MOLECULAR STUDIES ON MEAT QUALITY GENE IN BANDUR SHEEP

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CERTIFICATE

This is to certify that the thesis entitle "*Molecular Studies on Meat Quality Gene in Bandur Sheep"* submitted by **Mr. SUNIL KUMAR M.A I.D.No. MVHK- 840** in partial fulfillment of the requirement for the award of Master of Veterinary Science in Animal Genetics and Breeding of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work carried out by him during the period of his study in this university under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship fellowship or other similar titles.

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Date:

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(B.M. VEERE GOWDA)

Affectionately Dedicated to

my Parents, Sister, RBDGTC Hostel,

Teachers and Friends

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ABBREVIATIONS

A	-	Adenine
C	-	Cytosine
G	-	Guanine
T	-	Thymine
PCR	-	Polymerase Chain Reaction
Taq	-	*Thermus aquaticus*
DNA	-	Deoxyribo Nulceic Acid
cDNA	-	complementary DNA
RFLP	-	Restriction Fragment Length Polymorphism
bp	-	base pair
kb	-	kilo base pair
dNTP	-	deoxy Nucleotide Tri Phosphate

Introduction

1. INTRODUCTION

India stands eighth in the world's meat production with sheep contributing 5 per cent of the total meat production. The country has 61.4 million sheep as per 2003 livestock census and ranks sixth in the world. As per the 18th Quinquennial livestock census, Karnataka has 9.5 million sheep. Karnataka state is known for well-defined breeds and strains of sheep such as Bandur, Hassan, Deccani, Bellary, Chitradurga, Yelaga and nondescript local sheep, which are prevalent in specific parts of the state as adjunct to cropping pattern.

Bandur is one of the best mutton breeds of sheep in India. This breed originated from the Bannur village in Malavalli Taluk, Mandya District, Karnataka and is distributed in Mandya and adjoining districts of Mysore, Tumkur, Hassan and Bangalore rural. Bannur is a small to medium sized breed possessing distinguished characters such as Roman nose, long, leafy and drooping ears, short tail, short and stumpy legs and brown patches on the neck extending upto shoulder and powerful brisket region (Govindaiah, 2006). This breed is also known for its organoleptic characters and marbling effects of meat.

Producing tender meat in accordance with consumers' preference is one of the major problems facing the industry, since the meat tenderization during the postmortem period is highly variable among the carcasses. Tenderness, intramuscular fat (IMF) level and water holding capacity are meat quality traits with moderate heritability, however they are difficult to improve by conventional selection. Therefore, studies of biochemical

mechanisms for muscle breakdown are essential at the molecular level and there is a need to identify genetically superior animals for these traits (Mullen *et al*., 2006).

Meat quality describes properties and perceptions of meat. Eating quality of meat depends on several important characteristics including appearance, color, aroma, fat content, texture, juiciness, flavour and tenderness. Hence, consumer evaluation of eating quality is the major determinant of meat quality. However, the main source of consumer complaint and the primary cause of failure to repurchase is the variability in eating quality, especially tenderness (Tarrant, 1998; Bindon and Jones, 2001).

Meat quality is affected by postmortem tenderization, which is a complex structural and functional process that depends on species, genetic background, metabolic status of the animal, ante mortem and environmental factors. The rate of postmortem proteolysis of several important structural muscle proteins is considered a determinant factor in post mortem meat tenderization (Taylor *et al.,* 1995).

The physiological change in muscle structure during the postmortem period is complex (Koohmaraie, 1994). The calpain-calpastatin system apart from cathepsin is an endogenous, calcium- dependant proteinase system, hypothesized to mediate the proteolysis of key myofibrillar proteins during postmortem storage of carcass and cuts of meat at refrigerated temperatures (Koohmaraie *et al*., 1995b). Calpain is responsible for the breakdown of myofibrillar proteins, which are closely related to meat tenderness (Wheeler and Koohmaraie, 1994). Calpastatin (CAST) inhibits μ and m-calpain activity and therefore, regulates postmortem proteolysis. Increased postmortem CAST activity

has been correlated with reduced meat tenderness (Koohmaraie *et al*., 1995a; Pringle *et al.*, 1997).

The application of genetic markers can potentially alleviate many of these problems, since they can be measured at any time and in both sexes. The essence of using genetic markers in breeding programs is that they mark chromosomal regions (and sometimes individual genes) and so can follow the inheritance of these regions from parents to offspring (Visscher *et al*., 1988). Thus, if we know which chromosomal segments contain alleles of value, markers may be used to help identify animals that have inherited these alleles and hence the best of the genetic variation, whether or not we have progeny information on the animal.

Keeping this background in view, the present study was taken up with the following objectives:

1. To amplify gene associated with meat quality in Bandur sheep.
2. To study the polymorphism of this gene in Bandur sheep population by PCR-RFLP technique.
3. To study the genetic structure of Bandur sheep with respect to meat quality gene.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Bandur sheep

Bandur is one of the best mutton breeds of sheep in the world. This breed originated from the Bannur village in Malavalli Taluk, Mandya District, Karnataka.

Bandur is small to medium sized breed possessing distinguished characters such as Roman nose, long, leafy and drooping ears, short tail, short and stumpy legs and brown patches on the neck extending up to shoulder and powerful brisket region (Govindaiah, 2006) (Fig. 1 and Fig. 2).

2.2 Meat quality

For many effective breeding plans, a breeder should have sound knowledge about the existing genetic variation in animals. But unlike productivity, meat quality has largely been a product of natural selection over time immemorial. By effective and intense human selection, this operating factor has gradually been lost. Animal breeders have not considered meat quality as a factor in most of the improvement programmes. But by opening new vistas of molecular genetic studies, this area also started gaining due importance. It is an usual observation that meat productivity and quality are attributes of different individuals and often of different populations and breeds. There is an increasing demand for combining meat productivity with quality. The simplest and the most obvious course in this direction is of replacement of existing poor quality genes with high quality genes.

Fig. 1 : Typical Bandur Ram

Fig. 2 : Typical Bandur Ewe

Meat quality is a generic term used to describe properties and perceptions of meat. The critical point of appraisal of meat quality occurs when the consumer eats the product and it is this outcome, together with views of colour, healthiness and price determines the decision to repurchase (Boleman *et al*., 1997). Hence, consumer evaluation of eating quality is the major determinant of meat quality, with tenderness, juiciness and flavour of meat being the most important elements. However, the main source of consumer complaint and the primary cause of failure to repurchase is the variability in eating quality, especially tenderness (Tarrant, 1998; Bindon and Jones, 2001).

2.2.1 Meat quality genes

Meat tenderness is one of the most important factors leading to consumer satisfaction. Among the factors that have been identified as responsible for the postmortem meat tenderization process is the calpain proteolytic system. Two enzymes responsible for this process are the micromolar calcium-activated neutral protease, *calpain* (***CAPN1***), which is encoded by the *CAPN1* gene and its inhibitor, *calpastatin* (***CAST***), which is encoded by the *CAST* gene (Koohmaraie, 1996). To date several markers have been identified at the *CAST* gene (Barendse, 2002) and three markers have been identified at the *CAPN1* gene (Page *et al*., 2002; White *et al*., 2005). Previous studies (Barendse, 2002; Page *et al*., 2002, 2004; White *et al*., 2005) have independently evaluated markers at the *CAST* and *CAPN1* genes. These studies have shown an association of individual markers at *CAST* and *CAPN1* loci with meat tenderness in domestic animals.

2.2.2 Calpastatin gene

It was discovered during the initial studies on purification of m-calpain that muscle extracts having calpain activity also contained an inhibitor of this activity (Goll *et al.,* 1990). Okitani *et al.* (1976) established that this inhibitor was a heat-stable protein (to 100°C) and it has subsequently been shown that it is resistant to a wide variety of denaturing agents such as urea, SDS, or trichloroacetic acid (Geesink *et al*., 1998). The name, calpastatin, was proposed for this inhibitor in 1979 (Murachi, 1989). The early attempts to purify this inhibitor produced inconsistent and variable results and the inhibitor was described as a protein having molecular masses varying from 34 to 280–300 kDa (Goll *et al*., 1990).

Calpastatin is the endogenous and specific inhibitor of calpains, plays a central role in regulation in calpain activity in cells (Murachi, 1983; Forsberg *et al*., 1989) and is considered to be the major modulators of calpains. There is evidence that in different species, calpastatin is highly related to meat tenderness (Koohmaraie *et al.,* 1991; Sensky *et al*., 1998; Parr *et al*., 1999). It has been proved that this gene plays a major role in regulating meat tenderness (Palmer *et al.,* 1998 and Chung *et al*., 2001). At the protein structural level, Calpastatin is a five domain inhibitory protein (Killefer and Koohmaraie, 1994), of which the N-terminal leader (L) domain does not have any calpains inhibitory activity while the other domains (I-IV) are highly homologous and each of them are independently capable of inhibiting calpains (Cong *et al*., 1988). This indicates that the inhibitory domains of calpastatin contain three highly conserved regions, A, B and C, of which A and C bind calpain in a strictly Ca^{2+} dependant manner but have no inhibitory activity, whereas region B inhibits calpain on its own. It is also found that the removal of

the XL domain played a regulatory role by altering phosphorylation patterns on the protein (Takano *et al.,* 1999).

The CAST gene is located on chromosome 5 in humans (Inazawa *et al*., 1990), chromosome 2 in pigs (Ernst *et al*., 1998), chromosome 7 in cattle (Bishop *et al*., 1994) and chromosome 5 in sheep (Nassiry *et al*., 2006).

Studies during the past two decades have shown that, because of the use of different promoters (Cong *et al*., 1988: Imajoh *et al.,* 1987: Wang *et al*., 1994) or alternative splicing mechanisms (Lee *et al.,* 1992a: Lee *et al*., 1992b), a large number of different calpastatin isoforms ranging in molecular mass from 17.5 kDa (Wang *et al.,* 1994) to 46.35 kDa (Imajoh *et al*., 1987) to 84 kDa (Cong *et al*., 1988) are produced from this single gene.

Calpastatin does not inhibit any other protease with which it has been tested including the cysteine proteases, papain, cathepsin B, bromelin, or ficin in addition to proteases from other classes such as trypsin, chymotrypsin, plasmin, thrombin, pepsin, cathepsin D or thermolysin (Crawford, 1990).

2.3 Molecular Markers for Genome Analysis

A molecular marker is defined as any stable and inherited variation detectable or measurable by a suitable technique and which can be used to detect presence of a specific genotype or phenotype, which is otherwise not measurable or very difficult to detect. A piece of genetic material that bears or produces a distinctive feature is generally

considered to be a marker, usually a mutant gene, and can be either dominant or recessive (Strickberger, 1985).

'Molecular markers' or 'genomic markers' originated during the recent past due to tremendous progress in the field of molecular biology. A large amount of data have been generated since the first demonstration of RFLP (Grodzicker *et al.,* 1974) and the proposal to use them as markers in human genetics (Botstein *et al*., 1980), in genetic improvement of plants (Beckman and Soller, 1983; Burr *et al.,* 1983) and animals (Beckman and Soller, 1983). The use of PCR (Saiki *et al*., 1988) to amplify a DNA sequence of interest and subsequent restriction enzyme analysis (RFLP) of the amplified product (Pinder *et al.,* 1991) represented a milestone in this endeavour. An unlimited number of genetic polymorphisms at DNA sequence level have provided a number of genomic markers such as RFLP's (Botstein *et al*., 1980); minisatellites or variable number of tandem repeats (VNTR) (Jeffreys *et al*., 1985; Nakamura *et al*., 1987); minisatellites (Litt and Lutty, 1989; Weber and May, 1989; Tautz, 1990; Fries *et al*., 1990) and RAPD markers (Williams *et al*., 1990; Welsh and McClelland, 1990).

The use of molecular markers to define the genetic makeup (genotype) and predict the performance of an animal is a powerful aid in animal breeding (Beuzen *et al*., 2000). The selection based on these markers is known as Marker Assisted Selection (MAS). Several candidate genes for meat quality have been studied by various authors. Calpastatin gene is one of such candidate gene found to be associated with meat quality.

2.3.1 Restriction Fragment Length Polymorphism (RFLP)

Genetic polymorphism is the coexistence of multiple alleles at a locus and any site at which multiple alleles exists as stable components of the population is by definition polymorphic. Polymorphism in the genome can be detected by comparing the restriction maps of different individuals and a difference in restriction maps between two individuals is a restriction fragment length polymorphism or RFLP (Lewin, 2000). It can be used as a genetic marker; instead of examining some feature of the phenotype, a genotype as revealed by restriction map is assessed. RFLP refers to inherited differences in sites for restriction enzymes that result in differences in the length of the fragments produced by cleavage with the relevant restriction enzymes.

2.3.2 Restriction Endonucleases

Restriction endonucleases (RE) are a class of endonuclease enzymes able to cleave DNA at a specific nucleotide sequence. In some bacteria they form part of a defensive mechanism against infection by bacteriophage in which the bacterium will methylate specific sites in its own DNA to prevent cleavage by its intrinsic restriction enzyme which are still able to destroy foreign unmethylated DNA. Different enzymes, as obtained from a different range of organisms, have different specificities, often recognizing four or six base pairs. Because of this specificity, restriction enzymes cleave a sample of DNA into defined polynucleotide fragments which can then be separated according to their length. This procedure is sufficiently sensitive to be able to detect a difference of one base pair in certain circumstances and hence the enzymes are highly essential and a prerequisite for the procedures of genetic manipulation.

Three types of REs are known. Their grouping is based on the type of sequences recognized, the nature of the cut made in the DNA and the enzyme structure. Type I and Type III REs are not useful for molecular biology work because they cleave DNA at sites other then the recognition sites and thus cause random cleavage patterns. Type II endonucleases however, recognize specific sites and cleave just at these sites.

2.3.3 Principle of RFLP

RFLPs occur as a result of DNA base changes, deletions, insertions or rearrangements that either create, eliminate or translocate restriction enzyme cleavage site (Theilmann *et al.,* 1989). A point mutation, deletion or insertion can create or abolish the recognition site for a particular RE at the locus and thereby changes the size of the resulting restriction fragments. Inversion on the other hand, changes the distance between a pair of RE sites and therefore, causes changes in size of restriction fragments. The changes in DNA sequences associated with an allelic change at a locus can be visualized by the altered mobility of restriction fragments on gel-electrophoresis. Individuals carrying different allelic variants of the gene will show different distribution patterns. These differences in band numbers and sizes result from change in fragment size and are termed as restriction fragment length polymorphism (RFLP). Such variants are inherited in a Mendelian fashion and since gene expression is not required for RFLP analysis, variation in the flanking regions or introns of genes may also be detected. Consequently, the RFLP approach for analysis of genomic variation is potentially powerful (Theilmann *et al*., 1989).

2.3.4 Application of RFLP

Restriction Fragment Length Polymorphisms are an important tool for the identification of genetic variation, providing valuable markers for use in animal and plant genetics. RFLPs have been best exploited in human genetics where they comprise the basis of genetic map that can be used to diagnose and predict a number of diseases including cystic fibrosis (Bequedet *et al*., 1986), Duchene muscular dystrophy (Bakker *et al*., 1985), Huntington's chorea (Gussella *et al*., 1983) and retinitis pigmentosa (Bhattacharya *et al*., 1984). Genetics of domestic cattle stands to benefit significantly from the discovery and description of new RFLPs. Once a direct effect of a trait of economic importance has been assigned to a particular RFLP allele or haplotype, a few generations of selection would suffice to bring it to high frequency.

2.3.5 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is an in vitro method for enzymatically synthesizing defined sequences of DNA; the reaction normally uses two oligonucleotide primers that hybridize to opposite strands that flank the target DNA sequence that is to be amplified. The elongation of the primers is catalysed by Taq DNA polymerase that is isolated from the thermophilic eubacterium *Thermus aquaticus*. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by Taq DNA polymerase results in an exponential accumulation of specific DNA fragment.

2.3.6 PCR-RFLP Techniques

Standard southern blotting and hybridization techniques for conventional RFLP analysis require good DNA preparations and have long analysis time. Amplification of

desired sequence of DNA by using specific oligonucleotide primers by PCR followed by restriction enzyme digestion and separation of fragments on electrophoresis gel is termed PCR-RFLP (Pinder *et al*., 1991). Unlike other DNA based typing methods, PCR-RFLP does not involve the use of radioisotopes.

PCR-RFLP technique is reported as a powerful and sensitive technique for detecting of polymorphism in a functionally relevant domain of the Calpastatin gene (Palmer *et al*., 1998; Nassiry *et al*., 2007; Mohammadi *et al*., 2008).

2.4 Analysis of Polymorphism in CAST gene

2.4.1 Amplification of CAST gene

Palmer *et al.* (1998) carried out the amplification of exon 1C/1D of the Ovine CAST gene in a PCR cocktail consisting of 100 ng ovine genomic DNA. The reaction was cycled for 1 minute at 95 ^{o}C, 1 minute at 62 ^{o}C and 2 minute at 72 ^{o}C for 35 cycles.

Nassiry *et al.* (2006, 2007) achieved amplification of the exon 1C/1D from domain 1 region including the intron of the Ovine CAST gene to a 622 bp fragment by performing PCR in a 25 µl reaction mixture containing 2.5 µl PCR buffer 10X (200 mM (NH4)2SO4, 0.1 mM Tween 20, 750 mM Tris HCl, pH 8.8), 2.5 mM MgCl2, 200 µM dNTPs and 10 pM of each forward and reverse primers and 1U of Taq DNA polymerase. The thermal cycler profile was primary denaturation for 3 min at 95 ^{o}C followed by 35 cycles of 60s at 95 ^{o}C, 60s at 62 ^{o}C and 2 min at 72 ^{o}C and the final extension of 8 min at 72 ^{o}C.

Amplification of the exon and intron I from L domain of the ovine CAST gene to produce a 622 bp fragment was carried out by Mohammadi *et al*. (2008). The PCR was performed in a 25 µl reaction volume using a buffer PCR 1X, 200 µM dNTPs, 1.5 µM MgCl2, 10 pM each primer, 1.25 U Taq DNA polymerase. The amplification conditions comprised an initial denaturation of 95 ^{o}C for 5 minutes followed by 33 cycles of denatuaration at 94 ^{o}C for 1 minute, annealing at 60 ^{o}C for 1 minute and extension at 72 ^{o}C for 8 minutes.

2.4.2 Polymorphic studies of CAST gene in livesock

2.4.2.1 Sheep

Palmer *et al*. (1998) studied the nucleotide sequence of the 622 bp PCR amplimers of exon and intron regions from a portion of the first repetitive domain of the ovine calpastatin gene from six Dorset Down sheep. They reported the presence of two alleles *M* and *N* that differed by having a number of restriction sites present in one allele but not in the other. Digestion with restriction endonucleases *Msp*I and *Nco*I differentiated alleles *M* and *N*. The *Msp*I digested the allele *M* amplimer, but not allele *N*, where as *Nco*I had the reverse effect. The *Msp*I digestion of the allele *M* amplimer produced digestion products of 336 and 286 bp. The *Nco*I digestion of the allele *N* amplimer resulted in digestion products of 374 and 248 bp. The allele frequency for M and N alleles was 0.77 and 0.23 respectively.

Elyasi *et al*. (2005) reported that allelic frequency of both M and N alleles were 0.50 for CAST locus in Ghezel X Arkharomerino sheep. They observed MN genotype in

high value for Arkharomerino (47.62%) and Ghezel X Arkharomerino (46.67%). They did not detect NN genotype

Association of genetic polymorphism in the calpastatin (*CAST*) gene with average daily gain in Iranian purebred Kurdi sheep was examined by Nassiry *et al.* (2006). The genotypes for *CAST* were determined by the PCR-SSCP method. Three genotypes including *aa*, *ab* and *ac* with frequencies of 0.55, 0.32 and 0.13, were observed in this population with allelic frequencies of 0.78, 0.16 and 0.06 respectively for a, b and c alleles. The population was in Hardy-Weinberg equilibrium.

Nassiry *et al*. (2007) studied genetic variability and population structure in CAST loci in Iranian Kurdi sheep using PCR-RFLP. A 622 bp fragment from CAST I was amplified. Digestion with restriction endonuclease *Msp*I resulted in two alleles M and N. The *Msp*I was found to digest the allele M amplicon, but not allele N. The MM genotype exhibited two fragments of 336 and 286 bp, MN genotype produced 622, 336 and 286 bp fragments. The allelic frequencies were 88% and 12% for M and N, respectively. The genotype distribution in Kurdi sheep were 76, 24 and 0% for MM, MN and NN, respectively.

Polymorphism of calpastatin gene in Arabic sheep was analyzed by PCR-RFLP method by Mahammadi *et al.* (2008). Exon and intron I from L domain of the ovine calpastatin gene was amplified and a 622 bp fragment was digested by *Msp*I enzyme. A and B alleles were identified with a frequency of 0.85 and 0.5 respectively and the genotype frequencies of AA, AB and BB genotypes in the population were 0.70, 0.29 and 0.01 respectively.

2.4.2.2 Cattle

Bishop *et al.* (1993) analyzed the polymorphism of the Bovine CAST locus by using RFLP with three restriction enzymes. Two codominantly inherited fragments were detected using *Bam*HI with sizes of 9.0 and 5.0 kb plus one monomorphic band at approximately 15 kb. Fragment sizes for *Eco*RI and *Taq*I were 6.0 and 4.0 kb, and 1.9, 3.5**,** 4.0, and 5.0 kb, respectively. The frequencies of the *Bam*HI 9.0 and 5.0 kb alleles were 0.38 and 0.62 respectively.

Bovine calpastatin cDNAs coding for domains 2, 3, 4 and domains L and I were analyzed for polymorphism in *B. taurus* cattle by Lonergen *et al.* (1995). *Bam*HI and *Eco*RI enzymes produced RFLP with 9.0 and 5.0 kb and 6.0 and 4.0 kb fragments respectively. The *Bam*HI fragments had frequencies of 0.53 and 0.47 respectively and *Eco*RI frequently had frequencies of 0.43 and 0.57 respectively. The authors concluded that there was no relationship between these alleles and meat tenderness.

Schenkel *et al*. (2006) studied association of a single nucleotide polymorphism (SNP) in the calpastatin gene with carcass and meat quality traits of beef cattle. A SNP was identified in the CAST gene (a G to C substitution) and genotyped on crossbred commercially fed heifers (n = 163), steers (n = 226), and bulls (n = 61) from beef feedlots, and steers (n = 178). The CAST SNP allele C was more frequent (63%) in the crossbred population than allele G. Genotype CC yielded beef that was more tender than GG and CG had intermediate tenderness. The corresponding average allele substitution effect (G to C substitution) was also highly significant (−0.15 ± 0.05 kg, P = 0.002).

However, genotype CC had a greater fat yield (+1.44 ± 0.56%; *P* = 0.037) than genotype GG, with a corresponding allele substitution effect of 0.67 ± 0.27% (*P* = 0.015).

Majidi *et al.* (2009) studied bovine calpastatin gene in 41 Nelore cattle using PCR-RFLP in Malaysia. Forward and reversed primers amplified a 1552 bp fragment from calpastatin gene. *Xmn*I enzyme was used for restriction analysis of PCR products. Overall, the frequency of alleles A and B in the studied breeds were estimated as 0.42 and 0.58, respectively. The authors reported genotype frequencies of 12.2, 58.53 and 29.27% for AA, AB and BB respectively and also observed heterozygosity, expected heterozygosity and average value of heterozygosity as 0.58, 0.49 and 0.48 respectively. Highest frequency of allele was B (0.58) and lowest was A (0.42). This Nelore cattle population was in the Hardy-Weinberg equilibrium.

2.4.2.3 Pigs

Kuryl *et al*. (2003) studied the effect of calpastatin gene (*CAST*) polymorphism on carcass traits in pigs. They used three restriction enzymes (*Hinf*I, *Msp*I, *Rsa*I). An association between genotypes at *locus CAST* and carcass traits was analysed on 39 Stamboek castrated males free of *RYR1T* gene. Genotypes *DD* at *locus CAST*/*Msp*I and *EF* at *locus CAST*/*Rsa*I proved less fatty (thinnest backfat and lower weight of backfat with skin in loin) than the two remaining genotypes at each of these *loci*. Moreover, genotypes *DD* at *locus CAST*/*Msp*I and *EE* at *locus CAST*/*Rsa*I were the most advantageous for eye-muscle area when compared to the remaining genotypes at these *loci*. They concluded that *CAST* gene may be considered as a candidate gene for pig carcass quality.

Ciobanu *et al.* (2004) reported several missense and silent mutations in *CAST* using a three-generation intercross between Berkshire and Yorkshire pigs. Results demonstrated that one *CAST* haplotype was significantly associated with lower cooking loss and higher juiciness and, therefore, this haplotype is associated with higher eating quality. This study provides important evidence in favor of the presence of new alleles/haplotypes of the *CAST* gene associated with several important meat quality traits in pigs. By sequencing the entire coding region in BxY F3 individuals with extreme values for meat quality, eight mutations, including three missense mutations, were identified. Two of the missense mutations, *CASTArg249Lys* and *Ser638Arg*, are located in or close to subdomain C of their respective domains. This subdomain potentiates the inhibitory activity of CAST (Takano and Maki, 1999). It has been suggested that single mutations in conserved regions of any of the A or C subdomains might affect CAST activity (Ma *et al.*, 1994), even though they do not have inhibitory properties like Subdomain B. Both these substitutions (*Arg249Lys* and *Ser638Arg*) are outside the most conserved area of Subdomain C (Takano and Maki, 1999), but the *Ser638Arg* mutation is separated by just one AA from this sequence.

Kapelanski *et al.* (2004) found significant relations between the examined meat traits and polymorphic forms of the calpastatin gene in a group of 245 pigs of different breeds. Polymorphism at *locus CAST*/*Hinf*I was related to colour saturation and colour lightness of meat ($P<0.05$). Polymorphism of the calpastatin gene identified with endonucleases *Msp*I and *Rsa*I was found to influence other meat traits connected with water binding capacity and consistency of raw meat. It was found that at *locus CAST*/*Msp*I animals of *AA* genotype had firmer and springier meat ($P<0.05$) than those of

AB and *BB* genotypes. At *CAST*/*Rsa*I *locus* meat of pigs of *BB* genotype appeared firmer and springier than that of *AB* ($P<0.01$) or *AA* genotype pigs ($P<0.05$). Significantly lower drip loss during meat storage was observed in pigs of *AA* genotype at *locus CAST*/*Msp*I, and of *BB* genotype at *locus CAST*/*Rsa*I when compared to pigs of remaining genotypes ($P<0.05$).

Material and Methods

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Experimental Animals

The present investigation was carried out on 79 Bandur sheep. A random sample of Bandur sheep in the age group of one to four years formed the materials for this study. The experimental animals were selected from KVAFSU Sheep farm, Veterinary college, Hebbal, Bangalore and flocks owned by farmers of Hadli village near Malavalli Taluk of Mandya District.

3.1.2 Chemicals and Reagents

The chemicals and reagents used in the present study were of molecular biology grade and are presented in Appendix 1 and 3.

3.1.3 Primers

Primers were synthesized from Bangalore Genei, Bangalore.

3.1.4 Glassware and plastic-ware

All the glassware were thoroughly cleaned and sterilized as per the standard procedures. Micro-centrifuge tubes and micro tips were autoclaved before use.

3.1.5 Equipments

The major equipments used in the study are presented in Appendix 2.

3.2 Methods

3.2.1 Isolation of genomic DNA from venous blood

DNA isolation was carried out adopting the High salt method as described by Miller *et al*. (1988).

3.2.1.1 Procedure

About 10ml of venous blood was collected from external jugular vein in a 15ml sterile centrifuge tube containing EDTA at a concentration of 1.5mg/ml of blood. The blood samples were immediately stored in an icebox, transported to the laboratory and stored at 4^0C. Subsequent processing of blood was done within 48 hrs of collection. The blood samples were centrifuged at 4000 rpm for 20 minutes at RT. The buffy coat was removed along with small quantity of RBC and plasma and transported to another centrifuge tube. The buffy coat was resuspended in four volumes of RBC lysing buffer and kept at 4^oC for five min with occasional mixing. The mixture was centrifuged at 2000 rpm for five minutes. The resulting pellet was suspended in 10 ml TE buffer and carefully distributed with a tip of pipette. Proteinase-K and SDS were added at the final concentration of 0.2µg/ml and 0.5 per cent, respectively. The mixture was incubated at 55^oC for 60 minutes and then at 65^oC for 15 minutes, with occasional mixing.

After the incubation, three ml of 5M NaCl solution was added and mixed thoroughly. One volume of Chloroform: Iso amyl alcohol (24:1) was added and mixed thoroughly by brief vortexing using a cyclomixer for one minute. The mixture was centrifuged at 6000 rpm for 10 minutes. The upper aqueous phase was transferred carefully using a Pasteur pipette to a fresh tube. Two volumes of distilled absolute

alcohol was added to precipitate the DNA. The string of DNA was removed with micropipette tip and transferred in to 1.5 ml microfuge tube. The DNA was washed twice with 80 percent alcohol and dried in a vacuum drier. The DNA was resuspended in 0.5 ml TE (pH 7.0) and kept at -20^{o}C till further use.

3.2.2 Quality and quantity of genomic DNA

The purity and concentration of the isolated genomic DNA was estimated by using UV spectrophotometer and by electrophoresis on agarose gel.

3.2.2.1 UV spectrophotometry

The ratio of absorbance (A) at 260 and 280 nm was used as an indicator of DNA purity. A ratio between 1.6 and 1.9 was considered indicative of a relatively pure DNA sample. The genomic DNA concentration was measured by using the formula

Genomic DNA concentration in µg/µl = A_{260} X 50 X dilution factor (Sambrook and Russell, 2001).

3.2.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis (0.8%) was carried out for confirming the quality of the isolated DNA. Appropriate amount of agarose was dissolved in 1X tris acetate EDTA (TAE) buffer to make a final concentration of 0.8 per cent. The agarose was melted in a microwave oven. Ethidium bromide was added at a concentration of 0.5 µg/ml of agarose gel solution and the solution was allowed to cool sufficiently. The gel tray was sealed on either side by using adhesive tape and the comb was placed in proper position. The melted agarose solution was poured in to the gel tray carefully, avoiding formation of air

bubbles. After the gel had polymerized, the comb and the seal on either side of the gel tray were removed carefully. The gel tray was kept in an electrophoresis tank and 1X TAE buffer was poured to submerge the gel in the tank. The DNA samples were mixed with 1/6th volume of 6X gel loading buffer and loaded in to the wells using pipette. The gel was electrophoresed in 1X TAE buffer at 80 volts at room temperature for about half an hour. The gel was visualized under the UV trans-illuminator and photographed using gel documentation system.

The quality of the DNA was assessed based on the bands developed. Good quality DNA was used for further study.

3.3 PCR Amplification

3.3.1 PCR amplification of CAST gene

Exon and intron regions from a portion of the first repetitive domain of the ovine CAST gene were amplified by PCR.

3.3.2 PCR Primers

The details of the oligonucleotide primers (Mohammadi *et al*., 2008; Nassiry *et al.*, 2006; Palmer *et al*., 1998) used for PCR are given in Table 1.

Table 1 : Sequences of primers used for amplification of Calpastatin gene

Primer	Primer sequence	Expected product size (bp)
Forward	CAST 1C- (5' TGGGGCCCAATGACGCCATCGATG 3')	622
Reverse	CAST 1D - (5' GGTGGAGCAGCACTTCTGATCACC 3')	

3.4 PCR reaction:

PCR was carried out in a final reaction volume of 50 l. A master mix for minimum of 20 samples was prepared and was aliquoted to 45µl in each PCR tube. Five l genomic DNA (100 ng) was added in each tube to make a final volume of 50 l. Components used for each reaction volume are shown in Table 2.

Table 2 : Components used for each reaction volume

PCR component	Volume (l)	Final concentration
Taq Buffer	5.0	1 X
dNTP	4.0	400 M
Forward primer	2.0	10 pm/ l
Reverse primer	2.0	10 pm/ l
Taq polymerase enzyme	1.0	3U/ l
Genomic DNA	5.0	100 ng
FQW	31	To make 50 l reaction
Total	50.0	-

3.5. PCR Protocol:

The PCR protocol followed for the amplification of desired fragment of DNA is shown in the Table 3.

Table 3 : Polymerase Chain Reaction (PCR) protocol.

Step	Temperature	Time
Initial denaturation	95^{o}C	5 min
1.Denaturation	94^{o}C	1 min
2. Annealing	60^{o}C	1 min
3. Extension	72^{o}C	2 min
Steps 1-3 repeated for 33 cycles		
Final extension	72^{o}C	8 min

All the reactions were carried out in 0.2 ml thin walled PCR tubes. PCR tubes containing mixture were tapped gently and quickly spinned at 10,000 rpm for few seconds. The tubes were placed in a Master cycler gradient (Bio Rad) and subjected to PCR. One reaction tube without DNA was maintained as negative control.

3.6 Agarose gel electrophoresis of PCR products:

PCR amplification was confirmed by running 10 l of PCR product, mixed with 2 l of 6X gel loading dye from each tube on 1.5 per cent agarose gel at a constant voltage of 80 V for 45 to 60 min in 1X TAE buffer. 5µl of Ethidium bromide (1%) was incorporated in 100 ml for gel solution itself. The amplified products were visualized as a single compact fluorescent band of expected size under UV light and documented by gel documentation system.

3.7 Restriction enzyme digestion of the PCR products:

The PCR products of 10 l from each tube were digested with restriction enzymes along with the appropriate buffer supplied with the enzyme. 1.5 l of each enzyme was used in a digestion mixture of 31 l. The reaction mixture was centrifuged for few seconds for uniform mixing and then incubated at 37°C for 14 hr. The restriction enzymes *Msp*I and *Nco*I (Table 4) were used to digest the amplicons of Calpastatin. The details of the RE digestion mixture is given in Table 5.

Table 4 : The restriction enzymes used to digest the PCR products

Sl. No.	Restriction enzymes	Recognition site
1.	*Msp*I (*Morexella species*)	5'…C↓CGG…3' 3'…GGC↑C…5'
2.	*Nco*I (*Nocardia corallina*)	5'…C↓CATGG…3' 3'…GGTAC↑C…5'

Table 5 : Optimized RE digestion mixture:

Sl.No	Reaction components	Quantity
1.	Autoclaved triple distilled water	18 µl
2.	10 x assay buffer for RE	2 µl
3.	RE (10U/µl)	1 µl
4.	PCR product	10 µl
	Total	31 µl

3.8 Analysis of the PCR-RFLP Products:

After restriction enzyme digestion, the PCR products were elect -rophoresed on 2.4% agarose gel (according to the expected size of fragments) containing ethidium bromide (1%) at the rate of 5 l /100ml by submarine gel electrophoresis apparatus at constant voltage of 80V for 90 minutes using 1X TBE buffer. 6X gel loading dye was used to load digested PCR products. Gene Ruler™ (Bangalore Genei) 100bp DNA Ladder (Range, 100-1000 bp) was used as a molecular size marker. The bands were visualized under UV light and documented by gel documentation system. The band sizes were judged by comparing with molecular size marker and recorded. Genotyping of Calpastatin was carried out according to the band pattern of respective genotypes.

3.9 Sequencing of PCR products

The samples which showed different RFLP patterns were selected for sequencing. PCR products were sent to Chromous Pvt Ltd, Bangalore for sequencing and the results were analyzed.

3.10 Sequence Analysis

3.10.1 DNA Sequence Analysis

The CAST sequences were analyzed by BLAST search at NCBI site for homology using BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST). The BLAST programme compares a nucleotide query sequence against nucleotide sequences in the database. The CAST (622 bp) sequences of Bandur Sheep were submitted as query

sequences and selected the MegaBLAST programme for searching highly similar sequences from the DNA database.

3.10.2 Establishment of Gene Identity

The BLAST (Basic Local Alignment Search Tool) (http://ncbi.nih.gov/BLAST) analysis was used to find variation in the sequences obtained in the study with the reference sequences in ovines and with sequences of Calpastatin gene of other species.

3.11 Statistical analysis

The observed genotype frequencies based on RFLP patterns were tested for Hardy-Weinberg equilibrium by Chi- square test. The observed and expected frequencies were analyzed for goodness of fit at probability $p \leq 0.05$.

Results

4. RESULTS

The results obtained in the present study aimed at polymorphic analysis of ovine CAST gene in Bandur breed of sheep is as follows.

4.1 Isolation of genomic DNA

The DNA was isolated from venous blood of 79 animals of Bandur breed of sheep. The high salt DNA extraction procedure of Miller *et al*. (1988) was used with slight modifications.

4.1.1 Quantity and quality of DNA

About 300 µg of pure genomic DNA could be extracted by this technique from 10ml of blood sample. The OD 260/280 ratio was between 1.7 to 1.9 for most of the DNA sample which indicated their purity. The quality of isolated DNA was confirmed by agorose gel electrophoresis (0.8 %) and was of high molecular weight and appeared as single bands without sheared fragments.

4.2 PCR-RFLP of CAST gene

The PCR reactions were set up for the amplification of the exon 1C/1D from domain 1 region including the intron of the ovine CAST gene using the oligonucleotide primers ovine 1C and ovine 1D (Mohammadi *et al*., 2008; Nassiry *et al.*, 2006; Palmer *et al*., 1998).

4.2.1 Standardization of PCR procedure

The PCR technique was standardized to optimise the different PCR parameters *viz.,* template DNA concentration, dNTPs concentrations and annealing temperature. Two µl of genomic DNA, 200 µM dNTPs and annealing tempeature of 60ºC was found to be optimal to produce the desired 622 bp PCR product with less primer-dimer. A clear 622 bp fragment was amplified. Twenty picomol of each of the primers per reaction volume of 50 µl as employed by Nagaraja (1998) was used throughout the experiment as an optimum primer concentration. For complete and uniform amplification, 35 cycles of PCR was found to be ideal.

4.2.2 PCR-RFLP analysis of CAST gene

The oligonucleotide primers ovine 1C and 1D (Mohammadi *et al*., 2008; Nassiry *et al*., 2006; Palmer *et al*., 1998) were used for the amplification of the exon 1C/1D from domain 1 region including the intron of the Ovine CAST locus by PCR technique. In all the blood samples tested, the amplified product was approximately of 622 bp with no variation in size between the animals studied (Fig. 1). The RFLP patterns of the ovine CAST locus were generated by using *Msp*I and *Nco*I enzymes.

4.2.2.1 PCR-RFLP analysis of CAST gene using *Msp*I

*Msp*I digested the 622 PCR product into two fragments of sizes 336 and 286 bp. This RFLP pattern was denoted as A. The *Msp*I undigested pattern was denoted as B. These two RFLP patterns resulted in three genotypes. The animals with both alleles were assigned as AB genotype, where as those possessing only A or B alleles were designated as AA or BB genotypes respectively. AA genotype resolved two bands (336 and 286 bp)

while undigested single band animals were denoted as BB genotype. The animals with all three bands (622, 336 and 286 bp) were designated as AB genotype. AB animals displayed a pattern with all three bands (Fig. 2). Allelic pattern of CAST gene digested with *Msp*I restriction enzyme is given in table 6.

The gene and genotypic frequencies of CAST/ *Msp*I polymorphism in Bandur sheep are given in table 8. The gene frequencies for A and B alleles in Bandur sheep were 0.74 and 0.26 respectively. The genotypic frequencies of CAST/ *Msp*I polymorphism for AA, AB and Bb were observed as 0.54, 0.39 and 0.07 respectively.

Chi square test revealed that the population of Bandur sheep was in Hardy-Weinberg equillibrium (Table 8).

4.2.2.2 PCR-RFLP analysis of CAST gene using *Nco*I

The RFLP pattern was also resolved with another restriction endonuclease *Nco*I. This enzyme produced three restriction patterns (Fig. 3). This enzyme also resolved two alleles A and B. Allele was denoted as A when the restriction endonuclease did not digest the amplicon, while it was denoted as B when the enzyme digested amplicon into two fragnents of sizes 374 and 248 bp. Allelic Pattern of CAST gene digested with *Nco*I restriction enzyme is given in table 7.

The gene and genotypic frequencies of CAST/ *Nco*I polymorphism in Bandur sheep are given in table 9. The gene frequencies for A and B alleles in Bandur sheep were 0.697 and 0.303 respectively. The genotypic frequencies of CAST/*Nco*I polymorphism for AA, AB and BB were observed as 0.50, 0.395 and 0.105 respectively.

The population of Bandur sheep was found to be in Hardy-Weinberg equilibrium by comparing the observed frequencies with expected frequencies using chi square test (Table 9).

Table 6 : Allelic pattern of CAST gene digested with *Msp*I restriction enzyme in Bandur sheep

Sl. No	Allele type	Fragment size (bp)	Number of animals (n=79)
1.	AA	336, 286	43
2.	AB	622, 336, 286	31
3.	BB	622	5

Table 7 : Allelic pattern of CAST gene digested with *Nco*I restriction enzyme in Bandur sheep

Sl. No.	Allele type	Fragment size (bp)	Number of animals (n=38)
1.	AA	622	19
2.	AB	622, 374, 248	15
3.	BB	374, 248	4

Table 8 : Genotype and allele frequencies of CAST/*Msp*I polymorphism in Bandur sheep.

Population	Source	Genotypic frequency			$\chi 2$ value (df=2)	Allele frequency	
		AA	AB	BB		A	B
Bandur (79)	Number observed	0.54 (43)	0.39 (31)	0.07 (5)	0.00045^{NS}	0.74 (117)	0.26 (41)
	Number expected	0.55	0.38	0.068			

Figures in parenthesis are actual numbers
^{NS}- Not Significant
df- degrees of freedom

Table 9 : Genotype and allele frequencies of CAST/ *Nco*I polymorphism in Bandur sheep

Population	Source	Genotypic frequency			$\chi 2$ value (df=2)	Allele frequency	
		AA	AB	BB		A	B
Bandur (38)	Number observed	0.50 (19)	0.395 (15)	0.105 (4)	0.00252^{NS}	0.697 (53)	0.303 (23)
	Number expected	0.486	0.422	0.092			

Figures in parenthesis are actual numbers
^{NS}- Not Significant
df- degrees of freedom

4.3 DNA sequencing

Amplified products of CAST gene from Bandur sheep (one sample each from three genotypes corresponding to *Msp*I and *Nco*I restriction enzymes) were gel eluted, cloned and sequenced commercially.

4.3.1 Sequence analysis of CAST gene

The sequencing of the amplified products of CAST gene from Bandur sheep revealed two different CAST alleles having 622 bp each.

The BLASTn analysis of the nucleotide sequence of the exon 1C/1D from domain 1 region including the intron of the Ovine CAST locus of Bandur sheep is given in Table 10 . The sequence from Bandur sheep has revealed 98, 92, 98, 98 and 95 percent identity with *Ovis aries* (Accession # AF016006.1), *Bos taurus* (Accession # AY258325.1), *Capra hircus* (Accession # GU944861.1), *Bos grunniens* (Accession # EU009141.1) and *Bos taurus* x *Bos indicus* (Accession # EF529438.1), respectively.

Table 10 : Results of BLASTn showing the percentage of identity of the exon 1C/1D from domain 1 region including the intron of the Ovine CAST locus of Bandur sheep with other species

Accession no	Description	Maximum score	Total score	Query coverage (per cent)	Maximum Identity (per cent)
AF016006.1	Ovis aries calpastatin (CAST) gene, partial cds	977	977	88%	98%
AF016007.1	Ovis aries calpastatin (CAST) gene, partial cds	966	966	88%	98%
AF016008.1	Ovis aries calpastatin (CAST) gene, partial cds	961	961	88%	98%
EU486168.1	Ovis aries calpastatin (CAST) gene, CAST-e allele, exon 12, intron 12, exon 13 and partial cds	935	935	88%	97%
AY258325.1	Bos taurus calpastatin (CAST) gene, partial cds	821	821	90%	92%
AY834771.1	Bos taurus calpastatin (CAST) gene, exons 9 through 14 and 14t	813	813	90%	92%
HM053645.1	Capra hircus calpastatin transcript variant 2 mRNA, complete cds, alternatively spliced	137	137	12%	98%
GU944861.1	Capra hircus calpastatin (CAST) mRNA, complete cds	132	132	12%	97%
EU009141.1	Bos grunniens calpastatin (CAST) mRNA	128	128	11%	98%
EF529438.1	Bos taurus x Bos indicus calpastatin mRNA	117	117	11%	95%

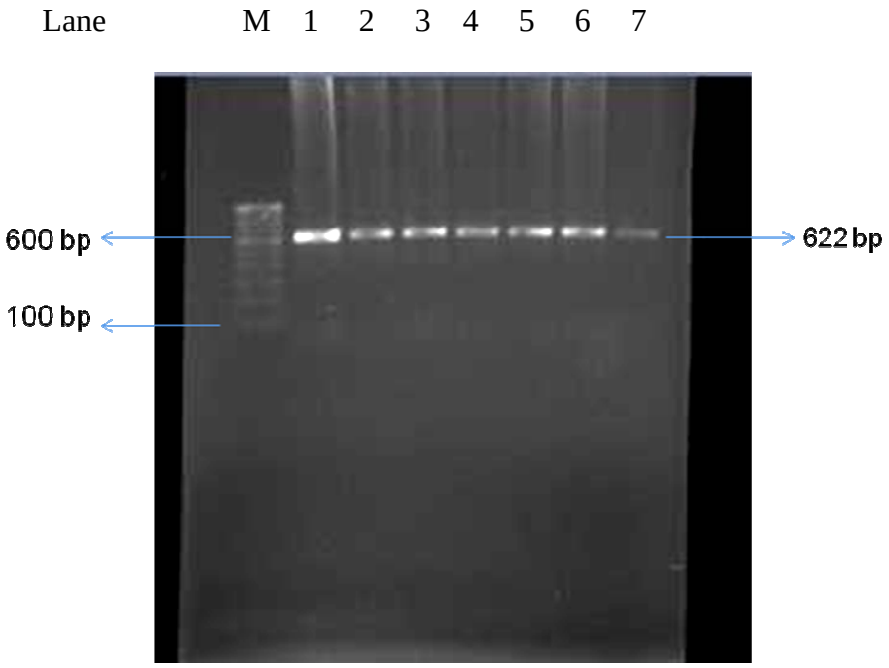

Fig. 3 : PCR amplification of the exon 1C/1D from domain 1 region including the intron of the ovine CAST locus in Bandur sheep

Lane 1-7 -------- Bandur sheep DNA samples

Lane M -------- Molecular marker

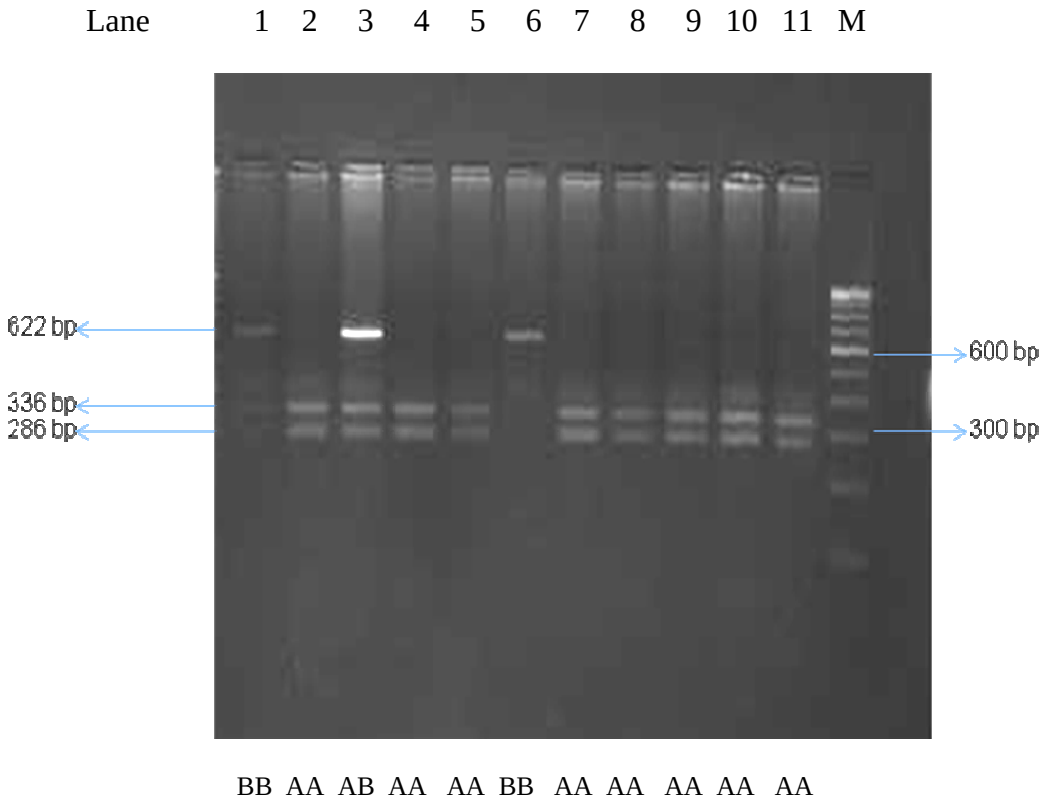

Fig. 4 : PCR-RFLP pattern of exon 1C/1D from domain 1 region including the intron of the Ovine CAST locus by *Msp*I restriction enzyme in Bandur sheep

Lane 1-11 -------- Bandur sheep DNA samples

Lane M -------- Molecular marker

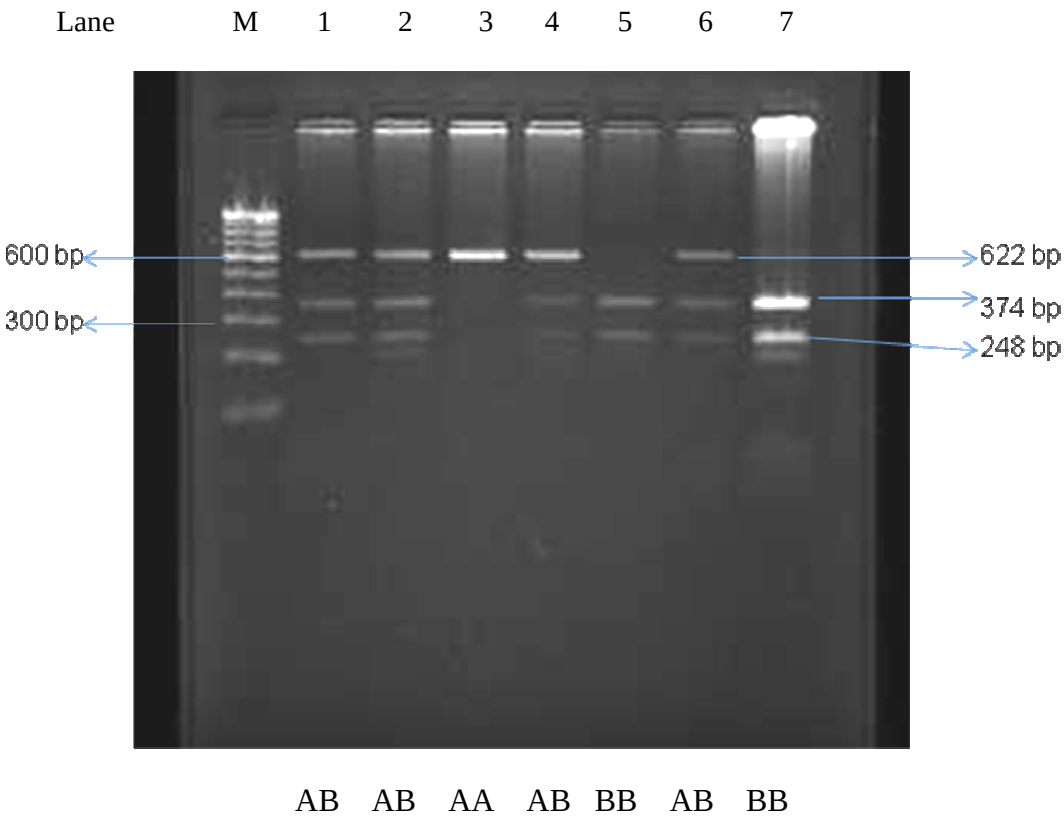

Fig. 5 : PCR-RFLP pattern of exon 1C/1D from domain 1 region including the intron of the Ovine CAST locus by *Nco*I restriction enzyme in Bandur sheep

Lane 1-7 -------- Bandur sheep DNA samples

Lane M -------- Molecular marker

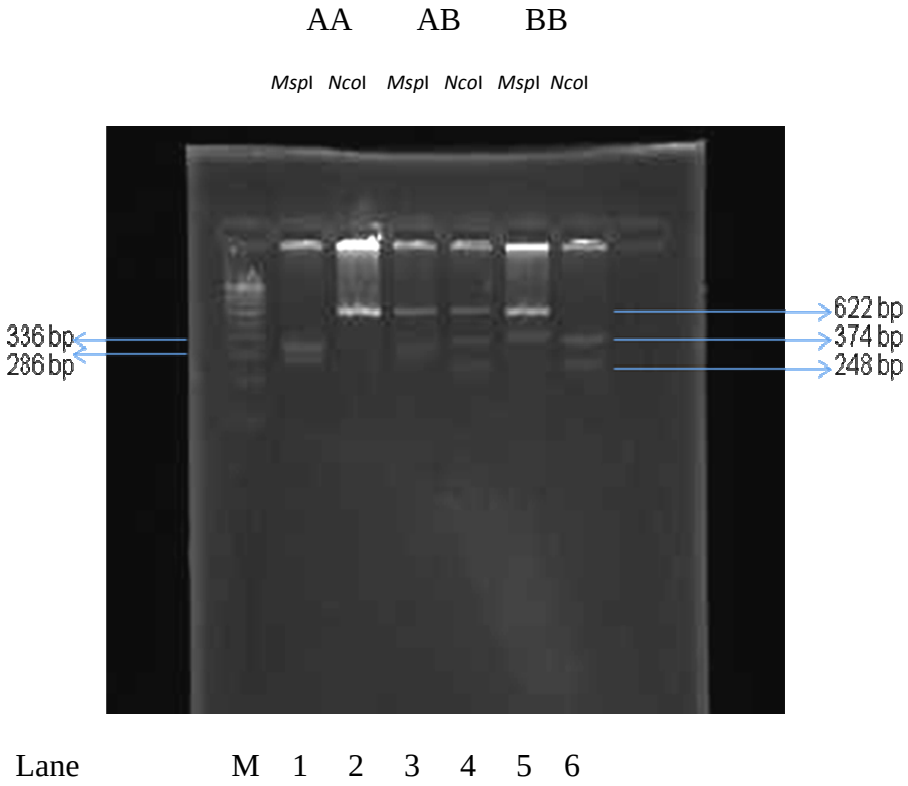

Fig. 6 : RFLP polymorphism analysis of the 622-bp exon 1C/1D amplimer from the CAST gene using *Msp*I and *Nco*I restriction enzyme in Bandur sheep

Lane 1-6 -------- Bandur sheep DNA samples

Lane M -------- Molecular marker

Discussion

5. DISCUSSION

The meat quality, especially the tenderness of meat determines the acceptibility of meat by the consumers. The tenderness of meat varies between breeds, between animals and within breeds. Breeders so far have not been successful in identification and selection of meat animals with desired tenderness, by the traditional selection methods. The recent advances in molecular genetics, kindled hopes of identification of molecular markers for such traits which are economically important, but difficult to genotype by traditional methods.

Calpastatin is one of the important genes responsible for improving the meat tenderness. Calpain-calpastatin system is responsible for the breakdown of myofibrillar protein which is closely related to meat tenderness. The present investigation was aimed at studying the genetic variation at CAST region in Bandur breed of sheep and to assess the possibility of developing a DNA marker system for meat tenderness, since it has been proved that this gene plays a major role in regulating meat tenderness (Palmer *et al.,* 1998 and Chung *et al*., 2001). Bandur is one of the best mutton breeds of sheep in India. This breed is also known for its organoleptic characters and marbling effects of meat.

5.1 Isolation of genomic DNA

The high salt DNA extraction procedure (Miller *et al.,* 1988) was used with slight modifications for the isolation of high molecular weight DNA from blood samples. This procedure was found to be suitable for purification of DNA from blood samples of sheep (Montgomery and Sise, 1990; Cushwa *et al*., 1996) and cattle (Gwakisa *et al*., 1994; Gelhaus *et al*., 1995).

5.1.1 Yield and quality of DNA

About 300 µg of pure genomic DNA could be extracted from 10ml of blood sample by high salt DNA extraction procedure. Montgomery and Sise (1990) reported higher yield of 640 µg DNA from 100 samples of 20 ml of sheep blood with high salt method.

Mitra (1994) obtained higher yields of 343 to 693 µg with an average of 403 µg per 10 ml of cattle and buffalo blood. Senthil (1995) reported 450 µg to 800 µg with an average of 625 µg per 15 ml of cattle blood using a similar protocol. Annapoorani (1996) obtained a yield of DNA ranging from 210 µg to 602 µg with an average of 400 µg per 100 ml of buffalo blood. Aravindakshan (1997) reported pure DNA in the range of 246 µg to 572 µg with an average of 360 µg per 10 ml of cattle blood and 300 µg to 707 µg with an average 452µg per 10 ml of buffalo blood. By the use of same protocol, Nagaraja (1998) extracted about 400µg of pure genomic DNA from 10-15 ml of cattle blood samples. The OD 260/280 ratio between 1.7 to 1.9 for most of the DNA sample indicated their purity.

5.2 PCR-RFLP of CAST gene

5.2.1 PCR technique

The PCR technique was followed as described by Mohammadi *et al*. (2008) for amplifying a selected region of CAST sequence and to compare the nucleotide variations within breeds.

5.2.2 Amplification of CAST gene by PCR

For specific amplification of the target sequence in the DNA and for obtaining specific quantity of the desired product, it is essential to optimize the different parameters.

The optimum parameters used for the amplification of the of the exon 1C/1D from domain 1 region including the intron of the Ovine CAST locus are given in Table… Annealing temperature and time optimized in this study was 60ºC for 60 seconds for 33 cycles. This is in accordance with the work of Mohammadi *et al.* (2008) who had also used 33 cycles of 60ºC for 60 seconds for optimum annealing. Palmer *et al*. (1998) and Nassiry *et al*. (2007) used 35 cycles of 62ºC for 60 seconds for optimum annealing. The variation in temperature could be in accordance with the particular laboratory conditions.

5.2.3 CAST gene polymorphism

A 622bp fragment of the of the exon 1C/1D from domain 1 region including the intron of the Ovine CAST locus was amplified by PCR using oligonucleotide primers. The size of amplification product was same for all the animals studied. This is in agreement with the results of Nassiry *et al*. (2007) in Iranian Kurdi sheep and Mohammadi *et al*. (2008) in Arabic sheep indicating that the CAST region in sheep is conserved.

5.2.4 Restriction patterns

The PCR-RFLP technique was followed to detect variation within the amplified genomic portion. Two restriction enzymes *Msp*I and *Nco*I which have a higher potential

for detecting polymorphism were used to digest the PCR product. A total of 3 different patterns were resolved by both *Msp*I and *Nco*I restriction endonuclease.

5.2.4.1 Restriction patterns with *Msp*I

The amplified DNA was digested with *Msp*I restriction enzyme and the fragments were separated by agarose gel electrophoresis. The amplified 622bp fragment was restricted into 336bp and 286bp fragments when a polymorphic *Msp*I site was present. This allowed the identification of two alleles *viz*., A (336 and 286bp fragment) and B (undigested fragment of 622bp).

Present study showed that the calpastatin locus had two alleles in Bandur sheep breed with *Msp*I restriction enzyme. Digestion with restriction enzyme *Msp*I differentiated alleles A and B. The *Msp*I digested the allele A amplicon, but not allele B. The AA genotype exhibited a 336 and 286 bp, AB genotype exhibited 622, 336 and 286 bp fragments and BB genotype exhibited a 622 bp fragment. This is in concurrence with the observations made earlier by Palmer *et al.,* (1998) and Chung *et al.,* (2001).

Among the 79 Bandur animals studied 43 were of AA, 31 were of AB and 5 were of BB genotypes with genotype frequencies of 0.54, 0.39 and 0.07 for AA, AB and BB, respectively. The gene frequencies were 0.74 and 0.26 for A and B respectively. The population was under Hardy- Weinberg equilibrium. The observed and expected heterozygosities were 0.39 and 0.38 respectively.

Similar result for calpastatin genotypes was obtained in Arabic sheep by Mohammadi *et al*. (2008), who however found a higher frequency of A allele (0.85) than

the B allele (0.15) with genotype frequencies of 70.27 for AA, 28.82 for AB and 0.9 for BB. Nassiry *et al.* (2007) reported the allelic frequencies of 88 per cent and 12 per cent for M and N respectively in Iranian Kurdi sheep with the genotype frequencies 76 and 24 per cent for MM and MN respectively. These workers also did not observe any Nn genotypes, as also observed in the present study. Allelic frequencies obtained in the present study are similar to the finding of Palmer *et al*. (1998) in Corriedale sheep.

In contrast to the present study, Elyasi *et al*. (2005) reported that allelic frequency of both M and N alleles were 0.50 for CAST locus in Ghezel X Arkharomerino sheep. They observed MN genotype in high value for Arkharomerino (47.62%) and Ghezel X Arkharomerino (46.67%). They also did not detect NN genotype. On the other hand Nassiry *et al.* (2006) reported six alleles of which three genotypes namely aa, ab and ac were observed with frequencies of 54.76, 32.14 and 13.09 per cent respectively in Iranian Kurdi sheep using PCR-SSCP. The bb, bc and cc genotypes were not observed. Allele frequencies for a, b and c were 0.78, 0.16 and 0.06 respectively.

In the present study, as well as in the earlier studies elsewhere, the frequency of NN genotypes was either zero or negligible. Significance of this outcome needs further study. It is possible that NN genotype may be associated with poor growth and hence might have been eliminated from the population by either natural selection or by selection against this genotype.

5.2.4.2 Restriction patterns with *Nco*I

The amplified DNA was also digested with *Nco*I restriction enzyme and the fragments were separated by agarose gel electrophoresis. The amplified 622bp fragment

was restricted into 374bp and 286bp fragments by *Nco*I enzyme. This allowed the identification of two alleles *viz*., A and B. which resulted in three genotypes AA, AB and BB. The AA genotype exhibited a 622 bp fragment, AB genotype exhibited 622, 374 and 248 bp fragments and BB genotype exhibited a 374 and 248 bp fragments.

The genotypic frequencies of Bandur sheep studied were 0.5, 0.395 and 0.105 for AA, AB and BB, respectively. The allelic frequencies of A and B were 0.697 and 0.303 respectively. The population studied was in Hardy-Weinberg equilibrium. The observed and expected heterozygosities were 0.395 and 0.422 respectively.

The *Nco*I allelic patterns found in the present study were in agreement with the findings of Palmer *et al.* (1998). They observed the allelic frequencies as 77% and 53% respectively for M and N alleles in Corriedale rams. Similar allelic patterns were also reported by Shahroudi *et al*. (2005), Mohammadi *et al.* (2008) and Nassiry *et al*. (2007) with *Msp*I restriction enzyme.

The population of Bandur sheep investigated in the present study was found to be under Hardy-Weinberg equilibrium when tested with two restriction endonuclease *viz.* *Msp*I and *Nco*I. Since most of the animals under study were being reared by farmers and since those farmers do not follow modern scientific methods of selection and breeding, it is possible that the animals are randomly bred. Hence there is equilibrium in the population.

The presence of M allele with a higher frequency in almost all the breeds may probably indicated its association to meat quality traits, which needs to be further investigated.

5.3 DNA sequence analysis of CAST gene

The amplified products of exon 1C/1D from domain 1 region including the intron of the CAST locus in Bandur sheep were gel eluted, cloned and sequenced at the DNA sequencing facility, Chromous Biotech Pvt. Ltd. Bangalore.

All the sequences shared high homology to the published ovine CAST sequences. There was query coverage to the extent of 95 per cent.

The RFLP and sequence data provided evidence that the Bandur sheep breed is polymorphic for calpastatin locus. This is the first such studiy in Bandur sheep. Further study is required to associate, if any, the meat quality traits of Bandur sheep to different genotypes, which may open future prospects of using these markers for selection programs.

Summary

6. SUMMARY

The present study was conducted with the objective of analyzing the genetic variation at the CAST region of Bandur breed of sheep by using PCR-RFLP technique. Genomic DNA was isolated from blood samples of 79 Bandur animals. DNA was extracted using high salt technique with slight modification and the quantity and quality of DNA was determined by spectrophotometer and agarose gel electrophoresis.

Two oligonucleotide primers CAST 1C and CAST 1D were used to amplify the exon 1C/1D from domain 1 region including the intron of the Ovine CAST gene. Amplification was carried out with initial denaturation of template DNA at 95^{o}C for five minutes followed by 33 cycles of denaturation of 94^{o}C for one minute, annealing at 60^{o}C for one minute and extension at 72^{o}C for two minutes. A final extension of 72 ^{o}C for eight minutes was included in the programme. Each 50 l PCR reaction consisted of 100 ng of genomic DNA, 20 p.mol of each primer, 400 M dNTPs, three units of *Taq* polymerase and 5 l of 10X assay buffer with 15 mM MgCl2.

The genetic diversity of ovine CAST locus was investigated by PCR amplification and restriction fragment analysis. A 622bp fragment of CAST gene was amplified by using specific primers. The size of the amplified product was same in all the animals studied indicating conservation of DNA at this locus.

Two restriction endonucleases *Msp*I and *Nco*I were used to detect the genetic variation of the experimental unit in the CAST gene. The digestion of PCR product was carried out at 37 ^{o}C with 15 units of restriction enzyme for fourteen hours of incubation.

The digested product was run on 2.4% agarose gel along with molecular weight markers and observed under transilluminator. The molecular weight of each digested fragment was calculated by analytical software using gel documentation unit.

CAST/*Msp*I polymorphism was observed as three patterns which allowed the identification of two alleles *viz.,* A (336 and 286 bp fragments) and B (undigested fragment of 622 bp). The gene frequencies for A and B alleles in the Bandur sheep were 0.74 and 0.26 respectively. The population studied was found to be in Hardy- Weinberg equilibrium with regard to CAST/*Msp*I polymorphism.

CAST/*Nco*I polymorphism also showed three genotypes with two alleles, *viz.*, A (undigested fragment of 622 bp) and B (374 and 248 bp fragments), the gene frequencies were 0.697 (A) and 0.303 (B). *Nco*I differentiated two alleles, A and B, similar to *Msp*I restriction enzyme, however the *Nco*I digested the allele B amplicon, but not allele A whereas *Msp*I had the reverse effect. The population studied was found to be in Hardy-Weinberg equilibrium with regard to CAST/*Nco*I polymorphism.

The sequence analysis indicated that there is high homology between the present result and the published ovine CAST sequences. There was query coverage to the extent of 95%.

Present results showed that PCR-RFLP is an appropriate tool for evaluating genetic variability. This study is the first of its kind carried out in native sheep breeds. Very little information is currently available to compare different Indian breeds.

The different regions of CAST gene could be further studied with larger number of animals, which are completely unrelated for establishment of any other markers. It is also worthwhile to use some more restriction endonucleases for such studies. Such extensive studies along with meat quality linkage studies may reveal breed specific marker, if any.

The present study revealed the genetic variability at CAST gene locus in Bandur sheep. Further study is suggested to understand the association of this gene with meat quality traits.

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Abstract

8. ABSTRACT

A study was conducted with the objective of analyzing the genetic variation at the CAST region of Bandur breed of sheep by using PCR-RFLP technique. Genomic DNA was isolated from blood samples of 79 Bandur animals. The genetic diversity of ovine CAST locus was investigated by PCR-RFLP. A 622 bp fragment of exon 1C/1D from domain 1 region including the intron was amplified. The size of the amplified product was same in all the animals studied indicating conservation of DNA at this locus. Two restriction endonucleases *Msp*I and *Nco*I were used to detect the genetic variation of the experimental unit in the CAST gene. CAST/*Msp*I polymorphism was observed as three patterns which allowed the identification of two alleles *viz.,* A and B. The allelic frequencies for A and B alleles were 0.74 and 0.26 respectively. The population studied was in Hardy- Weinberg equilibrium. CAST/*Nco*I polymorphism also showed three genotypes with two alleles, *viz.*, A and B, the gene frequencies were 0.697 and 0.303 respectively. The *Nco*I digested the allele B amplicon, but not allele A. The population studied was in Hardy- Weinberg equilibrium. The sequence analysis indicated that there is high homology between the present result and the published ovine CAST sequences. There was query coverage to the extent of 95%. The present study revealed the genetic variability at CAST gene locus in Bandur sheep. Further study is suggested to understand the association of this gene with meat quality traits.

Appendices

9. APPENDICES

ANNEXURE -1

Chemicals:

Chemical	Source
Absolute alcohol	Merck
Acetic acid	Qualigens
Agarose	Sigma
Ammonium chloride	Merck
Ammonium per Sulphate	Gibco BRL
Boric acid	Sigma
Bromophenol blue	Sigma
Chloroform	Sigma
dNTPs	Bangalore Genei
ddNTPs	USB
EDTA	Sigma
Ethidium bromide	Sigma
Isoamyl alcohol	Qualigens
Potassium chloride	SRL
Sodium chloride	Merck
SDS	SRL
Tris base	Sigma

Enzymes

Proteinase-K	Bangalore Genei
Taq DNA polymerase	Bangalore Genei
RE	Fermentas Life Sciences

Molecular size marker:

100 base pair DNA ladder	Bangalore Genei

ANNEXURE-2

Laboratory equipments

Equipments	Suppliers/Firm
Bench top centrifuge	Remi, India
Electric balance	Sartorius
Gel documentation system	Pharmacia Biotech
Gel drier	Bio-Rad, USA
Micro centrifuge	Beckman
Micropipettes	Nichipet
pH meter	Global, India
Sequencing Electrophoresis apparatus	Hoefer Scientific
Spectrophotometer	Shimadzu, Japan
Submarine electrophoresis unit	Bangalore Genei
Thermal cycler	Bio Rad
UV Transilluminator	Fotodyne
Vacuum pump	Minivac, Yamato
Water bath	Precision

ANNEXURE-3

A. Solutions or reagents for DNA isolation

EDTA (0.5M)

Disodium Ethylene Diamine Tetra Acetic acid (EDTA) : 18.61g

Triple glass distilled water to make up : 100 ml

pH adjusted to 8.0, autoclaved and stored at room temperature

Sodium Dodecyl Sulphate (SDS)

Sodium Dodecyl Sulphate : 10 g

Triple glass distilled water to make up to : 100 ml

5M NaCl

Sodium chloride : 29.27g

Triple glass distilled water to make up to : 100 ml

Filtered and stored at room temperature

Tris-EDTA buffer (TE)

Tris (hydroxyl methyl) aminomethane (10mM) pH 7.6 : 0.12114g

EDTA (0.1mM) : 0.03722g

Triple glass distilled water to make up to : 100 ml

pH adjusted to : 8.0

RBC lysing buffer

Ammonium chloride (1.7M) : 9.1g

Triple glass distilled water to make up to : 1000 ml

pH adjusted to 8.0, kept at 4^0C

B. <u>Solutions or Reagents for checking of DNA samples</u>

Tris acetate EDTA (TAE) buffer (50X)

Triple (hydroxyl methyl) aminomethane (10mM)pH7.6 : 48.4 g

Glacial acetic acid : 11.42 ml

0.5 M EDTA (pH 8.0) : 20 ml

Triple glass distilled water to make upto : 1000 ml

Agarose (0.8%)

Agarose : 0.64 g

TAE 50X : 1.6 ml

Triple glass distilled water to make up to : 80 ml

Agarose (1.5 %)

Agarose : 1.2 g

TAE (1X) to make up to : 80 ml

Agarose (2.4 %)

Agarose : 1.92 g

TAE (1X) to make up to : 80 ml

ANNEXURE-4

Sequence of ovine calpastatin gene compared with NCBI Genebank data

GENE ID: 443364 CAST | calpastatin [*Ovis aries*]

```
Query  10   AGCCTCTTCTGTAGATTTCTG-AAAATGAAATACAAAAGAAGGTTTGACTGGCAGATGAA  68
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  551  AGCCTCTTCTGTAGATTTCTGAAAAATGAAATACAAAAGAAGGTTTGACTGGCAGATGAA  492

Query  69   GGAAATGTATTAACCAGACGGTAAGAACCAGAAGTAAAAAGCCTTGGCATCTTGGGGCTT  128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  491  GGAAATGTATTAACCAGACGGTAAGAACCAGAAGTAAAAAGCCTTGGCATCTTGGGGCTT  432

Query  129  CCCTGGTGGCCAGTGGTTTAAGAGGCCGCCTTGCAACGCAGGGGACACGGGTTCGCTCCC  188
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  431  CCCTGGTGGCCAGTGGTT-AAGAGGCCGCCTTGCAACGCAGGGGACACGGGTTCGCTCCC  373

Query  189  TGGCCAGGGAAGATCCCACATGCCGTGGAGTAAGCCCTGTGCGCCACGACCACTGAGCCT  248
            |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  372  TGGCCAGGGAAGATCCCACATGCCGTGGAGTAAGCC-TGTGCGCCACGACCACTGAGCCT  314

Query  249  GTGCTCTAGAGCTCTCAAGCCACAACAACTGAAGCCTGTGCACCCAGAGCCCCGGGCTCT  308
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  313  GTGCTCTAGAGCTCTCAAGCCACAACAACTGAAGCCTGTGCACCCAGAGCCCCGG-CTCT  255

Query  309  GCAACAAGAGAAGCCACCTGCAGAAGGCTGCACATCGCAACTAGAGAATAGCCCTCGCTT  368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  254  GCAACAAGAGAAGCCACCTGCAGAAGGCTGCACATCGCAACTAGAGAATAGCCCTCGCTT  195

Query  369  GCCCCAACTAGAGGAAAGCCTGAACACAGCAATGAAGACCCAGCGAAGTCAAAAATAAAG  428
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  194  GCCCCAACTAGAG-AAAGCCTGAACACAGCAATGAAGACCCAGCGAAGTCAAAAATAAAG  136

Query  429  TTTaaaaaaaaGTTGTTGTCTTAAAGTGGGAGGTAGTTTCTAACAAGTTTCCCTGAGTGC  488
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  135  TTTAAAAAAAAAGTTGTTGTCTTAAAGTGGGAGGTAGTTTCTAACAAGTTTCCCTGAGTGC  76

Query  489  ATTAAAAACAATACCTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGGGGAACTGCAGGTG  548
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  75   ATTAAAAACAATACCTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGGGGAACTGCAGGTG  16

Query  549  AAGTCTGATGACAAG  563
            |||||||||||||||
Sbjct  15   AAGTCTGATGACAAG  1
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GENE ID: 443364 CAST | calpastatin [*Ovis aries*]

```
Query  10   AGCCTCTTCTGTAGATTTCTG-AAAATGAAATACAAAAGAAGGTTTGACTGGCAGATGAA  68
           |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  551  AGCCTCTTCTGTAGATTTCTGAAAAATGAAATACAAAAGAAGGTTTGACTGGCAGATGAA  492

Query  69   GGAAATGTATTAACCAGACGGTAAGAACCAGAAGTAAAAAGCCTTGGCATCTTGGGGCTT  128
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  491  GGAAATGTATTAACCAGACGGTAAGAACCAGAAGTAAAAAGCCTTGGCATCTTGGGGCTT  432

Query  129  CCCTGGTGGCCAGTGGTTTAAGAGGCCGCCTTGCAACGCAGGGGACACGGGTTCGCTCCC  188
           |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  431  CCCTGGTGGCCAGTGGTT-AAGAGGCCGCCTTGCAACGCAGGGGACACGGGTTCGCTCCC  373

Query  189  TGGCCAGGGAAGATCCCACATGCCGTGGAGTAAGCCCTGTGCGCCACGACCACTGAGCCT  248
           |||||||||||||||||||||||||||||||||| |||| ||||||||||||||||||||
Sbjct  372  TGGCCAGGGAAGATCCCACATGCCGTGGAGTAAGCC-TGTGAGCCACGACCACTGAGCCT  314

Query  249  GTGCTCTAGAGCTCTCAAGCCACAACAACTGAAGCCTGTGCACCCAGAGCCCCGGGCTCT  308
           |||||||||||||||||| |||||||||||||||||||||||||||||||| ||||
Sbjct  313  GTGCTCTAGAGCTCTCAAGTCACAACAACTGAAGCCTGTGCACCCAGAGCCCCGG-CTCT  255

Query  309  GCAACAAGAGAAGCCACCTGCAGAAGGCTGCACATCGCAACTAGAGAATAGCCCTCGCTT  368
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  254  GCAACAAGAGAAGCCACCTGCAGAAGGCTGCACATCGCAACTAGAGAATAGCCCTCGCTT  195

Query  369  GCCCCAACTAGAGGAAAGCCTGAACACAGCAATGAAGACCCAGCGAAGTCAAAAATAAAG  428
           |||||||||||||| |||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  194  GCCCCAACTAGAG-AAAGCCCGAACACAGCAATGAAGACCCAGCGAAGTCAAAAATAAAG  136

Query  429  TTTaaaaaaaaGTTGTTGTCTTAAAGTGGGAGGTAGTTTCTAACAAGTTTCCCTGAGTGC  488
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  135  TTTAAAAAAAAAGTTGTTGTCTTAAAGTGGGAGGTAGTTTCTAACAAGTTTCCCTGAGTGC  76

Query  489  ATTAAAAACAATACCTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGGGGAACTGCAGGTG  548
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  75   ATTAAAAACAATACCTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGGGGAACTGCAGGTG  16

Query  549  AAGTCTGATGACAAG  563
           |||||||||||||||
Sbjct  15   AAGTCTGATGACAAG  1
```

GENE ID: 281039 CAST | calpastatin [*Bos taurus*]

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Query  10   AGCCTCTTCTGTAGATTTCTG-AAAATGAAATACAAAAGAAGGTTTGACTGGCAGATGAA  68
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Sbjct  580  AGCCTCTTCTGTAGATTTCTGAAAAATAAAATACAGAAGAAGGTTTGACTGGCAGATGAA  521

Query  69   GGAAATGTATTAACCAGACGGTAAGAACCAGAAGTAAAAAGCCTTGGCATCTTGGGGCTT  128
            |||||  ||| ||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  520  GGAAACATATGAACCAAACGGTAAGAACCAGAAGTAAAAAGCCTTGGCATCTTGGGGCTT  461

Query  129  CCCTGGTGGCCAGTGGTTTAAGAGGCCGCCTTGCAACGCAGGGGACACGGGTTCGCTCCC  188
            |||||||||||||| |||| |||||||||||||||| |||||||||| ||||||||||
Sbjct  460  CCCTGGTGGCCAGCGGTT-AAGAGGCCGCCTTGCAATGCAGGGGACACAGGTTCGCTCCC  402

Query  189  TGGCCAGGGAAGATCCCACATGCCGTGGAGTAA----GCCCTGTGCGCCACGACCACTGA  244
            ||||||||||||||||||||||  ||||||||    |||| |||||||| |||||||||
Sbjct  401  TGGCCAGGGAAGATCCCACATGCATTGGAGTAACCATGCCC-GTGCGCCAAGACCACTGA  343

Query  245  GCCTGTGCTCTAGAGCTCTCAAGCCACAACAACTGAAGCCTGTGCACCCAGAGCCCCGGG  304
            |||||||||||||||| ||| |||||||||||||||||||||||| ||||||||||
Sbjct  342  GCCTGTGCTCTAGAGCCCTCGAGCCACAACAACTGAAGCCTGTGCACCTAGAGCCCCGGC  283

Query  305  CTCTGCAACAAGAGAAGCCACCTGCAGAAGGCTGCACATCGCAACTAGAGAATAGCCCTC  364
            | | ||||||||||||||| |||||||||| || |||||||||||||||||| || ||||
Sbjct  282  C-CAGCAACAAGAGAAGCCCCCTGCAGAAGCCTACACATCGCAACTAGAGAACAGGCCTC  224

Query  365  GCTTGCCCCAACTAGAGGAAAGCCTGAACACAGCAATGAAGACCCAGCGAAGTCAAAAAT  424
            |||||||||||||||| |||||| |||||||||||||||||| ||| ||||| ||||||
Sbjct  223  GCTTGCCCCAACTAGAG-AAAGCCCGAACACAGCAATGAAGACGCAGTGAAGTGAAAAAT  165

Query  425  AAAGTTTaaaaaaaa-GTTGTTGTCTTAAAGTGGGAGGTAGTTTCTAACAAGTTTCCCTG  483
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Sbjct  164  GAAGTTTAAAAAAAAAGTTGTTGTCTTAAAGTGGGAGGTAGTTTCTAACAAGCTTCCCTA  105

Query  484  AGTGCATTAAAAACAATACCTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGGGGAACTGC  543
            || |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  104  AGGGCATTAAAAACCATACCTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGGGGAACTGC  45

Query  544  AGGTGAAGTCTGATGACAAGGCATCGATGGCG  575
            |||||||||| ||||||||||||||||||||
Sbjct  44   AGGTGAAGTCGGATGACAAGGCATCGATGGCG  13
```

Capra hircus calpastatin transcript variant 2 mRNA

```
Query  503  CTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGGGGAACTGCAGGTGAAGTCTGATGACAA  562
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  863  CTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGGGGAACTGCAGGTGAAGTCTGATGACAA  804

Query  563  GGCATCGATGGCGGTCATT  581
            ||||||||||||| |||||
Sbjct  803  GGCATCGATGGCG-TCATT  786
```

Bos grunniens calpastatin (CAST) mRNA

```
Query  503   CTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGGGGAACTGCAGGTGAAGTCTGATGACAA 562
             |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  1002  CTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGGGGAACTGCAGGTGAAGTCGGATGACAA 943

Query  563   GGCATCGATGGCGGTCATT  581
             ||||||||||||  |||||
Sbjct  942   GGCATCGATGGCA-TCATT  925
```

Bos taurus x Bos indicus calpastatin mRNA

```
Query  503  CTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGGGGAACTGCAGGTGAAGTCTGATGACAA  562
            |||||||||||||||||||||||||||||| ||||||||| |||||||| ||||||||
Sbjct  312  CTCTTTCTCAGTTTTCTTTGCATCAGCTGTAGCGGAACTGCACGTGAAGTCGGATGACAA  253

Query  563  GGCATCGATGGCGGTCATT  581
            ||||||||||||  |||||
Sbjct  252  GGCATCGATGGCA-TCATT  235
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