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Association between Yearling Weight and Calpastatin and Calpain Loci Polymorphism in Iranian Zel Sheep

Research Article

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ABSTRACT

Genotypes of Iranian Zel sheep for Calpastatin (*CAST*) locus were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) methods and for Calpain (*CAPN*) locus by PCR-SSCP. Blood samples were collected from 200 purebred Zel sheep of Zel Breeding Station located in Golestan province in northeast of Iran. Extraction of genomic DNA was based on modified salting out method. The digestion of PCR products of *CAST* gene by *Msp*I and *Nco*I restriction enzymes revealed two alleles *M* and *N*, with frequencies 85.5 and 14.5%, respectively. Frequencies were 75, 21 and 4% for *MM*, *MN* and *NN* genotypes, respectively. Alternatively, using PCR-SSCP method, four genotypes including *AA*, *AB*, *BB* and *AC* with frequencies of 71, 21, 4 and 4%, respectively, were observed in this population. Analyzing *CAPN* gene by the PCR-SSCP method, revealed two different conformational patterns (*AA* and *AB*) with frequencies of 69 and 31% for *AA* and *AB*, respectively. Average heterozygosity for both loci was low (0.28 and 0.25% for *CAST* using PCR-SSCP and PCR-RFLP, and 0.26% for *CAPN*). Yearling weights (YW) were analyzed by a statistical model comprising PCR-SSCP and as a result *CAPN* genotypes had significant effect ($P<0.01$) on YW. A Chi-square test confirmed Hardy-Weinberg (H-W) equilibrium for the *CAST* locus using PCR-SSCP method but not for PRC-RFLP method and *CAPN* locus. Totally, the investigated herd had little genetic diversity and different factors disturb H-W equilibrium and PCR-RFLP and PCR-SSCP might be used successfully in these studies.

KEY WORDS calpain, calpastatin, molecular methods, sheep, yearling weight.

INTRODUCTION

Today, several DNA polymorphisms have been considered as potential tools for selecting dairy and meat producing ruminants. DNA-based molecular methods have made possible genotyping of animals of any age and sex for meat genes, thus providing a potentially more efficient selection tool. Selection efficiency, however, depends on allelic frequencies in the breeds and on the effect of these polymorphisms on meat traits and technological properties of meat. In case of sheep, research on genetic polymorphism of two candidate genes, calpastatin (*CAST*) and calpain (*CAPN*), have been performed (Palmer *et al.* 1999). The *CAPN* - *CAST* system (CCS) comprises a family of calcium dependent neutral proteases.

Calpastatin is a protein inhibitor that acts specifically on *CAPN* s and plays a regulatory role in postmortem beef tenderization and muscle proteolysis. Polymorphisms in the

bovine *CAST* gene have been associated with meat tenderness (Byun *et al.* 2009). Calpastatin and *CAPN* deserve special attention because of their major role in meat production and quality. The CCS is found in most animal tissues and influences on many important processes including muscle development and degradation, meat tenderization post mortem, cataract formation and fertility (Chung *et al.* 1999).

It has been shown that *CAPN* s play a major role in post mortem tenderization in beef, lamb and pork by degrading specific muscle structural proteins (Huff-Lonergan *et al.* 1996).

The *CAPN* system is also important in normal skeletal muscle growth (Palmer *et al.* 1997; Palmer *et al.* 1999). An increased rate of skeletal muscle growth can result from a decreased rate of muscle protein degradation and this is associated with a decrease in activity of the *CAPN* system due to principally a large increase in *CAST* activity. It is now accepted that *CAPN* mediated degradation of myofibrillar proteins that are responsible for meat tenderisation postmortem which occurs during storage at refrigeration temperature (Koohmaraie, 1988).

Dinparast Djadid *et al.* (2011) amplified and sequenced a fragment of approximately 1.5 kb of the L domain of *CAST* in Afshari sheep. They found the similarity of 89% between the Afshari sequences and the reported bovine sequences of this gene in (NCBI) website. Studies on genes which affect sheep meat quality are important in countries such as Iran due to the importance of sheep meat as a major source of protein from animal origin. Zel sheep, a native Iranian meat breed sheep, play a great role in sheep rearing activities in the north of Iran (Mason, 1996). Therefore, the aim of the present study was to consider detected polymorphisms of CAST and CAPN genes and the associations of their genetic variations with yearling weight in Zel sheep using molecular methods.

MATERIALS AND METHODS

Animals and DNA extraction

Blood samples were randomly collected from 200 Zel sheep from Shirang`s Zel Breeding Station in Fazel Abad city of Golestan province. DNA was extracted from blood as described by Miller *et al.* (1988). Quality and quantity of DNA were measured by visual and spectrophotometer methods.

PCR reaction

An aliquot of 100 ng genomic DNA was amplified in a total volume of 25 µl PCR mix. The PCR mix consisted of: 12.5 µl master mix (Sinaclon, Tehran), 1.2 µL forward and reverse primers (10 pmol/µL), and 9.1 µL ddH_2O.

Calpastatin PCR conditions

The PCR program included a preliminary denaturizing at 95 ^{o}C for 3 min, followed by 35 cycles, denaturing at 95 ^{o}C for 1 min, annealing at 59 ^{o}C for 1 min, extension at 72 ^{o}C for 2 min and 7 min at 72 ^{o}C as final extension. Exon and intron regions from a protein of the first repetitive domain of the ovine calpastatin gene were amplified to produce a 622 bp fragment using primers based on the sequences of the ovine *CAST* genes (Collingwood *et al.* 1992) and bovine *CAST* genes (Killefer and Koohmaraie, 1994), (GenBank accession no. L14450). Primer sequences (Palmer *et al.* 1998) were:

Ovine 1C: 5'-TGGGGCCCAATGACGCCATCGATG-3'
Ovine 1D: 5'-GGTGGAGCAGCACTTCTGATCACC -3'

Calpain PCR conditions

PCR program included a preliminary denaturizing at 95 ^{o}C for 3 min, followed by 35 cycles, denaturing at 94 ^{o}C for 45 sec, annealing at 59 ^{o}C for 60 sec, extension at 72 ^{o}C for 75 sec and 10 min at 72 ^{o}C as final extension. The ovine m-*CAPN* regulatory gene, exon 5 and 6 including intron (*CAPN*456), was amplified with primers designed according to the published bovine nucleotide cDNA sequence (Gene Bank accession no. J05065). Primer sequences were:

*CAPN*456F: 5'-AACATTCTCAACAAAGTGGTG-3'
*CAPN*456R: 5'-ACATCCATTACAGCCACCAT-3'

Products of amplification were recognized by electrophoresis using 1.5% agarose gel, stained with ethidium bromide.

Digestion reaction

Ten µl of PCR products were incubated for 10 h at 37 ^{o}C with 1 µL (10 units) of *Msp*I and *Nco*I enzymes for *CAST* gene, in separate reactions. Enzymes digest the amplimers in complementary manner. We used both restriction enzymes to determine genotypes more certainly. The *Msp*I digests the allele *M*, but not allele *N* and *Nco*I vice versa. Digestion products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide.

SSCP reaction

For genotyping of *CAPN* and *CAST* loci, a PCR-SSCP method was used. PCR products (3 µL) were diluted with 13 µL of running buffer included: 800 µL formamide 99%, 100 µL loading dye, 100 µL glycerol 98%, 3 µL 0.5 *M* EDTA and 2 µL 10 *M* NaOH. After heating at 95 ^{o}C for 5 min, they were immediately placed on ice for 10 min. Polymorphisms were detected using 12% and 8% non-denaturing polyacrylamide gel. The mixtures were electro-

phoresed for 8-9 h and 4 h at 250 V and 10 °C for *CAST* and *CAPN* genes, respectively. DNA fragments were visualized using a silver staining method (Benbouza *et al.* 2006).

Statistical analysis

Calculating genotypes and alleles frequencies, mean expected and observed heterozigosities and Chi-square test were performed using PopGene32 (ver 1.32), (Yeh *et al.* 2000).

Only 121 blood samples, 111 from female and 10 from male born in years 2006, 2007 and 2008, were used for statistical analysis. The selected animals were collected from different purebred herds of Zel sheep in the province and were used as parents for new herd. Yearling weight (YW) was analyzed using the fixed model of SAS (version 5.1; SAS Institute Inc, NC. USA) software and proc GLM (Generalized Liner Model) with the following statistical model:

$$Y_{ijkl} = \mu + S_i + YB_j + G_k + e_{ijkl}$$

Where:
Y_{ijklm}= mean value of the trait.
μ= general mean.
S_i= effect of sex (i=1 and 2).
D_j= effect of date-year-of birth (j=1, 2 and 3).
G_k= effect of genotype (k=1, 2, 3 and 4).
e_{ijkl}= random error.

RESULTS AND DISCUSSION

Calpastatin

A 622 bp fragment from *CAST* I was amplified. The *Msp*I and *Nco*I restriction enzymes digested the PCR products in complementary manner and alleles of *M* and *N* were detected. We used both restriction enzymes to determine genotypes more certainly. The *Msp*I digests the allele *M*, but not allele N and *Nco*I vice versa.

The *Msp*I digestion of the allele *M* produced digestion fragments of 336 and 286 bp. The *Nco*I digestion of the allele *N* resulted in fragments of 374 and 248 bp (Figure 1). The allelic frequencies were 85.5 and 14.5% for *M* and *N*, respectively. The genotype frequencies in Zel sheep were 75, 21, and 4% for *MM*, *MN* and *NN*, respectively (Table 1). Despite the significant effect of sex (P<0.05) on YW, no association was found between genotypes and YW (P>0.05) (Table 2). For sex effect, least square means from males (30.85±1.46 kg) were more than females (24.19±0.80 kg).

Under the SSCP analysis conditions, different conformations were recognized by electrophoresis on non-denaturing polyacrylamide gel (Figure 2). Three alleles, *A*, *B* and *C*, were observed with frequencies of 83.5, 14.5, and 2%, respectively. Genotype frequencies were 71, 21, 4, and 4% for *AA*, *AB*, *BB* and *AC*, respectively (Table 1). There was no significant relationship between genotypes and YW (P>0.05) (Table 2).

Table 1 Allele and genotype frequencies, and observed, expected and average heterozigosity for *CAST* and *CAPN* loci [a, b]

Locus	Allelic frequencies (%)			Genotype frequencies (%)						Heterozigosity			
	1	2	3	4	5	6	7	8	9	Obs	Exp	Ave	χ^2
CAST (SSCP)	83.5	14.5	2	71	21	4	4	0	0	0.25	0.28	0.28	6.01^{ns}
CAST (RFLP)	85.5	14.5	-	75	21	4	-	-	-	0.21	0.25	0.25	4.89^{*}
CAPN (SSCP)	84.5	15.5	-	69	31	0	-	-	-	0.31	0.26	0.26	6.61^{*}

a: 1, 2 and 3 are the respective *A*, *B* and *C* alleles for *CAST* (PCR-SSCP), and *A* and *B* alleles for *CAPN* (PCR-SSCP), and *M* and *N* alleles for *CAST* (PCR-RFLP).
b: 4, 5, 6, 7, 8 and 9 are the respective *AA*, *AB*, *BB*, *AC*, *BC* and *CC* genotypes for *CAST* (PCR-SSCP), and *AA*, *AB* and *BB* genotypes for *CAPN* (PCR-SSCP), and *MM*, *MN* and *NN* genotypes for *CAST* (PCR-RFLP).
Ns= non significant; *= significant at P<0.05.

Table 2 Least square means (LSM) and standard error (SE) for yearling weight (kg) resulted from *CAST* and *CAPN* genotypes

Loci[1]					
CAST (RFLP)		*CAST* (SSCP)		*CAPN* (SSCP)	
Genotype	LSM±SE	Genotype	LSM±SE	Genotype	LSM±SE
MM	28.48±0.66	*AA*	28.49±0.67	*AA*	27.92^{a}±0.65
MN	29.30±0.96	*AB*	29.30±0.97	*AB*	30.37^{b}±0.82
NN	24.79±2.33	*AC*	28.40±1.96		
	-	*BB*	24.79±2.34	-	
P>0.05		P>0.05		P<0.01	

[1] values in the same column with different superscript letters (a, b) differ significantly (P<0.01).

Significant difference (P<0.05) in YW was observed for sex effect. Least square means from males (31.08±1.40 kg) were more than females (24.41±0.78 kg).

Calpain

The ovine *CAPN* regulatory gene, exons 5 and 6 including intron (*CAPN*456), with 190 bp length was amplified. Under the SSCP analysis, different conformations were detected by electrophoresis on non-denaturing polyacrylamide gel (Figure 3).

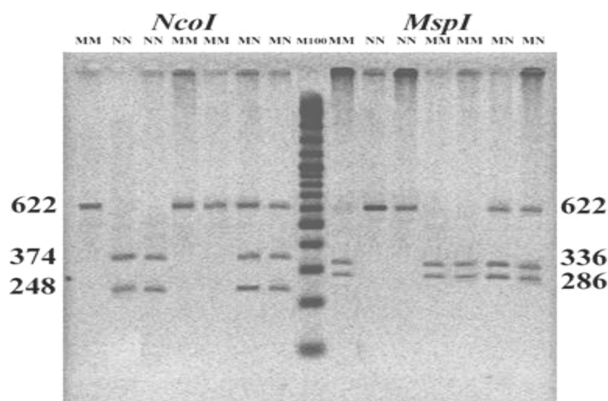

Figure 1 Restriction patterns of 622 bp fragments of *CAST*I after digesting with *Msp*I and *Nco*I in 2% agarose gel. Molecular marker was M100

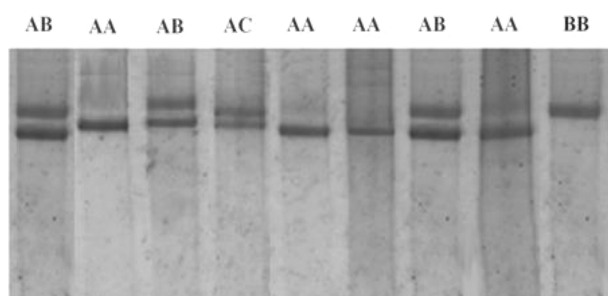

Figure 2 PCR-SSCP pattern of the ovine *CAST* gene in L region of exon 1 (622) on 12% non-denatured polyacrylamide gel. The letters *AA*, *AB*, *AC* and *BB*, demonstrating the 4 genotype patterns of *CAST* gene

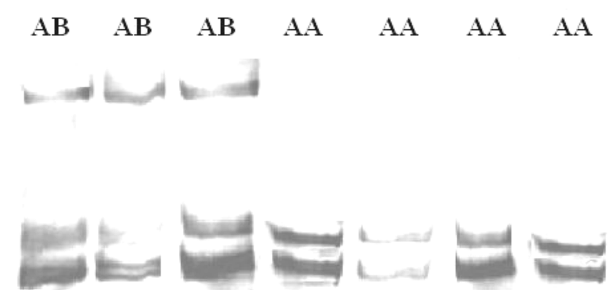

Figure 3 PCR-SSCP pattern of the ovine *CAPN* regulatory gene on 8% non-denatured polyacrylamide gel. The letters *AA* and *AB*, demonstrating the 2 genotype patterns of *CAPN* gene

Two alleles, *A* and *B,* were observed with frequencies of 84.5, and 15.5%, respectively. Genotype frequencies were 69, 31, and 0% for *AA*, *AB* and *BB*, respectively (Table 1). Significant effect of *CAPN* genotypes was observed for YW (P<0.01). The least square means of *AB* genotype (30.37 kg) were significantly different (P<0.01) from that of *AA* (27.91 kg) (Table 2). Significant associations (p<0.05) in YW were observed for sex and date-year- of birth effects. Least square means from males (32.62±1.20 kg) were more than females (25.66±0.38 kg) (P<0.01). The least square means of year 2007 (29.86±0.78 kg) were significantly different (P<0.05) from that of year 2006 (27.93±0.81 kg).

The results of this study showed that the two loci of *CAST* and *CAPN* are polymorphic in Zel sheep with *MM*, *MN* and *NN,* and *AA*, *AB*, *BB* and *AC* genotypes for *CAST* locus based on PCR-RFLP and PCR-SSCP methods, respectively. For *CAPN* locus, *AA* and *AB* genotypes were detected using PCR-SSCP method.

Statistic analysis indicated a significant association with YW for *CAPN* locus (P<0.01). However, this association for *CAST* locus was not significant (P>0.05). A genetic association was found between genotype *AB* for *CAPN* locus and YW trait. Similar results for *CAST* genotypes were obtained by Nassiry *et al.* (2006) and Mahdavi *et al.* (2009). Fakhr Kazemi *et al.* (2006) has reported a significant association between *CAST* genotypes and YW (P<0.05). Non-association may have been due to the environmental effects that exists and affects on this trait. In addition, mutation in intron region may be as a silent mutation. It also seems that introns, which have a role in the expression of genes and necessitate for physical instructors of DNA, do not have a major role in rank of amino acids and proteins' instructor (Matthews *et al.* 1997).

For alleles *A*, *B* and *C*, determined by PRC-SSCP method, only the alleles of *A* and *B* were digested by *Msp*I. Since the *Nco*I restriction enzyme had a reverse effect, samples which showed *MM* genotype in PCR-RFLP reaction were equal to *AA*, *AC* or *CC* genotypes in PCR-SSCP method. However, *MN* genotype determined by PCR-RFLP showed *AB* or *BC* genotypes by PCR-SSCP. Finally, the determined *NN* genotype by PCR-RFLP was equal to *BB* genotype in PCR-SSCP method. Palmer *et al.* (2000) also reported an association between PCR-SSCP and PCR-RFLP analysis at *CAST* locus. They directly sequenced nucleotides of the amplimers from homozygote sheep for each allele and found that there were differences between alleles *A*, *B* and *C* (GenBank Accession AF016006-8).

In this study conducted with Zel sheep population, except *CAST* locus under PCR-SSCP method, other loci did not show Hardy-Weinberg equilibrium, which can be due to the existence of the interference factors such as selection.

The investigated population, also, showed a low degree of genotypic variability for the *CAST* and *CAPN* loci. This may be explained by the conservation and breeding strategies in the herd with only a few rams. With respect to low effective number of population, inbreeding was expected to be high and so heterozigosity and genetic variability were low. Controlling breeding as planed matting may help in lowering inbreeding. Considering the low variability for genomic DNA, the results presented here provide evidence that Iranian's Zel sheep breed have a favorite polymorphisms for *CAST* and *CAPN* loci, which can be used for developing future selection programs especially based on marker-assistant selection aiming to improve weight gain and meat traits.

CONCLUSION

It can be concluded that although *CAPN* polymorphism was associated with yearling weight in Zel sheep at the Shirang`s Zel Breeding Station, but CAST genotypes did not show any effect on this trait. Despite the agreement that exists between the results of the present study with the previous ones, further studies, using a larger number of animals and the other breeds, are needed before making a definitive conclusion on the association between these genes and yearling weight, and the other growth traits.

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