Short Communication

Genetic variability of calpastatin and calpain genes in Iranian Zel sheep using PCR-RFLP and PCR-SSCP methods

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Abstract
The genotypes for calpastatin (CAST) and calpain (CAPN) loci were determined by PCR-RFLP and PCR-SSCP methods. Blood samples were collected from 200 pure-bred Zel sheep from Shirang's Zel sheep Breeding Station located in south-west of Golestan, Iran. Extraction of genomic DNA was performed based on the modified salting out method. Based on results, two investigated loci were polymorphic and had different gene variants. Heterozygosis was low for both loci. Chi-square test confirmed Hardy-Weinberg equilibrium only in CAST locus using SSCP method. Detected polymorphisms and associations of genetic variation with meat production and tenderness may help to find the effective genotypes of Zel sheep for the economic traits.
Keywords: Calpastatin; calpain; molecular methods; polymorphism; sheep

The calpain-calpastatin system (CCS) contains a family of Ca^{2+}-dependent neutral proteases. This system was found in most animal tissues and influences many important processes including muscle development and degradation, meat tenderization post mortem, cataract formation and fertility (Palmer *et al*., 1999). Calpastatin and calpain deserved special attention because of their major role in meat production and quality. Calpastatin is the endogenous and specific inhibitor of calpain proteases and regulates the rate and extent of post mortem tenderization (Kocwin and Kuryl, 2003). Calpains play a major role in post mortem tenderization in beef, lamb and pork by degrading structure of muscles (Huff-Lonergan *et al*., 1996). A number of studies have shown that the calpain system is also important in normal skeletal muscle growth (Goll *et al*., 2003; Palmer *et al*., 1999; 1997). An increased growth rate of skeletal muscle may be resulted from a decreased rate of muscle protein degradation due to reduction of calpain and increase in calpastatin activities.

Zel sheep is a native meat type breed and play a great role in sheep production activities in the North of Iran (Mason, 1996). The aim of the present study was to identify genotypes of calpastatin and calpain genes in this breed by employing molecular methods.

For this purpose, blood samples were randomly collected from 200 Zel sheep (190 ewes and 10 rams) of Shirang's Zel Breeding Station located in Fazel Abad of Golestan province. DNA was extracted from 3 ml of blood as described by Miller *et al*. (1988). The PCR program for both loci included a preliminary

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denaturizing at 95ºC for 3 min, followed by 35 cycles, denaturing at 95ºC for 1 min, annealing at 59ºC for 1 min and extension at 72ºC for 2 min and 7 min at 72ºC as a final extension. Exon and intron regions from a portion of the first repetitive domain of the ovine calpastatin gene were amplified to produce a 622 bp fragment using primers as suggested by Palmer *et al*. (1998): Ovine 1C: 5'-TGG GGC CCA ATG ACG CCA TCG ATG-3' and Ovine 1D: 5'-GGT GGA GCA GCA CTT CTG ATC ACC -3'. To amplify the ovine gene, containing exon 5, exon 6 and intron (CAPN456), two primers were used as suggested by chung *et al*. (2001): CAPN456F: 5'-AAC ATT CTC AAC AAA GTG GTG-3' and CAPN456R: 5'-ACA TCC ATT ACA GCC ACC AT-3'. Agarose gel electrophoresis on 1.5% agarose was used to recognize PCR products. PCR products were digested by *Msp*I and *Nco*I enzymes for calpastatin gene, in separate reactions. They were used to determine genotypes more carefully. The MspI digests the M allele, but not N allele. The NcoI has a reverse effect. It digests the N allele but not M allele. Digestion products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide (Fig. 1). For genotyping of calpain and calpastatin loci using PCR-SSCP method, 3 µl of PCR products were diluted with 13 µl of single strand conformation polymorphism (SSCP) buffer included: 800 µl formamide 99%, 100 µl loading dye, 100 µl glycerol 98%, 3 µl 0.5M EDTA and 2 µl 10M NaOH. After heating at 95 ºC for 5 min, samples were immediately placed on ice for 10 min. The mixture was electrophoresed on 12% and 8% non-denaturing polyacrylamide gels for 9 or 4 h at 250 V and 10ºC for calpastatin and calpain genes, respectively (Figs. 2 and 3). DNA fragments were visualized using a silver staining method (Benbouza *et al*., 2006). Calculating genotypes and alleles frequencies, expected and observed heterozygosity and examining Hardy-Weinberg equilibrium were performed using PopGene32 (Ver. 1.32), (Yeh *et al*., 2000), (Table 1).

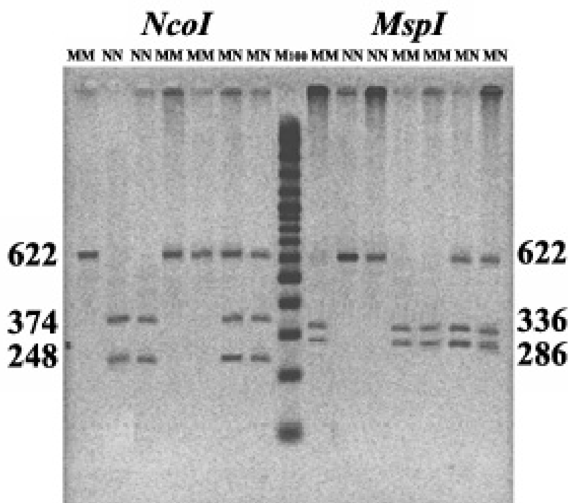

Figure 1. Restriction pattern of 622 bp fragments of CASTI gene in Zel sheep after digesting with MspI and NcoI on 2% agarose gel and staining with ethidium bromide. DNA ladder was M100.

The amplified region of calpastatin gene produced a 622 bp DNA fragment using *Msp*I and *Nco*I enzymes in RFLP method. Two alleles (M and N) and subsequently three genotypes were observed. The *Msp*I digestion of the allele M amplimer produced two digested products of 336 and 286 bp, but digestion of the allele N amplimer with *Nco*I enzyme produced the fragments 374 and 248 bp in length (Fig. 1). The allelic frequencies of M and N were 0.855 and 0.145, respectively. The genotype frequencies for MM, MN and NN were as 0.75, 0.21 and 0.04, respectively (Table 1). Under SSCP analysis conditions, different conformations were recognized by electrophoresis on non-denaturing polyacrylamide gel for this locus (Fig. 2). On the other hand, three alleles (A, B and C) were observed with frequencies of 0.835, 0.145 and 0.020, respectively. Genotype frequencies for AA, AB, BB

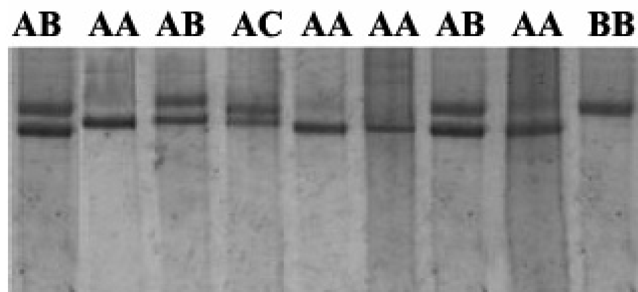

Figure 2. The SSCP pattern of the ovine CAST gene in L region of exon 1 (622 bp) in Zel sheep, on 12% non-denatured PAGE after silver nitrate staining.

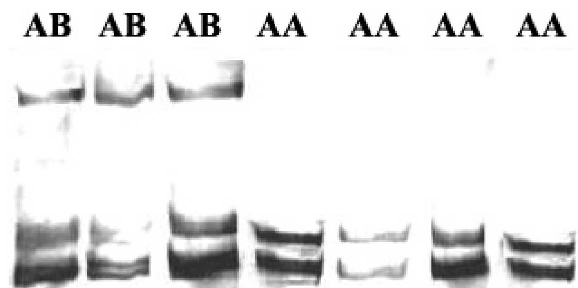

Figure 3. The SSCP patterns of the ovine CAPN regulatory gene in Zel sheep, on 8% non-denatured PAGE after silver nitrate staining.

Table 1. Allelic and genotype frequencies, observed, expected and average heterozigosity for CAST and CAPN loci in Zel sheep.

Locus	Allelic frequencies[a] (%)			Genotype frequencies[b] (%)						Heterozigosity			
	A	B	C	AA	AB	BB	AC	BC	CC	Obs.	Exp.	Ave.	X^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: A, and B correspond to M and N alleles for CAST locus (PCR-RFLP). b: AA, AB, and BB correspond to MM, MN and NN genotypes for CAST locus (PCR-RFLP). ns: non-significant. *: $P<0.05$.

and AC were as 0.71, 0.21, 0.04 and 0.04, respectively (Table 1).

The ovine calpain regulatory gene with 190 bp length was amplified by SSCP method. Different conformations were separated by electrophoresis on non-denaturing polyacrylamide gel (Fig. 3). Two alleles (A and B) were observed with frequencies of 0.845 and 0.155, respectively. Genotype frequencies for AA, AB and BB were as 0.69, 0.31 and 0.00, respectively (Table 1).

Similar result for calpastatin locus was observed in Iranian Kurdi sheep by Nassiry *et al* (2007), however they could not detect NN genotype. A high degree of calpastatin polymorphism has also been reported in studies on Dorset Down hoggets, Dorset Down×Coopworth sheep, Corriedale rams and Angus bulls (Chung *et al*., 1999; Palmer *et al*., 1997). Palmer *et al* (1997) were detected three genotypes (MM, MN and NN) in unrelated Corriedale rams for this locus which was in agreement with the present results. Chung *et al* (1999) observed AA, AB and BB genotypes for CASTI and CAST 5 loci, and AA, BB, CC, AB, AC and BC genotypes for CAST10 locus in Angus bulls.

In this study, CASTI locus was also examined by PCR-SSCP methods. Four different conformational patterns (AA, AB, BB and AC) were determined for this locus. The allelic frequencies for A, B and C were as 0.835, 0.145 and 0.020, respectively. Genotype frequencies for AA, AB, BB and AC genotypes were as 0.71, 0.21, 0.04 and 0.04, respectively. The results were similar to those reported by Nassiry *et al*. (2006) in Kurdi sheep, but in previous study BB genotype had been not determined. Tahmoorespour *et al*. (2006) found A, B and C alleles with frequencies of 0.70, 0.08 and 0.22 in Baluchi sheep, respectively. They also observed BC and CC genotypes in Baluchi sheep, which were not observed in Zel sheep. This inconsistency may be due to breed differences, population and sampling size, environmental factors, mating strategies, geographical position effect, and frequency distribution of genetic variants.

We also found that A and C alleles in SSCP method, could be digested by *MspI*, except for B allele. The *NcoI* restriction enzyme had a reverse effect. On the other hand, samples having MM genotype in RFLP reaction were equal to AA, AC or CC genotypes in SSCP method. Moreover, MN genotype in RFLP, showed AB or BC genotypes by SSCP. Finally, NN genotype (by RFLP), was equal to BB genotype in SSCP method. Palmer *et al* (2000) also reported this association between genotypes of PCR-SSCP and PCR-RFLP analysis for this locus. They directly sequenced nucleotides of the amplimers from homozygote sheep for each allele (GenBank Accession AF016006-8) and found that A and B alleles differed by 6 SNPs in the intron between 1C and ID exons. The C allele had 3 SNPs which differed from A allele. The A and C alleles differed by 9 SNPs, in total (Palmer *et al*., 2000).

In present study, calpain locus was considerated as a potential candidate gene affecting meat tenderness by PCR-SSCP method. Two different confor-mational patterns (AA, and AB) were found for this locus. The frequency for AA and AB genotypeswere as 0.69, and 0.31, respectively. This result was in contrast to study obtained byTahmoorespour *et al*. (2006), who found three genotypes (AA, AB, and BB) in Baluchi sheep. Totally, this population didn't show Hardy-Wein- berg equilibrium except calpastatin locus using SSCPmethod.

Based on our results the investigated population showed a low degree of genetic variability for both loci. This might be explained by the conservation and breeding strategies, which have been carried out. In this station, only a few rams have been used as sires in breeding plans. Due to small effective popula-tion size, inbreeding was high and as a concequence heterozigosity and genetic variability were low. Controlled breeding may help in lowering inbreeding.

Although low genetic variability was detected in the herd, but results showed acceptable polymorphism for calpastatin and calpain loci, which may open interesting prospects for future selection programs, especially using marker-assisted selection for improving weight gain and meat quality.

Furthermore, results showed that PCR-RFLP and PCR-SSCP techniques are appropriate tools for screening CAST and CAPN loci in sheep breeds. This study was one of the first studies on polymorphism of calpastatin and calpain loci in Zel sheep. Due to lack of suitable phenotypic records it was impossible to make association between different genotypes and animal production. Hence, detected polymorphisms and possible associations of genetic variation with meat production and tenderness may help to find the effective genotypes of Zel sheep for economic traits.

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