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RESEARCH ARTICLE

Characterization of Calpastatin Gene in Purebred and Crossbred Turkish Grey Steppe Cattle

Süleyman KÖK * Sertaç ATALAY ** Mustafa SAVAŞÇI ** Hasan Semih EKEN **

* Trakya University, Keşan Vocational High School, Department of Veterinary, TR-22800 Keşan, Edirne - TURKEY
** Keşan Food Control Laboratory, TR-22800 Keşan, Edirne - TURKEY

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Summary

Calpastatin (CAST) is a specific inhibitor of calcium-dependent neutral protease μ-calpain found in mammalian tissues. The genetic variants in the bovine CAST gene were analysed by using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The animal material of the study consisted of 132 bulls and heifers in Turkish Grey steppe cattle and Turkish Grey steppe x Brown Swiss crossbreds. C and G alleles are characterized by CAST/RsaI polymorphism were detected in the sample of animals were studied. Allele frequency C was significantly frequent in the crossbred group compared to the pure Turkish Grey steppe animals ($P<0.05$). In the total samples of animals, the average allele frequency C was 56.1%. Genotypic frequencies were estimated as 0.257, 0.499 and 0.243 in the purebred Turkish Grey steppe, and 0.388, 0.470 and 0.142 in the Turkish Grey steppe x Brown Swiss crossbred population for genotypes CC, CG and GG, respectively. As a result, genotypic distributions were equilibrium in both pure and crossbred examples of Turkish Grey steppe cattle.

Keywords: *PCR-RFLP, Grey steppe cattle, Calpastatin (CAST), Genetic marker, Polymorphism*

Safkan ve Melez Türk Boz Irk Sığırlarda Kalpastatin Geninin Karakterizasyonu

Özet

Kalpastatin (CAST), memeli dokularında bulunan ve kalsiyuma bağımlı doğal bir proteaz olan μ-kalpain'in spesifik bir inhibitörüdür. Sığır CAST geni içindeki genetik varyantlar, polimeraz zincir reaksiyonu-restriksiyon parça uzunluk polimorfizmi (PCR-RFLP) metodu kullanılarak analiz edildi. Çalışmada hayvan materyali olarak, Boz ırk ve Boz ırk x İsviçre Esmer melezlerinden oluşan 132 boğa ve düve kullanıldı. Çalışılan hayvan örneklerinde CAST/RsaI polimorfizminin C ve G alleleri karakterize edildi. C allel frekansı, saf Türk Boz ırklara nazaran melez grup içinde önemli bulunmuştur ($P<0.05$). Hayvan örneklerinin tümünde, C allel frekansı ortalaması %56.1'dir. Genotipik frekanslar, CC, CG ve GG genotipleri için sırasıyla, safkan Türk Boz ırkta; 0.257, 0.499 ve 0.243 ve Türk Boz ırk x İsviçre Esmer melezi popülasyonda; 0.388, 0.470 ve 0.142 olarak tahmin edildi. Sonuç olarak, genotipik dağılımlar Türk Boz ırk sığırı saf ve melez örneklerinin her ikisinde de dengededir.

Anahtar sözcükler: *PCR-RFLP, Boz ırk sığır, Kalpastatin (CAST), Genetik belirteç, Polimorfizm*

INTRODUCTION

According to the theoretical basis [1], the Grey steppe breed originated from the *Bos taurus primigenius*. There are similar and relative breeds to the Grey steppe in different countries such as Greece, Ukraine, Romania, Hungary, Serbia, Bulgaria and Italy [2,3]. This breed is named the Boz step or Plevne breed in Turkey. It is called Podolion in Italy, Iskar in Bulgaria and Grey steppe in English literature [3,4]. In Turkey, the breed was seen widely in the Trakya region and was used as a powerful draught animal for many years. Grey steppe breeds are preferred in rural regions because of their tolerance to cold and heat, and resistance to ecto- and endoparasites. This native breed was unprocessed beef in Turkey and was used extensively by poor farmers to produce beef in Trakya [4,5].

İletişim (Correspondence)
+90 284 7141794
koks@trakya.edu.tr

Meat tenderness has a major impact on consumer satisfaction is one of the most important issues in beef cattle production on consumer satisfaction [6]. Among the issues that have been identified as responsible for the post-mortem meat tenderisation process is the calpain proteolytic system. Calpastatin inhibits μ- and m-calpain activity and, regulates post-mortem proteolysis. Increased post-mortem calpastatin activity has been correlated with reduced meat tenderness [7]. Three enzymes responsible for this process are the μ-calpain (CAPN1) and m-calpain (CAPN2) and their inhibitor is calpastatin (CAST). A number of studies [8] have shown that the calpain-calpastatin system is also important in normal skeletal muscle growth.

There are different methods for the identification of the genetic structure of breed animals. The analysis of PCR is one of the effective methods to determine the genetic characterization of a population [9,10]. In *Bos taurus* and *Bos indicus*, two single nucleotide polymorphisms (SNPs) have been identified in the CAST gene in intron 5 between exons 5 and 6: a G/C SNP (known as UoG-CAST) [11-13], and an A/G SNP (known as CAST-T1) in the 3´UTR region in exon 30 [14,15]. These SNPs have been shown to be associated with meat tenderness and three commercially available genetic marker panels are currently used GeneSTAR Quality Grade, GeneSTAR Tenderness, and Igenity *Tender*-GENE. The UoG-CAST polymorphism was associated with shear force across days of post-mortem aging; genotype CC yielded beef that was more tender than GG, and CG had intermediate tenderness [13,14,16].

This study aims to determine the allele frequency of CAST/RsaI polymorphism related to meat tenderness in the calpastatin gene in the purebred and crossbred examples of Turkish Grey steppe cattle.

MATERIAL and METHODS

Animals: Samples of Turkish Grey steppe cattle (n=71) and first-generation (F_1) crossbreeds of Turkish Grey steppe x Brown Swiss (n=61) cattle were studied. The samples are taken from these cattle were bred in the rural feedlot sector from villages of the Edirne and Çanakkale in 2009 and 2010 and originated from the commercial rural herds (free range) of 20 farms. An extensive system is used in the rural feedlot sector. The 132 animals (32 females and 100 males) were bred according to the conditions in Turkey, and were slaughtered at 24 and 36 months of age are used in the study.

The research was carried out at Trakya rural feedyards, the slaughterhouse of Keşan, the Food Control Laboratory of Keşan and the laboratories of Keşan Vocational High School in Trakya University in Edirne in Turkey.

DNA Isolation: The sources of DNA were frozen steaks stored in the Keşan Vocational High School Laboratory at Trakya University. The DNA was isolated within the muscle tissue using the Fujifilm QuickGene mini-80 application tool and tissue kit (QuickGene, UK) as the manufacturer's instructions in the Food Control Laboratory of Keşan.

Amplifications of DNA Fragments: The CAST genotypes and gene polymorphisms were determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), a technique described by Maeda *et al.*[17]. For identification of alleles C and G of the calpastatin gene (GenBank Accession No. AY_008267.1:g.282C>G), a fragment of 523 base pair (bp) located in intron 5 was released with the primers 5′–cct cga ctg cgt acc aat tcc gaa gta aag cca aag gaa ca–3′ (forward) and 5′–att tct ctg atg gtg gct gct cac t–3′ (reverse) and cut with R*sa*I according to Schenkel *et al.*[13].

The PCR amplification reaction solution was performed in total volume of 25 μl containing 12.5 μl master mix 2X (Fermentas life sciences, Rott./Germany), 4.5 μL of distilled H_2O, 2 μL of forward primer (10 pmol/μL), 2 μL of reverse primer (10 pmol/μL) and 4 μL of DNA template (approximately 50 ng/μL).

The PCR cycling condition was 95°C (10 min) for one cycle; then 94°C (30 s); then from 69 to 62°C (30 s) and 72°C (30 s) for 8 cycles; then 94°C (30 s), 61°C (30 s) and 72°C (30 s) for 27 cycles; and finally 72°C (5.0 min) for one cycle, maintaining 4°C thereafter. The PCR reactions were performed on the My Genie 96 Thermal Block Bioner. The corresponding PCR products were amplified 523 bp fragments.

PCR-RFLP Genotyping: The PCR products were cut with the restriction endonuclease R*sa*I. 257 bp and 266 bp the specific allele fragments were amplified. In each PCR-RFLP reaction, a mixture containing 1 μL (3 units) of R*sa*I enzyme, 1 μl of restriction buffer, 2 μL of distilled H_2O and 6 μg of PCR product was incubated at 37°C for 4 h to ensure complete digestion.

DNA-fragments run on agarose gel to separate: the DNA fragments from the cutting PCR products were separated at a concentration 2% agarose gel containing ethidium bromide (0.4 μg/mL). Electrophoresis was performed in a 1xTBE buffer, at 125V for about 60 min. The genotype for each individual was read under ultraviolet light by using an appliance (DNR Bio-Imaging Systems MiniBİS Pro) and software was used for molecular analysis (Image Aide from Spectronics Corporation).

Statistical Analysis: The allelic and genotypic frequencies were calculated for each of the polymorphisms, according to Schenkel *et al.*[13]. The allele and genotype frequencies of each poly-morphism were calculated for deviations from the Hardy-Weinberg equilibrium by X^2 tests (significance based on $P<0.05$). Differences in allele frequencies between groups were evaluated by using probability tables [18].

RESULTS

In the studied genetic groups, two genetic variants (C and G) were found for the CAST/R*sa*I polymorphism. Three

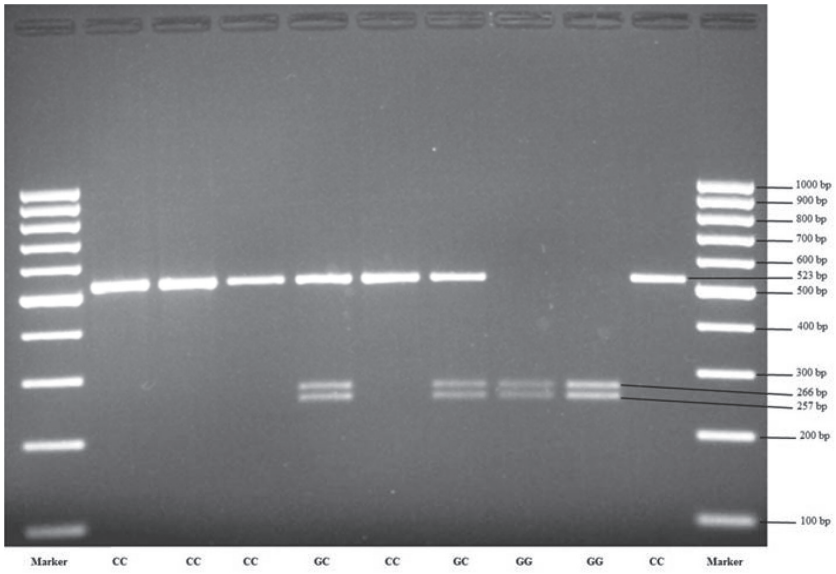

Fig 1. Genotyping for the bovine CAST gene polymorphism by the polymerase chain reaction restriction fragment-length polymorphism (PCR-RFLP) analysis. Samples were amplified by PCR followed by digestion with R*sa*I

Şekil 1. Sığır CAST geninin polimeraz zincir reaksiyonu-sınırlı parça uzunluk polimorfizmi (PCR-RFLP) ile genotiplendirilmesi

Table 1. *Allele and genotype frequencies of the CAST/RsaI polymorphism*
Tablo 1. *CAST/RsaI polimorfizminin allel ve genotip frekansları*

Breeds Genetic Groups	Number of Animals	CAST SNP/R*sa*I Allele frequency		Genotype Frequency *		
		C	G	CC	CG	GG
Greysteppe	71	0.507[b]	0.493[a]	0.257	0.499	0.243
Crossbred	61	0.623[a]	0.377[b]	0.388	0.470	0.142
Total (overall)	132	0.561	0.439	0.315	0.492	0.193

a, b; *Allele frequencies that differ significantly between genetic groups (P<0.05), * Genotype frequency was significantly different within breeds (P<0.05)*

unique banding patterns were identified, and either one or a combination of two different patterns was observed for individual cattle *(Fig. 1)*, which are consistent with homozygous or heterozygous genotypes, respectively, at the CAST locus. Genotype CC was characterised by the presence of single fragment of 523 bp, whereas genotype GG showed two fragments –257 and 266 bp. The heterozygotes (GC) were characterised by three fragments of 523, 257 and 266 bp.

The genotypic and allele frequencies reported for each of the SNPs analysis in the study are summarised in *Table 1*. Most of the animals were purebred, and so the allele frequencies for each breed could be thoroughly estimated. Allele frequencies for CAST were significantly different ($P<0.05$) between the Grey steppe and crossbred group. The G allele seemed lower among crossbred breeds.

Allele Frequencies were calculated according to the rule of the Hardy-Weinberg equilibrium *(Table 1)*. The allele frequency was significantly different ($P<0.05$) between purebred and crossbred groups. A greater frequency showed allele G of to the purebred Grey steppe than the crossbred. The present results show a higher frequency of allele C for the CAST/R*sa*I polymorphism in both pure and crossbred examples of Turkish Grey steppe cattle. This actuality indicates that the frequencies of alleles C and G were found to differ significantly between the purebred and crossbred genetic groups of Grey steppe cattle.

The overall results were observed *(Table 1)* of genotype frequencies (0.315, 0.492 and 0.193 for CC, CG and GG, respectively) which are in conformity with the Hardy-Weinberg equilibrium. Genotypic distributions were equilibrium (P>0.05) in examples of total cattle group. Genotype frequency was not significantly different within overall animal groups ($P>0.05$). Genotypic frequencies were identified as 0.257, 0.499 and 0.243 in the purebred Grey steppe breed, and 0.388, 0.470 and 0.142 in the crossbred population for genotypes CC, CG and GG, respectively. As a result, genotypic distributions were equilibrium in both pure and crossbred examples of Turkish Grey steppe cattle.

DISCUSSION

Schenkel *et al.*[13] reported allele frequencies for the CAST/R*sa*I SNP were 0.629 and 0.371 for allele C and G respectively, in *Bos taurus* cattle (Angus, Limousin, Charolais, Simmental), which were generally similar to frequencies found within the crossbreed cattle group at Trakya. In addition, Quaas *et al.*[19], Van Eenennaam *et al.*[14], Gill *et al.*[11], Curi *et al.*[20] and Reardon *et al.*[12] found average frequencies of the favourable allele C, as 72%, 72%, 64%, 69.3%, and 75% in crossbred populations of *B. taurus* breeds, respectively. On the other hand, in the present study the proportion of the allele C (50.7%) for the Grey steppe breed was lower than those were calculated in *B. taurus* breeds in other studies.

Van Eenennaam *et al.*[14] reported allele C frequencies ranging from 74% to 79% for genetic groups of *B. taurus* animals, and 79% for Brangus (5/8 *B. Taurus* + 3/8 *B. indicus*). The frequency of allele C on CAST /Rsal locus was reported as 0.64 in the Aberdeen Angus cattle by Gill *et al.*[11] and as 0.623 in the purebred Nellore cattle by Curi *et al.*[21]. These rates were similar to the Turkish Grey steppe crossbred

cattle group (0.623) in this study.

Gill *et al.*[11] reported genotypic frequencies as 0.41, 0.47 and 0.12 for CAST/R*sa*I SNP genotypes CC, CG and GG, respectively, in Aberdeen Angus cattle. Schenkel *et al.*[13] calculated genotypic frequencies 0.430, 0.398 and 0.172 for CC, CG, and GG SNP in a sample of *Bos taurus* cattle, respectively, and concluded that the frequencies are different between *Bos indicus* and *Bos taurus* cattle. In contrast, Curi *et al.*[21] estimated CAST genotypic frequencies as 0.377, 0.491 and 0.132 for CC, CG and GG, respectively in the purebred Nellore breed in *Bos indicus* cattle. In the present study, a proportional loss in the CC genotype frequency of the purebred Grey steppe was observed compared to the research results of the previous researchers.

SNP at the calpastatin and calpain loci appear to be associated with major gene effects for tenderness of meat according to Smith *et al*.[15]. The AY_008267.1:g282C>G SNP is corresponding with cross location change between cytosine and guanine in intron 5 of the bovine CAST gene, which was associated with meat tenderness in *Bos taurus* populations. Thus, Schenkel *et al*.[13] reported that the most favourable allele for CAST was C. Subsequently, CAST/R*sa*I polymorphism became part of a commercial test panel for the identification of animals that are superior in terms of meat tenderness [14]. Most of the published reports were indicated that CAST gene polymorphisms are associated with sheer force in the longissimus muscle [13,16,20,22,23].

In brief, Genetic marker technology is a promising tool for genetic improvement in livestock [24]. The findings of this study will contribute to the literature on the effect of CAST gene in the *Bos taurus* breed. CAST gene might be used as a candidate gene in the Grey steppe and its hybrid populations. Since CC genotype from CAST gene is related with meat tenderness, the meat quality in the Grey steppe might be improved by using Marker Assistant Selection (MAS).

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