Kafkas Univ Vet Fak Derg
21 (6): 789-794, 2015
DOI: 10.9775/kvfd.2015.13138

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Genetic Variability of CAST Gene in Native Sheep Breeds of Turkey [1]

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[1] *This study was presented as an oral presentation in 3rd Turkish-Bosnian Scientific Days within the Framework of the Partnership between the Veterinary Faculties of Sarajevo University and Istanbul University*

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Article Code: KVFD-2015-13138 Received: 13.02.2015 Accepted: 08.09.2015 Published Online: 09.09.2015

Abstract

The aim of this study is to determine the genetic variability of CAST gene in native sheep breeds of Turkey by PCR-RFLP method. Six different native sheep breeds; Kivircik, Imroz, Karayaka, Hemsin, Red Karaman and Karakul were used in this study. This study was the first report about CAST gene variation in Karayaka, Red Karaman and Hemsin sheep breeds. After DNA isolation and PCR amplification, RFLP was performed with *Msp*I enzyme. Two alleles M (336bp and 286bp) and N (622bp) were identified on 2% agarose gel electrophoresis. Allel and genotype frequencies, observed (Ho) and expected heterozygosity (He) and deviation from Hardy Weinberg Equilibrium were estimated by statistical analyses. The frequency of M allele was highest in Imroz (96%) and N allele was identified most frequently in Kivircik (30%) breed. Highest frequencies of MN genotype were identified in Kivircik (60%), MM in Imroz (92.6%) and NN in Red Karaman (7.1%) breeds respecitvely. Kivircik, Imroz, Karayaka and Karakul breeds were null from NN genotype. Kivircik sheep showed the highest heterozygosity (60%) and Imroz had the lowest (7.4%). The highest heterozygosity value was identified in Kıvırcık (60%), the lowest in Imroz (7.4%). All breeds except Kivircik and Hemsin were found in Hardy-Weinberg equilibrium. Absence of NN genotype in some breeds and high frequency of MN genotype in Kivircik breed might be resulted from the selection process of native sheep breeds in their breeding regions.

Keywords: *Calpastatin, Native sheep breeds, Genetic variation*

Türkiye Yerli Koyun Irklarında CAST Genine Ait Genetik Çeşitliliğin Belirlenmesi

Özet

Bu çalışmanın amacı, Türkiye yerli koyun ırklarında CAST genine ait genetik çeşitliliğin PCR-RFLP yöntemi ile belirlenmesidir. Çalışmada 6 farklı yerli koyun ırkı; Kıvırcık, İmroz, Karayaka, Hemsin, Morkaraman ve Karagül kullanılmıştır. Bu çalışma Karayaka, Morkaraman ve Hemsin koyun ırklarında CAST geni çeşitliliğini ortaya koyan ilk çalışmadır. DNA izolasyonu ve PZR ile yükseltgemenin ardından RFLP analizi *Msp*I enzimi ile gerçekleştirilmiştir. %2'lik agaroz jel elektroforezi ile M (336bç ve 286bç) ve N (622bç) olmak üzere iki allel saptanmıştır. İstatistiksel hesaplamalar ile allel ve genotip frekansları ile beklenen (Ho) ve gözlenen (Ho) heterozigotluk değerleri ile Hardy Weinberg dengesine uyum durumları saptanmıştır. En yüksek M allel frekansı İmroz (%96), N allel frekansı ise Kıvırcık ırkında tespit edilmiştir. MN, MM ve NN genotip frekansları en yüksek olarak sırasıyla Kıvırcık (%60), İmroz (%92.6) ve Morkaraman (%7.1) ırklarında belirlenmiştir. Kıvırcık, İmroz, Karayaka ve Karagül ırklarında NN genotipi bulunamamıştır. Heterozigotluk değeri Kıvırcık ırkında en yüksek (%60) iken, İmroz ırkında en düşük (%7.4) olarak belirlenmiştir. Kıvırcık ve Hemsin dışında tüm ırklar Hardy-Weinberg dengesine uyumlu bulunmuştur. Bazı ırkların NN genotipini bulundurmaması ve Kıvırcık ırkının MN genotipi için en yüksek genotip frekansına sahip olması; yerli koyun ırklarının yetiştirildikleri bölgelerdeki seleksiyon sürecinden kaynaklanmış olabileceği düşünülebilir.

Anahtar sözcükler: *Kalpastatin, Yerli koyun, Genetik çeşitlilik*

INTRODUCTION

Sheep is one of the most important red meat sources in Turkey [1]. Calpastatin (CAST) gene captures special attention for its major role in both meat tenderness and growth in animals. Therefore CAST is one of the most screened genes in livestock. Various studies have been performed to identify the CAST gene variation in goats [2] and its association with meat quality traits in pigs [3] and cattle [4-7]. CAST gene was first identified in sheep by

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Palmer et al.[8] and it was located on the 5th chromosome in sheep genome [9]. Calpastatin (CAST) enzyme is the specific inhibitor of calpain proteases which regulates the rate and extent of post mortem tenderization [10]. Calpain enzyme plays a key role in meat tenderness by degrading myofibrillar proteins after slaughter during the process of rigor mortis [11]. Calpain CAST system (CCS) is important in muscle growth. Reduction of calpain and increase in CAST activities may result to increase in growth rate of skeletal muscle. CAST gene was described as an important regulator on birth weight; its influence was shown on growth rate until weaning in Romney lambs [12]. Chung and Davis [13] reported that CAST gene has positive effect on both average daily gain and post weaning weight in Targhee sheep. However Dehnavi et al.[14] did not find any relation between CAST gene and yearling weight in Zel sheep. Variation of CAST locus in various sheep breeds were identified by using PCR-RFLP [10,14-26], PCR SSCP [9,27-29] and DNA sequencing [27,30-32] methods. A point mutation in intron 12 region of CAST gene causes the substitution of Guanin (G) nucleotide to Adenine (A) and diverges the CCGG nucleotides to CCAG sequence. Since CCGG is recognition site to *Msp*I enzyme, G-A substitution makes the site unrecognizable by the enzyme; therefor the mutation can be identified with PCR-RFLP method [31]. Two alleles (M and N) and three genotypes (MM, MN and NN) were described in CAST locus after PCR-RFLP analysis with *Msp*I enzyme [8].

The aim of this study was to identify CAST genotype variation in thin-tailed sheep breeds; Kivircik, Imroz and Karayaka, semi-fat tailed breed; Hemsin and fat-tailed sheep breeds; Red Karaman and Karakul by using PCR-RFLP method.

MATERIAL and METHODS

This study was approved by Ethic Committee of the Istanbul University Veterinary Faculty (Approval number: 2011/163).

Thin-tailed sheep breeds; Kivircik (n=25), Imroz (n=27) and Karayaka (n=22), semi-fat tailed breed; Hemsin (n=19) and fat-tailed sheep breeds; Red Karaman (n=14) and Karakul (n=15), in total 122 sheep were used as animal samples. Blood samples were taken from Vena jugularis into sterile vacuumed EDTA tubes in five different sheep breeds. Genomic DNA was isolated from blood by using ExiPrep^{TM} 16Plus automated nucleic acid extraction system (Bioneer Company, Chonbuk, Chonju, South Korea). DNA isolation in Karayaka sheep breed was performed from raw meat samples. DNA from meat samples were obtained by PureLink DNA isolation kit (Invitrogen, Carlsbad,CA, USA).

The region of the ovine CAST gene was amplified by using PCR with the forward primer 5'TGGGGCCCAATGA CGCCATCGATG3' and the reverse primer 5'GGTGGAGCA GCACTTCTGATCACC3', which captured a 622bp sized fragment from intron 12 and exon 13 (AF016006.1) [8,31].

PCR was carried out in a final volume of 50 µl containing; 100 ng genomic DNA, 20 pmol each primer, 200 mM dNTPs each, 1.5 mM $MgCl_2$, 10X PCR Buffer and 0.25U Taq polymerase (MBI Fermentas). PCR 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). PCR products were digested with 1 µl (10U) *Msp*I enzyme (MBI Fermentas). Samples were incubated at 37°C by overnight for *Msp*I digestion. After performing RFLP, band patterns were visualized by 2% agarose gel.

Samples showed polymorphic band pattern for *Msp*I enzyme were sequenced both forward and reverse directions by REFGEN gene research and biotechnology firm (www.refgen.com) with ABI 3100 avant automated DNA sequencer in order to confirm the haplotypes.

Allele and genotype frequency observed and expected heterozygosity and chi square test to analyze the deviation from Hardy-Weinberg equilibrium (HWE) were estimated by using PopGene32 software program version 1.31 [33].

The sequence results were compared with the reference sequence of ovine CAST gene (GenBank: AF016006.1) by using BioEdit sequence alignment editor *(http://www.mbio.ncsu.edu/BioEdit/bioedit.html)* software program.

RESULTS

Two alleles of CAST locus (M and N) were identified after digestion with *Msp*I enzyme. Band patterns of M allele (336bp and 286bp) and N allele (622bp), MM, MN and NN genotypes were viewed on 2% agarose gel stained with EtBr *(Fig. 1)*.

Allele frequencies, genotype frequencies, observed and expected heterozygosity, chi square and p values of CAST locus were given in *Table 1*.

Three different haplotypes; a (CCGGG), b (CCGGA) and c (CCAGA) were identified for CAST locus *(Fig. 2-A)*. Haplotypes that constitute MM (aa, ab), MN (ac) and NN (cc) genotypes were shown in *Fig. 2-B*. None of the MN genotype showed haplotype b. Only Hemsin and Red Karaman breed were carry haplotype b within their MM genotype.

DISCUSSION

The frequency of M allele was the highest in Imroz (96%) sheep. N allele was identified most frequently in Kivircik (30%) breed. The highest frequencies of MN genotype was observed in Kivircik (60%), MM in Imroz (92.6%) and NN in Red Karaman (7.1%) breeds respectively.

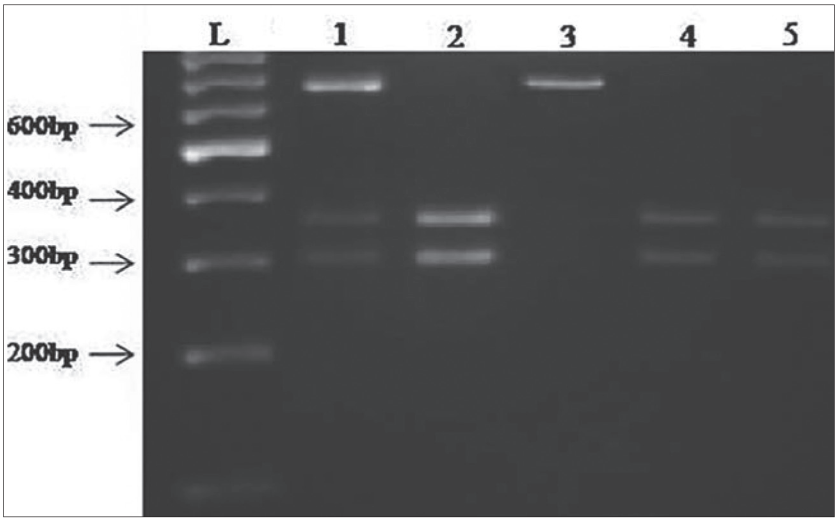

Fig 1. MM genotype (336bp and 286 bp in lane 2, 4, 5), MN genotype (622bp, 336bp and 286 bp in lane 1) and NN genotype (622 bp in lane 3) of CAST locus visualized on 2% agarose gel

Şekil 1. %2'lik agaroz jelde görüntülenen CAST lokusuna ait MN genotipi (622bç, 336bç ve 286 bç 1. kuyucukta) MM genotipi (336bç ve 286 bç 2., 4., 5. kuyucuklarda) ile NN genotipi (622 bç 3. kuyucukta)

Table 1. *Allele frequencies, genotype frequencies, observed and expected heterozygosity, chi square and p values for Hardy Weinberg Equation of CAST locus*

Tablo 1. *CAST lokusuna ait allel frekansları, genotip frekansları, gözlenen ve beklenen heterozigotluk, Hardy Weinberg Dengesi için ki kare ve p değeri*

Breed	**n**	**Allele Frequency (%)**		**Genotype Frequency (%)**			**Heterozygosity**			**P**
		M	***N***	***MM***	***MN***	***NN***	H_o	H_E	X^2	
Kivircik	25	70.0	30.0	40.0	60.0	0.0	0.600	0.429	4.235	0.040*
Imroz	27	96.3	3.7	92.6	7.4	0.0	0.074	0.073	0.020	0.889ns
Karayaka	22	90.9	9.1	81.8	18.2	0.0	0.182	0.169	0.162	0.688ns
Hemsin	19	89.5	10.5	84.2	10.5	5.3	0.105	0.194	5.139	0.023*
Karakul	15	73.3	26.7	46.7	53.3	0.0	0.533	0.405	1.697	0.193ns
Red Karaman	14	75.0	25.0	57.2	35.7	7.1	0.357	0.389	0.10	0.745ns

*ns = not significant P<0.05, * significant P<0.05*
*H_O = observed heterozygosity, H_E = expected heterozygosity, x^2 = Chi-square, **p** = probability*

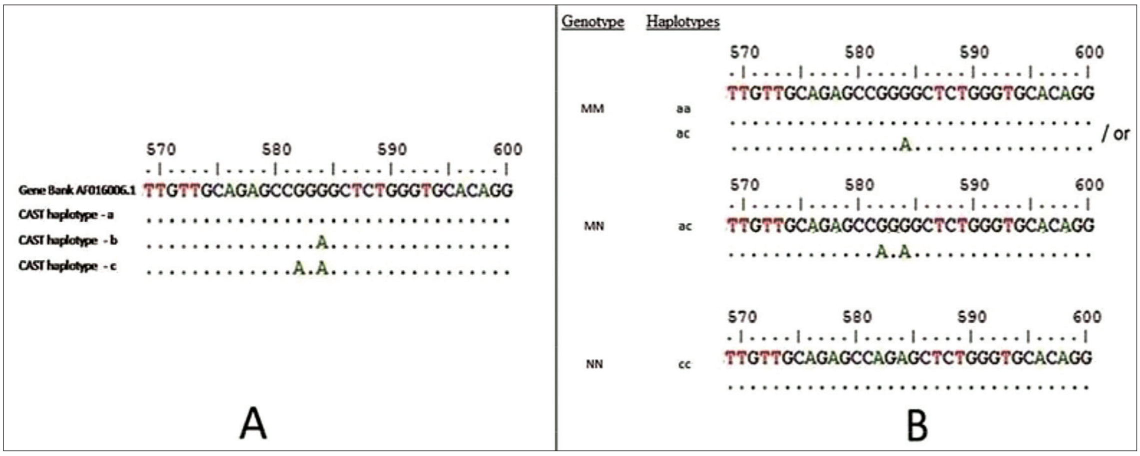

Fig 2. A- Three haplotypes (a,b and c) of CAST locus were identified in native sheep breeds of Turkey, **B**- Haplotypes constitute MM, MN and NN genotypes

Şekil 2. A- Türkiye yerli koyun ırklarında belirlenen CAST lokusuna ait üç haplotip (a,b ve c), **B**- MM, MN ve NN genotiplerini oluşturan haplotipler

Previous studies performed by other researchers for identifying genetic variation of CAST gene in different sheep breeds with RFLP method were summarized in *Table 2.*

According to the results of this study M and N allele frequencies and MM and MN genotype frequencies of Imroz sheep was found similar with Ile de France breed [22]. M and N allele frequencies of Red Karaman sheep were

Table 2. *Allele frequencies, genotype frequencies, heterozygosity values and compatibility of populations with HWE (p value) of CAST locus in different sheep populations reported by various researchers*

Tablo 2. *Çeşitli araştırmacıların allel frekansları, genotip frekansları, heterozigotluk değerleri, populasyonların HWE dengesine uyumları için ki kare ve p değeri*

Breed	N	Allele Frequency (%)		Genotype Frequency (%)			Heterozygosity			Reference
		M	*N*	*MM*	*MN*	*NN*	H_o	H_e	p	
Lori Sheep	100	63.8	36.2	40.7	46.2	13.1	ND	ND	ND	[15]
Kivircik	203	84.2	15.8	72.9	22.7	4.4	0.227	0.266	x	[16], [17]
	336	84.7	15.3	72.9	23.5	3.6	0.235	0.260	NS	
Karacabey Merino	248	80.0	19.9	66.9	26.2	6.9	0.262	0.320	xx	[17]
Chios (Sakız)	87	34.5	65.5	9.2	50.6	40.2	0.506	0.452	NS	
Imroz	49	98.9	1.02	97.9	2.04	0.0	0.020	0.020	NS	
Karya	90	54.4	45.6	54.3	38.8	6.9	0.577$^+$	0.496*	NS	[26]
Cine Capari	97	73.7	26.3	29.6	49.6	20.8	0.423$^+$	0.387*	NS	
Dalagh Sheep	110	55.5	44.5	36.0	38.0	26.0	0.380	0.490	x	[10]
	120	80.0	20.0	65.5	29.0	5.5	0.300	0.320	NS	[20]
Zel Sheep	200	85.5	14.4	75.0	21.0	4.0	0.210	0.250	x	[14]
Balkhi	300	88.0	12.0	74.0	24.0	0.0	0.240	0.210	NS	[18], [19]
Kajli	300	86.0	14.0	74.0	24.0	2.0	0.240	0.240	NS	
	100	90.0	10.0	80.0	2.0	0.0	0.100	0.310	NS	
Lohi	100	87.0	13.0	77.0	20.0	3.0	0.100	0.226	NS	[19]
Thalli	100	81.0	19.0	68.0	26.0	6.0	0.100	0.180	NS	
Atabi Sheep	120	81.0	19.0	67.5	27.5	5.0	0.275	0.306	ND	[21]
Polish Merino	82	76.2	23.8	56.1	40.2	3.7	ND	ND	ND	[22]
Berichon du Cher	41	92.7	7.3	85.4	14.6	0.0	ND	ND	ND	
Blackhead Mutton Sheep	59	81.4	18.6	71.2	20.3	8.5	ND	ND	ND	
Ile de France	30	95.0	5.0	90.0	10.0	0.0	ND	ND	ND	
Arabic Sheep	111	85.0	15.0	70.2	28.8	0.9	ND	ND	NS	[23]
Iranian Karakul Sheep	100	79.0	21.0	61.0	36.0	3.0	0.35	0.67	ND	[24]

x: *P<0.05,* ***xx:*** *P<0.01,* ***ND:*** *not determined,* ***NS:*** *not significant, * estimated from the referred article*
H_O *= observed heterozygosity,* H_E *= expected heterozygosity,* x^2 *= Chi-square,* **p** *= probability*

found similar with Cine Capari [26] and Polish Merino breed [22]. MM genotype frequency was also found similar with Polish Merino breed [22]. Observed heterozygosity and expected heterozygosity values of Red Karaman breed were found similar to both Iranian Karakul [24] and Cine Capari breed [26] respectively. M and N allele frequencies of Hemsin sheep breed were found similar with Balkhi [18] and Lohi [19] sheep breeds, on the other hand MM genotype frequency was found similar with Berichon du cher [22] sheep breed. NN genotype frequency of Hemsin breed was found similar with Dalagh [20] and Atabi sheep [21] breeds. Observed heterozygosity in Hemsin breed was found similar with Kajili, Lohi and Thalli [19] sheep breeds. Expected heterozgosity in Hemsin breed was also found similar with Thalli sheep breed. M and N allele frequencies of Karayaka sheep in this study were found similar with Kajili [18] and Berichon du cher [22] sheep breeds and MM and MN genotype frequencies were also similar to Kajili [18] and Blachead Mutton sheep breeds [22]. MM genotype frequency and observed heterozygosity of Kivircik breed were found similar with Lori sheep [10] and Karya sheep [26] respectively. M and N allele frequencies and MN genotype frequency of Karakul sheep were found similar with Cine Capari sheep breed [26]. Khan et al.[18] found that heterozygous (MN) genotype showed significantly higher weight gain from birth to eight months in Balkhi sheep and from birth to four months of age in Kajli sheep breeds respectively. Results of this study showed that Kivircik, Imroz, Karayaka and Karakul breeds were found null from NN genotype as; Balki [18], Kajli [19], Berichon du cher and Ile de France sheep [22] and Gokceada (Imroz) [17] sheep breeds were also reported. Yılmaz et al.[16] reported that animals with NN genotype showed lower average daily gain (ADG), back fat thickness (BT) and skin with back fat thickness (S+BT) values.

It can be concluded from the current study that, selection process of native sheep breeds in their breeding regions may occurred negatively for NN genotype in Kivircik, Imroz, Karayaka and Karakul sheep breeds however it may occurred positively for MN genotype in Kivircik breed. Kivircik is the most popular sheep breed for red meat source in Turkey.

Imroz, Red Karaman, Karayaka and Karakul populations were found in HWE. However Kivircik and Hemsin were not in HWE being similar to Kivircik [16], Dalagh [10] and Zel sheep [14] populations. The highest and lowest heterozygosity values were identified in Kivircik (60%) and Imroz (7.4%) breeds respectively. Since Imroz breed is originated from Imroz Island, low heterozygosity value is an expected outcome for this breed.

CAST gene haplotypes obtained with sequencing, were found very similar with the findings of Gregula-Kania [31]; only CAST-b haplotype (CCGGA) was divergent than reported data. CAST b haplotype located in border of recognition site of *Msp*I enzyme, was identified only in MM genotype of Hemsin and Red Karaman breeds. However structure of M and N alleles were formed respectively by haplotype a and c which were localized in *Msp*I recognition site.

Understanding the effect and selection variation of CAST locus may help to improve marker assistant selection (MAS) studies in sheep breeding, particularly in meat tenderness and growth. This was the first report about CAST gene variation in Karayaka, Red Karaman and Hemsin sheep breeds of Turkey. Further studies on CAST locus should be performed in different native sheep breeds to enlighten the genotype structure and candidacy profile for MAS studies of sheep genetic resources of Turkey.

Acknowledgments

The author is grateful to Assoc. Prof. Dr. Bülent TEKE and Assoc. Prof. Dr. Filiz AKDAĞ for their help in supplying meat samples of Karayaka sheep breed. Imroz, Karakul, Hemsin, Red Karaman and Kivircik sheep breeds were belonging to flock of Istanbul University Faculty of Veterinary Medicine Research and Education Farm. Special thanks to Prof. Dr. Ahmet ALTINEL for his scientific support in developing the study.

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