İSTANBUL
UNIVERSITY
PRESS

Determining Genetic Variation of Calpastatin Gene with MspI and NcoI Enzymes by Using PCR-RFLP Method in Kivircik Lambs

Kıvırcık Kuzularda PCR-RFLP Metodu Kullanılarak Calpastatin Geninin MspI ve NcoI Enzimleri ile Genetik Çeşitliliğinin Belirlenmesi

Kozet AVANUS 🆔

Department of Animal Breeding and Husbandry, İstanbul University Faculty of Veterinary Medicine, İstanbul, Turkey

ORCID IDs of the authors: K.A. 0000-0001-9886-7515.

Abstract

The aim of this study was to determine the genetic variation of calpastatin gene by using PCR-RFLP method with MspI and NcoI enzymes in Kivircik lambs. Blood samples of Kivircik lambs (n=153) that were collected from eight different farms located in Kirklareli province, were used for DNA isolation. After PCR amplification, products were digested with MspI and NcoI enzymes to differentiate M and N alleles on 2% and 3% agarose gel electrophoresis. The frequency of M and N alleles were found 88.2% and 11.8% for MspI locus and 98.7% and 1.3% for NcoI locus respectively. The frequencies of MM, MN and NN genotypes were identified 0.77, 0.22 and 0.01 respectively for MspI locus. The frequencies of MM and MN genotypes were determined 0.97 and 0.03 respectively for NcoI locus, NN genotype was not observed. Observed heterozygosity was higher in MspI (0.22) than NcoI locus (0.03). MspI and NcoI loci were found in Hardy-Weinberg equilibrium in Kivircik lambs raised in Kirklareli province.

Keywords: Calpastatin, native, sheep, Kivircik, genetic variation

Öz

Bu çalışmanın amacı Kıvırcık kuzularda PCR-RFLP yöntemi ile MspI ve NcoI enzimleri kullanılarak kalpastatin geninin genetik çeşitliliğini belirlemektir. Kırklareli ilinde bulunan sekiz farklı çiftlikten toplanan Kıvırcık kuzularının (n=153) kan örnekleri DNA izolasyonu için kullanılmıştır. PCR amplifikasyonundan sonra, ürünler %2'lik ve %3'lük agaroz jel elektroforezinde M ve N allellerini ayırt etmek için MspI ve NcoI enzimleri ile kesilmiştir. M ve N allellerinin frekansı MspI lokusunda sırasıyla %88,2 ve %11,8, NcoI lokusunda %98,7 ve %1,3 olarak bulunmuştur. MspI lokusu için MM, MN ve NN genotiplerinin frekansları sırasıyla 0,77, 0,22 ve 0,01 olarak tespit edilmiştir. NcoI lokusu için MM ve MN genotiplerinin frekansları sırasıyla 0,97 ve 0,03 olarak belirlenmiştir, NN genotipi ise gözlenmemiştir. Gözlenen heterozigotluk MspI lokusu için (0,22), NcoI lokusundan (0,03) daha yüksek olarak bulunmuştur. Kırklareli ilinde yetiştirilen Kıvırcık kuzuları MspI ve NcoI lokusları için Hardy-Weinberg dengesinde bulunmuştur.

Anahtar kelimeler: Kalpastatin, yerli, koyun, Kıvırcık, genetik varyasyon

Introduction

Kivircik is a native sheep breed and an essential source of red meat in Turkey (Ekiz and Altinel, 2005). For its major role in growth and meat tenderness, calpastatin gene captures particular attention in livestock. Association studies of calpastatin gene and meat quality traits in pigs (Ciobanu et al., 2004) and cattle (Casas et al., 2006; Curi et al., 2009) were analyzed by various studies. Calpastatin gene that was located on the 5th of the ovine chromosome (OAR5), was first genotyped in sheep by Palmer et al. (1998).

The level of calpastatin enzyme at slaugter, determines the calpain activity. Calpastatin inhibits calpain enzyme and involves in the degradation of myofibrilar proteins both in living and in post-mortem tissues. It regulates the extent of postmortem tenderization so that it has an influence on meat tenderness. (Page et al., 2002). A single nucleotide polymorphisms (C>T) identified in exon 27 of Calpastatin (CAST) gene in Korean cattle (Hanwoo) found highly correlated with meat tenderness (Chung and Davis, 2012[a]).

Calpastatin also has an effect on growth with promoting the proliferation of muscle fibers. It also influence on birth weight and growth rate until weaning in Romney lambs (Byun et al., 2008). Calpastatin gene has an affet on not only in average daily gain but also in post weaning weight of Targhee sheep (Chung and Davis, 2012[b]). Calpastatin locus variation was tried to figure out in different sheep breeds by using PCR-RFLP (Asadi et al., 2014; Ata and Cemal, 2008, Avanus, 2015; Azari et al., 2012; Dehnavi et al., 2012; Khan et al., 2012; Khederzadeh, 2011; Mohammadi et al., 2008; Nanekarani et al., 2011; Nassiry et al., 2006; Shahroudi et al., 2006; Suleman et al., 2012; Szkudlarek-Kowalczyk et al., 2011; Yılmaz et al., 2014), PCR-SSCP (Dagong et al., 2011; Palmer et al., 2000; Ranjbari et al, 2012; Zhou et al., 2007), and DNA sequencing (Aali et al., 2014; Djadid et al., 2011; Gregula-Kania, 2011; Zhou et al., 2007).

Gregula-Kania (2011) was reported two important SNPs in the intron1C and exon1D region of calpastatin gene. The one is G>A substitution and the other is C>T divergence. Restriction enzymes can be used to determine SNPs in the genome. Since recognition sites of MspI and NcoI enzymes were C**C**GG and CCA**T**GG, both can be used in identification of G>A and C>T SNPs in calpastatin gene. The aim of this study was to determine genetic variation of calpastatin gene for G>A and C>T SNPs in Kivircik lambs by PCR-RFLP method with using MspI and NcoI restriction enzymes.

Materials and Methods

This study was approved by Ethic Committee of the Istanbul University Veterinary Faculty (Approval number: 2013/24).

Animal material
Kivircik lambs (n=153) from eight different farms raised in Kirklareli region were used as animal material. Sterile vacuumed EDTA tubes and sterile double-ended nessles were used to take blood samples from Vena jugularis. An automated nucleic acid extraction system (ExiPrep™ 16Plus, Bioneer Company, South Korea) was used for genomic DNA isolation from blood samples of Kivircik lambs.

PCR components and conditions for calpastatin gene
The ovine caplastatin gene was amplified with 5'-TGGGGC-CCAATGACGCCATCGATG-3' and the reverse primer 5'-GGTG-GAGCAGCACTTCTGATCACC-3' as described by Palmer et al. (1999). Amplification of calpastatin gene was performed with

5 μL Ultra-Pure Taq PCR Master Mix (200 U/mL Ultra-Pure Taq DNA Polymerase, 1.25 mM dNTPs, 10 mM MgCl$_2$; Geneaid Biotech™, Taiwan), 0.5 μL 20 pmol each primer, 3 μL genomic DNA and 16 μL dH2O (AccuGENE™, Lonza, Belgium) in total volume of 25 μL. Polymerase chain reaction was performed with the following conditions; denaturing at 95°C in 3 min, 35 cycles of 95°C in 30 sec, 63°C in 50 sec, 72°C in 1 min and final extension at 72°C in 10 min (Bio-Rad T100, Bio-Rad Laboratories Inc., CA, USA).

Restriction analyzes with MspI and NcoI enzymes
Before starting to RFLP analyses, each PCR product was scaled according to a successful amplification for calpastatin gene. Amplicons of calpastatin gene were analyzed with RFLP method by using both MspI and NcoI enzymes. Incubation was performed to digest the PCR products with MspI and NcoI enzymes (MBI Fermentas) at 37°C by overnight. After performing the cleavage, band patterns for MspI and NcoI enzymes were visualized on 2% and 3% agarose gel respectively. Agarose gels were stained with ethidium bromide.

Statistical analysis
Frequencies for both alleles and genotypes, observed and expected heterozygosity and chi square (X^2) values for Hardy-Wienberg equilibrium (HWE) were estimated with PopGene32 software program version 1.31 (Yeh et al., 2000).

Results

Genomic DNAs that obtained from blood samples of Kivircik lambs were verified by observing gDNA bands on 0.8% agarose gel. Intron 1C and exon 1D constituent of the ovine calpastatin gene was amplified by PCR and screened as 622bp on 1% agarose gel electrophoresis.

Genotyping the gene with PCR-RFLP
Digesting the PCR products of calpastatin gene with MspI and NcoI enzymes were resulted with M and N alleles. Two fragments were observed (336 bp and 286 bp) for M allele, but N allele was retained unrestricted (622bp) after digesting with MspI enzyme (Figure 1). Frequencies of M and N alleles (88.2% and 11.8%), frequencies of MM, MN and NN genotypes (77.1%, 22.2% and 0.7%), observed heterozygosity and expected heterozygosity values (0.22 and 0.21) and chi square value (0.02) were given in Table 1 for MspI enzyme of calpastatin gene in Kivircik lambs.

Using NcoI enzyme resulted with two fragments for N allele (374 bp and 248 bp) and undigested fragment for M allele (622 bp) (Figure 1). Frequencies of M and N alleles (98.7% and 1.3%), frequencies of MM and MN genotypes (97.4% and 2.6%), observed heterozygosity and expected heterozygosity values (0.02 and 0.03) and chi square value (0.7) were shown in Table 1 for NcoI enzyme of calpastatin gene in Kivircik lambs. There were no NN genotype identified for NcoI enzyme.
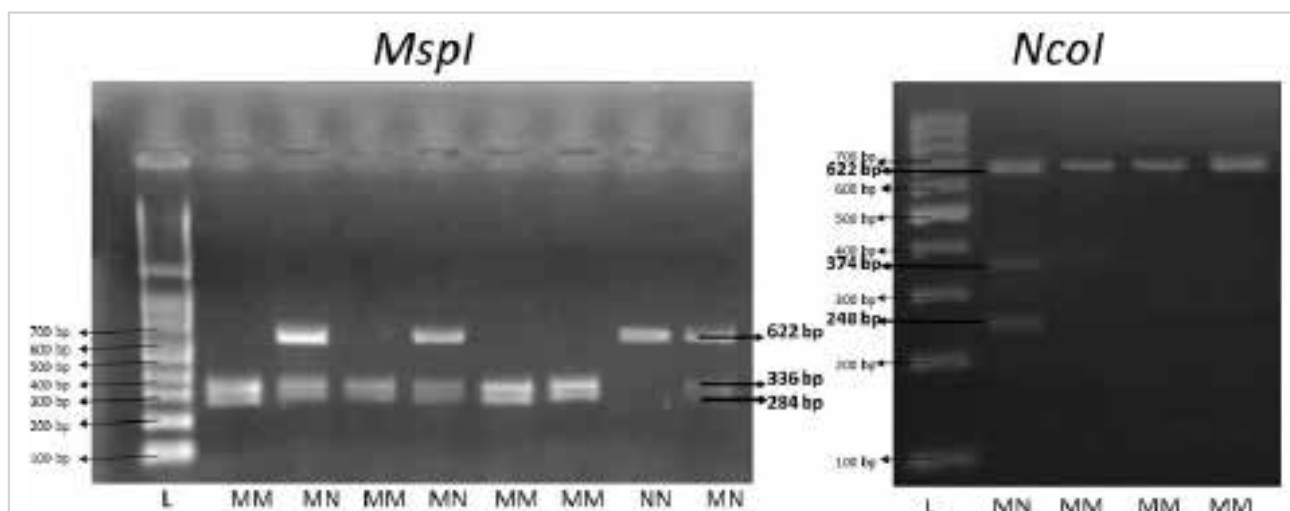
Figure 1. Band patterns of calpastatin gene genotypes (MM, MN and NN) observed after digested with MspI and NcoI restriction enzymes and visualized on 2% and 3% agarose gel electrophoresis in Kivircik lambs located in Kirklareli region

Table 1. Allele and genotype frequencies, observed and expected heterozygosity and chi square (X^2) values of calpastatin gene for MspI and NcoI enzymes in Kivircik lambs located in Kırklareli region

	Allele Frequency (%)			Genotype Frequency (%)			Heterozygosity		
Restriction Enzyme	n	M	N	MM	MN	NN	Ho	He	X^2
MspI	153	88.2	11.8	77.1	22.2	0.7	0.222	0.208	0.70[ns]
NcoI	153	98.7	1.3	97.4	2.6	-	0.026	0.025	0.02[ns]

ns: not significant (p>0.05)

Discussion

Kivircik lamb population raised in Kirklareli region was found in HWE. The frequencies (%) of M and N alleles in for MspI enzyme in this study were found similar to Zel (85.5 and 14.4) (Dehnavi et al., 2012), Balkhi (88.0 and 12.0), Kajili (88.0 and 12.0) (Khan et al., 2012), Lohi (87.0 and 13.0) (Suleman et al., 2012), Arabic (85.0 and 15.0) (Mohammadi et al., 2008) sheep breeds. The M and N allele frequencies were higher and lower respectively in Lori (63.8 and 36.2) (Asadi et al., 2012), Dalagh (55.5 and 44.5), (Azari et al., 2012), Thalli, Atabi (81.0 and 19.0) (Suleman et al., 2012; Nanekarani et al., 2011), Polish-Merino (76.2 and 23.8), Blackhead mutton (81.4 and 18.6) (Szkudlarek-Kowalczyk et al., 2011) and Iranian Karakul (79.0 and 21.0) (Shahroudi et al., 2006) sheep breeds. The frequencies of M and N alleles of the results of related study were determined lower and higher respectively in Berichon du Cher (92.7 and 7.3) and Il de France (95.0 and 5.0) (Szkudlarek-Kowalczyk et al., 2011) sheep breeds. The frequencies (%) of MM, MN and NN genotypes of this study were resemble Zel (75.0, 21.0 and 4.0) (Dehnavi et al., 2012), Kajli (74.0, 24.0 and 2.0) and Lohi (77.0, 20.0 and 3.0) (Suleman et al., 2012) sheep breeds.

Polish Merino, Bericchon du Cher and Il de France sheep breeds were not polymorphic for C>T transition in recognition site of NcoI enzyme and they all showed M allele. Blackhead Mutton sheep breed was reported as polymorphic for NcoI enzyme recognition site of calpastatin gene. The frequency of M and N alleles obtained from current study were found higher and lower for M (84.7) and N alleles (15.3) of Blackhead Mutton sheep breed (Szkudlarek-Kowalczyk et al., 2011). The frequency of MM (71.2), MN (27.1) and NN (1.7) genotypes of Blackhead Mutton sheep were very different from the outputs of related study.

Comparing the outputs of this study with the results of the studies that were obtained from native sheep breeds of Turkey showed that the M and N allele frequencies were found higher and lower respectively from Karacabey Merino (80.0 and 19.9), Chios (34.5 and 65.5) (Yılmaz et al, 2014b), Karya (54.4 and 45.6), Cine Capari (73.7 and 26.3) (Ata and Cemal, 2008) and Karakul (73.3 and 26.7) (Avanus, 2015) sheep breeds. The M and N allele frequencies were identified lower and higher respectively from Imroz (98.9 and 1.02; 96.3 and 3.7) (Yılmaz et al., 2014b; Avanus, 2015), Karayaka (90.9 and 9.1) and Hemsin (89.5 and 10.5) (Avanus, 2015) sheep breeds. Two Kivircik populations raised in Istanbul (Avanus, 2015) and Uşak (Yılmaz et al., 2014b) provinces of Turkey were reported for variation of calpastatin gene for MspI enzyme. Kivircik population was reported by Avanus (2015) from Istanbul province had

lower M (70.0) and higher N (30.0) allele frequencies, and its frequencies of MM (40.0), MN (60.0) and NN (0.0) genotyes were very different from Kivircik population raised in Kirklareli province. The frequencies of M (84.7) and N (15.3) allele and MM (72.9), MN (23.5) and NN (3.6) genotypes of Kivircik population located in Uşak (Yılmaz et al., 2014[b]). However calpastatin gene variation was not analysed with Nco I enzyme for native sheep breeds of Turkey. Analyzing calpastatin gene with NcoI enzyme resulted with M and N alleles, but there was no NN genotype observed for Kivircik breed located in Kirklareli region. Observed heterozygosity was higher in MspI than NcoI locus of calpastatin gene and both MspI and NcoI loci were found in Hardy-Weinberg equilibrium in Kivircik lambs raised in Kirklareli province.

Some association studies have been performed for calpastatin gene and different economically important traits in various sheep breeds. Balkhi sheep and Kajli sheep with heterozygous (MN) genotype for MspI enzyme exhibited higher weight gain from birth to four months and eight months of age respectively (Khan et al., 2012). However no significant difference was reported by Yılmaz et al. (2014) for MN genotype in Kıvırcık sheep breed for weaning weight. The average daily gain, back fat thickness and skin with back fat thickness values of Kivircik sheep with NN genotype for MspI enzyme was lower than MM and MN genotypes (Yılmaz et al., 2014). These undesirable effects of NN genotype might be also the reason of its lower frequency in Kivircik sheep breed in Kirklareli region compare to MM and MN genotypes.

This was the first report about calpastatin gene variation for NcoI locus in Kivircik sheep breed. No studies were found that focus on the relation between yield of quantitative economical traits and CAST/NcoI locus in the literature. In conclusion selection process in Kivircik sheep breed in Kirklareli region might be occurred negatively for NN genotype of both CAST/ MspI and CAST/NcoI loci. More association studies are need to perform between economically important traits and CAST/MspI and CAST/NcoI locus in Kıvırcık sheep breed. Further studies should be carried out to analyze calpastatin gene in different native sheep breeds of Turkey in order to understand its genetic structure and marker assisted selection candidacy profile in native sheep breeds.

References

Aali, M., Moradi-Shahrbabak, M., Sadeghi, M., 2014. Detecting novel SNPs and breed-specific haplotypes at calpastatin gene in Iranian fat- and thin-tailed sheep breeds and their effects on protein structure. Gene 537, 132-139. **[CrossRef]**

Asadi, N., Nanekarani, S., Khederzadeh, S., 2014. Genotypic frequency of calpastatin gene in lori sheep by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. African Journal of Biotechnology 13, 1952-1954. **[CrossRef]**

Ata, N., Cemal, I., 2008. Calpastatin gene polymorphism in Çine Çapari and Karya sheep. Scientific Papers Series D Animal Science 56, 48-51.

Avanus, K., 2015. Genetic Variability of CAST Gene in Native Sheep Breeds of Turkey. Kafkas Üniversitesi Veteriner Fakültesi Dergisi 21(6), 789-794.

Azari, M.A., Dehnavi, E., Yousefi, S., Shahmohamadi, L., 2012. Polymorphism of calpastatin , calpain and myostatin genes in native Dalagh sheep in Iran. Slovak Journal of Animal Science 12, 1-6.

Byun, S.O., Zhou, H., Forrest, R.H.J., Frampton, C.M., Hickford, J.G.H., 2008. Association of the ovine calpastatin gene with birth weight and growth rate to weaning. Animal Genetics 39, 572-573. **[CrossRef]**

Casas, E., White, S.N., Wheeler, T.L., Shackelford, S.D., Koohmaraie, R., Riley, D.G., Smith, T.P.L., 2006. Effects of calpastatin and μ-calpain markers in beef cattle on tenderness traits. Journal of Animal Science 84, 520-525. **[CrossRef]**

Chung, H., Davis, M., 2012[a]. Effects of genetic variants for the *calpastatin* gene on calpastatin activity and meat tenderness in Hanwoo (Korean cattle). Meat Science 90; 711-714. **[CrossRef]**

Chung, H., Davis, M., 2012[b]. PCR-RFLP of the ovine calpastatin gene and its association with growth. Asian Journal of Animal Science & Veterinary Advances 7, 641-652 **[CrossRef]**

Ciobanu, D.C., Bastiaansen, J.W.M., Lonergan, S.M., Thomsen, H., Dekkers, J.C.M., Rothschild, M.F., 2004. New alleles in calpastatin gene are associated with meat quality traits in pigs. Journal of Animal Science 82, 2829-2839. **[CrossRef]**

Curi, R., Chardulo, L.L., Mason, M.C., Arrigoni, M.D.B., Silveira, C., Oliveira, H.N., 2009. Effect of single nucleotide polymorphisms of CAPN1 and CAST genes on meat traits in Nellore beef cattle (Bos indicus) and in their crosses with *Bos taurus*. Animal Genetics 40, 456-462. **[CrossRef]**

Dagong, M.I., Sumantri, C., Noor, R.R., Herman, R., Yamin, M., 2011. Genetic polymorphisms of the coding region (exon 6) of calpastatin in Indonesian sheep. Media Peternakan 34, 190-195. **[CrossRef]**

Dehnavi, E., Ahani Azari, M., Hasani, S., Nassiry, M.R., Mohajer, M., Khan, A.R., Shahmohamadi, L., Yousefi, S., 2012. Association between yearling weight and calpastatin and calpain loci polymorphism in Iranian Zel sheep. Iranian Journal of Applied & Animal Science 2, 131-135.

Djadid, N.D., Nikmard, M., Zakeri, S., Gholizadeh, S., 2011. Characterization of calpastatin gene in Iranian Afshari sheep. Iranian Journal of Biotechnology 9(2), 145-149.

Greguła-Kania, M., 2011. New allelic variant of the ovine calpastatin gene. African Journal of Biotechnology 10, 13082-13085.

Khan, S., Riaz, M.N., Ghaffar, A., Ullah, M.F., 2012. Calpastatin (CAST) gene polymorphism and its association with average daily weight gain in Balkhi and Kajli sheep and Beetal goat breeds. Pakistan Journal of Zoology 44(2), 377-382.

Khederzadeh, S., 2011. Polymorphism of calpastatin gene in crossbreed Dalagh sheep using PCR-RFLP. African Journal of Biotechnology 10, 10839-10841. **[CrossRef]**

Mohammadi, M., Nasiri, M.T.B., Alami-Saeid, K., Fayazi, J., Mamoee, M., Sadr, A.S., 2008. Polymorphism of calpastatin gene in Arabic sheep using PCR- RFLP. African Journal of Biotechnology 7(15), 2682-2684.

Nanekarani, S., Khederzadeh, S., Kaftarkari, A.M., 2011. Genotypic frequency of calpastatin gene in Atabi sheep by PBR method. In: *Proc. International Conference on Food Engineering and Biotechnology IPCBEE, Bangkok, Thailand*, 9, 189-192.

Nassiry, M.R., Tahmoorespour, M., Javadmanesh, A., Soltani, M., Far, S.F., 2006. Calpastatin polymorphism and its association with daily gain in Kurdi sheep. Iranian Journal of Biotechnology 4(3), 188-192.

Page, B.T., Casas, E., Heaton, M.P., Cullen, N.G., Hyndman, D.L., Morris, C.A., Smith, T.P.L., 2002. Evaluation of single-nucleotide polymorphisms in CAPN1 for association with meat tenderness in cattle. Journal of Animal Science 80, 3077-3085. **[CrossRef]**

Palmer, B.R., Roberts, N., Hickford, J.G., Bickerstaffe, R., 1998. PCR-RFLP for *Msp*I and NcoI in the ovine calpastatin gene. Journal of Animal Science 76, 1499-1500. **[CrossRef]**

Palmer, B.R., Su, H.Y., Roberts, N., Hickford, J.G., Bickerstaffe, R., 2000. Single nucleotide polymorphisms in an intron of the ovine calpastatin gene. Animal Biotechnology 11, 63-67. **[CrossRef]**

Ranjbari, M., Hashemi, A., Mardani, K., Darvishzadeh, R., 2012. Allelic polymorphism of Makoei sheep calpastatin gene identified by polymerase chain reaction and single strand conformation polymorphism. Journal of Agricultural Science and Technology 14, 533-538.

Shahroudi, F.E., Nassiry, M.R., Valizadh, R., Heravi, A., 2006. Genetic poly-morphism at MTNR1A, CAST and CAPN loci in Iranian Karakul sheep. Iranian Journal of Biotechnology 4(2), 117-122.

Suleman, M., Khan, S.U., Riaz, M.N., Yousaf, M., Shah, A., Ishaq, R., 2012. Calpastatin (CAST) gene polymorphism in Kajli, Lohi and Thalli sheep breeds. African Journal of Biotechnology 4, 10655-10660.

Szkudlarek-Kowalczyk, M., Wiśniewska, E., Mroczkowski, S., 2011. Polymorphisms of calpastatin gene in sheep. Journal of Central European Agriculture 12, 425-432. **[CrossRef]**

Yeh, F., Yang, R.C., Boyle, T., 2000. Popgene (v.1.32) Microsoft Windows-based freeware for Population Genetic Analysis: http://www.ualberta.ca/~fyeh/Pop32.exe (Accessed 07.09.2017)

Yılmaz, O., Cemal, İ., Karaca, O., Ata, N., 2014a. Association of Calpastatin (CAST) gene polymorphism with weaning weight and ultrasonic measurements of loin eye muscle in Kivircik lambs. Kafkas Universitesi Veteriner Fakültesi Dergisi 20, 675-680. **[CrossRef]**

Yılmaz, O., Sezenler, T., Ata, N., Yaman, Y., Cemal, İ., Karaca, O., 2014b. Polymorphism of the ovine calpastatin gene in some Turkish sheep breeds. Turkish Journal of Veterinary & Animal Science 38, 354-357. **[CrossRef]**

Zhou, H., Hickford, J.G.H., Gong, H., 2007. Polymorphism of the ovine calpastatin gene. Molecular and Cellular Probes 21, 242-244. **[CrossRef]**