Assessment of calpastatin and insulin-like growth factor 1 genotypes in Tsigai sheep

Research Article

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ABSTRACT

The aim of the present study was to evaluate the genotypic distribution of calpastatin (*CAST*) and insulin-like growth factor 1 (*IGF1*) gene polymorphisms in the Tsigai sheep breed. Phenol-chloroform extraction procedures were applied to extract genomic DNA. A total of 56 sheep were genotyped by the PCR-RFLP method. Frequencies of the alleles/genotypes were calculated by the standard procedures. To evaluate the population-genetic properties, the Hardy–Weinberg Equilibrium (HWE) testing was performed. Moreover, genetic diversity was evaluated through the number of effective alleles (Ne), heterozygosity (He), polymorphism information content (PIC), the level of possible variability realization (*V*%), the fixation index (F_{IS}), and the Shannon-Weaver diversity index (*H´*) were estimated. Results revealed that the heterozygous genotype (0.64) frequency was remarkably higher than the homozygotes in the *CAST* locus. HWE testing showed a deviation ($P<0.05$) and the estimation of population genetic parameters indicated a moderate genetic variability in the *CAST* marker. Concerning *IGF1*, the Tsigai population was found to be monomorphic. In this context, all the animals were genotyped as the BB. The results provided by the present study may be useful in evaluating the genetic structure of the Tsigai sheep breed for which limited information is available.

Keywords: Tsigai breed, sheep, PCR-RFLP, *CAST*, *IGF1*

INTRODUCTION

Sheep breeding is one of the most significant sectors of livestock. It provides high-quality food and essential raw materials that are necessary for many branches of industry (Ilişiu et al., 2013; Yu et al., 2013; Sheriff and Alemayehu, 2018). In this context, many different sheep breeds have been developed worldwide concerning varying purposes. Tsigai sheep is a multi-purpose breed with a main focus on cheese production. This breed is one of the most important native sheep breeds in Central, South-Eastern, and Eastern Europe. They have white wool, brown, reddish, or white face, and legs. Tsigai is a medium-sized sheep that has an angular form, with a long and thin tail (Ilişiu et al., 2013). Although the homeland of this breed is Romania, it is imported and raised in different regions of Europe and Central Asia, such as Turkey.

Selection for low-heritability quantitative traits of economical importance is rather complicated and therefore difficult to benefit in breeding programs. Over the last decades, researchers in the field of livestock have been focused on the genotypic evaluation of these traits. Hereupon, the search for quantitative trait loci (QTL) and effective genetic markers significantly contribute to the variance of trait expression has been a primary interest for animal breeding and genetics.

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Various genetic markers have been known to be potentially associated with specific traits analyzed. Among them, calpastatin (*CAST*) is one of the important genes in livestock and it encodes the specific inhibitor of the calcium-dependent calpain protease family. Thus, it plays a fundamental role in muscle growth (Palmer et al., 1998; Schenkel et al., 2006; Gabor et al., 2009; Bayram et al., 2019). Ovine *CAST* gene is located on chromosome 5 and the length of its transcript is 2,701 bps (Ensembl, 2021). This gene has been reported to be a remarkable marker in selection aiming to improve meat quality and production (Casas et al., 2006; Schenkel et al., 2006; Zhou et al., 2007; Ardicli et al., 2017). Another important gene involves in physiological processes including reproduction and growth is insulin-like growth factor 1 (*IGF1*) which plays a pivotal role in mammalian fertility (Siadkowska et al., 2006; He et al., 2012; Bayram et al., 2019). It is thus a strong candidate gene for reproductive traits in livestock species. This gene has been mapped to chromosome 3 in sheep (Ensembl, 2021). The variation in the *IGF1* gene is associated with growth and reproductive traits in sheep (Curi et al., 2005; He et al., 2012; Ardicli et al., 2019).

The concept of breeding programs has been gradually improved by changing the main applications from phenotypical selection to genotypic selection by using molecular techniques. Genetic evaluation is needed to achieve desired sustainability and profitability and to implement an efficient livestock improvement. The knowledge of variation in the major genes in different breeds is one of the most indispensable constituents in genetic assessment. *CAST* and *IGF1* genes are significantly associated with growth and reproduction performance in livestock. These genes play fundamental roles in important traits of sheep as in other ruminants. In this study, the variation in the ovine *CAST* and *IGF1* genes has been investigated in the Tsigai breed.

MATERIAL and METHOD

Animals and sampling

In total 56 Tsigai sheep were investigated. All animals were raised in the 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). The study was carried out in compliance with the ethical requirements and was approved by the local Ethics Committee for Animal Research (App. No: 2020-05/12). Blood samples were collected into 5mL K_3EDTA vacutainer tubes from the vena jugularis of each sheep.

DNA isolation

Genomic DNA was isolated from blood samples using a phenol/chloroform method (Green and Sambrook, 2012). The spectrophotometric quantification of 1 µl of each DNA sample was carried out through the NanoDrop 2000c (Thermo Scientific, Wilmington, DE, USA).

Genotyping

Genotyping of the single nucleotide polymorphisms (SNPs) in the *CAST* and *IGF1* genes was performed by PCR-RFLP. PCR conditions, primer sequences, and restriction enzymes are shown in Table 1. PCR amplification was performed in a 25 µL reaction containing ~50 ng of genomic DNA, 12.50 µL PCR master mix (OneTaq Quick-Load 2x MM with Standard Buffer, New England BioLabs Inc., Ipswich, Cat#M0486S, USA), 1 µL (0.50 µM) of each primer, and 8 µL of nuclease-free water (Thermo Scientific Inc, USA, Cat#R0581). The PCR was performed using the Palm Cycler GC1-96 (Corbett Research, Australia). Amplification products were checked on a 2% agarose gel (migration for 50 min at 90 V) using 5 µL of PCR product and 2 µL of

loading buffer. RFLP analysis was performed by incubating a mixture of 15 µL of PCR product, 7.50 µL of nuclease-free water (Thermo Scientific), 3 µL of 10x enzyme buffer, and 0.50 µL of *Bst*H2I (RGCGC/Y; New England Biolabs, Beverly, MA, USA) at 37˚C for 16 h. Gels were visualized using a 3% agarose gel, photographed, and analyzed using a gel imaging system (DNR-Minilumi, DNR Bio-Imaging Systems, Israel).

Table 1. Primer sequences (from 5' to 3'), PCR conditions, and restriction enzymes that were used for genotyping the polymorphisms in the current study

Locus	Amplicon (bp)	Primers (5' to 3')	PCR conditions	Restriction enzyme	Reference
CAST	622	F: 5' TGGGGCCCAATGACGCCATCGATG 3' R: 5' GGTGGAGCAGCACTTCTGATCACC 3'	95°C 5′ (94°C 1′, 60°C 1′, 72°C 2′) 30 cycles, 72°C 10′	*Msp*I	Palmer et al.[6]
IGF1	294	F: 5'TGAGGGGAGCCAATTACAAAGC3' R: 5'CCGGGCATGAAGACACACACAT3'	94°C 6′ (94 °C 30s, 55°C 30s, 72°C 30s) 30 cycles, 72°C 10′	*Bst*H2I*	He et al.[12]

CAST: calpastatin; *IGF1*: insulin-like growth factor 1; * isoschizomer of *Bsp*143II

Evaluation of genotypic data

The allelic/genotypic frequencies were estimated using standard procedures (Falconer et al., 1996). Deviation from Hardy–Weinberg equilibrium (HWE) was calculated for each locus by using the chi-square goodness-of-fit test. Population genetic parameters including heterozygosity (He), effective allele numbers (Ne), and the polymorphism information content (PIC) were calculated according to Nei and Roychoudhury (1974) and Botstein et al. (1980). The level of possible variability realization (*V*%) was estimated according to Crow et al. (1970) as follows:

$V\% = (1\text{-}E) / (1\text{-}1/N) \times 100$

where:

E= expected homozygosity

N= number of individuals in a population regarding a particular locus

The fixation index (F_{IS}) was estimated from the values of theoretical (H_{the}) and experimental (H_{exp}) heterozygosities using the following formula:

$$Fis = \frac{Hthe - Hexp}{Hthe}$$

The Shannon-Weaver diversity index (*H′*) was calculated as follows:

$$H' = -\sum_{n=1}^{n} P_i^2 \ln P_i$$

where:

P_i is the proportion of each species/taxa/allele in the population, and ln is the natural logarithm (Ortiz-Burgos, 2016; Shannon and Weaver, 1949).

RESULTS

In the *CAST* gene, the reaction was performed to amplify partial regions of exon 1C and 1D and the intron between them. A 622 bp PCR product for the *CAST* gene (Figure 1) was digested with the *Msp*I restriction endonuclease and the reaction differentiated alleles M and N.

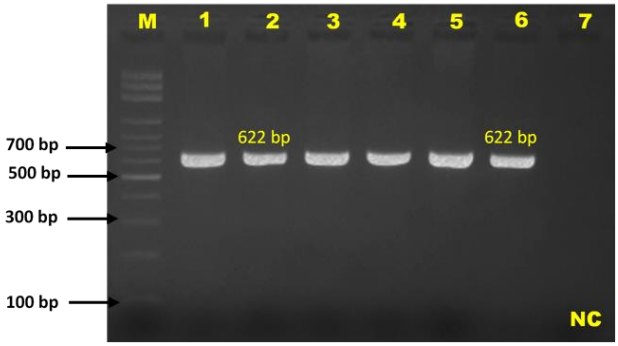

Figure 1. The electrophoresis pattern of PCR amplification (622 bp amplicon) for ovine *CAST* locus (M: Marker, 100-1500bp; NC: Negative control; bp: Base pair)

The results indicated three genotypes (MM, MN, and NN) were detected in Tsigai sheep (Figure

2). The homozygous genotype MM (336 bp, 287 bp) was detected in 14 sheep while the NN genotype (622 bp) was observed in six sheep. The heterozygous genotype MN (622 bp, 336 bp, 287 bp) was detected in 36 sheep.

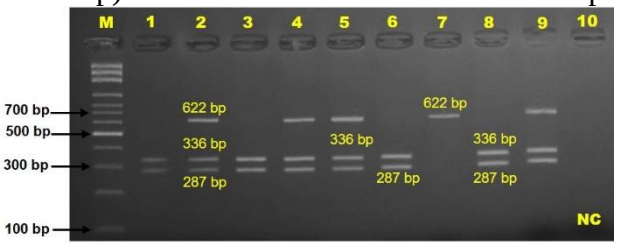

Figure 2. The electrophoresis pattern of restriction enzyme digestion of PCR product with *Msp*I for ovine *CAST* genotypes including NN and MN (M: Marker; NC: Negative control; bp: Base pair; Lines 1, 3, 6, and 8: MM; Line 7: NN; Lines 2, 4, 5, and 9: MN; Line 10: NC)

These results indicated a remarkable high frequency of heterozygous genotype carriers (64.29%). Notably, the NN genotype was very low (10.71%). The M allele frequency (0.57) was higher than the N allele frequency in the studied Tsigai population (Table 2). The genotypic distribution was not following the HWE (P<0.05). The calculation of population genetics indices revealed an admissible genetic variability regarding He (~0.49), Ne (~1.96), and PIC (~0.37). The F_{IS} and V% were found to be 0.2657 and 0.4990, respectively. The H' value indicated an acceptable diversity level (Table 3) in the Tsigai breed.

Table 2. Allele and genotype frequencies of *CAST* and *IGF1* in Tsigai sheep

Gene	Genotype Frequency*			Allele Frequency	
	MM	MN	NN	M	N
CAST	0.25 (14)	0.64 (36)	0.11 (6)	0.57	0.43
	AA	AB	BB	A	B
IGF1	0	0	1 (56)	0	1

*The number of animals per genotype is presented in parentheses

The amplification of the 5' regulatory region of the *IGF1* gene using the appropriate primers yielded a 294 bp amplicon. However, *Bst*H2I enzyme digestion (Figure 3) revealed that the B allele was fixed in the studied Tsigai sheep population because only the BB genotype was found (Table 2). Accordingly, the HWE and population genetic indexes for this locus were not estimated.

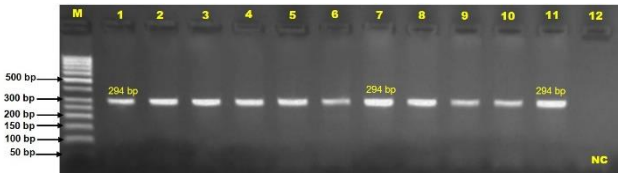

Figure 3. The electrophoresis pattern of the ovine *IGF1* locus. PCR revealed 294 bp amplicon; all samples remained undigested (*Bst*H2I restriction enzyme). Accordingly, all samples were genotyped as the BB (M: Marker, 50-1000bp; NC: Negative control; Lines 1-11: BB; Line 12: NC; bp: Base pair)

Table 3. Population genetic indices and compatibility with the Hardy–Weinberg equilibrium of calpastatin gene in Tsigai sheep

Population genetics parameters	
Heterozygosity (He)	0.4902
Number of effective alleles (Ne)	1.9616
Polymorphism information content (PIC)	0.3701
Level of possible variability realization (V%)	0.4990
Fixation index (F_{IS})	0.2657
Shannon index (H')[a]	0.6833
The compatibility with the Hardy–Weinberg equilibrium	
χ^2	5.4687
P	0.0193*

[a]Also referred to as the Shannon-Weaver diversity index, Shannon's information index, or Shannon entropy
*P<0.05; not consistent with the Hardy–Weinberg equilibrium

DISCUSSION

The presence of sufficient genetic diversity is a key point to the maintenance and long-term survival of most species including sheep and goats. Regarding recent animal production, research efforts into QTLs are underway internationally (Khan et al., 2012). This evaluation should be performed based on comprehensive information regarding the structure of the populations and the sources of genetic variability within and among populations (Sheriff and Alemayehu, 2018). The genotypic variation in livestock has been created by the forces of both natural and artificial selection (Groeneveld et al., 2010). The adaptation of different sheep breeds to a broad range of agroecology provides adequate variability that provides opportunities to meet the increased future demands for food and offers flexibility to respond to changing markets and needs (Sheriff and Alemayehu, 2018; Wollny, 2003). These processes have resulted in a wide variety of sheep breeds that were improved according to the demands of particular countries. Sheep are species able to adapt to a broad range of environments. This has enabled them to be easily reared in regions very different from the geography they originated from. However, this may result in genetic structures different from the original breed concerning natural processes that cause variation or crossbreeding. Genetic evaluation is necessary for not only pure/crossbreeds but also the breeds raised in different countries. This study was performed to evaluate the genetic variability regarding the *CAST* and *IGF1* genes in Tsigai sheep raised in Turkey conditions. It is important to note that the level of genetic variability may be remarkably high between breeds. In fact, genetic differences are present between different populations of the same breed (Ardicli et al., 2019). Evaluation of genotypic distribution of the major genes in livestock is required for providing a basic genetic data set through different sheep breeds raised in Turkey. In this respect, *CAST* is one of the best-known and popular major genes in livestock breeding. This gene encodes calpastatin which is an endogenous inhibitor of the calpains and its activity is associated with decreased rates of muscle protein turnover. This negative correlation results in increased levels of skeletal muscle growth (Chung et al., 2012; Goll et al., 1998). *CAST* plays important role in the formation of muscles, postmortem mechanisms related to muscle-specific systems, and thus, meat tenderness (Gabor et al., 2009). Hence, it has been shown to be a pivotal gene in meat quality evaluation (Schenkel et al., 2006). Therewithal, an association of the *CAST* with growth and live weight gain in sheep has been reported by Khan et al. (2012). The authors have reported that individuals with the heterozygous genotype were found to have higher weight gain compared to homozygotes in the native Balkhi and Kajli sheep breeds. However, the MN genotype frequency was quite lower in both sheep breeds they studied (Khan et al., 2012). Indeed, several studies have indicated that the MM genotype is predominant in various sheep breeds worldwide (Bayram et al., 2019; Dincel et al., 2015; Gabor et al., 2009; Gorlov et al., 2016; Khan et al., 2012). Gabor et al. (2009) have reported that the MM has the highest genotype frequency in Tsigai sheep and Tsigai × Lacaune crossbreds. They also reported that the NN genotype was not present in purebred Tsigai sheep. However, in the present study, we observed a quite different genotypic distribution compared to previous studies mentioned above. In this context, the predominant genotype was the *CAST-Msp*I-MN heterozygotes (0.64), and moreover, we have observed six sheep with the NN genotype. In accordance with the present results, Yılmaz et al. (2014) and Balcioğlu et al. (2014) have reported that the frequency of heterozygous genotype was the highest in Sakız and Karya sheep, respectively. Differences in the levels of genetic variation and the frequencies of alleles can be considered as common circumstances in genetic studies conducted on

different breeds or different populations of the same breed. However, remarkable differences in genetic variability in genetically well-known breeds or the absence of some common alleles may raise questions about breed purity. Nevertheless, this needs further investigation. Concerning the studied sheep breed in this study, Tsigai, there is limited genetic information even about very popular genetic markers for livestock. Hence, inconsistent results may not be surprising for this sheep breed.

In this study, there was a deviation from HWE regarding the selected *CAST* locus ($P<0.05$). This disequilibrium is a result of the substructure of populations under an intense selection process. Population genetic parameters are valuable indicators that provide knowledge on the genetic variation of populations concerning a particular gene or genes (Ardicli et al., 2019). Among these parameters, He and Ne express the quality and suitability of the genetic markers in a particular population. In this study, He approaches 0.50 whereas Ne approaches 2.00 in the Tsigai sheep population. Concerning the PIC observed (~0.37), the studied *CAST* marker can be considered as moderately informative (0.25<PIC<0.50) based on the classification suggested by Botstein et al. (1980). On the other hand, the indices of heterozygosity-homozygosity balance including F_{IS} and *V*% reflect eventual heterozygosity and they display the degree to which heterozygosity decreases (Duifhuis-Rivera et al., 2014; Miluchová et al., 2013). In this study, *V*% and F_{IS} were found to be 0.50 and 0.26, respectively. The *H′* is one widely used index for comparing diversity in biological systems (Ortiz-Burgos, 2016). This index can provide information to describe variation at multiple levels of genetic organization from SNPs, through whole species or larger taxonomic units to ecosystems (Konopiński, 2020). Estimation in the present study revealed that the *H′* amount was ~0.68, which represents a high genetic variation for the exon 1C/1D of the ovine *CAST* gene in the Tsigai breed. The *H′* value in this study was remarkably high compared to a previous study conducted on Tsigai (*H′*=0.30) and Tsigai × Lacaune (*H′*=0.33) sheep (Gabor et al., 2009). It is important to note that the usefulness of a marker is directly related to its level of polymorphism, and thus, the *CAST* *Msp*I marker may be evaluated as an admissible genetic variation constituent. The limitation of the present study is the small sample size, yet it should be considered that few animals are belonging to this breed raised in Turkey.

IGF1 gene plays a crucial role in the regulation of growth, development, and reproduction (Bakhtiar et al., 2017). In this study, polymorphism located on the 5' regulatory region of the *IGF1* gene was evaluated in Tsigai sheep. It is important to note that, although of limited importance, there is no information on *IGF1* in this breed. The results revealed that the studied population was monomorphic for the *IGF1* locus. All of the examined animals were found to be BB genotype carriers. Similarly, Bayram et al. (2019) have reported that lambs of the Akkaraman breed were found to have the monomorphic BB genotype. On the contrary, He et al. (2012) showed that all three genotypes (AA, AB, and BB) were present in Small Tail Han sheep and Hu sheep breeds. Dorset sheep were monomorphic regarding the AA genotype. The AA and AB genotypes were present in the Texel breed. Notably, Dorset and Texel sheep were characterized by very high AA genotype frequencies (100 and 93.80%, respectively). Grochowska et al. (2017) have indicated that the A allele was predominant (91.60%) resulting in the higher frequency of the AA (83.30%) compared to heterozygotes in Coloured Polish Merino sheep (the BB genotype was absent) concerning polymorphism in the 5' flanking region of the *IGF1* gene. Based on the results from previous studies, it may be plausible to interpret that the mentioned region of the ovine *IGF1* gene limitedly polymorphic. Nevertheless,

the differences among sheep breeds with varying genetic backgrounds should not be overlooked.

CONCLUSION

This paper focused on the genetic variability of the ovine *CAST* and *IGF1* in Tsigai sheep. Heterozygous genotype was found to be predominant in the *CAST* locus. The frequency of the NN genotype was the lowest in the population, which consequently resulted in the lower frequency of the N allele than the M. Population-genetic analysis showed that the *CAST Msp*I marker is substantially informative for the Tsigai breed. Concerning the *IGF1* locus, the sheep population was found to have the monomorphic BB genotype. The present results may contribute to the current genetic information on the Tsigai sheep breed.

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Conflict of interest: The authors declare no conflict of interest.

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