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LEPTIN GENE POLYMORPHISM IN VECHUR AND CROSSBRED CATTLE OF KERALA

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DECLARATION

I hereby declare that this thesis, entitled **"LEPTIN GENE POLYMORPHISM IN VECHUR AND CROSSBRED CATTLE 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.

Mannuthy
18-05-2009

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Certified that this thesis, entitled **"LEPTIN GENE POLYMORPHISM IN VECHUR AND CROSSBRED CATTLE OF KERALA"** is a record of research work done independently by **Dr. Lali F. Anand (2007-13-117)**, 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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Lali F. Anand

CONTENTS

LIST OF TABLES

LIST OF PLATES

INTRODUCTION

1. INTRODUCTION

India is a repository of domestic animal biodiversity. Diversified cattle breeds contribute remarkably to the dairy sector which safeguards the rural economy. In Kerala, dairying provides subsidiary income to the households. According to National Bureau of Animal Genetic Resources (NBAGR), there are 30 recognized cattle breeds in India, of which Vechur (*Bos indicus*) is the only one from Kerala.

Vechur cattle owe its name to the village of its origin, Vechur, a place near Vaikom in Kottayam District of Kerala, on the banks of the fresh water lake 'Vembanade'. Regardless of its low milk production, the smaller sized and docile natured Vechur cattle are famous as a household animal. This breed is considered as one of the smallest cattle breeds in the world. Vechur bull and cow measures an average of 99 and 89 cm in height and 104 and 93 cm in length, respectively (Iype and Venkatachalapathy, 2001). The characteristic features of Vechur cattle include high milk fat percentage (4.7), smaller sized milk fat globule (3.21 microns), low level of feed requirements and high disease resistance (Venkatachalapathy, 1996; Raghunandanan, 2006). Milk fat percentage, being an important criterion that decides milk pricing, the genetic basis of the same need to be investigated upon, which shall aid in proper selection and further improvement of the breed.

Selection is the most effective method to improve the performance of a varied population. One way to study genetic diversity is by the determination of genetic variability through polymorphism. Advancements in molecular biology devised efficient methodologies like microsatellite marker analysis, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), Single Strand Conformation Polymorphism (SSCP), Amplified Fragment Length Polymorphism (AFLP) and Amplification Refractory Mutation System - Polymerase Chain Reaction

(ARMS-PCR), to elucidate polymorphic patterns of various genes. Once these genes are proven to be associated with traits of interest in animals, these markers can be incorporated in marker assisted selection (MAS) in order to complement the traditional selection methods.

Various genes are found to be associated with production traits in cattle and leptin gene is one among them. Leptin is involved in the regulation of feed intake, energy metabolism, fertility and immune response (Houseknecht *et al*., 1998) which renders it as a potential candidate for Quantitative Trait Loci studies and MAS. If association between leptin gene polymorphism and milk production traits exists in the indigenous or crossbred cattle population of Kerala, it will be useful for selection based on molecular markers. The present study was designed to explore this possibility and to screen Vechur and crossbred cattle of Kerala for genetic polymorphism of leptin gene with the following objectives:

1. To reveal polymorphic pattern of leptin gene by PCR-RFLP and microsatellite analysis
2. To compare the allele frequencies of leptin gene loci in crossbred and Vechur cattle
3. To associate leptin genotypes with milk yield and milk fat percentage

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The word leptin is derived from the Greek root *leptos*, meaning thin. Leptin is a protein hormone that regulates food intake, energy expenditure, reproduction, tissue development and immune response. A nimiety of information exists regarding leptin which recommends it as a potential candidate for Quantitative Trait Locus (QTL) in Marker Assisted Selection (MAS).

2.1 LEPTIN

Leptin is a 16-kilodalton protein consisting of 146 amino acids and was discovered by Zhang *et al*. (1994) as leptin gene product in genetically obese mice. They cloned and sequenced leptin gene (LEP) for the first time.

Location of leptin gene in animals and human was illustrated by various scientists; in mice on chromosome 6 (Friedman and Leibel, 1992), human on chromosome 7 (Isse *et al*., 1995), cattle on chromosome 4 (Barendse *et al*., 1994; Stone *et al*., 1996b) and swine on chromosome 18 (Sasaki *et al*., 1996).

While cloning the human *ob* gene, Isse *et al*. (1995) studied the structural organization of the gene and demonstrated the presence of three exons and two introns. The coding region for leptin gene was included within exons two and three, and exon one was nontranslated.

Zhang *et al.* (1997) reported that leptin had 67% sequence identity among species such as human, gorilla, chimpanzee, orangutan, rhesus monkey, dog, cow, pig, rat and mouse.

Leptin gene had been cloned in most of the domestic animal species such as sheep (Dyer *et al*., 1997) , cow (Ji *et al*., 1998), swine (Ramsay *et al*., 1998), dog (Iwase *et al*., 2000), cat (Sasaki *et al*., 2001), horse (Buff *et al*., 2002), camel, Egyptian water buffalo (Sayed –Ahmed *et al*., 2003) and yak (Dongre *et al.,* 2009). Taouis *et al*. (1998) cloned and sequenced chicken leptin gene and Pitel *et al*. (1999) proved the location of *ob* gene in chicken microchromosome by linkage analysis.

Taniguchi *et al*. (2002) analyzed the exon-intron organization of the bovine leptin gene and reported that leptin gene consisted of three exons and two introns and spanned about 18.9 kb, equivalent to that of human or mouse gene.

The total length of leptin gene was reported as 4067 bp in *Bos taurus* cattle (Tellam, 2004) while 3897 bp in *Bos indicus* (Dubey, 2008).

2.1.1 Leptin-Structure

The hormone, leptin is classified in the family of helical cytokines along with interleukin-2 and growth hormone based on the three dimensional four-helix bundle structure illustrated by threading analysis (Madej *et al*., 1995).

According to Zhang *et al.* (1997), leptin was composed of four antiparallel helices and interconnecting loops. Parallel to the helix bundle, a large hydrophobic area was formed. Leptin's two cysteine residues at 96 and 146 positions established a disulphide bond. Both of these cysteines were conserved among species and mutation of either of them resulted in a biologically inactive form.

2.1.2 Leptin – A Pleiotropic Molecule

The most important function of this pleiotropic molecule is to orchestrate the whole body energy homeostasis to maintain the body lean. It is also involved in the regulation of appetite, energy expenditure, reproduction, wound healing and immune response. Zhang *et al*. (1997) first explained the importance of leptin while cloning and sequencing the leptin gene in genetically obese mice. They revealed the reason for genetic obesity as a single base mutation (Cytosine to Thymine) at position 105 of leptin gene which produced a biologically inactive, truncated form of leptin. Thus the genetically obese mice (*ob/ob* mice) were characterized by increased food intake, reduced weight loss and infertility.

2.1.2.1 Appetite regulation

Halaas *et al*. (1995) studied the effect of leptin treatment in *ob/ob* mice and found that daily intraperitoneal injections of either mouse or human recombinant obese protein (leptin) reduced the body weight of *ob/ob* mice by 30% after two weeks of treatment, with no apparent toxicity. The protein reduced food intake and increased energy expenditure in *ob/ob* mice.

Weigle *et al*. (1995) also reported reduction in body weight and feed intake by the administration of OB protein to *ob/ob* mice and suggested that this molecule could play a critical role in regulating total body fat content.

Detailed studies on physiological function of leptin in energy metabolism by Pelleymounter *et al*. (1995) and Campfield *et al*. (1995) propounded that intraperitoneal administration of recombinant leptin lowered their body weight, body fat percentage, food intake and serum concentrations of glucose and insulin. In

addition, metabolic rate, body temperature and activity levels were increased by this treatment and suggested that the OB protein normalized the metabolic status of the *ob/ob* mice.

The mechanism of action of leptin was elucidated by Banks *et al*. (1996). The researchers illustrated that leptin was synthesized and secreted from white adipocytes into blood and transported into brain where it mediated the release or inhibition of factors with a final outcome of reduction in food intake, increase in energy expenditure and physical activity. Additionally a negative feed back loop was also observed to inhibit further expression of leptin gene.

Schwartz *et al*. (1996) identified neuropeptide-Y (NPY) as the most important factor mediating leptin action. Two injections of leptin (3.5 mg intracerebroventricularly) significantly decreased NPY mRNA and down regulated the satiety centre.

According to Flier (1998) rising levels of leptin resisted obesity through brain signaling. When this signal was deficient, the brain promoted the physiological responses to increase appetite or decrease energy expenditure, resulting in energy storage and weight gain.

2.1.2.2 Reproduction

Barash *et al*. (1996) demonstrated the action of leptin on the reproductive endocrine system in both sexes of *ob/ob* mice and suggested that leptin might serve as a permissive signal to the reproductive system of normal animals. Leptin treated females had significantly elevated serum levels of Leutinizing Hormone (LH), increased ovarian and uterine weights and stimulated aspects of ovarian and uterine

histology compared to controls. Leptin treated males had significantly elevated serum levels of Follicle Stimulating Hormone (FSH), increased testicular and seminal vesicle weights, greater seminal vesicle epithelial cell height and elevated sperm counts compared to controls.

Experiments with recombinant human leptin in female *ob/ob* mice, Chehab *et al*. (1996) found that subsequent generations of female *ob/ob* pups were unable to reproduce following leptin withdrawal, demonstrating that correction of sterility required continuous leptin treatment.

While studying the action of leptin in obese mice, Ahima *et al*. (1996) stated that constant absence of leptin in circulation could interpret by the central neural networks as absence of energy stores and resulted in a constant pre-pubertal state and an ensuing shut down of reproduction.

Nagatani *et al.* (2000) proved the hypothesis that leptin was an important nutritional signal that modulated reproductive activity by regulating LH secretion in sheep. They reported that leptin administration prevented the fasting induced suppression of LH secretion. Chilliard *et al*. (2001) and Ehrhardt *et al*. (2001) explained the influence of leptin on embryonic development in ruminants. They found that leptin mRNA level was high during early pregnancy and reduced in late pregnancy and lactation in sheep.

According to Spicer (2001), leptin receptor and its mRNA were present in ovarian tissue of several species, including cattle. These functional receptors imparted direct effect on gonads by inhibiting insulin-induced steroidogenesis. Experiments in rodents revealed the presence of leptin receptor mRNA in the testes and it inhibited human chorionic gonadotropin induced testosterone secretion by Leydig cells.

2.1.2.3 Immune Response

The role of leptin in immune response was first observed by Lord *et al*. (1998). They reported that administration of leptin in mice reversed the immunosuppressive effects of starvation. They successfully illustrated the stimulatory effect of leptin on T-lymphocytes.

Sierra-Honigmann *et al*. (1998) conducted *in vivo* and *in vitro* assays and revealed the angiogenic activity of leptin. *In vivo,* leptin induced neovascularization in corneas of normal rats while *in vitro* cultures displayed tissue microvasculature. They suggested that by providing a local angiogenic signal, leptin improved the efficiency of lipid release from fat stores to maintain energy homeostasis.

Alteration in immune and inflammatory responses was detected in leptin or leptin receptor deficient animals by Fantuzzi and Faggioni (2000). Proliferative and apoptotic activities of T-lymphocytes, macrophages or haematopoietic proginators were regulated by leptin. They also noticed that leptin production was acutely increased during infection and inflammation.

Chan *et al*. (2007) elucidated the role of leptin in regulating neuroendocrine and immune functions in women. Fasting for 72 hours decreased leptin levels in women having normal weight by about 80% and consequent reduction in the number of circulating cells of immune system.

On the basis of studies in obese mouse, Claycombe *et al*. (2008) outlined the role of leptin in lymphopoiesis and myelopoiesis. The *ob/ob* mice had only 60% nucleated cells and 70% fewer B- lymphopcytes in bone marrow compared to lean

controls. They noticed doubling of B-cell number and normal percentage of monocytes and granulocytes after seven days of leptin treatment.

2.1.2.4 Tissue Development

Ahima *et al*. (1999), studying the brain development in obese mice, reported that leptin treatment for six weeks increased brain weight and suggested that leptin was required for normal neuronal maturation.

Ahima and Flier (2000) studied leptin level in starvation and concluded that homoestatic mechanisms to adapt the drastic changes in physiological status or environmental conditions were orchestrated by expression of leptin and leptin receptors.

Frank *et al*. (2000), studying the role of leptin in wound healing, stated that supplementation of leptin improved re-epithelialization of wounds in *ob/ob* mice. Leptin completely reversed the atrophied morphology of wound margins of leptin deficient animals into a well organized hyperproliferative epithelium. Ring *et al*. (2000) investigated the wound healing effect of leptin in *ob/ob* mice and opined that leptin accelerated wound healing.

Cao *et al*. (2001) explained leptin's role in the formation of fenestrated capillaries and increased permeability. Intradermal leptin administration was resulted in rapid vascular permeability response in *ob/ob* mice. The researchers suggested that the increased vascular permeability was due to the formation of fenestrated capillaries mediated by leptin.

Serum leptin level was considered as a regulator of bone mass by Elefteriou *et al*. (2004) and they demonstrated that increased serum leptin level in mice dramatically reduced bone mass. They suggested that leptin was a determinant of bone formation and its antiosteogenic function was conserved in vertebrates.

2.2 PCR-RFLP ANALYSIS IN BOVINE LEPTIN GENE

PCR-RFLP is an efficient method to detect Single Nucleotide Polymorphisms (SNPs) at various loci. SNPs are resulted due to a single base variation in the DNA sequence. In bovine leptin gene SNPs were detected in exonic, intronic and promoter regions (van der Lende *et al*., 2005).

2.2.1 C305T Mutation in Bovine Leptin Gene

Konfortov *et al*. (1999) resequenced leptin gene in diverse panel of cattle and displayed 20 SNPs. It was suggested that C/T variation at position 305 resulted in Arg to Cys substitution and it represented an abundant polymorphism with alleles C (0.59) and T (0.41).

Buchanan *et al*. (2002) uncovered one SNP in exon two and five SNPs in exon three of leptin gene. They propounded that all of those SNPs except one located at 73 bp from the start of exon two and another located 95 bp from the start of exon 3 were at silent codon positions. It was stated that the SNP at exon two was developed due to mutation of nucleotide cytosine to thymine which resulted in an aminoacid change from Arginine to Cysteine. They designed a PCR-RFLP with restriction enzyme *Kpn*21 to distinguish this functional mutation and identified two alleles as C (75 bp+19 bp) and T (94 bp) with frequencies 0.54 and 0.46, respectively in beef

breeds. The researchers succeeded in associating the T allele with fatter beef carcasses and the C allele to leaner beef carcasses.

Buchanan *et al*. (2003) extended the research on *Kpn*21 SNP in leptin gene to dairy cattle and found that animals homozygous for T allele (TT) produced more milk (1.5 kg/day) than CC animals. The increase in milk yield was most prominent in first 100 days of lactation (2.44 kg/day) declining to 1.74 kg/day between 101 and 200 days of lactation. They observed no significant reduction in fat percentage associated with T allele. A significant increase in milk protein yield and somatic cell count linear score was also detected in animals with TT genotype.

While investigating the association of leptin gene polymorphisms with leptin concentration, Liefers *et al*. (2003) genotyped Holstein Friesian cattle for three SNPs namely R4C (C/T SNP on exon two), A59V (C/T SNP on exon three) and C/T SNP on the second intron. R4C SNP was disclosed by single nucleotide extension and detected three genotypes (CC, CT and TT) with frequencies 0.46, 0.42 and 0.12. Of these, animals with CC genotype showed significantly higher serum leptin concentration.

The allele frequencies of *Kpn*21 polymorphism in Polish black and white breed were reported as C (0.54) and T (0.46) by Madeja *et al.* (2004). They found that the genotypes were distributed according to Hardy Weinberg law.

Choudhary *et al.* (2005) performed *Kpn*21 and *Bsa*A1 RFLPs in *Bos indicus, Bos taurus* and in their crossbreds and reported only CC genotype in indigenous breeds studied (Hariana, Sahiwal, Gir and Nimari), indicating the absence of C to T mutation. The frequencies for the C allele in crossbreds, Holstein Friesian and Jersey

cattle were 0.82, 0.60 and 0.44, respectively while the correspondent frequencies of T allele were 0.18, 0.40 and 0.56.

Kononoff *et al*. (2005), reported the genotype frequencies as 0.25 (CC), 0.51 (CT) and 0.25 (TT) in crossbred beef cattle for leptin SNP (C/T mutation in exon 2). They also observed association between leptin genotypes and quality grade, yield grade and weight of beef carcasses. According to Schenkel *et al*. (2005), T allele was found to be predominant over C allele for *Kpn*21 SNP (0.611 vs 0.389) in crossbred beef cattle. They opined that C allele was associated with less fat yield and grade fat and more lean yield.

Barendse *et al*. (2005) reported that the leptin C73T missense mutation (C to T mutation in exon 2) was not associated with marbling or fatness traits in Australian cattle.

Komisarek *et al*. (2005) analyzed the relationship between leptin gene polymorphism (*Kpn*21) and milk production traits in cattle and propounded that TT genotype had significantly higher milk and protein yield. But they failed to identify marked influence of this polymorphism on butterfat yield.

Schenkel *et al*. (2006) associated the leptin genotypes to carcass traits in beef cattle and reported a significant reduction in grade fat, subcutaneous fat and fat yield in CC genotypes of *Kpn*21 SNP.

While studying the SNPs of leptin gene in beef cattle, Lusk (2007) displayed the association of LEP/*Kpn*21 genotypes on body weight and backfat thickness.

Nassiry *et al*. (2007) studied the genetic variation in the exon 2 of leptin gene in Iranian native cattle breeds by restriction enzyme *Bsp*131 (isoenzyme of *Kpn*21) and failed to identify TT genotype in Golpayegani cattle. In Taleshi cows the genotype frequencies were 0.36 (CC), 0.36 (CT) and 0.27 (TT). The same group extended the research to more Iranian cattle breeds and propounded that TT genotype was present only in Taleshi, Sistani and Holstain cattle and confirmed the absence of TT genotype in Golpayegani (Nassiry *et al*., 2008).

Almeida *et al*. (2007) employed molecular marker analysis in leptin gene of beef cattle and reported that C and T allele frequencies of LEP/*Kpn*2I polymorphism were 0.42 and 0.58 in Charolais and 0.63 and 0.37 in Aberdeen cattle. They could not find any association between LEP/*Kpn*21 genotypes and weight gain.

Komisarek and Antkowiak (2007), studying the relationship of leptin gene polymorphisms and reproductive traits in Jersey cows, displayed C to T mutations by PCR-RFLP technique. Cows with TT genotype were characterized by shorter calving interval and lower number of inseminations per conception than animals with CT and CC genotype.

Sadeghi *et al*. (2008) performed *Kpn*21 RFLP in Iranian Holstein and found that the genotypes (CC, CT and TT) were in Hardy-Weinberg equilibrium. Association studies revealed that animals with TT genotype had higher milk, fat and protein yield compared to other genotypes.

The associations among single nucleotide polymorphisms in the *Kpn*21 locus in exon two of the leptin gene and lactation performance of Holstein cows were evaluated by Chebel *et al*. (2008) and found that cows homozygous for C allele (CC) had reduced milk, milk fat and milk protein yields compared to cows of CT and TT

genotypes. They also proposed that heterozygosity at leptin gene locus was associated with reduced incidence of diseases.

A significant increase in 60 and 100 day milk yield was reported by Alashawkany *et al*. (2008) for TT genotype of *Bsp*131 polymorphism (C3057) of leptin gene in Iranian Holstein. But, for 305 day milk yield, no significant difference was observed. They suggested that the increase in milk yield was prominent in early days of lactation.

Fischer (2008), studying the distributions and associations of SNPs in leptin gene, identified CC, CT and TT genotypes for *Kpn*21 SNP in *Bos taurus* and *Bos indicus* cattle. According to him T allele was associated with reduced gestation period.

Cattle breeds of Colombia, Harton Del Valle (HV) and Blanco Orejinegro (BON), were genotyped for *Kpn*21 polymorphism by Ceron –Munoz *et al*. (2008) and identified all three genotypes (CC, CT and TT). It was reported that *Kpn*21 polymorphism was associated with carcass traits.

DeVuyst *et al*. (2008) tested the effect of C305T SNP in leptin gene on beef calf weaning weights in crossbred and purebred beef cows. They found that crossbred cattle with CT and TT genotypes weaned significantly heavier beef calves (8.81 kg and 12.44 kg, respectively) than CC genotype.

Fortes *et al*. (2009) performed PCR-RFLP using *Kpn*21 in *Bos indicus* (Nelore) and crossbreds. In Nelore cattle the allele frequencies were reported as 0.96 for C and 0.04 for T whereas the genotype frequencies were 0.91 for CC and 0.09 for CT with the absence of TT genotype. The C/T allele frequencies in crossbred cattle

possessing distinct levels of *Bos indicus* blood (1/2 Nelore, 0.93/0.07; 3/8 Nelore, 0.61/0.39 and 7/16 Nelore, 0.74/0.26) showed an increase in T allele frequencies with the reduction of *Bos indicus* blood.

Pannier *et al*., 2009 reported that the Hereford breed displayed a frequency of 0.28 for TT genotype, while Salers cattle showed the absence.

Corva *et al*. (2009) employed Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) to display SNPs in leptin gene and reported the C and T allele frequencies as 0.504 and 0.496, respectively.

A recent study on cow- calf profitability and leptin genotyping, Mitchell *et al*. (2009) associated the TT genotype of SNP, C305T with increased calf weaning weights and cow productive life. They suggested that this genotypic information could be utilized for future selection programmes and mating decisions.

2.2.2 Other PCR-RFLPs in Bovine Leptin Gene

Pomp *et al*. (1997) identified two alleles for leptin gene by PCR-RFLP using the restriction enzyme *Sau*3A1 and confirmed the Mendelian inheritance of leptin gene.

Lein *et al*. (1997) analyzed polymorphisms in positions 1560 (C/T) and 1620 (G/A) of intron two of leptin gene by PCR-RFLP. The 522 bp fragment was digested with restriction enzymes *Hinf*1 and *Bsa*A1, specific for each mutation and obtained different polymorphic patterns. Using the same enzymes Tessane *et al*. (1999) performed PCR-RFLP in Angus bulls and explained that B and C alleles were correlated with kidney, pelvic and heart fat percentage.

A new mutation in exon two of bovine leptin gene was investigated by Haegeman *et al*. (2000) employing restriction endonuclease *Hph*1 and reported two alleles (331 bp and 311+20 bp).

Liefers *et al*. (2002) proposed that polymorphisms of leptin gene with enzymes *Sau*3A1 and *Hph*1 were distributed according to Hardy-Weinberg equilibrium. *Sau*3A1 genotypes (AA and AB) showed significant differences for milk, protein and lactose yield and food intake. A significant reduction in weight gain and lactose percentage in milk was reported for AA genotype of *Hph*1 polymorphism compared with other two genotypes (AB and BB).

SNPs in exon two and three of bovine leptin gene were studied by Lagonigro *et al*. (2003) using restriction endonucleases *Cla*1 and *Nru*1 and discovered a new mutation at position 252 in exon two. This A to T mutation was resulted in a nonconservative amino acid substitution from tyrosine (TAT) to phenylalanine (TTT). Animals of AT genotype of newly identified polymorphism had 19% greater mean feed intake than individuals with genotype AA.

Madeja *et al*. (2004) investigated *Hph*1 and *Sau*3A1 polymorphisms and examined the effect of leptin genotypes on milk production traits in Polish black and white breed. They found that animals with TT genotype of *Hph*1 polymorphism had significantly higher estimates of milk and protein yield.

Javanmard *et al*. (2005) genotyped Iranian cattle and buffalo populations for *Sau*3A1 polymorphism and found out highest frequencies for B allele and AB genotype in Dashtiyari cattle and Taleshi cattle, respectively. The same group, Javanmard *et al*. (2008), confirmed C to T mutation in intron two of leptin gene in

Iranian Sarabi cattle by *Sau*3A1 restriction endonuclease and reported three genotypes (AA, AB and BB).

Nkrumah *et al*. (2006) genotyped beef cattle for A59V SNP (C to T mutation in exon3) in leptin gene and found association between TT genotype and higher serum leptin concentration, feed efficiency and carcass traits.

2.3 MICROSATELLITE ANALYSIS IN LEPTIN GENE

Microsatellites are short tandem repeats of DNA sequences of only 1-6 base pairs in length usually repeated 15-30 times. Based on the number of repeat units, a population can be characterized. In leptin gene, various microsatellites and their association with production traits are reported.

Stone *et al*. (1996a) described for the first time a polymorphic microsatellite BM1500 at 3.6 kb downstream of leptin gene in cattle.

A tetranucleotide repeat at 3' flanking region of human obese gene was analyzed by Shintani *et al*. (1996) and they detected fifteen alleles with a heterozygosity of 0.85 and polymorphic information content of 0.83, indicating the highly informative nature of the marker.

A polymorphic microsatellite with 18 alleles (170-201 bp) was identified at the 5´ untranslated region of the leptin gene in *Bos taurus* and *Bos indicus* by Wilkins and Davey (1997).

While investigating Quantitative Trait Loci (QTL) for milk production traits in Swedish cattle, Lindersson *et al*. (1998) detected three alleles at BM1500 locus and

eight alleles at BM I501 locus. Twelve different haplotypes constituting these two microsatellites were recognized and tested for association with milk production traits. No significant overall effect of the obese locus was observed.

Fitzsimmons *et al*. (1998) characterized BM1500 microsatellite in beef bulls and obtained four alleles 138, 146, 148 and 140 bp with frequencies 0.47, 0.44, 0.09 and 0.003, respectively. Association studies with carcass traits disclosed that 138 bp allele was correlated with higher levels of fat, whereas the 146 bp allele had the opposite effect.

Tessane *et al*. (1999) detected two alleles (136 and 144 bp) from Stone microsatellite – BM1500 and six alleles (177-209 bp) from Wilkins and Davey microsatellite in Angus bulls and disclosed noteworthy relationships between Stone microsatellite genotypes and marbling score.

Performing BM1500 microsatellite analysis in Holstein cattle, Liefers *et al*. (2002) detected three alleles [136 bp (A), 144 bp (B) and 146 bp (C)] and six genotypes [AA (0.23), AB (0.38), BB (0.13), AC (0.13), BC (0.09) and CC (0.05)]. Liefers *et al*. (2003) suggested that presence of 136 bp allele was associated with lower leptin concentration and 144 bp allele with higher leptin concentration.

Almeida *et al*. (2007) opined that BMS1074 and BM1500 were positively associated with average weight gain. They also reported that carriers of BM1500 136 allele presented a higher average daily weight gain as compared to other animals (75g higher in Angus and 96g in Charolais).

Three alleles (136, 144 and 146) were detected for BM1500 microsatellite in Columbian cattle breeds by Ceron- Munoz *et al*. (2008) and obtained significantly low fat levels in animals with 146 bp allele.

2.4 SSCP IN BOVINE LEPTIN GENE

By SSCP and DNA sequencing techniques, Yang *et al*. (2007) illustrated leptin gene polymorphisms in Chinese cattle breeds and found that the individuals with genotype BB had higher indices for growth traits than with genotypes AA and AB.

Gupta *et al*. (2008) revealed high genetic variability of leptin gene in Sahiwal cattle (*Bos indicus*) with three different patterns for exon 2 and two for exon 3 by SSCP. They also suggested that SSCP variants could be sequenced and scanned for single nucleotide polymorphism markers for association studies and MAS.

2.5 VECHUR CATTLE

Girija (1994) reported the diploid chromosome number of dwarf cattle of Kerala as 60 with 29 pairs of acrocentric autosomes, submetacentric X chromosome and acrocentric Y chromosome. She stated that dwarf cattle of Kerala have unique characteristics and reiterated the necessity of conservation.

The milk composition of Vechur cattle was described by Venkatachalapathy (1996) who observed that the mean fat globule size in Vechur milk (3.12 μ) was lower than that of crossbred cattle (4.87 μ) and Murrah buffalo (5.85 μ), but higher than goat (2.6 μ). He suggested that increased phospholipid content in small fat globules aided in the nervous tissue development and fat digestion.

Venkatachalapathy and Iype (1997) reported that Vechur cattle had unique characteristics of its own with separate identity from other breeds of cattle not only by its small size but also due to its milk protein variants, composition of milk, size of fat globules and level of saturated fatty acids.

Iype and Venkatachalapathy (2001) prepared breed descriptor for Vechur cattle and explained that Vechur cattle was characterized by small size with an average height of 89.43 cm in bull and 87.75 cm in cow and average weight of about 170 kg for bulls and 130 kg for cows. These animals needed less feed as compared to crossbreds with a daily milk yield of 2-3 kg and they were adapted to hot and humid environment.

Anilkumar and Raghunandanan (2003) prepared breed descriptor for four genetic groups of cattle in Kerala, namely High range dwarf, Vechur, Vatakara and Kasargode.

Aravindakshan *et al*. (2003) analyzed the genetic polymorphism at the third exon of prolactin (PRL) gene in Vechur cattle by PCR-RFLP. They observed that PRL genotypes did not vary significantly for milk fat percentage, peak yield, first lactation yield and lactation length.

PCR-RFLP analysis of β -lactoglobulin locus in Vechur cattle revealed two alleles, A and B and the frequency of desirable B allele was found to be very high (Aravindakshan *et al*., 2004).

The Vechur animals were screened for the presence of the bovine leukocyte adhesion deficiency (BLAD), a genetic defect using PCR-RFLP and DNA sequencing methods and found that none of the 129 Vechur animals studied carried the BLAD allele (Aravindakshan *et al.,* 2006).

PCR-RFLP analysis at the third intron of growth hormone gene using restriction enzyme, *Msp*1 revealed two alleles namely (+) allele (612, 93 and 63 bp fragments) and (-) allele (705 and 63 bp fragments). The frequencies of *Msp*1(+)/*Msp*1(-) alleles were 0.35/0.65 and 0.48/0.52 for Vechur and Kasargode cattle, respectively. Association studies revealed that the genotypes were not significantly different for birth weight and body measurements (Aravindakshan *et al*., 2007).

Rajeev (2007) cloned and sequenced alpha-lactalbumin (α-LA) gene of Vechur cattle and reported 99% homology with *Bos taurus*, 98% with yak and 95% with sheep. He suggested α-LA as a genetic marker to increase milk production in Vechur cattle.

2.6 MILK PRODUCTION TRAITS IN VECHUR CATTLE

Girija (1994) reported the mean total lactation yield of 471 kg in Vechur cattle while the average daily milk yield reported was 2.17 kg. The average lactation length and peak yield recorded by her was 217 days and 3.71 kg, respectively.

Venkatachalapathy (1996) studied the milk composition of Vechur cattle and reported the average milk fat as 6.18% and total solids as 15.16%. The average milk fat percentages at 7^{th} and 13^{th} week of lactation were recorded as 5.56 and 5.77, respectively.

According to Venkatachalapathy and Iype (1997), the fat and total solids percentages of Vechur milk showed an increasing trend as the lactation advanced. The least squares means of milk fat, total solids and solids-not-fat in percentages were 6.13, 15.02 and 8.89, respectively for one to forty four weeks of lactation.

Iype and Venkatachalapathy (2001) recorded average daily milk yield (2.2 kg), peak yield (3.6 kg), lactation length (232 days), fat percentage (4.7) and solids not fat (8.87 %) in Vechur cattle.

The fat percentage of Vechur milk was reported by Raghunandanan (2006) as 4.5 to 5.0. He observed the average fat globule size in Vechur milk as 3.2 micron and recommended for infant feeding due to easy digestibility. The average lactation milk yield and daily milk yield in Vechur cows were reported as 561.1 kg (242 days of lactation) and 3 kg, respectively.

2.7 MILK PRODUCTION TRAITS IN CROSSBRED CATTLE

To improve the milk production, local cattle of Kerala were crossed with exotic breeds Jersey, Brown Swiss and Holstein Friesian. Now Kerala has a mosaic population of exotic and local cattle.

Chacko and Jose (1988) reported the average first lactation milk yield (2500 kg), first lactation length (274.3 days) and milk fat percentage (4) in crossbred cattle of Kerala.

Radhika (1997) evaluated the production performance of crossbred cattle population maintained in Kerala Agricultural University Farms and reported the average 305 day milk yield as 1829.68 kg. The milk fat percentages during early, mid and late lactation were reported as 3.46, 3.65 and 3.97, respectively.

The average daily milk yield of pooled population consisting of purebreds and crosses of Sahiwal, Jersey and Friesian breeds was reported as 8.64 kg by Kant and Prasad (1997).

Kannan *et al*. (2000) studied the production performance of crossbred dairy cattle and reported a mean 305 day lactation yield of 2113 kg.

Sathian (2001), studying the quality of milk in crossbred cows, observed an average value of 4.64 for milk fat percentage.

Thomas (2005) associated microsatellite markers with milk production traits in crossbred dairy cattle and reported that 305 day milk yield of crossbreds of Kerala was 2070.5 kg. An average milk fat percentage of 3.74 was also reported for cattle maintained at Cattle Breeding Farm, Thumburmuzhi and University Livestock Farm, Mannuthy.

According to Thirumurugan and Saseendran (2006), the average daily milk yields of crossbred cattle maintained in various housing systems in Kerala Agricultural University livestock farms ranged from 7.78 to 8.86 kg.

Chandran (2007) reported the mean 305 day milk yield of cattle from different zones of Kerala as 2406 kg.

3. MATERIALS AND METHODS

Blood samples collected from 74 Vechur cattle belonging to Vechur conservation unit of Kerala Agricultural University and 108 crossbred cattle, from University Livestock Farm, Mannuthy (68) and Cattle Breeding Farm, Thumboormuzhi (40) formed the materials for the present study on leptin gene polymorphism by PCR-RFLP and microsatellite analysis.

3.1 COLLECTION OF SAMPLES

From each animal, 5 ml of blood was collected from the jugular vein into sterile 15 ml polypropylene centrifuge tube containing Ethylene Diamine Tetra Acetic acid (EDTA) as anticoagulant (1 mg/ml of blood). The samples were brought to the laboratory at 4ºC, temperature being maintained with the aid of ice packs and stored at -20ºC until needed for DNA extraction.

Thirty milliliter of milk was collected consecutively for two days from animals in 65-90 days of lactation.

3.2 ISOLATION OF DNA FROM WHOLE BLOOD

DNA was extracted from whole blood using the 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 m*M* ammonium chloride, 10 m*M* potassium chloride and 0.1 m*M* 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 m*M* sodium chloride, 0.5 m*M* potassium chloride and 0.25 m*M* 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 m*M* sodium chloride and 35 *mM* EDTA). The cell suspension was incubated at 50ºC in water bath with 0.25 ml of 20 % sodium dodecyl sulphate (SDS) and 25 µl 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 4500 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 4500 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 4500 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% ethanol and air-dried.
11. Dried DNA was resuspended in 0.5 ml of Tris EDTA buffer (TE-10 m*M* Tris base, 0.1 *mM* EDTA) and stored at -20ºC.

3.3 QUANTIFICATION AND QUALITY CHECK OF DNA BY AGAROSE GEL ELECTROPHORESIS

The quality and molecular weight of DNA were measured electrophoretically using 0.8% agarose. An appropriate sized clean gel tray was selected and set with suitable combs. The agarose gel was prepared in 1X Tris EDTA buffer (TBE-0.045*M* Tris borate and 0.001 *M* EDTA) and allowed to cool to 50ºC. Five microliter of ethidium bromide (10 mg/ml) was added into the molten agarose and then poured into the tray to polymerize. After solidificatoin, the sealing and comb were removed gently and the gel with tray was immersed in the 1X TBE buffer in the electrophoresis tank with the wells towards the negatively charged electrode. One microliter of each DNA sample was mixed with one-sixth the volume of 6X gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% 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}).

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 PCR-RFLP ANALYSIS

In the present PCR-RFLP analysis, the point mutation, cytosine to thymine at position 305 of leptin gene in exon-two (C305T) was investigated using restriction endonuclease *Bsp*EI. The primer LEP-94 (Buchanan *et al*., 2002; Choudhary *et al*., 2005) was custom synthesized and used to amplify a 94 bp fragment from the start of exon-two of leptin gene which is composed of the specified mutation. A purposeful mismatch was introduced in the reverse primer to create the recognition site of the restriction enzyme.

Forward primer : 5´ATGCGCTGTGGACCCCTGTATC3´

Reverse primer : 5´TGGTGTCATCCTGGACCTTCC3´

The primers, obtained in the lyophilized form, were reconstituted in sterile distilled water to a concentration of 200 pmol/µl and 10 fold dilutions (20 pmol/µl) were used as working solutions for PCR.

3.4.1 Setting up of PCR

PCR was carried out in a final volume of 10 µl with concentrations of reagents as given in the Table 3.1. A peltier thermal cycler was pre-programmed for temperature and cycling conditions specified in Table 3.2 and used for amplification. The PCR products were cooled down to 4ºC and stored at ⁻20ºC till further analysis.

The amplification was checked by agarose gel electrophoresis. Two microliter of the product was subjected to electrophoresis using 2% agarose gel in 1X Tris Acetic Acid EDTA (TAE) buffer. The product size was confirmed using pUC18/ *Sau*3AI - pUC18/ *Taq*I digest as DNA size marker.

3.4.2 Restriction digestion of amplified products

Four microliter of the amplified product was digested with 5U of restriction enzyme *Bsp*E1 at 37ºC overnight in a dry bath. The composition of reaction mixture in a final volume of 10 µl was as follows

PCR product	-	4.0 µl
10X assay buffer	-	1.0 µl
*Bsp*E1 (10U/µl)	-	0.5 µl
Distilled water	-	4.5 µl
Total	-	10 µl

3.4.3 Non-denaturing Polyacrylamide Gel Electrophoresis (PAGE) for DNA samples

The PCR products after restriction digestion were separated by electrophoresis in 8% polyacrylamide gels in 1X TBE buffer with pUC18/ *Sau*3AI - pUC18/ *Taq*I digest 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% acrylamide and 0.5X TBE), 850 µl, 10% ammonium per sulphate (APS) and 50 µl N,N,N',N', Tetra methyl ethylene diamine (TEMED) in a beaker and poured. The gel was allowed to polymerize for 30 minutes.

After polymerization the comb was removed and the wells were filled with 1X TBE buffer. The digested samples were mixed with 2 µl of the 6X gel loading dye and loaded into the bottom of the wells. The gel sandwich was transferred into the electrophoresis tank and the 1X TBE buffer was poured into the top and bottom reservoirs. Electrophoresis was carried at 100 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 analyzed under UV transilluminator and documented in a gel documentation system (BioRad, Gel Doc 2000^{TM}).

3.4.4 Allelic and Genotype frequencies of LEP/*Bsp*E1 locus

The allelic and genotype frequencies at LEP/*Bsp*E1 locus were calculated by direct counting method for Vechur and crossbred cattle separately as well as for the pooled population. The variation of the allelic frequencies among the two populations was analyzed by the *Chi*-square test of significance as described by Snedecor and Cochran (1994) considering the allelic frequencies in a 2×2 table using the formula,

$$\chi^2 = \frac{(ad - bc)^2 N}{(a+c)(b+d)(a+b)(c+d)}$$

where a, b, c and d are allelic frequencies in different populations and N is the total number of alleles observed.

The distribution of LEP/*Bsp*E1 genotypes in Vechur and crossbred cattle population was checked for Hardy-Weinberg equilibrium by *Chi*-square test comparing the observed and expected frequencies.

3.5 MICROSATELLITE ANALYSIS

BM1500 microsatellite (Stone *et al*., 1996a; Fitzsimmons *et al*., 1998), located 3.6 kb down stream of leptin gene in cattle was analyzed using the following primers

Forward primer : 5´GATGCAGCAGACCAAGTGG3´
Reverse primer : 5´CCCATTGCTAGAACCCAGG3´

3.5.1 Incorporation of Radioactivity: End-Labelling of Forward Primer

The forward primer was radiolabelled using DNA-end-labelling kit (Bangalore Genei) at the 5' end with γ ^{32}P ATP to visualize the PCR products by autoradiography. For end-labelling the following components were mixed in a sequential manner in a 0.2 ml microcentrifuge tube.

10X Polynucleotide kinase (PNK) buffer	-	1.0 µl
Forward primer (200 pmol/µl)	-	1.0 µl
T_4 Polynucleotide kinase (5 U/µl)	-	0.5 µl
γ ^{32} P ATP (10 mCi/ml)	-	1.0 µl
Nuclease free water	-	6.5 µl

The mixture was incubated at 37ºC for 30 minutes. The final volume was made up to 40 µl with sterile water.

3.5.2 Setting up of PCR

The composition of reaction mixture and the standardized temperature and cycling conditions for PCR are given in Tables 3.1 and 3.2, respectively. The amplified products were checked electrophoretically using 1% agarose gel. The PCR products were subjected to denaturing PAGE along with M13 Bacteriophage DNA as marker.

3.5.3 Sequencing M13 Bacteriophage DNA

The procedure followed for sequencing the single stranded M13 phage DNA using Sequenase version 2, DNA sequencing kit (Amershan Pharmacia Biotech, USA) included preparation of annealing mixture, dilution of labelling mix and enzyme and labelling reaction.

1. Preparation of annealing mixture: The following solutions were mixed well, spun and incubated at 65ºC for 2 minutes. Then cooled to room temperature over 15-30 minutes and kept on ice.

M13 phage DNA (0.2 μg/μl)	-	5 μl
5X Sequenase reaction buffer	-	2 μl
Primer (5 pmol/μl)	-	1 μl
Sterile water	-	2 μl

2. Dilution of labelling mix: The labelling mix provided in the kit was diluted five fold as follows:

5X labelling mix	-	0.5 μl

Water	-	2.0 μl

3. Dilution of enzyme: The enzyme (Sequenase version 2.0) was diluted eight fold with Sequenase enzyme dilution buffer as follows:

Sequenase enzyme (13 U/μl)	-	0.5 μl
Sequenase dilution buffer	-	3.5 μl

4. Labelling reaction: To the ice cold annealed DNA mixture, the following solutions were added. The contents were mixed well, spun and incubated at room temperature for two to five minutes.

Dithiothreitol (DTT 0.1 M)	-	1 μl
Diluted labelling mix	-	2 μl
Diluted enzyme	-	2 μl
α ^{32}PdATP (10 μCi/μl)	-	2 μl

5. Four tubes labelled G, A, T and C were filled with 2.5 μl of each dideoxy termination mixtures (ddGTP, ddATP, ddTTP and ddCTP).

6. To each termination tubes (G, A, T, C), 3.5 μl of labelling reaction mixture was transferred, mixed well and incubated at 37ºC for 5 minutes.

7. The reaction was stopped by addition of 4 μl of stop solution provided in the sequencing kit and stored at –20ºC.

3.5.4 Denaturing Polyacrylamide Gel Electrophoresis

Denaturing PAGE was performed on a Vertical Sequencer (Consort, Belgium) using 6% denaturing polyacrylamide gel. The gels were set between two glass plates (41 x 33 cm) separated by 0.35 mm thick spacers. The gel was prepared by mixing 60 ml of 0.5X TBE gel mix (6% acrylamide, 6 *M* urea, 0.5X TBE) and 125 µl each of 10% APS and TEMED in a beaker. The gel was allowed to set for 30 min and assembled in the sequencer. The upper and lower electrode tanks were filled with 1X TBE buffer to the required level.

The PCR products were mixed with 3.5 µl formamide loading buffer (0.02% Xylene Cyanol, 0.02% bromophenol blue, 10 m*M* EDTA and 98% deionised formamide), denatured at 95ºC for 5 min and cooled immediately on ice. About 3 µl each of this mixture was loaded into each well. Sequenced products of M13 DNA, which were also denatured at 94ºC for 5 min, were loaded simultaneously in the middle or side wells.

The gels were electrophoresed at 40 W for three hours maintaining a temperature around 50ºC. The gel was carefully transferred to a filter paper, covered with cling film and dried in a vacuum heated gel drier (Scie-Plas, GD-4534) at 80ºC for one and a half hours.

3.5.5 Autoradiography

The cling film was removed after drying and the gel was set for autoradiography with X-ray film (Kodak, 35.6 x 43.2 cm) in a cassette (Kiran Hypercassete) fitted with an intensifying screen. The X- ray film was developed after 24 to 48 hours depending on the intensity of radioactive signal.

The X-ray film was developed by transferring the film serially into 1X developer solution (Kodak) for three to five minutes, one percent acetic acid for one minute followed by washing in distilled water and finally into fixer solution (Kodak)

for six to ten minutes. The developed film was washed thoroughly in running water and dried.

3.5.6 Microsatellite Typing

The genotypes of animals were determined for each microsatellite loci by comparing the sizes of alleles with M13 sequencing ladder. The G, A, T and C sequences were read from the bottom to the top in order. The allele sizes were assigned corresponding to the G, A, T, C bands. The frequency at each locus was determined by direct counting.

3.5.7 Direct Count Heterozygosity

Heterozygosity is a measure of usefulness of the marker. Heterozygosity was calculated in Vechur, crossbred and in pooled population by the method of Ott (1992).

$$He = 1 - \sum_{i=1}^{k} p_i^{\,2}$$

where p_i is the frequency of i^{th} allele at BM1500 locus.

3.5.8 Unbiased Heterozygosity

The unbiased heterozygosity of Vechur, crossbred and pooled population were calculated using the formula of Pandey *et al*. (2002)

$$He = \left[2n/(2n-1)\right]\left[1 - \sum_{i=1}^{k} p_i^{\,2}\right]$$

where p_i is the frequency of i^{th} allele at BM1500 locus and n is the number of observations.

3.5.9 Polymorphic Information Content (PIC)

The polymorphic information content expresses informativeness or usefulness of a marker for linkage studies. The PIC value of BM1500 was calculated for Vechur, crossbred and pooled population using the formula

$$PIC = 1-[\sum_{i=1}^{k} p_i^2] - \sum_{i=1}^{k-1}\sum_{j=i+1}^{k} 2p_i^2 p_j^2$$

where p_i and p_j are the frequencies of i^{th} and j^{th} alleles, respectively (Botstein *et al*., 1980).

3.6 MILK PRODUCTION TRAITS

Milk fat percentage was estimated by Gerber method as described in IS: 1224 (1977) part one. The information regarding the first lactation milk yield, lactation length and daily milk yield were collected from the records maintained in the farms. The lactation milk yield was standardized for 305 days to obtain 305 day milk yield (Rice *et al*., 1970).

3.7 EFFECT OF LEPTIN GENE POLYMORPHISMS ON MILK PRODUCTION TRAITS

The effect of LEP/*Bsp*E1 and BM1500genotypes on 305 day milk yield, daily milk yield, lactation length and milk fat percentage were determined by t- test and univariate analysis of variance (ANOVA) in Vechur and crossbred cattle.

Table 3.1. Optimized concentrations of PCR reagents for *Bsp*E1 and BM1500

Sl. no.	PCR reagents	*Bsp*E1		BM1500		Final concentration in 10 µl
		Concentration	Volume (µl)	Concentration	Volume (µl)	
1	Template DNA	50 ng/µl	1.00	50 ng/µl	1.00	50 ng
2	Dinucleotide phosphates	2.5 m*M*	0.80	2.5 m*M*	0.80	200 µ*M*
3	Magnesium chloride	10 m*M*	1.00	10 m*M*	1.00	1 m*M*
4	Forward primer	20 pmol/µl	0.25	5 pmol/µl	1.00	5 pmol
5	Reverse primer	20 pmol/µl	0.25	20 pmol/µl	0.25	5 pmol
6	10X PCR buffer	200m*M* Tris-HCl & 500m*M* KCl	1.00	200m*M* Tris-HCl & 500m*M* KCl	1.00	1X
7	*Taq* DNA polymerase	5 U/µl	0.06	5 U/µl	0.06	0.3 U
8	Distilled water		5.64		4.89	

Table 3.2 Standardized temperature and cycling conditions for amplification of *Bsp*E1 and BM1500 loci

Locus	Initial denaturation	35 Cycles of			Final extension
		Denaturation	Primer annealing	Primer extension	
*Bsp*E1	94 ºC for 5 min	94 ºC for 1 min	61.7 ºC for 1 min	72 ºC for 1 min	72 ºC for 5 min
BM1500	94 ºC for 5 min	94 ºC for 1 min	57.4 ºC for 1 min	72 ºC for 1 min	72 ºC for 5 min

4. RESULTS

4.1 ISOLATION OF GENOMIC DNA

Good quality DNA samples were obtained from the blood samples of Vechur and crossbred cattle using phenol chloroform extraction procedure. Single, clear band without shearing on agarose gel upon electrophoresis indicated the presence of good quality high molecular weight DNA (Plate1).

4.2 PCR-RFLP ANALYSIS OF LEPTIN GENE

The DNA samples of Vechur and crossbred cattle could be amplified at exon two of leptin gene without any major non-specific amplified products (Plate 2). In all the animals studied, the size of the amplified product was 94 bp, indicating conservation of DNA sequences at the leptin locus in *Bos indicus*, and their crossbreds with *Bos taurus*.

Digestion of DNA with restriction enzyme, *Bsp*E1 and further electrophoresis revealed three restriction digestion patterns in Vechur (Plate 3) as against two in crossbred cattle (Plate 4) with the identification of two alleles namely C and T. C allele was indicated by the presence of two bands of size 75 and 19 bp while T allele by a single band of size 94 bp.

The allele and genotype frequencies of LEP/*Bsp*E1 polymorphism in Vechur, crossbred and in the pooled population are presented in Table 4.1. The frequencies for C and T alleles in Vechur were noted as 0.82 and 0.18, respectively and for crossbreds the frequencies were recorded as 0.89 and 0.11, respectively. In the

pooled population C and T allele frequencies were obtained as 0.87 and 0.13, respectively.

In Vechur cattle the genotype frequencies of LEP/*Bsp*E1 polymorphism were observed as 0.71 (CC), 0.24 (CT) and 0.05 (TT) whereas in crossbred cattle the frequencies were 0.79 (CC) and 0.21 (CT). Of the 108 crossbreds typed, none of the animals were of TT genotype. The corresponding frequencies in the pooled population were recorded as 0.75 (CC), 0.23 (CT) and 0.02 (TT).

A comparison of the LEP/*Bsp*E1 alleles among Vechur and crossbred cattle populations, carried out using the *Chi*-square test, showed that C and T alleles were homogenously distributed in both the populations (Table 4.2). The Vechur and crossbred cattle populations were found to be in Hardy-Weinberg equilibrium (Table 4.3) by comparing the observed and expected frequencies by *Chi*-square test.

4.3 MICROSATELLITE ANALYSIS

DNA isolated from blood samples of Vechur and crossbred cattle could be amplified at BM1500 marker, 3.6 kb downstream of leptin gene by PCR. Autoradiographs of polymorphism at BM1500 marker are shown separately for Vechur and crossbred cattle populations in Plates 5 and 6, respectively.

In the pooled population, DNA amplified at BM1500 loci yielded six alleles of size 126 bp, 132 bp, 136 bp, 138 bp, 144 bp and 150 bp with frequencies 0.08, 0.43, 0.13, 0.03, 0.28 and 0.05, respectively. The alleles and corresponding frequencies for the marker BM1500 in Vechur cattle were 126 bp (0.05), 132 bp (0.40), 136 bp (0.13), 144 bp (0.32) and 150 bp (0.10) whereas in crossbreds, the alleles and frequencies were 126 bp (0.12), 132 bp (0.46), 136 bp (0.12), 138 bp

(0.06) and 144 bp (0.24). The 132 bp allele was the most abundant one in Vechur and crossbred cattle population. The 138 bp allele was absent in Vechur as against 150 bp allele in crossbreds. The allele frequencies of Vechur, crossbred and pooled population are summarized in Table 4.4.

In Vechur cattle population five genotypes (132/132, 136/126, 136/132, 144/132, 150/144) were observed as against seven genotypes (126/126, 132/132, 136/126, 136/132, 138/132, 144/132 and 144/136) in crossbred cattle population. The highest frequency was observed for 144/132 genotype in Vechur (0.44) as well as crossbred cattle (0.38). The lowest frequency was observed for 132/132 and 136/126 (0.07) in Vechur while 136/132 (0.05) in crossbred cattle. In the pooled population eight genotypes were noted and the highest and the lowest frequencies were reported for 144/132 (0.41) and 126/126 (0.04), respectively. The genotype frequencies of Vechur, crossbreds and pooled population are given in Table 4.5.

The direct count heterozygosity, unbiased heterozygosity and PIC for BM1500 marker were 0.7082, 0.7169 and 0.6595, respectively in Vechur and 0.6984, 0.7068 and 0.6560, respectively in crossbred cattle population. In the pooled population the direct count heterozygosity for BM1500 marker was found to be 0.7100 while the unbiased heterozygosity and PIC value were calculated as 0.7143 and 0.6666 (Table 4.6).

4.4 MILK PRODUCTION TRAITS

The average values for 305 day milk yield, daily milk yield, lactation length and milk fat percentage of Vechur and crossbred cattle are presented in Table 4.7.

4.4.1 305 Day Milk Yield

The average milk yield standardized for 305 days were 348.01±19.63 kg and 2106.78±56.13kg, respectively for Vechur and crossbred cattle.

4.4.2 Daily Milk Yield

The average daily milk yield for Vechur and crossbred cattle was recorded as 1.27±0.06 kg and 7.10±0.19 kg, respectively.

4.4.3 First Lactation Length

The average first lactation length was recorded in Vechur and crossbred cattle as 232.67±13.11 and 292.42±6.10 days, respectively.

4.4.4 Milk Fat Percentage

The averages milk fat percentage in Vechur and crossbred cattle in 65-90 days of lactation were 4.59±0.08 and 3.54±0.09, respectively.

4.5 LEP/*Bsp*E1 POLYMORPHISM AND MILK PRODUCTION TRAITS

The average values for milk production traits for Vechur and crossbred cattle carrying different LEP/*Bsp*E1 genotypes are presented in Table 4.8.

4.5.1 LEP/*Bsp*E1 Polymorphism and 305 Day Milk Yield

Genotypes of *Bsp*E1 polymorphism had significant effect on 305 day milk yield in the cattle populations studied. Vechur and crossbred cattle carrying T allele showed a significantly higher average for 305 day milk yield (387.91±26.43 kg and 2413.01±64.41) compared to animals wherein T allele was absent (298.14±23.1 kg and 1946.38±67.5).

4.5.2 LEP/*Bsp*E1 Polymorphism and Daily Milk Yield

The mean values for daily milk yield were significantly higher in Vechur and crossbred cattle carrying T allele of LEP/*Bsp*E1 polymorphism. The average daily milk yield for CT/TT and CC genotyped Vechur animals were 1.71±0.08 kg and 1.40±0.08, respectively and that of crossbred cattle were 7.90±0.23 kg and 6.68±0.23, respectively.

4.5.3 LEP/*Bsp*E1 Polymorphism and First Lactation Length

Vechur animals of CT/TT genotypes showed 235.87±16.03 days for first lactation length whereas CC genotypes had 228.67±22.48 days. The average lactation length of 318.14±6.01 days was observed for CT genotyped crossbreds while 278.95±8.13 days recorded for CC genotypes. A statistically significant increase was obtained for the first lactation length of T allele bearing crossbred cattle.

4.5.4 LEP/*Bsp*E1 Polymorphism and Milk Fat Percentage

The average milk fat percentage of 4.56±0.10 was observed for T allele bearing Vechur cattle while 4.62±0.12 recorded for animals without T allele.

Crossbred cattle of CT genotypes recorded 3.52±0.12 for milk fat percentage where as CC genotypes had 3.55±0.08. The reduction in milk fat percentage in CT/TT genotyped animals was not statistically significant.

4.6 BM1500 POLYMORPHISM AND MILK PRODUCTION TRAITS

Effects of alleles and genotypes of BM1500 microsatellite polymorphism on milk production traits were analyzed by Student's t-test and analysis of variance in Vechur and crossbred cattle populations. All alleles were included in the association study except 126 bp since data available on milk production traits was incomplete in animals carrying 126 bp allele. The association between BM1500 alleles and milk production traits in Vechur and crossbred cattle are summarized in Table 4.9 and Table 4.10, respectively.

Five genotypes (132/132, 136/132, 138/132, 144/132 and 150/144) were tested for the effect on milk production traits in cattle. Three genotypes (126/126, 144/136 and 136/126) were excluded from the association studies due to reduced frequency and incomplete availability of data. The association between BM1500 genotypes and milk production traits in Vechur and crossbred cattle are summarized in Table 4.11 and Table 4.12, respectively.

Vechur and crossbred cattle carrying 136 bp allele showed highest estimates of 378.39±42.44 and 2142.61±104.4 kg, respectively for average 305 day milk yield. The 305 day milk yields (kg) associated with BM1500 alleles other than 136 bp were 346.2±30.6 (132 bp), 337.33±24.05 (144 bp) and 339.46±30.31 (150 bp) in Vechur and 1915.94±99.55 (132 bp), 1624.75±117.9 (138 bp) and 1943.71±128.6 (144 bp) in crossbred cattle.

The average values for 305 day milk yield (kg) in 136/132, 144/132 and 150/144 genotyped Vechur cattle were 363.20±53.78, 352.56±43.45 and 334.76±25.06, respectively while the values for 132/132, 138/132 and 144/132 genotyped crossbred cattle were 2028.55±134.5, 1624.74±137.9 and 1992.61±137.4 kg, respectively.

The average daily milk yields in Vechur cattle carrying different alleles were noted in descending order as 1.43±0.12 kg (136 bp), 1.26±0.10 kg (132 bp), 1.23±0.26 kg (144 bp) and 1.2±0.23 kg (150 bp). The corresponding values for 136/132, 144/132 and 150/144 genotyped Vechur cattle were recorded as 1.51±0.14 kg, 1.27±0.10 kg and 1.05±0.16 kg, respectively.

Crossbred cattle bearing 136 bp allele (7.31±0.38 kg) and 138 bp allele (5.38±0.67 kg) of BM1500 marker showed the highest and the lowest averages for daily milk yield. The corresponding values in various BM1500 genotyped crossbred cattle were observed as 6.72±0.64 (132/132), 5.38±0.67 (138/132) and 6.67±0.49 kg (144/132).

The lactation lengths (days) can be assumed in descending order as 246.83±25.73, 217.19±19.32, 210.88±20.95 and 192.86±28.00, respectively in 150, 132, 144 and 136 bp allele bearing Vechur animals. The average lactation lengths (days) of 291.83±8.04, 282.2±15.21, 304±6.72 and 301.58±14.16, respectively were noted in crossbreds bearing 132 bp, 136 bp, 138 bp and 144 bp alleles of BM1500 marker. The highest lactation lengths of 219.67±23.79 and 304±6.72 days were observed in 150/144 genotyped Vechur and 138/132 genotyped crossbreds, respectively.

The Vechur cattle possessing 136 bp allele (4.56±0.26%) showed highest milk fat percentage, while in crossbreds bearing 138 bp allele (3.56±0.13%) gave highest milk fat percentage. The average milk fat in percentage for different alleles of BM1500 marker were 4.24±0.18 (132 bp), 4.18±0.12 (144 bp) and 4.37±0.13 (150 bp) in Vechur and 3.42±0.08 (132 bp), 3.51±0.11 (136 bp) and 3.35±0.09 (144 bp) in crossbreds.

The milk fat percentage in descending order in different BM1500 genotypes were 4.55±0.37 (136/132), 4.53±0.24 (150/144) and 4.08±0.17 (144/132) in Vechur and 3.58±0.14 (138/132), 3.48±0.09 (144/132) and 3.41±0.08 (132/132) in crossbreds. A statistically significant difference in milk fat percentages was noticed between 138/132 and 132/132 genotypes of crossbred cattle.

Table 4.1 Genotype and allele frequencies of LEP/*Bsp*E1 polymorphism in Vechur and crossbred cattle of Kerala

Population	Genotype frequency			Allele frequency	
	CC	CT	TT	C	T
Vechur cattle (74)	0.71 (52)	0.24 (18)	0.05 (4)	0.82 (122)	0.18 (26)
Crossbred cattle (108)	0.79 (85)	0.21 (23)	-	0.89 (193)	0.11 (23)
Pooled population (182)	0.75 (137)	0.23 (41)	0.02 (4)	0.87 (315)	0.13 (49)

Figures in parenthesis are actual numbers

Table 4.2 Comparison of frequencies of LEP/*Bsp*E1 alleles in Vechur and crossbred cattle of Kerala

Population	C allele	T allele	χ^2 value (df=1)
Vechur	0.82 (122)	0.18 (26)	3.61^{NS}
Crossbred cattle	0.89 (193)	0.11 (23)	

^{NS} – Not significant
Figures in parenthesis are number of observations

Table 4.3 Testing of genotypes of LEP/*Bsp*E1 polymorphism for Hardy – Weinberg equilibrium in Vechur and crossbred cattle

Population	Source	Genotypes			χ^2 value (df=2)
		CC	CT	TT	
Vechur	Number Observed	52 (0.71)	18 (0.24)	4 (0.05)	1.8016^{NS}
	Number Expected	51.06	20.78	2.22	
Crossbred cattle	Number Observed	85 (0.79)	23 (0.21)	0	1.1719^{NS}
	Number Expected	85.32	21.6	1.08	

^{NS} – Not significant
df – degrees of freedom
Figures in parenthesis are genotype frequencies

Table 4.4 Allele frequencies of BM1500 polymorphism in Vechur and crossbred cattle of Kerala

Sl no.	Population	Alleles and Frequencies					
		126 bp	132 bp	136 bp	138 bp	144 bp	150 bp
1.	Vechur (41)	0.05 (4)	0.40 (33)	0.13 (11)	-	0.32 (26)	0.10 (8)
2.	Crossbred cattle (42)	0.12 (10)	0.46 (39)	0.12 (10)	0.06 (5)	0.24 (20)	-
3.	Pooled Population (83)	0.08 (14)	0.43 (72)	0.13 (21)	0.03 (5)	0.28 (46)	0.05 (8)

Figures in parenthesis are number of observations

Table 4.6 Direct count heterozygosity, Unbiased heterozygosity and PIC value at BM1500 locus in Vechur and crossbred cattle of Kerala

Population	No. of alleles	Direct count heterozygosity	Unbiased heterozygosity	PIC
Vechur (41)	5	0.7082	0.7169	0.6595
Crossbred (42)	5	0.6984	0.7068	0.6560
Pooled population (83)	6	0.7100	0.7143	0.6666

Number of observations in parenthesis

Table 4.5 Genotype frequencies of BM1500 microsatellite in Vechur and crossbred cattle of Kerala

Population	Genotypes and Frequencies							
	126/126	132/132	136/126	136/132	138/132	144/132	144/136	150/144
Vechur (41)	-	0.07 (3)	0.07 (3)	0.22 (9)	-	0.44 (18)	-	0.2 (8)
Crossbred cattle (42)	0.07 (3)	0.1 (8)	0.1 (4)	0.05 (2)	0.12 (5)	0.38 (16)	0.2 (4)	-
Pooled population (83)	0.04 (3)	0.13 (11)	0.08 (7)	0.13 (10)	0.06 (5)	0.41 (34)	0.05 (4)	0.1 (8)

Figures in parenthesis are actual number

Table 4.7 Milk production traits in Vechur and crossbred cattle

Sl. no.	Milk production traits	Population (Mean±SE)	
		Vechur cattle (35)	Crossbred cattle (64)
1	305 day milk yield (kg)	348.01±19.63	2106.78±56.13
2	Daily milk yield (kg)	1.27±0.06	7.10±0.19
3	First lactation length (days)	232.67±13.11	292.42±6.10
4	Milk fat in percentage	4.59±0.08	3.54±0.09

Number of observations in parenthesis

Table 4.8 LEP/*Bsp*E1 polymorphism and milk production traits

Sl. no.	Milk production traits	Genotype of Vechur cattle (Mean±SE)			Genotype of Crossbred cattle (Mean±SE)		
		CT/TT (18)	CC (17)	t-value	CT (22)	CC (42)	t-value
1	305 day milk yield (kg)	387.91±26.43	298.14±23.1	2.23*	2413.01±64.41	1946.38±67.5	4.47**
2	Daily milk yield (kg)	1.71±0.08	1.40±0.08	2.39*	7.90±0.23	6.68±0.23	3.34**
3	First lactation length (days)	235.87±16.03	228.67±22.48	0.86^{NS}	318.14±6.01	278.95±8.13	3.24*
4	Milk fat in percentage	4.56±0.10	4.62±0.12	0.84^{NS}	3.52±0.12	3.55±0.08	0.72^{NS}

Number of observations in parenthesis

** indicates that the values were significantly different at 1% level ($p \leq 0.01$) between genotypes in each population

* indicates that the values were significantly different at 5% level ($p \leq 0.05$) between genotypes in each population

^{NS} indicates that the values were not significantly different

Table 4.9 Milk production traits of Vechur cattle carrying specific alleles of BM1500 marker

Sl. no.	Milk production traits	Allele (Mean±SE)			
		132 bp (16)	136 bp (7)	144 bp (17)	150 bp (6)
1	305 day milk yield (kg)	346.2±30.60	378.39±42.44	337.33±24.05	339.46±30.31
2	Daily milk yield (kg)	1.26±0.10	1.43±0.12	1.23±0.26	1.2±0.23
3	First lactation length (days)	217.19±19.32	192.86±28.00	210.88±20.95	246.83±25.73
4	Milk fat in percentage	4.24±0.18	4.56±0.26	4.18±0.12	4.37±0.13

Milk production traits were not significantly different between animals carrying a specific allele

Table 4.10 Milk production traits of crossbred cattle carrying specific alleles of BM1500 marker

Sl. no.	Milk production traits	Allele (Mean±SE)			
		132 bp (24)	136 bp (5)	138 bp (5)	144 bp (12)
1	305 day milk yield (kg)	1915.94±99.55	2142.61±104.4	1624.75±117.9	1943.71±128.6
2	Daily milk yield (kg)	6.38±0.31	7.31±0.38	5.38±0.67	6.49±0.45
3	First lactation length (days)	291.83±8.04	282.2±15.21	304±6.72	301.58±14.16
4	Milk fat in percentage	3.42±0.08	3.51±0.11	3.56±0.13	3.35±0.09

Milk production traits were not significantly different with alleles between animals carrying a specific allele

Table 4.11 Milk production traits of Vechur cattle carrying specific genotypes of BM1500 marker

Sl. no.	Milk production traits	Genotype (Mean±SE)		
		136/132 (5)	144/132 (11)	150/144 (3)
1	305 day milk yield (kg)	363.20±53.78	352.56±43.45	334.76±25.06
2	Daily milk yield (kg)	1.51±0.14	1.27±0.10	1.05±0.16
3	First lactation length (days)	197.80±27.31	210.64±22.44	219.67±23.79
4	Milk fat in percentage	4.55±0.37	4.08±0.17	4.53±0.24

Observed numbers in parenthesis
Milk production traits were not significantly different between animals carrying specific genotypes

Table 4.12 Milk production traits of crossbred cattle carrying specific genotypes of BM1500 marker

Observed numbers in parenthesis

Sl. no.	Milk production traits	Genotype (Mean±SE)		
		132/132 (5)	138/132 (5)	144/132 (11)
1	305 day milk yield (kg)	2028.55±134.5	1624.74±137.9	1992.61±137.4
2	Daily milk yield (kg)	6.72±0.64	5.38±0.67	6.67±0.49
3	First lactation length (days)	291.20±7.62	304±6.72	296.27±14.33
4	Milk Fat in percentage	3.41 ± 0.08^{a}	3.58 ± 0.14^{b}	3.48 ± 0.09^{a}

Different superscripts indicate that the values were significantly different between genotypes

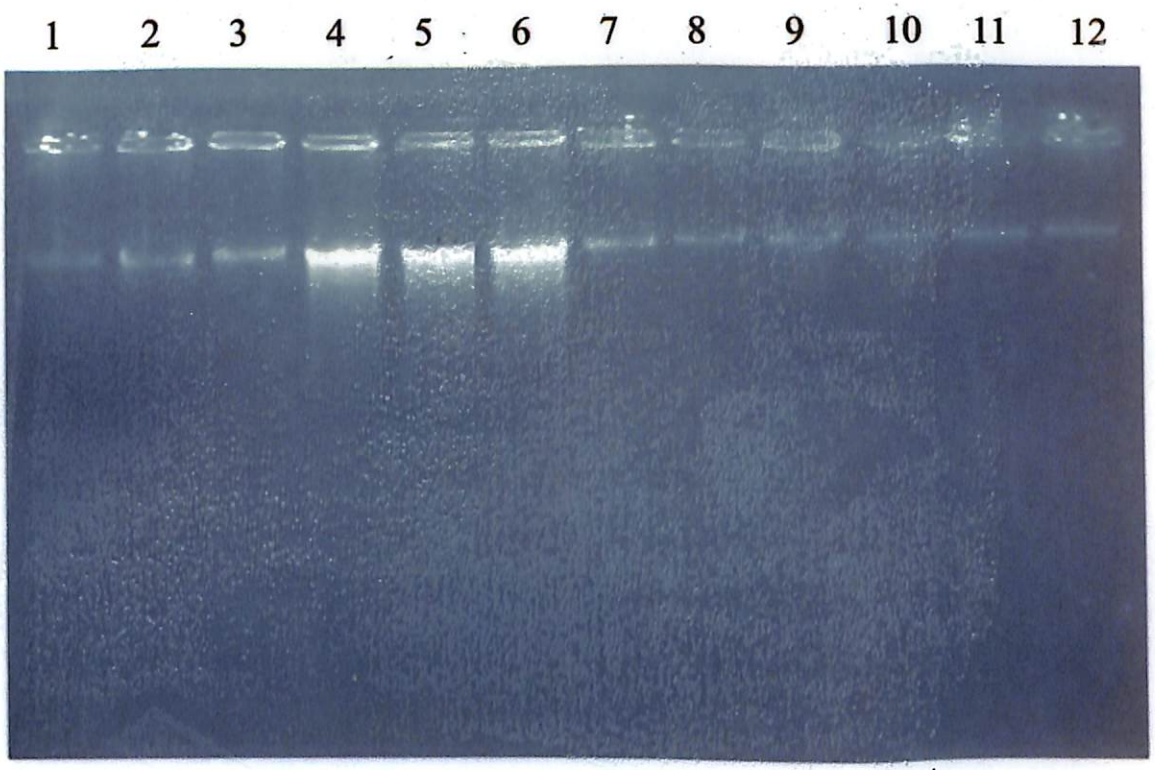

Plate 1. DNA isolated by phenol chloroform extraction procedure on 0.8 % agarose gel

Lane 1-3, 7-12 : PCR template
Lane 4-6 : Stock DNA

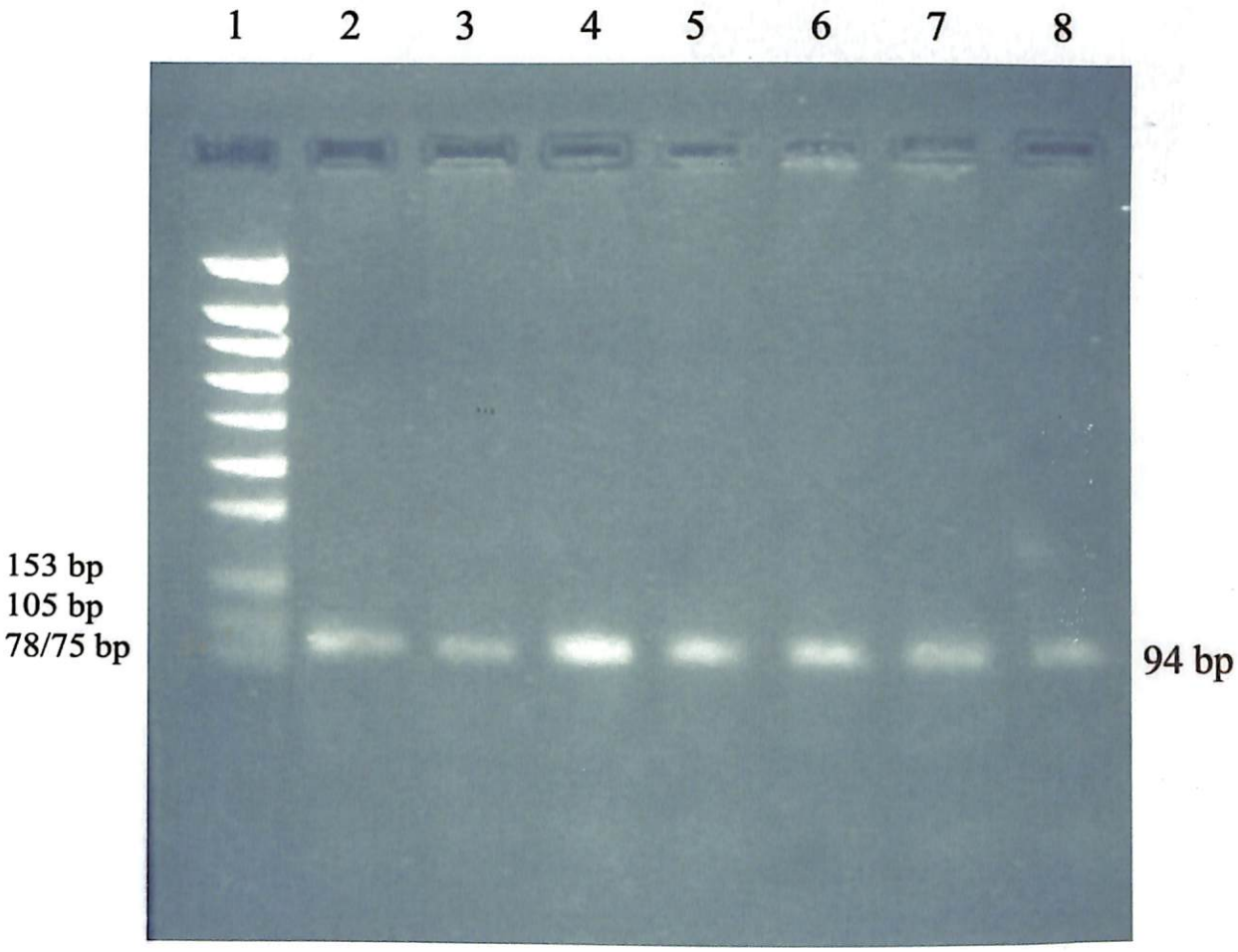

Plate 2. The 94 bp fragment of the bovine leptin gene amplified from genomic DNA by PCR on 2% agarose gel

Lane 2-8 : PCR product of size 94 bp
Lane 1 : pUC18/ *Sau*3AI - pUC18/ *Taq*I digest as DNA size marker

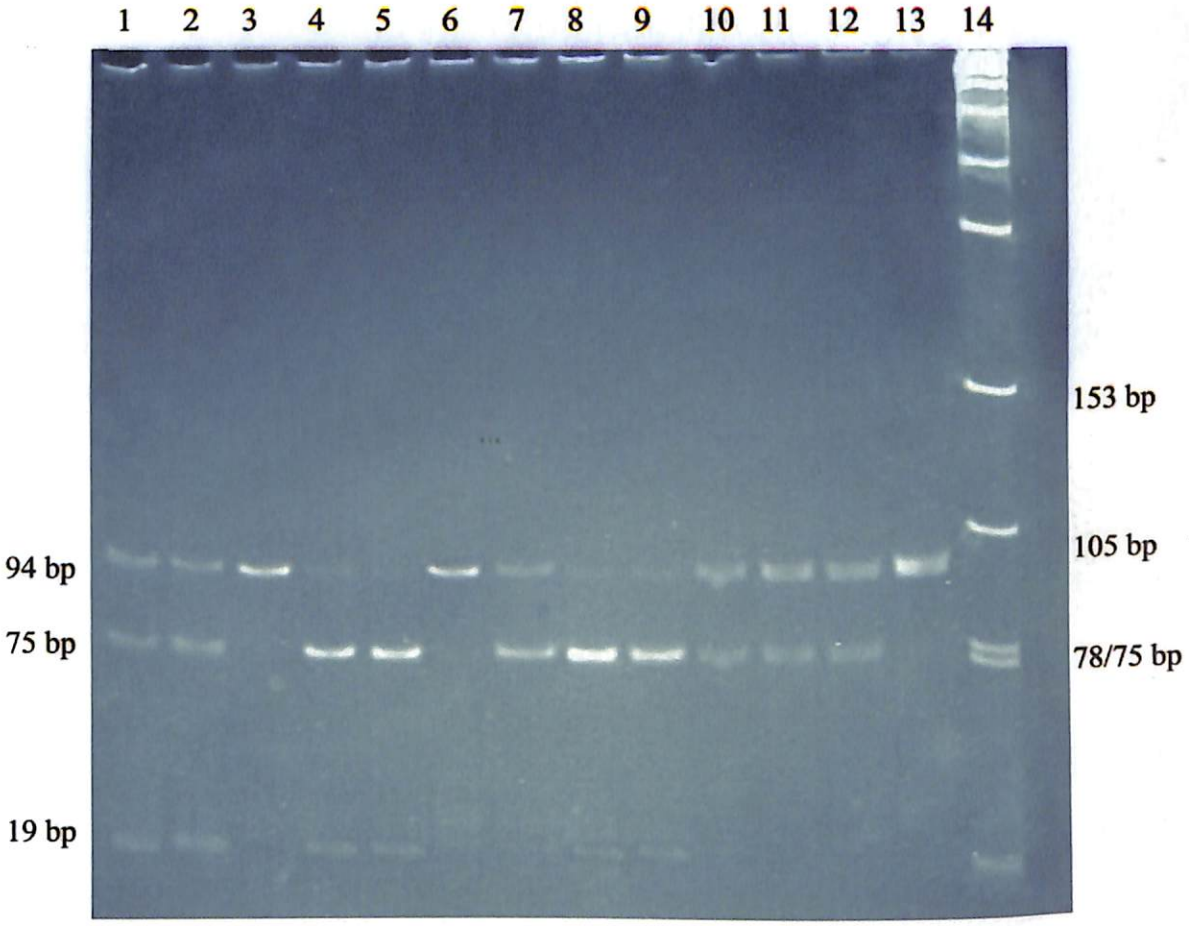

Plate 3. The LEP/*Bsp*E1 genotypes of leptin gene in Vechur cattle resolved on 8% nondenaturing polyacrylamide gel

Lane 4,5,8,9 : CC genotype with 75 and 19 bp fragments
Lane 1,2,7,10-12 : CT genotype with 94, 75 and 19 bp fragments
Lane 3,6 : TT genotype with 94 bp
Lane 13 : PCR product
Lane 14 : pUC18/ *Sau*3AI - pUC18/ *Taq*I digest as DNA size marker

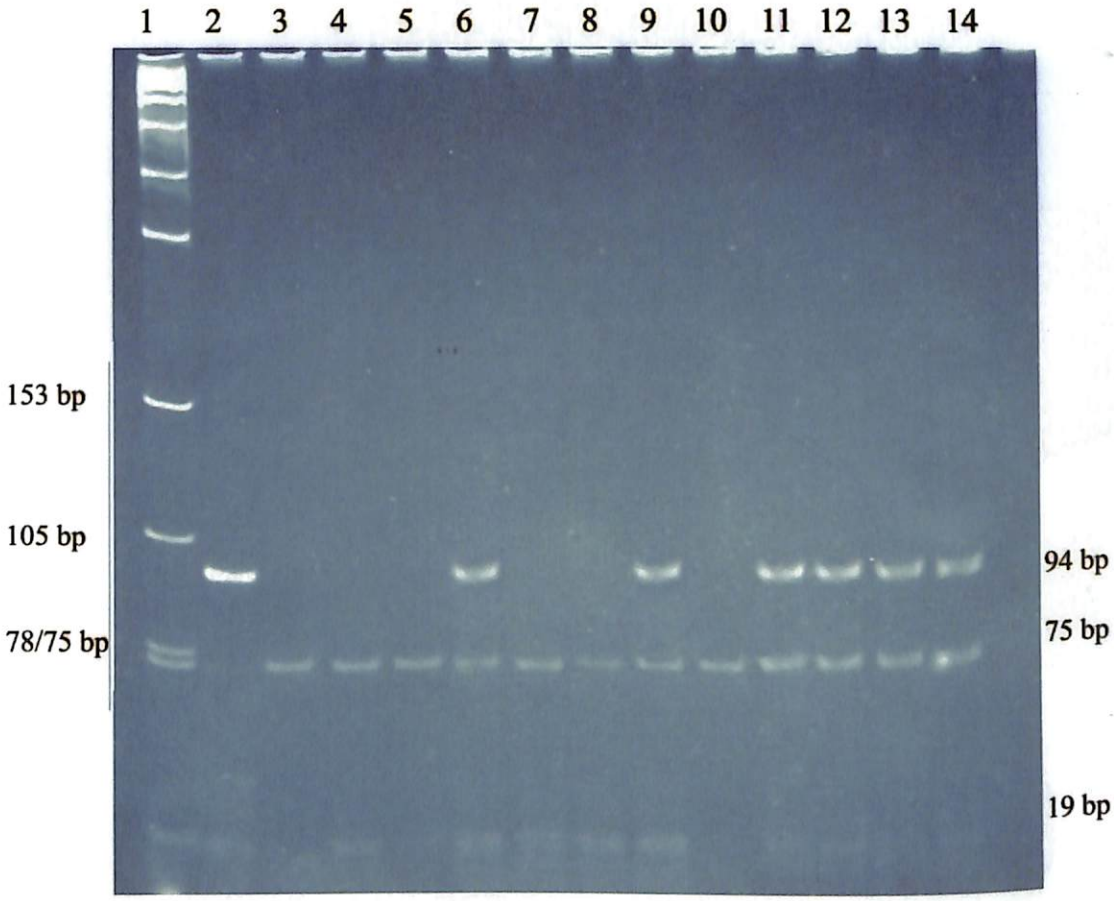

Plate 4. The LEP/*Bsp*E1 genotypes of leptin gene in crossbred cattle of Kerala resolved on 8% nondenaturing polyacrylamide gel

Lane 1 : pUC18/ *Sau*3AI - pUC18/ *Taq*I digest as DNA size marker
Lane 2 : PCR product
Lane 3-5,7,8,10 : CC genotype with 75 and 19 bp fragments
Lane 6,9,11-14 : CT genotype with 94, 75 and 19 bp fragment

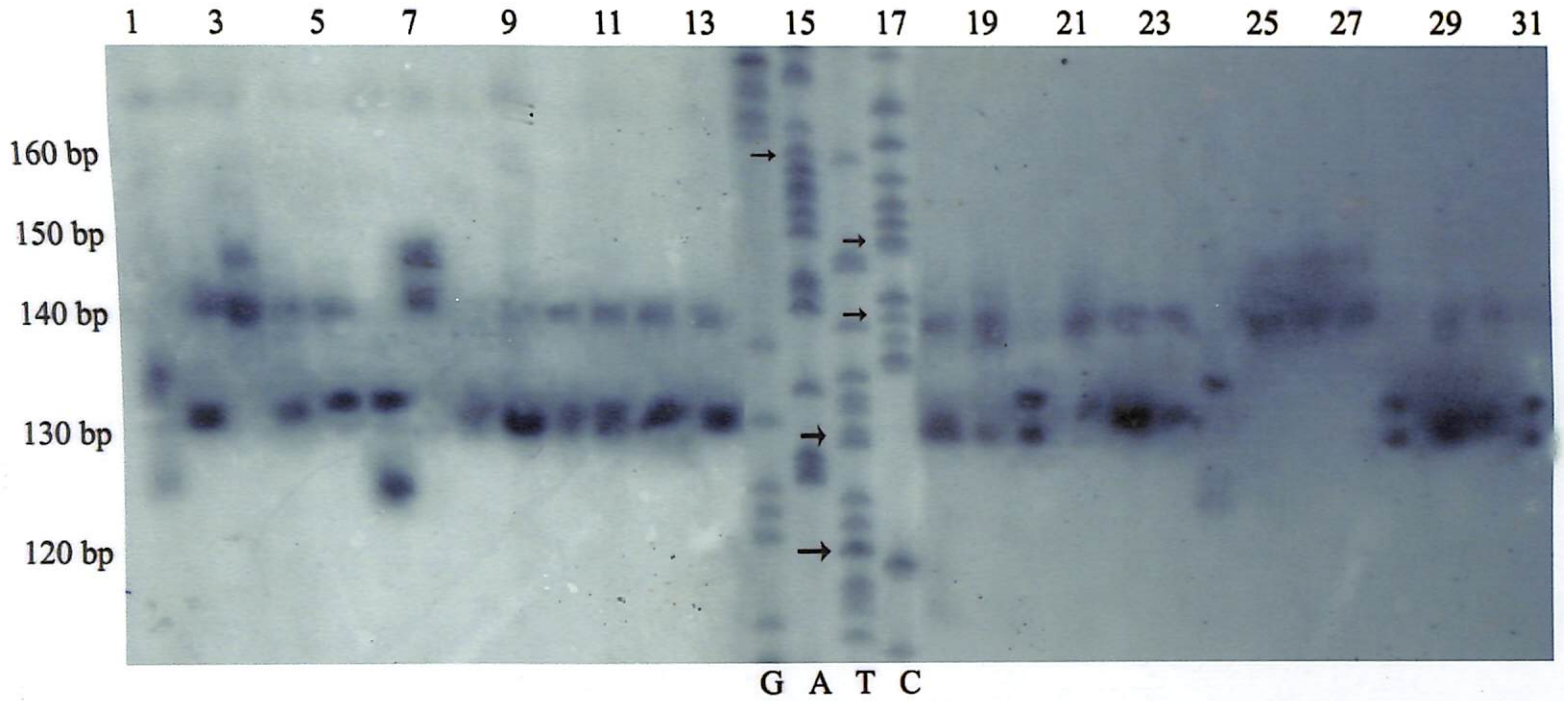

Plate 5. Autoradiograph of BM1500 genotypes of leptin gene in Vechur cattle

Lane 1-13, 18-31 : Samples
Lane 14-17 : G,A,T,C sequences of M13 DNA

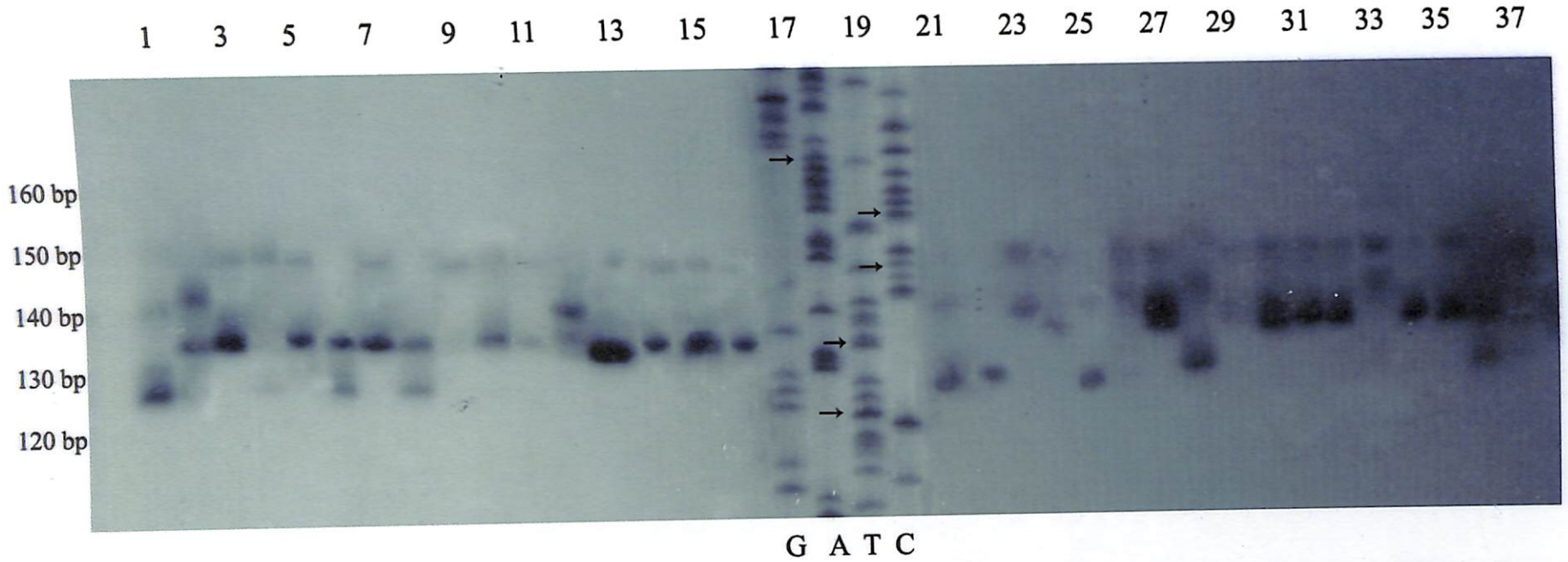

Plate 6. Autoradiograph of BM1500 genotypes of leptin gene in crossbred cattle of Kerala

Lane 1-16, 21-37 : Samples
Lane 17-20 : G,A,T,C sequences of M13 DNA

5. DISCUSSION

5.1 POLYMORPHISM OF LEP/*Bsp*E1

The polymorphism of leptin gene was investigated in Vechur and crossbred cattle of Kerala by PCR-RFLP using restriction enzyme, *Bsp*E1. In all the animals tested, the size of the amplified product was 94 bp indicating conservation of DNA sequences at the leptin locus in *Bos indicus* and their crossbreds with *Bos taurus*. The same sized PCR products were reported earlier by Barendse *et al*. (2005) and Choudhary *et al*. (2005) in various purebreds and crossbreds of cattle using the same primers.

Analysis of amplified DNA by *Bsp*E1 revealed three restriction digestion patterns indicative of two alleles namely C (94 bp fragment) and T (75 and 19 bp fragments), the names of alleles corresponding to the nitrogenous bases (Cytosine or Thymine) at position 73 of exon two of leptin gene which created *Bsp*E1 polymorphism (Buchanan *et al*., 2002). The above findings are in close agreement with the reports of Lusk (2007) and Fischer (2008).

In Vechur cattle the frequencies of C and T alleles of LEP/*Bsp*E1 polymorphism were observed as 0.82 and 0.18, respectively indicating the predominance of C allele in the population. Fortes *et al*. (2009) made similar findings in Nelore cattle (*Bos indicus*) and reported C and T allele frequencies as 0.96 and 0.04, respectively. Perusal of literature revealed that the allele frequencies of LEP/*Bsp*E1 polymorphism were different in various breeds of *Bos taurus*, *Bos indicus* and their crossbreds.

The presence of T allele of LEP/*Bsp*E1 polymorphism was also reported in *Bos indicus* breeds outside India. The frequencies of C and T alleles of Brahman cattle were reported as 0.81 and 0.19, respectively by Barendse *et al*. (2005) and 0.83 and 0.17, respectively by Fischer (2008). Nassiry *et al*. (2008) reported higher frequencies for T allele in various Iranian *Bos indicus* breeds like Sarabi (0.32), Taleshi (0.45), Sistani (0.31) and Gopayegani (0.29).

A contradictory result was obtained by Choudhary *et al*. (2005), who reported the absence of T allele at LEP/*Bsp*E1 locus in various *Bos indicus* breeds in India namely Hariana, Sahiwal, Gir and Nimari.

Crossbred cattle of Kerala, used in the present study have a mosaic genetic make up of Jersey, Brown Swiss, Holstein Friesian and local cattle. The C and T allele frequencies of LEP/*Bsp*E1 polymorphism for crossbred cattle of Kerala were obtained as 0.89 and 0.11, respectively. The present findings are in close agreement with Choudhary *et al*. (2005), who reported the allele frequencies in *Bos taurus* and *Bos indicus* crossbreds (1/2 Holstein Friesian × 1/2 Hariana) as 0.82 for C and 0.18 for T allele.

Fortes *et al*. (2009) reported the C/T allele frequencies in crossbred cattle possessing distinct levels of *Bos indicus* blood (1/2 Nelore, 0.93/0.07; 3/8 Nelore, 0.61/0.39 and 7/16 Nelore, 0.74/0.26). They detected an increasing trend in the T allele frequencies with the increase in the levels of *Bos taurus* blood and opined that the T allele was contributed by *Bos taurus* cattle.

The C/T allele frequencies in *Bos taurus* purebreds were 0.55/0.45 in Brown Swiss (Buchanan *et al*., 2003), 0.44/0.56 in Jersey (Choudhary *et al*., 2005) and

0.75/0.35 in Holstein Friesian cattle (Banos *et al*., 2008). These breeds were used for crossbreeding programmes in Kerala.

In the pooled population, the study of LEP/*Bsp*E1 polymorphism revealed C and T allele frequencies of 0.87 and 0.13, respectively. Similar findings have been reported by Fortes *et al.* (2009) who recorded the frequencies of C and T alleles of pooled cattle population consisting of *Bos indicus* and their crossbreds with *Bos taurus* as 0.81 and 0.19, respectively.

The genotype frequencies of LEP/*Bsp*E1 polymorphism in Vechur cattle were recorded as 0.71 (CC), 0.24 (CT) and 0.05 (TT). Similar results were reported by Fischer (2008) as 0.73 for CC, 0.20 for CT and 0.07 for TT in *Bos indicus* cattle (Brahman). Lower frequency for CT genotype was recorded in Nelore breed (0.09) by Fortes *et al.* (2009).

In contradiction to the present findings in Vechur cattle with regard to the genotype frequencies of LEP/*Bsp*E1 polymorphism, absence of TT genotype was reported in *Bos indicus* cattle, *viz.* Hariana, Sahiwal, Gir and Nimari by Choudhary *et al*. (2005) and Nelore by Fortes *et al.* (2009).

Higher proportion of T allele bearing animals among Vechur cattle under study than those reported in other *Bos indicus* cattle may be due to higher rate of inbreeding practised in the Vechur conservation project or lesser sample size.

In crossbred cattle of Kerala the genotype frequencies of LEP/*Bsp*E1 polymorphism were found to be 0.79 for CC and 0.21 for CT. The TT genotype was absent in the crossbred population studied. Similar results were recorded by Fortes *et*

al. (2009) in crossbreds of *Bos indicus* and *Bos taurus* with genotype frequencies of 0.85 and 0.15, respectively for CC and CT genotypes.

As against the present findings of only two genotypes (CC and CT) for LEP/*Bsp*E1 polymorphism in crossbreds, three genotypes (CC, CT and TT) were reported in 1/2 Holstein Friesian × 1/2 Hariana (0.68, 0.27 and 0.05) crossbreds by Choudhary *et al.* (2005) and 1/2 *Bos taurus* × 1/2 Nelore (0.37, 0.47 and 0.16) by Fortes *et al*. (2009).

The CC, CT and TT genotype frequencies in *Bos taurus* purebreds were reported as 0.43, 0.45 and 0.12 in Holstein Friesian by Banos *et al*. (2008) while 0.18, 0.52 and 0.30 in Jersey by Choudhary *et al*. (2005). These breeds were extensively used for crossbreeding programmes in Kerala.

The reason for the absence of TT genotype among crossbreds could be attributed to reduced sample size or the process of natural or artificial selection going on against TT genotypes in the population. Chebel *et al*. (2008) reported increased incidence of postparturient diseases and displacement of abomasum and suggested increased risk for multiple diseases and morbidity in TT genotyped Holstein Friesian animals.

5.2 POLYMORPHISM OF BM1500 MICROSATELLITE MARKER

Microsatellites are the powerful tools to differentiate breeds and they are giving the correct answer regardless of whether the breeds are closely related or not (Saitbekova *et al*., 1999).

Microsatellite analysis at BM1500 marker located 3.6 kb downstream of leptin gene, revealed six alleles and eight genotypes in the pooled population, comprising Vechur and crossbred cattle of Kerala. Five alleles each were detected in Vechur (126, 132, 136, 144 and 150 bp) as well as crossbred cattle population (126, 132, 136, 138 and 144 bp) in the present study. At BM1500 locus, various researchers reported varied number of alleles and genotypes in diverse panel of cattle, four alleles and five genotypes in beef breeds comprising Angus, Charolais, Hereford and Simmental (Fitzsimmons *et al*., 1998), three alleles and six genotypes in Holstein Frisian (Liefers *et al*., 2002), six alleles in Angus and four alleles in Charolais (Almeida *et al*., 2007).

The alleles of BM1500 marker in the cattle population under study were 126, 132, 136, 138, 144 and 150 bp. In comparison with the alleles reported by various researchers, 136 and 144 bp by Tessane *et al*. (1999), 136, 144 and 146 bp by Liefers *et al*. (2002) and 136, 138, 142, 144, 146, 148 and 150 bp by Almeida *et al*. (2007), two novel alleles of size 126 and 132 bp were detected at BM1500 locus in the present study.

The frequencies of alleles at BM1500 locus observed in Vechur cattle in descending order were 0.40 (132 bp), 0.32 (144 bp), 0.13 (136 bp), 0.10 (150 bp) and 0.05 (126 bp). Almeida *et al*. (2007) reported frequencies of 0.25 and 0.31 for 136 bp and 144 bp alleles, respectively whereas Tessane *et al.* (1999) reported higher frequencies of 0.49 and 0.51, respectively in Angus breed. The frequency of 150 bp allele in Charolais breed was reported as 0.07 which is comparable with the frequency of 150 bp allele in Vechur cattle.

The frequencies of 126, 132, 136, 138, and 144 bp alleles at BM1500 locus in crossbred cattle population were 0.12, 0.46, 0.12, 0.06 and 0.24, respectively. In

Holstein Friesian, one of the purebred *Bos taurus* cattle used for the breeding programme in Kerala, the allele frequencies of 0.41 (136 bp), 0.45 (144 bp) and 0.14 (146 bp) were reported (Liefers *et al*., 2003).

The highest frequency at BM1500 locus was observed for 132 bp allele in Vechur (0.40) crossbred (0.46) and pooled population (0.43) under study. Contradictory results were obtained by Liefers *et al*. (2003), who reported highest frequency for 144 bp allele (0.45) in Holstein Friesian cattle while Almeida *et al*. (2007) recorded highest frequency for 142 bp allele (0.46) in Charolais breed.

Alleles of size 142 and 146 bp at BM1500 locus, which were absent in the present study, were reported by Almeida *et al*. (2007) and Liefers *et al*. (2003) in Angus and Holstein Friesian, respectively.

The genotypes at BM1500 locus observed in Vechur cattle were 132/132, 136/126, 136/132, 144/132 and 150/144. In crossbred cattle population the genotypes observed were 126/126, 132/132, 136/126, 136/132, 138/132, 144/132 and 144/136. While examining the genotypes reported by Fitzsimmons *et al*. (1998), Liefers *et al*. (2003) and Almeida *et al*. (2007), six new genotypes (126/126, 132/132, 136/126, 136/132, 138/132 and 144/132) were identified at BM1500 locus in the present study.

In Vechur cattle the highest genotype frequency was observed for 144/132 (0.44) while the lowest for 132/132 as well as 136/126 with a frequency of 0.07. The 144/132 genotype topped with a frequency of 0.38 whereas 136/132 genotype (0.05) showed the lowest frequency in crossbred cattle. All the genotypes other than 144/136 (Liefers *et al*., 2003) observed in the crossbred cattle population, have not yet been reported upon.

Difference in number and frequencies of alleles and genotypes at a microsatellite marker are indicative of genetic variability and form the basis of all genetic diversity indices (Bindu, 2006).

Botstein *et al*. (1980) opined that a marker was highly informative if its PIC value was greater than 0.5. In the present study, the polymorphic information content of BM1500 marker was obtained as 0.6595 in Vechur, 0.6560 in crossbred and 0.6666 in the pooled population. Thus the BM1500 marker is considered as highly informative in Vechur and crossbred cattle population.

The direct count heterozygosity and unbiased heterozygosity in Vechur were 0.7082 and 0.7169, while in crossbred cattle 0.6984 and 0.7068, respectively. In the pooled population the direct count heterozygosity and unbiased heterozygosity were reported as 0.7100 and 0.7143, respectively.

5.3 MILK PRODUCTION TRAITS

5.3.1 305 Day Milk Yield

The recorded average 305 day milk yield of Vechur cattle was 348.01±19.63kg in the present study as against 471 kg reported by Girija (1994).

In crossbred cattle of Kerala the average 305 day milk yield was recorded as 2106.78±56.13 kg. Similar result was reported by Kannan *et al*. (2000) in crossbreds of Kerala (2113±54.98 kg). A higher average value was reported by Chandran (2007) for 305 day milk yield of cattle from different zones of Kerala (2406±16.6 kg).

5.3.2 Daily Milk Yield

The average daily milk yield of Vechur cattle was recorded as 1.27±0.06 kg in the present study. Girija (1994) and Iype and Venkatachalapathy (2001) reported higher averages for daily milk yield in Vechur cattle as 2.17 kg and 2.2 kg, respectively.

In crossbred cattle of Kerala the daily milk yield (kg) was recorded as 7.10±0.19. The above finding is in close agreement with Thirumurugan and Saseendran (2006), who reported the average daily milk yield (7.78±0.12 to 8.86±0.13 kg) of crossbred cattle maintained in various housing systems in Kerala Agricultural University livestock farms.

The average daily milk yield of pooled population consisting of purebreds and crosses of Sahiwal, Jersey and Friesian breeds was reported as 8.64 kg by Kant and Prasad (1997).

5.3.3 First lactation Length

The average first lactation length of Vechur cattle was recorded as 232.67±13.11 days in the present study. Girija (1994) reported a lower value (217 days) where as Raghunandanan (2006) recorded a higher value (242±9.4 days) for lactation length in Vechur cattle.

In crossbred cattle under study the average first lactation length (days) was recorded as 292.42±6.10. The above finding is in agreement with Chacko and Jose (1988) and Bhadoria *et al.* (2004) who reported 274.3±1.97 and 292.43±4.35 days of lactation in crossbred cattle and Gir, respectively.

5.3.4 Milk Fat Percentage

The average milk fat of Vechur animals in 65-90 days of lactation was recorded in percentage as 4.59±0.08. A comparable average milk fat percentage of 5.56±0.13 in Vechur cattle at seventh week of lactation was reported by Venkatachalapathy (1996). Similar observations were made by Iype and Venkatachalapathy (2001) and Raghunandanan (2006) who reported the average fat percentages as 4.7 and 4.5, respectively.

In the present study the average milk fat percentage in crossbred cattle was 3.54±0.09. This is in agreement with Radhika (1997) and Thomas (2005) who reported the milk fat percentages in crossbred cattle during mid lactation as 3.65±0.04 and 3.74±0.08, respectively. A higher milk fat percentage (4.64) of crossbred cattle in Kerala during mid lactation was reported by Sathian (2001).

5.4 LEP/*Bsp*E1 POLYMORPHISM AND MILK PRODUCTION TRAITS

Various LEP/*Bsp*E1 genotypes in Vechur and crossbred cattle of Kerala were associated with milk production traits like 305 day milk yield, daily milk yield, lactation length and milk fat percentage.

5.4.1 LEP/*Bsp*E1 Polymorphism and 305 Day Milk Yield

Vechur animals bearing T allele showed an increase of 89.77 kg (13.08%) for 305 day milk yield (387.91±26.43 kg) than animals where T allele was absent (298.14±23.1 kg). Reports of association studies of LEP/*Bsp*E1 polymorphism are scanty in Indian cattle breeds.

In crossbred cattle of Kerala, 305 day milk yield (kg) recorded was 2413.01±64.41 and 1946.38±67.5 for CT and CC genotyped animals, respectively. An increase of 466.63 kg (10.7%) of 305 day milk yield obtained in CT genotyped crossbred cattle is in line with the findings of Chebel *et al.* (2008) who reported 282 kg increase in 305 day milk yield in Holstein Friesian carrying CT genotype than CC genotype. Similar observations were made by Komisarek *et al*. (2005) who reported significantly increased milk yield in cattle possessing T allele.

These findings point to the fact that CT genotypes of LEP/*Bsp*E1 polymorphism are correlated with increased milk production. Hence selection in that direction may contribute to rise in milk production in the State.

In contrast to the present findings, Madeja *et al*. (2004) reported that milk yield was not associated with LEP/*Bsp*E1 polymorphism in Polish black and white cattle.

5.4.2 LEP/*Bsp*E1 Polymorphism and Daily Milk Yield

The average daily milk yield for CT/TT and CC genotyped Vechur animals were 1.71±0.08 and 1.40±0.08 kg, respectively while for crossbreds 7.90±0.23 and 6.68±0.23 kg, respectively. A significant increase of 0.31 kg (9.96%) and 1.22 kg (8.38%) for average daily milk yield was noticed in CT/TT genotyped Vechur and CT genotyped crossbred cattle (1.71±0.08 and 7.90±0.23 kg), respectively than CC genotyped animals (1.40±0.08 and 6.68±0.23 kg). Similar results were detected by (Buchanan *et al*., 2003) who reported an increase of 1.5 kg for average daily milk yield in Holstein Friesian cattle bearing T allele. Sadeghi *et al.* (2008) also reported the association of T allele with increased milk yield in Iranian Holstein cattle.

In contrast to the present results, Alashawkany *et al*. (2008) could not find a significant relationship between LEP/*Bsp*E1 genotypes and average daily milk yield in Holstein cattle.

The reason for remarkable variation in milk yield was attributable to the functional alteration in the leptin gene product (leptin) associated with C to T mutation at position 305 (C305T) of leptin gene and subsequent amino acid change (arginine to cysteine) in the α-helix of leptin molecule which might disrupt the binding of leptin to its receptor (Buchanan *et al*., 2002). They proposed that the presence of unpaired cysteine in the altered molecule might destabilize the disulfide bridge which is critical for its biological function.

Liefers *et al.* (2003) explained that higher milk yield was related to lower leptin concentration. This fact reiterates the effect of C to T mutation at leptin gene locus, producing the altered molecule which in turn reduces the concentration of active leptin in the circulation.

Buchanan *et al*. (2003) opined that T allele possessing animals had increased body fat and adipose tissue reserves indirectly contributing to the increased milk yield. They speculated that the increased feed intake associated with T allele of LEP/*Bsp*E1 polymorphism might affect indirectly on significant increase in milk yield in T allele possessing cattle.

Another reason attributable to changes in lactation performance in cattle of different LEP/*Bsp*E1 genotypes may likely be the result of differences in partition of nutrients with CT cows prioritizing milk yield as opposed to CC genotyped cattle (Chebel *et al.*, 2008).

Silva *et al.* (2002) demonstrated a direct inhibitory effect of leptin on proliferation of a mammary epithelial cell line (Mac T cells) *in vitro*. They suggested that the altered leptin molecule could not bind with the receptors resulting in the down regulation of leptin action which remove the inhibitory effect on mammary epithelial cells.

5.4.3 LEP/*Bsp*E1 Polymorphism and First Lactation Length

The average first lactation lengths (days) recorded in Vechur and crossbred cattle possessing T allele were 235.87±16.03 and 318.14±6.01, respectively while 228.67±22.48 and 278.95±8.13 were recorded for lactation lengths in Vechur and crossbred cattle without T allele of LEP/*Bsp*E1 polymorphism. The average first lactation lengths (days) were higher in T allele bearing Vechur (1.55%) and crossbred cattle (6.54%) compared to animals without T allele. A statistically significant increase was obtained for the first lactation length in crossbred cattle possessing T allele.

5.4.4 LEP/*Bsp*E1 Polymorphism and Milk Fat Percentage

In the present study the mean milk fat percentages were lower in Vechur (0.66%) and crossbred cattle (0.42%) possessing T allele of *Bsp*E1 polymorphism compared to CC genotypes. The reduction in milk fat percentage associated with T allele was not statistically significant. The above finding is in accordance with Buchanan *et al.* (2003) who also reported a nonsignificant reduction in milk fat percentage in TT (0.1%) and CT (0.07%) genotyped animals compared to CC genotypes.

In contrast to the above finding, Madeja *et al.* (2004) reported the lack of association between LEP/*Bsp*E1 polymorphism and milk fat percentage in Polish black and white cattle breed. Chebel *et al.* (2008) reported significant reduction in milk fat yield associated with CT/TT genotype of *Bsp*E1 polymorphism in Holstein Friesian cattle.

Buchanan *et al*. (2002) reported the association of T allele with increase in fat deposition in beef cattle. DeVuyst *et al*. (2008) reported that beef cattle with CT and TT genotypes weaned significantly heavier beef calves (8.81 and 12.44 kg, respectively) than CC genotype.

Extensive association studies regarding LEP/*Bsp*E1 polymorphism and milk fat percentage are required to investigate the influence of T allele on milk fat percentage in *Bos indicus* and crossbreds.

5.5 BM1500 POLYMORPHISM AND MILK PRODUCTION TRAITS

In the association study, polymorphism of BM1500 microsatellite with milk production traits, the highest 305 day milk yield and daily milk yield were recorded in Vechur (378.39±42.44 and 1.43±0.12 kg) and crossbred cattle (2142.61±104.4 and 7.31±0.38 kg) possessing 136 bp allele and the increase in milk yield was not statistically significant. The reason for increased milk yield may be due to lower leptin concentration associated with 136 bp allele as reported by Liefers *et al.* (2003).

Almeida *et al*. (2007) detected a positive correlation of 136 bp allele of BM1500 marker with increased average weight gain in beef cattle and live weight in Holstein Friesian cattle.

Liefers *et al.* (2002) could not find significant association between polymorphism at BM1500 marker and milk production traits in Holstein Friesian cattle.

The highest milk fat percentage was noticed in crossbred cattle carrying 138 bp allele (3.56±0.13) compared to animals carrying 136 (3.51±0.11), 132 (3.42±0.08) and 144 bp alleles (3.35±0.09). The genotype 138/132 topped with a milk fat percentage of 3.58±0.14 in the crossbred cattle population. Similar observations were made by Fitzsimmons *et al.* (1998) in beef cattle who reported an association of 138 bp allele with fat deposition.

The average lactation length was highest in 138 bp allele carrying crossbred cattle (304±6.72 days) while in Vechur cattle the highest value was observed in 150 bp allele carrying animals (246.83±25.73 days).

The crossbred cattle possessing 144 bp allele had higher average for 305 day milk yield (1943.71±128.6 kg) and daily milk yield (6.49±0.45 kg) compared to animals bearing 132 and 138 bp alleles.

In Vechur cattle 136/132 genotyped animals had highest averages for 305 day milk yield (363.20±53.78 kg), daily milk yield (1.51±0.14 kg) and milk fat percentage (4.55±0.37) whereas in crossbreds 132/132 genotyped animals had highest averages for 305 day milk yield (2028.55±134.5 kg) and daily milk yield (6.72±0.64 kb).

In the present study milk fat percentage in crossbred cattle of Kerala was significantly different in 138/132 (3.58±0.14) and 132/132 (3.41±0.08) genotype carrying cattle.

In the present study Vechur and crossbred cattle of Kerala possessing T allele for LEP/*Bsp*E1 polymorphism produced significantly higher average for 305 day milk yield (13.08% and 10.7%) and daily milk yield (9.96% and 8.38%) compared to animals lacking T allele. Animals carrying T allele are also associated with increased disease resistance, reduced risk of displacement of abomasum and post parturient diseases and reduced gestation length. Animals with 136 bp allele of BM1500 locus was also associated with increased 305 day and daily milk yield compared to others. Milk production in crossbred and Vechur cattle can be improved by increasing the frequency of animals having T allele of LEP/*Bsp*E1 and 136 bp allele of BM1500 polymorphism.

LEP/*Bsp*E1 marker can be suggested as a valuable candidate for marker assisted selection for milk production in cattle population of Kerala. The molecular markers can successfully complement traditional selection methods and increase the efficiency of selection.

The research on leptin gene polymorphism should be extended to cattle population of different geographical regions of the State to confirm the influence of LEP/*Bsp*E1 marker on milk production traits. The reduced milk yield is the most important factor for the reluctance of rearing of Vechur cattle, the only recognized cattle breed in Kerala. Exploitation of LEP/*Bsp*E1 marker for selection of Vechur animals will improve the milk production and increase the value of the native breed.

6. SUMMARY

New molecular techniques focused on genome analysis, open new possibilities for accurate evaluation of economically important traits in dairy cattle. Milk production traits are typical quantitative characteristics controlled by a number of genes. Mutations in their sequences may alter lactation performance of animals. In the present study the associations of polymorphisms of LEP/*Bsp*E1 and BM1500 markers at leptin locus with milk production traits were analyzed in Vechur and crossbred cattle of Kerala.

DNA was isolated from blood samples of 74 Vechur and 108 crossbred cattle of Kerala using phenol chloroform extraction procedure. All DNA samples obtained were suitable for analysis.

For PCR-RFLP analysis of leptin gene, 94 bp fragment from the start of exon two was amplified with specific primers. The PCR products were digested with restriction enzyme, *Bsp*E1 at 37ºC overnight in dry bath. On non- denaturing polyacrylamide gel (8%), three restriction digestion patterns in Vechur as against two in crossbred cattle were revealed with the identification of two alleles namely C (75 bp and 19 bp fragments) and T (94 bp fragment). The allele sizes were confirmed using pUC18/ *Sau*3AI - pUC18/ *Taq*I digest as DNA size marker.

The C/T allele frequencies in Vechur and crossbred cattle population were 0.82/0.18 and 0.89/0.11, respectively indicating the predominance of C allele. *Chi*-square test revealed that the C and T alleles were uniformly distributed in both populations.

The genotype frequencies of LEP/*Bsp*E1 polymorphism were 0.71 (CC), 0.24 (CT) and 0.05 (TT) in Vechur whereas 0.79 (CC) and 0.21 (CT) in crossbreds with the absence of TT genotype. The populations of Vechur and crossbred cattle under study were in Hardy-Weinberg equilibrium with regard to LEP/*Bsp*E1 locus.

Microsatellite analysis was performed at BM1500 marker, located 3.6 kb downstream of leptin gene in Vechur and crossbred cattle of Kerala by PCR amplification with endlabelled forward primer, fractionation by denaturing PAGE (6%) and visualization by autoradiography. The sizes of alleles were determined by comparing with G, A, T and C sequences of M 13 phage DNA.

Five alleles each were detected in Vechur (126, 132, 136, 144 and 150 bp) as well as crossbred cattle population (126, 132, 136, 138 and 144 bp) in the present study. The highest frequency was observed for 132 bp allele in Vechur (0.40) and crossbred cattle (0.46) while lowest for 126 bp allele in Vechur (0.05) and 138 bp allele in crossbred cattle (0.06). The 138 bp allele was absent in Vechur as against 150 bp in crossbreds. At BM1500 locus two novel alleles, *viz.* 132 bp and 126 bp were detected in Vechur and crossbred cattle of Kerala.

Five genotypes (132/132, 136/126, 136/132, 144/132 and 150/144) were observed at BM1500 locus in Vechur as against seven (126/126, 132/132, 136/126, 136/132, 138/132, 144/132 and 144/136) in crossbred cattle population. The highest frequency was observed for 144/132 genotype in Vechur (0.44) as well as crossbred cattle (0.38). The lowest frequency was observed for 132/132 and 136/126 (0.07) in Vechur while 136/132 (0.05) in crossbred cattle. All the genotypes except 144/136 observed in the crossbred cattle population under study have not yet been reported upon.

The highly informative nature of the microsatellite marker, BM1500 was established by PIC values of 0.6595 in Vechur and 0.6560 in crossbred cattle population of Kerala. The direct count heterozygosity and unbiased heterozygosity in Vechur were 0.7082 and 0.7169, while 0.6984 and 0.7068, respectively in crossbred cattle.

Milk samples of Vechur and crossbred cattle in 65-90 days of lactation were analyzed for milk fat percentage using Gerber's method. The milk fat percentage of Vechur cattle (4.59±0.08%) was significantly higher than crossbred cattle (3.54±0.09%). The average milk yield (kg) standardized to 305 days was recorded as 348.01±19.63 in Vechur and 2106.78±56.13 in crossbred cattle. The average daily milk yield (kg) and lactation length (days) in Vechur cattle were 1.27±0.06 and 232.67±13.11, while 7.10±0.19 and 292.42±6.10 in crossbred cattle.

The association of LEP/*Bsp*E1 and BM1500 markers with milk production traits like 305 day milk yield, daily milk yield, lactation length and milk fat percentage was studied using student's t-test and analysis of variance in Vechur and crossbred cattle population.

Vechur animals bearing T allele showed 13.08% (89.77 kg) increase for 305 day milk yield (387.91±26.43 kg) than animals where T allele was absent (298.14±23.1 kg). A significant increase of 9.96% for average daily milk yield was noticed in T allele possessing Vechur cattle (1.71±0.08 kg), than CC genotyped animals (1.40±0.08 kg). Average lactation length was also higher (1.55%) in T allele bearing Vechur animals compared to animals lacking T allele even though the difference was non significant. Though reduced milk fat percentage was observed for T allele bearing Vechur animals than CC cattle, the reduction noticed was found to be not significant.

In the case of crossbred cattle, similar observations were found wherein T allele bearing animals showed higher averages for 305 day milk yield (10.7%), daily milk yield (8.38%) and lactation length (6.54%) compared to animals lacking T allele. All the said observations in T allele possessing crossbred cattle were significantly different from animals wherein T allele was absent.

Analysis of microsatellite marker polymorphism at BM1500 locus revealed that allele 136 bp was associated with higher 305 day milk yield, daily milk yield and milk fat percentage in Vechur . Higher values for the said parameters were observed in animals with 136/132 genotype. It was also observed that highest average lactation length was observed for animals possessing 150 bp allele.

In the case of crossbred cattle also animals carrying 136 bp allele topped for 305 day milk yield and daily milk yield. Average lactation length and milk fat percentage were higher in 138 bp allele bearing animals. Microsatellite marker analysis at BM1500 loci in Vechur and crossbred cattle of the State prove that animals possessing 136 bp allele for the said locus topped with lactation yield and daily milk production. The milk fat percentage in crossbred cattle of Kerala was significantly different in 138/132 (3.58±0.14) and 132/132 (3.41±0.08) genotypes.

While going for marker assisted selection for milk production in cattle population of Kerala, LEP/*Bsp*E1 and BM1500 markers could be made use of to a greater degree. The research on leptin gene polymorphism should be extended to cattle population of different geographical regions of the State to confirm the influence of LEP/*Bsp*E1 marker on milk production traits. Microsatellite marker analysis should be extended to a sizeable population making use of a higher number of markers with a view to achieve a relevant scientific confirmation.

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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 (2%)

Weighed 2 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^{o}C in a dark coloured bottle.

Formamide dye / Stop buffer

Deionised formamide	98 %
Xylene cyanol	0.025 %
Bromophenol blue	0.025 %
0.5 *M* EDTA	10 m*M*

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^{o}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^{o}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^{o}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^{o}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^{o}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^{o}C.

TBE Gel mix (0.5X)

40% Acrylamide	150 ml
10X TBE buffer	50 ml
Urea	450 g

Mixed well in 700 ml distilled water, volume made up to 1000 ml and stored at 4^{o}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^{o}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^{o}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
Di-sodium hydrogen orthophosphate	-	SRL, Bombay
dNTPs	-	Finn Enzymes
EDTA	-	SRL, Bombay
Ethanol	-	Merck
Ethidum bromide	-	BDH lab, England
6X gel loading buffer	-	Bangalore Genei Pvt. Ltd.
Glacial acetic acid	-	BDH-E, Merck (India) Ltd.
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

pUC18/ *Sau*3AI - pUC18/ *Taq*I digest	-	Bangalore Genei Pvt. Ltd.
M13 sequencing ladder	-	Amersham Pharmacia Biotech, USA.

(D) ENZYMES

Restriction endonuclease, *Bsp*E1	-	Labmate (Asia) Pvt. Ltd.
Taq DNA polymerase	-	Bangalore Genei Pvt. Ltd.
Proteinase-K	-	Bangalore Genei Pvt. Ltd.
Poly Nucleotide Kinase	-	Bangalore Genei Pvt. Ltd.

(E) KITS

DNA-End-labelling kit - Bangalore Genei Pvt. Ltd.

Sequenase version 2.0 DNA sequencing kit - Amersham Pharmacia Biotech, USA.

(F) ISOTOPES

γ^{32} P-ATP	-	BRIT, Bombay
α^{35} S-dATP	-	BRIT (Jonaki), Hyderabad.

ANNEXURE – III

ABBREVIATIONS

LEP	Leptin gene
ob/ob mice	Genetically obese mice
RFLP	Restriction Fragment Length Polymorphism
SSCP	Single Strand Conformation Polymorphism
PCR	Polymerase Chain Reaction
DNA	Deoxyribo Nucleic Acid
MAS	Marker Assisted Selection
QTL	Quantitative Trait Loci
PAGE	Polyacrylamide Gel Electrophoresis
PIC	Polymorphic Information Content
EDTA	Ethylene Diamine Tetra acetic Acid
TEMED	N, N, N´, N´ Tetra methyl ethylene diamine
APS	Ammonium Persulphate
PNK	Polynucleotide Kinase
He	Heterozygosity
µl	microlitres
µg	microgram
mg	milligram
m*M*	millimolar
cm	centimeter
nm	nanometer
pmol	picomols
mCi	millicurie

kb	Kilo basepair
bp	base pair
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
dNTP	Deoxy Nucleotide Triphosphate
ddATP	Dideoxy Adenosine Triphosphate
ddCTP	Dideoxy Cytosine Triphosphate.
ddGTP	Dideoxy Guanosine Triphosphate
ddTTP	Dideoxy Thymidine Triphosphate

LEPTIN GENE POLYMORPHISM IN VECHUR AND CROSSBRED CATTLE OF KERALA

LALI F. ANAND

Abstract of the thesis submitted in partial fulfilment of the requirement for the degree of

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ABSTRACT

Representative population comprising 74 heads of Vechur and 108 numbers of crossbred cattle of Kerala were investigated for leptin gene polymorphisms and their associations with milk production traits. LEP/*Bsp*E1 and BM1500 are the polymorphic markers used for PCR-RFLP and microsatellite analysis, respectively.

For PCR-RFLP analysis, the amplified PCR product was digested with restriction enzyme *Bsp*E1 which revealed three genotypes in Vechur (CC, CT and TT) and two in crossbred cattle (CC and CT) indicating the presence of C (75 bp and 19 bp fragments) and T (94 bp fragment) alleles. The C/T allele frequencies were 0.82/0.18 and 0.89/0.11, respectively in Vechur and crossbred cattle. The genotypes of LEP/*Bsp*E1 polymorphism were distributed according to Hardy-Weinberg equilibrium with frequencies 0.71 (CC), 0.24 (CT) and 0.05 (TT) in Vechur and 0.79 (CC) and 0.21 (CT) with the absence of TT genotype in crossbred cattle under study.

The microsatellite polymorphism at BM1500 locus was analyzed in Vechur and crossbred cattle and two novel alleles of size 126 and 132 bp were detected. Allele frequency was highest for 132 bp and in Vechur (0.40) as well as crossbred cattle (0.46) under study. The polymorphic information content of BM1500 marker in Vechur was 0.6595 while 0.656 in crossbred cattle.

In the present study, the significant influence of LEP/*Bsp*E1 polymorphism on milk production traits. The T allele bearing Vechur and crossbred cattle showed 13.08% (89.77 kg) and 10.7% (466.63 kg) increase for 305 day milk yield, respectively whereas 9.96% (0.31 kg) and 8.38% (1.22 kg), respectively for average daily milk yield. A nonsignificant reduction for milk fat percentage was also

observed associated with T allele in both populations. Increased body fat reserves, adipose tissue reserves, feed intake and favorable change in partitioning of nutrients in T allele possessing animals might have contributed to increased milk yield in cattle.

For BM1500 marker, highest averages of 305 day milk yield and daily milk yield were observed for Vechur and crossbred cattle possessing 136 bp allele. The highest milk fat percentages were recorded in 136 bp allele bearing Vechur animals and crossbreds possessing 138 bp allele. The association of BM1500 marker with milk production traits can be established only by expanding the research to an extensive cattle population.

The selection of Vechur and crossbred cattle, heterozygous for C and T alleles for LEP/*Bsp*E1 polymorphism can significantly improve milk yield. The use of molecular techniques provides new opportunities and challenges for building and using more accurately predictive and efficient selection procedures for livestock improvement.