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

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

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Abstract

The aim of this study was to determine the genetic variation of calpastatin gene by using PCR-RFLP method with MspI and Ncol enzymes in Kivircik lambs. Blood samples of Kivircik lambs (n=153) that were collected from eight different farms located in Kırklareli province, were used for DNA isolation. After PCR amplification, products were digested with MspI and Ncol 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 Ncol 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 Ncol locus, NN genotype was not observed. Observed heterozygosity was higher in MspI (0.22) than Ncol locus (0.03). MspI and Ncol loci were found in Hardy-Weinberg equilibrium in Kivircik lambs raised in Kırklareli province.

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

Öz

Bu çalışmanın amacı Kıvırcık kuzularda PCR-RFLP yöntemi ile MspI ve Ncol enzimleri kullanılarak kalpastatin geninin genetik çeşitliliğini belirlemektir. Kırklareli ilinde bulunan seksiz farklı çiftlikte 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 allelerinin ayrı etmek için MspI ve Ncol enzimleri ile kesildiği M ve N allelerinin frekansı MspI lokusunda sırasıyla %88,2 ve %11,8, Ncol 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. Ncol 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), Ncol lokusundan (0,03) daha yüksektir olarak bulunmuştur. Kırklareli ilinde yetiştirilen Kıvırcık kuzuları MspI ve Ncol lokusları için Hardy-Weinberg dengesinde bulunmuştur.

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

Introduction

Kıvırcık 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 (OARS), was first genotyped in sheep by Palmer et al. (1998).
The level of calpastatin enzyme at slaughter, 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). Calpastatin also has an effect on growth with 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 effect on not only in average daily gain but also in post weaning weight of Targhee sheep (Chung and Davis, 2012). Calpastatin locus variation was tried to figure out in different sheep breeds by using PCR-RFLP (Asadi et al., 2014; Ate 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; Nasiriy et al., 2006; Shahroud et al., 2006; Suleman et al., 2012; Szkudlarek-Kowalczyk et al., 2011; Yılmaz et al., 2014), PCR-SSCP (Djadid et al., 2014; Djadid et al., 2011; Gregula-Kania, 2011; Zhou et al., 2007), and DNA sequencing (Aali et al., 2014; Djadid et al., 2014; Ata and Cemal, 2008, Avanus, 2015; Azari et al., 2014; 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 ofMspI and Ncol enzymes are CCGG and CCATGG, both can be used in identification ofG>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 Ncol 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 Kirkareli region were used as animal material. Sterile vacuumed EDTA tubes and sterile double-ended nessesles 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 calpastatin gene was amplified with 5’-TGGGGC-CCAATGACGCCATCGT-3’ and the reverse primer 5’-GGTG-GAGGACACTTCTTGATCACC-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 MgCl2; Geneaid Bio-tech™, 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 Ncol enzymes

Before starting to RFLP analyzes, 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 Ncol enzymes. Incubation was performed to digest the PCR products with MspI and Ncol enzymes (MBI Fermentas) at 37°C by overnight. After performing the cleavage, band patterns for MspI and Ncol 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 (X2) 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 Ncol enzymes were resulted with two fragments for N allele. Digesting the PCR products of calpastatin gene with MspI and Ncol enzymes were resulted with two fragments for N allele. Incubation was performed to digest the PCR products with MspI and Ncol enzymes (MBI Fermentas) at 37°C by overnight. After performing the cleavage, band patterns for MspI and Ncol enzymes were visualized on 2% and 3% agarose gel respectively. Agarose gels were stained with ethidium bromide.

Using Ncol 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 (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. There were no NN genotype identified for Ncol enzyme.
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), Kajli (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 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, Berichon du Cher and II de France sheep breeds were not polymorphic for C>T transition in recognition site of Ncol enzyme and they all showed M allele. Blackhead Mutton sheep breed was reported as polymorphic for Ncol 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) (Yilmaz 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) (Yilmaz et al., 2014a), 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 (Yilmaz et al., 2014a) provinces of Turkey were reported for variation of calpastatin gene for MspI enzyme. Kivircik population was reported by Avanus (2015) from Istanbul province had

Table 1. Allele and genotype frequencies, observed and expected heterozygosity and chi square (X²) values of calpastatin gene for MspI and Ncol enzymes in Kivircik lambs located in Kirklareli region

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Allele Frequency (%)</th>
<th>Genotype Frequency (%)</th>
<th>Heterozygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>M</td>
<td>N</td>
</tr>
<tr>
<td>MspI</td>
<td>153</td>
<td>88.2</td>
<td>11.8</td>
</tr>
<tr>
<td>Ncol</td>
<td>153</td>
<td>98.7</td>
<td>1.3</td>
</tr>
</tbody>
</table>

ns: not significant (p>0.05)
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) genotypes were very different from Kivircik population raised in Kirkklareli 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). However calpastatin gene variation was not analysed with NcoI enzyme for native sheep breeds of Turkey. Analyzing calpastatin gene with Ncol enzyme resulted with M and N alleles, but there was no NN genotype observed for Kivircik breed located in Kirkklareli region. Observed heterozygosity was higher in Mspl than Ncol locus of calpastatin gene and both Mspl and Ncol loci were found in Hardy-Weinberg equilibrium in Kivircik lambs raised in Kirkklareli 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 Mspl 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 Yilmaz et al. (2014) for MN genotype in Kivircik 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 Mspl enzyme was lower than MM and MN genotypes (Yilmaz et al., 2014). These undesirable effects of NN genotype might be also the reason of its lower frequency in Kivircik sheep breed in Kirkklareli region compare to MM and MN genotypes.

This was the first report about calpastatin gene variation for Ncol locus in Kivircik sheep breed. No studies were found that focus on the relation between yield of quantitative economical traits and CAST/Ncol locus in the literature. In conclusion selection process in Kivircik sheep breed in Kirkklareli region might be occurred negatively for NN genotype of both CAST/ Mspl and CAST/Ncol loci. More association studies are need to perform between economically important traits and CAST/Mspl and CAST/Ncol locus in Kivircik 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.

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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]


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]

Dagost, M.L., 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]


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]