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Allelic Polymorphism of Makoei Sheep Calpastatin Gene Identified by Polymerase Chain Reaction and Single Strand Conformation Polymorphism

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ABSTRACT

Calpastatin (*CAST*) is a specific inhibiter of calpains, playing a role in meat tenderization and myogenesis. In the present study, the polymorphism of the *CAST* gene of Makoei sheep was investigated by polymerase chain reaction and single strand conformation polymorphism technique (PCR–SSCP). Genomic DNA was extracted from whole blood samples collected from 100 sheep. A 622 bp *CAST* exon 1 segment was amplified by standard PCR, using the locus specific primers. PCR products were subjected to a non-denaturing gel electrophoresis. Four SSCP patterns, representing four different genotypes, were identified. The frequencies of the observed genotypes were 0.31, 0.04, 0.63 and 0.02 for *AA, BB AB* and *AC*, respectively. Allele frequencies were 0.6313, 0.3586 and 0.01 for *A*, *B* and *C,* respectively. The Observed heterozygosity (H_{obs}) value for *CAST* gene was 0.4728. The chi-square test showed significant (P< 0.01) deviation from Hardy-Weinberg equilibrium for this locus in Makoei sheep population.

Keywords: *CAST* gene, Makoei sheep, PCR, SSCP.

INTRODUCTION

The improvement in meat quality is the main goal of livestock production. Meat tenderness is one of the most important factors for quality assessment of the meat. The calpain proteolytic system has been identified as a factor for postmortem meat tenderization process through the proteolysis of myofibrillar and associated proteins (Koohmaraie, 1992; Taylor *et al*., 1995). Variation in meat tenderness is due to the genetic variation, biological and physiological differences during slaughter, and chemical differences during post-mortem aging (Koohmaraie 1996).

Calpastatin (CAST) gene located on the fifth chromosome of sheep encodes a specific calpain inhibitor that plays important roles in the formation of muscle, degradation, and meat tenderness after slaughter (Gabor *et al*., 2009; Palmer *et al*., 1999). Polymorphisms in the bovine (Casas *et al*., 2006; Schenkel *et al*., 2006) and pigs (Ciobanu *et al*., 2004) *CAST* gene have been associated with meat tenderness, making the *CAST* gene an excellent candidate for controlling meat traits in livestock.

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Genetic polymorphism identification of the *CAST* gene and its relation to the meat quality could be used as a tool to predict meat tenderness in animals allowing breeders to enhance the trait (Seiler, 1994). In addition, genotyping animals by employing this molecular marker will help to classify carcasses based on eating quality before slaughter (Lonergan *et al*., 1995). It was demonstrated that the favorable effect of the variants of *CAST* gene on pig carcass quality traits depends on the cut. It was also reported that post-mortem changes in different periods depends on the *CAST*/*Rsa*I genotype. It seems that the *BB* genotype is related to the rate of glycolysis immediately after slaughter while the *AA* genotype is related to the rate of glycogenolysis in the process of muscles conversion into meat (Krzecio *et al*., 2008).

Palmer *et al*. (1998) have described two allelic systems of polymorphic variants (M and N) in a region of the bovine *CAST* by the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method. Using a molecular genetics approach to study meat quality in sheep, Palmer *et al*. (1999) chose the ovine *CAST* gene as a candidate gene for meat quality. A three allelic system of polymorphic variants (a, b, and c) have also been described by PCR and single strand conformation polymorphism (PCR-SSCP) in a region of the ovine and cattle *CAST* (Chung *et al*., 1999; Palmer *et al*., 2000).

The present study aimed to evaluate the genotype and gene frequencies at the ovine *CAST* gene of ˝Makoei˝ sheep breed in west Azerbaijan Breeding Station, Iran.

MATERIALS AND METHODS

Sheep Blood Sample Collection and Genomic DNA Extraction

Makoei sheep examined in this study were fat-tailed sheep with medium body size and white color with black spots on face and feet. They are raised in East and West Azerbailan Provinces of Iran and their main products are meat and wool (Saadat-Noori and Siah-Mansoor, 1992). Blood samples (approximately 2-3 ml) were obtained from 100 unrelated Makoei sheep from different parts of West Azerbaijan province and stored in EDTA-coated tubes. Genomic DNA was extracted from 0.3 ml of blood using the genomic DNA purification kit (Fermentas, EU) according to manufacturer's instructions. Quality and quantity of extracted DNA was measured by agarose gel (0.8 %) electrophoresis.

Amplification of the Exon 1 of *CAST* Gene

The DNA amplification of the *CAST* gene was achieved by PCR. Two primers exon 1C (5´-TGGGGCCCAATGACGCCATCGATG-3´) and exon 1D (5´ GGTGGAGCAGCACTTCTGATCACC-3´) targeting a fragment of 622 bp was employed as described by Palmer *et al*. (1998). The PCRs were carried out in 50 µl volumes using PCR mastermix kit (Cinnagen, Iran) containing 2.5 units Taq DNA Polymerase in reaction buffer, 4 mM $MgCl_2$, 50 µM each of dATP, dCTP, dGTP and dTTP, 0.5 µM of each primer and about 100 ng of extracted DNA as template. Amplification was performed in Mastercycler (Eppendorf, Germany) using 35 cycles of incubation at 95°C for 45 seconds, 62°C for 1 minUTE, and 72°C for 75 seconds, with a final extension at 72°C for 7 minutes.

Single Strand Confirmation Polymorphism (SSCP)

PCR products were mixed with 8 µl of denaturing loading dye [95% (w/v) deionized formamide, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue and 0.02 M EDTA] in a total volume of 15 µl. The mixture was denatured at 95ºC for 5 minutes and was snap chilled on ice (Pipalia

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et al., 2004). The total volume was applied in a 15% polyacrylamide gel, as described by Herring *et al*. (1982). The electrophoresis was performed in 0.5 X TBE buffer (Tris 100 mM, Boric Acid 9 mM, EDTA 1 mM) at room temperature (18ºC) and constant 200 V for 3 hours. Polyacrylamide gels were stained with silver nitrate according to the protocol described by Herring *et al*. (1982).

Statistical Analysis

The allelic and genotypic frequencies, expected means, observed and expected Nei's heterozygosities ($HE=1-\Sigma P_i^2$, where P_i is the frequency of allele i) and Hardy-Weinberg equilibrium were calculated using PopGene32 program, ver 1.31, Canada (Yeh *et al*., 1997).

RESULTS

PCR-SSCP Analysis of *CAST* Gene

The amplification of a 622 bp fragment of the *CAST* exon 1 gene was successful in our first attempt. All extracted DNAs from sheep blood samples yielded a specific single band PCR product without any nonspecific band. Therefore, the PCR products were directly used for SSCP analysis.

The allelic variation in the *CAST* gene was examined by PCR-SSCP. The non-denaturing gel electrophoresis enabled visualization of ssDNA and analyzed for SSCP band patterns. In this study a total of four SSCP patterns were observed in the examined sheep (Figure 1). The frequencies of the observed genotypes were 0.31, 0.04, 0.63 and 0.02 for *AA, BB, AB* and *AC*, respectively. Allele frequencies were 0.63, 0.36 and 0.01 for *A*, *B* and *C* respectively (Table 1).

Statistically estimated parameters for *CAST* locus in Makoei sheep have been presented in table 2. The chi-square test showed significant ($P< 0.01$) deviation from Hardy-Weinberg equilibrium for this locus in the studied population.

DISCUSSION

In the present study, three alleles (A, B, and C) and four genotypes (AA, AB, BB, and AC) were observed for *CAST* gene in "Makoei" sheep breed in West Azerbaijan, Iran. The most frequent allele and genotype in the "Makoei" sheep breed were 63.13% and 31% for allele *A* and genotype *AB*, respectively. The results obtained from this study revealed the polymorphism in the

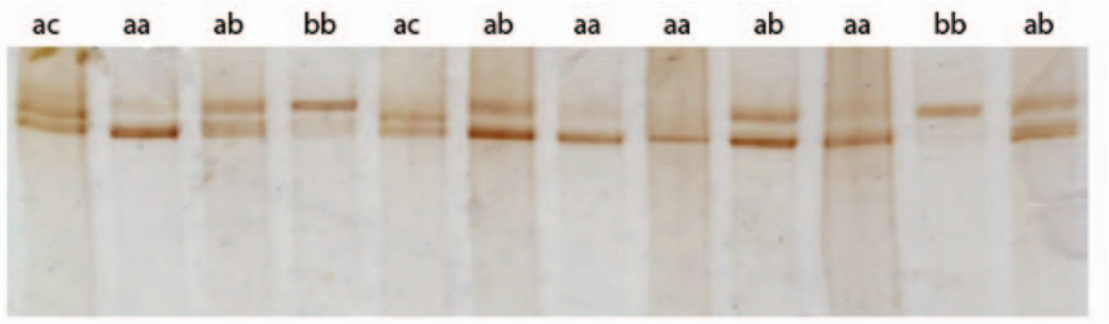

Figure 1. SSCP polymorphism of Makoei sheep *CAST* gene. Four different PCR-SSCP patterns (genotype) were identified.

Table 1. Observed allele and genotypic frequencies for *CAST* locus in Makoei sheep**.**

A	*B*	*C*	*AA*	*BB*	*AB*	*AC*
0.6313	0.3586	0.0101	0.31	0.04	0.63	0.02

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Table 2. Statistically estimated parameters for *CAST* locus in Makoei sheep.

Exp-Het	Exp-Hom	Het $_{(Nei)}$	Ave-Het	Obs-Hom	Obs-Het
0.4752	0.5248	0.4728	0.4728	0.3434	0.6566

CAST gene of Makoie sheep. Variation in non-coding and coding regions of the ovine *CAST* gene has been reported by several researchers (Palmer *et al*., 1998; Palmer *et al*., 2000; Roberts *et al*., 1996; Zhou *et al*., 2007).

Polymorphism study on the same region of the *CAST* gene in Kurdi sheep by PCR-SSCP revealed three genotypes including *aa, ab* and *ac* (Nassiry *et al*., 2006). The polymorphism in the exon 1 of the CAST in sheep was also reported by other researchers using PCR-RFLP technique (Gabor *et al*., 2009; Mohammadi *et al*., 2008; Palmer *et al*., 1998). In goats and bovine the exon 6 of CAST gene were investigated for polymorphisms and a number of allelic variants were identified in these species (Zhou and Hickford 2008; Zhou *et al*., 2007). Fortest (2007) reported higher frequencies of *CAST* gene's allele *A* compared to the allele *B* in Nellore (0.66), Rubia Gallega (0.72), Canchim (0.62), Brangus (0.78) and Pardo Suico (0.80) cattle.

There are several studies on the association of *CAST* gene polymorphism and meat quality in animals. Schenkel *et al*. (2006) reported a significant association between allele *C* of bovine *CAST* gene and meat tenderness. Kuryl *et al*. (2003) reported that *CAST* gene may be considered as a candidate gene for pig carcass quality. Association between allele *D* and *F* of porcine *CAST* gene and meat quality traits was also reported by Kapelański *et al*. (2004).

Palmer *et al*. (1999) found allelic frequencies of 0.69 and 0.70 for allele *A* in Dorset Down and Coopworth, respectively, which was in close agreement with the frequency of the allele *A* in Makoei sheep in the present study. In contrast, they reported that frequencies of alleles *A* and *B* in Corriedale and Ruakura were 0.27 and 0.41, respectively. Different frequencies for the alleles of the *CAST* gene have been reported in Iranian Baluchi sheep with 0.70 for allele *A*, 0.08 for allele *B,* and 0.22 for allele *C*. Genotypes *BC* and *CC*, which presented, respectively, the 0.03 and 0.04 frequencies in Baluchi sheep, were not observed in Makoei sheep (Tahmoorespur *et al*., 2007). Two allelic systems of polymorphic variants (M and N) in the region of ovine *CAST* locus have been described by PCR-RFLP method (Palmer *et al*., 1998; Shahroodi *et al*., 2005). According to Palmer *et al*. (1998), allelic frequencies were 77% and 12% for the *M* and *N* in Corriedale sheep, respectively.

The present study was the first attempt for identification of *CAST* gene variation in Iranian Makoei sheep. Further studies are required to investigate the relationship between *CAST* gene polymorphisms and the performance traits in Makoei sheep.

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شناسایی چندشکلی های ژن کالپاستاتین در گوسفند ماکویی با استفاده از تکنیک PCR-SSCP

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چکیده

کالپاستاتین (CAST) یک مهارکننده اختصاصی کالپین ها می باشد که در تردی و ساختن سلولهای عضلانی نقش دارد. در مطالعه حاضر چند شکلی *CAST* در گوسفندان نژاد ماکویی با استفاده از تکنیک PCR-SSCP مورد ارزیابی قرار گرفت. DNA ژنومی از نمونه های خون کامل تعداد ۱۰۰ رأس گوسفند ماکویی تهیه گردید. قطعه‌ای به اندازه ۶۲۲ جفت باز از ناحیه اگزون یک با بکارگیری آغازگرهای اختصاصی لوکوس *CAST* تکثیر شد. با استفاده از روشSSCP ، محصولات PCR ژن *CAST* بر روی ژل غیر دناچوره کننده الکتروفورز شدند. چهار الگوی SSCP که بیانگر چهار ژنوتیپ مختلف بودند شناسایی گردید. فراوانی ژنوتیپهای مشاهده شده *AA* ، *BB*، *AB*و *AC* به ترتیب عبارت بودند از: ۰/۳۱، ۰/۰۴، ۰/۶۳ و۰/۰۲ .فراوانی آللی برای آللهای *A*، *B*و *C* به ترتیب عبارت بودند از: ۰/۶۳۱۳، ۰/۳۵۸۶ و ۰/۰۱. مقدار هتروزیگوسیتی مشاهده شده (Obs-Het) ژن *CAST* برابر ۰/۴۷۲۸ بود. آزمون مربع کای انحراف از تعادل Hardy-Weinberg را برای لوکوس *CAST* در گوسفند ماکویی معنی دار نشان داد.

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