were visualized electrophoresis a 2% agarose gel for 1 h at 73 V.

Model of power supply for electrophoresis was PAC1000 (Bio-Rad company; USA). The gel was stained with ethidium bromide. A sample of digested PCR products was sequenced by Bioasia Biological and Technology Co. Ltd. (Beijing, China).

Allele and genotype frequency

Genotype and allele frequencies were calculated using the computer software package PopGene 3.2 (Yeh *et al.* 1997). A chi-square test of SPSS software (version 17.0) was performed for significant analysis of allele frequencies between breeds.

RESULTS AND DISCUSSION

PCR- RFLP

The PCR amplified product of 412 bp visualized on 2% agarose gel is shown in Figure 2. The amplified 412 bp product upon digestion by *EarI*, yielded three restriction patterns; one fragment of 416 bp, two fragments of 163 and 253 bp and three fragments of 163, 253 and 416 bp corresponding to BB, AA and AB genotypes respectively (Figure 3).

Allele and genotype frequency

The genotypic and allelic frequencies of MC1R gene in all seven-goat breeds are illastrated in Table 1. The highest frequency of allele in indigenous breeds of Khalkhal, Naeini, Najdi and Tali was for A and in Markhor and exotic breeds of Cashmere and Saanen was for B.

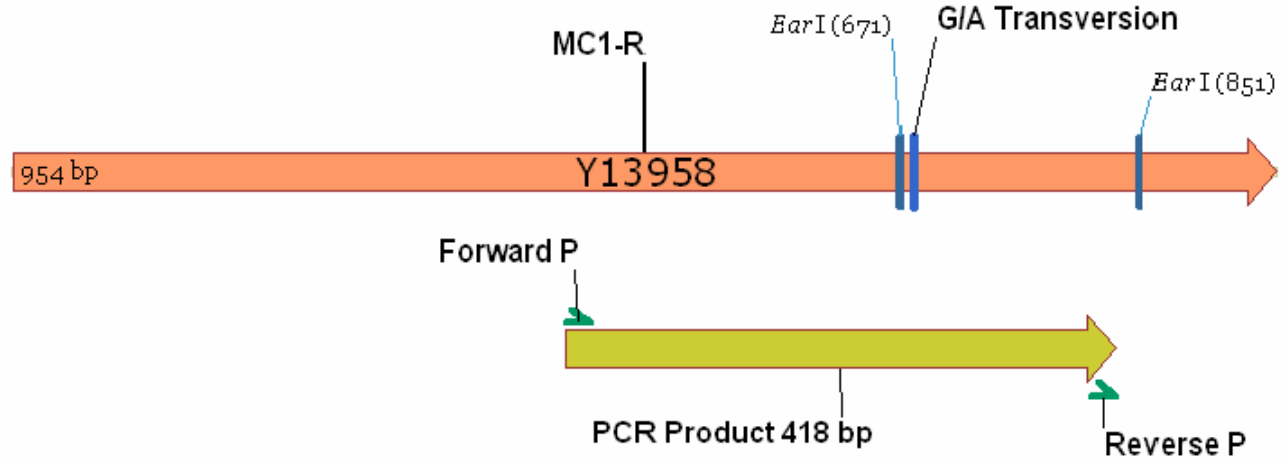

Figure 1 The target sequence and position of annealing of primers in MC1R gene used in this study

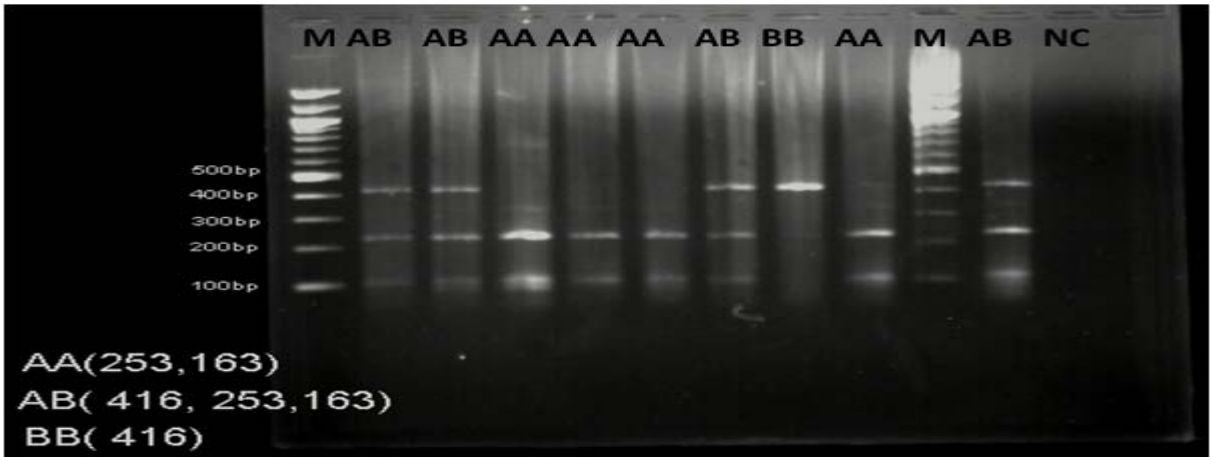

Figure 2 The gel picture for PCR-RFLP analysis of MC1R loci showing either AA, AB and BB genotypic variation. UD is undigested control product. Different fragment size (bp) of each genotype is represented at the side of the picture

In contrast to Saanen, Cashmere and Markhor with more BB genotype, indigenous breeds had more AA but less BB genotypes. Only AA and AB genotypes were observed in Tali breed, while the AA genotype was not detected in Cashmere goat.

Sequencing of the MC1R gene

Sequence analysis showed the presence of a single nucleotide polymorphisms located in the CDs region of the MC1R gene. Overall, allele B sequence was similar to the sheep coding region except for one mutation which was identified in Lys to Glu (Lysine/Glutamine) mutation at position 676. Since identified mutation located in the *EarI* recognition site allow distinguishing the B allele from the A allele and this finding double confirmed the a missense mutation K226E (Lys226Glu) variant in Saanen White goat sequence.

Statistical analysis

Comparison of allele frequency between different indigenous and exotic breeds of goats using χ^2 test showed significant ($P<0.01$) differences between colored and white coat phenotypes. The illustrated dendrogram using UPGMA in Popegene software also separated these breeds into two clusters, including exotic breeds with white / yellow coat color and indigenous breeds with black/brown phenotypes (Figure 4). The values of the chi-square (χ^2) test for genotype frequencies indicated that the locus considered in our present study was in Hardy-Weinberg equilibrium.

Local endangered breeds should be investigated and need to be preserved because they represent an important gene pool of rare alleles and allelic combinations that have disappeared in highly selected breeds (Gandini and Villa, 2003). In this study, we investigated the pattern and variability of MC1R gene in several goat populations. Our survey showed that indigenous goat breeds in the present study, the AA genotype was perfectly in accordance with black/brown coat color which could suggested A allele of MC1R gene is associated with such a phenotype and B allele is dealing with white / yellow coat color.

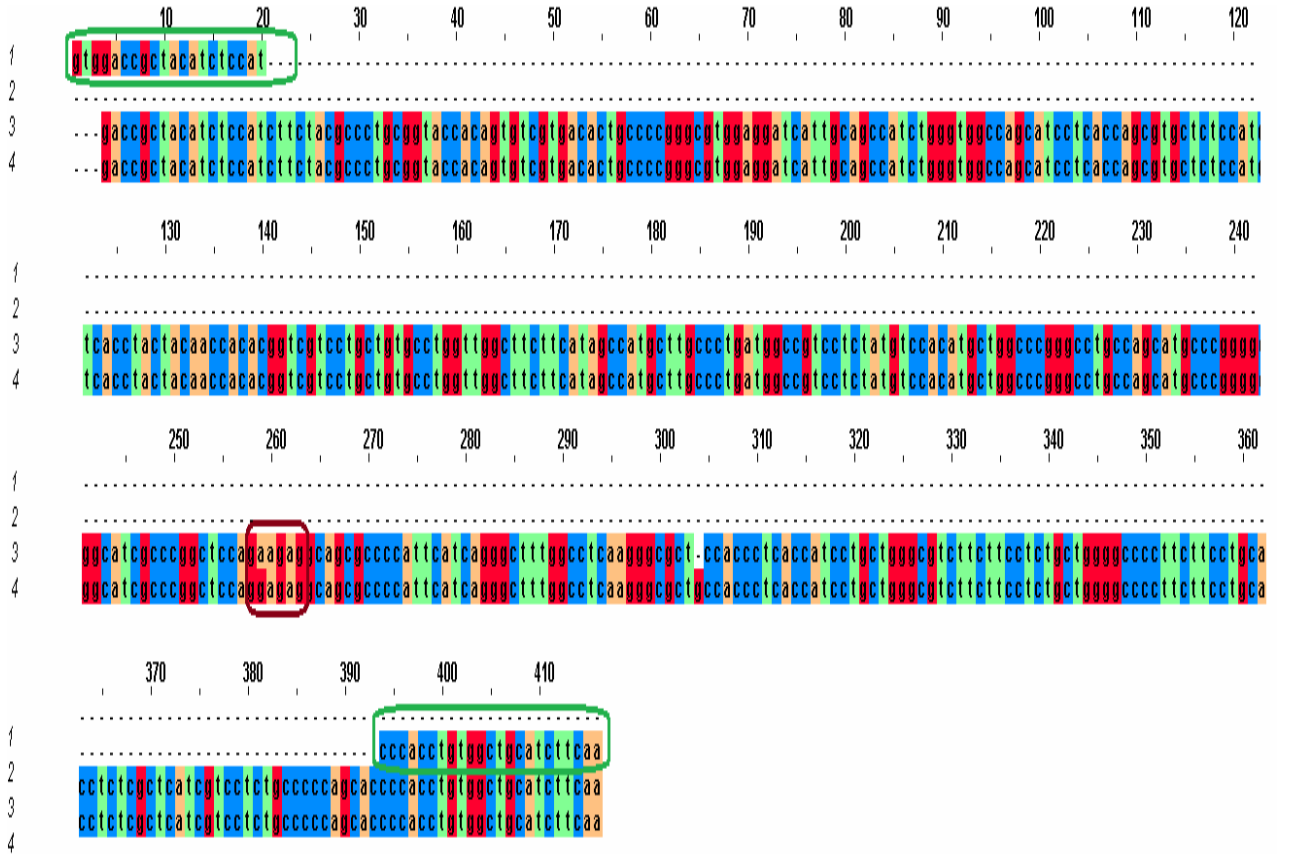

Figure 3 The target sequence and position, location of primers and identified SNP in MC1R gene investigated in this study

Table 1 Genotype and allele frequencies for the MC1R gene in seven breeds of goats used in this study (sample size in each breed=30)

Goat breed	Genotype frequency			Allele frequency		P-value from χ^2 test
	AA	AB	BB	A	B	
Saanen	0.1	0.13	0.76	0.17	0.83	0.60
Cashmere	0.0	0.1	0.9	0.05	0.95	0.59
Najdi	0.7	0.16	0.13	0.78	0.22	0.01
Markhor	0.16	0.2	0.63	0.26	0.74	0.00002
Khalkhal	0.6	0.16	0.23	0.68	0.32	0.00005
Tali	0.9	0.1	0.00	0.95	0.05	0.00001
Naeini	0.83	0.1	0.06	0.88	0.12	0.00001

Homozygote AA was defined when base A existed at position 676 forming GAAGAG being recognized by *EarI*; homozygote BB was defined when base G existed at this position, forming GGAGAG not being recognized by *EarI* and heterozygote AB was defined when A and G existed at the same position of the homologous chromosome.

Functions and mutations of MC1R gene have been widely investigated by different researchers. For example, analyzing the sheep MC1R gene by Vage *et al.* (1999) identified two missense mutations (p.M73K and p.D121N) determining the dominant black (E^D) allele in the Norwegian Dala breed.

The presence of these two mutations was also observed in other sheep breeds: Corriedale, Damara, Black Merino, Black Castellana and Karakul (Vage *et al.* 2003; Royo *et al.* 2008).

Pharmacological characterization of these two amino acid substitutions revealed that the p.M73K substitution alone was able to constitutively activate the receptor, probably increasing the stability of the high affinity activated state.

Our results are consistent with findings from earlier work on colour based genotyping by Russo *et al.* (2007); Deng *et al.* (2009); Fontanesi *et al.* (2009); Fontanesi *et al.* (2010);

Hepp *et al.* (2012) and Xi *et al.* (2012) in different livestock in this candidate gene.

In contrast to our finding some studies found monomorphic pattern in their targeted breeds. As logic justification, the reason for a difference in the present result with other findings seems particularly due to different breeds and geographical, molecular markers, candidate gene, different restriction enzyme and even more by technical staff, which may influence the analysis.

Zhao-Long *et al.* (2006) suggested that a missense mutation (K226E amino acid substitution) in the MC1R gene was associated with the presence of the red head phenotype in Tangshan dairy goats.

They explained that A is a recessive allele of MC1R gene and the color trait of this allele which is be long to autosomal inheritance can be explained by Mendelian law. Their results provided some interesting findings regarding to coat color in goats.

Deng *et al.* (2009) studied the entire coding region of the MC1R gene and showed two silent mutation sites located at A12G and G144C.

Thus, it seems that, at least in some goat breeds, the mechanisms of determination of the red coat color might be similar to those already described in other species, in which mutations in the MC1R gene are involved in determining this phenotype.

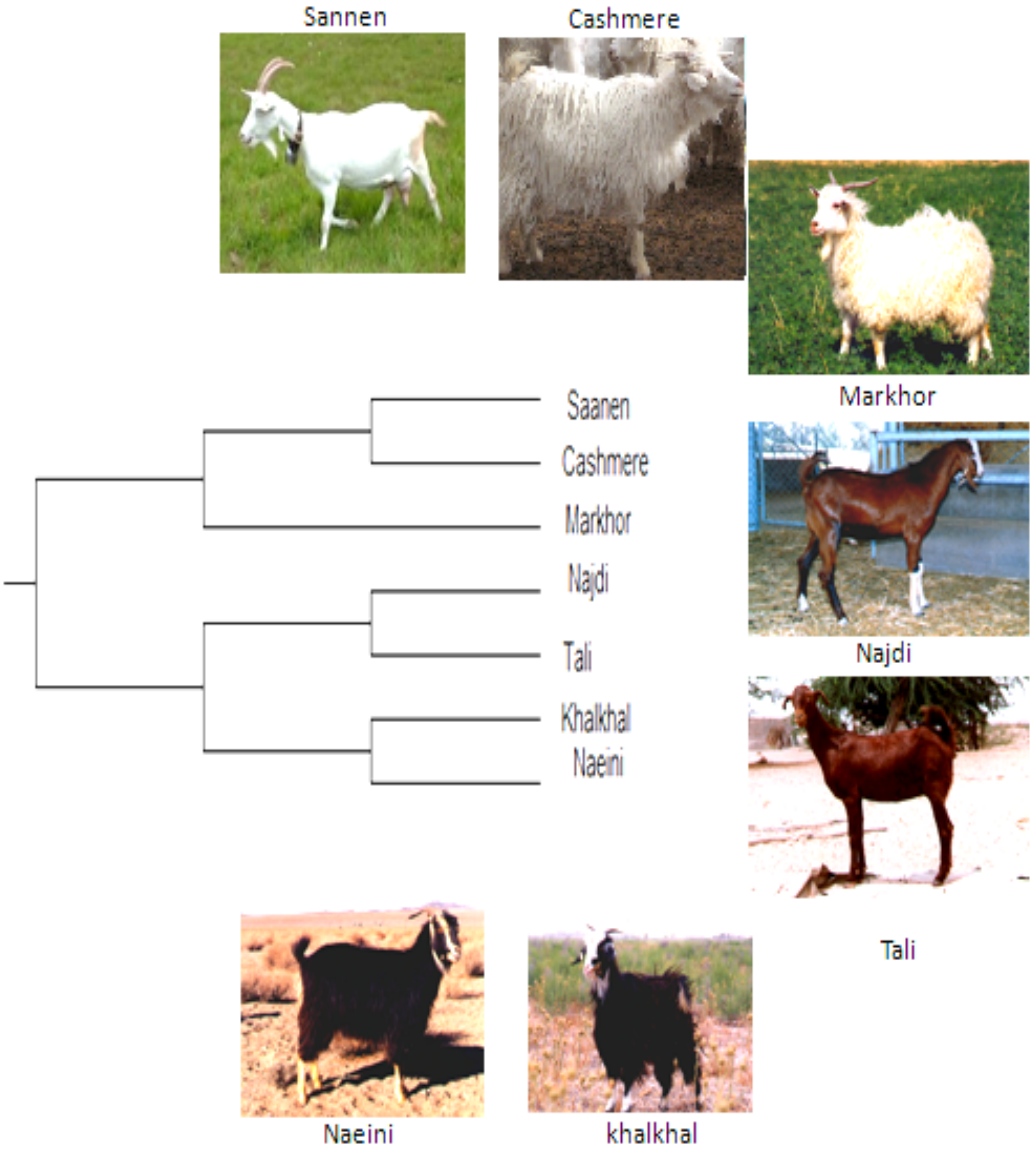

Figure 4 Dendogram showing two clusters of white and color coat phenotypes in seven studied goat breeds
In the goat breeds with black / brown coat color AA genotype showed higher frequency than white color goat breeds
Tali had AA and AB but no BB genotype and the AA genotype was not observed in cashmere

Some other studies have indicated that variations at the Agouti locus may be the main source of color variability within and between breeds (Fontanesi *et al.* 2009). The existence of a dominant E^D black allele and a recessive e red allele has been suggested in few breeds. In other goat populations, epistatic effects of Agouti alleles might mask and confound the action of the Extension locus. On the other hand, the wild type E^+ allele, the most common form supposed at this locus, should make the phenotypic effects of the different Agouti alleles possible, as observed in other species (Fontanesi *et al.* 2009).

Because the limitations of current study, the pattern and variability of MCR1 gene were only assessed in one small part of gene and not at different exons. Therefore, it is not surprising that in this study the valditiy of cluster for classification of the breeds based on molecular data are not high. Second, this result will be reasonable if used different candidate gene genotyping for search specific colour based SNP for white and coloured goat breed.

CONCLUSION

The present study demonstrated that the coat color can be an indirect selection marker for the MC1R genotype or a genotype at a closely linked gene. In conclusion, our results suggest that this locus is a potential candidate marker for a genetic tractability test that could be used to certify typical livestock production. It may, also be used in other breeds of local importance as a population-specific marker. However, future investigations into the limitations research and any other research questions to improve our knowledge are necessary.

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