BETA LACTOGLOBULIN POLYMORPHISM IN GOATS OF KERALA

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DECLARATION

I hereby declare that this thesis, entitled **"Beta lactoglobulin Polymorphism in Goats of Kerala"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **"Betalactoglobulin Polymorphism in Goats of Kerala"** is a record of research work done independently by **Dr. Sudina. K.**, under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Introduction

1. INTRODUCTION

India's vast genetic resources in goats (*Capra hircus*) are reflected by the availability of 20 breeds of goat, according to National Bureau of Animal Genetic Resources (NBAGR). Goats are important species of livestock for India with a total population of 120.0 million (Livestock census, 2003).

The goats contribute greatly to the agrarian economy, especially in areas where crop and dairy farming are not economical, and play an important role in the livelihood of a large proportion of small, landless and marginal farmers.

Goat production in Kerala is centered mainly on the native breed Malabari or Tellicherry of northern Kerala, a dual purpose goat breed with high prolificacy. This breed is probably originated centuries ago by mixing of native feral goats with Arab, Surti, and Mesopotamian goats along with native goats of Western Coast (Kaura, 1952). The main physical characteristics of this breed have been well studied and documented. The milk production is 0.5-1.0 kg per day but animals yielding 1.0-1.5 kg are not rare.

Apart from Malabari, Kerala is having Attappady black goats which are found exclusively in Attappady area, an isolated hilly region in Palghat District of Kerala and are popular as the goats of tribes in Attappady. These goats have unique characteristics and reared entirely on grazing for meat purpose. The total population of Attappady black in its breeding tract was estimated to be 9350 (Stephen *et al.*, 2005). This breed has all potential to be developed into an excellent meat breed.

Variations exist between and within goat populations with regard to economically important traits. These variations have to be studied in detail for

further improvement of the breed and for which polymorphism studies were attempted. Protein polymorphisms were once used as molecular markers for studying variation. However the level of polymorphism observed in proteins is often low, which has reduced the applicability of protein typing in diversity studies. With the development of Polymerase Chain Reaction (PCR) and sequencing technologies, DNA-based polymorphisms are now the markers of choice for the molecular-based surveys of genetic variation. Broadly two experimental strategies have been developed for this purpose: linkage studies and candidate gene approach.

Linkage studies rely on the genetic map knowledge and search for quantitative trait loci (QTL) by comparing segregation patterns of genetic markers and the trait being analysed.

The second approach focuses on the study of the genetic polymorphism of candidate genes by marker studies. Markers have been classified into two categories; hybridization based markers and PCR based markers. Hybridization based markers include Dynamic Allele Specific Hybridization, Molecular beacons, SNP microarrays etc. The PCR based markers include PCR-RFLP, PCR-SSCP, AS-PCR, ARMS-PCR etc. The most commonly used strategy for detecting point mutations is to amplify fragment of interest by PCR, scan the products for the presence of mutations by a rapid procedure and then sequence the PCR products that were positive by the scanning techniques like RFLP or SSCP.

Milk proteins can be considered as important markers for milk production traits. Milk proteins of mammals can be divided into two classes: the caseins and whey proteins. The caseins ($\alpha s1$, $\alpha s2$, β and κ) comprise the major protein component of ruminant milk. The whey proteins of milk correspond to the protein fraction that remains in solution after precipitation of casein micelles and fat

globules and are constituted principally by beta lactoglobulin (β-LG) and alpha lactalbumin (α-LA). Beta lactoglobulin is the major whey protein in ruminant milk and alpha lactalbumin is part of enzyme system involved in lactose synthesis.

The detection of genetic polymorphism of milk proteins offered new explanations for some of the variations in dairy traits. The genetic variants of major milk proteins have been demonstrated to influence the milk yield, fat percentage and solids not fat (SNF). Nowadays pricing of milk depends on fat and SNF. The association between β-LG polymorphism and milk production or composition traits, if exists can be useful for selection of goats based on molecular markers. So the present study was designed with following objectives:

1. Analyse the polymorphism of the β-LG gene in Malabari, Attappady black and Malabari crossbred goats of Kerala by PCR-RFLP
2. Determination of allelic frequencies of β-LG gene and
3. Analyse the relationship between various genotypes of β-LG with peak milk yield, fat percentage, total solids and SNF.
4. In addition to the above mentioned gene, a related gene alpha lactalbumin (α-LA) also will be studied for its association with milk production and composition traits.

Review of Literature

2. REVIEW OF LITERATURE

2.1 BETA LACTOGLOBULIN

Beta lactoglobulin (β-LG) is the major whey protein secreted in the milk of ruminants like cow and sheep as well as monogastrics like pig, horse, dog, cat, kangaroo and dolphin (Perez and Calvo, 1995). It is absent in the milk of human and rodents. The β-LG constitutes about 14 per cent of the total milk protein and 53 per cent of total whey protein in bovines (Kuzdzal-Savoie *et al.*, 1966).

In ruminant milk, β-LG is found as a dimer with a molecular weight of 36.4 kDa corresponding to 162 amino acids. In most other species in which it has been found, β-LG appeared to be monomeric. This protein belongs to lipocalin family whose sequence is closest to the human placental protein 14 or pregnancy-associated endometrial glycoprotein (Hambling *et al.*, 1992).

2.1.1 Structure of Beta Lactoglobulin

Mc Kenzie (1977) found that β-LG 'A' could form octamers under conditions of low temperature, higher concentrations and at pH close to the iso-ionic point.

The primary structure of β-LG revealed internal homology between species. A comparison of the primary structure of ovine β-LG with those of bovine and caprine revealed variations at six and one positions, respectively. (Kolde and Brunitzer, 1983).

According to Monaco *et al.* (1987), the core of β-LG molecule consisted of a very short alpha-helix segment and eight strands of antiparallel beta sheet, which wraped around to form an antiparallel beta-barrel.

The folding pattern of β-LG molecule was found to be remarkably similar to that of serum retinol-binding protein (Cowan *et al.*, 1990), later termed as the lipocalin fold (Flower *et al.*, 1993).

Bewley *et al.* (1997) suggested that the core of the β-LG molecule was highly conserved among the genetic variants; 75 per cent of residues, covering all of the β strands, the three turn α helix and some loops.

Bonomi *et al.* (2003) studied the variation in immunoreactivity of bovine β-LG upon combined physical and proteolytic treatment. The residual immunochemical reactivity of the products of combined pressure-enzyme treatment was assessed by ELISA and Western Blotting. The reactivity was found to be significantly reduced on treatment.

Creamer *et al.* (2004) reported that, the structure, stability and hydrolysis characteristics of β-LG 'A' were different from those of either β-LG 'B' or β-LG 'C'. They also opined that the rate of hydrolysis was in the order β-LG 'A'>β-LG 'B' > β-LG 'C' under most circumstances.

Chakraborty *et al.* (2009) investigated the effect of chemical modification by acetylation and succinylation on secondary and tertiary structures of bovine β-LG where both derivatives showed higher electrophoretic mobility compared to native β-LG.

2.1.2 Biochemical Role of Beta Lactoglobulin

Goddovac-Zimmerman (1988) and Cowan *et al.* (1993) reported that β-LG belonged to the large and diverse lipocalin family, found in higher organisms as well as prokaryotes. They also opined that lipocalins were a group of extracellular transport proteins characterised by a range of different molecular-recognition properties such as ability to bind small hydrophobic molecules, as well as to specific cell-surface receptors and formation of macromolecular complexes.

According to Said *et al.* (1989), β-LG enhanced retinol uptake in the jejunum and eleum of suckling rats.

The amino acid composition of β-LG suggested high nutritional value of β-LG but the molecular properties, particularly the acid stability, lead to the supposition that some additional specific function could exist (Hambling *et al.*, 1992).

Perez *et al.* (1992) suggested the biological role of ruminant β-LG being more closely related to milk fatty acid metabolism than to retinol transport. The bovine β-LG could participate in the digestion of milk lipids during the neonatal period, by enhancing the activity of pregastric lipase, through removal of the fatty acids that inhibited this enzyme.

The existence of low homology between ruminant β-LG with those of horse and pig could lead to the existence of functional differences among species (Perez and Calvo, 1995).

Neurath *et al.* (1996) reported that β-LG acquired anti viral properties upon acylation.

Gutierrez-adán *et al.* (1999) produced transgenic mice which expressed bovine β-LG gene. They described position-dependent, copy number-related and high expression of bovine protein β-LG in the milk of six lines of transgenic mice.

The anti viral effect of β-LG increased with the increase in induced negative charges, particularly against enveloped viruses (Swart *et al.*, 1999).

Claeys *et al.* (2002) studied the kinetics of β-LG denaturation in milk with different fat content and reported that β-LG denaturation kinetics differed significantly in milk with different fat content.

The concentration of β-LG at first post partum milking in goat was reported to be 30.7±10.4 mg/ml with a range of 9.3-49.8 mg/ml which reduced abruptly in the subsequent milking (Levieux *et al.*, 2002).

High pressure induced heat denaturation of α-LA and β-LG in dairy systems showed higher bioresistance of β-LG compared to α-LA (Huppertz *et al.*, 2004).

Chen *et al.* (2005) reported 90 per cent loss of β-LG in processed and dry milk during its processing. The loss was presumably associated with the heating procedure. Essentially, β-LG was the only major fraction converted to aggregates in milk when heated at 95°C for 30 min.

Liu *et al.* (2007) suggested the antioxidant property of bovine β-LG, where the activity was higher than that of vitamin E and probucol. The conversion of monomer to dimer was responsible, in part, for the action in protecting low density lipoproteins against copper induced oxidation.

The bovine β-LG acquired antibacterial properties upon amidation. The positively charged amidated β-LG, appeared to have stronger affinity to the negatively charged microbial cell membrane (Pan *et al.*, 2007).

According to Almaas (2008), caprine whey obtained after reaction with human duodenal juice was found to be effective against cells of Listeria monocytogenes. It was also reported that digestion of caprine whey by human duodenal juice left most of β-LG intact.

2.1.3 The β-LG Protein Polymorphism

Erhardt (1989) described the phenotypes of β-LG (*viz.*, AA, BB, AB, CC, AC, BC) by Poly Acrylamide Gel Electrophoresis (PAGE) and Iso Electric Focusing (IEF) in various Hungarian sheep breeds. The gene frequencies varied in different breeds studied.

Godovac-Zimmerman *et al.* (1996) identified a total of seven variants of bovine whey protein β-LG –A, B, C, D, I, J and W.

According to Bonvillani *et al.* (1998), the frequency of bovine β-LG 'A' allele was 0.43 and that of 'B' was 0.57 in Argentinian Holstein. The allelic and genotypic frequencies indicated great homogeneity among populations.

Garg *et al.* (2009) analysed the genetic polymorphism of milk proteins in Barbari goat by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE). The electrophoretic pattern of β-LG showed the presence of two

alleles 'A' and 'B' and the corresponding frequencies were 0.910 and 0.090, respectively.

2.1.4 The β-LG Protein Variants-Associations

Aleandri *et al.* (1990) reported that cheese yield on fixed amount of milk and fat percentages were significantly related to β-LG phenotype with highest estimate obtained for β-LG BB.

Lunden *et al.* (1997) analysed the association of protein variants of β-LG with the composition of milk in Swedish Holstein cows. The results suggested a positive additive effect of the β-LG 'B' allele on casein content and the ratio of casein to total protein.

Ng-Kwai-Hang (1997) reviewed the relationship of β-LG polymorphism with milk composition and milk production performances in bovines and opined that 'A' variant of β-LG was associated with higher milk yield, more total protein but lower concentrations of casein and fat.

Winkleman (1997) associated the bovine β-LG protein variants with 305-day milk yield, fat and protein where cows with AA recorded higher yields of milk, fat and protein compared to CC genotypes.

Ng-Kwai Hang (1998) reported that, replacement of β-LG 'B' allele by 'A' allele was followed by an increase of 0.05, 0.07 and 0.08 percentages of protein for the three lactation periods. The milk yield and fat content were not influenced by β-LG phenotypes.

Amigo *et al.* (2000) reviewed the three protein variants (A, B, and C) of ovine β-LG and their relationship with lactation yield, milk composition, rennetability, cheese yield and heat denaturation. The 'A' variant appeared to be related to higher total solids ($p<0.05$), fat, protein and casein content ($p<0.05$) with a relatively higher percentage of κ-casein and β-casein while the β-LG 'B' allele was associated with higher milk yield.

Jeichitra *et al.* (2003) reported higher allelic frequency of β-LG 'B' than β-LG 'A' in Kangayam cattle.

Tsiaras *et al.* (2005) reported that β-LG polymorphism was significantly associated with milk yield (AB>AA), fat yield (BB and AB>AA), fat content (BB>AA and AB), lactose yield (AB>AA) and protein yield (AB>AA) in Holstein cows.

According to Mele *et al.* (2007), the frequency of β-LG AB was higher than AA or BB and the polymorphism was significantly associated with milk fatty acid composition and not with milk composition.

Meza-Nieto *et al.* (2007) reported that β-LG 'B' was found to be associated with casein prior to renneting whereas both β-LG 'A' and β-LG 'B', either alone or mixed, had a profound influence on the mechanical strength and coagulation kinetics of the rennet-induced casein gels.

Bobe *et al.* (2009) opined that on 'low-fat' control diet, cows with β-LG 'B' allele had greater milk yield than cows with 'A' allele, whereas no differences by β-LG phenotype were observed in cows on tallow supplemented diet.

According to Heck *et al.* (2009), β-LG genotype was associated with the relative concentrations of β-LG (A>B) and of α-LA, αS1-CN, αS2-CN, β-CN, and κ-CN (B >A) but not with any milk production trait.

2.1.5 Beta Lactoglobulin Gene

The β-LG encoding gene consisting of 7379 bp of genomic DNA was cloned and sequenced in sheep (Harris *et al.*, 1988).

Alexander *et al.* (1989) sequenced the full length cDNA sequence of bovine β-LG 'A' variant. It was 91% similar at nucleotide level to ovine β-LG cDNA. The β-LG leader peptides of these two species differed in length due to the deletion of two codons in the bovine β-LG cDNA following position 69.

Porcine β-LG cDNA of 768 bp length, encoding a pre-protein of 178 amino acids was isolated and sequenced by Alexander and Beattie (1992). Porcine cDNA exhibited sequence similarity of 79.6 per cent and 78 per cent to bovine and ovine β-LG cDNA, respectively. Porcine β-LG was 66.9 per cent similar to both bovine and ovine β-LG and 65 per cent similar to caprine β-LG at protein level.

The β-LG gene had been mapped to short arm of chromosome 3 in sheep and chromosome 11 in goats and cows by a combination of non-isotopic *in situ* hybridization and simultaneous fluorescent R-banding. The results were in agreement with the high degree of banding pattern similarity, previously reported among cattle, sheep, and goat karyotypes (Hayes and Petit, 1993).

Folch *et al.* (1994) sequenced the 4698 bp transcription unit of seven exons encoding caprine β-LG and, 2148 bp and 1242 bp of the 5' and 3' flanking regions,

respectively. The overall structure of the caprine β-LG gene was similar to that of its ovine and bovine counterparts; sizes of introns and exons were well conserved.

Ballester *et al.* (2005a) performed radiation hybrid mapping using a pig specific primer in intron 1 of porcine β-LG gene. The porcine β-LG was found to be localized on the telomeric region of chromosome 1.

2.1.5.1 The Beta Lactoglobulin Gene Polymorphism

The bovine β-LG 'A' and 'B' alleles were differentiated on the basis of the presence or absence of a *Hae* III recognition site in exon IV (Medrano and Aguilar, 1990).

Jadoth *et al.* (1992) genotyped bovine β-LG into 'A' and 'B' variants by molecular hybridization using probes containing the site of mutation. The results perfectly matched with the data obtained by electrophoretic mobility of milk proteins.

Tee *et al.* (1992) detected single base substitutions (A to G transition) in exon III known to differentiate alleles 'A' and 'B' of the bovine β-LG gene. He could amplify the exon III of the β-LG gene and detect the variation by temperature gradient gel electrophoresis (TGGE). On silver staining of the amplified product, separate bands could be obtained.

Wilkins and Kuys (1992) genotyped bovine β-LG into both 'A' and 'B' variants by PCR based RFLP using *Hph* I enzyme. Non denaturing PAGE analysis of milk protein gave genotypes identical to those deducted from DNA analysis. The frequencies of 'A' and 'B' alleles were found to be 0.45 and 0.55, respectively.

A new allele β-LG 'I' in the milk of Polish red cattle was identified by amplification created restriction site using *Sma* I endonuclease (Prinzenberg and Erhardt, 1997).

The bovine β-LG 'D' variant differed from 'B' by a single mutation (G to C) change in codon GAG. This mutation created a *Pvu* II recognition site in the β-LG 'D' allele. Polymorphism at this site was screened by PCR-RFLP method (Braunschweig *et al.*, 1999).

Folch *et al.* (1999) studied the differential expression of β-LG 'A' and 'B' alleles in mammary epithelial cell line. The relative expression levels were 57 per cent for β-LG 'A' and 43 per cent for β-LG 'B' promoter. An allele specific mutation had been reported to have a differential binding affinity to the activator protein-2 between the β-LG 'A' and 'B' promoters.

Lion *et al.* (1999) found the frequencies of β-LG variants in Nordic cattle breeds and opined that 'B' allele was predominant in most Nordic cattle breeds. Exceptions were the Danish Black Pied and Western Red Polled cattle with higher frequencies of β-LG 'A'.

Vohra *et al.* (2006) investigated the β-LG genetic variants and their association with milk composition traits in riverine buffalo by single strand conformation polymorphism. Polymorphism was detected in two fragments of the gene on exon I, IV and intron IV, where frequency distributions were different in different breeds of buffaloes.

Braunschweig (2007) studied genetic polymorphism in the 5′-flanking region of the β-LG gene. Comparison of DNA sequences showed that the investigated 5′-flanking region was highly conserved between ruminants, and the duplication (CTCTCGC from −1885 to −1879) and the substitution (A to G at +1888) were found only in the β-LG A and D alleles in cattle. The cytosine at position −1957 and the thymines at positions −2008 and −2049 were found only in β-LG 'B' alleles of cattle.

2.1.5.2 The β-LG Gene Polymorphism in Goat

Pena *et al.* (2000) detected two new variants in goat β-LG gene at cDNA level and confirmed at genomic level. The two poymorphisms were located on exon 7 of the gene. One of the polymorphic sites was produced by a single nucleotide substitution in position +4601, allowing PCR-RFLP procedure to be developed. The other was a 10 bp long insertion at position +4641, detected by capillary electrophoresis of the PCR product amplified with fluorescent primer. The distribution and association of these polymophisms were studied in Spanish and French goat breeds.

Yahyaoui *et al.* (2000) detected polymorphism (C to T) at -60 of the proximal promoter region of the β-LG gene using base excision sequence scanning method in Spanish and French goat breeds. The same polymorphism was detected in a large number of goats by PCR-RFLP procedure using *Sma* I endonuclease.

Graziano *et al.* (2003) studied the mutations affecting the transcription level of the gene in β-LG promoter region by sequencing the PCR products. A PCR-RFLP procedure using *Bfa* I or *Sma* I endonuclease was applied to detect a transition of T to C at -341 of β-LG promoter region in various Italian goat breeds.

Veress *et al.* (2004) detected a single nucleotide polymorphism at -60 position (C to T) in Hungarian milk goat by PCR-RFLP and AS-PCR method and opined that, -60T allele was found to be lower in Hungarian milk goat than in Saanen breed.

Ballester *et al.* (2005b) sequenced the proximal promoter and first six exons containing the entire coding region for the β-LG gene in 11 goat breeds from Spain, France, Italy, Switzerland, Senegal and Asia to identify genetic variants. Fifteen polymorphisms were detected, nine in the promoter region and six in the exons.

Kumar *et al.* (2006) analysed the polymorphism at +4601 position, in exon 7 of the β-LG gene using *Sac* II endonuclease by PCR-RFLP and revealed two alleles namely S_1 and S_2 and three genotypes, S_1S_1, S_1S_2 and S_2S_2 in various Indian goat breeds. The frequencies of S_1S_1, S_1S_2 and S_2S_2 genotypes were found to be 0.0 to 0.23, 0.0 to 0.94 and 0.0 to 1.0, respectively.

Elmaci *et al.* (2009) studied the polymorphism in the exon 7 to the 3' flanking region of the β-LG gene in Turkish hair goat populations using PCR-RFLP. Digestion of amplified product with *Sac* II endonuclease revealed two alleles namely S_1 and S_2. The S_2S_2 was found to be lower than the other genotypes in the studied population.

2.1.5.3 The β-LG Gene Variants-Associations

Genetic variants of β-LG had been shown to influence milk curd formation and thereby cheese-making properties (Jacob and Puhan, 1992).

Sabour *et al.* (1996) studied the associations of β-LG genetic variants of bulls with daughters yield deviations for milk, fat and protein using mixed model analysis. Results suggested that, increasing the frequency of the β-LG 'A' variant in young bulls improved the protein yield.

The genotype β-LG BB produced milk containing more casein in cows, which was more suitable for milk processing (Hill, 1997).

Prosser *et al.* (1997) reported that the ratio of β-LG to α-LA mRNA in mammary tissue from β-LG AA cows was 1.9 fold greater than β-LG BB cows, and reflected 1.6 fold greater ratio for proteins in milk.

According to Ikonen *et al.* (1999), the 'B' variant of β-LG had significant favourable effect on milk coagulation properties.

Robitaille *et al.* (2002) verified the possible interaction between the effects of β-LG gene polymorphism and stage of lactation on expression of 'A' and 'B' variants in milk samples collected, at different times during lactation, from cows of β-LG AB. The β-LG AA allele was significantly associated with an increased proportion of β-LG in the whey protein fraction. There was no interaction between genotype and stage of lactation for the major whey proteins.

Kuss *et al.* (2003) studied on a polymorphic position (R10) in an Activator-Protein-2 (AP-2) binding site of the bovine β-LG gene promoter region and quantitative traits of individual milk proteins. The milk samples were analyzed with alkaline Urea-PAGE in combination with densitometry for quantification of individual milk proteins and a positive association ($P<0.001$) with milk yield had been observed.

According to Tsiarus *et al.* (2005), β-LG loci had significant effect on lactation yield and milk composition (*viz.*, lactose and fat yield as well as fat, protein and lactose content) and reproductive performance (*viz.*, gestation length, calving interval, age at first, second calving and number of services per conception). The lactation yield, fat content as well as fat and lactose yield was higher for β-LG AB, BB, BB and AB, respectively. The β-LG locus had no significant effect on protein content.

Luhar *et al.* (2006) studied the association of β-LG genetic variants with mastitis resistance in dairy cows. The RFLP pattern of β-LG genotypes in this study indicated the presence of 'B' (BB, AB) allele in more than 95 per cent cows suffering from mastitis suggesting the association of 'B' allele with the occurrence of mastitis.

Hallen *et al.* (2007) reported that the frequency of β-LG 'B' variant was found to be higher compared that of 'A' where the genetic variants of β-LG had no effect on the milk constituents in Swedish cattle.

Zhang *et al.* (2007) studied the association of polymorphism in β-LG and β-LG 5' flanking region with milk production traits and mastitis related traits in Chinese Holstein. The β-LG genotypes had a significant effect on protein percentage and the ratio of fat percentage to protein percentage. Polymorphism in β-LG flanking region was associated with 305 day standard milk yield, protein percentage, fat percentage, pre-somatic cell count and somatic cell count.

Dario *et al.* (2008) analysed the distribution of β-LG variants in Leccese dairy ewes and its effect on milk parameters like milk yield, fat, protein, lactose, total

nitrogen, casein content, casein number, whey protein and ash. Milk composition did not differ considerably among the β-LG genotypes, except for fat and whey protein content.

Szulc *et al.* (2008) investigated the effect of protected methionine and fish meal on milk yield, composition and physical properties as well as the proportion of individual casein fractions, on cows with different milk β-LG genotypes (AA, AB and BB). The addition of protected methionine to the diet of cows with the BB β-LG genotype resulted in significant increase in the content of crude protein and production of β-casein.

2.2 ALPHA LACTALBUMIN

Ebner and Brodbeck (1970) discovered that α-LA was one of the two proteins required for the lactose synthesis in mammary gland by interacting with enzyme UDP-galactosyl-transferase, giving rise to heterodimer enzyme lactose synthase.

Shewale *et al.* (1983) determined the complete amino acid sequence of α-LA from a marsupial, *Macropus rufogriseus* and also reported changes in sequence at two regions of bovine and goat α-LA.

The structure of gene encoding α-LA had been reported in rat (Qasba and Safaya, 1984).

Vilotte *et al.* (1988) sequenced the gene encoding α-LA in bovine.

The gene encoding α-LA had been sequenced in goat (Vilotte *et al.*, 1991).

Hambling *et al.* (1992) suggested that α-LA is a calcium metalloprotein and one of the major serum-proteins in ruminant milk.

The goat α-LA transcription unit, located on chromosome 5, organized in 4 exons varying in length from 75 nucleotides (3rd exon) to 329 nucleotides (4th exon) coded for a 123- amino acid polypeptide chain (Hayes *et al.*, 1993).

2.2.1 Alpha Lactalbumin Polymorphism

Amigo *et al.* (2000) reviewed two variants α-LA 'A' and 'B' where variant 'B' was rare and confined to very specific breeds.

Bastos *et al.* (2001) found three variants of α-LA gene with genotypic frequencies of 57.5, 22.5 and 20 by PCR-SSCP analysis in Portugese indigenous sheep.

Rincon and Medrano (2003) reported single nucleotide polymorphism (A to G) in bovine α-LA gene at -753 position by Amplification Refractory Mutation System (ARMS) -PCR method.

Mroczkowski *et al.* (2004) reported that α-LA protein was monomorphic in the sheep population studied.

Lan *et al.* (2007) detected a T to C mutation at +1897 position by PCR-RFLP analysis using *Msp* I enzyme and found the allelic frequencies of this polymorphism (A_1 and A_2 alleles) in various Chinese goat breeds like Inner Mongolia White Cashmere (0.983 and 0.017) Xinong saanen dairy (1.0 and 0.0) Laoshan dairy (1.0

and 0.0), Guanzhong dairy (0.976 and 0.024), Leizhou (1.0 and 0.0) and Guizhou Black (0.976 and 0.024).

Ramesha *et al.* (2008) investigated single nucleotide polymorphisms in the coding region of α-LA gene by PCR-SSCP followed by sequencing analysis and found an A to G substitution (1264) in South Kanara buffalo and C to T substitution (+864) in both South Kanara and Murrah.

Garg *et al.* (2009) detected genotypic and allelic frequencies of α-LA as 0.933 (AA) and 0.067 (AB), and 0.966 (A) and 0.034 (B) by sodium dodecyl sulphate polyacrylamide gel electrophoresis in Barbari goat.

Jain *et al.* (2009) performed PCR-SSCP of all four exons of α-LA and reported a total of 9 phenotypes of Jamunapari goat. They also analysed the nucleotide and amino acid variations within breeds of Indian goat and homology between caprine, ovine, bovine, bubaline and human. They had described two novel gene variants on the goat α-LA gene exon 4.

Yardibi *et al.* (2009) reported allelic frequencies of α-LA gene as 0.14 (A) and 0.86 (B) in South Anatolian red cattle and 0.46 (A) and 0.54 (B) in East Anatolian red cattle by PCR-RFLP using *Msp* I enzyme.

2.2.2 Alpha Lactalbumin Variants-Associations

Bleck and Bremel (1993) found that α-LA BB genotype is associated with higher protein and fat percentage than α-LA AA in Holstein cattle whereas α-LA AB genotype had intermediate values for these traits.

Lunden and Lindersson (1997) reported that α-LA polymorphism was associated with milk yield ($p<0.1$), energy corrected milk yield ($p<0.05$), fat ($p<0.05$), protein ($p<0.05$) and lactose ($p<0.05$) in Swedish red and white breeds.

Martin *et al.* (2002) reviewed that α-LA 'A' allele is associated with higher milk yield, protein yield and fat yield where as 'B' is associated with higher percentage of protein and fat.

Dayal *et al.* (2006) performed PCR-SSCP analysis in α-LA gene of Murrah and Bhadavari buffalo and found that polyporphism at this locus was significantly associated with total milk yield and daily milk yield.

Materials and Methods

3. MATERIALS AND METHODS

Blood samples collected from 20 Malabari, 25 Attappady black and 30 Malabari crossbred goats belonging to University Goat and Sheep Farm, Mannuthy as well as 45 Malabari goats from the native tract of Malabari breed formed the materials for the present study.

3.1 COLLECTION OF SAMPLES

Five millilitre of blood from jugular vein was collected from each animal in sterile 15 ml polypropylene centrifuge tube containing Ethylene Diamine Tetra Acetic acid (EDTA) as anticoagulant (1 mg/ml of blood). The tubes were transported in boxes containing ice packs to the laboratory and stored in deep freezer at -20°C.

Thirty milliliter of milk was collected consecutively for two days from goats in 60-90 days of lactation. The milk samples from field were preserved by adding hydrogen peroxide (3-4 drops/250 ml) to freshly collected milk (Mathur and Arora, 2004).

3.2 ISOLATION OF DNA FROM WHOLE BLOOD

DNA was extracted from whole blood using standard phenol chloroform extraction procedure (Sambrook and Russell, 2001) with modifications. The procedure followed was

1. To 5 ml blood, double the volume of ice cold RBC lysis buffer (150 mM ammonium chloride, 10 mM potassium chloride and 0.1 mM EDTA) was

added and kept in ice cold condition with occasional mixing for 10 min for complete lysis of red blood cells.

2. The leukocytes were pelleted by centrifuging at 4000 rpm for 10 min, and the supernatant containing lysed RBCs was discarded.
3. The pellet was resuspended in ice-cold RBC lysis buffer and the above two steps were repeated till a clear pellet was obtained.
4. The pellet was then washed twice with Tris buffered saline (TBS-140 mM sodium chloride, 0.5 mM potassium chloride and 0.25 mM Tris base) by vigorous vortexing followed by centrifugation at 3000 rpm for 10 minutes.
5. The white blood cell pellet was resuspended in 3 ml saline EDTA buffer (SE-75 mM sodium chloride and 35 mM EDTA). The cell suspension was incubated at 50°C in water bath with 0.25 ml of 20 per cent sodium dodecyl sulphate (SDS) and 25µl of proteinase-K (20 mg/ml) for a minimum of three hours. Swirled the viscous solution occasionally.
6. The digested samples were cooled to room temperature, 300 µl of 5 M sodium chloride was added and mixed. An equal volume of phenol (pH 7.8) saturated with Tris-hydrochloride was added, mixed by gentle inversion for 10 min and centrifuged at 4000 rpm for 10 minutes.
7. The aqueous phase containing DNA was collected in fresh tubes, to which an equal volume of saturated phenol: chloroform: isoamyl alchohol (25:24:1) was added. The contents were mixed and centrifuged at 4000 rpm for 10 minutes.
8. The aqueous phase was transferred in fresh tubes, equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed and centrifuged at 4000 rpm for 10 minutes.
9. The supernatant was transferred to a sterile 50 ml beaker and one tenth volume of 3 M sodium acetate (pH 5.5) was added and mixed.

10. To this mixture, equal volume of chilled isopropyl alcohol was added and the precipitated DNA was spooled out using a fresh micropipette tip, washed in 70 per cent ethanol and air-dried.
11. Dried DNA was resuspended in 0.5 ml of Tris EDTA buffer (TE-10 mM Tris base, 0.1 mM EDTA) and stored at -20°C.

3.3 CHECKING OF QUALITY, PURITY AND CONCENTRATION OF DNA

The genomic DNA isolated from the blood samples was checked for quality, purity and concentration. DNA samples having good quality, purity and concentration were used for further analysis.

3.3.1 Quality of Genomic DNA

Horizontal submarine agarose gel electrophoresis was performed to check the quality of genomic DNA using 0.8 per cent agarose gels. The agarose gel was prepared in 1X Tris Borate EDTA buffer (TBE- 0.045 M Tris borate and 0.001 M EDTA). Five microliter of ethidium bromide (10 mg/ml) was added into the molten agarose.

One microliter of each DNA sample was mixed with one-sixth the volume of 6X gel loading dye (0.25 per cent bromophenol blue, 0.25 per cent xylene cyanol and 40 per cent sucrose) and loaded into the wells. Horizontal electrophoresis unit was set at 80 V for 2 h and the gel was visualized after the electrophoresis under UV Transilluminator (Hoefer MacroVue^{TM}).

3.3.2 Purity of Genomic DNA

The purity of genomic DNA was checked using UV-Spectrophotometry. About 6 µl of genomic DNA of each sample was dissolved in 294 µl of triple distilled water and spectrophotometric reading at OD_{260} and OD_{280} were taken against 300 µl triple distilled water as blank. Only the genomic DNA samples lying in the ranges of OD ratio (260:280) between 1.7 to 1.9 were considered good and were used for further study and those showing value beyond this range were reprocessed by phenol: chloroform extraction method.

3.3.3 Concentration of Genomic DNA

The formula used for estimating the concentration of genomic DNA was as follows.

$$\text{DNA concentration } (\mu g/\mu l) = \frac{OD_{260} \times \text{dilution factor} \times 50}{1000}$$

(1 OD value at 260nm is equivalent to 50 ng dsDNA/µl)
Template DNA for PCR was prepared by diluting the DNA stock solution with sterile triple distilled water to a concentration of 50 ng/µl.

3.4 AMPLIFICATION OF GENE FRAGMENTS BY PCR

A 426 bp fragment of caprine β-LG gene from exon 7 to proximal 3' flanking region enclosing the polymorphic site was amplified using the forward (B_1) and reverse (B_2) primers as reported by Pena *et al.* (2000) and Kumar *et al.* (2006).

A 268 bp fragment spanning from exon 3 to flanking region of α-LA also was amplified using the forward (A_1) and reverse (A_2) primers as reported by Lan *et al.* (2007) (Table 3.1).

3.4.1 PCR Primers

The primers, obtained in lyophilized form were stored at -20°C. The stock solution was made by diluting the same with sterile distilled water to make a concentration of 200 pmol/μl. Ten fold dilution of stock solution was used as working primer solution with a final concentration of 20 pmol/μl.

Table 3.1 Primer sequences

Primers	Sequences	Length	Amplified gene
B_1	5'CGGGAGCCTTGGCCCTCTGG 3'	20	β-LG
B_2	5' CCTTTGTCGAGTTTGGGTGT 3'	20	β-LG
A_1	5' TCATCTAAAAGGCAACAGGTA 3'	21	α-LA
A_2	5' ATAGTGCTGGGGCGAAAA 3'	18	α-LA

3.4.2 Reaction Mixture

PCR was standardized using various combinations of reaction chemicals. The standardized concentrations of the components used in the PCR reaction mixture are given in Table 3.2

Table 3.2 PCR reaction mixture for β-LG and α-LA gene fragments

SI No.	Reaction components	Concentration	Volume (µl)	
			β-LG	α-LA
1	Template DNA	50 ng/µl	1.00	1.0
2	dNTPs mix	2.5 mM	1.60	1.2
3	$MgCl_2$	10.0 mM	2.50	1.5
4	Forward Primer	20 pmol/µl	0.50	0.50
5	Reverse primer	20 pmol/µl	0.50	0.50
6	10 x PCR assay buffer	200mM Tris-HCl & 500mM KCl	2.00	1.50
7	Taq DNA polymerase	5U/µl	0.12	0.12
8	Distilled water		11.78	8.68
	Total		20	15

3.4.3 Setting Up of PCR Reaction

A PCR master mix containing all the reaction components except genomic DNA, was prepared. The master mix was mixed gently, followed by spinning by table top micro centrifuge. Master-mix of 19 µl for β-LG and 14 µl for α-LA was added to each PCR tubes containing 1µl (50ng) of good quality diluted genomic DNA. Finally, the contents of the tube were gently mixed and spinned at 2000 rpm for 10 sec. Then, the PCR tubes were kept in a preprogrammed peltier thermal cycler. PCR products obtained, after the completion of the programme, were kept at 4°C in refrigerator till further analysis.

3.4.4 PCR Programme

The annealing temperature was standardized for the best amplification of the desired fragments. The standardized programme is given in Table 3.3

Table 3.3 PCR reaction conditions for β-LG and α-LA gene fragments

	Steps	β-LG		α-LA	
		Temperature (°C)	Time	Temperature (°C)	Time
1	Initial denaturation	93 °C	4 min	93 °C	4 min
2	Denaturation	94 °C	1 min	94 °C	1 min
3	Annealing	55.4 °C	1 min	58.5	1 min
4	Extension	72 °C	1 min	72 °C	1 min
5	Repeat	35 Cycles			
6	Final extension	72 °C for	10 min	72 °C	10 min
7	Hold	4 °C	30 min	4 °C	30 min

3.4.5 Checking of the Amplified Product

Horizontal submarine agarose gel electrophoresis was carried out to check the amplified product. A 1.5 per cent w/v agarose (Low EEO) gel was prepared in 1.0 X TAE and subsequently 5 µl PCR product was mixed with 1 µl of 6 X gel loading dye (Bromophenol blue and xylene cyanol) and loaded along with 100 bp ladder as a marker in a separate lane. The electrophoresis was done at 60 volt for 2 hr. The gel was examined for the presence of the amplified product and documented in gel documentation system (BioRad, Gel Doc 2000^{TM}).

3.5 RESTRICTION ENZYME DIGESTION

The PCR products were digested with restriction enzyme and a point mutation of caprine β-LG gene in exon-seven was investigated using restriction endonuclease *Sac* II.

A single nucleotide polymorphism in the α-LA gene was detected using *Msp* I endonuclease.

3.5.1 Setting up of Restriction Analysis

Ten microliter of the β-LG amplified product was digested with 10U of restriction enzyme *Sac* II at 37° C overnight in a dry bath. The reaction was carried out in a final volume of 15 µl. The compositions of various reaction components with their respective amounts are given in Table 3.4.

Restriction digestion of α-LA gene was carried out using 10 U of *Msp* I endonuclease for a period of 3 hours at 37 °C in dry bath (Table 3.5).

Table 3.4 *Sac* II RE digestion of 426 bp fragment of β-LG gene

SI No	Reaction components	Amount
1	PCR product	10.0 µl
2	Distilled water	3.0 µl
3	10X assay buffer	1.5 µl
4	*Sac* II (20U/µl)	0.5 µl
	Total	15.0 µl

Table 3.5. *Msp* I RE digestion of 268 bp fragment of α-LA gene

SI No	Reaction components	Amount
1	PCR product	10.0 µl
2	10x assay buffer	2.0 µl
3	*Msp* I (10U/ µl)	1.0 µl
4	Distilled water	7.0 µl
	Total	20 µl

All the reagents were added as per the order given in the Tables in a 0.5 ml eppendorf tube, except PCR product and were mixed. The PCR products were added to 0.2 ml PCR tubes and the master mix was dispensed into corresponding tubes. The restriction enzyme digestion was carried out for 16 hours at 37°C and 3 hours at 37°C in a drybath for *Sac* II and *Msp* I respectively. After that, the digested products were kept in refrigerator at 4°C till further study.

3.5.2 Non-Denaturing Poly Acrylamide Gel Electrophoresis (PAGE) of Digested PCR Product

The PCR products after *Sac* II restriction digestion were subjected to non-denaturing polyacrylamide gel electrophoresis. It was carried out in in 8 per cent polyacrylamide gels in 1X TBE buffer with 100bp DNA ladder as size marker.

Non-denaturing PAGE was performed in a vertical electrophoresis system (Hoefer SE 600 Series). The glass plates (18 x 16 cm) were assembled with Teflon spacers and comb (1.5 mm thick) and mounted over the gel casting assembly. The gel was prepared by mixing 70 ml of TBE gel mix (8 per cent acrylamide and 0.5X

TBE), 850 µl, 10 per cent ammonium per sulphate (APS) and 50 µl N,N,N',N', Tetra methyl ethylene diamine (TEMED) in a beaker. The gel was allowed to polymerize for 30 minutes.

The gel sandwich was transferred into the electrophoresis tank and the 1X TBE buffer was poured into the top and bottom reservoirs. The digested samples were mixed with 2 µl of the 6X gel loading dye and loaded into the bottom of the wells. Electrophoresis was carried at 60 V till the bromophenol blue dye reached the opposite end of the gel. After disconnecting the electrodes from power packs, the gel was separated from glass plates and stained with ethiduim bromide (0.5 µg/ml of 1X TBE) for 30-45 minutes. The restriction pattern was documented in a gel documentation system (BioRad, Gel Doc 2000^{TM}).

3.5.3 Agarose Gel Electrophoresis of Digested PCR Product

The *Msp* I digested PCR product was electrophoresed in 2.5 per cent w/v agarose gel for 3 hours at 60V in 1X TBE buffer. About 15 µl of digested product was mixed with 3 µl of 6X gel loading dye and then loaded into the corresponding wells. After completion of gel electrophoresis, the digested products were visualized by keeping the gel over UV transilluminator and documented in gel documentation system (BioRad, Gel Doc 2000^{TM}). For better comparison, an undigested sample was also loaded in the gel. Around 5µl (500ng) of 100 bp DNA marker was also run parallel to the digested product in one of the wells to determine the size of the fragments obtained. Photographs of all the gels were stored in computer system and used for further analysis of genotype pattern of different animals.

3.6 ALLELIC AND GENOTYPIC FREQUENCIES

The genotypes were identified by seeing the PCR-RFLP pattern of each sample in the gel. The allelic and genotypic frequencies were estimated for Malabari, Attappady black and Malabari crossbred goats separately as well as for the pooled population using standard procedure. (Falconer and Mackey, 1996):

$$\text{Genotypic frequency} = \frac{\text{Total No. of individual of a particular genotype}}{\text{Total No. of individuals of all genotypes}}$$

$$\text{Allelic frequency} = \frac{D + \frac{1}{2}H}{N}$$

Where,

D= No. of homozygotes

H = No. of heterozygotes

N= Total no. of individual

The variation of the allelic frequencies among the three populations was analysed by the *Chi*-square test of significance as described by Snedecor and Cochran (1994) considering the allelic frequencies in a 3×2 table using the formula,

$$\chi 2 \text{ value} = \sum \frac{(O - E)^2}{E}$$

where 'O' is the observed and 'E' is the expected frequencies in different populations.

The distribution of genotypes in Malabari, Attappady black and crossbred goat population was checked for Hardy-Weinberg equilibrium by *Chi- square* test, comparing the observed and expected frequencies.

3.7 DNA SEQUENCING

Amplified products of both Malabari and Attappady black samples were selected for sequencing. Sequencing was carried out commercially (Bioserve Hyderabad, Pvt. Ltd.) by the dideoxynucleotide sequencing method using an automated DNA sequencer (Applied Biosystems, USA).

3.7.1 Sequencing Analysis of Caprine β-LG Gene

The sequences obtained from different genotypes were first blasted (www.ncbi.nlm.nih.gov/BLAST) to ascertain that sequences were of β-LG gene.

3.8 EFFECT OF β-LG GENE POLYMORPHISMS ON DAIRY TRAITS

Information regarding dairy traits like peak yield, milk fat percentage, total solids and solids not fat were obtained. The peak yield data was collected from the records maintained in the farm. Milk fat percentage was estimated by Gerber method as described in IS: 1224 (1977) part one and total solids in percentage were determined as per Gravimetric method (IS: 1479, 1961). The solids not fat were estimated by subtracting milk fat percentage from the total solids in percentage.

The effect of population and β- LG/*Sac* II genotypes on peak milk yield, milk fat percentage, total solids and solids not fat was checked by univariate analysis of variance (ANOVA).

Results

4. RESULTS

4.1 ISOLATION OF GENOMIC DNA

A total of 120 genomic DNA samples were obtained from whole blood collected from Malabari, Attappady black and Malabari crossbred goats using phenol chloroform extraction procedure.

4.2 QUALITY AND PURITY OF GENOMIC DNA

Single, clear band without shearing on agarose gel upon electrophoresis indicated the presence of good quality high molecular weight DNA (Plate1). The ratio of optical density at the two wavelengths (OD_{260}/OD_{280}) was in between 1.7 to 1.9, indicative of the good quality of genomic DNA without protein or RNA.

4.3 AMPLIFICATION OF THE β-LG GENE FRAGMENT

Beta lactoglobulin gene fragment from exon 7 to proximal 3' flanking region could be amplified without any non-specific amplified products from the DNA samples of Malabari, Attappady black and crossbred goats (plate 2). In all the animals studied, the size of the amplified product was 426 bp, indicating conservation of DNA sequences at the β-LG locus in Malabari, Attappady black and crossbred goats.

4.3.1 Restriction Enzyme Digestion

Electrophoresis of digested PCR products with restriction enzyme *Sac* II revealed three restriction digestion patterns in Malabari, Attappady black and

crossbred goats (plate 3) with the identification of two alleles namely S_1 and S_2. Allele S_1 was indicated by the presence of two bands of 349 and 77 bp while S_2, by a single band of size 426 bp.

4.3.2 Allelic and Genotypic Frequencies

The allelic and genotypic frequencies of β-LG/*Sac* II polymorphism in Malabari, Attappady black, crossbred goats as well as pooled population are presented in Table 4.1. The frequencies for S_1 and S_2 alleles were noted as 0.37 and 0.63, 0.34 and 0.66, 0.18 and 0.82 in Malabari, Attappady black and crossbred goats, respectively. In the pooled population, the corresponding frequencies were 0.32 and 0.68, respectively.

The genotypic frequencies of β-LG/*Sac* II polymorphism in Malabari goats were observed as 0.14 (S_1S_1), 0.46 (S_1S_2) and 0.40 (S_2S_2), Attappady black as 0.04 (S_1S_1), 0.60 (S_1S_2) and 0.36 (S_2S_2) and crossbred goats as 0.10 (S_1S_1), 0.17 (S_1S_2) and 0.73 (S_2S_2). The genotypic frequencies in the pooled population were recorded as 0.11 (S_1S_1), 0.42 (S_1S_2) and 0.47 (S_2S_2) (Table 4.1).

A comparison of the β-LG/*Sac* II alleles among Malabari, Attappady black and crossbred goat populations using *Chi*-square analysis arrived at a non-significant χ^2 value (3.35, df=2), indicated that S_1 and S_2 alleles were homogenously distributed in all the populations (Table 4.2).

On *Chi*-square analysis of the genotypic frequencies, Malabari goat population (χ^2 value 0.005, df=2) was found to follow Hardy-Weinberg equilibrium (H-W equilibrium) (Table 4.3). Malabari goat samples were further grouped according to the region as Tellicherry, Badagara and University farm population and

genotypic as well as allelic frequencies were found (Table 4.4). The frequencies for S_1 and S_2 alleles were noted as 0.39 and 0.61, 0.32 and 0.68, 0.39 and 0.61 in Tellicherry, Badagara and University farm population, respectively. The region wise analysis of allelic frequencies proved that S_1 and S_2 alleles were homogenously distributed in all the populations (Table 4.5). The *Chi*-square analysis of the genotypic frequencies proved that S_1S_1, S_1S_2 and S_2S_2 genotypes in Tellicherry (0.14, 0.50 and 0.36), Badagara (0.2, 0.25 and 0.55) and University farm (0.09, 0.61 and 0.30) populations were in H-W equilibrium (Table 4.6).

The *Chi*-square analysis of the observed and expected genotypic frequencies in Attappady black (χ^2 value 2.20, df=2) and crossbred goats populations (χ^2 value 5.88, df=2) proved that both populations were in H-W equilibrium (Table 4.3).

4.3.3 DNA Sequencing

For sequencing various alleles of β-LG gene were selected on the basis of their RFLP patterns. The S_2S_2 genotype from Malabari and S_1S_1 from Attappady black were selected for sequencing. These alleles were sequenced by Sanger's dideoxy chain termination sequencing method in an automated DNA sequencer. The sequencing results revealed 391 bp and 393 bp fragments, respectively for Malabari and Attappady black samples of the gene from exon 7 to proximal 3' flanking region. The nucleotide sequence of the amplified region of β-LG gene in both Malabari and Attappady black were presented in Fig.1 and 2.

4.3.3.1 DNA Sequence Analysis

Sequencing confirmed the amplification of β-LG gene fragment in Malabari and Attappady black goat. The sequences obtained were subjected to NCBI nBLAST

and the similar sequences were downloaded from the Internet. After comparing with other available sequences of other species the amplified fragments were confirmed.

The BLAST analysis of the nucleotide sequence of the β-LG gene of both Malabari (Table 4.7) and Attappady black (Table 4.8) goat obtained through sequencing revealed 99 per cent identity with that of *Capra hircus* (Accession # Z33881.1) gene encoding β-LG. The sequence was also found to be having 93 per cent identity with that of bovine β-LG variant B (Accession # Z48305.1), 90 per cent with sheep β-LG (*Ovis aries*; X12817.1), 91 per cent with that of buffalo β-LG mRNA (*Bubalus bubalis*; AJ005429.1) and 91 per cent with that of yak β-LG (*Bos grunniens*; #AF194982.1).

The sequenced region of both Malabari and Attappady black β-LG gene varied at 7062 position (C to A) from the published sequence (Accession # Z33881.1) of β-LG. The Malabari goat β-LG sequence (S_2S_2 genotype) varied from that of Attappady black (S_1S_1 genotype) at 6751 (C to T) which formed the basis for β-LG/*Sac* II polymorphism.

4.4 AMPLIFICATION OF THE α-LA GENE FRAGMENT

A 268 bp fragment of caprine α-LA gene spanning from exon 3 to flanking region was amplified (plate 4). In all the groups studied size of the amplified product was the same.

4.4.1 Restriction Enzyme Digestion

On agarose gel electrophoresis, the *Msp* I endonuclease digested PCR products appeared to be monomorphic. The entire population under study was found to be of A_1A_1 genotype (plate 5).

4.4.2 Allelic and Genotypic Frequencies

The α-LA/*Msp* I allelic and genotypic frequencies in Malabari, Attappady black, crossbred goats as well as pooled population are presented in Table 4.9. The frequencies of A_1 and A_2 alleles were noted as 1.0 and 0.0 in all the populations under study. The genotype frequencies of A_1A_1, A_1A_2 and A_2A_2 were also the same (1.0, 0.0 and 0.0) in Malabari, Attappady black and crossbred goat populations.

4.5 β-LG/*Sac* II POLYMORPHISM AND MILK PRODUCTION TRAITS

The average values for milk production traits for Malabari, Attappady black and crossbred goats carrying different β-LG/*Sac* II genotypes are presented in Tables 4.11, 4.12, 4.13 and 4.14.

4.5.1 β-LG/*Sac* II Polymorphism and Peak Yield

The average peak milk yield for Malabari, Attappady black and crossbred goats were 440.22±35.02 ml, 400±58.78 and 637.21±47.50 ml, respectively (Table 4.10). Significant variation existed between breeds ($p<0.05$) where the average peak yield was found to be higher in crossbred population compared to Malabari and Attappady black.

Genotypes of β-LG/*Sac* II polymorphism had significant effect on Peak milk yield in the Malabari goat populations studied (Table 4.11). The Malabari goats carrying S_2 allele showed a significantly higher average ($p<0.05$) for Peak milk yield (420±74.24 ml and 501.79±46.47 ml) compared to animals with S_1 allele (250±13.36 ml and 501.79±46.47 ml). The average values of peak yield for S_1S_1, S_1S_2 and S_2S_2 in crossbred goats were found to be 800.0±115.47, 780.0±198.49 and 602.86±50.08, respectively with no significant association detected (Table 4.11). The average values of peak yield for each genotype was not taken into consideration in Attappady black population studied due to less sample size.

4.5.2 β-LG/*Sac* II Polymorphism and Fat Percentage

The average milk fat percentages in Malabari, Attappady black and crossbred goats in 60-90 days of lactation were recorded as 4.10±0.14, 3.76±0.26 and 4.14±0.22, respectively (Table 4.10). The average milk fat percentages did not vary significantly between the breeds under consideration.

The mean values for milk fat percentage were found to be 3.99±0.25, 4.21±0.26 and 4.02±0.18 for S_1S_1, S_1S_2 and S_2S_2, respectively in Malabari goat (Table 4.12). In case of Attappady black breed, 5.6±0.0, 3.71±0.2 and 3.50±0.65 for S_1S_1, S_1S_2 and S_2S_2, respectively (Table 4.12) were recorded. The crossbred population showed 3.2±0.25, 4.66±0.54 and 4.16±0.27 for S_1S_1, S_1S_2 and S_2S_2, respectively (Table 4.12). The mean values were not significantly related to β-LG/*Sac* II polymorphism.

4.5.3 β-LG/*Sac* II Polymorphism and Total Solids

The average value for total solids in percentage were recorded in Malabari, Attappady black and crossbred goats in 60-90 days of lactation as 14.15±0.25, 13.70±0.38 and 14.32±0.31, respectively (Table 4.10). The average value for total solids did not vary significantly between the breeds under consideration.

The mean values for milk total solids in percentage were 13.69±0.45, 14.16±0.42 and 14.38±0.37 for S_1S_1, S_1S_2 and S_2S_2, in Malabari, 15.95±0.0, 13.43±0.09 and 13.66±2.17, in Attappady black and 15.3±1.88, 14.57±0.72 and 14.13±0.32, in crossbred goat population, respectively (Table 4.13). The values were not significantly associated with β-LG/*Sac* II polymorphism.

4.5.4 β-LG/*Sac* II Polymorphism and Solids Not Fat

Population averages for milk solids not fat in percentage in Malabari, Attappady black and crossbred goats in 60-90 days of lactation were 10.05±0.18, 10.04±0.29 and 10.18±1.75, respectively (Table 4.10). The average value for milk solids not fat did not vary significantly between the breeds under consideration.

The mean values of solids not fat for genotypes S_1S_1, S_1S_2 and S_2S_2 were 9.71±0.34, 9.96±0.30 and 10.35±0.28 in Malabari, 10.35±0.0, 9.85±0.25 and 10.60±1.51, in Attappady black and 12.1±1.75, 9.91±0.30 and 9.98±0.29, in crossbred goat population, respectively (Table 4.14). The values were not significantly related to β-LG/*Sac* II polymorphism.

Table 4.1 Genotypic and allelic frequencies of β-LG/*Sac* II polymorphism in Malabari, Attappady black and crossbred goats

Population	Genotypic frequency			Allelic frequency	
	S_1S_1	S_1S_2	S_2S_2	S_1	S_2
Malabari (65)	0.14 (9)	0.46 (30)	0.40 (26)	0.37 (48)	0.63 (82)
Attappady black (25)	0.04 (1)	0.60 (15)	0.36 (9)	0.34 (17)	0.66 (33)
Crossbred (30)	0.10 (3)	0.17 (5)	0.73 (22)	0.18 (11)	0.82 (49)
Pooled population (120)	0.11 (13)	0.42 (50)	0.47 (57)	0.32 (76)	0.68 (164)

Figures in parenthesis are actual numbers

Table 4.2 Comparison of frequencies of β-LG/*Sac* II alleles in Malabari, Attappady black and crossbred goats

Population	S_1 allele	S_2 allele	χ^2 value (df=2)
Malabari	0.37 (48)	0.63 (82)	3.35^{NS}
Attappady black	0.34 (17)	0.66 (33)	
Crossbred	0.18 (11)	0.82 (49)	

^{NS} – not significant
df – degrees of freedom
Figures in parenthesis are number of observations

Table 4.3 Testing of genotypes of β-LG/*Sac* II polymorphism for H–W equilibrium in Malabari, Attappady black and crossbred goats

Population	Source	Genotypes			χ^2 value (df=2)
		S_1S_1	S_1S_2	S_2S_2	
Malabari	Number Observed	9 (0.14)	30 (0.46)	26 (0.4)	0.005^{NS}
	Number Expected	8.86	30.28	25.86	
Attappady black	Number Observed	1(0.04)	15 (0.6)	9 (0.36)	2.20^{NS}
	Number Expected	2.89	11.22	10.89	
Crossbred	Number Observed	3(0.10)	5(0.17)	22(0.73)	5.88^{NS}
	Number Expected	1.01	8.98	20.01	

^{NS} – not significant
df – degrees of freedom
Figures in parenthesis are genotypic frequencies

Table 4.4 Genotypic and allelic frequencies of β-LG/*Sac* II polymorphism in Tellicherry, Badagara and University farm populations

Population	Genotypic frequency			Allelic frequency	
	S_1S_1	S_1S_2	S_2S_2	S_1	S_2
Tellicherry (22)	0.14 (3)	0.50 (11)	0.36 (8)	0.39 (17)	0.61 (27)
Badagara (20)	0.2 (4)	0.25 (5)	0.55 (11)	0.32 (13)	0.68 (27)
University farm (23)	0.09 (2)	0.61 (14)	0.30 (7)	0.39 (18)	0.61 (28)
Pooled population (65)	0.14 (9)	0.46 (30)	0.40 (26)	0.37 (48)	0.63 (82)

Figures in parenthesis are number of observations

Table 4.5 Comparison of frequencies of β-LG/*Sac* II alleles in Tellicherry, Badagara and University farm populations

Population	S_1 allele	S_2 allele	χ^2 value (df=2)
Tellicherry	0.39 (17)	0.61 (27)	0.244^{NS}
Badagara	0.32 (13)	0.68 (27)	
University farm	0.39 (18)	0.61 (28)	

^{NS} – not significant

Figures in parenthesis are number of observations

Table 4.6 Testing of genotypes of β-LG/*Sac* II polymorphism for H-W equilibrium in Tellicherry, Badagara and University farm populations

Population	Source	Genotypes			χ^2 value (df=2)
		S_1S_1	S_1S_2	S_2S_2	
Tellicherry	Number Observed	3 (0.14)	11 (0.5)	8 (0.36)	0.065^{NS}
	Number Expected	3.29	10.43	8.28	
Badagara	Number Observed	4 (0.2)	5 (0.25)	11 (0.55)	3.69^{NS}
	Number Expected	2.11	8.78	9.11	
University farm	Number Observed	2 (0.09)	14 (0.67)	7 (0.30)	1.77^{NS}
	Number Expected	3.52	10.96	8.52	

^{NS} – not significant
df – degrees of freedom
Figures in parenthesis are genotypic frequencies

Table 4.7 Results of BLASTn of Malabari goat's β-LG gene nucleotide sequence showing the percentage identity with other species

Accession	Description	Maximum score	Total score	Query coverage (per cent)	Maximum identity (per cent)
# DQ489319.1	*Bos taurus* β-LG variant B precursor	577	577	100	93
# Z48305.1	*Bos taurus* β-LG gene variant B	577	577	100	93
#AF194982.1	*Bos grunniens* β-LG gene exon 6,7 and partial cds.	538	538	100	91
# X12817.1	*Ovis aries* β-LG gene	505	505	100	90
# Z19571.1	*Capra hircus* β-LG mRNA	483	483	66	100
# Z36937.1	*Bos. taurus* β-LG pseudogene	296	296	51	93
# AJ005429.1	Bubalus bubalis β-LG mRNA	213	213	39	91

Table 4.8 Results of BLASTn of Attappady black goat's β-LG gene nucleotide sequence showing the percentage identity with other species

Accession	Description	Maximum score	Total score	Query coverage (per cent)	Maximum identity (per cent)
# DQ489319.1	*Bos taurus* β-LG variant B precursor	575	575	100	93
# Z48305.1	*Bos taurus* β-LG gene variant B	575	575	100	93
#AF194982.1	*Bos grunniens* β-LG gene exon 6,7 and partial cds.	536	536	100	91
# X12817.1	*Ovis aries* β-LG gene	514	514	100	90
# Z19571.1	*Capra hircus* β-LG mRNA	483	483	67	99
# Z36937.1	*Bos. taurus* β-LG pseudogene	307	307	51	94
# AJ005429.1	Bubalus bubalis β-LG mRNA	213	213	40	91

Table 4.9 Genotypic and allelic frequencies of α-LA/*Msp* I polymorphism in Malabari, Attappady black and crossbred goats

Population	Genotypic frequency			Allelic frequency	
	A_1A_1	A_1A_2	A_2A_2	A_1	A_2
Malabari (65)	1.0 (65)	0.0 (0)	0.0 (0)	1.0 (130)	0.0 (0)
Attappady black (25)	1.0 (25)	0.0 (0)	0.0 (0)	1.0 (50)	0.0 (0)
Crossbred (30)	1.0 (30)	0.0 (0)	0.0 (0)	1.0 (30)	0.0 (0)
Pooled population (120)	1.0 (120)	0.0 (0)	0.0 (0)	1.0 (240)	0.0 (0)

Figures in parenthesis are actual numbers

Table 4.10 Dairy traits in Malabari, Attappady black and crossbred goats

SI. no.	Milk parameters	Population (Mean±SE)			
		Malabari (47)	Attappady black (11)	Crossbred (30)	F-value
1	Peak yield (ml)	440.22±35.02	400±58.78	637.21±47.50	22.30*
2	Milk fat in percentage	4.10±0.14	3.76± 0.26	4.14±0.22	0.59NS
3	Total solids in percentage	14.15±0.25	13.70±0.38	14.32±0.31	0.66NS
4	Solids not fat in percentage	10.05±0.18	10.04±0.29	10.18±1.75	0.095NS

* ($p \leq 0.05$)
NS – not significant
df – degrees of freedom
Number of observations in parenthesis

Table 4.11 β-LG/*Sac* II polymorphism and peak yield

Sl. no.	Population	Genotype (Mean±SE)			
		S1S1	S1S2	S2S2	F-value
1	Malabari	250±13.36[b] (13)	501.79±46.47[a] (17)	420±74.24[a] (7)	4.02*
2	Crossbred	800.0±115.47 (22)	780.0±198.49 (5)	602.86±50.08 (3)	1.156[NS]

* ($p \leq 0.05$)
[NS] indicates that the values were not significantly different
Number of observations in parenthesis

Table 4.12 β-LG/*Sac* II polymorphism and Milk fat in percentage

Sl. no.	Population	Genotype (Mean±SE)			
		S1S1	S1S2	S2S2	F-value
1	Malabari	3.99±0.25 (13)	4.21±0.26 (17)	4.02±0.18 (7)	0.243[NS]
2	Attappady black	5.60±0.0 (1)	3.71±0.20 (8)	3.50±0.65 (2)	-
3	Crossbred	3.2±0.25 (22)	4.66±0.54 (5)	4.16±0.27 (3)	1.373[NS]

[NS] indicates that the values were not significantly different
Number of observations in parenthesis

Table 4.13 β-LG/*Sac* II polymorphism and total solids in percentage

Sl. no.	Population	Genotype (Mean±SE)			
		S1S1	S1S2	S2S2	F-value
1	Malabari	13.69±0.45 (13)	14.16±0.42 (17)	14.38±0.37 (7)	0.464^{NS}
2	Attappady black	15.95±0.0 (1)	13.43±0.09 (8)	13.66±2.17 (2)	-
3	Crossbred	15.3±1.88 (22)	14.57±0.72 (5)	14.13±0.32 (3)	0.69^{NS}

Number of observations in parenthesis
^{NS} indicates that the values were not significantly different

Table 4.14 β-LG/*Sac* II polymorphism and solids not fat in percentage

Sl. no.	Population	Genotype (Mean±SE)			
		S1S1	S1S2	S2S2	F-value
1	Malabari	9.71±0.34 (13)	9.96±0.30 (17)	10.35±0.28 (7)	0.901^{NS}
2	Attappady black	10.35±0.0 (1)	9.85±0.25 (8)	10.6±1.51 (2)	-
3	Crossbred	12.1±1.75 (22)	9.91±0.30 (5)	9.98±0.29 (3)	2.841^{NS}

Number of observations in parenthesis
^{NS} indicates that the values were not significantly different

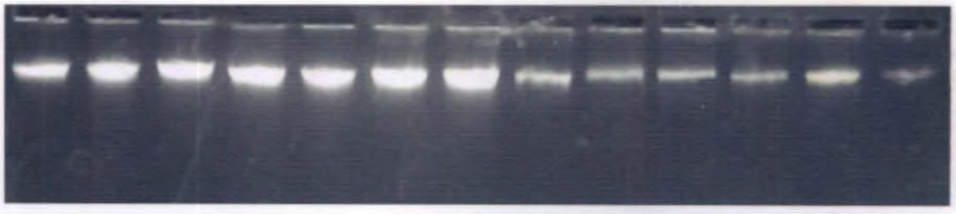

Plate 1. PCR template on 0.8 % agarose gel

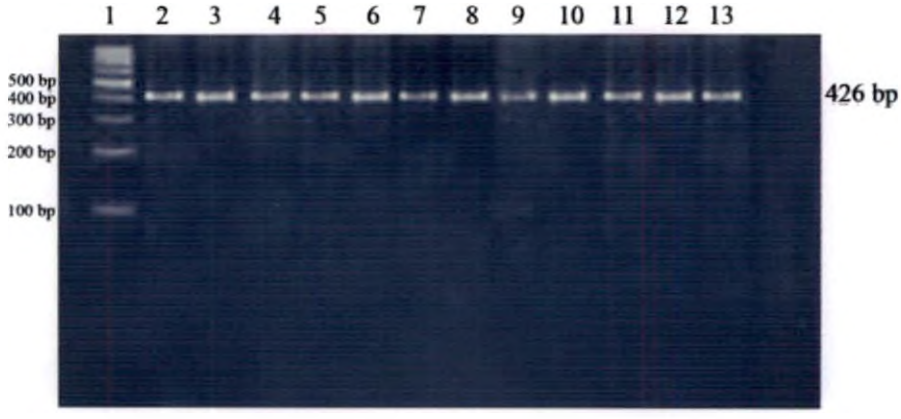

Plate 2. The amplified fragment of caprine β-LG gene on 1.5% agarose gel

Lane 1: 100 bp DNA size ladder
Lane 2-13 : Amplified fragment of size 426 bp

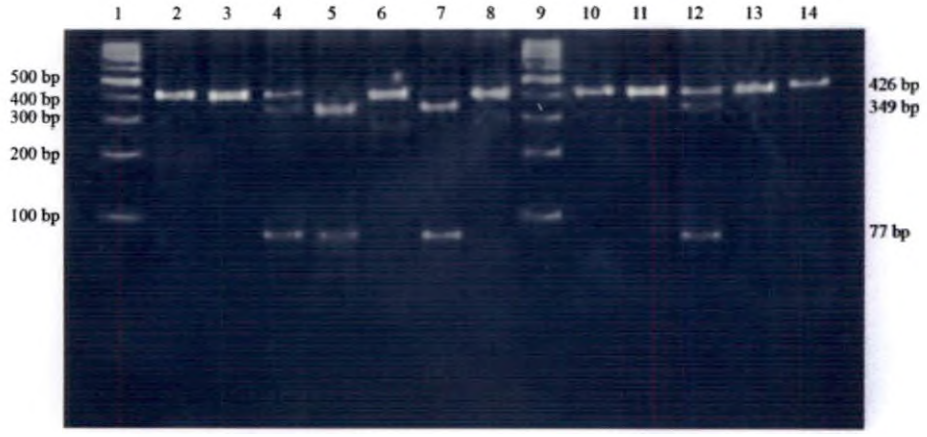

Plate 3. The genotypes of β-LG/Sac II polymorphism on 8% nondenaturing polyacrylamide gel
Lane 5, 7 : S1S1 genotype with 349 and 77 bp fragments
Lane 4, 12 : S1S2 genotype with 426, 349 and 77 bp fragments
Lane 2, 3, 6, 8, 10, 11 : S2S2 genotype with 426 bp fragment
Lane 13, 14 : PCR product 426 bp
Lane 1, 9 : 100 bp DNA size ladder

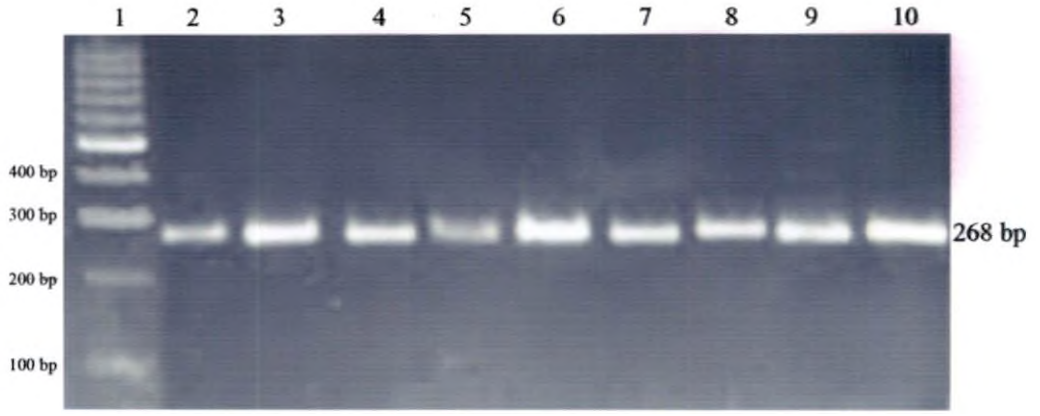

Plate 4. The amplified fragment of the caprine α-LA gene on 2.5% agarose gel
Lane 2- 10 : Amplified fragment of size 268 bp
Lane 1: 100 bp DNA size ladder

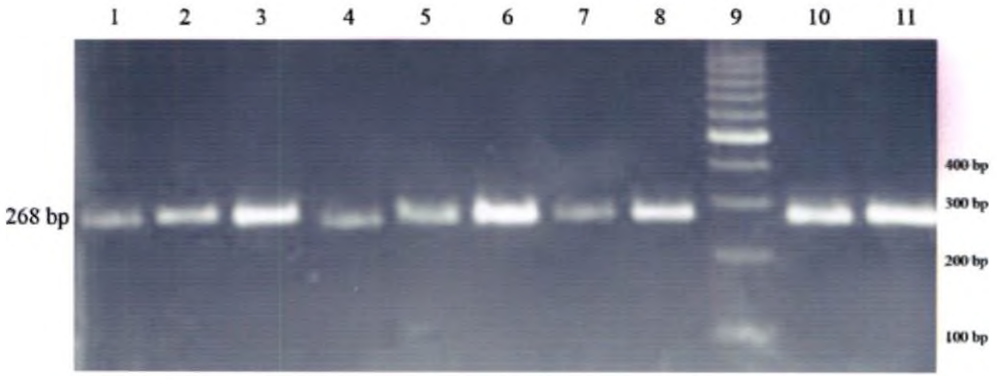

Plate 5. The α-LA/Msp I genotypes on 2.5% agarose gel
Lane 1-8 : A1A1 genotype with 268 bp fragment
Lane 10,11 : PCR product
Lane 9 : 100 bp DNA size ladder

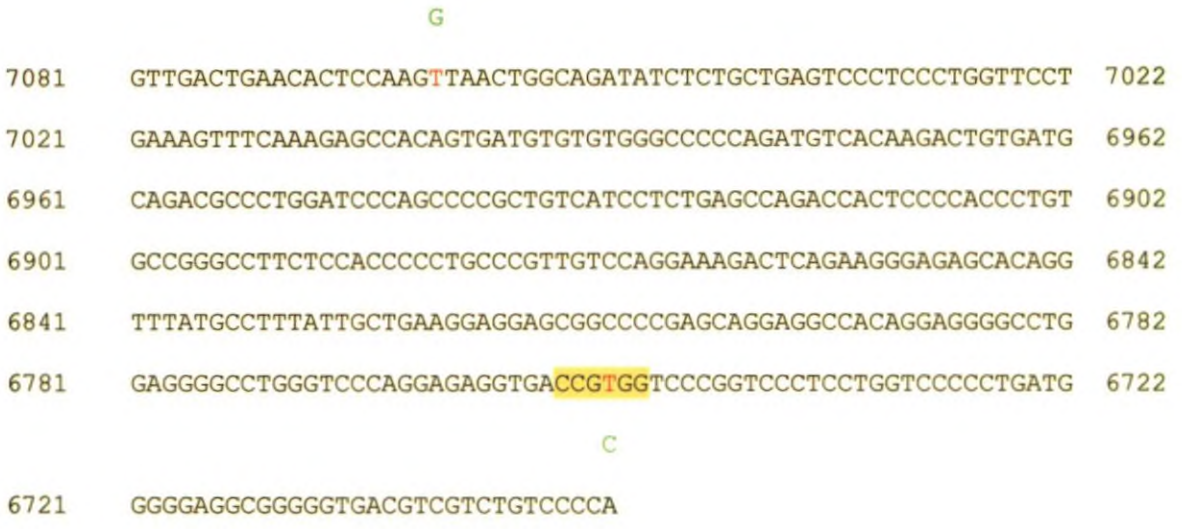

Fig 1 Malabari goat's β-LG gene nucleotide sequence from exon 7 to flanking region

Fig 2 Attappady black goat's β-LG gene nucleotide sequence from exon 7 to flanking region

Discussion

5. DISCUSSION

The variation in DNA sequence, as it causes the variation in the performance of animals, is the basic material for improving livestock through selection. Dairy traits are important criterion under consideration for improvement of livestock. Beta lactoglobulin and Alpha lactalbumin were considered as important marker genes influencing dairy traits. So variation in these loci will be an important tool in selection for dairy traits.

5.1 AMPLIFICATION OF THE β-LG GENE FRAGMENT

In all the animals tested, the size of the amplified product was 426 bp fragment, indicating conservation of DNA sequences at that fragment of caprine β-lactoglobulin locus. The same sized PCR products were reported earlier by Pena *et al.* (2000) and Kumar *et al.* (2006) in various exotic and Indian goats breeds using the same primers.

5.1.1 The β-LG/*Sac* II Gene Polymorphism

Analysis of amplified DNA by the endonuclease *Sac* II revealed three restriction digestion patterns indicative of two alleles namely S_1 (349 and 77 bp fragments) and S_2 (426 bp fragment), which created *Sac* II polymorphism. The above findings are in close agreement with the reports of Pena *et al.* (2000), Kumar *et al.* (2006) and Elmaci *et al.* (2009).

The frequencies of S_1 and S_2 alleles of β-LG/*Sac* II polymorphism were 0.37 and 0.63 in Malabari, 0.34 and 0.66 in Attappady black, respectively indicating the predominance of S_2 allele in the population. In the pooled population also, the

frequency of S_2 allele (0.68) was higher than S_1 (0.38). Kumar *et al.* (2006) made similar findings in Surti goats and reported the frequencies of S_1 and S_2 alleles (0.41 and 0.59). The frequencies of S_1 and S_2 alleles in crossbreds were 0.18 and 0.82, respectively. Similar findings were observed in Barbari (0.16 and 0.84) and Marwari (0.17 and 0.83) by Kumar *et al.* (2006). Infinitesimal frequencies of S_1 allele were reported by the same author in various Indian goat breeds like Beetal (0.08), Black Bengal (0.05), Sirohi (0.05), Gaddi (0.05), Jamunapari (0.03), Jakhrana (0.02) and Chegu (0.0). Probable reason for higher frequency of S_2 might be the selective advantage of this allele in Indian goat breeds having linkage to any favourable character.

Contradictory to the present result, predominance of S_1 allele was reported by Pena *et al.* (2000) in exotic goats like Spanish (0.703) and French (0.687) and Elmaci *et al.* (2009) in Turkish hair goat breed (0.67).

The genotype frequencies of S_1S_1 was the highest in Malabari (0.14) followed by crossbred goats (0.10) and Attappady black (0.04). The frequency of S_1S_1 genotype in the pooled population was recorded as 0.11. Similar results were observed in Surti (0.23), Gaddi (0.06), Marwari (0.04) whereas S_1S_1 was found to be absent in Jamunapari, Barbari, Sirohi, Jakhrana, Beetal, Chegu, Osmanabadi and Black Bengal (Kumar *et al.*, 2006). Contradictory results were reported by Pena *et al.* (2000) in Spanish (0.37) and French (0.44) and Elmaci *et al.* (2009) in Turkish hair goat (0.45) where higher frequencies of S_1S_1 were observed. Probable reason for higher frequency of S_1S_1 genotype could be association with traits of some economic importance.

The frequency of S_1S_2 was the highest in Attappady black (0.60) followed by Malabari (0.46) and pooled population (0.42). Similar results were reported in exotic

breeds like Spanish (0.52), French (0.47) by Pena *et al.* (2000) and in Turkish hair goat breeds (0.44) by Elmaci *et al.* (2009). But in crossbred population the frequency of heterozygotes was too low (0.17). Similar findings were observed in Black Bengal (0.09), Sirohi (0.11), Beetal (0.16) and Gaddi (0.14). The values were higher in Osmanabadi (0.24), Marwari (0.27), Surti (0.35) and Barbari (0.32) as reported by Kumar *et al.* (2006).

Cross bred population showed the highest frequency for S_2S_2 genotypes (0.73) followed by Malabari (0.40) and Attappady black (0.36). The frequency of S_2S_2 in pooled population was recorded as 0.47. Comparable values were reported in Jamunapari (0.94), Sirohi (0.89), Beetal (0.84), Gaddi (0.80), Osmanabadi (0.76), Marwari (0.69), Barbari (0.68) and Surti (0.42) by Kumar *et al.* (2006). Lower frequencies for S_2S_2 genotypes were reported by Pena *et al.* (2000) in French (0.09) and Spanish (0.11) and Elmaci *et al.* (2009) in Turkish hair goat (0.11).

5.1.2 β-LG Nucleotide Sequence Analysis

The sequencing of β-LG gene fragment was performed by an automated DNA sequencer at the DNA Sequencing Facility, Bioserve, Biotechnologies (India), Pvt. Ltd. Hyderabad. A few nucleotides were lost from both ends of the amplified products. So the size of the resultant sequence (391 bp and 393 bp fragments, respectively for Malabari and Attappady black samples) was lesser than that of the amplified product (426 bp).

The 99 per cent identity with the published *Capra hircus* (Accession # Z33881.1) gene encoding β-LG on nBLAST of the resultant sequences confirmed the amplification of β-LG gene in all the samples studied. The 1 per cent reduction in identity could be due to C to A transversion at 7062 position.

The present sequence analysis revealed G to A transition at 6751 position which is responsible for the restriction fragment length polymorphism. Pena *et al.* (2000) also reported the same nucleotide change at the same position in Spanish and French breeds.

5.2 AMPLIFICATION OF THE α-LA GENE FRAGMENT

The size of the amplified product (268 bp) was the same in all the DNA samples under study confirming the conservation of DNA sequence at that locus. The same sized PCR products were reported by Lan *et al.* (2007) in Chinese goat breeds.

5.2.1 Restriction Enzyme Digestion of the α-LA Gene Fragment

On restriction analysis with the endonuclease *Msp* I monomorphic pattern with a single allele A_1 was obtained. Lan *et al.* (2007) reported similar results in Xinong saanen dairy, Laoshan dairy and Leizhou breeds of China. But presence of A_2 allele at a very low frequency was reported in Inner Mongolia White Cashmere (0.017), Guizhou Black (0.024) and Guanzhong dairy (0.024) by the same author.

5.3 β-LG POLYMORPHISM AND DAIRY TRAITS

A significant number of studies have been conducted on association of protein polymorphism of β-LG with milk production and composition traits. Ng-Kwai-Hang (1997) as well as Winkleman (1997) opined that 'A' variant of β-LG was associated with higher milk yield in cattle compared to the 'C' variant whereas Tsiaras *et al.* (2005) reported that β-LG genotype AB was associated with higher milk yield than AA in Holstein cows.

According to Ng-Kwai Hang (1990) fat content was not influenced by protein variants of β-LG. But Winkleman (1997) and Amigo *et al.* (2000) associated the bovine β-LG protein variants with fat and concluded that cows with AA recorded higher fat value compared to CC genotypes. Contradictory to the above findings, Tsiaras *et al.* (2005) reported that β-LG BB and AB were significantly associated with higher fat than AA genotype. Amigo *et al.* (2000) reviewed that out of the three protein variants (A, B, and C) of ovine β-LG, the 'A' variant exhibited higher total solids ($p<0.05$). Though several association studies were reported for protein variants of β-LG with milk production traits, no such study has been yet reported for β-LG gene polymorphism with milk production traits in goats.

The average peak milk yield for Malabari, Attappady black and crossbred goats were 440.22±35.02 ml, 400±58.78 and 637.21±47.50 ml, respectively. The minimum Peak yield in all the above mentioned populations was 200 ml where as the maximum values were 1200, 800 and 1500 ml in Malabari, Attappady black and crossbred goats, respectively. A significantly higher yield was observed in crossbred goat population of the university farm, indicative of selection for milk production in the farm.

Present study revealed that Malabari goats carrying S_2 allele showed a significantly higher average ($p<0.05$) for peak milk yield (420±74.24 ml and 501.79±46.47 ml) compared to animals homozygous for S_1 allele (250±13.36 ml). Even though the average peak yield values for S_1S_1 (800.0±115.48), S_1S_2 (780.0±198.49) and S_2S_2 (602.86±50) were higher in crossbred than Malabari, no significant association between the genotype and peak yield could be observed.

In the present study the average milk fat in percentage was 4.10±0.14, 3.76± 0.26 and 4.14±0.22 in Malabari, Attappady Black and crossbred goats, respectively.

The mean milk fat percentage for S_1S_2 genotype (4.21±0.26) was found to be higher than that of S_1S_1 (3.99±0.25) and S_2S_2 (4.02±0.18) in Malabari goat. In case of Attappady black breed S_1S_1 genotype (5.60±0.0) showed a higher fat value compared to S_1S_2 (3.71±0.2) and S_2S_2 (3.50±0.65) whereas crossbred population showed a lower value for S_1S_1 (3.2±0.25) compared to S_1S_2 (4.66±0.54) and S_2S_2 (4.16±0.27). All these differences were without any statistical significance.

The average value of milk total solids in percentage of Malabari, Attappady black and crossbred goats were found to be 14.15±0.25, 13.70±0.38 and 14.32±0.31. Even though Attappady black showed a lesser value than the other two populations, the difference was not significant.

The average total solids value was higher for S_2S_2 (14.38±0.37) than S_1S_2 (14.16±0.42) and S_1S_1 (13.69±0.45) in Malabari goat. But in crossbred goat population a lesser value for S_2S_2 (14.13±0.32) than S_1S_1 (15.3±1.88) and S_1S_2 (14.57±0.72) genotypes was recorded whereas in Attappady black population the value was lesser for S_1S_2 (13.43±0.09) than S_1S_1 (15.95±0.0) and S_2S_2 (13.66±2.17). All these differences were without any statistical significance. Lower sample size in case of Attappady black and crossbred goat population could be the reason for higher value of S_1S_1.

In the present study, the average value for milk solids not fat in 60-90 days of lactation was 10.05±0.18, 10.04±0.29 and 10.18±1.75 in Malabari, Attappady black and crossbred goats, respectively.

A higher mean for solids not fat value was obtained for S_2S_2 (10.35±0.28) than S_1S_1 (9.71±0.34) and S_1S_2 (9.96±0.30) in Malabari. Attappady black breed also followed the same trend with the highest value for S_2S_2 (10.6±1.51) followed by S_1S_1 (10.35±0.0) and S_1S_2 (9.85±0.25). Contradictory results were obtained in crossbred goat population with the highest value for S_1S_1 (12.1±1.75) followed by S_2S_2 (9.98±0.29) and S_1S_2 (9.91±0.30). All these differences were not statistically significant.

Beta lactoglobulin has been recognized as an important dairy trait marker in ruminants. From the present study a significant association could be detected for β-LG polymorphism with peak milk yield in Malabari breed. A statistically significant association could not be established between β-LG polymorphism and the other milk production traits. The significance of β-LG polymorphism as a dairy trait marker in goats can be improved by extending the study to the goat populations of entire state. The result of present study can definitely be used to complement the present breeding programme in goats and thereby improve the production potential of our goat population.

Summary

6. SUMMARY

With the development of Polymerase Chain Reaction (PCR) and sequencing technologies, DNA-based polymorphisms are now the markers of choice for evaluation of economically important traits in livestock. Milk production traits being polygenic, are controlled by a number of genes. Variations in those genes can have associations with milk production traits. In the present study the associations of polymorphisms of β-LG/*Sac* II and α-LA/*Msp* I with dairy traits were analyzed in Malabari, Attappady black and crossbred goats of Kerala.

DNA was isolated from blood samples of 65 Malabari, 25 Attappady black 30 cross bred goats using phenol chloroform extraction procedure. For PCR-RFLP analysis of β-LG gene, 426 bp fragment from exon 7 to proximal 3' flanking region enclosing the polymorphic site was amplified with specific primers. The PCR products were digested with restriction enzyme, *Sac* II at 37°C overnight in dry bath. On non-denaturing polyacrylamide gel (8 per cent), three restriction digestion patterns were revealed with two alleles namely S_1 (two bands of 349 and 77 bp) and S_2 (a single band of 426 bp). The allele sizes were confirmed using 100bp DNA ladder as the size marker.

In addition to the above study a 268 bp fragment of caprine α-LA gene spanning exon 3 and a part of the flanking region was amplified. The amplified fragments were subjected to digestion with *Msp* I enzyme at 37°C for 3 hours. On agarose gel electrophoresis (2.5 per cent) only a single allele A_1 (a single band of 268 bp) could be obtained in the entire population under study suggesting the monomorphic nature of the locus.

The S_1/S_2 allele frequencies in Malabari, Attappady black and crossbred goat population were 0.37/0.63, 0.34/0.66 and 0.18 /0.82, respectively indicating the predominance of S_2 allele. *Chi*-square test revealed that the S_1 and S_2 alleles were uniformly distributed in Malabari, Attappady black and crossbred goat populations.

The genotype frequencies of β-LG/*Sac* II polymorphism were 0.14 (S_1S_1), 0.46 (S_1S_2) and 0.40 (S_2S_2) in Malabari, 0.04 (S_1S_1), 0.60 (S_1S_2) and 0.36 (S_2S_2) in Attappady black and 0.10 (S_1S_1), 0.17 (S_1S_2) and 0.73 (S_2S_2) in crossbreds. All the populations under study were in Hardy-Weinberg equilibrium with regard to β-LG/*Sac* II locus. Malabari goat samples were further grouped according to the region as Tellicherry, Badagara and University farm population. The region wise analysis of allele frequencies proved that S_1 and S_2 alleles in Tellicherry (0.39 and 0.61), Badagara (0.32 and 0.68) and University farm (0.39 and 0.61) population were homogenously distributed in all the populations. *Chi*-square analysis proved that the genotype frequencies of S_1S_1, S_1S_2 and S_2S_2 in Tellicherry (0.14, 0.50 and 0.36), Badagara (0.2, 0.25 and 0.55) and University farm (0.09, 0.61 and 0.30) populations were in Hardy-Weinberg equilibrium.

The average peak yield in crossbred population (637.21±47.50 ml) was significantly higher ($p<0.05$) than Malabari (440.22±35.02 ml) and Attappady black goat (400±58.78 ml) population. The Malabari goats carrying S_2 allele showed a significantly higher average ($p<0.05$) for peak milk yield (420±74.24 ml and 501.79±46.47 ml) compared to animals homozygous for S_1 allele (250±13.36 ml). The average values of peak yield for S_1S_1, S_1S_2 and S_2S_2 in crossbred goats were found to be 800.0±115.47, 780.0±198.49 and 602.86±50.08, respectively with no significant association detected between β-LG/*Sac* II polymorphism and peak milk yield.

The average milk fat percentage was the highest in crossbred goats (4.14±0.22) followed by Malabari (4.10±0.14) and Attappady black (3.76±0.26) but without any statistically significant difference between mean values. The mean values for milk fat percentage were found to be 3.99±0.25, 4.21±0.26 and 4.02±0.18 for S_1S_1, S_1S_2 and S_2S_2, respectively in Malabari goat. In case of Attappady black breed, 5.6±0.0, 3.71±0.2 and 3.50±0.65 for S_1S_1, S_1S_2 and S_2S_2, respectively. The crossbred population showed 3.2±0.25, 4.66±0.54 and 4.16±0.27 for S_1S_1, S_1S_2 and S_2S_2, respectively. The mean values were not significantly related to β-LG/*Sac* II polymorphism.

The average total solids value in crossbred (14.32±0.31) goats was higher than Malabari (14.15±0.25) and Attappady black (13.70±0.38) but not statistically significant. The mean values for milk total solids in percentage were 13.69±0.45, 14.16±0.42 and 14.38±0.37 for S_1S_1, S_1S_2 and S_2S_2 in Malabari, 15.95±0.0, 13.43±0.09 and 13.66±2.17 in Attappady and 15.3±1.88, 14.57±0.72 and 14.13±0.32 in crossbred goat population, respectively. The values were not significantly related to β-LG/*Sac* II polymorphism.

Population averages for milk solids not fat in percentage in Malabari, Attappady black and crossbred goats in 60-90 days of lactation were 10.05±0.18, 10.04±0.29 and 10.18±1.75, respectively but the differences between mean values were not statistically significant. The mean values of solids not fat for genotypes S_1S_1, S_1S_2 and S_2S_2 were 9.71±0.34, 9.96±0.30 and 10.35±0.28 in Malabari, 10.35±0.0, 9.85±0.25 and 10.6±1.51, in Attappady and 12.1±1.75, 9.91±0.30 and 9.98±0.29, in crossbred goat population, respectively. The values were not significantly related to β-LG/*Sac* II polymorphism.

From the present study a significant association could be detected for β-LG polymorphism with peak milk yield in Malabari breed. But β-LG polymorphism was not significantly associated with other milk production traits in the population under study. So the β-LG polymorphism can be considered as a useful dairy trait marker in goat selection.

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Annexures

ANNEXURE – 1

COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

Acrylamide (30%)

Acrylamide	29 g
N_1N – Methylene bisacrylamide	1 g
Water to	100 ml

Acrylamide (40%)

Acrylamide	380 g
N_1N – Methylene bisacrylamide	20 g
Water to	1000 ml

Agarose (0.8%)

Weighed 0.8 g of agarose powder and mixed with 100 ml of 1X TBE buffer in a conical flask. Solution was heated in a microwave oven until boiling and cooled slowly.

Agarose (1.5%)

Weighed 1.5 g of agarose powder and mixed with 100 ml of 1X TAE buffer in a conical flask. Solution was heated in a microwave oven until boiling and cooled slowly.

Ammonium persulphate (10 %)

Ammonium persulphate	100 mg
Water to	1 ml

Denaturing Polyacrylamide Gel

0.5 X TBE Gelmix	60 ml

TEMED	0.125 ml
Ammonium persulphate (10 %)	0.125 ml

Mixed well without air bubbles.

EDTA (0.5*M*, pH 8.3)

Dissolved 18.61 g of EDTA (disodium, dihydrate) in 80 ml of distilled water by bringing the pH to 8.3 with NaOH solution and volume made upto 100 ml. Stored at room temperature after filtration and autoclaving.

Ethidium Bromide (10 mg/ml)

Dissolved 100 mg ethidium bromide in 10 ml of distilled water. Solution stored at 4°C in a dark coloured bottle.

Gel loading buffer

Bromophenol blue	0.25 %	50 mg
Xylene cyanol	0.25 %	50 mg
Sucrose	40 %	8 g

Components stirred well in 20 ml distilled water and stored at 4°C.

Nondenaturing Polyacrylamide Gel

TBE Gelmix	70 ml
TEMED	0.05 ml
Ammonium persulphate (10 %)	0.85 ml

Mixed well without air bubbles.

Phenol (Saturated, pH 7.8)

Commercially available crystalline phenol melted at 65°C in a water bath. Hydroxyquinolone added to a final concentration of 0.1 percent. To the molten phenol,

0.5 *M* Tris HCl (pH 8.0) was added in equal volume and stirred for 30 minutes in a magnetic stirrer. The contents were transferred into a separating funnel. Lower phenolic phase was collected and mixed with equal volume of 0.1 *M* Tris HCl (pH 8.0) and stirred again for 30 minutes. The phenolic phase was collected and extraction repeated with 0.1 *M* Tris HCl (pH 8.0) until the pH of phenolic phase was more than 7.8. Finally 0.1 volume of 0.01 *M* Tris HCl (pH 8.0) added and stored in a dark bottle at 4°C.

RBC lysis buffer

Ammonium chloride	150 m*M*	8.0235 g
Potassium chloride	10 m*M*	0.7455 g
EDTA	0.1 m*M*	0.0372 g

Dissolved the contents in distilled water and volume made up to 1000 ml. Stored at 4°C after filtration and autoclaving.

Sodium acetate

Dissolved 40.824 g of Sodium acetate in 70 ml of distilled water and pH adjusted to 5.5 with glacial acetic acid. Volume made up to 100 ml, autoclaved and stored at room temperature.

Sodium chloride (5 *M*)

Dissolved 29.22 g of sodium chloride in 80 ml distilled water and volume made up to 100 ml. Solution filtered and stored at room temperature.

Sodium chloride- EDTA (SE) buffer (pH, 8.0)

Sodium chloride	75 m*M*	4.383 g
EDTA	35 m*M*	9.306 g

Dissolved the contents in 900 ml distilled water and pH adjusted to 8.0. Made up the volume to 1000 ml, filtered, autoclaved, stored at 4°C.

Sodium dodecyl sulphate (SDS) 20 %

SDS	20 g
Distilled water make up to	100 ml

Stirred, filtered and stored at room temperature.

Tris Acetate EDTA (TAE) buffer (50X)

Tris base	48.4 g
Glacial acetic acid	11.42 ml
0.5 M EDTA (pH 8.0)	20 ml
Distilled water up to	1000 ml

Autoclaved and stored at room temperature.

Tris-Borate (TBE) buffer pH 8.3) 10X

Tris base	108.0 g
Boric acid	55.0 g
EDTA	9.3 g

Dissolved in 700 ml of distilled water and pH adjusted to 8.3. Volume made up to 1000 ml, autoclaved and stored at room temperature.

TBE Gel mix for Nondenaturing PAGE

30% Acrylamide	106.8 ml
5X TBE buffer	80.0 ml
Distilled water to	400 ml

Mixed well and stored at 4°C.

Tris Buffered Saline (TBS) pH 7.4

Sodium chloride	140 m*M*	8.18 g
Potassium chloride	0.5 m*M*	0.0373 g
Tris base	0.25 m*M*	0.0303 g

Dissolved in 900 ml of distilled water and pH adjusted to 7.4. Made up the volume to 1000 ml, filtered, autoclaved and stored at 4°C.

Tris EDTA (TE) buffer (pH 8.0)

Tris base	10 m*M*	1.2114 g
EDTA	0.1 m*M*	0.3722 g

Dissolved in 900 ml of distilled water and adjusted the pH to 8.0. Made up the volume to 1000 ml, filtered, autoclaved and stored at 4°C.

Tris 1M (pH 8.0)

Tris base	121.14 g

Added distilled water up to 1000 ml, pH adjusted to 8.0, filtered and stored at room temperature.

ANNEXURE – II

SOURCES OF IMPORTANT CHEMICALS AND ENZYMES USED IN THIS STUDY

(A) CHEMICALS

Acrylamide (Molecular biology grade)	-	SRL, Bombay
Agarose (Low EED)	-	Bangalore Genei Pvt. Ltd.
Ammonium chloride	-	SRL, Bombay
Ammonium per sulphate	-	SRL, Bombay
N-N-Methylene bis acrylamide	-	SRL, Bombay
Boric acid	-	SRL, Bombay
Chloroform	-	Merck
Crystalline phenol	-	Merck
dNTPs	-	Sigma
EDTA	-	SRL, Bombay
Ethanol	-	Merck
Ethidum bromide	-	BDH lab, England
6X gel loading buffer	-	Bangalore Genei Pvt. Ltd.
Glacial acetic acid	-	SRL, Bombay
Hydroxy quinolone	-	Qualigens Chemicals, Mumbai
Isoamyl alcohol	-	Merck
Methanol	-	SRL, Bombay
Potassium chloride	-	SRL, Bombay
Sodium acetate	-	SRL, Bombay
Sodium chloride	-	SRL, Bombay
Sodium dodecyl sulphate (SDS)	-	SRL, Bombay
TEMED	-	SRL, Bombay
Tris base	-	SRL, Bombay
Urea	-	SRL, Bombay

(B) PRIMERS -Integrated DNA technologies

(C) MOLECULAR MARKERS

100 bp DNA size ladder - Bangalore Genei Pvt. Ltd.

(D) ENZYMES

Restriction endonuclease, *Sac* II	-	Labmate (Asia) Pvt. Ltd.
Restriction endonuclease, *Msp* I	-	Bangalore Genei Pvt. Ltd.
Taq DNA polymerase	-	Bangalore Genei Pvt. Ltd.
Proteinase-K	-	Bangalore Genei Pvt. Ltd.

ANNEXURE – III

ABBREVIATIONS

α-LA	Alpha lactalbumin
β-LG	Beta lactoglobulin
RFLP	Restriction Fragment Length Polymorphism
PCR	Polymerase Chain Reaction
DNA	Deoxyribo Nucleic Acid
PAGE	Polyacrylamide Gel Electrophoresis
EDTA	Ethylene Diamine Tetra acetic Acid
TEMED	N, N, N′, N′ Tetra methyl ethylene diamine
APS	Ammonium Persulphate
µl	microlitres
µg	microgram
mg	milligram
m*M*	millimolar
cm	centimeter
nm	nanometer
pmol	picomols
kb	Kilo basepair
bp	base pair
SDS	Sodium Dodecyl Sulphate
dNTP	Deoxy Nucleotide Triphosphate

BETA LACTOGLOBULIN POLYMORPHISM IN GOATS OF KERALA

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ABSTRACT

Beta lactoglobulin gene polymorphism and its association with various milk production traits were investigated in DNA samples isolated from the blood of 20 Malabari, 25 Attappady black and 30 crossbred goats of University goat farm as well as 45 Malabari goats from the native tract of the breed. Study was conducted by PCR-RFLP using *Sac* II restriction endonuclease. Apart from this study a related gene α-lactalbumin was also analysed by the same technique using a different enzyme *Msp* I.

Digestion of the amplified PCR product with *Sac* II restriction endonuclease revealed three genotypes (S_1S_1, S_1S_2 and S_2S_2) in the goat population under study, indicating the presence of S_1 (349 and 77 bp fragments) and S_2 (426 bp fragment) alleles. The S_1/S_2 allele frequencies were 0.37/0.63, 0.34/0.66 and 0.18/0.82, respectively in Malabari, Attappady black and crossbred goats. The Malabari goat population under study with genotype frequencies 0.14 (S_1S_1), 0.46 (S_1S_2) and 0.40 (S_2S_2), was found to follow H-W equilibrium. This population was further classified into three and the genotypic frequencies of S_1S_1, S_1S_2 and S_2S_2 in Tellicherry (0.14, 0.50 and 0.36), Badagara (0.2, 0.25 and 0.55) and University farm (0.09, 0.61 and 0.30) were in Hardy-Weinberg equilibrium. But the genotypes of β-LG/*Sac* II polymorphism were distributed according to Hardy-Weinberg equilibrium with frequencies 0.04 (S_1S_1), 0.60 (S_1S_2) and 0.36 (S_2S_2) in Attappady black and 0.10 (S_1S_1), 0.17 (S_1S_2) and 0.73 (S_2S_2) in crossbred goats under study. Restriction analysis of α-LA gene revealed a single genotype (A_1A_1) in the entire population under study indicating the monomorphic nature of the locus.

From the present study a significant association could be detected for β-LG polymorphism with peak milk yield in Malabari breed. Malabari goats carrying S_2

allele showed a significantly higher average ($p<0.05$) compared to animals homozygous for S_1 allele. A statistically significant association could not be established between β-LG polymorphism and other milk production traits. The results of the present study suggest the β-LG gene polymorphism as an important dairy trait marker to complement the present breeding programme in goats.