Indian Journal of Animal Sciences **86** (8): 907–912, August 2016/Article

Association of genetic polymorphisms of *Calpain* and *Calpastatin* genes with growth traits in Bandur sheep

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Received: 31 October 2015; Accepted: 11 January 2016

ABSTRACT

The study was undertaken to determine genetic polymorphisms of *Calpain* and *Calpastatin* genes and to study their association with growth traits (birth weight, weaning weight, 6-month body weight and ADG) in Bandur ram lambs (100) distributed over core area of the home tract as well as from Livestock Research and Information Centre (Sheep), KVAFSU, Nagamangala. Miller's high salt method was used for isolation of genomic DNA from venous blood. A 192 bp of *CAPN* (exons 5 and 6) and 620 bp of *CAST* (exons 1C/1D) genes were amplified by PCR using published primers. The PCR-SSCP analysis of *CAPN* gene revealed genotype frequency for AA (0.672) and AB (0.295) and allele frequency for A (0.820) and B (0.180), respectively. Alignment of A and B allele of *Calpain* by CLC Main Workbench 6.8.1 showed addition/insertion of one nucleotide 'A' in B allele. The PCR-RFLP analysis of *CAST* gene revealed frequency for genotypes MM (0.24), MN (0.59) and NN (0.17), and for alleles M (0.535) and N (0.465), respectively. The Chi-square test indicated that the studied population was in Hardy-Weinberg equilibrium. Five SNPs were identified in the intron between exon 1C and 1D of *CAST*. The association of *CAPN* and *CAST* genotypes with growth traits was analysed by ANOVA using GLM procedures of SAS 9.3. No significant associations were observed between polymorphisms of *CAPN* and *CAST* loci and growth traits.

Key words: Bandur Sheep, *Calpain*, *Calpastatin*, Growth traits, Polymorphism

Among all sheep breeds, Bandur sheep (another popular name is Mandya sheep) is acclaimed as one of the best indigenous mutton type breed in Karnataka and India (Govindaiah *et al*. 2006). The *calpain* system is found in most of the animal tissues and influences many important processes including muscle development and degradation, regulation of protein degradation and rebuilding, cell cycle, organogenesis, post-mortem meat tenderization, cataract formation and cell death (Merin *et al.* 1998). *Calpain* activity depends on Ca^{2+}, and proteolysis of myofibrils by calpain plays a significant role in muscle growth and tenderness of slaughtered meat (Sensky *et al*. 2000), whereas *Calpastatin* is the most variable component of *calpain* system and its activity in skeletal muscle is highly related to the rate of muscle protein turnover and post-mortem tenderisation. Ovine *Calpain* and *Calpastatin* genes may be considered as potential candidate genes for growth, carcass and meat quality traits (Palmer *et al*. 1999a).

Nassiry *et al.* (2006) found that AB genotype had higher daily weight gain in Iranian Kurdi sheep, and Palmer *et al*. (1999a) reported that AC genotype was having increased live weight (123 g/day) at 18% more than AA genotype in the crossbred Dorset and Coopworth sheep. The *Calpain* gene was investigated as a potential candidate gene for quantitative trait locus (QTL) affecting meat tenderness (Koohmaraie 1992). Two *CAPN* alleles (A and B) from exons 5 and 6 (including intron between them) were easily detected by PCR amplification and SSCP process (Chung *et al*. 1999). Acharya (1982) opined that Bandur sheep is one of the best meat breeds of the country as far as conformation is concerned, although body weights, weight gains and feed conversion efficiency are not very superior to most other breeds. The present study was conducted to determine the *Calpain* and *Calpastatin* gene polymorphisms and their association with growth traits in Bandur sheep.

MATERIALS AND METHODS

Blood samples and isolation of genomic DNA: Blood samples were collected from Bandur ram lambs (100) distributed over the villages of Malavalli taluk, Mandya district as well as from Livestock Research and Information Centre (Sheep), KVAFSU, Nagamangala. About 5 ml of blood was collected in vacutainer containing EDTA and stored at 4°C. The blood samples were processed within 24 h of collection. Genomic DNA was isolated by following Miller's High salt method (Miller *et al.*1988) with necessary

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modifications. The quality of the isolated DNA was ascertained by spectrophotometer reading and electrophoretic analysis using 0.8% agarose gel.

PCR amplification: Exons 5, 6 and intervening intron (*CAPN* 456) of Ovine m-*Calpain* regulatory gene and exons 1C/1D and the intervening intron between them from a portion of the first repetitive domain of the ovine *Calpastatin* gene were amplified by PCR. The details of the selected primers used for amplification of Ovine *CAPN* and *CAST* gene are presented in Table 1.Thermal cycler was used for PCR amplification of *CAPN* and *CAST* gene using Red PCR Master Mix.

CAPN *gene:* The amplification was done in 25 µl reaction mixture which consisted of 12.5 µl of Red PCR master mix, 1 µl (10 pmol/ µl) each of forward and reverse primer, 9.5 µl of PCR grade water and 1 µl (100 ng) of template DNA. The PCR reaction was carried out with an initial denaturation temperature of 95°C (3 min), 35 cycles of 94°C (45 sec), 59°C (1 min) and 72°C (90 sec), followed by final extension at 72°C (10 min).

CAST *gene:* The reaction mixture of 25 µl consisted of 12.5 µl of Red PCR Master Mix, 1 µl (10 pmol/ µl) each of forward (Exon 1C) and reverse (Exon 1D) primer, 9.5 µl of PCR grade water and 1 µl (100 ng) of template DNA. The PCR reaction was carried out with an initial denaturation temperature of 95°C (5 min), 33 cycles of 94°C (1 min), 60°C (1 min) and 72°C (2 min), followed by final extension at 72°C (8 min). The PCR amplified products were confirmed by resolving them on 1.5% agarose in parallel with 100 bp DNA ladder. Gel electrophoresis was carried out at 100 V for 60 min in 1× TAE buffer.

Genotyping of CAPN *gene by PCR-SSCP*: The PCR products were mixed with denaturing formamide dye consisting of formamide (95%), xylene cyanol (0.025%), bromophenol blue (0.025%) and 0.5 M EDTA. The mixture was denatured at 95°C for 5 min and snap cooled on ice for 3 min. Polymorphism of *Calpain* gene was determined by using 12% polyacrylamide gel. The electrophoresis was carried out at 100 V for 3 h and the resolved bands were visualized after silver staining (Bassam *et al*. 1991).

Genotyping of CAST *gene by PCR-RFLP:* RFLP analysis of PCR amplified *CAST* gene was carried out using *Msp*I and *Nco*I restriction enzymes. Restriction enzyme digestion was carried out in a total reaction mix of 10 µl, which consisted of 3.5 µl of autoclaved triple distilled water, 1 µl of 10 × assay buffer for RE, 0.5 µl of restriction enzyme (10U/µl) and 5 µl of PCR product. The digestion mixture was incubated overnight at 37°C. The digested products were resolved along with 100 bp DNA ladder on 2.5% agarose gel electrophoresis and the resultant fragments that were stained with ethidium bromide were analysed under gel documentation system.

Statistical analysis: The genotypes were determined by scoring the bands under the gel documentation system. The allele number, allele frequency and genotype frequency observed and expected heterozygosities were calculated as per Rosner (2005). The observed genotype frequency were tested for Hardy-Weinberg equilibrium by Chi-square test.

Growth performance: Growth traits such as birth weight, weaning weight and 6 months body weight were recorded on ram lambs (100) that were randomly selected for genotyping. The weight gain from birth to weaning (0–3 months ADG), from weaning to 6 months age (3–6 months ADG) and from birth to 6 months age (0–6 months ADG) were calculated based on the growth traits measured.

Sequencing of PCR products: The PCR amplified gene products showing different patterns, in SSCP and RFLP were custom sequenced by single pass sequencing method using primers which were employed for amplification of respective products. The sequencing was done and the resultant sequences were analyzed using CLC Main Workbench software (CLC BIO, 2011).

BLAST analysis: The BLAST analysis was applied to identify variations in the *CAPN* and *CAST* sequences

Table 1. Sequence of primers used for amplification of Ovine *CAPN* and *CAST* gene

Gene		Primer sequence	Expected product size (bp)	References
CAPN	F	5' AAC ATT CTC AAC AAA GTG GTG 3'	190	Shahroudi *et al*. (2006); Dehnavi *et al*. (2012)
	R	5' ACA TCC ATT ACA GCC ACC AT 3'		
CAST	F	5' TGGGGCCCAATGACGCCATCGATG 3'	622	Palmer *et al*. (1998); Nassiry *et al.* (2006)
	R	5' GGTGGAGCAGCACTTCTGATCACC 3'		

Table 2. Allele and genotypic frequency of *CAPN* and *CAST* gene in Bandur sheep

Locus	Allele frequency		Genotype frequency			Heterozygosity		χ^2 (P<0.05)
	A_1	A_2	G_1	G_2	G_3	Observed	Expected	
CAPN	0.820	0.180	0.672	0.295	-	0.360	0.295	
CAST	0.535	0.465	0.240	0.590	0.170	-	-	3.45^{NS}

A_1 and A_2 correspond to A and B for *CAPN* locus; M and N for *CAST* locus; G_1, G_2 and G_3 correspond to AA, AB and BB for *CAPN* locus; MM, MN and NN for *CAST* locus.

obtained in comparison with the reference sequences.

Association studies: The association of *CAPN* and *CAST* genotypes with growth traits (birth weight, weaning weight, 0–3 months ADG, 3–6 months ADG and 0–6 months ADG) were analysed by ANOVA using GLM procedure (Linear fixed model) of SAS (SAS 9.3). The fixed effect of genotype was included as independent variables in the linear model.

RESULTS AND DISCUSSION

Analysis of CAPN *gene:* A 192 bp fragment of exons 5 and 6 including intervening intron of ovine calpain regulatory gene was amplified by PCR. The PCR-SSCP analysis of PCR products revealed 2 patterns, viz. AA and AB with frequency of 0.672 and 0.295, respectively (Fig. 1). The allele frequency for A (0.820) and B (0.180), respectively. The observed and expected heterozygosities were 0.360 and 0.295, respectively. The allele and genotype frequency observed and expected heterozygosities for *CAPN* gene are presented in Table 2. The alignment of A and B allele of *CAPN* by CLC Main Workbench 6.8.1 showed addition of one nucleotide 'A' in B allele at 89 bp position. The PCR amplified sequence of *CAPN* gene (192 bp) was used as query and subjected to nucleotide blast at NCBI, which revealed 100, 99, 97, 97 and 89% identity with *Ovis aries* (AF309634.1), *Capra hircus* (AY935995.1), *Bos taurus* (EF139087.1), *Bubalus bubalis* (XM_006068758.1) and *Sus scrofa* (AJ410870.2), respectively.

The occurrence of 2 genotypes confirmed the reports of Shahroudi *et al*. (2006), Nassiry *et al*. (2007) and Dehnavi *et al*. (2012) in Karakul, Kurdi and Zel sheep of Iran, respectively. The genotypic frequency for AA and AB were 0.70 and 0.30, 0.92 and 0.08, and 0.69 and 0.31, for the above mentioned breeds, respectively, whereas the respective allele frequencies were 0.85 and 0.15, 0.96 and 0.04, and 0.845 and 0.151 for A and B. The observed and expected heterozygosities were 0.2963 and 0.254 in Iranian Karakul sheep (Shahroudi *et al*. 2006), and 0.0824 and 0.0794 in Kurdi sheep (Nassiry *et al*. 2007), respectively.

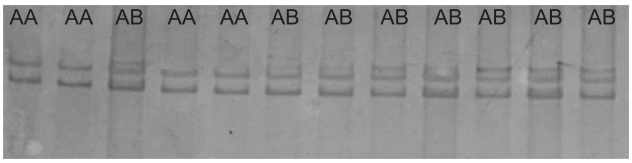

Fig. 1. Poly acrylamide gel electrophoresis (12%) showing SSCP patterns of exons 5 and 6 and intervening intron of *Calpain* gene.

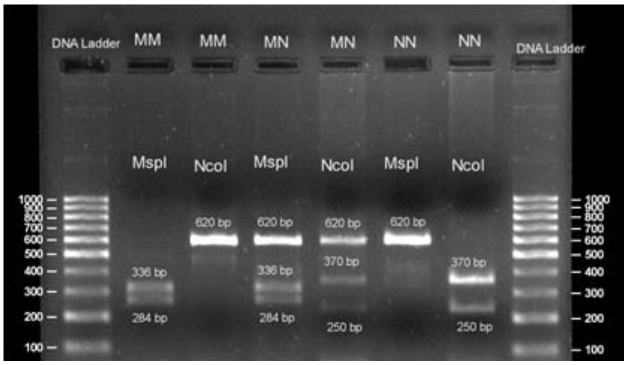

Fig. 2. Agarose gel electrophoresis (2.5 %) showing the PCR-RFLP pattern of *Calpastatin* gene under gel documentation system. Lanes 1 and 8, 100 bp Ladder; lanes 2, 4, 6- *CAST*/*Msp*I genotypes MM, MN and NN; and lanes 3, 5, 7- *CAST*/*Nco*I genotypes MM, MN and NN.

Azari *et al*. (2012) observed 3 different patterns (G1, G2 and G3) with the genotypic frequency of 0.082, 0.892 and 0.027, respectively, in Dalagh sheep of Iran and the results were in accordance with the reports of Tahmoorespour (2005), who reported 3 genotypes (AA, AB and BB) with allele frequency for A (0.56) and B (0.44) alleles, respectively, in Baluchi sheep. Arora *et al*. (2014) found allele frequency for A (0.603) and B (0.397) alleles, respectively, and observed genotype frequency of 0.388, 0.429 and 0.183 % for AA, AB and BB genotypes, respectively. Further the observed and expected heterozygosities were 43.5 and 56.9 %, respectively, in 11 Indian sheep breeds. These results were in contrast to those obtained in this study. Arora *et al*. (2014) identified SNP g.44C > T in *CAPN4* gene of Indian sheep breeds. Zhou *et al*. (2007) reported presence of three SNPs in exon 10 region of Ovine *CAPN* 3.

Analysis of CAST *gene:* A 620 bp fragment was amplified by PCR, comprising the exons 1C/1D and the intervening introns of the ovine *CAST* gene. RFLP analysis of PCR products yielded 2 alleles, viz. M and N. The presence of these two alleles in *CAST* gene was differentiated using two restriction enzymes having differing restriction sites. The enzyme *Msp*I digested only M amplimer but not the N, whereas, enzyme *NcoI* digested only N amplimer. *Msp*I digestion of M amplimer yielded digestion products of 284 and 336 bp whereas, *Nco*I digestion of N amplimer resulted in 2 fragments of size 370 and 250 bp (Fig. 2).

The Bandur ram lamb population showed 3 genotypes MM, MN and NN with frequency of 0.24, 0.59 and 0.17, respectively. The allelic frequency was 0.535 and 0.465 for alleles M and N, respectively (Table 2). The selected PCR product corresponding to 3 genotypes: MM, MN and NN were sequenced and the expected size of 620 bp fragment was confirmed. Alignment of M and N allele sequences of *CAST* by CLC Main Workbench 6.8.1. showed 5 SNPs: G→A transition at 286 and 352 bp, C→T transition at 373 and 526 bp and A→G transition at 443 bp position. Further, the PCR amplified sequence of *CAST* gene (620 bp) was used as query and subjected to nucleotide blast at NCBI, which revealed 100, 94 and 98 % identity with *Ovis aries* (AF016006.1), *Bos taurus* (AY834771.1) and *Capra hircus* (JQ739234.1), respectively.

Palmer *et al*. (1998) described 2 allele systems of polymorphic variants (M and N) in a region (Exon 1C/1D) of the ovine *CAST* by PCR-RFLP method in Dorset Down sheep. They concluded that digestion with restriction enzymes *MspI* and *NcoI* differentiates alleles M and N. Our

study confirmed the presence of these alleles as also observed by others (Gabor *et al*. 2009, Gharahveysi *et al*. 2012, Khan *et al*. 2012, Mohammadi *et al*. 2008, Nanekarani *et al*. 2011a, Nanekarani *et al*. 2011b, Shahroudi *et al*. 2006, Szkudlarek- Kowalczyk *et al*. 2011, Suleman *et al*. 2012) in different sheep breeds. Our results are in agreement with the observations in different breeds of sheep. Azari *et al*. (2012) reported an allele frequency of 55.45 and 44.55% for A_1 (M) and A_2 (N), respectively, in Dalagh sheep of Iran. Elyasi *et al*. (2009) observed an allele frequency of 69, 48 and 50% for M allele in Ghezel, Arkhamerino and Ghezel × Arkhamerino sheep, respectively. Nanekarani *et al*. (2011a) recorded frequency of 63.8 and 36.2% for M and N alleles, respectively, in Lori Sheep, whereas Ata and Cemal (2013) observed allele frequency of 54.4 and 45.6% for M and N alleles, respectively, in synthetic Karya sheep of Turkey. Further, higher MN genotype was observed in Arkhamerino (47.62%) and Ghezel × Arkhamerino sheep (46.67%), Lori sheep (46.2%) and Dalgh sheep (38%).

Several reports were in contrast to the present study, viz. wherein, Palmer *et al.* (1998) reported M and N allele frequency as 77 and 23%, respectively, in Corriedale rams. Similar results were also reported in different sheep breeds of Iran (Shahroudi *et al*. 2006 and Gharahveysi *et al.* 2012), Slovakia (Gabor *et al*. 2009), Pakistan (Suleman *et al.* 2012) and Poland (Szkudlarek-Kowalczyk *et al*. 2011). Further, with respect to the genotypic frequency in the above studies, MM genotype was higher followed by MN and NN. However, breeds like Berrichon du Cher, lle de France (Szkudlarek-Kowalczyk *et al*. 2011) and Slovakia (Gabor *et al*. 2009) showed only 2 genotypes MM and MN, whereas in our study, the frequency of MN genotype was higher followed by MM and NN.

Growth performance: Growth traits such as birth weight, weaning weight and 6 months body weight were recorded on 100 ram lambs that were randomly selected for genotyping of *CAPN* and *CAST* gene. The average daily gain for 0–3 months, 3–6 months and overall 0–6 months was estimated. The weight at birth, weaning, 6 months and average daily gain are presented in Table 3.

Association of CAPN *and* CAST *genotypes with growth traits:* The growth parameters viz. birth weight, weaning weight, 6 months body weight, 0–3 months ADG, 3–6 months ADG and overall 0–6 months ADG for different genotypes of *CAPN* and *CAST* are presented in Table 4. No significant associations were observed between *CAPN* and *CAST* polymorphisms with growth traits.

In accordance to our study, Sutikno *et al*. (2011) and Nikmard *et al*. (2012) found no significant association between *CAST* genotypes and growth traits in local sheep of Indonesia and Afshari sheep of Iran, respectively. However, Palmer *et al.* (1999a) observed an association of the *CAST* genotype 'ac' with increased live weight gain (+12–17%, P<0.05) compared to sheep with *CAST* genotype 'aa' in Dorset Down × Coopworth lambs. Palmer *et al*. (1999b) concluded that the ovine *CAST* gene is a major gene which can affect weight gain in sheep. Chung *et al*. (2001) opined that Angus calves of AB and BB genotypes of *CAST* had significantly higher (P<0.05) weight at day 56 than that of AA genotype. Nassiry *et al*. (2006) observed a significant (P<0.05) association between AB genotype of *CAST* and higher daily gain (birth to weaning) in Iranian Kurdi sheep. The association of *CAST* genotype with the growth traits were also confirmed by the reports of Byun *et al*. (2008), Gregula-Kania (2012) and Chung and Davis (2012) in Romney lambs, synthetic lambs of Poland and Suffolk sheep, respectively.

The present study successfully demonstrated the molecular genetic polymorphism of *CAPN* and *CAST* gene in Bandur sheep through PCR-SSCP and PCR-RFLP analysis, respectively. These techniques are suitable for detecting single nucleotide changes after DNA sequence analysis of the investigated genomic region. Further, the results unwrap remarkable prospects for future selection programmes, especially marker assisted selection though, in our study no associations were found between genetic

Table 3. Growth performance of Bandur ram lambs (100)

Birth weight (kg)	Weaning weight (kg)	Six months weight (kg)	0-3 months ADG (g)	3–6 months ADG (g)	Overall (0-6 m) ADG (g)
2.01 ± 0.04	9.47 ±0.17	13.73 ±0.16	82.84 ±1.83	47.39 ±1.40	65.11±0.87

Table 4. Growth performance of different *CAPN* and *CAST* genotyped Bandur ram lambs

Growth trait	*CAPN* genotypes		*CAST* genotypes		
	AA (64)	AB (36)	MM (24)	MN (59)	NN (17)
Birth weight	2.02 ± 0.052[a]	1.99 ± 0.056[a]	2.031±0.079[a]	2.00±0.050[a]	2.02±0.089[a]
Weaning weight	9.30 ± 0.277[a]	9.00 ± 0.382[a]	9.96±0.382[a]	9.45±0.221[a]	8.85±0.395[a]
6 months weight	13.15 ± 0.291[a]	12.93 ± 0.349[a]	14.28±0.346[a]	13.62±0.214[a]	13.37±0.283[a]
0-3 months ADG	80.84 ± 2.883[a]	77.91 ± 4.115[a]	88.08±3.988[a]	82.71±2.341[a]	75.88±3.932[a]
3-6 months ADG	42.83 ± 2.044[a]	43.70 ± 4.002[a]	48.03±3.263[a]	46.30±1.750[a]	50.23±3.272[a]
Overall ADG	61.15 ± 1.677[a]	60.80 ± 1.971[a]	68.06±1.762[a]	64.51±1.179[a]	63.05±1.609[a]

Means bearing same superscripts are not significantly different at P<0.05; Figures in the parenthesis are the number of animals.

variability and growth traits. However, the genetic variability observed in the *Calpain* and *Calpastatin* gene could be utilized to find association with carcass and meat quality traits.

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