



## Identification of four novel single nucleotide polymorphisms of CAPN1 gene in Indian goat

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### ABSTRACT

The CAPN1 (calcium-activated neutral proteases), is thought to be the main candidate marker gene in the quality of beef meat. Corresponding protein product is nonlysosomal, intracellular cysteine protease which act as primary enzyme in the postmortem tenderization process of meat. SNPs (single nucleotide polymorphisms) in exon9 and 14 of CAPN1 in cattle have been associated with meat tenderness. Data on SNPs in the caprine CAPN1 do not exist. In this study, variations and new polymorphisms are detected in exon9 and exon14 along with their boundary regions in caprine CAPN1 by sequencing of amplified gene fragments in a panel of 7 Indian goat breeds, reared in different regions of country for various production traits. Sequenced caprine genomic segments share >90% similarity with corresponding bovine and pig sequences. Thirty four nucleotide differences were recorded in studied region of caprine CAPN1 gene as compared with that of *Bos taurus* (AF 252504S1 and AF 252504S2). Four novel SNPs were identified, 1 in exonic region (5707, C/T) and 3 in intronic region (intron 8; 5543 T/C and intron14; 4638 T/C, 4689 C/T). The SNP 5707 in exon 9 corresponds to nonconservative substitution (CGG to TGG) that changes an amino acid (Arg to Trp) in the protein sequence. Genotype and allelic frequency of polymorphic sites were determined. These polymorphisms can be used in further search for associations between gene polymorphisms and enzyme activity as well as caprine meat quality traits.

**Key words:** CAPN1, Indian Goat, Meat tenderness, SNPs

Meat quality is of great importance to the meat industry where the consumer is willing to pay more for superior products (Shackelford 2001). Traditional trait improvement has centred on quantitative genetics; using statistical analysis of phenotypic data to determine animals with the highest genetic merit (Dekkers and Hospital 2002). Meat quality traits can usually be measured post-slaughter and often have low heritability (Marshall 1999), therefore making progress using direct measurement is difficult for these traits. Identification of the genes/polymorphisms underlying such traits and an understanding of how these genes/polymorphism interact with environment or other genes affecting economic traits might be the key to successful application of Marker-assisted selection in commercial animal production (Cheong *et al.* 2008).

Tenderness of meat is the most important quality attribute

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of meat (Aviles *et al.* 2009). Davis *et al.* (2008) discussed the status of DNA test for meat tenderness in cattle and so far only 2 genes have been reported to have consistent effects on meat tenderness, i.e. Calpain (calcium-activated neutral proteases gene; CAPN1) and Calpastatin (inhibitor of CAPN1 gene) (White *et al.* 2005). The CAPN1 gene, encodes a cysteine protease thought to be primary enzyme in post-mortem tenderization of meat (Koohmaraie M and Gecsink 2006). Two non-synonymous single nucleotide polymorphisms (SNPs) in the CAPN1 were found associated with meat quality traits in *Bos taurus* beef cattle (Page *et al.* 2004): CAPN316 (C/G), in exon 9 and CAPN530 (A/G), in exon 14, which are responsible for amino acid substitutions p.gly316Ala and p.val530Ile respectively (Curi *et al.* 2009). Polymorphisms with insertion and deletion of nucleotides in intron 17 were found in Hereford, Brahman, and Braford cattle (Iglesias *et al.* 2010). Therefore, CAPN1 might be considered a candidate gene associated with meat quality traits in goats as well. So far no information is available on the status of polymorphism in CAPN1 gene of goat. Therefore, we investigated CAPN1 gene of Indian native goat to discover polymorphisms/ SNPs in exon 9 and exon 14 along with their flanking regions.

## MATERIALS AND METHODS

**Blood sampling and DNA extraction:** To explore the genetic variations with in goat (*Capra hircus*) CAPN1 gene, blood samples were collected from a panel of 42 animals belonging to 7 Indian goat breeds, viz. Beetal, Barbari, Ganjam, Black Bengal, Sirohi, Osmanabadi and Malabari. These breeds were selected due to difference in agro-climatic location as well as utility type (Acharya 1982). The information on these parameters is summarized in Table 1. Blood was collected from jugular vein into EDTA (0.2%) containing vacutainer tubes (BD Bioscience, Germany) and the genomic DNA for PCR amplification was isolated from white blood cells using standard Phenol–chloroform extraction method (Sambrook *et al.* 1989).

**Primer and PCR amplification:** Variation and polymorphism were identified in the potential region of CAPN1 gene following Corva *et al.* (2007). A product of 709bp was amplified for exon 9 and adjoining introns with the primers CCAGGGCCAGATGGTGAA (forward) and CGTCGGGTGTCAGGTTGC (reverse). For sequencing exon 14 along with partial intron 13 and intron 14, 787bp fragment was amplified with exon 14 primer pair AGCGCAGGGACCCAGTGA (forward) and CCCCTGCCAGTTGTCTGAAG (reverse). The PCR amplification was performed in a total volume of 25 µl containing ~ 100ng of genomic DNA, 10pmol of each primer, 200µM of each dNTP, 2.5 µl of 10X buffer with 1.5 mM MgCl<sub>2</sub> and 1U Taq DNA polymerase. Amplification was performed using BIO-RAD i cycler with thermal cycling conditions of initial denaturation at 95°C for 5 min followed by 35 cycle of 94°C for 45s, annealing temperature for 45s and 72°C for 45s followed by final extension at 72°C for 5 min. The annealing temperature was 62.5°C and 62 °C for exon 9 and exon 14 amplification, respectively.

**DNA sequencing and analysis:** The PCR product was visualized by electrophoresis through 1.8% (w/v) agarose gel by staining with ethidium bromide. The PCR product was purified with the NP PCR purification kit and sequenced directly on ABI 3100 genetic analyzer. Nucleotide BLAST

program at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used for sequence homology searches in public databases. Sequence data were edited manually using Chromas Ver.2.33, (<http://www.technelysium.com.au/chromas.html>). Nucleotide sequence of exon 9 and exon14 of Caprine CAPN1 gene was submitted to GenBank (NCBI). Multiple sequence alignments were performed with MegAlign program of LASERGENE software. Allele and genotype frequencies of single nucleotide polymorphism (SNPs) were determined by direct counting method. The coding DNA sequence of exon 9 and exon 14 were conceptually translated to amino acid sequence using DNASTAR (EDIT Seq) software. Cluster analysis was performed following Neighbour-joining procedure using CLC free workbench software.

## RESULTS AND DISCUSSION

SNP analysis is a well-established tool for the identification of genes associated with traits of economic interest in livestock populations (He *et al.* 2009). In the first report on polymorphisms of the Caprine CAPN1 gene, 4 novel SNPs were identified in Indian native goat breeds. The sequences for exon 9 and exon 14 were deposited in GenBank database with accession number GU585590 and GU784846, respectively. We identified thirty four nucleotide changes in CAPN1 gene as compared with that of *Bos taurus* (AF 252504S1 and AF 252504S2). These variations included 20 in exon 9 and their flanking regions; of which 13 were transitions (4 C/T, 3 T/C, 2 G/A, 4 A/G), and 7 were transversions (3 C/G, 2 G/C and 1 T/A and C/A). In the sequenced region covering exon 14 along with the sequence of intron–exon boundary a deletion (C), 1 transversion (A/C) and 12 transitions, viz. 7 C/T, 3 T/C, 2 A/G were recorded. Transversion in the exon 9 region (30%) was higher than that in exon 14 region (10%).

Four variations have been located in exon 9 as compared to cattle CAPN1 sequence (AF 252504S1) (Table 2). Except 1 (5709, C/G), others were non-synonymous substitutions. Nucleotide variation at position 5723 (A/G) and 5741 (G/A) correspond to his to arg (ATG to GTG) and gly to asp (GGG

Table 1. Characteristics of Indian goat breeds selected for SNP identification in CAPN1 gene

| Name <sup>a</sup> | Location <sup>a</sup>                    | Meat: body weight <sup>a</sup> |             | Conformation <sup>a</sup> | Utility <sup>b</sup> |
|-------------------|------------------------------------------|--------------------------------|-------------|---------------------------|----------------------|
|                   |                                          | 6 months                       | 12 months   |                           |                      |
| Barbari           | Uttar Pradesh                            | 7.8±0.48                       | 12.56±1.22* | Small, compact body       | Dual purpose         |
| Beetal            | Punjab                                   | 12.18                          | 21.83       | Large animal              | Dual purpose         |
| Ganjam            | Orissa                                   | 9.52±0.11                      | 11.69±0.16  | Tall, leggy animals       | Meat                 |
| Black -Bengal     | West-Bengal, Bihar, Orissa and Jharkhand | 8.80±0.11                      | 12.60±0.63  | Small animals             | Meat                 |
| Sirohi            | Rajasthan                                | 13.4±0.19                      | 21.27±0.23  | Compact, medium sized     | Dual purpose         |
| Osmanabadi        | Maharashtra                              | 11.07±0.05                     | 15.12±0.24  | Tall animals              | Dual purpose         |
| Malabari          | Kerala                                   | 8.73±0.09                      | 14.12±0.21  | Medium sized animals      | Dual purpose         |

\*9-month data; <sup>a</sup> Acharya (1982); <sup>b</sup>Tomar (2004).

Table 2. Nucleotide variations and SNPs of exon 9 and flanking region in CAPN1 gene of Indian goat

| Region   | Nucleotide Position | Exotic cattle (AF252504S1) | Indian goat | Amino acid change | Genotype Frequency   | Allele frequency   | Type of change |
|----------|---------------------|----------------------------|-------------|-------------------|----------------------|--------------------|----------------|
| Intron 8 | 5496                | G                          | A           |                   | 1                    |                    | Transition     |
|          | 5499                | C                          | T           |                   | 1                    |                    | Transition     |
|          | 5500                | A                          | G           |                   | 1                    |                    | Transition     |
|          | 5503                | C                          | T           |                   | 1                    |                    | Transition     |
|          | 5519                | C                          | T           |                   | 1                    |                    | Transition     |
|          | 5520                | C                          | G           |                   | 1                    |                    | Transversion   |
|          | 5541                | G                          | C           |                   | 1                    |                    | Transversion   |
|          | 5543                | T                          | T/C         |                   | TT-0.143<br>CT-0.857 | T-0.572<br>C-0.428 | Transition     |
|          | 5584                | T                          | A           |                   | 1                    |                    | Transversion   |
|          | 5587                | C                          | A           |                   | 1                    |                    | Transversion   |
|          | 5607                | C                          | G           |                   | 1                    |                    | Transversion   |
|          | 5609                | T                          | C           |                   | 1                    |                    | Transition     |
|          | 5654                | A                          | G           |                   | 1                    |                    | Transition     |
|          | 5680                | G                          | C           |                   | 1                    |                    | Transversion   |
|          | 5687                | T                          | C           |                   | 1                    |                    | Transition     |
|          | 5688                | A                          | G           |                   | 1                    |                    | Transition     |
| Exon 9   | 5707                | C                          | C/T         | Arg>trp           | CC-0.727<br>CT-0.273 | C-0.863<br>T-0.137 | Transition     |
|          | 5709                | C                          | G           |                   | 1                    |                    | Transversion   |
|          | 5723                | A                          | G           | His>arg           | 1                    |                    | Transition     |
|          | 5741                | G                          | A           | Gly>asp           | 1                    |                    | Transition     |

to GTA) substitution at position 11 and 17 of conceptualized protein, respectively. One novel SNP has been identified in Indian goat, SNP 5707, corresponding to a C/T transition in exon 9. This SNP generated a non-synonymous codon (CGG to TGG) that would cause an Arg to Trp substitution at position 6 of the corresponding CAPN1 protein. Genotypic frequencies for SNP 5707 are presented in Table 2. Frequencies for alleles at SNP 5707 were not balanced, the C allele being predominant over the T allele. The T allele was present in Barbari, Beetal, Ganjam, Sirohi, Malabari and Osmanabadi but was absent in meat type Black Bengal goat where only C allele is present.

SNPs in exon 9 of CAPN1 have previously been associated with meat quality traits in cattle. This change may provoke functional alterations in the proteolytic activity of  $\mu$ -calpain, with consequent variations in postmortem myofibrillar degradation (Curi *et al.* 2010). The C allele of 331G>C, also known as the 316 marker (exon 9) has been associated with muscle tenderness in various studies (Cheong *et al.* 2008, Aviles *et al.* 2009, Smith *et al.* 2009). No SNP was recorded in Exon14 of indigenous goat although 2 SNPs (A/G, G/A) have been reported and associated with meat tenderness trait in cattle (Corva *et al.* 2007, Cheong *et al.* 2008). Two variations were identified in exon 14 at position 4575 (C/T) and 4578 (C/T), compared with cattle CAPN1 sequence (AF 252504S2). These variations were synonymous in nature and thus should have no effect on the goat protein of this gene.

SNPs in exon 9 and 14 were predicted to alter the protein sequence of calpain I in cattle. The SNPs in exon 9 and 14 were predicted to change amino acid sequence in domain II and domain III, respectively. The polymorphism in exon 9 of the Calpain I gene in Indian goats is of biological interest as it encodes part of domain II of protein, which has been identified to possess calcium dependent cysteine-protease activity (Costello *et al.* 2007). This SNP reflects in the change of amino acid from arginine, a positively charged amino acid to Tryptophan an aromatic ring amino acid, having no net charge at physiological pH.

The partial intronic regions flanking either side of the coding region (intron 8, intron 13 and intron 14) showed 20 transitions, 7 transversions, and 1 deletion. Three SNPs were also detected in introns. One SNP in intron 8 (5543, T/C) and 2 SNPs downstream of exon14 at position 4638(T/C) and 4689 (C/T) (Table 3) were recorded. SNPs involving T/C transition has been reported in intron 14 of Irish cattle by Costello *et al.* (2007), as well as in Korean cattle (Cheong *et al.* 2008). Nevertheless, the significance of such single nucleotide polymorphisms cannot be denied as it was found that mutations which are predicted to be translationally silent can alter gene expression by influencing splicing accuracy/efficiency or codon biasness (Chamary *et al.* 2006).

Extreme frequencies of any allele at a given marker locus make selection inefficient (Corva *et al.* 2007), so that consideration of allele frequencies is an important consideration for any SNP to be used as marker for MAS

Table 3. Nucleotide variations and SNPs of exon 14 and flanking region in CAPN1 gene of Indian goat

| Region    | Nucleotide Position | Exotic cattle (AF252504S1) | Indian goat | Amino acid change | Genotype Frequency     | Allele frequency   | Type of change |
|-----------|---------------------|----------------------------|-------------|-------------------|------------------------|--------------------|----------------|
| Intron 13 | 4530                | C                          | -           |                   | 1                      |                    | Deletion       |
| Exon 14   | 4575                | C                          | T           | -                 | 1                      |                    | Transition     |
|           | 4578                | C                          | T           | -                 | 1                      |                    | Transition     |
| Intron 14 | 4585                | C                          | T           |                   | 1                      |                    | Transition     |
|           | 4614                | C                          | T           |                   | 1                      |                    | Transition     |
|           | 4622                | C                          | T           |                   | 1                      |                    | Transition     |
|           | 4638                | T                          | T/C         |                   | TT- 0.095<br>CT- 0.906 | T-0.548<br>C-0.452 | Transition     |
|           | 4650                | A                          | G           |                   | 1                      |                    | Transition     |
|           | 4654                | A                          | G           |                   | 1                      |                    | Transition     |
|           | 4689                | C                          | C/T         |                   | CT- 0.930<br>TT- 0.070 | C-0.933<br>T-0.067 | Transition     |
|           | 4713                | T                          | C           |                   | 1                      |                    | Transition     |
|           | 4729                | C                          | T           |                   | 1                      |                    | Transition     |

(marker's assisted selection). There are 20 well recognized breeds of goat in India. These breeds were developed to produce milk, meat and fibre through natural selection and have adapted to diversified agro-climatic conditions based on their utility. Seven different breeds were selected for the present study to present comprehensive picture regarding CAPN1 polymorphism. Overall C allele exhibited preponderance over T allele in SNP of exon 9 and one SNP of intron14 (4689, C/T). Overall allelic frequencies of other 2 intronic SNPs were not skewed in present study. However it is important to mention the different behaviour of Black Bengal goat from other goat breeds in the panel (results not shown, available on request). Black Bengal goat was monomorphic with only 1 allele present at each SNP site (C, T, C, T allele at SNP loci 5543 (T/C), 5707 (C/T), 4638 (T/C) and 4689 (T/C) respectively). Black Bengal is considered as important meat breed of India. Further, these alleles showed higher proportions in Ganjam and Malabari goat breeds, used for meat, having very low milk.

The exon 9 sequence of different breeds of Indian goat was further subjected to basic local alignment search to know the sequence homology with the corresponding region of other species. The BLAST results showed more than 90% similarity with cattle, viz. 94% with *Bos taurus* x *Bos indicus*, 93% with *Bos taurus* and 90% with *Sus scrofa*.

The phylogenetic tree constructed based on Neighbor-Joining method of this region also revealed the clustering together of prominent meat type goat breeds, viz. Ganjam and Black Bengal. Indian goat formed an altogether different group from the *Bos taurus* and *Sus scrofa* (Fig. 1).

This is the first study in goat reporting SNPs in CAPN1 gene. However, further efforts are required to analyze novel SNPs for possible associations with meat tenderness and other quality traits in indigenous goat by using populations from different genetic backgrounds and increasing the size of sample.

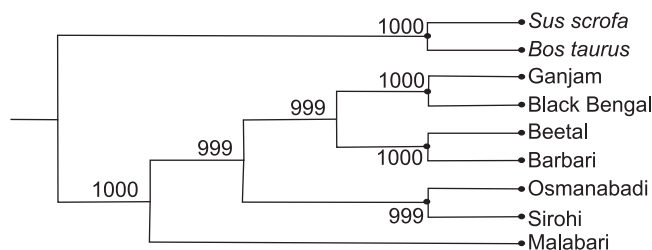


Fig 1. Phylogenetic tree constructed based on exon 9 sequence of CAPN1 gene (Figures at nodes represented bootstrap values of 1000 resampled data sets).

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