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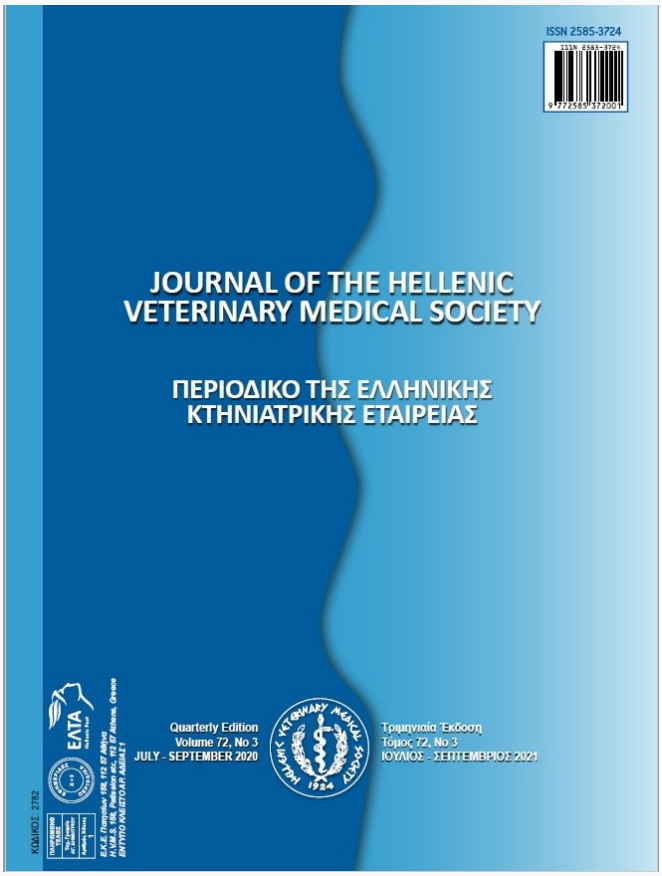

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Research article
Ερευνητικό άρθρο

Association of the caprine calpastatin *Msp*I polymorphism with growth and reproduction traits in Saanen goats

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ABSTRACT: This study was designed to evaluate the effects of calpastatin (*CAST*) *Msp*I polymorphism on some growth and reproduction traits, including birth weight, first breeding weight, litter size, and average daily weight gain in Saanen goats. In this sense, blood samples obtained from 73 purebred female Saanen goats were used for genotyping. Genomic DNA was isolated by the phenol-chloroform method and used to determine *CAST* genotypes, including MM, MN, and NN, by means of the PCR-RFLP method. The population genetic parameters were estimated based on allelic distribution and the data were statistically analysed using analysis of variance (ANOVA) using a general linear model (GLM). Results revealed that N allele frequency was remarkably high (0.64) and the MM genotype was not present. The frequency of the heterozygous genotype was 59.62%. Concerning ANOVA results, significant differences were found between genotypes of the *CAST* locus concerning birth weight ($P<0.05$). In this respect, animals with the NN genotype were associated with higher birth weight means (2.85±0.29 kg) compared to heterozygous animals (2.53±0.24 kg). There was no significant association between the *CAST* marker and any of the remaining phenotypic traits evaluated. The present results suggest that focusing on this genomic region may be particularly useful in improving birth weight in goats which can be considered as an early indicator of post-weaning animal growth and survival.

Keywords: Saanen, birth weight, *CAST*, single nucleotide polymorphism

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INTRODUCTION

Goat breeding is preferred worldwide for dairy production, meat, leather, and hair, and moreover, selection for increased levels of reproductive performance and disease resistance is also included in breeding objectives (Korkmaz Ağaoğlu et al., 2012; Rupp et al., 2016).

Growth and reproduction, along with profitability in production, are significant functional traits in sustainable goat breeding programs. Even with goats reared primarily for milk, such as the Saanen breed, desired reproduction parameters make an important contribution by influencing the herd size and providing a profitable dairy goat management concerning decreased culling rates (Shelton, 1978). Growth traits are important indicators in evaluating the sustainability and profitability of goat production systems because they contribute to current and future production through influencing post-weaning animal growth (Hanford et al., 2006; Menezes et al., 2016).

From a biological standpoint, the genetic basis of growth and reproduction traits is a complex one. Numerous genes contribute to the expression of these functional traits, directly or indirectly. In this context, the discovery of new mutations/polymorphisms in functional genes or identification of novel associations between previously reported genotypic variations and growth/reproduction traits are not surprising circumstances. The calpastatin (*CAST*) gene, which encodes an endogenous inhibitor of the calpains (m- and μ calpain), has been reported to be widely expressed in reproductive tissues or organs and it regulates the calpain activity of cells (Chung and Davis, 2012; Garcia et al., 2006). As a Ca^{2+}-dependent cytosolic cysteine protease, calpain play pivotal roles in proteolytic modulation of Ca^{2+} mediated intracellular mechanisms, such as cell cycle and differentiation, signal transduction, and apoptosis (Hata et al., 2001). Calpain proteinase system, involving m-calpain, μ calpain, and calpastatin, has a significant role in normal postnatal skeletal muscle growth (Goll et al., 1998). Increased calpastatin activity is associated with decreased rates of muscle protein turnover, and hence, this negative correlation results in increased levels of skeletal muscle growth (Chung and Davis, 2012; Goll et al., 1998; Parr et al., 1992; Pringle et al., 1993). These investigations impel researchers to focus on the genotypic structure of the *CAST* gene for growth and reproductive traits in livestock. Caprine *CAST* gene is located on chromosome 7 (14,437,312-14,567,828 reverse strand) (Ensembl Genome Browser, 2021). Evaluating this genomic region may provide useful information about variations of growth in goat breeding based on the calcium-binding domain which is the major regulator of the calpastatin activity.

CAST gene is mainly associated with muscle development to the formation of the fibers and thus it may influence the growth performance of mammals (Goll et al., 1998).Polymorphisms in particular genes that affect complex quantitative traits may affect multiple traits. This pleiotropy is the main cause of the genetic correlations between corresponding traits (Bolormaa et al., 2014). For instance, polymorphisms associated with increased milk yield may also increase the weight or age at puberty (Collis et al., 2011).The potential relationship between *CAST* and reproduction traits was studied on the bovine genome (Collis et al., 2011; Bolormaa et al., 2014; Ortega et al., 2017). However, the current knowledge on the associations of caprine *CAST* variations with growth and reproduction traits is quite limited. A comprehensive evaluation of this genomic region for not only muscle development and growth but also essential reproduction traits may provide adequate genotypic consideration of dairy goat reproduction performance. Taken altogether, this study was, therefore, performed to evaluate the effects of *CAST Msp*I polymorphism on birth weight, first breeding weight, litter size, and average daily weight gain in Saanen goats.

MATERIALS AND METHODS

Animals, sampling, and DNA extraction

A total of 73 purebred female Saanen goats that were grown on the same farm, Bursa Uludag University, Faculty of Veterinary Medicine Practice and Research Farm, located in the South Marmara region of Turkey (40° 14′ N and 28° 52′ E) were used. All of the animals were raised with the same feeding and management conditions. Animals were housed indoor and were fed with a concentrate feed in pellet form which contains 18% crude protein, 12 MJ/kg metabolizable energy, and alfalfa as roughage. During the experiment, goats had free access to water and had 0.50 kg/per animal concentrate feed and *ad libitum* roughage. All animals were fed twice daily (at 09:00 and 16:00). Measurement of phenotypic traits was performed according to Rashidi et al. (2011). Litter size was the number of kids born per parturition. The birth weight was the weight of the kids and it was recorded within 12 hours of birth. Average daily weight

gain (ADWG) was calculated from birth (BW) to the time reaching the first breeding weight (FBW) and it was calculated as ADWG = (FBW−BW)/age of goat at first breeding. The first breeding weight was the weight of goats when reaching the breeding age. Ethical approval was received from Bursa Uludag University local Research Ethics Committee(App. No: 2020-02/09). Blood samples (approximately 4 mL) were taken from jugular veins. Genomic DNA was extracted using a phenol-chloroform method according to Green and Sambrook (2012). To evaluate DNA quantification (ng/μL) and purity (260/280) analysis, a NanoDrop spectrophotometer (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA) was used.

Genotyping

In this study, genotyping of the SNP in the caprine *CAST* gene was performed by PCR-RFLP. A 622 bp fragment in the exon 1C/1D region of the *CAST* gene was amplified from the purified genomic DNA. PCR reactions were performed in a total volume of 25 μL, using 2.50 μL DNA sample (approximately 60 ng genomic DNA) as a template, 12.50 μL PCR master mix (OneTaq Quick-Load 2x MM with Standard Buffer, New England BioLabs Inc., Ipswich, Cat#M0486S, USA), 1 μL (0.5 μM) of each primer, and 8 μL of nuclease-free water (Thermo Scientific). Primer sequences were as follows:

Forward: 5'-TGGGGCCCAATGACGCCATC-GATG-3'

Reverse: 5'-GGTGGAGCAGCACTTCTGAT-CACC-3'

The primers were used based on the ovine sequences (Palmer et al., 1998) and were verified for caprine genome specificity by conducting BLAST searches of the NCBI Gen-Bank database. The PCR profile included an initial denaturation step at 95°C for 5 min, 30 cycles of 94°C (1 min), 60°C (1 min) and 72°C (2 min), and a final extension step of 8 min at 72°C. The PCR quality control was verified by electrophoresis (85-90 V for 45 min) using 10 μL of the amplified product in 2% (w/v) agarose gels (Sigma Aldrich, Steinheim, Germany) stained with ethidium bromide (Sigma Aldrich) with the concentration of 1 μg mL^{-1}. Afterward, 15 μL of PCR product (if verified) was digested with 10 U of *Msp*I restriction enzyme (New England BioLabs, Cat#R0106S), with 10x NEB buffer (New England BioLabs, Cat#B7004S) by incubating at 37 °C overnight. The digested products were then subjected to 3% (w/v) agarose gel electrophoresis. PCR-RFLP reactions were performed in a thermal cycler (Palm Cycler GC1-96, Corbett Research, Australia). The electrophoresis patterns were visualized by a gel imaging system (DNR-Minilumi, DNR Bio-Imaging Systems, Israel). Allele sizes were estimated by comparison to a 100-bp ladder (100-1500 bp, Biomatik Corporation, Ontario, Canada).

Statistical analysis

Estimation of allelic and genotypic frequencies and the Hardy-Weinberg equilibrium (HWE) testing (α=0.05) were performed by using Cervus v3.0 software. Indices of genetic diversity (effectiveness of allele incidence) including observed (experimental) heterozygosity (H_{exp}) / homozygosity (Ho) and the polymorphism information content (PIC) were estimated based on the formulas indicated by Nei and Roychoudhury (1974) and Botstein et al. (1980), respectively. The expected (theoretical) heterozygosity (H_{the}), the effective number of alleles (Ne), and the level of possible variability realization (LVPR-*V*%) were calculated as described by Crow and Kimura (1970). The fixation index (F_{IS}) was estimated from the values of theoretical (H_{the}) and experimental (H_{exp}) heterozygosities using the following formula:

$$F_{IS} = (H_{the} - H_{exp}) / H_{the}$$

The phenotypic traits were birth weight, first breeding weight, litter size, and average daily weight gain. Minitab (Minitab, Pennsylvania, USA, v17.1.0) was used as statistical software. The data were evaluated utilizing ANOVA using a general linear model (GLM) according to the following statistical models:

Model [1] was used to test the effects of *CAST* genotypes on the birth weight of kids:

$$Y_{ijklmn} = \mu + B_i + S_j + C_k + G_l + I_m + e_{ijklmn}$$

where: Y_{ijklmn} = the studied trait, μ = the overall mean, B_i = Birth year (i=2012-2018), S_j = Season (j=spring, summer, winter), C_k = Litter size (k=single, twin, triplet), G_l = *CAST* genotypes (l=MN, NN), I_m = two-way interactions, e_{ijklmn} = random error.

Model [2] was used to test the effects of *CAST* genotypes on first breeding weight, and average daily weight gain:

$$Y_{ijklmn} = \mu + B_i + S_j + G_k + \beta W_l + I_m + e_{ijklmn}$$

where: Y_{ijklmn} = the studied trait, μ = the overall

mean, B_i = Birth year (i=2012-2018), S_j = Season (j=spring, summer, winter), G_k = *CAST* genotypes (k=MN, NN), βW_l = regression effect of birth weight, I_m=two-way interactions, e_{ijklmn} = random error.

Model [3] was used to test the effects of *CAST* genotypes on litter size:

$$Y_{ijklm} = \mu + B_i + S_j + G_k + I_l + e_{ijklm}$$

where: Y_{ijklm} = the studied trait, μ = the overall mean, B_i = Birth year (i=2012-2018), S_j = Season (j=spring, summer, winter), G_k = *CAST* genotypes (k=MN, NN), I_l=two-way interactions, e_{ijklm} = random error.

A probability level of $P<0.05$ was considered statistically significant. Two-way interactions were not statistically significant ($P>0.05$) and they will not be discussed further.

RESULTS

PCR-RFLP patterns

The amplification of the gene encoding calpastatin using the appropriate primers yielded a 622bp amplicon (Figure 1). The cleavage of the PCR product with the *Msp*I nuclease resulted in three bands (622bp, 336bp, and 286bp) for heterozygous genotype (MN). The DNA amplified from NN animals remained undigested (622 bp) with the corresponding restriction enzyme (Figure 2). MM genotype (336bp and 286bp) was not observed in the present study.

Genetic variability

The frequency of allele N (0.64) of the caprine *CASTMsp*I polymorphism was much higher than M (0.36). The frequency of heterozygous genotype was quite high (59.62%) and the MM genotype was unseen, as shown in Table 1.

Table 1. Genotype and allele and frequencies of *CAST Msp*I polymorphism in caprine *CAST* gene, population genetic indices (H_{the}, H_{exp}, Ho, F_{IS}, LPVR, Ne, PIC) and compatibility with the Hardy-Weinberg equilibrium (HWE).

Locus	*CAST*		
Genotypes	NN	MN	MM
n	21	52	0
GF	40.38	59.62	0
EGF	30.30	33.50	9.30
Alleles	N		M
AF	0.64		0.36
H_{the}		0.4657	
H_{exp}		0.4608	
Ho		0.5392	
F_{IS}		0.0205	
LPVR (*V%*)		0.4499	
Ne		1.8546	
PIC		0.3546	
χ^2(HWE)		22.3395	
P		0.000*	

CAST - calpastatin. *n* - number of experimental goats. GF - genotype frequency. EGF - the expected genotype distribution according to HWE. AF - allele frequency. H_{the} - theoretical heterozygosity. H_{exp} - experimental heterozygosity Ho - homozygosity. F_{IS} - fixation index. LPVR - level of possible variability realization. Ne - number of effective alleles. PIC - polymorphism information content. χ^2(HWE) - Hardy-Weinberg equilibrium χ^2 value.
*not consistent with HWE.

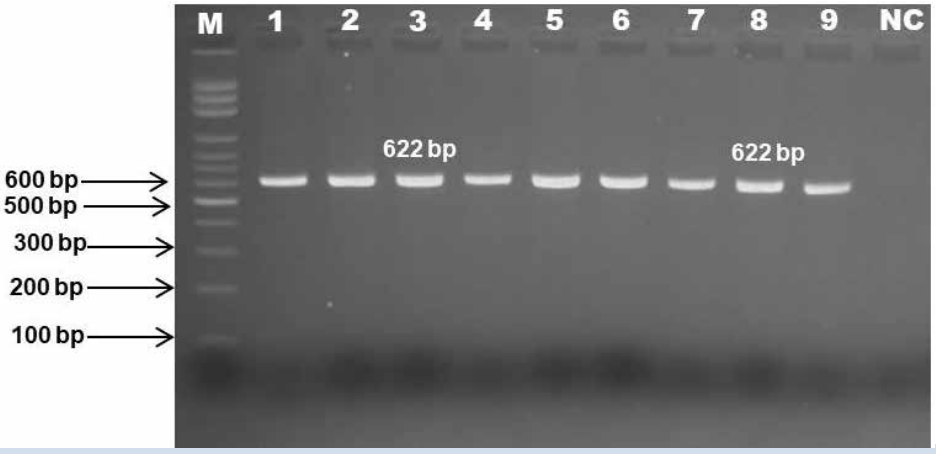

Figure 1. The electrophoresis pattern of PCR amplification (622 bp amplicon) for caprine *CAST* locus (M: Marker, 100-1500bp; NC: Negative control; bp: Base pair)

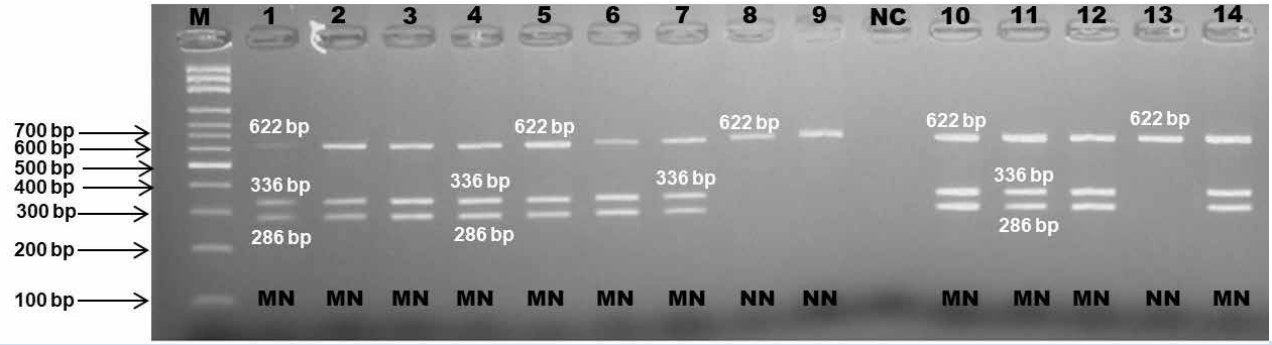

Figure 2. The electrophoresis pattern of restriction enzyme digestion of PCR product with *Msp*I for caprine *CAST* genotypes including NN and MN (M: Marker; NC: Negative control; bp: Base pair; Lines 8, 9, and 13: NN; Lines 1-7, 10-12, and 14: MN)

Table 2. The least-square means for the genotype effects of *CAST Msp*I polymorphism on studied traits (*n*=73)

Traits	NN Genotype		MN Genotype		Significance (*P*-value)
	Mean	Standard error	Mean	Standard error	
Birth weight (kg)	2.851	0.286	2.532	0.243	**0.032**
First breeding weight (kg)	40.191	4.322	42.460	3.160	0.490
Average daily weight gain (kg)*	0.050	0.005	0.053	0.003	0.723
Litter size	2.190	0.120	2.021	0.079	0.242

* Calculated based on the period between birth and reaching the first breeding weight.

HWE testing revealed that the population was determined not to be compatible with the equilibrium (P<0.001). The population genetic indices are presented in Table 1. Moderate levels of these indices were distinctly observable. The number of heterozygous genotype carriers was remarkably high (n=52) and the minor allele frequency was 0.36 (M allele). This resulted in the desirable genetic variability of Ne (1.85), although only two genotypes were observed (NN and MN).

Marker associations

Table 2 shows the least-squares means, standard errors, and the levels of significance concerning the effects of the *CAST* marker on birth weight, first breeding weight, litter size, and average daily weight gain. Significant differences were found between the genotypes of the *CAST* locus about birth weight (P<0.05). Animals with the NN genotype were associated with higher birth weight means (2.85±0.28 kg) compared to heterozygous animals (2.53±0.24 kg). There was no association between the marker and any of the remaining phenotypic traitsevaluated (first breeding weight, litter size, and average daily weight gain).

DISCUSSION

Genetic diversity

The present results indicated that there was a deviation from HWE for the *CAST* marker (P<0.001). Population characteristics in relation to selection process dynamics and inbreeding levels may cause this disequilibrium (Lacorte et al., 2006). Moreover, the typical structure of dairy herds which are under intense selection with a few sires should be considered when evaluating HWE. Population genetic parameters are very important indicators in the assessment of population structure for genetic variation. They also indicate the quality and suitability of genetic markers in a particular population (Ardicli et al., 2019). The low levels of H_{exp} indicate high inbreeding rates and it should be considered as a potential problem for the herd and should be accompanied by detailed pedigree information. On the other hand, the effectiveness of selected loci is determined by Ne values (Trakovická et al., 2013). In this study, the H_{exp} value was 0.4608, whereas, Ne value was 1.8546, and thus, the results indicated an admissible level of genetic variability in the analysed Saanen population at the considered locus. This interpretation was partially confirmed by a relatively low level of F_{IS} (0.0205) because this value can be considered as a good indicator for eventual heterozygosity and it displays the degree to which heterozygosity decreases (Duifhuis-Rivera et al., 2014). LVPR value, which is associated with homozygosity in the considered population (Miluchová et al., 2013), was determined to be 0.4499. Besides, present results

revealed that *CASTMsp*I polymorphism was moderately informative according to the classification of PIC values (high polymorphism if PIC>0.50, moderate polymorphism if 0.25<PIC<0.50, and low polymorphism if PIC<0.25) suggested by Botstein et al. (1980) (Table 1). Another important point is that the MM genotype was not present. This situation partially causes a negative impact on population impact on population genetic indices which is directly related to allele frequency distributions.

Marker effect on trait means

The relationship of caprine *CASTMsp*I polymorphism to birth weight, litter size, first breeding weight, and average daily weight gain was evaluated in this study. A member of the calpain-calpastatin system was chosen because it plays a crucial role in growth regulation. As Goll et al. (1998) indicated skeletal muscle growth is significantly associated with muscle protein synthesis and degradation and size/ number of skeletal muscle cells. There is fair evidence of a relationship between increased skeletal muscle growth and decreased muscle protein degradation (Chung and Davis, 2012; Goll et al., 1998). This is a cause of decreased levels of calpain activity which is regulated by calpastatin activity. Thus, the genetic mechanisms underlying the impacts of the calpain-calpastatin system on growth traits may partially explain the variations between individuals. Byun et al. (2008) suggested that *CAST* is an excellent candidate gene for controlling growth in livestock. The results of this study suggest that there is a significant association between the *CASTMsp*I polymorphism and the birth weight of kids in Saanen goats (P<0.05). Animals with the NN genotype had +0.319 kg heavier birth weight compared to the MN genotype. The birth weight of kids is one of the most important and reliable indicators of breed efficiency in the breeding plans for commercial goat production systems. This trait is known to be highly variable and is significantly affected both by genetic and environmental factors (Mioč et al., 2011). It is important to note that birth weight is associated with kid mortality which generally occurs at birth and from birth to weaning compared to mortality weaning to breeding age. Evaluating the effectiveness of preweaning management is imperative to have a desired kid survival rate (Awemu et al., 1999; Hailu et al., 2006). The present study indicates that selecting animals with the NN genotype of the *CASTMsp*I polymorphism induced higher means of birth weight and this application may contribute to well-handled preweaning management. However, there was no association of *CAST* marker with first breeding weight, average daily weight gain, and litter size. As in the present study, Byun et al. (2008) suggested that the *CAST* gene is an important regulator of birth weight, but has only a limited effect on growth rate to weaning in Romney lambs. Similar results were presented by Chung and Davis (2012) indicating a potential relationship between ovine *CAST* and birth weight. There is limited information about the relationship of the *CAST* marker to growth and reproduction parameters in goats. To the best of the author's knowledge, this is the first study suggesting a significant association between caprine *CASTMsp*I polymorphism and birth weight. The application area of genomic selection is quite limited in goat breeding compared to sheep and, especially, cattle. Thus, there is plenty of room for improvement in evaluating the genetic base of quantitative traits regarding the caprine genome.

Litter size or the number of kids born per parturition is an important indicator of productivity in goat breeding. In the present study, the litter size for the NN genotype was 2.19 whereas it was determined to be 2.02 in heterozygous animals. On the other hand, the heterozygous animals seemed to be heavier than the NN genotype concerning the first breeding weight of the goats (42.46 kg and 40.19 kg, respectively). However, this difference was not substantiated in association analysis (P>0.05). A similar implementation may be considered for the average daily weight gain from birth to first breeding. Growth and reproductive traits are known to show wide variations among breeds or populations and even different populations of the same breed in the same environmental conditions. It is important to note that considering different combinations of the polymorphisms based on the genotypic interactions through epistasis, genetic linkage, and pleiotropy may be worthy to provide a broad aspect in understanding the genetic basis of the quantitative traits. Recently, the trend of selection has gradually evolved from traditional procedures to marker-assisted selection or genomic selection in developed countries. These genomic methods allow breeders or researchers to use quantitative trait loci and candidate genes, which directly or indirectly affect the phenotype of individuals (Trakovická et al., 2013).

Improvement of production traits to increase economic gain is the preeminent aim of livestock. Never-

theless, the importance of functional traits including growth and reproduction cannot be underestimated to achieve a sustainable production system, especially in small ruminant breeding.

CONCLUSIONS

This paper points out the need for genetics research on the caprine genome regarding economically important quantitative traits and it also indicates a potential association between *CASTMsp*I polymorphism and birth weight in Saanen breed. The NN genotype may have a favourable influence on the birth weight of kids, and thus, we first suggested that caprine *CAST* could be regarded as a candidate molecular marker for birth weight. Further studies with larger populations are required to understand the complex biological nature of genetic basis on growth and reproduction traits in goats.

CONFLICT OF INTEREST STATEMENT

None of the authors of this article has any conflict of interest.

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