Study on Genetic Variation in Milk Genes and Their Association with Milk Performance Traits in Indigenous Cattle Breeds

देशी गौवंशीय नस्लों में दुग्ध जीनों में आनुवांशिक विभिन्नताओं एवम् दुग्ध उत्पादन सम्बंधि गुणों पर उनके प्रभाव का अध्ययन

VIJAY KUMAR AGRAWAL
M.V.Sc

THESIS

DOCTOR OF PHILOSOPHY
(Animal Genetics and Breeding)

।पशुधनं नित्यं सर्वलोकोपकारकम्।

2017

Department of Animal Genetics and Breeding
College of Veterinary and Animal Science
Rajasthan University of Veterinary & Animal Sciences,
Bikaner-334001 Rajasthan

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THESIS

Submitted to the
Rajasthan University of Veterinary & Animal Sciences, Bikaner
in partial fulfillment of the requirement for
the degree of

DOCTOR OF PHILOSOPHY
(Animal Genetics and Breeding)

By

VIJAY KUMAR AGRAWAL
M.V.Sc

2017

Rajasthan University of Veterinary & Animal Sciences, Bikaner
College of Veterinary and Animal Science, Bikaner

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This is to certify that **Vijay Kumar Agrawal** had successfully completed the **comprehensive examination** held on **12/11/2016** as required under the regulation for PhD degree.

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Date……..……

This is to certify that this thesis entitled **STUDY ON GENETIC VARIATION IN MILK GENES AND THEIR ASSOCIATION WITH MILK PERFORMANCE TRAITS IN INDIGENOUS CATTLE BREEDS** submitted for the degree of **DOCTOR OF PHILOSOPHY** in the subject of **Animal Genetics and Breeding** embodies bonafide research work carried out by **Mr. Vijay Kumar Agrawal**, under my guidance and supervision and that no part of this thesis has been submitted for any other degree. The assistance and help received during the course of investigation have been fully acknowledged. The draft of the thesis was also approved by the advisory committee on **16/06/2017**.

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Date: Vijay Kumar Agrawal

Place: Bikaner

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ABBREVIATIONS

ANOVA	Analysis of variance
bp	Base pairs
cm	Centi meter
DNA	Deoxy ribonucleic acid
dNTP	Deoxy nucleotide triphosphate
Eae I	*Enterobacter aerogenes*
EDTA	Ethylene diamine tetra acetic acid
Kb	Kilo base
Kg	Kilo gram
M	Molar
mg	Milli gram
ml	Milli liter
mM	Milli molar
ng	Nano gram
NJ	Neighbour Joining
nm	Nano meter
NS	Non significant
OD	Optical density
PCR	Polymerase chain reaction
pmol	Pico mols
QTL	Quantitive trait loci
RBC	Red blood cells
RE	Restriction enzymes
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute
Rsa I	*Rhodopseudomonas sphaeroides*
SE	Standard error
SNP	Single nucleotide polymorphism
SSCP	Single strand conformation polymorphism
TBE	Tris-boric acid-EDTA
TE	Tris-EDTA
UV	Ultra violet
µl	Microliter

1. INTRODUCTION

Milk contains almost all the nutrients vital for life with biological value greater than 94%. India, the largest producer of milk in the world, contributes about 2.7 per cent (127.2 million tonnes) of the world milk production (Dhaka *et al*., 2015). As per Livestock Census (2012), 37.28% of the total livestock population (190.90 million) belongs to cattle. The indigenous cattle hold great promise and potential for milk production and constitute about 79% (151.17million) of the total cattle population (Livestock Census, 2012). The great importance of indigenous cattle and their superiority over exotic cattle is owed to their great adaptive capacity to tropical climate, excellent nutritional benefits and resistance to the diseases. Unlike exotic germplasm, indigenous breeds are least affected by genotype-environment interactions. Recent census has witnessed an increase of 0.16% in population of milking indigenous cattle from 48.04million to 48.12million (Livestock Census, 2012). Native dairy cows also produces 100 per cent A2 type of milk and hence are a source for safe milk (Mishra *et al*., 2007) than exotic cattle breeds like HF and Jersey which produces around 60% A1 milk that is considered to be responsible for many human disorders like Type1 diabetes, autism and heart diseases (Sodhi *et al*., 2001; Mishra and Joshi, 2009).

India is the homeland and origin place of many elite cattle breeds of the world and possesses around 40 well recognized breeds of cattle (Breed Survey, 2013). Sahiwal, one of the most renowned milch breeds (Tolenkhomba and Yadav, 2012) of India, constitutes about 3.23% of total indigenous cattle population (Breed Survey, 2013) (**Fig. 1.1**). The breed is famous for higher milk production (2000-2500kg per lactation), massive body growth and excellent adaptability for heat stress and tick infestation (Khan *et al*., 2008; Ganguly *et al*., 2013). Many synthetic breeds (Australian Milking Zebu, Frieswal, Jamaica Hope, Karan Swiss, and Taurindicus) throughout the world have been developed from animals of Sahiwal breed (Rehman *et al*., 2008). Rathi is another distinct cattle breed that possesses variable degree of milk production and has not yet been fully explored for its production potential. The overall standard lactation yield of Rathi was observed to be 1726.08 kg in an average lactation length of 275 days (Dhaka *et al*., 2015). The majority of cattle population in north-western part of Rajasthan especially in Bikaner and Sri-Ganganagar districts is represented by Rathi cattle (0.82% of indigenous cattle) that have good milk potential

and adaptability in the desert and draught prone areas (Dhaka *et al*., 2015). Kankrej is considered as best dual purpose breed (Ankuya *et al*., 2016) of north-western region of India with population estimates of around 2% of total indigenous cattle (Breed Survey, 2013).

Increased milk production has emerged as one of the main dairy breeding goals (Meredith *et al*., 2012) throughout the world. However, newer breeding goals especially on milk composition traits have recently been identified following the demands of a healthier human diet (Krovvidi *et al*., 2013). The genetic improvement in milk performance traits of native breeds could increase their potential values and improve production levels. The effective genetic improvement requires genetic information about the genetic variability and their effects on milk production. The importance of specific alleles or genotypes and allelic richness has been also advocated for conservation purposes (Gandini and Villa, 2003). The potential value of indigenous livestock breeds for milk production traits must be analyzed and conserved to maximize the milk production so that they can become a self-sustainable resource. Livestock selection for improved production of milk has also influenced the evolution of animal breeds (Beja-Pereira *et al*., 2003) on global basis.

Studies on genes influencing the genetic variation in milk performance traits in our indigenous cattle breeds have gained importance in the recent times. The genetic variation in gene affects the physiological pathways and phenotype and moreover the proportion of genetic and phenotypic variation affecting the traits (Mohammed *et al*., 2015). The rate of genetic progress for economically important traits can be approximately doubled (Schefers and Weigel, 2012) through increase in accuracy of selection and reduction in general interval after identification of genes underlying the genetic variability of milk production traits that are useful and could be implemented in breeding programs (Szyda and Komisarek, 2007). The incorporation of genotypic information as fixed factor in estimation of breeding value through traditional methods can significantly improve selection responses (Naqvi, 2007).

The candidate gene approach (Kale *et al*., 2014) for the identification and characterization of specific DNA markers for various milk productivity traits (El-Magd *et al*., 2013; El-Magd *et al*., 2014) and to assess genetic variability would promote improvement in the animal productivity and will ultimately contribute in the nation economics (Choudhary *et al*., 2003). More than 100 different genes such as prolactin, casein, growth hormone, FASN, FABP and DGAT genes affects milk production of which few candidate genes have been identified on the basis of knock

out trials and their proximity with quantitive trait loci (QTL) region. The candidate genes related with the milk traits are called as lactogenic genes. Candidate genes such as prolactin (b*PRL*) gene (Korwin-Kossakowska *et al*., 2006) and diacylglycerol aminotransferase (*DGAT*) gene (Winter *et al*., 2002) are potential genes that were identified to have significant effect on milk production (Brym *et al.,* 2005a*;* Signorelli *et al*., 2009) and milk composition traits in dairy cattle (Komisarek and Dorynek, 2009) due to their location in or near the (QTL) region.

The prolactin, also called as "versatilin or omnipotin" hormone (Bern and Nicoll, 1968) is a single chain polypeptide hormone primarily transcribed in the specialized lactotrophs cells of the anterior pituitary gland and tissues like mammary gland (Feng *et al*., 2006). The hormone is known to have over 300 separate biological functions (Bole-Feysot *et al*., 1998). The bovine prolactin gene (*bPRL*) is traditionally regarded as a good candidate gene for marker-assisted selection (MAS) (Weller, 2001) for milk production parameters, because it has been located to chromosome 23 at 43 cM, close to the quantitative trait loci region (QTLs) (36, 41, and 42 cM) (Bennewitz *et* al., 2004). The prolactin gene invariably and directly affects growth and development of mammary gland (mammogenesis), synthesis of milk (lactogenesis) (Dahl, 2008) and maintenance of milk secretion (galactopoiesis) (Dong *et al*., 2013). In addition, it is known that the binding of the *bPRL* gene product with its receptor (*PRLR*) initiates a signaling cascade that activates the transcription of a number of genes, including expression of milk protein, lactose and lipid genes (Othman *et al*., 2011) via Janus kinases/signal transducers and activators of transcription (JAK STATs) signal transduction pathway (Hu *et al*., 2009). The indirect effect of prolactin gene on milk production traits occurs through its modulatory role on thermoregulatory mechanisms during hot climates (Alamar, 2011) and regulatory role in calcium homeostasis through enhanced intestinal calcium absorption (Charoenphandhu and Krishnamra, 2007). Reduction in serum prolactin level was also found to be associated with depressing effect on milk protein and lactose levels (Singh and Ludri, 1999). Prolactin also influences carbohydrate metabolism and insulin sensitivity through effects on insulin receptors (Goffin *et al*., 2002).

Several polymorphic sites have been detected in the coding regions of exon 3 and exon 4 of prolactin gene. One of them, recognized by *Rsa I* restriction endonuclease enzyme, has become a popular genetic marker for the genetic characterization of cattle populations for milk production traits (Dong *et al*., 2013). Statistically significant associations between bovine prolactin gene (*bPRL*) variants and milk production traits have been described in dairy cattle (Vaclavicek *et al.,* 2006).

Another candidate gene, *DGAT* located on the centromeric end of the bovine chromosome 14 in the QTL region (Farnir *et al*., 2002) is considered to be directly responsible for 50% variation in milk fat content and milk yield in dairy cattle (Banos *et al*., 2008). *DGAT* has strong functional and positional role in milk traits such as milk yield and fat content in cattle (Furbass *et al*., 2006). The *DGAT* gene has become a candidate gene after experiments have shown reduced or inhibited milk secretion in *DGAT* knock-out mouse lines (Smith *et al.* 2000). *DGAT* gene encodes an integral microsomal membrane enzyme that catalyses the final step of triglyceride synthesis in the mammary gland (Farese *et al*., 2000; Anton *et al*., 2012). A di-nucleotide polymorphism (GC/AA) in exon eight of *DGAT1* gene harbours a non-conservative lysine to alanine substitution (*K232A*) with profound effect on milk fat content (Winter *et al*., 2002) and fat deposition in different bovine breeds (Sorensen *et al.*, 2006). The presence of lysine (*AA*) variant at amino acid position 232 of *DGAT1* enzyme synthesizes 1.5 times greater triglycerides amount than alanine variant (*GC*) due to its more efficient binding of acyl CoA than neutral hydrophobic alanine. The polymorphic status of *DGAT1* gene and their association with milk yield and milk fat has been amply reported in exotic cattle, however, there have been scant studies in Indian cattle. The largest negative effect on milk fat was detected for the variant *AA* of the *DGAT1* gene (Molee *et al*., 2015). The variability in *DGAT1* gene also implicates the health status of the cow mammary gland (Liu *et al.* 2007).

Polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP) analysis are two most powerful approaches to detect genetic variation in a large population. RFLP's are the most abundant type of ideal polymorphic genetic markers for detection of genetic variation in polygenic traits (Jalving *et al*., 2004) and detects single nucleotide polymorphisms (SNPs) located at restriction site, easy to conduct and less cumbersome. SSCP analysis is more efficient and polymorphic in nature and is the method of choice for the detection of SNPs (Orita *et al.* 1989). SSCP detects single base sequence changes by abnormal electrophoretic migration of one or both single strands in a non-denaturing polyacrylamide gel (Gruszczynska *et al.*, 2005). The SNP's identification through SSCP is becoming the preferred marker for the elimination of sequence errors and misaligned bases during high density mapping (Vignal *et al*., 2002). SNP's are considered as useful polymorphic and ideal genetic markers for genetic studies of polygenic traits and are most abundant type of polymorphism in the genome (Jalving *et al*., 2004). Gene sequencing and sequence analysis of the PCR product is the most powerful approach to identify the absolute location and nature of SNPs.

The identification of specific pattern of allele and genotypic frequencies in indigenous cattle breeds may result in detection of causal factors responsible for variation in performance for milk production. Most of the earlier studies have reported SNP's within the *bPRL* and *DGAT* gene either on the basis of restriction fragment length polymorphism (RFLP) or single stranded conformation polymorphism (SSCP) patterns (Hart *et al*., 1993) without explaining their nature and locations. In addition, few researches have been conducted for the exploration of genetic variation in prolactin and *DGAT* gene in indigenous breeds of Rathi, Sahiwal and Kankrej cattle and their association with milk yields and milk composition traits (Kayastha *et al*., 2008). Therefore, the present investigation was undertaken with the following objectives:

(i) To study the genetic variability in exon 3 and exon 4 region of Prolactin (*PRL*) gene; and Diacylglycerol acyltransferase (*DGAT*) gene in indigenous cattle breeds of Rathi, Sahiwal and Kankrej.

(ii) To estimate the gene and genotypic frequencies of exon 3 and exon 4 region of Prolactin (*PRL*) gene; and Diacylglycerol acyltransferase (*DGAT*) gene in indigenous cattle breeds of Rathi, Sahiwal and Kankrej.

(iii) To study the association between different genotypic pattern of Prolactin (*PRL*); and Diacylglycerol acyltransferase (*DGAT*) genes with milk performance and composition traits in indigenous cattle breeds of Rathi, Sahiwal and Kankrej.

2. REVIEW OF LITERATURE

Milk is one of the most essential and complete nutrition sources for mammals. Milk and milk products, both their quantity and quality are important for the economy of the country. Some of the domesticated animals for instance cattle were employed for milk production for the humankind in the last ten millennia. Variation in genetic make-up of dairy animals could leads to variation in milk and milk composition even in the animals of same breed (Pasha and Hayat, 2012). In many regions of the world, techniques have been practiced in molecular genetics in combination with conventional animal breeding to improve animal breeding programmes, ensuing higher milk yield and fat content due to better genetics (Afzal, 2010). Improvement of animals via genetics has been proven effective (Bilal and Sajid, 2005). Some of the confirmed markers (*DGAT* and Prolactin gene) could not be validated in indigenous animals like cattle.

Breeds of cattle have differentiated gene pools. Differentiation was under the effects of natural selection, random genetic drift and environmental selection. The tracking of genes which are known to be associated with the properties of the milk could detect the status of the breed in relation to the property under consideration. Two of the genes (*PRL, DGAT1*) were known to be associated with milk traits in cattle (Dybus, 2002; Sanders *et al.,* 2006). Genotype data in dairy cattle have the potential to be used as tools to improve milk production.

2.1 Genetic Markers

RFLP markers were established in a number of studies as an ideal marker for the detection of small alterations that happen naturally in the genome from changes due to deletions or insertions of one or more pairs of nucleotides (Lewin *et al*., 1992; Skinkyte *et al*., 2005). The sensitivity of earlier methods, such as single-stranded conformation polymorphism analysis (SSCP) (Kunhareang *et al,* 2009) to detect single nucleotide polymorphism (SNPs) are low (70% to 80%) and require considerable skill and labour. Single base mutation could result change in the folded conformation of the single strand DNA leading to differences in electrophoretic mobility patterns (Hayashi and Yandell, 1993). Thus direct gene sequencing has been considered as powerful method for identification of nucleotide sequence variation in amplified DNA and is considered as the gold-standard approach for

genotyping analysis and expected to have almost 100% sensitivity (Laurie and George, 2009). The DNA based markers associated with a quantitative trait loci (QTL), contribute to variations in phenotype. DNA based markers could be used as tool for marker based selection. The MAS method was used to eliminate deleterious gene alleles or select for favorable conditions based on some marker information (Eggen, 2012).

2.2 Quantification of Genetic Diversity

Gene and genotypic frequencies are considered as basic genetic parameters and their assessment could demonstrate the possible genetic structure of the population and their evolution over time in addition to comparison one population with other populations in the sense that population with different genotype frequencies could have identical allele frequencies (Mayr 1963). Such knowledge will be helpful in selection and mating strategy, development of knockout technology and understanding the structure, function and evolution of the gene.

F-statistics of Sewal Wright could reveal the correlation or non-independence among alleles in non-HWE populations having deficit or excess of heterozygotes.

The fixation index measures the Hardy-Weinberg disequilibrium. The value of fixation index (F_{IS}) indicates the level of inbreeding in the population probably as a result of long term selection and the maintenance of small group of closely related sires (Hansen *et al*., 2003). Wright's fixation index (F_{IS}), a measure of the inbreeding coefficient, being low when genetic diversity was high (FIS < 0.05) and medium or high when the heterozygosity was low (FIS > 0.10). A bias towards heterozygote excess was inferred from the negative FIS values that could be confirmed by the differences between observed and expected heterozygosity.

Shete *et al*. (2000) identified polymorphism information content (PIC) value as most common measure of polymorphism for a marker locus for use in linkage analysis. Initially derived by Botstein *et al*. (1980), PIC value is often considered as a measure of informativeness of a genetic marker for linkage studies.

2.3 Role of Prolactin in Lactation

Prolactin is a peptide hormone released by the anterior pituitary gland; however, its secretion is also attributed to central nervous system, immunological system, and mammary gland (Bole-Feysot et al., 1998). This hormone is considered

as pleiotropic and more than 300 biological functions have been attributed to PRL (Bole-Feysot et al., 1998). Prolactin plays a key role in the initiation and maintenance of lactation in mammals. Genotypes in pathway were found to be associated with milk production (Lu *et al*., 2010). The suppression of prolactin hormone was found to inhibit the milk secretion in mammals (Miltiadou *et al*., 2016). In dairy ruminants, a good body of evidence has shown that *PRL* is galactopoietic and increases feed intake to provide the nutrients necessary to support lactation (Lacasse and Ollier, 2015). Therefore, PRL could be regarded as a functional candidate gene associated with milk production and composition. In cattle, the prolactin hormone was reported to function through interaction with the target cells through binding with *PRL* receptor (Zukiewicz *et al*., 2012). The *PRL* hormone was also reported for its involvement in adaptive response to stress in mammals through its analgesic effects mimicked by a number of neurotransmitters (Bole-Feysot *et al*., 1998).

2.4 Bovine Prolactin (*bPRL*) Gene

The hormone genes are excellent candidate genes for linkage analysis with quantitative trait loci (QTL) because of their biological significance on the quantitive traits of interest. The prolactin hormone gene as one of the potential genetic marker and QTL region in dairy animals was found to be primarily responsible for the initiation and maintenance and regulation of lactation (Bernichtein *et al*., 2010) with key role in synthesis of milk components, including milk proteins, lactose, lipids (Le Provost *et al*., 1994; Dahl, 2008) in addition to regulation of reproductive functions, fluid balance, cellular growth and differentiation (Kelly *et al*., 1991; Brand *et al*., 2004). Experimental trials in several animals revealed the expression of prolactin gene in pituitary gland and several other sites including the central nervous system, immune system and the mammary gland (Sinha, 1995; Ben-Jonathan *et al*. 1996) which indirectly reflects its role in adaptation of animals to their surroundings. Extensive evidence suggests that lymphocyte can be a source of prolactin as well (Gala and Shevach, 1994). It was observed in a trial that secretion, metabolism and distribution of prolactin hormone increases between days 30 to 150 of lactation (Collier *et al*., 1984). The prolactin gene affects the each stage of milk protein gene expression, i.e., transcription, translation, and post translational modifications of the proteins (Zwierzchowski *et al*., 1998). Prolactin is also a common mediator of the immunoendocrine network, where nervous, endocrine and immune system communicate each other (Goffin *et al*., 1998).

2.5 Structure of *bPRL* Gene

The bovine *PRL* gene has been cloned and mapped on chromosome 23 (23q21 position) (Hallerman *et al*., 1988; Brym *et al.,* 2005a). Camper *et al*. (1984) detected five exons and four introns in *PRL* gene with an overall length of about 9.4kb, encoding a 229-amino-acid prolactin precursor. The mature bovine *PRL* is composed of 199 amino acids along with a signal peptide of 30 amino acids (Wallis, 1974; Cao *et al*., 2002).

A number of studies had explored the polymorphisms in bovine prolactin gene (*bPRL*) (Udina *et al*., 2001) and concluded that prolactin could be an excellent candidate gene for linkage analysis with quantitative trait loci (QTL) affecting milk production traits (Brym *et al*., 2005a). *PRL* alleles had emerged due to a silent A→G mutation in prolactin gene that resulted in the appearance of polymorphic *Rsa*I restriction site and was found to affect the milk production traits like milk yield, milk fat and milk protein (Lewin *et al*., 1992).

The transition mutation *A/G* at the codon for amino acid 103 in exon-3 of bovine *PRL* gene was known to produce (Mitra *et al*., 1995) two allelic variants *G* and *A* at DNA level, based on *Rsa* I polymorphism (Alipanah *et al*., 2007b). The *G* allele is usually more frequent than *A* allele (Wojdak-Maksymiec *et al*., 2008). Though the identified mutation was silent in nature, many researchers have observed its tight linkage with QTL for milk production (Dong *et al*., 2013). It was noteworthy that even silent mutations in introns may turn to be QTL loci as observed in pigs (Van Laere *et al*., 2003). Many studies in different cattle breeds throughout the world have identified the site as poplar genetic marker for genetic characterization of cattle population through RFLP methods (Dybus *et al*., 2005; Bukhari *et al*., 2013). An association between *PRL* marker and milk production was established in the studies of Alipanah *et al*. (2008), Maksymiec et *al*. (2008) and Ghasemi *et al*. (2009).

2.6 Genetic Polymorphism in *bPRL* Gene

Seven nucleotide substitutions based on the sequences of 4 cDNA clones were detected in *bPRL* gene (Sasavage *et al*., 1982) of which one nucleotide substitution recognized by *Rsa*I restriction enzyme has become the most commonly used genetic marker for the genetic characterization of multiple cattle populations and associations with milk performance traits. However, Kaminski *et al*. (2005) identified five SNPs in *bPRL* gene. Six SNPs were detected by Brym *et al*. (2005b)

through sequencing of three different SSCP patterns in exon 4 of *bPRL* gene, one of which was shown to be associated with milk yield and fat content. Three SNP's mutations were observed in the exon-4 of PRL gene at positions 8362, 8377 and 8398 and three mutations were detected at 8307 and 8314 in intron 3; and 8510 in intron 4 (Brym *et al*., 2005b).

Halabian *et al*. (2008) reported four SSCP patterns and detected four SNPs in 120 Iranian Holstein cows (*Bos taurus*) in exon 3 of prolactin gene after sequencing. Four SNP's were detected by Halabian *et al*. (2008) at positions 6237, 6263, 6268 and 6297. Two of the SNPs identified at positions 6237 and 6268 were found to be non-synonymous and caused change in the amino acid sequences of the protein. One SNP was found to be silent and the last SNP was present in intron 3 region. A study conducted in 586 Chinese Holstein cows by Dong *et al*. (2013) identified three different SSCP patterns and eight different SNP's in prolactin exon-4 gene that were found to be in complete linkage with significant linkage disequilibrium. The frequencies of allele *G* and *A* were estimated as 0.8754 and 0.1246, respectively. Among all the SNPs identified in earlier studies, SNP located in position 8398 of *bPRL* exon 4 is the most popular genetic marker for genetic characterization of cattle populations by mean of PCR-RFLP (Brym *et al*., 2005b). Hu *et al*. (2009) also detected genetic polymorphism in exon 4 of prolactin (*PRL*) gene in Chinese Holstein cattle through PCR SSCP and confirmed the A>G mutation at position 8398 in exon 4 of *PRL* gene through gene sequencing. The frequencies of allele *A* and *G* were found to be 0.894 and 0.106, respectively. Chinese Holstein cows with genotype GG were observed to be high milk yielders than genotypes AA and AG ($P<0.05$).

The gene frequencies of PRL^A and PRL^G allele in 57 Indian Sahiwal cattle screened by Mitra *et al*. (1995) through PCR-RFLP technique were observed to be 0.49 and 0.51. Chung *et al*. (1996) observed higher frequency of 0.73 for PRL^A allele. However, a considerable higher frequency of PRL^A (0.95) in Holstein breed was observed by Chrenek *et al*. (1998). Breed differences in frequencies were reported for prolactin exon 3 *Rsa*I loci by Dybus *et al*. (2005) in a study conducted on 427 cows of Holstein and Jersey cattle who revealed significantly different gene and genotypic frequency between breeds. The genotypic and gene frequencies observed for Holstein cattle were 0.711 (*AA*), 0.285 (*AG*), 0.004 (*GG*); 0.85 (PRL^A) and 0.15 (PRL^G) whereas the respective frequencies for Jersey cattle were found to be 0.092 (*AA)*, 0.432 (*AG),* 0.476 (GG); 0.3081 (PRL^A) and 0.6919 (PRL^G). Similarly Kumari *et al*. (2008) observed significant deviation in gene and genotypic frequencies of

prolactin exon 3 *Rsa*I loci in Sahiwal and Kankrej cattle. The study observed significantly lower value of *GG* genotype in indigenous cattle breed of Sahiwal and Kankrej. The study reported genotypic frequencies of 0.77, 0.23, 0 (Sahiwal), 0.31, 0.62, 0.07 (Kankrej) for AA, AG and GG genotype, respectively for *PRL* exon-3 *Rsa I* loci with respective gene frequencies of *A* and *G* alleles were 0.88 and 0.12 (Sahiwal) and 0.60 and 0.40 (Kankrej). Sodhi *et al*. (2011) observed significant differences in the frequency of allele *G* between populations and between breeds of *Bos indicus* and cattle breeds from other countries, with high allele frequency >0.60 of allele G in Indian native cattle.

2.7 Association of *bPRL* Genotypes with Milk Trait

Inconsistent and different results were observed by many workers across the globe for the effect of prolactin gene on milk production performance which could be ascribed to breed or species difference (Brym *et al*., 2005a). The inconsistent results might also be due to age dependent absorption of calcium under influence of prolactin. SNPs occurring within the *PRL* gene may influence the chemical composition of milk or at least be an effective DNA marker of a sub region of dairy cattle genome (He *et al*., 2006).

Significant associations between *PRL-Rsa*I polymorphism and milk production traits were observed by various workers. Chung *et al*. (1996) reported that Holstein-Friesian cows with *AA* genotype at *PRL Rsa*I locus had significantly higher milk yield and higher fat percentage than cow of *GG* genotype. However, Dybus *et al*. (2005) observed statistically significant differences between individuals of different *PRL* genotypes in terms of milk fat yield, milk fat content and sum of fat and protein content. Statistically significant differences between individuals of different *PRL* genotypes for milk fat content were found in the first lactation ($P \leq 0.01$). The study observed production of less milk fat (-15.9 and –14.6 kg) in cows of *AA* genotype than *AG* and *GG* individuals ($P \leq 0.01$). The cows with the *AA* genotype produced milk with lower fat content (-0.19 and -0.18%) than *AB* and *BB* individuals, respectively. The association was observed in Jersey cows whereas such association was not observed for Holstein cows. The study also observed statistically significant differences ($P \leq 0.01$) in sum of milk fat and protein (%). During third lactation, cows with *AG* genotype had higher fat and protein content (+0.44%) than *AA* an individual which was in contrast with the study conducted by Dybus (2002) who observed that cows with the *AA* genotypes of the *PRL RsaI* gene had higher milk protein content than *AG* individuals. Similarly, the negative impact of genotype

AA on milk yield in Montbéliarde cows and the highest milk yield in Holstein cows with genotype AG had been observed (Brym *et al*., 2005a; Ghasemi *et al*., 2009). The breed difference between Russian Black Pied cattle and Russian Red Pied cattle for *PRL* gene were reported by Alipanah *et al*. (2008). Dong *et al*. (2013) observed that genotypes vary significantly with respect to the milk performance traits ($P < 0.05$). Cows with allele *AA* at locus 7545 had a higher milk yield at 305 days (8457 ± 938 kg) in comparison to those with the *AG* allele (7537 ± 1278 kg; $P < 0.01$) and *GG* allele (7757 ± 1174 kg; $P < 0.05$). However, the percentage of fat and protein in milk did not differ between the genotypes ($P > 0.05$). The study corroborates the finding in Russian Red Pied cattle, where the AA genotype also had a positive effect on milk yield (Alipanah *et al*., 2007). No statistically significant differences between the cows with different *PRL* genotype were observed in Brown Swiss cattle by Chrenek *et al*. (1999).

2.8 Diacylglycerol Acyltransferase (*DGAT1*) Gene

The *DGAT* activity was first described by Weiss *et al*. (1956) and Kennedy *et al*. (1957). The *DGAT1* enzyme was found to play fundamental role in the metabolism of cellular triacylglycerol during physiological processes, such as intestinal fat absorption, lipoprotein assembly, adipose tissue formation and lactation (Cases *et al*., 1998). The gene was also found to be related with intramuscular fat deposition in cattle (Thaller *et al*., 2003). Functionally, the *DGAT1* gene was identified as one of at least two genes that encodes *DGAT* enzyme which catalyzes the final step of triglyceride synthesis in eukaryotic cells (Yen *et al*., 2008). The *DGAT1* gene became a candidate gene for milk production traits in dairy cows after experiments showed reduced or inhibited milk secretion in *DGAT1* knock-out mouse lines (Smith *et al*., 2000).

2.9 Structure of Diacylglycerol acyltransferase (*DGAT1*) Gene

Many studies in dairy cattle have shown that a quantitative trait locus (QTL) with major influence on milk production and composition is located in the centromeric end of chromosome 14 (Boichard *et al*., 2000; Looft *et al*., 2001). The fluorescence *in situ* hybridization and radiation hybridization method identified one such gene, *DGAT1* in this QTL region having profound effect on milk production (Winter *et al*., 2002) and primarily responsible for milk fat variation in dairy animals (Dokso *et al*., 2015). *DGAT1* was the first identified gene of about 14,117 bp and 17 exons (Mohammed *et al*., 2015) that encodes a protein with *DGAT* activity (Cases *et al*.,

1998). A quantitative trait loci (QTL) region of *DGAT1* gene was identified in the centromeric region of the bovine chromosome 14 (BTA14) (Grisart *et al*., 2002) in which a missense mutation *K232A* (Lys^{232}→Ala) was shown to be significantly associated with variation in milk fat (Winter *et al*., 2002).

Grisart *et al*. (2004) confirmed the functional and genetic causality of *K232A* at position 10433-10434 in exon 8 of *DGAT1* gene for fat content on BTA14 through positional cloning approach and analysis of the expression of both alleles separately in virus expression systems. The study demonstrated the substitution effect of *K→A* on the *DGAT* enzymatic activity and observed that the amount of triglycerides synthesized by the lysine variant was approximately 1.5 times greater than alanine variant. The enzyme encoded by the *K* allele was characterized by a higher velocity rate (V_{max}) in production of triacylglycerols than the A allele. The *K* allele of *DGAT1* gene have specific role in attachment of fatty acids to position 3, the only place on the triacylglycerol molecule where the C4 and C6 fatty acids are found whereas the *A* allele may be associated with a lower proportion of these short chained fatty acids. The magnitude of the K232A mutation on milk traits though found to be differed between populations but the direction was usually found to be same. The mathematical prediction model of Shorten *et al*. (2004) also suggested an increase of 120% in acylation rate of DGAT1 enzyme on substitution of alanine allele with lysine allele.

2.10 Genetic Polymorphism in *DGAT1* Gene

The haplotype analysis indicated that lysine coding allele *K* of *DGAT1* gene appeared to be ancestral allele and the *K232A* substitution probably occurred early in the history of cattle domestication or even before domestication after the divergence of the *Bos indicus* and *Bos taurus* lineages (Winter *et al*., 2002). The reported *K232A* polymorphism in different breeds throughout the world is presented in **Table 2.1**.

A wide range of distribution of the *K232A* polymorphism in 38 *Bos indicus* and *Bos taurus* breeds has been observed by Kaupe *et al.* (2004). Several workers reported variation in allelic frequency of '*K*' allele of *DGAT1* gene which was found to vary from 0.19 (Manga and Riha, 2011), 0.25 (Fontanesi *et al*., 2007), 0.32 (Berry *et al*., 2010), 0.37 (Gautier *et al*., 2007), 0.59 (Patel *et al*., 2009) to 0.79 (Mashhadi *et al*., 2012) between breeds

Table 2.1 *K232A* polymorphism of *DGAT1* gene in different cattle breeds

Breed	KK	KA	AA	K	A	References
Friesian Cattle	0.11	0.58	0.31	0.40	0.60	Strzalkowska *et al*. (2005)
Montebeliarde	0	14	370	0.04	0.96	Gautier (2007)
Swedish HF	0.03	0.20	0.76	0.86	0.14	Naslund *et al*. (2008)
German HF	0.19	0.50	0.31	0.44	0.56	Rahmatalla (2010)
Girolando cattle	0.27	0.54	0.19	0.54	0.46	Cardoso *et al*. (2011)
Czech Holstein	0.01	0.36	0.63	0.19	0.81	Manga and Riha (2011)
Frieswal	0.38	0.52	0.10	0.64	0.36	Ganguly *et al*. (2013)
Sahiwal	0.92	0.08	0.00	0.96	0.04	Ganguly *et al*. (2013)
Czech Holstein	0.06	0.43	0.51	0.28	0.72	Kadlecova *et al*. (2014)
Czech Fleckvieh cattle	0.08	0.92	0.00	0.54	0.45	Rychtarova *et al*. (2014)
Turkish TG	0.53	0.33	0.13	0.70	0.30	Unal *et al*. (2015)
Turkish EAR	0.56	0.44	0.00	0.78	0.22	Unal *et al*. (2015)
Turkish AB	0.48	0.21	0.31	0.58	0.42	Unal *et al*. (2015)
Turkish SAR	0.60	0.40	0.00	0.80	0.20	Unal *et al*. (2015)
Crossbred Holsteins	0.11	0.48	0.41	0.35	0.65	Molee *et al*. (2015)
Simmental	0.293	0.293	0.060	0.616	0.384	Dokso *et al*. (2015)
Holstein	0.637	0.637	0.088	0.775	0.225	Dokso *et al*. (2015)
Brown Swiss	0.329	0.329	0.027	0.651	0.349	Dokso *et al*. (2015)
Polish HF	0.154	0.491	0.356	0.4	0.6	Komisarek and Kolenda (2016)

The nearly fix nature of A allele of *DGAT1* gene was observed by Kaupe *et al*. (2004) in few of the *Bos taurus* breeds such as Belgian Blue, Gelbvieh, Hereford, Pinzgauer and Slavonian Syrmian breeds whereas the reverse fixation of *DGAT1K* allele has been noticed in six cattle (*Bos indicus*) and five buffalo (*Bubalus bubalis*) breeds of India by Tantia *et al.* (2006). Likewise, Kaupe *et al*. (2004) and Ganguly *et al*. (2013) reported very high *DGAT1 K* allele frequency of 0.99 and 0.96 in Nellore and Sahiwal cattle, respectively. Low frequency of A allele of *DGAT1* gene was

reported in Gyr and Red Sindhi animals with complete absence in Nellore cattle (Lacorte *et al*., 2006). In crossbred animals of Frieswal cattle, slightly higher than intermediate gene frequency of 0.64 was reported for *K* allele (Ganguly *et al*., 2013). The *K232A* polymorphism has been observed in most of the Holstein-Friesian breed throughout the world (Nowacka-Woszuk *et al*., 2008). A study in German and Polish Holstein-Friesian cattle revealed *DGAT1 K* frequency of 0.54 (Nowacka-Woszuk *et al.,* 2008). The information on allele frequency gives an indication on the genetic variance due to *DGAT1 K232A* polymorphism.

2.11 Association of *DGAT1* Genotypes with Milk Traits

The effect of genes depends on genetic structure of the population. The ApA to GpC dinucleotide substitution of a positively charged lysine residue with a neutral hydrophobic alanine residue in the DGAT1 gene (Sanders *et al*., 2006; Kaupe *et al*., 2007) was reported to exert major effect on fat content and other milk characteristics (Farnir *et al*., 2002; Koopaei *et al*., 2012). The lysine coding allele K was found to enhance milk fat compared to alanine coding allele 'A' (Winter *et al*., 2002; Gautier *et al*., 2007) and was reported to negatively affect the milk yield and protein yield (Berry *et al*., 2010). The *K232A* polymorphism was reported to have much larger additive effect for milk fat and sires selected for DGAT1 KK genotype were observed to have higher breeding values for milk fat content and lower breeding values for milk yield and milk protein contents (Szyda and Komisarek, 2007; Nowacka-Woszuk *et al*., 2008). The K allelic variant was found to negatively affect the amount of milk yield with concurrent increase in fat and protein content (Kaupe *et al*., 2004). This positive effect of K variant of DGAT1 gene was reported by Thaller *et al*. (2003) with largest content of milk fat was observed for KK genotype (Leskova *et al*., 2013). The opposite supportive evidence was provided by various workers which suggested an increase in milk yield on addition of A allele in DGAT1 gene (Sanders *et al*., 2006; Oikonomou *et al*., 2008). An increase of 548kg in milk yield and 12.6 kg protein yield with simultaneous decrease of 15.4kg milk fat was reported in Holstein animals homozygous for A allele of *DGAT1* gene (Hradecka *et al*., 2008). A study in Butana cattle and Friesian cattle indicated significantly higher milk and protein yield in heterozygous KA genotype than KK homozygous cows however non significant variation for fat yield was reported for similar genotypes (Strzalkowska *et al*., 2005; Rahmatalla *et al*., 2015).

Spelman *et al*. (2002) observed an allele substitution effect of -110 litre milk, +5.76 kg milk fat and -2.45kg milk protein in HF cattle and -130 litre milk, +3.30kg

milk fat and -2.48kg milk protein in HF cattle on substitution of A allele with K allele in the population. Similar observation on average effect of allele substitution was reported by Grisart *et al*. (2002). Although the effect of DGAT1: K232A alleles was established in the same direction in many cattle populations throughout the world but the magnitude of effects was variable between breeds (Suchocki *et al*., 2010), parity (Thaller *et al*., 2003) and during lactation (Szyda *et al*., 2014). The significant impact of lactation stage on K232A effects was confirmed by Bovenhuis *et al*. (2015). Recently, Akbar *et al*. (2013) reported that the expression of this gene in the liver is reduced during the hot season and increased during the spring season. The seasonal effect of K232A genotype on some fatty acids in the milk was also reported by Duchemin *et al*. (2013). Thus declined expression in summer season results in lesser polymorphism effect of K232A substitution (Komisarek and Kolenda, 2016). However, the large magnitude effect of K232A amino acid substitution as Quantitative Trait Nucleotide (QTN) (Mackay, 2001) for milk performance traits was supported by the studies of Rahmatalla *et al*. (2015).

Few studies also documented the pleiotropic effect of K232A amino acid substitution on reproduction parameters (Ashwell *et al*., 2004) which could be due to its correlated effect with production traits (Demeter *et al.,* 2009). The A allele was reported to decrease conception rate with increase in number of inseminations per conception and greater chance of reproductive problems in first 305 day of lactation (Oikonomou *et al*., 2009). The 'KK' genotype of *DGAT1* gene was found to be associated with better results for days open and calving interval (Rychtarova *et al*., 2014). According to Schennink *et al*. (2007), an increase frequency of 'A' allele could lead to a decrease in C16:0 fraction and an increase in the unsaturated C18 fraction by approximately 5 to 10%.

In contrast, Dokso *et al*. (2015) observed the non significant effect of K allele on the milk fat and milk protein levels in Holstein, Simmental and Brown Swiss animals. An opposite of the established findings was reported by Manga and Riha (2011) who demonstrated favourable effect of K allele on milk yield in Czech Holstein cows. Similar analogous results were reported in Hungarian Holstein (Anton *et al*., 2008) and Mexican Holstein (Hori-Oshima *et al*., 2002). The investigation of Rychtarova *et al*. (2014) in Czech Fleckvieh cattle demonstrated increase in estimated breeding value for protein and fat percentage for lysine variant of *DGAT1* gene.

3. MATERIALS AND METHODS

3.1 Experimental Design

The animals for the present experimental trials were selected at random with no preference over others. Animals completed at least one parity were selected for the study.

3.2 Selection of Animals

An overall 225 animals were selected for the present study from pure breeding population of Rathi (n=75), Sahiwal (n=75) and Kankrej (n=75). Only milking cows with minimum of 120 days lactation were included in the study. Animals of particular breed were selected from the same farm to exclude the farm effect. Apparently healthy animals were identified for the present study with no evidence of any chronic ailments.

3.3 Location of Farm

The Rathi cattle for the present trial were selected from Livestock Research Station situated in the campus of Rajasthan University of Veterinary and Animals Sciences, Bikaner (Rajasthan). The animals from Sahiwal and Kankrej breeds were selected from Livestock Research Station, Kodamdesar, Bikaner (Rajasthan). Both farms are located in the similar geographical region of Bikaner in the grid position of 28.02ºN and 73.31ºE with mean annual rainfall of 277.55 mm. The region is characterized as semi arid, sandy and harsh with extreme adverse climatic conditions. All the animals under study experienced similar environmental conditions of temperature and humidity.

3.4 Management of Farm Animals

Animals in both the farms were managed under similar feeding and housing conditions. Routine vaccination and regular deworming was uniformly applied to all the animal of the farm. The milking animals were fed appropriate concentrate mixtures to maintain lactation performance. Milking in both farms was carried out twice with equal interval of 12 hours.

3.5 Collection of Phenotypic Information

The phenotypic information on various lactation traits such as total milk yield (TMY), 305-day milk yield, average daily milk yield (ADMY) and lactation length (LL) was recorded on parity basis from the official records maintained in the farm.

3.6 Standardization of Phenotypic Information

The phenotypic information on different lactation parameters were standardized to reduce systematic errors and to make the data set uniform. Information on outliers animals and aberrant lactation was excluded from the present study. Animals with history of mastitis or dystokia were also excluded for the collection of biological material.

3.7 Collection of Biological Materials

The biological materials such as milk and blood for analysis were collected from the same animals included in the study in accordance with the standard ethical procedures.

3.7.1 Collection of milk samples

Morning milk sample (100 ml) from each animal was collected in clean, dry, grease free and labeled milk collection vials. The milk sample was collected during mid milking stage to avoid sampling fluctuations in milk composition data. No milk collection was carried out from the animals which were in terminal or very early stage of lactation. The collection of milk samples was replicated on the same test day of each month for three consecutive months to obtain an average value of milk fat and other compositional traits. The collected milk samples were then refrigerated at 4ºC soon after collection till further analysis.

3.7.2 Collection of blood samples

About 2ml of blood was taken from jugular vein in a sterile vacutainer tube containing Ethylene Diamine Tetra Acetic acid (EDTA) as an anticoagulant under strict aseptic conditions from all the animals of Rathi, Sahiwal and Kankrej cattle. Samples were refrigerated at 4ºC and preserved until processing.

3.8 Analysis of Milk Samples

The collected milk samples were transported under ice to Milk Analysis Laboratory of Uttari Rajasthan Milk Union Limited (URMUL) situated in Bikaner for the analysis of different milk constituents. The samples were initially kept at constant

temperature of 37ºC in water bath for 30 minutes. The samples were then properly mixed and homogenized before analysis. The different fractions of milk samples, *viz*., milk fat, protein, lactose, SNF and total solid were then analyzed through Automated Milkosan Tester (Bentley Instruments Inc., Chaska, USA). The analyzed value from each animal was recorded in proper tabulated form.

3.9 Extraction of Genomic DNA

Genomic DNA from the whole blood sample was extracted through spin column method as per standard method (Sambrook and Russell, 2001) under manufacturer's protocols with slight modification through Genomic DNA Isolation Kit supplied by Himedia Pvt Ltd.

The purity (OD ratio 260/280) and concentration (ng/µl) of extracted genomic DNA was determined by Nano-drop spectrophotometer. The quality of genomic DNA was assessed through 0.8% agarose electrophoresis to detect fragmentation or shearing if any. Only the good quality genomic DNA was allowed to be processed further. The extracted DNA samples after proper markings were stored at -20ºC till further analysis.

3.10 Selection of Genomic Region for Genetic Variation Analysis

Three highly informative and polymorphic loci belong to *PRL* gene and *DGAT1* gene were identified through extensive review of literature. The exon-3 and exon-4 region of *PRL* gene and exon 8 of *DGAT1* gene with corresponding flanking regions were selected for the present study. The well established A/G nucleotide substitution in exon 3 and exon 4 of *PRL* gene and a dinucleotide AA/GC substitution in exon 8 of *DGAT1* gene were selected as genetic marker for the differentiation of population in different breeds.

3.11 Selection of Primers

Three pairs of primers used in the present study for the selected genomic regions, *viz.,* exon-3 and exon-4 region of *PRL* gene and exon-8 of *DGAT1* gene, were constructed on the basis of available sequences of *PRL* and *DGAT I* gene in NCBI GenBank database. The sequences of primers, the accession number of reference sequence and expected fragment length of different selected region are represented in **Table 3.1.**

Table 3.1 Primer sequences and expected fragment sizes of PCR products of selected genomic regions

Selected Region	Primer Sequences	GenBank Accession No.	Expected Fragment Length	Refere-nces
PRL exon-3 region	Forward (5'-CGAGTCCTTATGAGCTTGATTCTT-3') Reverse (3'-GCCTTCCAGAAGTCGTTTGTTTTC-5')	NM173953	156-bp	Mitra *et al*. (1995)
PRL exon-4 region	Forward (5'-CCAAATCCACGAATTATGCTT-3') Reverse (3'-ACAGAAATCACCTCTCTCATTCA-5')	AF426315	294-bp	Brym *et al*. (2005)
DGAT 1 exon-8 region	Forward (5'-GCACCATCCTCTTCCTCAAG-3') Reverse (GGAAGCGCTTTCGGATG-3')	AJ318490	411-bp	Winter *et al*. (2002)

3.12 Amplification of Genomic DNA

The allele-specific polymerase chain reaction was carried out to amplify selected genomic regions of *PRL* and *DGAT1* gene using specific desalted oligonucleotide primers custom synthesized in accordance with Lewin *et al*. (1992).

3.12.1 Amplification of exon-3 of *PRL* gene

A 156-bp fragment of exon-3 of *PRL* gene was amplified by polymerase chain reaction in a final reaction volume of 25µl. PCR reaction mixture used for amplification of genomic DNA was 5X PCR buffer (5µl), 1.5mM $MgCl_2$ (3µl), 10 Mm dNTP's mix (1µl), forward primer 70pmol/µl (1µl), reverse primer 70 pmol/µl (1µl), genomic DNA 25 ng/µl (4µl), Taq DNA polymerase 5U/µl (0.2µl) and DNAase free water (10.8µl). The gradient PCR programme was used to find out the appropriate annealing temperature that was further used for the amplification of all samples. The reactions were carried out in programmable thermocycler (Chino Scientific Gradient Thermocycler). Amplification was carried out in PCR thermo cycler with programme illustrated in **Table 3.2**. After the amplification the PCR product were stored at -20˚c for further analysis.

Table: 3.2 PCR programming for amplification of exon-3 of *PRL* gene

Steps	Temperature	Time	No. of Cycle
I. Initial Denaturation	95°C	5 min.	1 cycle
II. Cycle (i) Denaturation (ii) Annealing (iii) Synthesis	 95°C 59°C 72°C	 45 sec 45 sec 1 min.	35 cycles
III. Final extension	72°C	10 min.	1 cycle
IV. Hold	4°C	5 min	1 cycle

3.12.2 Amplification of exon-4 of *PRL* gene

The 294-bp region of exon-4 of *PRL* gene including part of intron 3 and intron 4 was amplified in a reaction mixture of 25 µl containing 5X PCR buffer (5µl), 1.5mM $MgCl_2$ (3µl), 10 Mm dNTP's mix (1µl), forward primer 80pmol/µl (0.5µl), reverse primer 80pmol/µl (0.5µl), genomic DNA 25ng/ µl (5µl), Taq DNA polymerase 5U/µl (0.3µl) and DNAase free water (9.7µl). The PCR reaction was carried out in Master cycler. The amplification programme adopted is depicted in **Table 3.3**.

Table: 3.3 PCR programming for amplification of exon-4 of *PRL* gene

Steps	Temperature	Time	No. of Cycle
I. Initial Denaturation	95°C	5 min.	1 cycle
II. Cycle (i) Denaturation (ii) Annealing (iii) Synthesis	 95°C 55°C 72°C	 1 min. 1 min. 1 min.	35 cycles
III. Final extension	72°C	10 min.	1 cycle
IV. Hold	4°C	5 min	1 cycle

3.12.3 Amplification of exon-8 of *DGAT1* gene

A region of the cattle *DGAT1* gene spanning parts of exon 7 and exon 9 (including intron 7, exon 8 and intron 8 entirely) was amplified by PCR. 5% dimethyl sulphoxide (DMSO) was added to the amplification mixture for equal amplification of

both alleles as suggested by Winter *et al*. (2002). Seventy pico mol primer concentration of each primer was observed suitable for optimum amplification with all other components were similar to PCR mix of exon-3 of *PRL* gene. The PCR programme used for the amplification of *DGAT1* gene is presented in **Table 3.4**.

Table: 3.4 PCR programming for amplification of exon-8 of *DGAT1* gene

Steps	Temperature	Time	No. of Cycle
I. Initial Denaturation	95^{o}C	5 min.	1 cycle
II. Cycle (i) Denaturation (ii) Annealing (iii) Synthesis	 95^{o}C 52^{o}C 72^{o}C	 1 min. 30 sec. 1 min.	 40 cycles
III. Final extension	72^{o}C	10 min.	1 cycle
IV. Hold	4^{o}C	5 min	1 cycle

3.13 Quality Check of Amplified DNA Fragments

The quality and size of the PCR amplicons for different studied locus were assessed on 1.5% agarose gel containing ethidium bromide (1% solution). 5µl PCR product of each amplified sample was mixed with 1µl of 6X gel loading dye. The samples were then loaded on precast 1.5% agarose gel plate along with standard molecular weight marker. Electrophoresis was carried out at a constant voltage of 120 V in 0.5X TBE buffer till the dye reaches to opposite ends. The size of the amplified DNA fragments was assessed by comparison with standard molecular weight marker.

3.14 Detection of Genetic Variation

The genetic variation in the selected genomic regions of *PRL* and *DGAT1* gene was identified through three different approaches in a phase wise manner:

3.14.1 Restriction fragment length polymorphism (RFLP) assay

The initial screening for polymorphism at specified nucleotide position within the selected genomic regions was carried out by RFLP method. Amplicons representing different regions of *PRL;* and *DGAT1* gene were digested with specific restriction endonuclease enzymes, diagnostic for the probable nucleotide substitution.

In relation to methodology, RFLP analysis of *PRL* gene was planned to be done by the procedure given by Dybus (2002). However, this procedure was slightly modified in the present study. For better resolution of the bands, polyacrylamide gel instead of agarose was preferred. The digestion of the amplified fragment of exon-3 and exon-4 of *PRL* gene was carried out through *Rsa* I restriction enzyme, which recognized and cut the nucleotide sequence at specific site of GT↓AC. Restriction digestion of the amplified products of exon-3 and exon-4 of *PRL* gene was carried out separately in a 30 µl reaction mixture containing 10X buffer (2µl), amplified product (10µl), *Rsa* I 10 units (1µl) and nuclease free water (17 µl). Uniform mixing of the reaction mixture was assured through spinning of samples for few seconds before incubation at 37ºC for 6 hr in water bath. The separation of the restricted products was carried out on 8% polyacrylamide gel at 120 V to obtain the clear picture of the digested samples. The polyacrylamide gel after 2/3 migration of the digested sample, was stained in gel tray containing 1% ethidium bromide solution. The sizes of the digested fragments were compared with standard DNA ruler. The digested bands were visualized under UV light and documented by gel documentation system. The determination of the prolactin genotypes for exon-3 and exon-4 region was carried out as per protocols described by Udina *et al*. (2001). The frequencies of different genotypic patterns were recorded properly for exon-3 and exon-4 of *PRL* gene. Genotypes for *PRL* exon-3 region was determined based on size and number of the digested fragments: (*GG* 156 bp), *AG* (156 bp, 82 bp, 74 bp) and *AA* (82bp, 74 bp); whereas the banding patterns for *PRL* exon-4 region was: *GG* 294 bp, *AG* (294 bp, 162 bp, 132 bp) and *AA* (162bp, 132 bp).

The dinucleotide polymorphism (GC/AA) responsible for alanine to lysine (*K232A*) polymorphism in *DGAT1* gene was detected with the use of restriction endonuclease enzyme, *Eae* I having restriction site of Y^GGCC_R. The 411-bp amplicons for the respective *DGAT1* gene were digested with 5U of *Eae* I (1µl) restriction enzyme in a 40µl reaction mixture containing 10X buffer (5µl) and nuclease free water (35µl). The reaction mixture was mixed properly through spinning and kept under water bath at 37ºC for overnight digestion. The different *DGAT1* genotypes were analyzed as per method of Winter *et al*. (2002) on 8% polyacrylamide gel. The results of electrophoretic separation were visualized and documented through GelDOC Unit after staining with ethidium bromide dye.

Cleavage by *Eae* I restriction enzyme was diagnostic for the alanine-bearing allele. An undigested fragment of 411 bp indicates *K* (lysine) allele and two

co-migrated fragments (208 and 203 bp) indicates the *A* (alanine) allele. Genotypes for *DGAT1* gene were determined based on number/length of amplified DNA fragments: (*KK* 411 bp), *KA* (411 bp, 208 bp, 203 bp) and *AA* (208 bp, 203 bp).

3.14.2 Single strand conformation polymorphism (SSCP) analysis

The single-strand conformation polymorphism (SSCP) method was used to scan mutations within the amplified regions (Zhang *et al*. 2007) apart from the restriction site. In order to detect mutations, SSCP analysis (Orita *et al*., 1989) was performed according to guidelines described by Hayashi and Yandell (1993) with slight modifications. Aliquots of 5µL PCR products were mixed with 5µL denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene cyanole and 0.025% bromophenol blue), denatured for 10 min at 95℃ fol lowed by a rapid chill on ice for 10 min. Denatured PCR products were subjected to 8% polyacrylamide gel electrophoresis in Tris-Borate-EDTA buffer and constant voltage (120 V) for 15 h at a constant temperature of 4℃, and then gels were sta ined with 1% ethidium bromide solution and visualized with under UV light and documented by gel documentation system. Individual genotypes were defined according to band patterns. The frequencies of different electrophoretic patterns were recorded under each breed.

3.14.3 Sequence analysis and putative SNP's detection

At least three representative PCR products for each electrophoresed SSCP patterns under each breed were purified through Exosap method and sequenced in both direction in a commercial laboratory (X Celris Genomics Pvt. Ltd, Ahmedabad, India) in an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) through Sanger dideoxy chain termination method. The raw sequences were analysed with Chromas 1.45 (http://www.technelysium.co.au) software for the detection of sequencing anomaly, if any on the basis of chromatogram generated. Forward and reverse sequence of each gene fragment was assembled against the most closely related reference sequence of respective gene to obtain total sequence length and similarity was looked in to the non-redundant database of Gene Bank with BLAST algorithms http://www.ncbi.nlm.nih.gov/BLAST). The assembled sequences were compared with reference sequence for similar regions available at NCBI GenBank database to detect single nucleotide polymorphisms (SNP's) through Bioedit Sequence Alignment Editor (*ver.* 7.0.5.3) software. Sequences with maximum similarity were adopted as reference sequences for the detection of SNP's.

All sequence variants were confirmed by independent amplification, sequencing and genotyping reactions. Difference between query sequence and the reference sequence available in the GenBank were classified as SNP's. Multiple sequence alignment for each of the sequenced region, *viz.,* exon-3 and exon-4 region of *PRL* gene and *DGAT1* gene was carried out through Clustal W software to detect the polymorphic location of nucleotide sequences among the three different breeds.

3.14.4 Submission of generated sequences to NCBI database

An overall nineteen sequences were generated for region of *PRL* exon-3 (n=7), *PRL* exon-4 (n=6) and *DGAT1* (n=6) gene under different breeds and were submitted to National Center for Biotechnology Information (NCBI) GenBank database, an international repository of gene and genomic sequences, through online gateway of BankIt submission.

3.15 Phylogenetic analysis

Phylogenetic tree was constructed using Neighbourhood Joining (NJ) method of bootstrap test of phylogeny in MEGA7 (Kumar *et al*., 2016) software to evaluate the evolutionary relationships of studied indigenous breeds with other farm animals of *Bos taurus*, *Bubalus bubalis*, *Camelus dromedarius*, *Capra hircus* and *Ovies aries* for each of the studied locus. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and implemented with bootstrap test involving simple stepwise addition.

3.16 Statistical Analysis

3.16.1 Genetic analysis of studied population

The genetic structure of different studied population for gene and genotypic frequencies, observed heterozygosity (H_o), expected heterozygosity (H_E) and expected unbiased heterozygosity (H_E unbiased), shannon index (I), effective number of alleles and Nei's genetic distances was analyzed through POPGENE program (version 3.1) of Yeh *et al*. (1999). The Hardy-Weinberg equilibrium for gene and genotypic frequencies was tested using chi-square ($\chi 2$) and maximum likelihood ratio test (G^2 test). Probability value less than 0.05 were considered to be significant. Standard error of allelic frequency was calculated as $[p\ (1-p)/2n]^{1/2}$ where n is the sample size and p is the frequency of allele (Spiess, 1989). The expected polymorphism information content (PIC) value of each locus was calculated by using

the method of Botstein *et al*. (1980). Wright's fixation index (F_{IS}) was estimated according to FSTAT v.2.9.3.2 package program (Goudet, 2002) to measure within-population inbreeding level through measurement of heterozygote deficiency or excess and to reveal the reproductive structure of the population analyzed.

3.16.2 Analysis of lactation performance for selected animals of Rathi, Sahiwal and Kankrej cattle for milk traits

The selected animals of Rathi, Sahiwal and Kankrej were analyzed for different lactation traits such as total milk yield (TMY), 305-day milk yield (MY), average daily milk yield (ADMY) and lactation length (LL); and milk composition parameters such as milk fat, protein, lactose, solid not fat (SNF) and total solid. The information on different lactation traits was analyzed for first and second parity. The average of TMY, 305-day MY, ADMY and LL was also calculated over all the completed lactation for each animal to obtain an estimate of the parameter value irrespective of the parity.

3.16.3 Analysis for association of genetic marker information with lactation traits and milk composition parameters

The lactation records and the milk composition values from 225 cows included in the analyses were tested for significance of association with genetic marker information using least square method of SPSS *ver.* 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical analysis was carried out under single gene model to estimate the effect of genotype on the traits. The following univariate analysis of variance was carried out through general linear model (GLM) procedure to analyse the differences among genotypes:

$$X_{ij} = \mu + g_i + e_{ij}$$

Where-

X_{ij} = mean observed value of milk performance parameter

μ= general mean

gi= fixed effect of the i^{th} genotype

e_{ij}= random error, e_{ij} ~NID (0, σ_e^2)

Least squares means for each genotype and their corresponding standard errors were computed. The least significant difference between the means for each group was used to compare the effects of each genotype. Duncan multiple range test as described by Kramer (1957) was used to compare the mean of different genotypes.

4. RESULTS AND DISCUSSION

The present study was conducted to detect genetic variation in *bPRL* and *DGAT1* genes through different molecular marker approaches such as RFLP, SSCP and SNP analysis. Genes encoding *PRL* hormone and *DGAT1* enzyme are considered as key links in the gene networks constituting the hereditary component of milk productivity. Gene disruption experiments have proved the mandatory role of prolactin gene in mammary gland development, lactogenesis, and expression of milk protein genes (Horseman *et al*., 1997) whereas similar knock out trial in mouse lines established the features of reduced or inhibited milk secretion in *DGAT1* gene deficient mouse (Smith *et al*., 2000). Therefore, the two excellent candidate genes, *bPRL* and *DGAT1* gene having crucial role in milk production were included in the present investigation. As suggested by Alipanah *et al*. (2007a), an integrated approach through different marker systems in conjunction with sequence analysis was adopted in the present study to detect the genetic variability in the form of haplotypes which could be used as more informative marker in association studies of candidate genes with milk production traits.

4.1 Isolation of Genomic DNA

Good quality genomic DNA as assessed through 0.8% agarose check and nanospectrophotometer was isolated from all the collected blood samples of three different indigenous cattle breeds, *viz*., Rathi, Sahiwal and Kankrej. The DNA concentration among breeds varied between 50 to 75ng/µl. The OD 260/280 ratio, an indicator of purity was observed in the range of 1.6 to 1.8 which indicated the suitability of the extracted DNA samples for hassle free *in vitro* amplification. All samples employed in the present investigation were found to be devoid of fragmentation as evidenced by the absence of smearing on gel and presence of intact bright genomic DNA band (**Fig. 4.1)**.

4.2 Genetic Variation in Exon-3 of *PRL* Gene

4.2.1 Amplification of exon-3 of *PRL* gene

The PCR procedure for amplification of exon-3 of *PRL* gene was standardized for different PCR parameters, *viz*., template DNA concentration, dNTP's concentration and annealing temperature. Seventy pico mol (1µl) of each forward

and reverse primer per reaction volume of 25 µl was observed to be optimum concentration. An annealing temperature of 59ºC was found to be optimal for desired amplification of 156-bp PCR product after comparing the amplifications and primer dimer resulting at varying temperature. The procedure outlined by Mitra *et al*. (1995) in Indian cattle was followed for amplification of exon-3 of *PRL* gene. Thirty five cycles of amplification was required for complete and uniform amplification. A clear cut amplified band of 156-bp with no smearing was obtained in all the studied animals (**Fig. 4.2**). The PCR product of same size was obtained by Kumari *et al*. (2008) for exon-3 of *PRL* gene.

4.2.2 RFLP analysis of exon-3 of *PRL* gene

The RFLP analysis of 156-bp fragment of *PRL* gene was carried out to assess the polymorphic status of A ⟶ G nucleotide substitution. The restriction enzyme *Rsa* I having recognition sequence and restriction site of GT↓AC was used in the present study to reveal the presence of *A/G* SNP at the restriction site and to establish the polymorphic nature of exon-3 of *PRL* gene. About 10 units of *Rsa* I restriction enzyme was found suitable for complete digestion of 5 µl PCR product. The different genotypic patterns of 156-bp *PRL* gene were resolved on 8% polyacrylamide gel. The *Rsa* I enzyme digests the *A* allele but not *G* allele. The restriction site present in *A* allele produced two bands of 82 and 74-bp on digestion whereas the non digested *G* allele was revealed as single band of 156 bp. The technique was found successful in detection of polymorphic status of exon-3 of *PRL* locus in a number of studies (Dybus *et al*., 2005). The present study detected only two different genotypes *GG* and *AG* for exon-3 of *PRL* gene in each of the studied breed (**Fig. 4.3**). The genotypic pattern *AA* was not detected in any of the studied breeds. The homozygotic pattern *GG* was identified by the presence of an intact band of 156-bp whereas the heterozygous genotypic pattern *AG* was recognized by the presence of three separate intact bands of 156-bp, 82-bp and 74-bp. The allele *A* was detected in heterozygous form only. Similar RFLP patterns using *Rsa* I restriction enzyme were reported by Kumari *et al*. (2008) in exotic and Zebu cattle, Sacraverty *et al*. (2008) in Kankrej cattle and Ghasemi *et al*. (2009) in Montebeliard cows.

4.2.2.1 Gene and genotypic frequency of exon-3 of *PRL* gene

The genetic structure of different breeds in terms of gene and genotypic frequency for exon-3 of *PRL* gene as detected through RFLP marker are presented in **Table 4.1**. The variants of the analyzed loci were found in all the three breeds and therefore it is the allelic distribution that characterizes the differences between breeds (Liron *et al*., 2002).

Table 4.1 Gene and genotypic frequencies of exon-3 of *PRL* gene detected through RFLP analysis

Locus	Breed	N	Genotypic frequencies			Allele frequencies		Std. Error
			GG	AG	AA	G	A	
***PRL* exon-3**	Rathi	75	0.28 (21)	0.72 (54)	---	0.64	0.36	0.039
	Sahiwal	75	0.19 (14)	0.81 (61)	---	0.59	0.41	0.040
	Kankrej	75	0.36 (27)	0.64 (48)	---	0.68	0.32	0.038
	Overall	225	0.28 (62)	0.72 (163)	---	0.64	0.36	0.023

The genotypic frequency of *GG* genotype in three indigenous breeds ranged from 0.19 to 0.36, whereas such frequency for *AG* genotypic pattern ranged from 0.64 to 0.81. The observed genotypic frequencies of *GG* and *AG* genotype for different studied breeds was 0.28, 0.72 for Rathi; 0.19, 0.81 for Sahiwal; and 0.36, 0.64 for Kankrej, respectively. The overall frequency of different genotypes irrespective of breed was observed to be 0.28 and 0.72 for *GG* and *AG* genotypic patterns, respectively. An excess of heterozygotes over homozygotes in all the three studied breeds indicates that heterozygotes are preferred in the indigenous breeds which could be the effect of natural selection over the years of evolution. The estimated gene frequency of *G* allele as observed for Sahiwal cattle (0.59) was less than Rathi cattle (0.64) and Kankrej cattle (0.68). Therefore, the *A* allele was found to be more prevalent in Sahiwal cattle (0.41) than Rathi (0.36) and Kankrej cattle (0.32). The average gene frequency of *G* and *A* allele of *PRL* exon-3 locus in overall population corresponds sharply with respective gene frequency of Rathi cattle. The minor differences in allele frequencies among different breeds may have resulted from different histories of selection during course of evolution and utilization in different breeding programmes. Thus, it could be concluded that the three studied breeds have nearly similar genetic structures in term of analyzed *A/G* SNP despite

their different origins. However, the relatively higher percentage of heterozygotes in Sahiwal breed could be due to over exploitation of the pure bred animals of this precious genetic resource which is also reflected in the breed wise statistical information (Breed Survey, 2013).

The results of the present study are supported by Kumari *et al*. (2008) who reported higher frequencies for genotypes *AG* in different breeds studied: Jersey (0.65), Kankrej (0.62), Gir (0.49), Red Sindhi (0.62), Hariana (1.0) and Dangi (1.00) with frequency of allele *G* as 0.88 (Sahiwal), 0.60(Kankrej), 0.61 (Gir), 0.46 (Red Sindhi) and 0.50 (Dangi) . Aravindakshan *et al*. (2004) also observed the similar findings in Vechur cattle. In a similar study on 54 Sahiwal based crossbred cattle 'Frieswal', an excess of heterozygotes (0.63) was observed over homozygotes, with very less number of animals (n=3) were detected for *AA* genotypes (Bukhari *et al*., 2013). A study on 720 HF cows revealed very high frequency of heterozygotes (0.79) with gene frequency of 0.58 for *G* allele (Wojdak-Maksymiec *et al*., 2008). Low frequency of genotypic pattern *AA* was also observed by Alipanah *et al*. (2007b) in Red Pied cattle.

The gene frequency observed in the present study for *G* and *A* allele in indigenous breeds of Rathi, Sahiwal and Kankrej cattle is also in sharp agreement with the findings (*G*=0.63; *A*=0.37) of Bukhari *et al*. (2013). Unal *et al*. (2015) in a similar type of study reported complete absence of *AA* genotype and significantly higher frequency of *AG* genotype (0.60-0.64) in at least two of the four studied breeds. Similar results were reported by Brym *et al*. (2005a) in Jersey and Black and White cattle; and Skinkyte *et al*. (2005) in Lithuanian Black and White and Lithuanian Red cattle. Very low frequency of *AA* genotype with significantly higher frequency of *G* allele was reported by Alipanah *et al*. (2007a) in Red Pied cattle. The results obtained in the present study are in contrast to those reported by Alipanah *et al*. (2007b) and Alipanah *et al*. (2008) in Russian Pied cattle. The prevalence of genotype *GG* (0.776) was observed in the American Swiss cattle than *AG* (0.174) and *AA* (0.026) pattern with gene frequency of 0.88 and 0.12 for *G* and *A*, respectively (Alfonso *et al*., 2012). The Montebeliard cattle of Iran was also reported to be nearly deficient of *AA* genotype as observed in the present investigation (Ghasemi *et al*., 2009). Breed difference in gene and genotypic frequency was reported in Najdi cattle in which the frequency of allele *G* (0.571) and *A* (0.429) were observed in nearly intermediate state with genotypic frequency of 0.29, 0.57 and 0.14 for *GG*, *AG* and *AA* genotype, respectively (Sharifi *et al*., 2010).

4.2.2.2 Hardy-Weinberg equilibrium for exon-3 of *PRL* gene

The population under each breed was tested for Hardy-Weinberg equilibrium through two different test; chi square and G square statistic to establish the role of selection, mutation, migration and mating behavior on the observed genetic structure of population for exon-3 of *PRL* gene (**Table 4.2**).

Table 4.2 Hardy-Weinberg equilibrium for exon-3 of *PRL* gene

***PRL* exon-3 region**						
Breed	**Chi^2**	**p value**	**Significance**	**G^2**	**p value**	**Significance**
Rathi	23.22	0.000	**	31.65	0.000	**
Sahiwal	34.58	0.000	**	45.23	0.000	**
Kankrej	16.20	0.000	**	23.03	0.000	**
Overall	72.06	0.000	**	97.76	0.000	**

Both the Chi^2 and G^2 test value observed for *PRL* exon-3 gene in Rathi, Sahiwal and Kankrej cattle revealed highly significant deviation from Hardy-Weinberg equilibrium ($p<0.001$) which indicates that animals differ in their genotypic distribution with respect to gene frequency. Deviations were observed not to be accumulated within one breed but uniformly distributed in all the three studied breeds. The absence of *AA* genotypic pattern and the preference for heterozygote indicates higher fitness of heterozygotes over either of the homozygotes and could be a possible reason for the deviation of equilibrium state.

Similar absence of Hardy-Weinberg equilibrium was reported by Bukhari *et al*. (2013) in Frieswal cattle. The existence of equilibrium state for exon-3 of *PRL* gene was also not observed in American Swiss cattle (Alfonso *et al*., 2012) where bulls and cattle from outside the herd were frequently inducted to improve the quality of herds. Similarly, Unal *et al*. (2015) observed significant deviation of Hardy-Weinberg equilibrium in two naturally adapted native Turkish breeds suggesting the role of natural selection in maintenance of genetic diversity. In contrast, Sharifi *et al*. (2010) reported presence of equilibrium in gene and genotypic frequency of exon-3 of *PRL* gene in Iranian cattle breed, Najdi. Nonsignificant chi^2 values were also observed for *PRL* exon-3 locus in Black Pied and Red Pied by Alipanah *et al.* (2007b). The Montebeliard cattle of Iran were observed in Hardy-Weinberg equilibrium for *PRL* exon- 3 loci (Ghasemi *et al*., 2009). The variability of results in different studies could be due to differences in breed and the reduced number of analyzed samples ($n<50$),

leading to poor and inefficient representation of different genotypes (Brym *et al*., 2005a).

The adaptive effects of a particular allele, domestication and population origin affects the gene and genotypic frequency between breeds (Medjugorac *et al*., 1994; Mac-Hugh *et al*., 1997). The presence of significant variation in gene and genotypic frequency for *PRL* exon-3 locus in the present study also suggest the active role of natural selection on exon-3 of *PRL* gene in indigenous cattle. Keeping in view, the significant role of prolactin hormone in milk production, the observed deviation might have been linked with natural fitness of our genetic resources for milk related traits. Even *G* allele though present in higher frequency, was predominately appeared in heterozygous state.

4.2.3 SSCP variation in exon-3 of *PRL* gene

SSCP analysis of Orita *et al*. (1989) is considered as a fast, economic and accurate method for the initial screening of a large number of samples to detect polymorphism in sequences of amplified fragments. Animals under each restriction pattern were analyzed further for single strand sequence polymorphism to detect SNP's present at any position in addition to the restricted site. The genotypic frequencies of different SSCP patterns under each restriction pattern have been represented in **Table 4.3.** Three different SSCP bands patterns (P1-P3) were observed for 156-bp fragment of *PRL* gene in Rathi cattle whereas two different conformation patterns were observed in both Sahiwal and Kankrej cattle (**Fig 4.4**).

All the animals having *GG* restricted genotype in Rathi cattle revealed one SSCP banding pattern termed as 'P1' whereas animals representing *AG* genotype in Rathi cattle demonstrated two different SSCP genotypic patterns, 'P2' and 'P3'. The P2 pattern was observed abundant in nature and nearly 80% of the *Rsa*I *AG* genotyped animals were identified as 'P2' genotypic pattern. The detection of an extra genotype 'P3' under *AG* restriction pattern in Rathi cattle through SSCP analysis denotes the presence of sequence variation in *A* allele of *PRL* exon-3 locus and is suggestive of multiple allelic nature of this region. This multiple allelic states could have generated in Rathi cattle due to admixture of blood from different breeds during the course of evolution. Thus polymorphism contained in the 156-bp fragments of *PRL* gene in Rathi cattle was revealed more clearly through SSCP method in combination with RFLP analysis.

Table 4.3 Genotypic frequencies of SSCP patterns detected under restriction patterns for exon-3 of *PRL* gene

Exon-3 of *PRL* gene							
Breed	**Rathi**			**Sahiwal**		**Kankrej**	
RFLP pattern	**GG**	**AG**		**GG**	**AG**	**GG**	**AG**
Rsa I frequency	0.28 (21)	0.72 (54)		0.19 (14)	0.81 (61)	0.36 (27)	0.64 (48)
SSCP pattern	**P1**	**P2**	**P3**	**P4**	**P5**	**P1**	**P6**
SSCP frequency	0.28 (21)	0.57 (43)	0.15 (11)	0.19 (14)	0.81 (61)	0.36 (27)	0.64 (48)

Both Sahiwal and Kankrej cows produced two different SSCP banding patterns representing restricted genotype *GG* and *AG*. The SSCP pattern of Sahiwal was designated as 'P4' and 'P5' equivalent to *GG* and *AG* restriction pattern. The genotypic pattern revealed by SSCP marker in Kankrej cows were considered as P1 and P6 for their respective *GG* and *AG* genotypic patterns on the basis of their electrophoretic migration. The electrophoretic mobility of 'P1' pattern of Kankrej cattle was detected similar to 'P1' pattern of Rathi cattle. The different SSCP patterns of *PRL* exon-3 gene showed variation in electrophoretic movement as a result of sequence variation and conformational change in single stranded DNA. The genotypic frequency of identified SSCP banding pattern in Sahiwal and Kankrej cattle was found similar in nature to RFLP frequency whereas the genotypic frequency of *AG* genotype in Rathi cattle was redistributed according to SSCP banding pattern due to additional information generated for exon-3 of *PRL* gene. Very few studies have conducted till date on SSCP based detection of variation in exon-3 of *PRL* gene However; many workers have suggested the suitability of SSCP marker for the detection of polymorphism in prolactin gene.

As per available literature, a study conducted by Halabian *et al*. (2008) identified four different SSCP banding patterns in Iranian Holsteins for exon 3 region of *PRL* gene. Likewise, Zhang *et al*. (1994) detected three genotypes for exon 1 of *PRL* gene through SSCP analysis whereas Hart *et al*. (1993) observed four alleles in the 5'flanking region of b*PRL* gene. An integrated approach adopted in the present study revealed the sharing of common gene pool among indigenous cattle breeds which might have occurred due to common ancestry. Rathi and Kankrej cattle shared the common gene pool for *G* allele as reflected by similar SSCP pattern (P1). The partial similarity in band distance covered between P3 and P5 SSCP patterns

indicates an exchange of genes between Rathi and Sahiwal. The SSCP method was observed to be more informative in detection of genetic variation in exon-3 of *PRL* gene than RFLP method.

4.2.4 Genetic diversity in exon-3 of *PRL* gene

The quantification of genetic variation within breed and the knowledge about its pattern among different breeds is necessary to distribution of genotype under the factors affecting Hardy Weinberg equilibrium. Different parameters were suggested to measure the genetic variation between populations.

4.2.4.1 Observed and expected heterozygosity (H_E)

The heterozygosity is a measure of genetic variation within a natural population. High heterozygosity values within a natural population or breed denotes long term selection for adaptation, or historic mixing of different populations (Kotze and Muller, 1994). H_E is also called as gene diversity (D) and accounts for both the richness and evenness of alleles in the population.

Animals under the studied breeds reflected dominance of heterozygote individuals in population. Around 72.4 per cent of total population was identified as heterozygote. The observed genetic diversity (H_o) for *PRL* exon-3 region was significantly higher than expected heterozygosity and Nei's expected level in population of Rathi, Sahiwal and Kankrej cattle. The results indicate the presence of sufficient genetic variation at this locus though in the form of heterozygote combination (**Table 4.4**).

Table 4.4 Within-population heterozygosity estimates, PIC and F_{IS} values of Rathi, Sahiwal and Kankrej cattle for exon-3 of *PRL* gene

Breed	Sample size (N)*	Observed Hetero-zygosity	Expected Hetero-zygosity	Nei's Unbiased Hetero-zygosity	PIC	Fixation index
		H_o	H_E	H_e		F_{IS}
Rathi	75	0.720	0.463	0.431	0.3546	-0.562
Sahiwal	75	0.813	0.483	0.459	0.3668	-0.685
Kankrej	75	0.640	0.435	0.459	0.3405	-0.471
Overall	225	0.724	0.463	0.462	0.3546	-0.568

The present study replicated the results obtained in native Turkish breed where significantly higher heterozygosity greater than 0.5 was observed in three of

the four breeds (Unal *et al*., 2015). In contrast to present study, very low degree of heterozygosity (H_E=0.196) was reported in American Swiss cattle (Alfonso *et al*., 2012). The heterozygosity frequency in different European breeds was observed to be lower than level of 0.5 which could be due to trends adopted for artificial selection. Skinkyte *et al*. (2005) found heterozygosity value of 0.33 and 0.23 in Black and White and Red cattle. Ghasemi *et al*. (2009) reported heterozygosity value of 0.15 in Montebeliard cattle. Kalashnikova *et al*. (2009) observed values of 0.40 in Black Pied cattle whereas Alipanah *et al*. (2007a) reported 0.39 in Russian Red Pied cattle.

4.2.4.2 Polymorphism information content (PIC)

The PIC has become the most widely applied method to measure the information content of molecular markers in genetic studies since its first application (Nagy *et al*., 2012). It reflects the quality or informativeness of polymorphism to be used as genetic marker for linkage analysis. According to classification of PIC, low polymorphism occurs if PIC value < 0.25, medium polymorphism if PIC value < 0.5 and high polymorphism if PIC value >0.5. The PIC value for exon-3 of *PRL* gene in studied breeds ranged from 0.3405 to 0.3668 (**Table 4.4**). All the studied breeds showed medium polymorphism for *PRL* exon-3 region with PIC value greater than 0.25 and less than 0.50. Kankrej cattle showed lowest PIC value whereas highest PIC was observed for Sahiwal cattle.

4.2.4.3 Fixation Index (F_{IS})

Wright's fixation index (F_{IS}) is an indirect measure of inbreeding coefficient and is suggestive of level of genetic variation in the population. Though the study observed highly significant deviation of gene and genotypic frequency in exon- 3 of *PRL* gene, the negative FIS value within population (**Table 4.4**) suggest nonsignificant status of inbreeding in the population and reflects that samples were not from an inbred gene pool (Unal *et al*., 2015). The low and negative value of fixation index observed in the present study in all the studied breeds and also on overall basis suggests the presence of sufficient genetic variation in the population to maintain the genetic diversity.

4.2.4.4 Effective number of alleles

The effective number of alleles (n_e) observed for *PRL-3* region in Rathi, Sahiwal and Kankrej cattle were 1.932, 1.932 and 1.770, respectively (**Table 4.5**). Lowest n_e values were observed for Kankrej cattle for *PRL-3* region. The results

indicate that comparatively less number of number of alleles are required in Kankrej cattle to maintain genetic diversity than Rathi and Sahiwal animals.

Table 4.5 Observed and effective number of alleles; and Shannon information index for exon-3 of *PRL* gene

Breed	Sample size of alleles	Observed number of alleles	Effective numbers of alleles	Shannon's Information Index
	N	n_a	n_e	I
Rathi	150	2.00	1.932	0.675
Sahiwal	150	2.00	1.932	0.675
Kankrej	150	2.00	1.770	0.627
Overall	450	2.00	1.858	0.654

4.2.4.5 Shannon's information index

The degree of heterozygosity as measured by Shannon Information Index (I) (Weir, 1990) indicates equal level of heterozygosity in Rathi and Sahiwal than Kankrej (**Table 4.5**). The trend of Shannon Index towards unity indicates the presence of sufficient heterozygosity and reflects the suitability of primer pair used for genetic diversity analysis. The observed value of Shannon Index in studied indigenous cattle breeds was remarkably higher than value (0.37) observed for exotic American Swiss cattle (Alfonso *et al*., 2012).

4.2.4.6 Genetic distance among studied breeds

Nei's genetic identity is the probability that two alleles chosen at random from population are identical in nature. A significantly higher genetic identity was observed among the three studied breeds for exon-3 of *PRL* gene. Nei's genetic distance value is based on the gene frequency values rather than different proportion of genotypes and is considered as a reliable estimator of genetic diversity. The Nei's genetic distance analysis amongst the three cattle population for *PRL* exon-3 region showed similar and minimum genetic distance between Rathi and Sahiwal (0.003); and Rathi and Kankrej cattle (0.003). Sahiwal was observed to be relatively more diverged from Kankrej cattle as reflected from the genetic distance values (0.013) (**Table 4.6**). Nei's genetic distance value considers both monomorphic and polymorphic locus information and thus it is frequently used in the construction of phylogenetic tree. Variation in genetic distances between herds of American Swiss was also reported by Alfonso *et al*. (2012).

Table 4.6 Pair wise genetic distance matrix between studied cattle breeds for exon-3 of *PRL* gene

***PRL*-3 region**			
Breed	**Rathi**	**Sahiwal**	**Kankrej**
Rathi	****	0.996	0.997
Sahiwal	0.003	****	0.987
Kankrej	0.003	0.013	****

* Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

4.2.5 SNP's analysis of exon-3 of *PRL* gene

4.2.5.1 Generation of gene sequences

The forward and reverse raw sequences generated through Sanger sequencing were checked for accuracy through Chromas software. The sequences were assembled with the help of reference sequence already available at NCBI database (GenBank Accession No. NM173953). The assembled sequence of 156-bp, generated for three studied breeds of indigenous Rathi, Sahiwal and Kankrej cattle were submitted to the NCBI GenBank through online BankIt gateway after proper annotation and accordingly accession numbers were obtained (**Table 4.7**).

Table 4.7 List of accession numbers obtained for different SSCP patterns of exon-3 of *PRL* gene

b *PRL* exon-3 Region				
Breed	**Restricted Allele**	**SSCP Pattern**	**Product size**	**Accession Number**
Rathi	G	P1	156-bp	KX863742
	A	P2	156-bp	KX863741
	A	P3	156-bp	KY777607
Sahiwal	G	P4	156-bp	KY777608
	A	P5	156-bp	MF045466
Kankrej	G	P1	156-bp	MF045467
	A	P6	156-bp	KY777609

4.2.5.2 SNP's detection

The detection of sequence variations is the core of all genetic analysis that ultimately leads to variation discovery across candidate gene that may affect the trait of interest (Nickerson *et al*., 1997). Single nucleotide polymorphisms (SNPs) or one

base changes including deletion, insertion and substitution, may play important roles in the regulation of genes transcription and amino acids sequences of mature proteins, which could be used for the association studies between candidate genes and complex traits in domestic animals (Yoon *et al*., 2005). The different SSCP banding patterns representing different nucleotide sequences of Rathi, Sahiwal and Kankrej were sequenced in triplicate to identify the position and nature of possible SNP's responsible for sequence variation.

The multiple sequence alignment (MSA) of generated sequences (query sequences) was carried out with the help of Clustal W software to detect the position of SNP's in amplified fragments through comparison with reference sequence of cattle (**Fig. 4.5**). The study revealed that polymorphism contained in the different SSCP patterns of 156-bp fragments of *PRL* gene in Rathi, Sahiwal and Kankrej cattle were the result of substitution of four bases at four different positions in nucleotide sequences. The four identified SNP's were observed to be located at 39th, 58th, 59th and 75th position in amplified fragments of 156-bp of *PRL* gene. The SNP identified at 75th position was responsible for the occurrence of restriction site in prolactin gene of bovines. The SNP's observed at 39th, 58th and 75th positions were observed transition in nature whereas the SNP detected at 59th position was transversion mutation (**Table 4.8)**.

Table 4.8 List of position and nature of SNP's observed in exon-3 of *PRL* gene

Exon-3 of *PRL* gene			
Base position	**Region**	**Change**	**Nature of SNP's**
39	Coding	C>T	Transition
58	Coding	C>T	Transition
59	Coding	C>A	Transversion
75	Coding	A>G	Transition

The present study is in sharp agreement with the study conducted by Halabian *et al*. (2008) which also reported four SNP's in a similar fragment of 156-bp in Iranian Holsteins cows of which two SNP's were reported to alter the predicted sequence of amino acids. Similarly Sasavage *et al*. (1974) identified seven possible nucleotide substitutions in prolactin gene on the basis of sequence analysis of four different cDNA clones. One of SNP (A/G), recognized by the *Rsa* I endonuclease, has become a popular genetic marker used for genetic characterization of cattle

populations by means of PCR-RFLP (Dybus, 2001). In agreement, Kaminski *et al*. (2005) genotyped b*PRL* gene and identified five SNP's.

4.2.5.3 Construction of haplotype/haplotype analysis

An overall five different haplotypes were constructed on the basis of detected SNP's in 156-bp amplified fragment of exon-3 of *PRL*-3 gene as per Winter *et al*. (2002) (**Fig. 4.6**). The haplotype were designated on the basis of restricted base. The haplotype, Hap-*A1* was unique to the animals of Rathi breed and was present in 43 of the 75 animals studied. The second haplotype denoted as Hap-*A*2 was common in Rathi and Sahiwal and was represented in 11 and 61 animals of Rathi and Sahiwal cattle, respectively. The haplotype labeled as Hap-*A*3 differed from *A*1 haplotype at 59th SNP position and was present in animals of Kankrej only. The fourth haplotype considered as Hap-*G*1 was observed in 21 Rathi and 27 Kankrej animals. The haplotype, Hap-*G*2 was observed in animals of Sahiwal only. Haplotype Hap-*A*2 varied from Hap-*A*3 and Hap-*G*2 at SNP position 39th and 75th, respectively. It could be supposed that origin of Hap-*G*2 haplotype might have occurred from Hap-*A*2 haplotype during the course of evolution.

The frequency of Hap-*A*1, Hap-*A*2 and Hap-*G*1 haplotype were estimated to be 0.29, 0.07 and 0.64, respectively in Rathi cattle. Similarly, the Hap-*A*2 and Hap-*G*2 haplotype in Sahiwal cattle were present in the frequency of 0.41 and 0.59; and Hap-*A*3 and Hap-*G*1 in frequency of 0.32 and 0.68 in Kankrej cattle. Haplotypes Hap-*A*1, Hap-*A*3 and Hap-*G*2 were novel and breed specific in nature. Halabian *et al*. (2008) reported less number of haplotypes and observed only two haplotypes for similar region in Iranian Holsteins cattle. Intragenic haplotypes constructed for the first time in the present study in indigenous cattle breeds could be used as more informative markers in polygenic and association studies. Haplotype-based association analysis has proved to be a powerful approach to identify genes affecting complex traits (Knoblauch *et al*., 2002).

4.2.6 Phylogenetic Analysis

The FASTA converted nucleotide sequences of exon-3 of *PRL* gene of indigenous breeds of Rathi, Sahiwal and Kankrej cattle were tested for their evolutionary relationship with the different available sequences of *Bos indicus*, *Bos taurus*, *Bubalus bubalis*, *Camelus dromedarius*, *Capra hircus* and *Ovies aries*.

4.2.6.1 Evolutionary divergence among breeds/species

The evolutionary divergence between pair of different breeds/species is depicted in **Table 4.9**. The overall mean genetic distances among different breeds/species was observed as 0.0208±0.0072 for exon-3 of *PRL* gene. The intraspecies evolutionary divergence among the different alleles of studied *Bos indicus* cattle, *viz.,* Rathi, Sahiwal and Kankrej, was observed lower than mean value and ranged between 0.0000 to 0.0197 whereas the observed interspecies divergence between indigenous cattle and referenced *Bos taurus* cattle was higher for some sequences than the mean value and ranged between 0.0130 to 0.0335. The different alleles of indigenous cattle genetically diverged from buffalo in evolutionary range of 0.0130 to 0.0197. The divergence of exon-3 of *PRL* gene of camel from different identified alleles of indigenous cattle ranged from 0.0065 to 0.0265. The *Capra hircus* reflected evolutionary divergence of either 0.0197 or 0.0265 to any of the detected alleles of exon-3 of *PRL* gene in indigenous cattle. *Ovies aries* reflected highest genetic divergence (0.0335 to 0.0405) with indigenous cattle for *PRL* exon-3 region.

4.2.6.2 Construction of phylogenetic tree

Dendrogram are considered as an effective way to represent the genetic distances through construction of tree based diagrams which represents the statistically proved graphical overview of the relationship among the studied populations (Alfonso *et al*., 2012). The evolutionary analysis was conducted through MEGA7 software of Kumar *et al*. (2016). Phylogenetic tree was constructed through algorithm based Neighbor-Joining (NJ) method of Nei and Saitou (1987) for the studied breeds and different species/or breeds of ruminants to observe the evolutionary relationship between species/breed for *PRL* exon-3 region (**Fig. 4.7**). The population from different breeds or species was clustered through hierarchical method on the basis of their genetic characteristics for phylogenetic studies.

The Jukes-Cantor model (Jukes and Cantor, 1969) having minimum Bayesian Information Content (BIC) value (842.602) was selected out of 24 different maximum likelihood substitution models for the construction of dendrogram. Bootstrapping was carried out to obtain confidence statements about the grouping of breeds and to test the validity of the clusters obtained. The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The NJ tree showed bootstrap values ranged

Table 4.9 Evolutionary divergence among breed /species for *PRL* exon-3 region

Breed/Species	RAB	RAC	SAA	SAB	KAA	KAB	BIKP	BTAH	BTAH	BTAF	BBDQ	CDKX	CHAY	OAKC
Rathi Allele A_KX863741 (RAA)														
Rathi Allele B_KY777607 (RAB)	0.013													
Rathi Allele C_KX863742 (RAC)	0.0197	0.0197												
Sahiwal Allele A_KY777607 (SAA)	0.013	0	0.0197											
Sahiwal Allele B_KY777608 (SAB)	0.0197	0.0065	0.013	0.0065										
Kankrej Allele A_KY777609 (KAA)	0.0065	0.0065	0.013	0.0065	0.013									
Kankrej Allele B_KX863742 (KAB)	0.0197	0.0197	0	0.0197	0.013	0.013								
Bos indicus_ KP 635966 (BIKP)	0.013	0	0.0197	0	0.0065	0.0065	0.0197							
Bos taurus _AH013356 (BTAH)	0.0265	0.013	0.0335	0.013	0.0197	0.0197	0.0335	0.013						
Bos taurus _AF426315 (BTAF)	0.0265	0.013	0.0335	0.013	0.0197	0.0197	0.0335	0.013	0					
Bos taurus _AH001155 (BTAH)	0.0335	0.0335	0.0265	0.0335	0.0265	0.0265	0.0265	0.0335	0.0197	0.0197				
Bubalus bubalis_ DQ287249 (BBDQ)	0.0197	0.0197	0.013	0.0197	0.013	0.013	0.013	0.0197	0.0335	0.0335	0.0265			
Camelus dromedarius _KX863739 (CDKX)	0.0265	0.013	0.0197	0.013	0.0065	0.0197	0.0197	0.013	0.0265	0.0265	0.0335	0.0197		
Capra hircus _AY517553 (CHAY)	0.0265	0.0265	0.0197	0.0265	0.0197	0.0197	0.0197	0.0265	0.0405	0.0405	0.0335	0.0065	0.0265	
Ovis aries_ KC764410 (OAKC)	0.0405	0.0405	0.0335	0.0405	0.0335	0.0335	0.0335	0.0405	0.0265	0.0265	0.0197	0.0197	0.0405	0.013

Mean genetic distance = 0.0208±0.0072

from 15 to 78. The different allele of indigenous cattle shared different branches. Sheep and goat revealed the less distant ancestral relationship for *PRL* exon-3 region.

4.2.6.3 Conserved regions among breeds/species

The identification of conserved region among studied breeds; and between indigenous breeds and other species was detected through Bio Edit software (ver. 7.0.5.3) (**Table 4.10**). The detection of consensus region among different breeds/species for *PRL* exon-3 region highlights the sequence fragments which have been subjected to least evolutionary pressure. Four conserved regions were identified among the Rathi, Sahiwal and Kankrej cattle breeds whereas three different conserved regions were identified between studied breeds and exotic cattle or other species. In overall, nucleotide positions 3-27, 76-91 and 93-156 were identified as highly conserved among diverse range of species.

Table 4.10 Conserved regions among breeds/species for *PRL* exon-3 region

Attributes	No. of conserved regions	Position of conserved regions				
Between studied breeds	4	1-38	40-57	60-74	76-156	-----
Between studied cattle breeds and exotic cattle	3	3- 38	-------	60-74	76-156	-----
Between studied cattle breeds and other species	3	3-27	------	--------	76-91	93-156

4.3 Genetic Variation in Exon-4 of *PRL* Gene

The herds of Rathi, Sahiwal and Kankrej were screened for another locus of *PRL* gene, i.e., exon-4, to detect additional mutations in prolactin gene and to evaluate the nucleotide substitutions as potential markers of milk performance traits.

4.3.1 Amplification of exon-4 of *PRL* gene

The 294-bp region of *PRL* gene encompassing the whole exon-4 and parts of introns 3 and 4 was amplified through specific primers based on cattle sequences available at NCBI database (GenBank Accession No. AF426315). The PCR reaction was carried out as per standard protocol of Brym *et al*. (2005a) with slight modifications. Eighty pico mol of each forward and reverse primer was observed suitable for proper amplification with no primer dimer formation. The annealing temperature was standardized to 55ºC to obtain bright, intact and smear free band of 294-bp (**Fig. 4.8**). The number of cycles required to get optimum amplification were similar to exon-3 of *PRL* gene.

4.3.2 RFLP analysis of exon-4 of *PRL* gene

All the amplified samples (n=225) having fragment length of 294-bp length for exon-4 of *PRL* gene were digested with restriction enzyme *Rsa* I to reveal the polymorphic nature of SNP *g.8398G>A*. The transition of *G* into *A* at position 163 of amplified fragment created a restriction site for *Rsa*I endonuclease enzyme. Digestion of the 294-bp PCR product with *Rsa* I enzyme resulted in two restriction fragments of 162 and 132 bp for *AA* homozygotes, one uncut fragment of 294 bp for *GG* homozygotes, and all three fragments for *AG* heterozygotes (**Fig. 4.9**). All the three indigenous breeds investigated in the present study revealed the presence of each of the three genotypic patterns. The polymorphism in *bPRL* gene has not been investigated earlier in Rathi cattle whereas a single study in exon-3 region of *PRL* gene was carried out in Kankrej cattle by Sacraverty *et al*. (2008). Brym *et al*. (2005a), Mehmannavaz *et al*. (2009), Othman *et al*. (2011) also reported similar restriction patterns in Black and White cows, Iranian Holsteins and Egyptian buffalo, respectively.

4.3.2.1 Gene and genotypic frequency

The breed wise gene and genotypic frequency observed for RFLP restricted genotypic patterns are represented in **Table 4.11**. The three genotypes *GG*, *AG* and *AA* were distributed in the frequency of 0.21, 0.47 and 0.32 in Rathi cattle, 0.24, 0.61 and 0.15 in Sahiwal cattle; and 0.17, 0.60 and 0.23 in Kankrej cattle, respectively. Lowest frequency of *AA* genotype was observed in Sahiwal cattle. The overall genotypic frequency irrespective of breed corresponded with Rathi cattle for *GG* genotype and Kankrej cattle for *AA* genotype.

Table 4.11 Gene and genotypic frequencies of exon-4 of *PRL* gene detected through RFLP analysis

	Breed	N	GG	AG	AA	G	A	Std. Error
***PRL* exon-4**	**Rathi**	75	0.21 (16)	0.47 (35)	0.32 (24)	0.45	0.55	0.041
	Sahiwal	75	0.24 (18)	0.61 (46)	0.15 (11)	0.55	0.45	0.041
	Kankrej	75	0.17 (13)	0.60 (45)	0.23 (17)	0.47	0.53	0.041
	Overall	225	0.21 (47)	0.56 (126)	0.23 (52)	0.49	0.51	0.023

The allele *A* was found to be less frequent in Sahiwal animals (0.45) than Rathi (0.55) and Kankrej cattle (0.53) with an overall frequency of 0.51. The corresponding frequency of *G* allele in Rathi, Sahiwal and Kankrej cattle was observed as 0.45, 0.55 and 0.47, respectively. The distribution of *Rsa* I alleles of *PRL* exon-4 region was observed in nearly intermediate range for all the studied breeds. The studied breeds of *Bos indicus* in the present study exhibited considerably less variation in gene frequency with preponderance of heterozygotes in the population. In contrast to the above findings, the *G* allele of *PRL* exon-4 was observed predominant in nature in most European breeds of cattle. The observed allele frequency of *PRL Rsa* I locus (0.45 to 0.55) for *G* is in sharp contrast to those reported previously by Boleckova *et al*. (2012). The *G* allele was found to be dominant (0.88) in Czech Fleckvieh cattle population with frequency of different genotypes observed as 0.77, 0.22 and 0.01 for genotype *GG*, *AG* and *AA*, respectively (Boleckova *et al*., 2012). In disagreement to present study, Iranian Holstein cattle reflected a very low frequency of heterozygotes (0.12) with frequency of *G* allele towards the extreme end (0.87) (Mehmannavaz *et al*., 2009). The breed specific variation in gene frequency was not pronounced in Indian cattle for exon-4 of *PRL* gene. Brym *et al*. (2005a) reported a quite variable nature of *A* and *G* allele frequencies in two different European breeds with frequencies of 0.113 and 0.706 for *A* allele in *PRL* exon-4 gene of Black and White cows; and Jersey cattle, respectively. Lower frequency of the *G* allele was observed in Jersey breed (Dybus *et al*., 2005). The frequency of *G* allele varied from 0.29 in Jersey cows (Brym *et al*., 2005a) to 0.95 in Argentina Creole cows (Golijow *et al*., 1999) which could be explained by different history of breeds, long term geographical isolation and selection towards high fat and protein percents of milk.

4.3.2.2 Hardy-Weinberg equilibrium of population studied for exon-4 of *PRL* gene

The test for Hardy-Weinberg equilibrium for exon-4 region of prolactin gene reflected a quite variable picture in different studied breeds (**Table 4.12**). Significant deviation (p□0.05) in gene and genotypic frequency of *PRL* exon-4 gene from Hardy Weinberg equilibrium was observed in Sahiwal cattle whereas Rathi and Kankrej breeds showed the state of equilibrium for the identified alleles. Similarly, Brym *et al.* (2005a) observed nonsignificant deviation in gene and genotypic frequency of exon-4 of PRL gene in Black and White; and Jersey cattle. The studies in Iranian Holstein by Mehmannavaz *et al*. (2009) also denoted maintenance of Hardy-Weinberg equilibrium (p<0.05). Likewise, Boleckova *et al*. (2012) observed distribution of genotypes according to Hardy-Weinberg equilibrium in Czech Fleckvieh cattle. The comparative analyses of the present study with different studies have shown that locus is usually presented in equilibrium state in most of the studied breeds. The absence of equilibrium state in Sahiwal cattle might be the result of over exploitation of this precious genetic resource.

Table 4.12 Hardy-Weinberg equilibrium for exon-4 of *PRL* gene

***PRL*-4 region**						
Breed	**Chi^2**	**p value**	**Significance**	**G^2**	**p value**	**Significance**
Rathi	0.294	0.587	NS	0.294	0.587	NS
Sahiwal	3.994	0.045	*	4.049	0.044	*
Kankrej	2.902	0.088	NS	2.926	0.087	NS
Overall	3.150	0.075	NS	3.158	0.075	NS

4.3.3 SSCP variation in exon-4 of *PRL* gene

The SSCP marker analysis was also carried out in addition to RFLP marker to detect the presence of nucleotide sequence polymorphism within exon-4 of *PRL* gene in indigenous cattle. The PCR-SSCP analysis of 294-bp product of *PRL* gene in three studied breeds revealed the presence of three unique patterns in each of the three breeds reflecting their respective restricted genotypic pattern (**Fig. 4.10**). The SSCP pattern resolved for animals of Rathi cattle were considered as P1 to P3 whereas P4-P6 genotypic patterns with differential gel mobility were obtained for Sahiwal animals. Likewise, the SSCP patterns obtained for the animals of Kankrej

cattle were termed as P7, P8 and P9 corresponding to *GG*, *AG* and *AA Rsa* I genotype of Kankrej animals. The PCR products representing SSCP patterns P1 ad P3 (Rathi); P4 and P6 (Sahiwal); and P7 and P9 (Kankrej) were shown to be opposite homozygotes. The banding patterns P2, P4 and P6 were identified as heterozygotes. None of the banding pattern among breeds reflected similar electrophoretic movement on polyacrylamide gel. The frequency of different SSCP patterns corresponded with respective genotypic frequency of RFLP patterns for each breed in strict order (**Table 4.13**).

Table 4.13 Genotypic frequencies of SSCP patterns detected under restriction patterns for exon-4 of *PRL* gene

***PRL* Exon-4**									
Breed	**Rathi**			**Sahiwal**			**Kankrej**		
RFLP pattern	**GG**	**AG**	**AA**	**GG**	**AG**	**AA**	**GG**	**AG**	**AA**
Rsa I frequency	0.21 (16)	0.47 (35)	0.32 (24)	0.24 (18)	0.61 (46)	0.15 (11)	0.17 (13)	0.60 (45)	0.23 (17)
SSCP pattern	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**
SSCP frequency	0.21 (16)	0.47 (35)	0.32 (24)	0.24 (18)	0.61 (46)	0.15 (11)	0.17 (13)	0.60 (45)	0.23 (17)

The dissimilar gel movement of different patterns under each breed indicates an independent evolution of exon-4 of *PRL* gene in studied cattle breeds. The present study is in agreement with findings of Brym *et al*. (2005a) who observed three different SSCP patterns representing the three different restriction patterns of exon-4 of *PRL* gene in Black and White; and Jersey cattle. In a quite similar study, Dong *et al*. (2013) also observed three different SSCP banding patterns for exon-4 of PRL gene in a study on 586 randomly selected Chinese Holstein cows.

4.3.4 Genetic diversity in exon-4 of *PRL* gene

4.3.4.1 Expected heterozygosity (H_E)

The value of the expected heterozygosity was observed in the intermediate range in all the three breeds. The exon-4 region of *PRL* gene demonstrated higher observed heterozygosity than expected in Sahiwal and Kankrej cattle. The animals of Rathi revealed slightly lower observed heterozygosity than expected heterozygosity which could be due to sampling variation (**Table 4.14**).

4.3.4.2 Polymorphism Information Content (PIC)

The PIC value of the haplotype block under each breed indicates that the ten SNP's should be considered as polymorphic. A medium PIC value indicates the presence of sufficient genetic variation at this locus (**Table 4.14**). Nearly uniform PIC value was observed in breeds of Rathi, Sahiwal and Kankrej.

Table 4.14 Within-population heterozygosity estimates, PIC and F_{IS} values of Rathi, Sahiwal and Kankrej cattle for exon-4 of *PRL* gene

Breed	Sample size (N)*	Observed Hetero-zygosity	Expected Hetero-zygosity	Nei's Unbiased Hetero-zygosity	PIC	Fixation index
		H_o	H_E	H_e		F_{IS}
Rathi	75	0.4667	0.4976	0.4943	0.3725	0.0559
Sahiwal	75	0.6133	0.4990	0.4956	0.3725	-0.2374
Kankrej	75	0.6000	0.5019	0.4986	0.3741	-0.2034
Overall	225	0.560	0.501	0.4998	0.3749	-0.1206

4.3.4.3 Fixation index

A bias towards heterozygote excess was inferred from the negative F_{IS} value in Sahiwal and Kankrej cattle for *PRL* exon-4 region that was confirmed by the difference between observed and expected heterozygosity (**Table 4.14**). High F_{IS} (0.0559) observed for *PRL*-4 region (Rincon *et al*., 2006) in Rathi cattle indicates the less number of heterozygotes in the population.

4.3.4.4 Effective number of alleles (n_e)

The number of expected alleles required to maintain genetic variation in exon-4 of *PRL*-4 gene was observed to be higher in all the three breeds studied (**Table 4.15**). The number of observed alleles (n_a) and effective allele (n_e) are in quite conformity with each other. The effective number of alleles observed in Rathi (1.977), Sahiwal (1.982) and Kankrej cattle (1.994) were nearly similar to each other.

4.3.4.5 Shannon Information Index (I)

The Shannon index value ranged between 0.687 to 0.691 with an overall value of 0.692 (**Table 4.15**). The values observed were greater than intermediate values in all the studied breeds and suggests the appropriateness of primer for study of polymorphism in this region.

Table 4.15 Observed and effective number of alleles; and Shannon information index for exon-4 of *PRL* gene

Breed	Sample size of alleles	Observed number of alleles	Effective numbers of alleles	Shannon's Information Index
	N	n_a	n_e	I
Rathi	150	2.00	1.977	0.687
Sahiwal	150	2.00	1.982	0.688
Kankrej	150	2.00	1.994	0.691
All	450	2.00	1.999	0.692

4.3.4.6 Genetic Distance

In contrast to the results observed for *PRL*-3 region, the Nei's genetic distance analysis for *PRL*-4 region reflected a higher genetic distance between Rathi and Sahiwal (0.020) than compared with the genetic distance for Rathi and Kankrej (0.001); and Sahiwal and Kankrej (0.011) (**Table 4.16**).

Table 4.16 Pair wise genetic distance matrix between studied cattle breeds for exon-4 of *PRL* gene

Breed	Rathi	Sahiwal	Kankrej
Rathi	****	0.980	0.998
Sahiwal	0.020	****	0.989
Kankrej	0.001	0.011	****

* Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

4.3.5 SNP's analysis of *PRL* exon-4 region

4.3.5.1 Generation of gene sequences

The polymorphism detected through RFLP and SSCP marker in *PRL* exon-4 gene of Rathi, Sahiwal and Kankrej breed was tested for nature and position of individual mutation through sequencing and sequence analysis of amplified fragments representing the polymorphic elements. Six different homozygotic patterns representing different restricted alleles and SSCP patterns of studied breeds were sequenced. The generated sequences were submitted to NCBI GenBank database under accession numbers, MF045465; and KY777610 to KY777614 (**Table 4.17**).

Table 4.17 List of accession numbers obtained for different SSCP patterns for exon-4 of *PRL* gene

b *PRL*-4 Region				
Breed	**Restricted Allele**	**SSCP Pattern**	**Product size**	**Accession Number**
Rathi	G	P1	294-bp	MF045465
	A	P3	294-bp	KY777610
Sahiwal	G	P4	294-bp	KY777612
	A	P6	294-bp	KY777611
Kankrej	G	P7	294-bp	KY777614
	A	P9	294-bp	KY777613

4.3.5.2 SNP's detection

The polymorphic nature of 294-bp product of prolactin gene observed in the present study is suggestive to compare the observed sequence with other cattle breeds or ruminant species to reveal the presence of any intra and inter species polymorphism. The multiple sequence alignment of the respective amplified products from all the selected animals revealed polymorphism. The position of individual SNP was identified through comparison with reference sequence (AF426315) available in NCBI GenBank database (**Fig. 4.11**). A total of ten different SNP's were detected on alignment of representative sequences with reference sequence of which eight SNP's were observed in non coding region (**Table 4.18**).

Table 4.18 List of position and nature of SNP's observed in exon-4 of *PRL* gene

Exon-4 of *PRL* gene			
Base position	**Region**	**Change**	**Nature of SNP's**
36	Intron-3	C>T	Transition
39	Intron-3	A>C	Transversion
40	Intron-3	C>T	Transition
46	Intron-3	A>T	Transversion
71	Intron-3	C>T	Transition
72	Intron-3	T>C	Transition
79	Intron-3	C>T	Transition
142	Exon-4	G>C	Transversion
163	Exon-4	A>G	Transition
275	Intron-4	T>C	Transition

The intron 3 region was found to be highly variable than other regions of the studied fragment as seventy per cent of detected SNP's were located in this region. Only two SNP's were observed in coding region of exon-4 of *PRL* gene at position 142 and 163. The position of the individual mutations was identified. The SNP's located in the exon-4 codon region at position 163 was found to be responsible for RFLP polymorphism. Most of the identified mutation in amplified fragments of 294-bp were observed to be transition in nature except mutation at position 39th, 46th and 142nd which were transversion in nature. The SNP's identified at referred position 8377 and 8398 were located in exon-4 region, whereas SNP's at referred position 8271, 8274, 8275, 8281, 8306, 8307, 8314 were located in intron 3 and SNP at 8510 was located in intron-4 of PRL gene.

4.3.5.3 Construction of haplotype/haplotype analysis

Six haplotype were constructed on the basis of detected SNP's (**Fig 4.12**). Three haplotypes *A1*, *A2* and *A3* were related with *Rsa* I restriction allele *A* whereas *G1*, *G2* and *G3* haplotypes represented restricted allele *G*. Each breed was characterized with distinct haplotypic pattern that was not replicated in other breed. The result indicates independent evolution of different haplotypes during period of time. The haplotypes *A1* and *A3* were most nearest in term of base composition with differences of only two SNP's. Four SNP's each were observed between haplotypes of *G1* and *G2*; *G1* and *G3*; and *A2* and *A3*. The average diversity observed among the different *G* haplotype was found to be greater than observed diversity among different *A* haplotypes. The haplotypic frequency within each breed corresponded to the frequency of restriction allele observed in RFLP analysis of related breed.

The present study is in correspondence with findings of Dong *et al*. (2013) who observed eight different SNP's in a similar region of *PRL* exon-4 with four SNP's in intron-3, two in exon-4 and two in intron-4 in Chinese Holstein cattle. The number of SNP's reported by Brym *et al*. (2005a) was less in numbers and six SNP's were observed in similar 294-bp fragment of exon-4 of *PRL* gene in Black and White cows and Jersey cattle. The present study also identified five of the six SNP's reported at 8307, 8314, 8377, 8398 and 8510 position by Brym *et al*. (2005a) and four of the eight SNP's reported at 8307, 8377, 8398 and 8510 position by Dong *et al*. (2013) in the studied Indian cattle breeds. Two of the SNP's, *i.e.,* 8377 and 8398, overlapped with mutation reported previously by Sasavage *et al*. (1982). Mutation observed in positions 8271, 8274, 8275, 8281, and 8306 were found as novel mutations. SNP's identified in the present study could be used for the construction of intragenic

haplotypes which could be used as more informative markers in near future for construction of bovine haplotype map as a new strategy for QTL mapping. The eight SNP's detected in the haplotype block by Dong *et al*. (2013) were found to be in complete linkage with each other so the detected SNP's in the present study could be tested for linkage with each other in the near future.

4.3.6 Phylogenetic analysis

The phylogenetic relationship analysis of different breeds of indigenous cattle and exotic cattle, along with buffalo, sheep and goat was carried out through MEGA7 software.

4.3.6.1 Evolutionary divergence among breeds

The mean evolutionary distance between different breeds or species was observed as 0.0117±0.0051 (**Table 4.19**). The distance between alleles of studied breeds ranged below the mean value and was observed as 0.0000 to 0.0116. The indigenous cattle diverged from exotic cattle in the range of 0.0000 to 0.0176 depending on the breed of exotic cattle. The divergence of different cattle breeds from buffalo was estimated 0.0058 to 0.0237. Goat diverged more from bovids animals (0.0116-0.0298) than sheep (0.0058-0.0237) for exon-4 region of *PRL* gene.

4.3.6.2 Construction of Phylogenetic Tree

Phylogenetic analysis was performed to evaluate the rate of evolution for prolactin gene in various livestock species. The phylogenetic tree was constructed on the basis of lowest BIC value based on 24 different nucleotide substitution models. The T92 model with gamma distribution was observed to reveal lowest BIC values**.** The NJ phylogenetic tree was constructed on the basis of evolutionary relationships among the submitted sequences and other ruminants (**Fig 4.13**). Different breeds or species were clustered through hierarchical method on the basis of their genetic characteristics. The ovine and caprine sequences shared the same clade which indicates higher level of relationship between these two species for exon-4 region of *PRL* gene. The *A* allele of all the three breeds shared the clade directly or indirectly with *Bos taurus* sequence. The allele *G* of Rathi shared the different branch than allele *G* of Sahiwal and Kankrej cattle.

Table 4.19 Evolutionary divergence among breed /species for *PRL* exon-4 region

Breed/Species	RAG	SAA	SAG	KAA	KAG	BTAF	BTAH	BTJF	BTAH	BBEU	OAKC	CHEU
Rathi_Allele_A_KY777610 (RAA)												
Rathi_Allele_G_MF045465 (RAG)	0.0058											
Sahiwal_Allele_A_KY777612 (SAA)	0.0000	0.0058										
Sahiwal_Allele_G_KY777611(SAG)	0.0058	0.0000	0.0058									
Kankarej_Allele_A_KY777614 (KAA)	0.0000	0.0058	0.0000	0.0058								
Kankarej_Allele_G_KY777613 (KAG)	0.0116	0.0058	0.0116	0.0058	0.0116							
Bos_taurusAF426315 (BTAF)	0.0058	0.0117	0.0058	0.0117	0.0058	0.0176						
Bos_taurus_AH013356 (BTAH)	0.0058	0.0117	0.0058	0.0117	0.0058	0.0176	0.0000					
Bos_taurus_JF826522 (BTJF)	0.0116	0.0058	0.0116	0.0058	0.0116	0.0000	0.0176	0.0176				
Bos_taurus_AH001155 (BTAH)	0.0116	0.0058	0.0116	0.0058	0.0116	0.0000	0.0176	0.0176	0.0000			
Bubalus_bubalis_EU340420 (BBEU)	0.0116	0.0058	0.0116	0.0058	0.0116	0.0116	0.0176	0.0176	0.0116	0.0116		
Ovies_aries_KC764410 (OAKC)	0.0176	0.0116	0.0176	0.0116	0.0176	0.0175	0.0237	0.0237	0.0175	0.0175	0.0058	
Capra_hircus_EU256165 (CHEU)	0.0236	0.0175	0.0236	0.0175	0.0236	0.0236	0.0298	0.0298	0.0236	0.0236	0.0116	0.0058

Mean genetic distance = 0.0117±0.0051

4.3.6.3 Conserved regions among breeds/species

Three conserved regions were identified between the studied cattle breeds and the other species in the fragment region of 93-126 bp, 164-179 bp and 181-258 bp (**Table 4.20**).

Table 4.20 Conserved regions among breeds/species for *PRL* exon-4 region

Attributes	Conserved regions	Position of conserved regions					
Between studied cattle breeds	6	1-35	47-70	80-141	143-162	164-274	276-294
Between studied cattle breeds and exotic cattle	6	1-35	47-70	80-126	143-162	164-274	276-294
Between studied cattle breeds and other species	3	93-26	164-179	181-258	--	--	---

4.4 Genetic Variation in *DGAT1* Gene

The detection of genetic variation in indigenous breeds of Rathi, Sahiwal and Kankrej cattle was carried out in 411-bp product (spanning through exon-7 to exon-9) of *DGAT1* gene containing a dinucleotide base pair substitution GC/AA in exon-8 earlier reported for variation in milk fat, protein and yield. The investigation of genetic variation in *DGAT1* was carried out through multidimensional approach to obtain reliable and valid information.

4.4.1 Amplification of Exon-8 of *DGAT1* Gene

The 411-bp product of specific length and position was amplified through primers based on *DGAT1* gene sequence available at NCBI database (GenBank Accession No. AJ 318490) (**Fig. 4.14)**. The PCR reactions were carried out as per procedure described out by Winter *et al*. (2002). Seventy pico mol concentration of each primer was identified as an optimal concentration for amplification of *DGAT1* gene. Five per cent DMSO was added in each reaction volume to get an equal amplification of the both strands. Forty cycles of amplification was carried out to obtain desired amplification. The PCR gradient method was adopted to cross check

the yield of products at different temperature before finalize the annealing temperature of 52ºC.

4.4.2 RFLP analysis of *DGAT1* gene

The RFLP analysis enables interpretation of genetic information on wider basis for the above mentioned QTL on BTA14 for GC/AA dinucleotide base pair substitution leading to non conservative lysine to alanine amino acid substitution (*K232A*). The RFLP analysis of *DGAT1* gene through *Eae* I restriction enzyme revealed the presence of single genotypic pattern '*KK*' in all the 225 studied animals of Rathi, Sahiwal and Kankrej breed and no animal of either '*KA*' or '*AA*' genotype was observed in any of the studied breed (**Fig. 4.15**). The *Eae* I restriction enzyme digests the '*A*' allele but not '*K*' allele required for alanine and lysine production, respectively. Only one undigested intact band of 411-bp, representative of '*KK*' genotype, was observed (**Table 4.21**). Thus, the allelic frequency of allele '*K*' was found to be fixed in Indian dairy cattle breeds of Rathi, Sahiwal and Kankrej with complete absence of *DGAT1* '*A*' allele.

Table 4.21 Gene and genotypic frequencies of exon-8 of *DGAT1* gene detected through RFLP analysis

Locus	Breed	N	Genotypic frequencies			Allele frequencies		Std. Error
			KK	KA	AA	K	A	
DGAT1	Rathi	75	1.00	0.00	0.00	1.00	0.00	0.00
	Sahiwal	75	1.00	0.00	0.00	1.00	0.00	0.00
	Kankrej	75	1.00	0.00	0.00	1.00	0.00	0.00
	Overall	225	1.00	0.00	0.00	1.00	0.00	0.00

The fix nature of allele '*K*' was initially identified by Kaupe *et al*. (2004) in Nellore cattle. An analogous finding about fixed nature of '*K*' allele was reported in 20 animals of six Indian dairy cattle breeds of Rathi, Sahiwal, Tharparkar, Deoni, Red Kandhari and Punganur by Tantia *et al*. (2006). The present study is in agreement with reports of Ganguly *et al.* (2013) who also observed nearly fix nature of allele '*K*' in 51 Sahiwal cattle. Most of the earlier studies which established the conserve nature of *DGAT1* gene in indigenous cattle were conducted on limited number of animals. In this context, the present study is supposed to be one of the first study conducted on large number of milking animals to establish the fix nature of *DGAT1*

gene in indigenous cattle breed of Rathi, Sahiwal and Kankrej. The fixation of allele '*K*' in Indian cattle breeds such as Rathi, Sahiwal and Kankrej cattle could be due to effect of genetic drift acting upon the population during evolution as well as by the founder effect. The absence of genetic diversity at GC/AA position of *DGAT1* gene most likely reflects the selective breeding being carried out in indigenous breeds for increase milk fat content (Kaupe *et al*., 2003). Similarly, very high frequency of allele '*K*' (0.7) of *DGAT1* gene was observed in New Zealand Holsteins, selected with special emphasis for fat content (Grisart *et al*., 2001). The markedly higher frequency of '*K*' allele was also detected in German and Polish Holstein (Thaller *et al*., 2003; Bennewitz *et al*., 2004). The lowest frequency of *DGAT1* '*K*' allele was observed in HF animals of US and Dutch origin which were selected primarily for milk yield (Grisart *et al*., 2001; Spelman *et al*., 2002). Likewise, in contrast to the present findings, Simmental breed was traditionally selected for '*A*' variant of *DGAT1* gene (Kaupe *et al*., 2004; Scotti *et al.,* 2010)

The results from this and other studies implies that the frequencies and genotypes of *DGAT1* gene in different breeds are diverse worldwide (Lacorte *et al*., 2006; Ripoli *et al*., 2006). Previous studies in many exotic cattle breeds throughout the world have reported differences in breed composition for '*K*' and '*A*' allele with wide interval frequency in *DGAT1* gene (Patel *et al*., 2009; Berry *et al*., 2010; Manga and Riha, 2011; Mashhadi *et al*., 2012; Dokso *et al*., 2015). The *DGAT1* allele '*A*' and genotype '*KA*' was reported to be most frequent in crossbred population (Molee *et al*., 2015).

The present study indicates the purity of the sampled animals maintained under farm conditions as observed by the preservance of fat enhancing '*K*' allele in native population of Rathi, Sahiwal and Kankrej cattle. Therefore, the estimation of frequency of '*A*' allele in indigenous cattle breeds could be utilized as an indicator test for level of cross breeding in the population (Souza *et al*., 2014). In addition, considering the fixed nature of *DGAT1* '*K*' allele in Indian breeds, the frequency of allele '*K*' could be modulated in future selection programmes to optimize the milk yield potential of Indian dairy cows. An increase in frequency of *DGAT1* '*K*' allele from 0.21 to 0.58 was reported in Turkish cattle selected over a period of 10 years for increase in fat yield through introgression of Zebu cattle blood in native Turkish cattle population (Unal *et al*., 2015). Moreover, it has been shown that '*K*' allele is related to an increase in saturated and decrease of unsaturated fatty acids in milk which may impose a negative effect on human health (Schennink *et al.,* 2007). Thus fatty acid

profile of milk could be altered through selective breeding in near future to realize the concept of designer milk (Demeter *et al*., 2009).

4.4.3 SSCP marker analysis of *DGAT1* gene

The observed monomorphic nature of *DGAT1* gene established through RFLP analysis for *K232A* polymorphism was tested for polymorphism at any other nucleotide position in 411-bp product of *DGAT1* gene apart from GC/AA site through SSCP marker. The technique is considered as golden approach for detection of nucleotide variation in DNA sequences based on the conformational changes in sequences of DNA. The SSCP analysis of *DGAT1* gene spanning from exon-7 through exon-9 revealed five different conformational banding patterns in studied animals of Rathi, Sahiwal and Kankrej breed (**Fig. 4.16**).

The first banding pattern (P1) was characterized by the presence of two bands and was designated as '*K1K1*'. It was observed in 40 animals of Rathi and 32 animals of Sahiwal cattle. The second banding pattern (P2) was referred as '*K1K2*' type and was identified by the presence of four bands on 8% acrylamide gel. This pattern was identified in 35 animals of Rathi breed only. The electrophoretic distance covered by the four bands of '*K1K3*' banding pattern (P3) was partially different from earlier two detected banding patterns and was observed in 43 animals of Sahiwal cattle. The fourth banding pattern (P4) termed as '*K2K2*' was characterized by the presence of only two bands and was detected in 38 animals of Kankrej whereas the last genotypic pattern (P5) referred as '*K2K4*' revealed four bands in all the remaining 37 animals of Kankrej breed. The genotypic patterns '*K1K1*' and '*K2K2*' were considered as homozygotes whereas the banding patterns '*K1K2*', '*K1K3*' and '*K2K4*' were identified as heterozygotes.

4.4.3.1 Gene and genotypic frequency

The breed wise distribution of genotypic pattern of lysine variants has been represented in **Table 4.22**. The lysine allele though observed to be fixed, the frequency and type of different variants of lysine allele varied between breeds. The animals of Rathi were identified as either '*K1K1*' or '*K1K2*' whereas Sahiwal cattle were categorized into two lysine variants, namely '*K1K1*' and '*K1K3*'. Similarly all the studied animals of Kankrej cattle were classified as '*K2K2*' and "*K2K4*'.

Table 4.22 Genotypic frequencies of SSCP patterns detected for lysine variants of *DGAT1* gene

Breed	Genotypic frequency of *DGAT1 K* variants					Gene frequency of *DGAT1 K* variants			
	K1K1	***K1K2***	***K1K3***	***K2K2***	***K2K4***	***K1***	***K2***	***K3***	***K4***
Rathi (n=75)	0.53 (40)	0.47 (35)	---	---	---	0.77	0.23	---	---
Sahiwal (n=75)	0.43 (32)	---	0.57 (43)	---	---	0.71	---	0.29	---
Kankrej (n=75)	---	---	---	0.51 (38)	0.49 (37)	---	0.75	---	0.25
Over all (n=225)	0.32 (72)	0.16 (35)	0.19 (43)	0.17 (38)	0.16 (37)	0.49	0.33	0.10	0.08

The overall frequency of '*K1K1*' genotype was estimated to be highest (0.32). The '*K1*' and '*K2*' lysine variants were observed in dominating gene frequency of 0.49 and 0.33, respectively in the studied population. The lysine variant '*K3*' and '*K4*' were novel and present only in animals of Sahiwal and Kankrej breeds, respectively. The results indicates that fix nature of '*K*' allele at GC/AA locus was accompanied by mutation at other locations which ultimately resulted in different conformation of single stranded DNA on acrylamide gel. The nearly similar gene frequency of '*K1*' lysine variant in Rathi (0.76) and Sahiwal cattle (0.71) indicates the evolutionary relationship between these two breeds.

The present study is supported by the findings of many workers that have shown that QTL for fat content on *BTA14* is determinated by more polymorphic elements than just the *K232A* polymorphism (Kuhn *et al*., 2004; Gautier *et al*., 2007). The evidence for other polymorphic site was reported by Kaupe *et al*. (2007) who analysed the haplotypes for SNP's originating in *DGAT1* locus and other linked genes. Further, Kuehn *et al*. (2007) found different effects of maternal and paternal inherited haplotypes composed of *K232A* alleles of the *DGAT1* gene and inferred about the existence of non additive interactions in *DGAT1* polymorphism. A study on haplotype analysis of *DGAT1* gene in different breeds of cattle and buffalo by Winter *et al*. (2002) also indicated that ancestral lysine variant (*AA* allele) existed in two or more allelic variant with different breeding value for milk fat.

4.5.3.2 Hardy-Weinberg equilibrium of population studied for *DGAT1* loci

The population under each breed was tested for Hardy Weinberg equilibrium through chi square and G square statistic to establish the role of selection, mutation, migration and mating behavior on the observed genetic structure of population for studied loci (**Table 4.23**).

Table 4.23 Hardy-Weinberg equilibrium for lysine variants of *DGAT1* gene

***DGAT1* region**						
Breed	**Chi^2**	**p value**	**Significance**	**G^2**	**p value**	**Significance**
Rathi	6.71	0.009	**	10.51	0.001	**
Sahiwal	11.78	0.000	**	13.37	0.000	**
Kankrej	7.78	0.005	**	12.01	0.000	**
Overall	145.44	0.000	**	165.02	0.000	**

Both chi square and G^2 likelihood test ratio revealed highly significant departure of gene and genotypic frequency of variants of *DGAT1 K* gene from expected Hardy-Weinberg equilibrium (HWE) ($p<0.001$). The highly significant deviation from HWE is suggestive of long term and differential selection pressure for fat bearing '*K*' allele variants over the period of evolution. The demand of Indian consumers for fat rich milk could be a possible reason for fixation of '*K*' allele in the population. The Rathi and Sahiwal cattle shares nearly 70% variant of K allele which reflects the ancestral relationship between the two breeds. The distribution of different variants of *DGAT1* lysine variant was not independent among breeds. The results also supports the popular view that *K* allele was a wild allele and allele A substitution probably occurred after the divergence of *Bos taurus* and *Bos indicus* (Kaupe *et al*., 2004).

4.4.4 Genetic Diversity in *DGAT1* gene

The quantification of genetic variation within and between population was carried out through different approaches to obtain reliable and valid information.

4.4.4.1 Observed and expected heterozygosity (H_E)

The observed genetic variation within population (H_o) was found to be higher than expected heterozygosity (H_E) in all the three breeds which indicates presence of sufficient genetic diversity in Rathi, Sahiwal and Kankrej for *DGAT1 K* variants. Nei's

unbiased heterozygosity values were also observed to be lower than observed heterozygosity in studied breeds. The higher heterozygosity value indicates the long term natural selection for adaptation and the polymorphic nature of this amplified region (**Table 4.24**).

Table 4.24 Within-population heterozygosity estimates, PIC and F_{IS} values of Rathi, Sahiwal and Kankrej cattle for lysine variant of *DGAT1* gene

Breed	Sample size (N)*	Observed Heterozygosity	Expected Heterozygosity	Nei's Unbiased Heterozygosity	PIC	Fixation index
		H_o	H_E	H_e		F_{IS}
Rathi	75	0.467	0.360	0.358	0.2915	-0.304
Sahiwal	75	0.573	0.411	0.409	0.3270	-0.402
Kankrej	75	0.493	0.374	0.372	0.3047	-0.327
Overall	225	0.511	0.634	0.633	0.5707	0.192

4.4.4.2 Polymorphism Information Content (PIC)

The PIC value of lysine variants of *DGAT1* gene was observed as 0.2915, 0.3270 and 0.3047 for Rathi, Sahiwal and Kankrej breed, respectively. Medium level of polymorphism was observed for the different variants of *DGAT1* gene. On overall basis, irrespective of breed, the PIC value of 0.5707 indicated highly polymorphic status of *DGAT1* gene for lysine variants (**Table 4.24**)

4.4.4.3 Fixation Index (F_{IS})

Genetic divergence among populations of the same or different breeds is usually quantified by fixation indices or *F* statistics (Wright, 1955). Wright's fixation index (F_{IS}), an indirect measure of inbreeding coefficient, is suggestive of level of genetic variation in the population. The different variants of *DGAT1 K* allele were observed in heterozygote excess in studied breeds that ultimately lead to low and negative F_{IS} values (**Table 4.24**). The F_{IS} values in different studied breeds ranged from -0.304 to -0.402. The present study is in agreement with the findings of Rincon *et al*. (2006) who also observed negative estimates of F_{IS} for *DGAT1* loci in Uruguayan Creole cattle population.

4.4.4.4 Effective number of alleles

The effective number of alleles (n_e) for lysine variants of *DGAT1* gene ranges from 1.557 (Rathi) to 1.692 (Sahiwal) (**Table 4.25**). The n_e values indicate the minimum number of alleles required to achieve given level of gene diversity. The lowest n_e values observed for Rathi cattle indicates high level of gene diversity in Rathi cattle than Sahiwal cattle which needs more number of effective alleles to maintain genetic diversity. The gene diversity in Kankrej cattle is also nearly equivalent to Rathi cattle.

Table 4.25 Observed and effective number of alleles; and Shannon information index for lysine variants of *DGAT1* gene

Breed	Sample size of alleles	Observed number of alleles	Effective numbers of alleles	Shannon's Information Index
	N	n_a	n_e	I
Rathi	150	2.00	1.557	0.543
Sahiwal	150	2.00	1.692	0.599
Kankrej	150	2.00	1.591	0.559
Overall	450	4.00	2.721	1.144

4.4.4.5 Shannon's Information Index (I)

The Shannon index for Rathi cattle was observed lowest among the three breeds with value of 0.543 (**Table 4.25**). Slightly higher index value was recorded for Kankrej cattle with highest value was found in Sahiwal cattle. The greater than intermediate value of Shannon index in all the three breeds suggests the suitability of primer pair for the exploration of genetic variation in indigenous cattle.

4.4.4.6 Genetic distance among studied breeds

The pair wise genetic distance matrix between indigenous breeds revealed low genetic distance values between Rathi and Sahiwal (0.119) than Rathi and Kankrej (1.284) (**Table 4.26**).

Table 4.26 Pair wise genetic distance matrix between studied cattle breeds for lysine variants of *DGAT1* gene

***DGAT1* region**			
Breed	**Rathi**	**Sahiwal**	**Kankrej**
Rathi	****	0.887	0.276
Sahiwal	0.119	****	0.000
Kankrej	1.284	0.000	****

The high value of pair wise genetic identity (0.8877) between Rathi and Sahiwal for *DGAT1* gene indicates high level of genetic similarity and is suggestive of genetic admixture of the two populations. The genetic relationship between Sahiwal and Kankrej could not be established which might be due to differences in type of lysine variant present in the two breeds.

4.4.5 SNP's analysis of lysine variants of *DGAT1* gene

4.4.5.1 Generation of gene sequences

The samples with different PCR-SSCP patterns were selected and sequenced to identify the underlying SNP's responsible for differences in migration behavior of single stranded DNA. The assembled sequences were submitted to GenBank database of NCBI, to which accession numbers were assigned (**Table 4.27**).

Table 4.27 List of accession numbers obtained for different SSCP pattern of lysine variants of *DGAT 1* gene

***b DGAT1* Region**				
Breed	**Restricted Allele**	**SSCP Pattern**	**Product Size**	**Accession Number**
Rathi	K	K1K1	411-bp	MF045468
	K	K1K2	411-bp	MF045469
Sahiwal	K	K1K1	411-bp	MF045470
	K	K1K3	411-bp	MF069167
Kankrej	K	K2K2	411-bp	MF069168
	K	K2K4	411-bp	MF069169

4.4.5.2 SNP's detection

The multiple sequence alignment (MSA) of Rathi Sahiwal and Kankrej *DGAT1* gene was carried out and the assembled sequences were compared with the reference sequence (GenBank Accession No. AJ 318490) of Winter *et al*. (2002) for

the identification of SNP's in studied breeds (**Fig 4.17**). The present study detected 8 different SNP's in 411-bp product of *DGAT1* gene in animals of Rathi, Sahiwal and Kankrej breeds in addition to two SNP's responsible for alanine to lysine amino acid substitution in all animals. Three of the eight SNP's were observed in exon-7, four in intron-7 and one in exon-8 of *DGAT1* gene (**Table 4.28**). The SNP's (A-A) observed at 200-201 position in sequenced region of 411-bp in all the animals of three breeds was found to responsible for K232A polymorphism.

Table 4.28 List of position and nature of SNP's observed in *DGAT1* gene

Exon-8 of *DGAT1* gene			
Base position	**Region**	**Change**	**Nature of SNP's**
62	Exon-7	G>C	Transversion
84	Exon-7	A>T	Transversion
85	Exon-7	C>G	Transversion
86	Intron-7	A>G	Transition
132	Intron-7	C>G	Transversion
157	Intron-7	C>G	Transversion
185	Intron-7	C>G	Transversion
188	Exon-8	C>T	Transition
200	Exon-8	A>G	Transition
201	Exon-8	A>C	Transversion

4.4.5.3 Construction of haplotype/haplotype analysis

Four different haplotypes were identified on the basis of detected SNP's in the studied breeds (**Fig. 4.18**). The first haplotype was observed in Rathi and Sahiwal breed and was designated as '*K1*' whereas the second haplotype (*K2*) was detected in animals of Rathi and Kankrej breed. Similarly third (*K3*) and fourth haplotype (*K4*) were detected in Sahiwal and Kankrej, respectively. Seven transversion at base positions 62, 84, 85, 132, 157,185 and 201; and three transitions at base positions 86, 188, and 200 were observed in 411-bp length of *DGAT1* gene.

The non synonymous mutational replacement of guanine with cytosine residue at 62nd base position in Kankrej cattle (*K4*) was predicted to produce arginine (R) instead of glycine (G) amino acid. Similarly another non synonymous mutation detected at 84th base position in exon 7 of Sahiwal cattle (*K3*) was predicted to

produce amino acid glutamine (Q) instead of leucine (L) of other haplotypes. The SNP's at position 200-201 were responsible for production of lysine variant of *DGAT1* gene. All other mutations in the sequence were observed as either synonymous in nature or located in noncoding intron regions. The two most abundant haplotype '*K1*' and '*K2*' were found to be similar in nature in terms of predicted amino acid sequence which suggests the conserved nature of gene function in these two haplotypes.

The presence of allelomorphs for lysine allele of *DGAT1* gene was reported by many workers (Komisarek and Kolenda, 2016). The study is in agreement with the findings of Winter *et al*. (2002) who reported at least five different haplotypes of lysine allele of which three were identified in Indian cattle breeds of Hariana, Sahiwal and Tharparkar through direct sequencing and sequence analysis of *DGAT1* gene from pooled DNA samples. The study revealed significant frequency shifts at six variable positions between groups of animals with high and low breeding values for milk fat content in different breeds. SNP's present at location other than GC/AA position in the same or different exon or intron region could influence the expression of *DGAT1* gene. A novel single nucleotide mutation observed in exon splicing enhancer of *DGAT1* gene was found to induce exon 16 skipping and resulted in enzymatically inactive *DGAT1* protein (Lehnert *et al*., 2015). The SNP's present at other location apart from '*K232A*' position may impart variance in milk fat and proteins among breeds.

In addition, the traditionally observed differences in milk fat and protein contents in Rathi, Sahiwal and Kankrej cattle by farming community also suggests the presence of another variations in the genomic region of *DGAT1* gene among different cattle populations (Spelman *et al*., 2002; Thaller *et al*., 2003).

4.4.6 Phylogenetic Analysis

The population from different breeds or species was clustered through hierarchical method on the basis of their genetic characteristics for phylogenetic studies. Dendograms have been created on the basis of genetic distances between breeds. In a dendrogram, the horizontal axis lists the observations in a particular order. The vertical axis shows the successive steps or cluster numbers. Therefore, the phylogenetic trees summarize evolutionary relationships among breeds or populations and categorize cattle populations into distinct genetic groups. The trees consist of nodes and branches. The nodes are the breeds and the branch lengths between breeds are graphical estimates of the genetic distance between the breeds and give an indication of genetic relationships between breeds. Bootstrapping is

usually done to provide confidence statements about the groupings of the breeds as revealed by the dendrogram and hence test the validity of the clusters obtained. The bootstrap values are given in percentages and the higher the value, the higher is the confidence in the grouping.

4.4.6.1 Evolutionary divergence among breeds/species

The evolutionary divergence between different breeds/species was calculated through MEGA 7 algorithm program of Kumar *et al*. (2016) **(Table 4.29).** The gene sequences related to *DGAT1* region of different species or genus were retrieved in FASTA format from NCBI database and were aligned with the generated sequences of indigenous breeds to reveal the divergence of *DGAT1* gene during evolution.

The mean evolutionary distance estimated between pair of breed/ species was observed as 0.0177±0.0035. The evolutionary distance between any two lysine variant in indigenous cattle observed below mean distance and ranged between 0.000 to 0.01746. Similarly the evolutionary distance between indigenous and *Bos taurus* ranged below mean value and was found to be 0.00244-0.1746. The distance between different lysine variant of studied breeds and buffalo was slightly higher than mean value with range of 0.00738-0.02260. The *Ovies aries* and *Capra hircus* diverged significantly from indigenous cattle breeds and showed values of 0.1746-0.03045 and 0.00244-0.04402, respectively. *Camelus dromedarius* diverged most from indigenous cattle and other species and demonstrated the evolutionary distance of 0.00738 to 0.04950.

4.4.6.2 Construction of Phylogenetic Tree

The phylogenetic tree for the different identified alleles of *DGAT1* gene and other species or genus of ruminants groups such as *Bos taurus, Bubalus bubalis, Ovies aries*, *Capra hircus* and *Camelus dromedarius* was constructed through NJ method. The phylogenetic tree was constructed with the support of MEGA7 software. Out of 24 different maximum likelihood substitution models based on various parameters, the T92 model with gamma distribution was selected on the basis of lowest BIC value of 1821.222 to construct phylogenetic tree (**Fig 4.19**). Bootstrap consensus tree was constructed to obtain confidence statements and for validity of clusters. Different lysine variant of indigenous cattle shared different branches with other species or breed. Sheep and goat were observed more near than camel from cattle. The two lysine variant in Kankrej shared the common cluster whereas the buffalo shared the cluster with *Capra hircus*.

Table 4.29 Evolutionary divergence among breed/species for *DGAT1* gene

Breed/Species	RBMF	SAMF	SBMF	KAMF	KBMF	BTEU	BTHMF	BBKX	BBMF	OAEU	CHLT	CHMMF	CDAMF	CDBMF
Rathi Allele A_MF045468 (RAMF)														
Rathi Allele B_MF045469 (RBMF)	0.00490													
Sahiwal Allele A_MF045470 (SAMF)	0.01490	0.01491												
Sahiwal Allele B_MF069167 (SBMF)	0.00490	0.00490	0.01490											
Kankrej Allele A_MF069168 (KAMF)	0.00737	0.00244	0.01746	0.00737										
Kankrej Allele B_MF069169 (KBMF)	0.00490	0.00000	0.01491	0.00490	0.00244									
Bos taurus EU077528 (BTEU)	0.00245	0.00244	0.01238	0.00245	0.00490	0.00244								
Bos taurus Holstein_MF069174 (BTHMF)	0.01238	0.01240	0.01746	0.01238	0.01494	0.01240	0.00988							
Bubalus bubalis KX965992 (BBKX)	0.01238	0.01238	0.02260	0.01238	0.01491	0.01238	0.00987	0.02003						
Bubalus bubalis MF069172 (BBMF)	0.00738	0.01238	0.01238	0.00738	0.01491	0.01238	0.00987	0.01491	0.02001					
Ovis aries EU178818 (OAEU)	0.02261	0.02259	0.03310	0.02261	0.02519	0.02259	0.02001	0.03045	0.01746	0.03044				
Capra hircus LT221856 (CHLT)	0.03331	0.03322	0.04402	0.03331	0.03587	0.03322	0.03059	0.03849	0.02798	0.04130	0.01496			
Capra hircus Marwari_MF069173 (CHMMF)	0.00988	0.01491	0.01490	0.00988	0.01746	0.01491	0.01238	0.01746	0.02260	0.00244	0.03310	0.04401		
Camelus dromedarius Allele A_MF069170 (CDAMF)	0.02001	0.02003	0.01494	0.02001	0.02263	0.02003	0.01746	0.02263	0.02783	0.01238	0.03847	0.04950	0.01490	
Camelus dromedarius Allele B_MF069171 (CDBMF)	0.01745	0.01746	0.01240	0.01745	0.02003	0.01746	0.01491	0.02003	0.02520	0.00987	0.03577	0.04674	0.01238	0.00738

Mean genetic distance = 0.0177±0.0035

4.4.6.3 Conserved Regions among Breeds/Species

The conserved regions in 411-bp fragment of *DGAT1* gene was identified between different species and breed to detect the regions that were least affected by evolutionary pressure under selection (**Table 4.30**). Six different conserved regions were identified among different identified alleles of *DGAT1* gene in studied breeds whereas five conserved zones were detected between indigenous and *Bos taurus* cattle. On the basis of minimum conserved length of 15-bp, seven conserved regions were detected between studied cattle breeds and other species.

Table 4.30 Conserved regions among breed/species for *DGAT1* gene

Attributes	No. of conserved regions	Position of conserved regions						
Between studied breeds	6	1-61	63-83	87-131	133-156	158-184	189-411	--
Between studied cattle breeds and exotic cattle	5	1-61	87-131	133-156	165-184	202-411	--	--
Between studied cattle breeds and other species	7	1-23	25-61	99-119	165-184	189-274	295-355	357-411

4.5 Analysis of Lactation Performance in selected animals of Rathi, Sahiwal and Kankrej Cattle Breeds for Milk Traits

The mean lactation performance of the selected milking animals for first, second and overall average for all parity for different lactation traits was analyzed and the breed wise results are presented in **Table 4.31**. Similarly, the least square mean and standard error for milk fat, protein, lactose, SNF and total solid were estimated for the three breeds and the results are presented in **Table 4.32**. A significant difference (p□0.05) in TMY in 1st parity was observed. Rathi cattle displayed maximum milk yield ($p<0.05$) than Sahiwal cattle in 1st parity whereas Kankrej breed exhibited nonsignificant differences with either of the two breed for TMY. The differences among the breeds became disappeared in the next parity. The mean TMY averaged over all the parity revealed significantly higher TMY in Kankrej cattle than Sahiwal cattle.

The 305-day MY was observed nonsignificant among the breeds for the 1st parity and over average of all parity however significant differences were observed during 2nd parity with significantly higher mean was observed for Rathi cattle. Similar

pattern to that of 305-day MY was observed among the breeds for ADMY in which Rathi cattle produced significantly higher ADMY than Sahiwal cattle. In terms of lactation length, studied animals of different breeds were varied nonsignificantly with each other and numerically highest mean lactation length was observed for the animals of Rathi cattle. The compositional analysis of milk revealed that the protein and SNF content of Sahiwal and Kankrej was significantly higher than Rathi breed, whereas the total solid content was observed to be significantly higher in Rathi breed than Sahiwal and Kankrej breed. The fat content of Rathi cattle was observed numerically highest than the other two breeds. Similarly, the lactose concentration in milk of three breeds revealed nonsignificant differences among each other.

These obvious differences in the three breeds for milk performance and compositional traits could be ascribed to some underlying genetic factors as the breeds were managed under the similar feeding and housing conditions. The nationwide higher number of graded animals in Sahiwal population (77.62%) than Rathi (30%) and Kankrej (35.76%) cattle could be a contributing factor for low performance of Sahiwal animals (Breed Survey, 2013). The relatively higher natural adaptation of Rathi cattle for the geographical condition of the region (breeding tract) might affect the performance of Rathi cattle in a positive manner.

In addition, the recent introduction of Sahiwal cattle in genetic improvement programme in LRS, Kodamdesar than genetically improved Rathi cattle might have affected the different performance parameters. The genetic potential of Sahiwal cattle for milk production could not be neglected as revealed by statistically similar 305-day MY, ADMY and lactation length for the studied breeds over average of all parity. Thus the present study was designed to unmask the relevant genetic factors responsible for variation in milk performance traits of Rathi, Sahiwal and Kankrej cattle.

4.6 Association Analysis of Selected Genomic Region with Lactation Performance and Milk Composition Traits

4.6.1 Association analysis of exon-3 of *bPRL* gene with lactation performance and milk composition traits

The associations of different genotypic patterns of exon-3 of *PRL* gene with lactation performance and milk composition traits was estimated and presented in **Table 4.33** and **4.34**.

Table 4.31 Lactation performance (LSM±SE) of studied animals in different parity

Breed	Total milk yield (kg)	Milk yield-305 day (kg)^{NS}	Daily milk yield (kg) ^{NS}	Lactation length (days) ^{NS}
1st Parity				
Rathi (75)	1574.00^{b} ±80.20	1788.79 ±52.53	5.87 ±0.17	261 ±8.56
Sahiwal (75)	1339.68^{a} ±58.27	1699.70 ±49.44	5.57 ±0.16	239 ±6.46
Kankrej (75)	1425.38^{ab} ±66.30	1754.46 ±43.34	5.75 ±0.14	243 ±8.27
P-value	0.050	0.427	0.427	0.110
2nd Parity				
Rathi (75)	1670.49 ±62.02	2017.32^{b} ±44.33	6.61^{b} ±0.15	253 ±7.40
Sahiwal (75)	1478.65 ±67.33	1857.34^{a} ±48.62	6.10^{a} ±0.16	239 ±6.60
Kankrej (75)	1514.01 ±54.48	1930.58^{ab} ±40.94	6.33^{ab} ±0.13	237 ±6.30
P-value	0.066	0.042	0.042	0.203
Over all Parity				
Rathi (225)	1600.48^{ab} ±71.47	1869.28 ±53.27	6.13 ±0.17	255 ±6.78
Sahiwal (225)	1441.80^{a} ±52.68	1824.89 ±47.44	5.98 ±0.16	240 ±4.95
Kankrej (225)	1623.32^{b} ±47.10	1942.99 ±36.37	6.37 ±0.12	253 ±4.89
P-value	0.050	0.191	0.192	0.121

Means in the same column bearing different superscripts are significantly different ($p<0.05$).
NS Non-significant

Table 4.32 Milk composition (LSM±SE) of studied animals of Rathi, Sahiwal and Kankrej

Breed	Fat ^{NS} (%)	Protein ^{NS} (%)	Lactose ^{NS} (%)	SNF ^{NS} (%)	Total solids (%)
Rathi (75)	4.53 ±0.09	2.88^{a} ±0.03	4.68 ±0.08	8.48^{a} ±0.04	16.79^{b} ±0.21
Sahiwal (75)	4.38 ±0.05	3.18^{b} ±0.04	4.77 ±0.05	8.78^{b} ±0.04	16.17^{a} ±0.14
Kankrej (75)	4.45 ±0.07	3.17^{b} ±0.03	4.79 ±0.03	8.74^{b} ±0.03	16.73^{a} ±0.12
P-value	0.343	0.001	0.383	0.001	0.013

Means in the same column bearing different superscripts are significantly different ($p<0.05$).
NS Non-significant

4.6.1.1 Association analysis of *PRL* exon-3 region with milk performance traits in Rathi, Sahiwal and Kankrej breeds

Significant effect of *PRL* exon-3 genotypic patterns was observed on milk yield in all the studied breeds for 1st, 2nd and over all the parities except in Rathi cattle in which detected genotypic patterns did not produce any variation in TMY averaged over all parity. The genotypic pattern *AG* significantly yielded more milk in each lactation than *GG* genotype in animals of Rathi, Sahiwal and Kankrej cattle. The results clearly indicate that presence of allele *A* in heterozygote for *PRL* exon-3 region affects the milk yield in a positive way. Failure to detect genotypic pattern *AA* restricted the present study to compare the effect of the two opposite homozygotes. The observed genotypic patterns on cumulative basis also reflected significant differences between the two genotypes for 1st, 2nd and average yield over parity.

The 305-day MY for the two observed genotypic patterns was found significant (p□0.05) for Rathi cattle in the first lactation with mean milk yield of 1855.07 kg for animals having *AG* genotypic pattern of *PRL*-3 gene. The genotypic pattern *GG* yielded about 280 kg less milk than *AG* genotype. The standardized milk yield for 305 days was observed nonsignificant in Rathi cattle for 2nd lactation and over mean of all lactation. Sahiwal and Kankrej cattle also demonstrated nonsignificant differences in mean value of 305-day milk yield for the studied parity however numerically higher values were observed for *AG* genotype. The observed difference in Rathi than other breeds for 305 day performance could be due to long term improvement carried out in herd of Rathi cattle and the presence of novel *A*1 haplotype in Rathi population. In terms of overall effect on 305-day milk yield irrespective of the breeds, animals with heterozygote *AG* genotype were found to be superior in milk production than homozygote *GG* animals in the first parity.

The ADMY also reflected the similar pattern to that 305-day MY. The ADMY was found to be significantly higher for heterozygote pattern *AG* in first lactation of Rathi cattle. The other two breeds could not demonstrate any difference in ADMY for the two detected genotype during any of the lactation. The variability in results among the breeds indicates the greater role of SNP's detected at variable position in alleles of *G* and *A* restricted patterns and thus the role of haplotype on milk production could not be neglected.

The length of lactation in addition to milk yield bears an important effect on the economy of dairy cattle farming. Higher number of lactation days enhances the feasibility of dairy animals rearing thus the present study estimated the effect of

genotype on the lactation length. On overall basis irrespective of breeds, the heterozygote *AG* produced higher number of lactation days than *GG* genotype in all the parity and revealed lactation length of 257, 251 and 254 days for 1st, 2nd and over all parity. Significant and varied effect on deviation in lactation length was observed between breeds.

Rathi exhibited significantly higher LL in first lactation for *AG* genotypic pattern whereas Sahiwal reflected higher LL for second lactation and over average of all parity for heterozygote pattern *AG*. In contrast to Rathi and Sahiwal, Kankrej demonstrated significantly higher LL for *AG* genotype for all the lactation period studied. The differences in breed performances could be due to the presence of specific haplotype present in the particular breed and their specific combination. The significant difference between genotypes for lactation length in Kankrej breed could be due to the presence of specific haplotype *A*3 and its combination with *G*1 haplotype. The *A*2 haplotype in combination with *G*2 haplotype significantly affected the mean LL in subsequent lactation except first lactation in Sahiwal cattle. The *A*1 haplotype was able to express its effect only in first lactation. The variable results for studied breeds over various parity suggest the differences in fitness of haplotype and possible genotype-environment interactions. Out of the four SNP's detected in Holstein dairy cattle by He *et al*. (2006), one SNP was found significantly associated with milk yield.

The association of prolactin alleles with milk yield was initially suggested by Lewin *et al*. (1992). The significant role of *PRL* exon-3 *A* allele on milk production of Kankrej cattle from second to fourth lactation was observed by Sacravarty *et al*. (2008). The *AB* genotype of *PRL* exon-3 locus in Black Pied cattle was found to produce 356.37kg and 761.17kg more milk than *GG* and *AA* genotyped animals whereas the *AA* genotype in Red Pied cattle was observed to be superior in milk production than *AB* genotype (Alipanah *et al*., 2007a). The results are in concordance with findings of Dybus *et al*. (2005) who observed favorable effect of *AG* genotype on the first lactation however genotype *GG* was found to be suitable for second and third lactations in Jersey cattle, and both genotype *GG* and *AG* were observed favorable for Black and White. In contrast to present study, Bukhari *et al*. (2013) observed non-significant effect of genotype on mean lactation length and lactation yield in Frieswal cattle though the *AG* genotype were observed superior in performance for lactation yield. Similar non-significant association of lactation length was reported by Aravindakshan *et al*. (2004) in Vechur and Kassargode cattle; Alipanah *et al*. (2008) in Russian Black Pied and Russian Red Pied cows; and

Ghasemi *et al*. (2009) in Montebeliard cows. Chrenek *et al*. (1999) found nonsignificant differences among cows of Brown Swiss with diverse *PRL* genotypes.

Table 4.33 Effect of genotypic patterns of exon-3 of *PRL* gene on lactation performance in different parity

Breed	Lactation Performance					
	1st Lactation		2nd Lactation		All Lactation	
	GG	GA	GG	GA	GG	GA
Total milk yield (kg)						
Rathi (75)	1265.88[a] ±146.56	1693.77[b] ±91.39	1461.30[a] ±114.43	1751.84[b] ±71.36	1550.81 ±135.82	1619.80 ±84.69
Sahiwal (75)	1085.20[a] ±131.70	1398.09[b] ±63.10	1184.39[a] ±152.19	1546.19[b] ±72.92	1225.85[a] ±119.52	1491.37[b] ±57.26
Kankrej(75)	1231.10[a] ±107.56	1534.66[b] ±80.67	1356.77[a] ±88.48	1602.00[b] ±66.36	1475.77[a] ±76.04	1706.32[b] ±57.03
Over all (225)	1209.93[a] ±74.27	1536.28[b] ±45.80	1353.25[a] ±66.47	1630.89[b] ±40.99	1444.75[a] ±63.92	1597.22[b] ±39.42
Milk yield-305 day (kg)						
Rathi (75)	1572.69[a] ±96.50	1855.07[b] ±60.18	1931.48 ±83.52	2050.69 ±52.08	1847.67 ±101.31	1877.68 ±63.18
Sahiwal (75)	1542.33 ±113.40	1735.82 ±54.32	1770.55 ±112.73	1877.26 ±54.00	1722.18 ±109.74	1848.47 ±52.57
Kankrej(75)	1689.17 ±72.11	1791.18 ±54.08	1868.11 ±68.09	1965.73 ±51.07	1868.04 ±60.05	1985.16 ±45.03
Over all (225)	1632.03[a] ±52.75	1791.63[b] ±32.53	1867.54 ±49.52	1967.77 ±30.54	1828.21 ±50.95	1898.40 ±31.42
Daily milk yield (kg)						
Rathi (75)	5.16[a] ±0.32	6.08[b] ±0.2	6.33 ±0.27	6.72 ±0.17	6.06 ±0.33	6.16 ±0.21
Sahiwal (75)	5.06 ±0.37	5.69 ±0.18	5.81 ±0.37	6.12 ±0.18	5.65 ±0.36	6.06 ±0.17
Kankrej(75)	5.54 ±0.24	5.87 ±0.18	6.13 ±0.22	6.45 ±0.17	6.12 ±0.20	6.51 ±0.15
Over all (225)	5.35[a] ±0.17	5.87[b] ±0.1	6.12 ±0.16	6.43 ±0.10	5.99 ±0.17	6.22 ±0.10
Lactation length (days)						
Rathi (75)	230[a] ±15.74	273[b] ±9.81	231 ±13.75	261 ±8.58	251 ±13.00	257 ±8.00
Sahiwal (75)	218 ±14.81	244 ±7.09	208[a] ±14.88	245[b] ±7.13	219[a] ±11.2	245[b] ±5.37
Kankrej(75)	221[a] ±13.50	256[b] ±10.1	219[a] ±10.22	247[b] ±7.66	238[a] ±7.9	262[b] ±5.9
Over all (225)	223[a] ±8.45	257[b] ±5.22	220[a] ±7.29	251[b] ±4.50	238[a] ±6.15	254[b] ±3.79

Means in the same row depicts effect of genotypic pattern in different parity ($p<0.05$).

An positive association between 305-day TMY and genotypic pattern GG was reported in American Swiss cattle (Alfonso *et al*., 2012). Alipanah *et al*. (2008) analyzed the different factors affecting milk production and observed that *PRL Rsa* I

polymorphism affects 13.57%, 37.03% and 4.77% of total variation in milk yield, milk fat and protein, respectively.

The *A*/*G* polymorphism as reported by Brym *et al*. (2005) was found to associate with decrease in somatic cell count (SCC). Wojdak-Maksymiec *et al*. (2008) found a statistically significant association between SCC and *PRL* genotype (p=0.01). The highest SCC cell count was recorded in milk of *AA* cows while the lowest SCC was found in *GG* cows. Thus the maintenance of exemplary resistance of our indigenous cattle to subclinical and clinical mastitis without having depression effect on milk fat indicates the rationale behind the favor of heterozygotes individuals in the population. The observed association might be the result of linkage disequilibrium between identified mutation and other genes on the same chromosome.

4.6.1.2 Association of exon-3 of *PRL* gene with milk composition traits

The difference in milk fat content of studied breeds was not found to be affected by the *Rsa* I alleles of the *PRL*-3 genes. Nonsignificant difference in average milk fat content was observed between *GG* and *GA* genotypes of the *PRL* exon-3 genes in Rathi, Sahiwal and Kankrej cattle. The average milk fat was nonsignificantly higher in Sahiwal and Kankrej cattle than Rathi cattle for *GG* genotype. The milk fat of Kankrej was observed to be higher than other studied breeds. The milk protein in Rathi, Sahiwal and Kankrej cattle showed varied values for two identified genotypic patterns. The *AG* genotype was found to enhance the milk protein contents in all the breeds and on overall basis also.

The *AG* genotype in Sahiwal cattle having haplotype combination of *A*2*G*2 revealed significantly higher milk protein content than *GG* genotype which indicate significant role of *A*2 haplotype on milk protein secretion. Kankrej cattle having *A*3*G*1 haplotype combination revealed comparable protein content in milk to that of Sahiwal for heterozygotic combination. Rathi cattle also revealed the similar trend and exhibited significantly higher protein content in animals of A*G* genotype.

The lactose content was found to be nonsignificantly higher in animals having *GG* genotype irrespective of the breed. Rathi, Sahiwal and Kankrej cattle produced 4.75%, 4.90% and 4.81% of milk lactose, respectively for *GG* genotype whereas the *GA* genotype produced 4.66% (Rathi), 4.73% (Sahiwal) and 4.79% (Kankrej) milk lactose. The variation in SNF content of milk was not observed to be related with

different genotypic patterns. The total solid content for detected genotypic pattern also revealed nonsignificant variation in studied breeds.

Table 4.34 Effect of genotypic patterns of exon-3 of *PRL* gene on milk composition parameters

Breed	Genotype		P- value
	GG	GA	
Fat (%)			
Rathi (75)	4.47±0.17	4.55±0.11	0.661
Sahiwal (75)	4.43±0.12	4.37±0.06	0.669
Kankrej (75)	4.50±0.12	4.44±0.09	0.865
Over all (225)	4.46±0.08	4.45±0.05	0.945
Protein contents (%)			
Rathi (75)	2.78^{a}±0.05	2.93^{b}± 0.03	0.022
Sahiwal (75)	2.98^{a}±0.10	3.23^{b}±0.05	0.027
Kankrej (75)	3.09^{a}±0.04	3.21^{b}±0.03	0.023
Over all (225)	2.96^{a}±0.04	3.12^{b}±0.03	0.001
Lactose (%)			
Rathi (75)	4.75±0.16	4.66±0.10	0.647
Sahiwal (75)	4.95±0.12	4.73±0.06	0.113
Kankrej (75)	4.81±0.04	4.79±0.03	0.623
Over all (225)	4.82±0.07	4.72±0.04	0.207
Solid not fat (%)			
Rathi (75)	8.42±0.07	8.51±0.04	0.282
Sahiwal (75)	8.80±0.10	8.77±0.05	0.824
Kankrej (75)	8.80±0.05	8.70±0.04	0.080
Over all (225)	8.67±0.04	8.66±0.03	0.846
Total solids (%)			
Rathi (75)	16.61±0.52	16.67±0.33	0.924
Sahiwal (75)	16.23±0.34	16.15±0.16	0.839
Kankrej (75)	16.37±0.21	16.15±0.16	0.400
Over all (225)	16.64±0.17	16.50±0.10	0.479

Means in the same row bearing different superscripts are significantly different ($p<0.05$).
NS Non-significant

Very few studies have investigated the effect of *PRL* genotypes on the milk composition traits. Similar to present investigation, results in Red Pied cattle breed showed that cows with *AA* genotype had higher milk yield, fat yield, protein yield, but lesser fat percentage ($p<0.05$) (Alipanah *et al*., 2008). In contrast, Chung and Kim

(1997) reported high milk fat for *GG* and *AG* genotypic pattern. Nonsignificant association of milk fat for different *PRL*-Rsa I genotypes was reported by Alipanah *et al*. (2007b). Significant association observed for milk protein content and genotypic patterns of exon-3 of *PRL* gene could be due to activation of JAK-STAT pathway leading to activation of promoters regions of milk protein genes (Bole-Feysot *et al*., 1998). Variable results observed for exon 3 of *PRL* gene could be due to breed difference, different history of adaptation and variation in structure of prolactin receptor (Van-Rens *et al*., 2003).

4.6.2 Association of exon-4 of *PRL* gene with lactation performance and milk composition traits

4.6.2.1 Association analysis of exon-4 of *PRL* gene with lactation performance

The association of identified genotypes with different lactation traits was performed for different parity and the results are represented in **Table 4.35**. The genotypic patterns detected in all the studied breeds significantly influenced the TMY.

The genotypic pattern having restricted allelic pattern *AA* was found to significantly enhance the TMY in each parity taken into account. The effect of genotype *AA* was more pronounced in Rathi cattle which could be due to the effect of specific haplotype (A1) present in Rathi for this region. TMY under the genotype *AA* showed an increasing trend with increase in parity number. The specific haplotype (G2) present in GG restricted genotype of Sahiwal contributed less increase in milk compared to G1 and G3 haplotype present in Rathi and Kankrej cattle. The presence of G2 haplotype in Sahiwal also observed to reduce the TMY in heterozygote combination in 1st lactation. The specific SNP combination (A3 haplotype) present in Kankrej cattle also enhanced the TMY (p□0.05) in line with the A1 haplotype of Rathi cattle. An overall low TMY was observed in GG genotype of all the animals studied irrespective of breed.

The 305-day MY was not found to be affected by the genotypic patterns in Rathi and Kankrej cattle in any parity though a high difference in GG and AA genotypic pattern was observed. Sahiwal displayed a significantly enhanced 305-day MY in first parity for *AA* genotyped animals which indicates its inherent potential to achieve substantial milk production and further warrants for genetic improvement required in the herd. The absence of this effect in subsequent lactation could be due to confounding effect of environment on milk yield.

Table 4.35 Effect of genotypic patterns of exon-4 of *PRL* gene on lactation performance in different parity

Breed	Lactation Performance								
	1st Lactation			2nd Lactation			All Lactation		
	GG	GA	AA	GG	GA	AA	GG	GA	AA
Total milk yield (kg)									
Rathi (75)	1275.71^{a} ±167.49	1519.88^{ab} ±113.25	1851.67^{b} ±136.76	1521.56^{a} ±130.07	1582.97^{a} ±87.95	1897.41^{b} ±106.21	1513.91^{ab} ±150.28	1464.87^{a} ±101.61	1855.98^{b} ±122.71
Sahiwal (75)	1169.29^{a} ±114.52	1323.79^{a} ±71.64	1684.98^{b} ±146.49	1204.27^{a} ±132.27	1510.94^{ab} ±82.74	1792.63^{b} ±169.21	1265.56^{a} ±103.46	1439.75^{a} ±64.72	1738.81^{b} ±132.34
Kankrej(75)	1221.03^{a} ±154.00	1371.32^{ab} ±82.77	1724.74^{b} ±134.67	1318.73^{a} ±125.74	1474.06^{a} ±67.58	1769.11^{b} ±109.95	1478.36^{a} ±109.91	1589.57^{ab} ±59.08	1823.52^{b} ±96.12
Over all (225)	1219.82^{a} ±83.46	1395.26^{a} ±50.98	1774.91^{b} ±79.35	1343.94^{a} ±74.72	1517.78^{a} ±45.63	1833.30^{b} ±71.04	1408.96^{a} ±70.92	1500.23^{a} ±43.32	1820.58^{b} ±67.43
Milk yield-305 day (kg)									
Rathi (75)	1647.11 ±112.91	1776.40 ±76.34	1901.32 ±92.19	2063.60 ±96.59	1968.02 ±65.31	2058.35 ±78.86	1848.76 ±113.97	1779.96 ±77.06	2013.23 ±93.06
Sahiwal (75)	1617.99^{a} ±95.50	1643.82^{a} ±59.74	2067.09^{b} ±122.16	1811.64 ±98.92	1831.56 ±61.88	2039.98 ±126.54	1773.65 ±94.80	1783.85 ±59.30	2080.35 ±121.27
Kankrej (75)	1629.13 ±103.42	1748.67 ±55.59	1865.63 ±90.44	1747.06 ±96.68	1956.90 ±51.97	2001.26 ±84.55	1774.51 ±85.22	1953.77 ±45.81	2043.32 ±74.52
Over all (225)	1630.98^{a} ±59.74	1718.10^{a} ±36.48	1924.72^{b} ±56.79	1879.55 ±56.72	1914.23 ±34.64	2035.80 ±53.92	1799.46^{a} ±57.38	1843.46^{a} ±35.05	2037.26^{b} ±54.55
Daily milk yield (kg)									
Rathi (75)	5.4±0.37	5.83±0.25	6.24±0.30	6.77±0.32	6 .45±0.21	6.75±0.26	6.06±0.37	5.84±0.25	6.60±0.31
Sahiwal (75)	5.31^{a}±0.31	5.39^{a}±0.20	6.78^{b}±0.40	5.94±0.32	6.00±0.20	6.69±0.41	5.82±0.31	5.85± 0.19	6.82±0.40
Kankrej(75)	5.34±0.34	5.73±0.18	6.12±0.30	5.73±0.32	6.42±0.17	6 .56±0.28	5.82±0.28	6.41±0.15	6.70±0.24
Over all (225)	5.35^{a}±0.20	5.63^{a}±0.12	6.31^{b}±0.19	6.16±0.19	6.28±0.11	6.67±0.18	5.90^{a}±0.19	6.04^{a}±0.12	6.68^{b}±0.18
Lactation length (days)									
Rathi (75)	225^{a}±17.88	259^{ab}±12.10	288^{b}±14.60	227^{a}±15.46	247^{ab}±10.45	279^{b}±12.62	240±14.29	247±9.67	278±11.67
Sahiwal (75)	221±13.14	246±8.23	236±16.80	200^{a}±12.59	248^{b}±7.87	260^{b}±16.10	217^{a}±9.75	247^{b}±6.10	249^{b}±12.47
Kankrej(75)	217^{a}±19.30	237^{ab}±10.37	278.±16.87	229^{a}±14.62	228^{a}±7.86	267^{b}±12.79	251±11.64	247±6.25	270±10.18
Over all (225)	221^{a}±9.64	246^{b}±5.89	274^{c}±9.17	217^{a}±8.21	241^{b}±5.02	271^{c}±7.81	234^{a}±6.93	247^{a}±4.24	269^{b}±6.59

The observed difference in ADMY values in Rathi and Kankrej cattle for the three different genotypes was found statistically similar and nonsignificant ($p>0.05$) in each parity. The SNP's present at specific position in AA genotype of Sahiwal cattle significantly enhanced the ADMY in first parity. The homozygote genotype *GG* and heterozygote genotype *AG* in Sahiwal cattle were nonsignificant in their role on ADMY.

The effect of different haplotype of allele *A* on lactation length was clearly evident in studied breeds of cattle. The allele *A* present in homozygotic combination in Rathi and Kankrej cattle significantly enhanced the lactation length in first and second parity. The effect of haplotypic combination *A2A2* on lactation length in Sahiwal cattle was observed in 2nd lactation and on average of all parity.

The *A* allele was found positively associated with milk yield by Mehamannavaz *et al*. (2009). The present study is in strong agreement with similar findings in Chinese Holstein cattle where allelic combination *AA* produced significantly higher milk yield in comparison to those with *AG* genotype and *GG* allele combination (Dong *et al*., 2013). Previous association analysis of this polymorphism with milk performance traits did not present consistent results probably due to lack of concerted efforts. In contrast to the results observed in the present study in *Bos indicus* cattle, Brym *et al*. (2005a) reported significant effect of genotype *AG* on milk yield in first lactation in Black and White cattle but no significant role of any of the genotype on milk yield in Jersey cattle. The *GG* and *AG* genotype of *PRL* exon-4 in Czech Fleckvieh cattle were observed superior in milk production than *AA* genotype with genotype effect of 252.95 kg for *GG* genotype (Boleckova *et al*. 2012). Conversely, the association of this polymorphism with milk productions was not confirmed in other studies (Chrenek *et al*., 1999). The possible linkage of *Rsa*I/*PRL* mutation and causative polymorphism in *PRL* gene regulatory sequences as suggested by Brym *et al*. (2007) could not be neglected for possible variation in results.

4.6.2.2 Association of genotypic patterns of exon-4 of *PRL* Gene with milk composition traits

The different genotypic patterns detected for exon-4 of *PRL* gene were analyzed for their association with milk composition parameters and the results are presented in **Table 4.36.** The effect of different genotypes of *PRL* exon-4 region on milk fat could not be established for any of the breed included in the present study. However, nonsignificantly higher mean fat content was observed for GG genotype in

all the studied breeds. The absence of discernible effect of genotype on milk fat might be due to masking effect of genes present at other locus.

Table 4.36 Effect of genotypic patterns of exon-4 of *PRL* gene on lactation performance in different parity

Breed	Genotype			P- value
	GG	GA	AA	
Fat (%)				
Rathi (75)	4.75±0.19	4.47±0.13	4.46±0.16	0.435
Sahiwal (75)	4.59±0.11	4.29±0.06	4.43±0.13	0.056
Kankrej (75)	4.68±0.17	4.34±0.09	4.60±0.15	0.112
Over all (225)	4.67^{b}±0.09	4.36^{a}±0.06	4.50^{ab}±0.08	0.012
Protein contents (%)				
Rathi (75)	2.81^{a}±0.06	2.85^{ab}±0.04	2.99^{b}±0.05	0.048
Sahiwal (75)	2.99^{a}±0.09	3.22^{ab}±0.05	3.32^{b}±0.11	0.041
Kankrej (75)	3.12^{a}±0.06	3.13^{a}±0.03	3.30^{b}±0.06	0.022
Over all (225)	2.96^{a}±0.05	3.09^{b}±0.03	3.16^{b}±0.05	0.010
Lactose (%)				
Rathi (75)	4.74±0.19	4.67±0.13	4.66±0.15	0.939
Sahiwal (75)	4.91±0.11	4.73±0.07	4.72±0.14	0.349
Kankrej (75)	4.82±0.06	4.81±0.03	4.74±0.05	0.533
Over all (225)	4.83±0.08	4.74±0.05	4.70±0.07	0.454
Solid not fat (%)				
Rathi (75)	8.41±0.08	8.54±0.05	8.46±0.06	0.343
Sahiwal (75)	8.73±0.09	8.84±0.05	8.59±0.11	0.107
Kankrej (75)	8.78±0.07	8.72±0.04	8.77±0.06	0.650
Over all (225)	8.63±0.05	8.71±0.03	8.59±0.05	0.061
Total solids (%)				
Rathi (75)	17.01±0.60	16.72±0.41	16.31±0.49	0.650
Sahiwal (75)	16.51±0.29	16.04±0.18	16.14±0.38	0.415
Kankrej (75)	16.50±0.30	16.08±0.16	16.40±0.26	0.361
Over all (225)	16.81±0.19	16.38±0.12	16.67±0.18	0.098

Means in the same row bearing different superscripts are significantly different ($p<0.05$).
NS Non-significant

The percentage of protein present in milk varied significantly among detected genotypes within each breed. The genotypic pattern *AA* was observed to be significantly better than *GG* genotypic pattern in Rathi, Sahiwal and Kankrej cattle.

The protein yield of *GG* genotypic pattern ranged from 2.99% (Rathi) to 3.32% (Sahiwal). The magnitude of difference between genotypes within breed was found to be highest in Sahiwal cattle (*AA*-*GG*). The difference in magnitude of *GG* genotype in different breed might be due to haplotype difference as SNP's located at position other than restricted site might impart variance in milk protein content. The values of milk lactose, SNF and total solids were nonsignificantly varied between genotypes in all the studied breeds. The results suggest that exon-4 of *PRL* gene imparts variance in protein component only. An overall positive effect of *GG* genotype irrespective of breed was observed on milk fat percentage which is in agreement with the similar findings on Black and White cattle of Poland whereas Jersey cattle reflected higher fat yield for *AA* genotype (Brym *et al*., 2005a).

As observed in present study, significant negative impact of genotype *GG* on milk protein percentage and positive impact of *A* allele was observed in Czech Fleckvieh cattle by Boleckova *et al*. (2012). It was reported that animals having *AA* genotype yields more milk and protein (Alipanah *et al*., 2007a) whereas more fat was observed in animals of *GG* and *AG* genotype (Khatami *et al*., 2005). The association of allele *A* with milk yield was indirectly revealed by Othman *et al*. (2011) who observed only *G* allele in buffalo population producing fat rich milk. The unfavorable effect of *G* allele and favourable effect of *A* allele milk protein yield was established by Mehmannavaz *et al*. (2009) through allele substitution study. Dong *et al*. (2013) could not observe any association of different genotypic pattern with percentage of milk fat and proteins.

In a number of studies, the candidate genes affected the genetic trend in a significant way. At molecular level, genetic trends for traits depends on allelic frequency variation during years and the rate of independent effect of each gene on the trait. The results of the present study indicated that *PRL* polymorphism significantly influenced the genetic trend for milk related traits. The significant effect of *PRL* genotypes observed in the present study especially for milk production and milk proteins traits might be due to combined effect on numerous biological processes due to pleiotropic nature of *PRL* gene (Bole-Feysot *et al*., 1998). Since most of the earlier studies were concentrated on the polymorphism of *Rsa*I restricted SNP, the present study observed that other SNP's present in haplotype should also be evaluated for their influence on milk production and composition traits across the different cattle breeds. The non observance of fat effect with the different genotypic

pattern of exon 3 and exon 4 of *PRL* gene could be due to interactions with different background genes in the three breeds.

4.6.3 Association analysis of lysine variants of *DGAT1* gene with milk performance traits and milk composition traits

4.6.3.1 Association of *DGAT1* lysine variants with milk performance traits

An association between genotype of *DGAT1* gene and milk performance could not be established for *K232A* polymorphism through RFLP analysis due to absence of alanine coding allele '*A*' in each of the studied breeds examined. However, the different variants of lysine coding allele '*K*' as detected through SSCP marker were analyzed for their association with traits of lactation performance (**Table 4.37)**. The present investigation could not observe any association between SSCP patterns of lysine variant and lactation performance traits during any parity for TMY, 305-day MY, ADMY and LL. On the basis of obtained results, it could be inferred that none of the lysine haplotype identified in the present study affected the traits related to milk yield and lactation length in indigenous breeds of Rathi, Sahiwal and Kankrej cattle. Despite being differential mobility on polyacrylamide gel, the nonobservance of lysine variant effect on milk yield and lactation length in Rathi, Sahiwal and Kankrej cattle could be due to masking effect of VNTR polymorphism located in the promoter region of *DGAT1* gene (Kuhn *et al.* 2004). The reports of Bennewitz *et al*. (2004) also indicated that *K232A* polymorphism of *DGAT1* is not sole responsible for all the genetic variation in QTL located at the centromeric end of chromosome 14 for milk related traits.

4.6.3.2 Association of lysine variant of *DGAT1* gene with milk composition traits

The complete absence of alanine coding allele restricted the present study to analyse the difference in milk composition traits between alanine coding and lysine coding allele. Dokso *et al*. (2015) also failed to detect the cause of variation in milk fat in three Russian cattle breeds having similar '*KK*' genotype. Most of the earlier studies conducted on *DGAT1* gene were restricted towards estimation of effect of dinucleotide GC/AA base substitution effect on milk composition.

The detection of different variants of lysine coding *K* allele through fine screening of amplified products of *DGAT1* gene by SSCP and SNP's methods permitted to analyze their effect on milk composition estimates of studied breeds

(**Table 4.38**). Significant differences in least square means of milk fat and protein was observed in Sahiwal and Kankrej cattle and SNF in Sahiwal cattle. The SSCP pattern '*K1K1*' and '*K1K2*' observed in Rathi cattle were not found to affect variability in milk fat, protein, lactose, SNF and total solids in a significant manner. Non observance of effect of haplotype '*K1*' and '*K2*' on milk composition could be attributed to nature and position of SNP's.

Table 4.37 Effect of genotypic patterns of lysine variants of *DGAT1* gene on lactation performance in different parity

Breed	Lactation Performance					
	Rathi (75)		Sahiwal (75)		Kankrej (75)	
	K1K1	***K1K2***	***K1K1***	***K1K3***	***K2K2***	***K2K4***
Total milk yield (kg)						
1st Lactation	1567.66 ±110.84	1581.16 ±117.85	1219.92 ±78.65	1428.81 ±81.19	1417.75 ±101.37	1433.20 ±86.40
2nd Lactation	1698.28 ±87.89	1638.72 ±88.08	1512.50 ±110.28	1453.47 ±84.96	1600.89 ±80.86	1424.78 ±70.91
All Lactation	1659.63 ±99.17	1532.89 ±103.34	1381.04 ±88.86	1487.02 ±63.85	1649.98 ±61.56	1595.94 ±72.13
Milk yield-305 day (kg)						
1st Lactation	1788.04 ±77.02	1789.65 ±52.53	1633.67 ±61.81	1748.85 ±72.66	1748.57 ±57.09	1760.53 ±66.23
2nd Lactation	2013.75 ±66.84	2021.39 ±57.52	1863.56 ±85.86	1852.71 ±56.68	1960,65 ±55.85	1899.71 ±60.33
All Lactation	1958.15 ±71.64	1767.71 ±77.04	1771.57 ±78.88	1864.57 ±58.39	1933.24 ±48.79	1953.02 ±54.72
Daily milk yield (kg)						
1st Lactation	5.86±0.24	5.87±0.25	5.36±0.20	5.73±0.24	5.73±0.19	5.77±0.22
2nd Lactation	6.60±0.22	6.63±0.19	6.11±0.28	6.07±0.18	6.43±0.18	6.23±0.20
All Lactation	6.42±0.23	5.80±0.25	5.81±0.26	6.11±0.19	6.34±0.16	6.40±0.18
Lactation length (days)						
1st Lactation	260±12.10	261±8.56	227±10.11	248±8.23	240±12.38	246±11.08
2nd Lactation	258±10.83	246±9.95	235±9.25	238±6.60	246±9.48	227±8.08
All Lactation	254±9.42	257±9.89	244±6.95	240±4.95	258±6.37	247±7.43

The two genotypes '*K1K1*' and '*K1K3*' observed in Sahiwal cattle were remarkably varied (p□0.05) in terms of milk fat, protein and SNF. The substitution of '*K1*' lysine variant with '*K3*' haplotype in '*K1K3*' genotypes of Sahiwal cattle resulted in significant (p□0.05) reduction of milk fat content with subsequent increase in

protein and SNF content of milk. The reason for reduced breed average for milk fat in Sahiwal cattle could be ascribed to the presence of '*K3*' lysine variant in Sahiwal population. The differences in SNP's between '*K1*' and '*K3*' haplotype in Sahiwal cattle might have affected the enzymatic behavior of *DGAT1* gene. An overall higher breed average for milk protein content (p□0.05) in Sahiwal cattle also indicates the presence of less fat efficient *DGAT1* '*K*' variant in the population. Another novel haplotype '*K4*' observed in Kankrej cattle contributed significantly in enhancement of milk fat from 4.29% to 4.62% with concurrent decrease in milk protein from 3.22% to 3.11% in '*K2K2*' and '*K2K4*' genotypic pattern, respectively.

The presence of alternate form of high and low fat bearing '*K*' allele in Sahiwal and Kankrej cattle population agrees with the findings of Winter *et al*. (2002) who also observed differences in breeding value for milk fat and protein in between haplotypes of lysine variant of *DGAT1* gene. The presence of milk fat enhancing and lowering variants in different haplotype combination (Winter *et al*., 2002) in Indian breeds of Rathi, Sahiwal and Kankrej cattle indicates the presence of multiple alleles of *DGAT1* gene '*K*' variant. It may be possible that SNP's present at other positions may influence the expression of *DGAT1* '*K*' allele in different breeds. The absence of significant variation (p>0.05) in milk fat among studied breeds could be due to relatively low and restricted frequency of '*K3*' and '*K4*' haplotype in the population and very high frequency of common haplotype '*K1*' and '*K2*' in overall population in addition to large environmental deviation. Significant difference (p□0.05) observed in the breed average for milk protein content in Rathi, Sahiwal and Kankrej cattle could be due to effect of different variant of lysine allele present in the studied breed. The low milk protein in indigenous dairy cattle in comparison to exotic cattle breeds could be attributed to the negative impact of *DGAT1* '*K*' variants and absence of '*A*' allele in Indian cattle (Molee *et al*., 2015).

The fix nature of '*K*' allele in indigenous cattle breeds of Rathi, Sahiwal and Kankrej reflects their exceptional adaptive value. The greater overall immunity of indigenous cattle against many diseases and their survival adaptation to coarse roughage with low requirement of green could be due to enhanced acyltransferase activity of *DGAT1* gene which catalyzes the synthesis of retinol esters and thus regulates the synthesis of Vitamin A or retinol. The presence of a QTL affecting the somatic cell count (SCC) in the centromeric region on BTA14 was also reported by Zhang *et al.* (1998). The significant and favourable association of *DGAT1* '*K*' allele with low somatic cell count in lactating cattle was reported by Manga and Riha (2011)

that partially explains the exceptional genetic resistance of indigenous cattle against mastitis as the triglycerides are also one of the main constituent of cell membrane in immunocompetent cells (Liu *et al*., 2007).

The role of another mammalian gene *DGAT 2* (Cases *et al*., 2001) on milk composition and yield could not be neglected to estimate the precise role of *DGAT1* gene however the more significant effect of *DGAT1* gene on milk fat could not be denied (Smith *et al*. 2000). Similarly, an allelic variant of VNTR in promoter region of *DGAT1* gene was identified as fat enhancer in Holstein and Butiana dairy cattle (Rahmatalla *et al*., 2008; Rahmatalla *et al*., 2015) whereas another allele in same promoter region (VNTR) of *DGAT1* reflected its significant role on lactose and milk energy content (Sanders *et al.,* 2006). The VNTR alleles in promoter region of *DGAT1* gene was reported to have effect on potential sp1 binding sites and therefore might have an impact on *DGAT1* expression and ultimately on content of fat in milk (Furbass *et al*., 2006). The presence of another neighborhood conserved lysine residue near lysine residue at 232 positions was found to affect interaction of CoA moiety with *DGAT* enzyme and could be responsible for fraction of variance in milk fat contents (Gautier *et al.* (2007).

Table 4.38 Effect of lysine variants of *DGAT1* gene on milk composition parameters

Attributes	Milk composition parameters					
	Rathi (75)		Sahiwal (75)		Kankrej (75)	
	K1K1	***K1K2***	***K1K1***	***K1K3***	***K2K2***	***K2K4***
Fat (%)	4.51±0.12	4.55±0.14	4.50^{b}±0.10	4.29^{a}±0.50	4.29^{a}±0.08	4.62^{b}±0.11
Protein contents (%)	2.93±0.04	2.83±0.04	3.08^{a}±0.06	3.26^{b}±0.06	3.22^{b}±0.04	3.11^{a}±0.04
Lactose (%)	4.61±0.16	4.76±0.04	4.67±0.12	4.85±0.03	4.81±0.04	4.78±0.04
Solid not fat (%)	8.54±0.04	8.43±0.06	8.66^{a}±0.06	8.87^{b}±0.06	8.75±0.04	8.72±0.04
Total solids (%)	16.71 ±0.30	16.89 ±0.29	16.04 ±0.27	16.26 ±0.15	16.52 ±0.15	16.94 ±0.19

5. SUMMARY AND CONCLUSION

Milk yield and its quality has been a major selection criterion for genetic improvement in livestock species such as cattle. Role of *prolactin* and *DGAT1* gene in determining milk quantity and quality is very important. In this context, genetic profiling of *prolactin* and *DGAT1* gene in indigenous cattle breeds of India was performed and potential genetic markers were identified. The association analysis of prolactin and *DGAT1* gene with different lactation and milk composition traits illustrated worth of this gene for marker-assisted selection of dairy cattle. Series of wet and dry lab experimentation was performed to identify the genetic marker.

Three proven genomic regions, *viz.,* exon-3 and exon-4 of *PRL* gene; and exon-8 of *DGAT1* gene were selected on the basis of their significant role in lactation as revealed in a number of studies and through knock out trials. The genetic variations in selected genomic regions were identified through two different marker systems, RFLP and SSCP. Sequencing of the selected gene products for different molecular variants was carried out to detect the SNP's present in the identified markers. Haplotypes were constructed on the basis of SNP's to reveal the variation in different milk related parameters. The evolutionary divergence between different species of livestock and indigenous cattle was estimated and phylogenetic tree was constructed to depict the relationship between groups of livestock species for selected genomic regions. Identified markers for different gene were checked for their association with lactation and milk composition parameters.

A total of 225 milking animals true to breed representatives of indigenous cattle, viz., Rathi (n=75), Sahiwal (n=75) and Kankrej (n=75) were selected from the Livestock Research Stations of Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, for the collection of blood and milk samples. The genomic DNA was isolated from whole blood through spin column method to obtain the pure and quality DNA. The concentration and quality of DNA was determined through NanoDrop spectrophotometer and 0.8% agarose gel electrophoresis. The composition of different milk fractions was estimated through Automated Milkoscan Machine at three different intervals.

The 156 bp region of exon-3 of *PRL* gene was amplified through species specific primers at an annealing temperature of 59ºC. The restriction digestion of the amplicons with *Rsa* I restriction enzyme revealed the presence of two genotypes, *GG*

and *AG* with frequency of 0.28 and 0.72 (Rathi); 0.19 and 0.81 (Sahiwal); 0.36 and 0.64 (Kankrej), respectively. The other homozygotic pattern *AA* was not observed in any of the studied breeds. The allele frequency of *G* and *A* was 0.64 and 0.36 (Rathi); 0.59 and 0.41 (Sahiwal); 0.68 and 0.32 (Kankrej), respectively. The Chi^2 and G^2 test for Hardy-Weinberg equilibrium revealed highly significant deviation of gene and genotypic frequency from equilibrium. Application of other marker such as SSCP revealed more genetic variation in the studied 156 bp fragments in studied breeds. Rathi cattle demonstrated three SSCP genotypic markers (P1-P3) whereas both Sahiwal (P4 and P5) and Kankrej cattle (P1 and P6) produced two SSCP markers. The restricted genotype *AG* in Rathi cattle revealed variation in conformational pattern and produced two different SSCP patterns. The frequency of *AG* restricted genotype was redistributed in Rathi cattle for SSCP marker with frequency of 0.57 and 0.15 under pattern P2 and P3, respectively.

The genetic diversity measured in 156 bp region revealed higher observed heterozygosities (Rathi, 0.720; Sahiwal, 0.813; and Kankrej, 0.640) than expected levels. The PIC value ranged from 0.3405 to 0.3668. The fixation index F_{IS} was negative and low (-0.471 to -0.685). The effective numbers of alleles ranged from 1.770 to 1.932. The Shannon Information Index as reflection of suitability was primer was quite high and observed as 0.675, 0.675 and 0.627 for Rathi, Sahiwal and Kankrej cattle. The genetic distance between Rathi and Sahiwal (0.003); and Rathi and Kankrej cattle (0.003) was observed lower than Sahiwal and Kankrej cattle (0.013).

Sequencing of the representative SSCP markers of 156 bp *PRL* region generated seven nucleotide sequences which were submitted to NCBI GenBank database for the allotment of accession numbers. Sequence analysis of the 156 bp *PRL* region through Clustal W and Bioedit software revealed the presence of three transitions and one transversion type of SNP. Haplotype construction and analysis detected presence of five different haplotype on the basis of four identified SNP's. Haplotypes Hap-*A1* (Rathi), Hap-*A3* (Kankrej) and Hap-*G*2 (Sahiwal) were novel and breed specific in nature.

The mean evolutionary distance between different species of livestock was 0.0208±0.0072 for exon-3 of *PRL* gene with distance between different alleles of studied breeds ranged between 0.00 to 0.0197 whereas the interspecies divergence between indigenous studied breed and exotic cattle was 0.0130 to 0.0335. Highest genetic divergence of 0.0335 to 0.0405 was observed between *Ovies aries* and indigenous cattle. NJ based bootstrapped dendrogram was constructed based on the

Jukes-Cantor model with minimum BIC value of 842.602 out of 24 different maximum likelihood substitution models for the construction of dendrogram. The evolutionary distance in *PRL* exon-3 sequence in *Bos taurus* revealed least divergence among themselves whereas indigenous cattle breeds revealed more distance among themselves. Three conserved regions were identified between studied indigenous cattle and other livestock species.

Association analysis of *PRL* exon-3 gene with lactation traits identified significantly higher total milk yield for *AG* genotype in first and second parity in all the three breeds. 305-day MY was observed significantly higher in Rathi cattle in 1st parity with nonsignificant variation in all other parity and in other breeds. The ADMY also reflected the similar pattern to that 305-day MY. The 1st parity in Rathi cattle, 2nd parity in Sahiwal cattle and; 1st and 2nd parity in Kankrej cattle revealed significantly higher lactation length for *AG* genotype of *PRL* exon-3 region. Nonsignificant difference in milk fat with identified genotype was observed in all studied breeds. Significantly higher milk protein content was observed for *AG* genotype than *GG* genotype in all the breeds. Lactose, SNF and total solid were found to be nonsignificantly associated with genotypic patterns of *PRL* exon-3 region.

A second region of prolactin gene, *i.e.,* exon 4 was also screened genetic variation and its possible association with lactation and milk composition traits. Species specific primers were used to amplify the 294 bp length of *PRL* exon-4 region at an annealing temperature of 55ºC. The RFLP marker analysis was carried out with similar *Rsa* I restriction enzyme to detect the A/G mutation at restriction site. Three different genotypic patterns *GG*, *AG* and *AA* were detected in all the three breeds. The genotypic frequency of 0.21, 0.47 and 0.32 in Rathi cattle, 0.24, 0.61 and 0.15 in Sahiwal cattle; and 0.17, 0.60 and 0.23 in Kankrej cattle was observed for *GG*, *AG* and *AA* restricted patterns, respectively. The allelic frequency of G and A was 0.45 and 0.55 in Rathi; 0.55 and 0.45 in Sahiwal; and 0.47 and 0.53 in Kankrej cattle, respectively. Both Rathi and Kankrej population were observed in Hardy-Weinberg equilibrium however Sahiwal population deviated from the equilibrium status as evident from Chi^2 and G^2 test statistic. SSCP marker analysis also generated the similar banding patterns on 8% polyacrylamide gel in the form of three SSCP patterns, though the electrophoretic movement of bands in each breed was dissimilar with other breed suggesting the presence of sequence variation in exon-4 region of *PRL* gene in different indigenous breeds. The frequency of SSCP marker was similar to that of RFLP marker frequency.

The observed heterozygosity in exon-4 region of *PRL* gene was greater than the expected heterozygosity in Sahiwal and Kankrej cattle whereas Rathi cattle were observed less heterozygous than their expected values. Medium values of PIC, *viz.,* 0.3725, 0.3725 and 0.3741 was observed in Rathi, Sahiwal and Kankrej cattle, respectively. The fixation index revealed low and negative F_{IS} values in Sahiwal and Kankrej cattle whereas the positive F_{IS} value observed in Rathi cattle could be due to heterozygote deficit. The number of effective alleles in each breed observed was 1.977, 1.982, 1.994 in Rathi, Sahiwal and Kankrej breed, respectively. The Shannon Index value were greater than intermediate values with highest values of 0.691 was observed for Kankrej cattle. Lowest pair wise genetic distance was found between Rathi and Kankrej (0.001), followed by Sahiwal and Kankrej (0.011) and; Rathi and Sahiwal (0.020).

Six different sequences were generated through sequencing of amplified product of 294 bp length by Sanger's chain termination method and aligned to get variations in genomic region. The sequences were submitted to NCBI GenBank database under accession numbers, MF045465; and KY777610 to KY777614. The multiple sequence alignment of the generated sequences with referenced sequence (Accession No. AF426315) was carried out to identify the position and nature of SNP's. Ten different SNP's were identified in 294 bp fragment with eight SNP's were observed in intron region. Two SNP's were identified in the coding region of exon-4. Seven of the ten mutations were observed as transition mutation and three mutations were identified as transversion. A total of six haplotypes were constructed on the basis of identified SNP's with two haplotypes in each breed were recognized.

Phylogenetic analysis was performed to evaluate the rate of evolution in exon-4 of *PRL* gene in various livestock species. Mean evolutionary distance among livestock species was observed to be 0.0117±0.0051 with distances between different alleles of studied breeds was 0.0000 to 0.0116. Evolutionary divergence between indigenous and exotic cattle ranged from 0.0000 to 0.0176 depending on the breeds. Goat diverged more from bovine animals (0.0116-0.0298) than buffalo (0.0058 to 0.0237) and sheep (0.0058-0.0237). The *A* allele of all the three breeds shared the clade of phylogenetic tree directly or indirectly with *Bos taurus* sequence. The allele *G* of Rathi shared the different branch than allele *G* of Sahiwal and Kankrej cattle. Three conserved regions were observed between studied cattle breeds and other livestock species.

Association analysis of genotypic patterns of exon-4 of *PRL* gene revealed significant positive effect of *AA* genotype with TMY in all the three breeds in both parity. The 305-day MY however was found significantly higher for *AA* genotype in Sahiwal cattle in the first parity. The ADMY was also observed to be significantly higher in *AA* genotypes of Sahiwal cattle in the first parity. Significantly higher lactation length was observed in 1st parity of Rathi cattle and 2nd parity of Sahiwal and Kankrej cattle for animals identified with *AA* genotype. The total fat, lactose, SNF and total solids of milk samples were found to be indifferent with the type of genotypic patterns. The protein content of milk was found significantly higher in animals characterized with *AA* genotype in all the three breeds.

The knock out trials in mouse lines validated the role of *DGAT1* gene on performance of various milk related traits. The 411bp product of *DGAT1* gene was successfully amplified through primers based on gene sequence of Accession No. AJ 318490 at an annealing temperature of 52ºC. The RFLP analysis was carried out through *Eae* I restriction enzyme, which is diagnostic for GC/AA dinucleotide base pair substitution and produces non conservative *K232A* polymorphism, revealed the presence of single genotypic pattern '*KK*' in all the 225 studied animals of Rathi, Sahiwal and Kankrej breed. Thus allele '*K*' was found to be fixed in Indian cattle breeds of Rathi, Sahiwal and Kankrej with complete absence of *DGAT1* '*A*' allele. The SSCP marker analysis was utilized to test for polymorphism at any other nucleotide position apart from restricted base position and revealed the presence of five different conformational banding patterns in studied animals. Rathi cattle were characterized by the presence of SSCP patterns '*K1K1*' and '*K1K2*' whereas Sahiwal cattle revealed genotypic patterns '*K1K1*' and '*K1K3*'. Likewise, Kankrej cattle were genotyped for '*K2K2*' and '*K2K4*' SSCP marker. The genotypic frequency of *K1K1* marker was observed as 0.53 and 0.43 in Rathi and Sahiwal, respectively. The *K1K2* and *K1K3* pattern appeared in the frequency of 0.47 and 0.57 in Rathi and Sahiwal, respectively. The *K2K2* and *K2K4* SSCP marker in Kankrej cattle appeared in the frequency of 0.51 and 0.49, respectively. The overall frequency of *K1*, *K2*, *K3* and *K4* irrespective of breed, was observed as 0.49, 0.33, 0.10 and 0.08, respectively. Both Chi^2 and G^2 likelihood test ratio revealed highly significant departure of gene and genotypic frequency of lysine variants of *DGAT1* gene from expected Hardy-Weinberg equilibrium (HWE) ($p<0.001$).

The observed heterozygosity for different lysine variants of *DGAT1* gene was found higher than expected heterozygosity in all the studied breeds. Nei's unbiased

heterozygosity was also lower than observed heterozygosity. Medium PIC value was observed in studied breeds with values ranged from 0.2915 (Rathi) to 0.3270 (Sahiwal). Negative fixation index value in Rathi (-0.304), Sahiwal (-0.402) and Kankrej (-0.327) denoted the presence of sufficient genetic variation for lysine variants in indigenous cattle. The effective number of alleles for lysine variant of DGAT1 gene ranged from 1.557 to 1.692 in different breeds. Shannon index in Rathi, Sahiwal and Kankrej cattle reflected value of 0.543, 0.599 and 0.559, respectively. The genetic distance for lysine variant in Rathi and Sahiwal was lower (0.119) than Rathi and Kankrej cattle (1.284) whereas lysine variants of Kankrej cattle were not found to be related with lysine variants of Sahiwal cattle.

Sequencing and sequence analysis of lysine variants for respective SSCP patterns in different breeds generated six nucleotide sequences which were submitted to NCBI database for allotment of accession numbers. Multiple sequence alignment through Clustal W software and its comparison with reference sequence (AJ 318490) detected the presence of eight SNP's in addition to two SNP's responsible for *K232A* polymorphism. Three SNP's in exon 7, four in intron 7 and one in exon 8 of *DGAT1* gene were identified. SNP's A-A were observed at 200-201 position in exon-8 of *DGAT1* gene. Four different haplotypes (*K1 to K4*) were identified on the basis of detected SNP's. Haplotype *K1*and *K2* were detected in animals of both Rathi and Kankrej breed whereas *K3* and *K4* were novel and detected in Sahiwal and Kankrej, respectively. Seven transversion at base positions 62, 84, 85, 132, 157,185 and 201; and three transitions at base positions 86, 188, and 200 were observed in 411 bp product of *DGAT1* gene.

Mean evolutionary distance of 0.0177±0.0035 was observed for *DGAT1* gene in different livestock species. The evolutionary distance between lysine variants in indigenous cattle was observed below mean distance (0.000 to 0.01746). *Bos taurus* evolutionary diverged from studied indigenous cattle in the range of 0.00244-0.1746. *Ovies aries* and *Capra hircus* diverged in the range of 0.1746-0.03045 and 0.00244-0.04402, respectively whereas *Camelus dromedarius* diverged most from indigenous cattle and other species and demonstrated the evolutionary distance of 0.00738 to 0.04950. The T92 model with gamma distribution on the basis of lowest BIC value of 1821.222 was used to construct phylogenetic tree for *DGAT1* gene. Different lysine variant of indigenous cattle shared different branches with other species or breed. The two lysine variant in Kankrej shared the common cluster whereas the buffalo shared the cluster with *Capra hircus*. Seven conserved region in 411 bp fragments were identified in between studied cattle breeds and other livestock species.

The present investigation could not observe any association between SSCP patterns of lysine variants and lactation performance traits during any parity for TMY, 305-day MY, ADMY and LL. Significant differences in least square means of milk fat and protein was observed in Sahiwal and Kankrej cattle and SNF in Sahiwal cattle. The SSCP pattern '*K1K1*' and '*K1K2*' observed in Rathi cattle were not found to affect variability in milk fat, protein, lactose, SNF and total solids in a significant manner. The substitution of '*K1*' lysine variant with '*K3*' haplotype resulted in significant (p□0.05) reduction of milk fat content. A novel haplotype '*K4*' observed in Kankrej cattle contributed significantly in enhancement of milk fat from 4.29% to 4.62% with concurrent decrease in milk protein from 3.22% to 3.11% in '*K2K2*' and '*K2K4*' genotypic pattern, respectively.

Conclusion

The present study reported for the first time the nucleotide sequence of the bovine *PRL* gene in Rathi and Kankrej cattle and sequence of *DGAT1* gene in Rathi cattle. A critical analysis of the three important loci responsible for milk production and milk composition traits in the present study concluded that our indigenous breeds were naturally evolved and adapted for optimized milk production without comprise for production of milk fat which could be seen as survival advantage through production of energy rich fat required for neonatal survival during period of scarcity and drought. Heterozygotes for *PRL* exon-3 were favored to produce average milk containing adequate amount of fat. The present study is different in nature than most of the earlier studies which have only described RFLP or SSCP marker without explaining the nature and location of SNP's. Haplotypes for *PRL and DGAT1* gene were constructed for the first time in indigenous cattle breeds of Rathi, Sahiwal and Kankrej. The effect of *PRL* genotypes on milk composition traits was also not covered in earlier studies on Indian breeds. The non polymorphic nature of *DGAT1 K232A* locus established in a large number of animals in Indian cattle breeds of Rathi, Sahiwal and Kankrej creates great possibilities to accomplish change in milk protein composition and increase in milk yield without affecting the milk fat content. Though, the frequency of different haplotype needs to be modulated to accomplish this. The results of this study confirmed that *PRL* and *DGAT1* could be a strong candidate gene for application of marker assisted selection for the improvement of dairy traits. The results of the present study will also contribute sharing of polymorphism information on indigenous cattle genetic resources on national and global basis.

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Study on Genetic Variation in Milk Genes and Their Association with Milk Performance Traits in Indigenous Cattle Breeds

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Abstract

The present study was undertaken to explore the genetic variability in exon-3 and exon-4 of prolactin (*PRL*); and *DGAT1* gene and their association with milk performance and milk composition traits in indigenous cattle breed of Rathi, Sahiwal and Kankrej. 225 apparently healthy and lactating cows from breeds of Rathi (n=75), Sahiwal (n=75) and Kankrej (n=75) were selected randomly for the collection of blood and milk samples. The phenotypic information on various lactation traits was recorded from official records maintained in the farm. 100 ml milk from selected animals was collected for the analyses of different milk constituents. About 2ml jugular vein blood was collected in sterile vacutainer from all the selected animals. Genomic DNA from whole blood was extracted through spin column method. The purity (OD ratio 260/280) and concentration (ng/µl) of extracted genomic DNA was determined by Nano-drop spectrophotometer and 0.8% agarose electrophoresis. Species specific primers were used to amplify the 156 bp, 294 bp and 411 bp region of *PRL* exon-3, *PRL* exon-4 and *DGAT1* gene, respectively. Two approaches such as restriction fragment length polymorphism (RFLP), single strand conformation polymorphism (SSCP) was carried out to detect the genetic variation in selected gene regions. Bioinformatics tools such as Chromas, Bioedit and Clustal W were used for single nucleotide polymorphisms (SNPs) detection and analysis after gene sequencing. Generated sequences were submitted to international database, NCBI GENBANK for assignment of accession number. Neighbourhood Joining (NJ) phylogenetic tree was constructed through bootstrap test method in MEGA7 bioinformatics software. The genetic structure of the population and genetic variation was analysed through POPGENE program. Univariate analysis of variance was carried out through general linear model (GLM) procedure of SPSS software to detect the association of different lactation performance and milk composition traits with detected genetic variation in selected genomic regions. The RFLP analysis of *PRL* exon-3 through *Rsa* I enzyme detected two genotypic patterns in all the studied breeds in the genotypic frequency of 0.28 and 0.72 for *GG* and *AG* genotype, respectively and gene frequency of 0.64 (*G* allele) and 0.36 (*A* allele) on overall basis. SSCP analysis detected three different genotype in Rathi cattle whereas two genotype each in Sahiwal and Kankrej cattle. *AG* genotype of *PRL* exon-3 gene in Rathi cattle demonstrated two different SSCP genotypic patterns, ‘P2’ and ‘P3’. The RFLP frequency of *AG* genotype (0.72) in Rathi was redistributed for P2 (0.57) and P3 (0.15) SSCP pattern. Sufficient genetic variation in the form of heterozygosity, PIC, fixation index and Shannon information index was detected for *PRL* exon-3 locus however all the populations were significantly deviated from Hardy-Weinberg equilibrium. The genetic distance between Rathi and Sahiwal was observed lower than between distance of Sahiwal and Kankrej. Three transition and one transversion type of SNP were detected in *PRL* exon-3 locus of studied breeds. Five different haplotypes were constructed on the basis of detected SNP’s in 156-bp amplified fragment of exon-3 of *PRL*-3 gene. The evolutionary divergence among the different alleles of studied breeds was observed lower than mean

distance. Four conserved regions were identified among studied breeds. Both RFLP analysis of *PRL* exon-4 locus with *Rsa* I enzyme and SSCP analysis revealed three different genotypic patterns in an overall frequency of 0.21, 0.56 and 0.23 for *GG*, *AG* and *AA* genotype with gene frequency of 0.49 and 0.51 for *G* and *A* allele, respectively, in Hardy-Weinberg equilibrium except in Sahiwal cattle. Observed (H_O) and expected heterozygosity (H_E), PIC, fixation index and Shannon information index revealed sufficient genetic variation in *PRL* exon-4 locus however Rathi revealed positive F_{IS} value. The genetic between Sahiwal and Kankrej was less than distance between Rathi and Sahiwal. SNPs detection revealed seven transitions and three transversion SNPs in *PRL* exon-4 locus with the presence of six difference haplotypes. The distance between alleles of studied breeds ranged below the mean value of 0.0117±0.0051. The *A* allele of *PRL* exon-4 locus in all the three breeds shared the clade directly or indirectly with *Bos taurus* sequence. Six conserved regions were identified in between studied breeds. RFLP analysis of *DGAT1* gene through *Eae* I restriction enzyme revealed monomorphic pattern *KK* in all the samples whereas SSCP analysis revealed five different conformational banding patterns for lysine (*KK*) variants in studied animals with an overall genotypic frequency of 0.32, 0.16, 0.19, 0.16 and 0.17 for *K1K1*, *K1K2*, *K1K3*, *K2K2 and K2K4*, respectively. The gene frequency of *K1*, *K2*, *K3* and *K4* lysine variant was observed 0.49, 0.33, 0.10 and 0.08, respectively in Hardy-Weinberg disequilibrium. Observed (H_O) and expected heterozygosity (H_E), PIC, fixation index and Shannon information index revealed sufficient genetic variation in lysine variant of *DGAT1* gene. The genetic distance between Rathi and Sahiwal was observed less than Rathi and Kankrej cattle or lysine variants of *DGAT1* gene. SNP analysis reflected three transition and seven transversion mutation which revealed four different haplotype sequences. The evolutionary distance between any two lysine variant in indigenous cattle observed below mean distance and ranged between 0.000 to 0.01746. Different lysine variant of indigenous cattle shared different branches with other species or breed on phylogenetic tree. Six different conserved regions were identified among different identified alleles of *DGAT1* gene in studied breeds. Association analysis revealed significantly higher total milk yield (TMY) and milk protein content for *AG* genotype of *PRL* exon-3 and *AA* genotype of *PRL* exon-4 locus in all studied breeds whereas breed specific variable effect was observed for 305-day milk yield (305-day MY), daily milk yield (DMY) and lactation length (LL) in different parity. No significant effect of lysine variants of *DGAT1* gene was observed on different lactation traits however significant effect was observed on milk fat, protein and SNF. Thus the present study concluded the significant effect of prolactin gene on milk yield and protein percentage and *DGAT1* gene on milk fat, protein and SNF.

देशी गौवंशीय नस्लों में दुग्ध जीनों में आनुवांशिक विभिन्नताओं एवम् दुग्ध उत्पादन सम्बंधि गुणों पर उनके प्रभाव का अध्ययन

पशु आनुवांशिकी एवम् प्रजनन विभाग
पशु चिकित्सा एवं पशु विज्ञान महाविद्यालय
राजस्थान पशुचिकित्सा और पशु विज्ञान विश्वविद्यालय, बीकानेर–334 001

शोधकर्ता : **विजय कुमार अग्रवाल**
मुख्य समादेष्टाः **प्रो. जी.सी. गहलोत**

अनुक्षेपण

वर्तमान शोध में देशी गौवंशीय नस्लों में प्रोलेक्टिन जीन के एक्जान 3 व एक्जान 4 तथा डीगेट 1 जीन में आनुवांशिक विभिन्नताओं तथा दुग्ध उत्पादन एवम् दुग्ध संगठक सम्बंधि गुणों पर उनके प्रभाव का अध्ययन किया गया। राठी (75), साहीवाल (75) व कांकरेज (75) गौवंश के 225 दुधारू एवम् स्वस्थ पशुओं का रक्त व दुग्ध के नमूनों के लिए यादृच्छिक चयन किया गया। सभी चयनित पशुओं की दुग्ध संबंधि सूचना संकलित की गई। सभी पशुओं से असंक्रमित तरीकें से 2 मी.ली. रक्त लिया गया। सम्पूर्ण रक्त से स्पीन कालम तरीके से डी.एन. ए. प्राप्त किया गया। नैनो ड्राप स्पेकट्रोफोटोमीटर एवम् 0.8 प्रतिशत अगार जेल द्वारा प्राप्त डी. एन.ए. की मात्रा व गुणवत्ता की जांच की गई। जाति विशिष्ट प्राइमर द्वारा प्रोलेक्टिन एक्जान 3, प्रोलेक्टिन एग्जान 4 व डीगेट 1 जीन के क्रमशः 156 बी.पी. , 294 बी.पी. व 411 बी.पी. भाग को एम्पीफाई किया गया। आर एफ एल पी व एस एस सी.पी. तकनीक द्वारा चयनित जीनों के उक्त भागों की आनुवांशिक विभिन्नताओं का अध्ययन किया गया। जीन सिक्वेसिंग के बाद विभिन्न जैव सूचना तकनीक जैसे क्रोमा, बायोएडिट व कलस्टल डब्लू द्वारा एस.एन. पी की जाँच एवं विश्लेषण किया गया। उत्पादित जीन सिक्वेंस को एक्शेसन संख्या प्राप्त करने हेतु अंतर्राष्ट्रीय संस्था एन.सी.बी.आई. में जमा कराया गया। मेगा 7 साफ्टवेयर द्वारा एन जे प्रकार का जैव आनुवांशिक वृक्ष बनाया गया। चयनित पशुओं की आनुवांशिक संरचना व विभिन्नताओं का अध्ययन पॉपजीन द्वारा किया गया। एस.पी.एस. साफ्टवेयर द्वारा आनुवांशिक विभिन्नताओं का विभिन्न दुग्ध सम्बंधि गुणों पर प्रभाव का अध्ययन किया गया। प्रोलेक्टिन एक्जॉन 3 में आर एस ए एन्जाइम द्वारा जो जीनोटाईप ज्ञात हुए उनकी आवृत्ति 0.28 (जी.जी.) व 0.72 (ए.जी.) पायी गयी तथा जीन आवृत्ति 0.64 (जी) व 0.36 (ए) एलील हेतु पायी गयी। एस.एस.सी.पी. तकनीक द्वारा राठी के ए.जी. जीनोटाईप के दो प्रतिरूप सहित कुल 3 जीनोटाईप तथा साहीवाल व कॉकरेज में दो–दो कुल जीनोटाईप दिखाई दिए। विभिन्न तकनीकों द्वारा उचित मात्रा में इस जीन हेतु आनुवांशिक विभिन्नता देखने को मिली हांलाकि हार्डी–विनबर्ग साम्य अनुपस्थित मिला। राठी व साहीवाल में आनुवांशिक दूरी साहीवाल व कांकरेज के मध्य दूरी से

कम मिली। प्रोलेक्टिन एक्जॉन 3 में तीन ट्रांजिशन व एक ट्रान्सवर्जन के आधार पर कुल चार भिन्न हेप्लोटाइप बनाए गए। चयनित नस्लों के मध्य जैव दूरी माध्य दूरी से कम पायी गई तथा कुल चार संरक्षित क्षेत्रों की पहचान हुई। प्रोलेक्टिन एक्जॉन–4 में दोनों तकनीकों द्वारा ही तीन जीनोटाईप की पहचान हुई। जिनकी आवृत्ति 0.21, (जी.जी.), 0.56 (एजी) व 0.23 (एए) पायी गयी तथा जीन आवृति 0.49 (जी) व 0.51 (ए) पायी गयी। साहीवाल को छोड़कर सभी नस्लें हार्डी विन्बर्ग साम्य में पायी गयी। सभी नस्लों में उचित मात्रा में आनुवांशिक विभिन्नता देखने को मिली। हालांकि राठी में एफ. आई. एस. परिमाण अधिक पाया गया। साहीवाल व कॉकरेज के मध्य आनुवांशिक दूरी राठी व साहीवाल से कम दूरी पायी गयी। सात ट्राजिसन व तीन टान्सवर्जन एस.एन.पी. के आधार पर छह विभिन्न हेप्लोटाईप प्राप्त हुए। विभिन्न जीन एलील के मध्य दूरी 0.117 पायी गयी तथा छह संरक्षित क्षेत्र पाए गए। ई.ए.ई एन्जाइम द्वारा डीगेट 1 जीन के देखी नस्लों में एकल रूपी (के.के.) होने का पता चला। एस.एस.सी.पी. अध्ययन द्वारा पाँच प्रतिरूप ज्ञात हुए जिनकी जीनोटाईप आवृत्ति 0.32 (के1 के1), 0.16 (के 1 के 2), 0.19 (के 1 के 3), 0.16 (के 2 के 2) तथा 0.17 (के 2 के 4) तथा जीन आवृत्ति 0.49 (के 1), 0.33 (के 2) 0.10 (के 3) तथा 0.08 (के 4) पायी गयी। सभी नस्लों में हार्डी–विनबर्ग असाभ्य पाया गया। डीगेट 1 के प्राप्त लाइसिन प्रतिरूप के लिए उचित आनुवांशिक भिन्नता पाई गयी। राठी व साहीवाल के मध्य डीगेट 1 जीन हेतु आनवांशिक दूरी राठी व कॉकरेज के मध्य दूरी से कम पायी गई। एस.एन. पी विश्लेषण द्वारा तीन ट्रांजिसन तथा सात टांसवर्जन उत्परिवर्तन ज्ञात हुए जिसके आधार पर चार हेप्लोटाइप निर्मित किए गए। विभिन्न लाइसिन एलिल के मध्य दूरी 0. 00 से 0.01746 तक पायी गयी तथा छह संरक्षित क्षेत्रों की पहचान हुई। सहकारक विश्लेषण द्वारा कुल दुग्ध उत्पादन तथा दुग्ध प्रोटीन मात्रा पर प्रोलेक्टिन एक्जान 3 के ए जी जीनोटाईप तथा प्रोलेक्टिन एक्जान 4 के ए ए जीनोटाईप का विशिष्ट प्रभाव ज्ञात हुआ जबकि अन्य दुग्ध गुणों जैसे 305–दिन दुग्ध मात्रा, तथा दुग्ध स्रावक दिनों की संख्या में नस्ल अनुसार विभिन्नता देखने को मिली। डीगेट 1 जीन के विभिन्न लाइसिन प्रतिरूपों का का दुग्ध उत्पादन गुणों पर कोई प्रभाव नहीं दिखा यद्यपि दुग्ध वसा, प्रोटीन व एस.एन.एफ. पर विशिष्ट प्रभाव पाया गया। अतः वर्तमान शोध द्वारा देशी गौवंश में दुग्ध मात्रा व प्रोटीन पर प्रोलेक्टिन का तथा दुग्ध वसा, प्रोटीन व एस.एन.एफ. पर डीगेट 1 जीन का प्रभाव देखा गया।

APPENDIX-I

MOLECULAR BIOLOGICAL AND CHEMICALS USED IN THE STUDY

Acrylamide	Himedia
Agarose	SRL
Ammonium chloride	SRL
N, N'-methylene bis acrylamide	Sigma
Boric acid	Qualigens
Bromophenol blue	Himedia
dNTP's	Fermentas
Denaturing solution	Amresco
EDTA	Qualigens
Ethanol	Merck
Ethidium bromide	SRL
Formaldehyde	Qualigens
Formamide	SRL
Glacial acetic acid	SRL
Ladder DNA marker 100 bp	Bioenzyme
Ladder DNA marker 50 bp	Fermentas
Magnesium chloride	Fermentas
Oligonucleotide primers	Fermentas
Potassium chloride	Qualigens
Proteinase K	Fermentas
Sodium acetate	Qualigens
Sodium chloride	Qualigens
Taq DNA Polymerase	X celris
Taq DNA Polymerase buffer	X Celris
TRIS base	Qualigens
TRIS HCl	Qualigens
Methanol	Merck
Nuclease free water	Ambion
TRIS saturated phenol	SRL
50X TAE	Himedia

APPENDIX-II

THE MAJOR EQUIPMNTS USED IN THE STUDY ARE

1	Agar gel electrophoresis system	Bangalore Genei
2	Cyclomixer	Bangalore Genei
3	Deep freezer	Vest Frost
4	Dessicator	Techno instruments
5	DNA thermal cycler	Chino Scientific Limited
6	Drying oven	Emkay, India
7	Electronic balance	Sartorius Switzerland
8	Incubator	Galenkamp
9	Laboratory centrifuge	Remi, India
10	Laboratory incubator	Newtronic India
11	Microscope	Leitz
12	pH meter	Global, India
13	Refrigerator	Godrej, India
14	Refrigerated ultra centrifuge	Ohermile, Germany
15	Spectrophotometer	Shimadz, Japan
16	UV lluminator	Biometra TI-3

APPENDIX-III

COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

1. Ethidium bromide (10mg/ml)

Dissolved 100gm of Ethidium bromide in 10 ml of distilled water and store in a dark bottle at 4ºC.

2. Gel loading dye (6X)

Bromophenol Blue (0.22%)	50mg
Xylene cyanole (0.25%)	50mg
Sucrose (40%)	8g

Stir well in 20ml distilled water and store at 4ºC.

3. Proteinase-K

Proteinase-K	20mg
Distilled water	1ml

4. RBC lysis buffer

Ammonium chloride (150mM)	8.0235g
Potassium chloride (10mM)	0.7455g
EDTA (0.1mM)	0.0372g

Add distilled water up to 1000ml, stir, filter, autoclave and store at 4ºC.

5. Sodium acetate (3M, pH 5.5)

For 100 ml, dissolve 40.824g of sodium acetate in 70ml of distill water. Adjust pH to 5.5 with glacial acetic acid. Make up the vlume to 100ml, autoclave and store at 4ºC.

6. Tris-Borate-EDTA (TBE) buffer (pH 8.3) 10X

Tris	108.0g
Boric acid	55.0g
EDTA	9.3g

Dissolve in 700ml distilled water. Adjust the pH to 8.3. Make up the volume to 100ml, autoclave and store at 4ºC.

7. 6X Gel loading dye

Bromophenol blue	0.25%
Xylene cyanole	0.25%
Sucrose in water	40%

Mix and store at 4ºC.

8. 10% Ammonium persulphate (APS)

APS	100mg
ddH_2O	1ml

Store at -20 ºC.

Fig. 1.1 Rathi, Sahiwal and Kankrej cattle under farm conditions

Rathi

0.82% of total indigenous cattle
Pure=70% Graded=30%

Sahiwal

3.23% of total indigenous cattle
Pure=22.38% Graded=77.62%

Kankrej

2% of total indigenous cattle
Pure=64.24% Graded=35.76%

Fig. 4.1 Genomic DNA of studied breeds

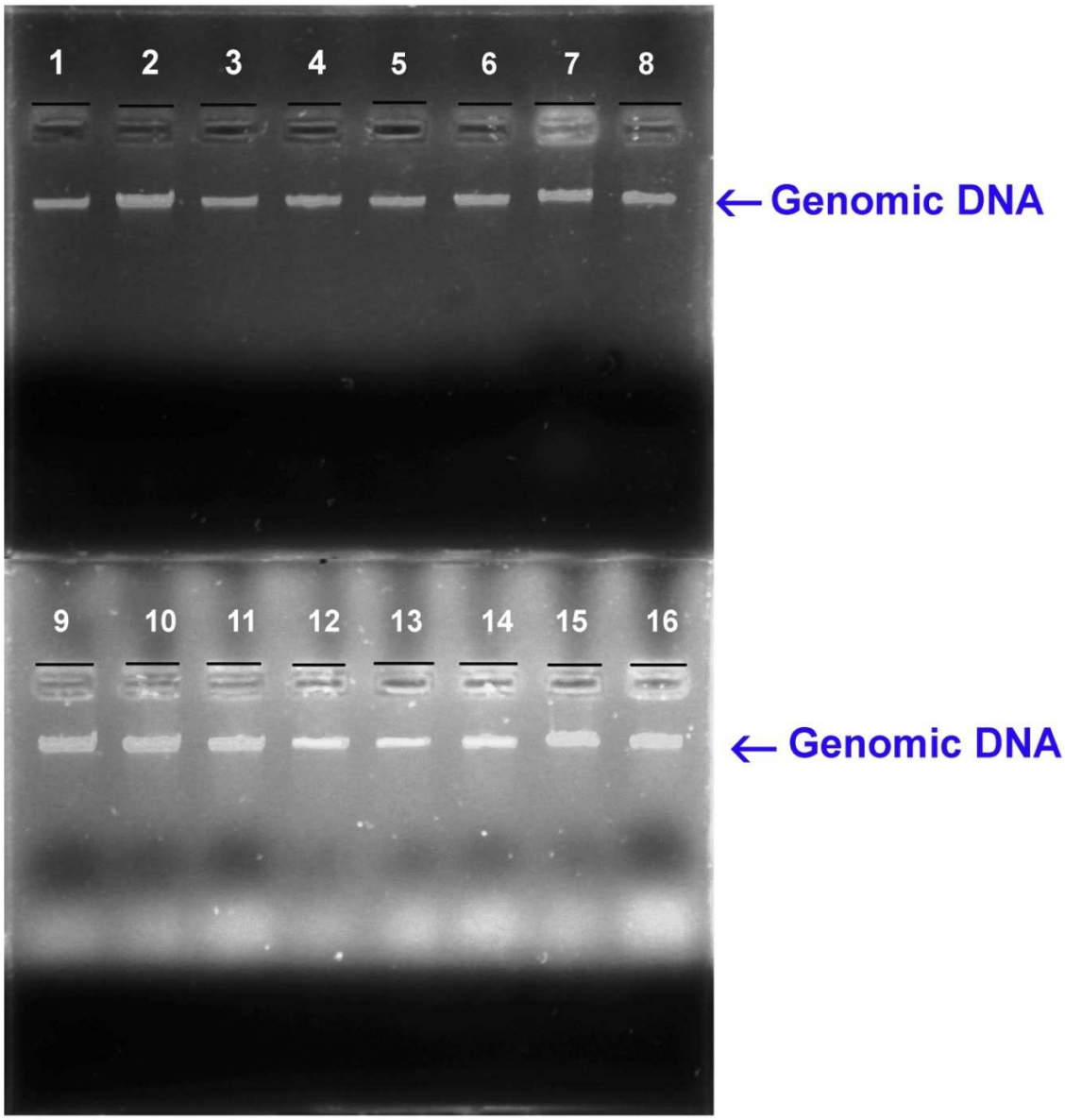
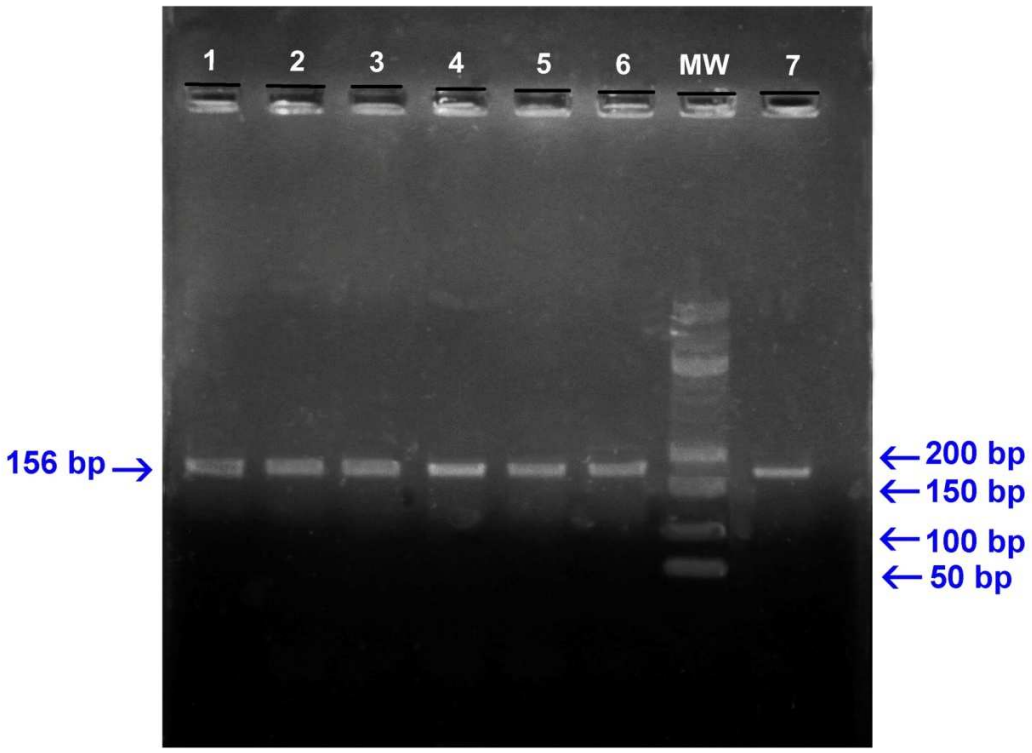
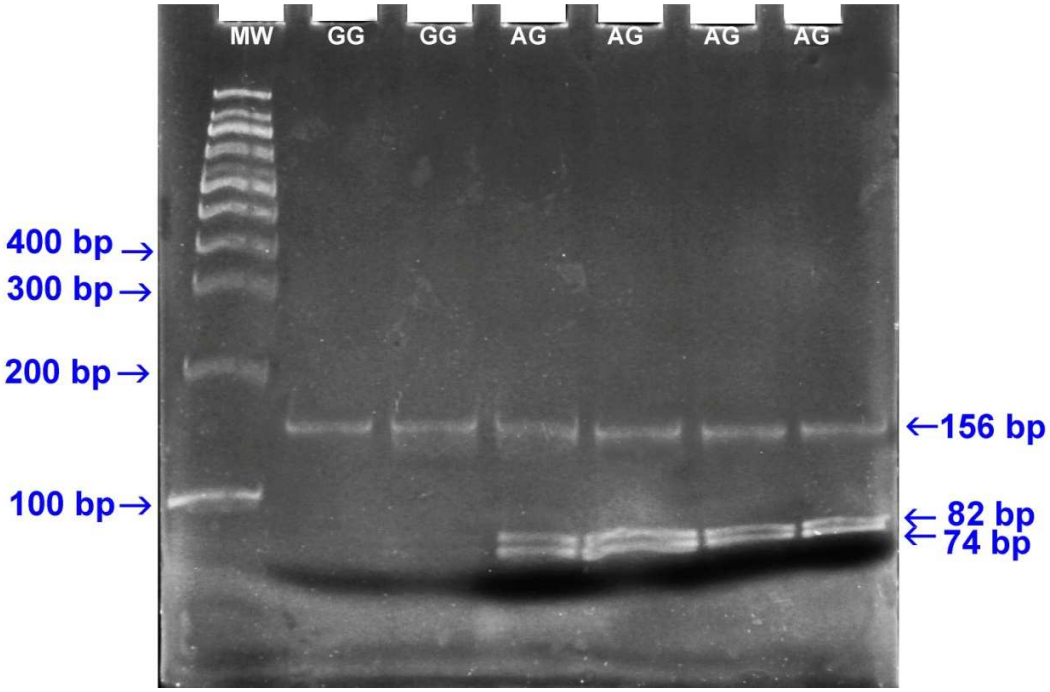


Fig. 4.2 PCR amplicons of exon-3 of *PRL* gene



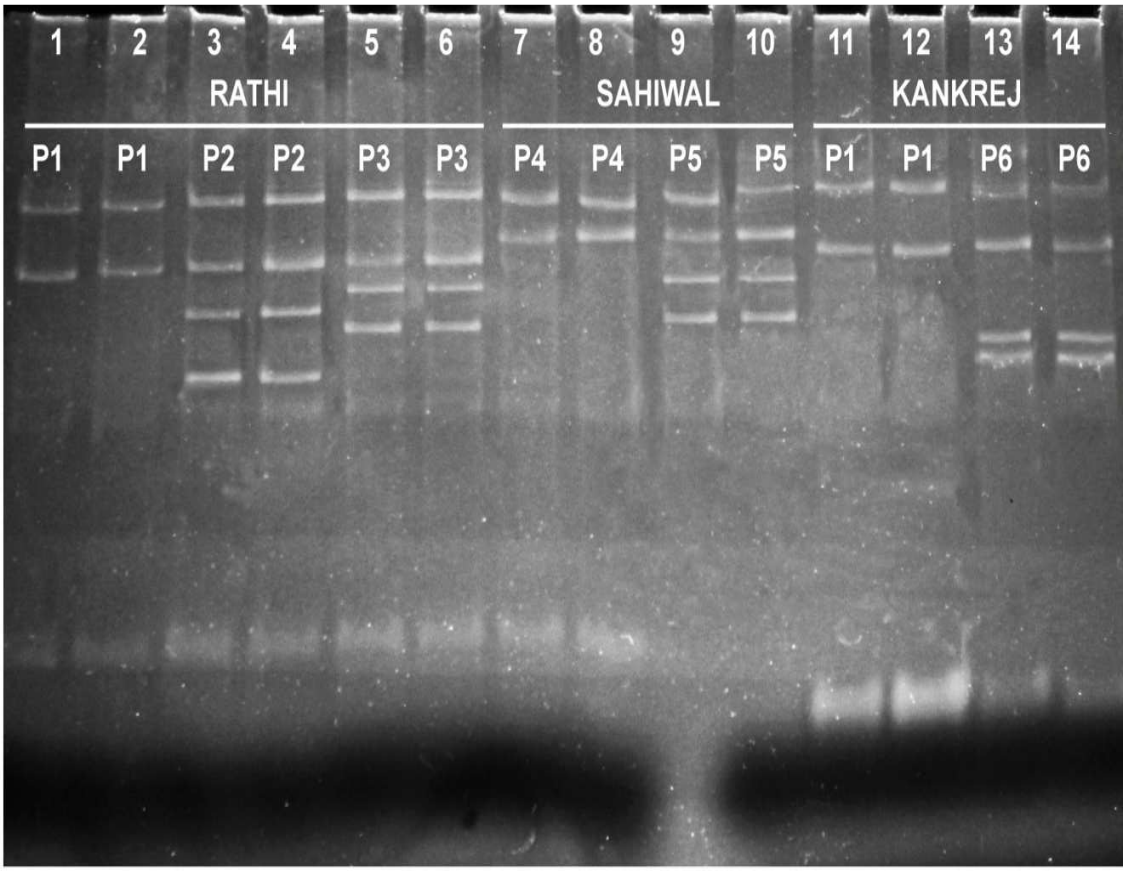
Lane 1- 7: PCR Amplicons of 156 bp, MW: Molecular weight marker (50 bp ladder)

Fig. 4.3 Restriction fragment analysis of exon-3 of *PRL* gene



Lane 1: Molecular weight marker, Lane 2-3: genotype *GG*, Lane 4-7: genotype *AG*

Fig. 4.4 SSCP analysis of exon-3 of *PRL* gene



Lane 1-6: SSCP patterns in Rathi breed

Lane 7-10: SSCP patterns in Sahiwal breed

Lane 11-14: SSCP patterns in Kankrej breed

Fig. 4.5 Multiple sequence alignment of exon-3 of *PRL* gene

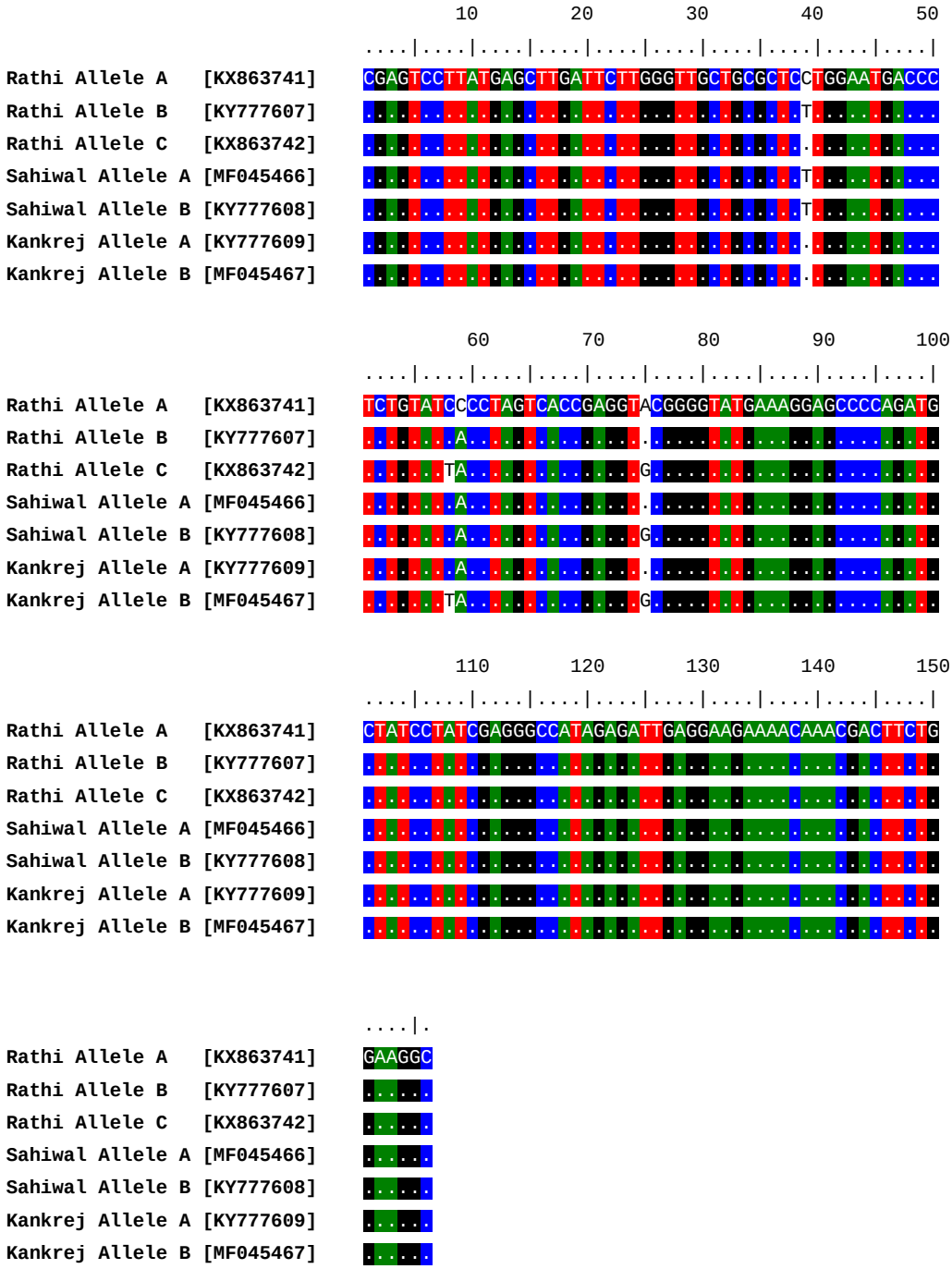


Fig 4.6 Haplotypes of exon-3 of *PRL* gene based on nucleotide positions and gene frequency estimates of different *Rsa* I genotypes

PRL Exon-3 region	Base position	Base substitution*				
Exon-3	75	A	A	A	G	G
Exon-3	59	C	A	A	A	A
Exon-3	58	C	C	C	T	C
Exon-3	39	C	T	C	C	T

Breeds	A1	A2	A3	G1	G2	n	Gene frequency of *Rsa*I allele
							0.2 0.4 0.6 0.8 1.0
Rathi						75	
Sahiwal						75	
Kankrej						75	

*****Based on sequence in GenBank, accession number NM173953

Haplotype were named according to base present on restricted site

Fig. 4.7 NJ Phylogenetic tree of exon-3 of *PRL* gene

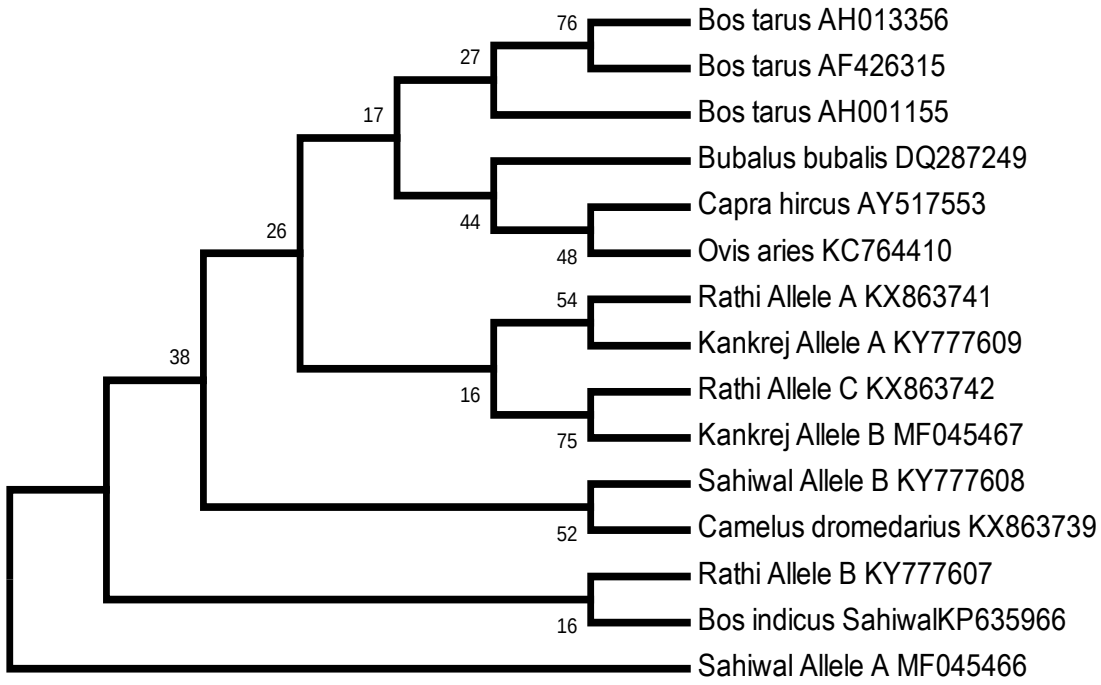
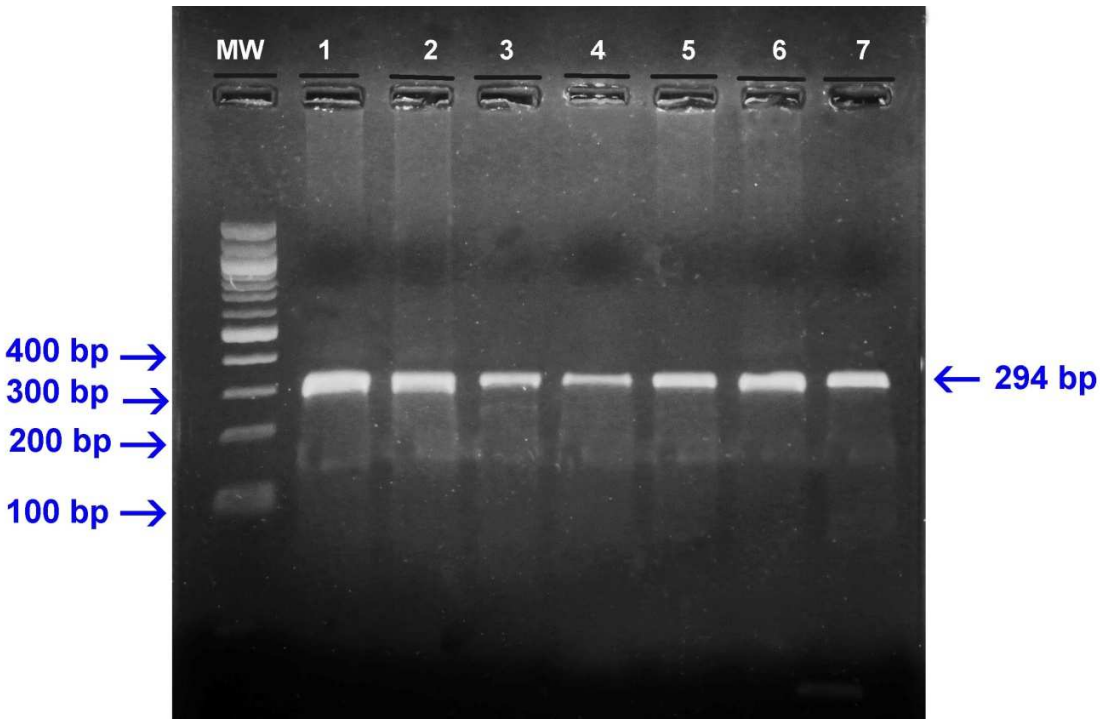
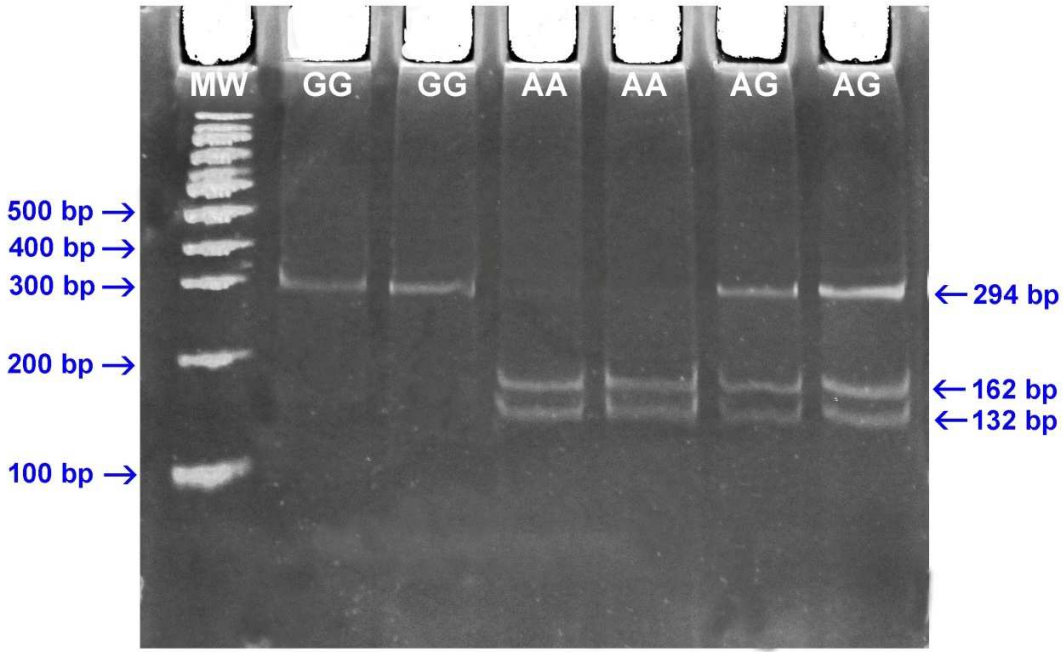


Fig. 4.8 PCR amplicons of exon-4 of *PRL* gene



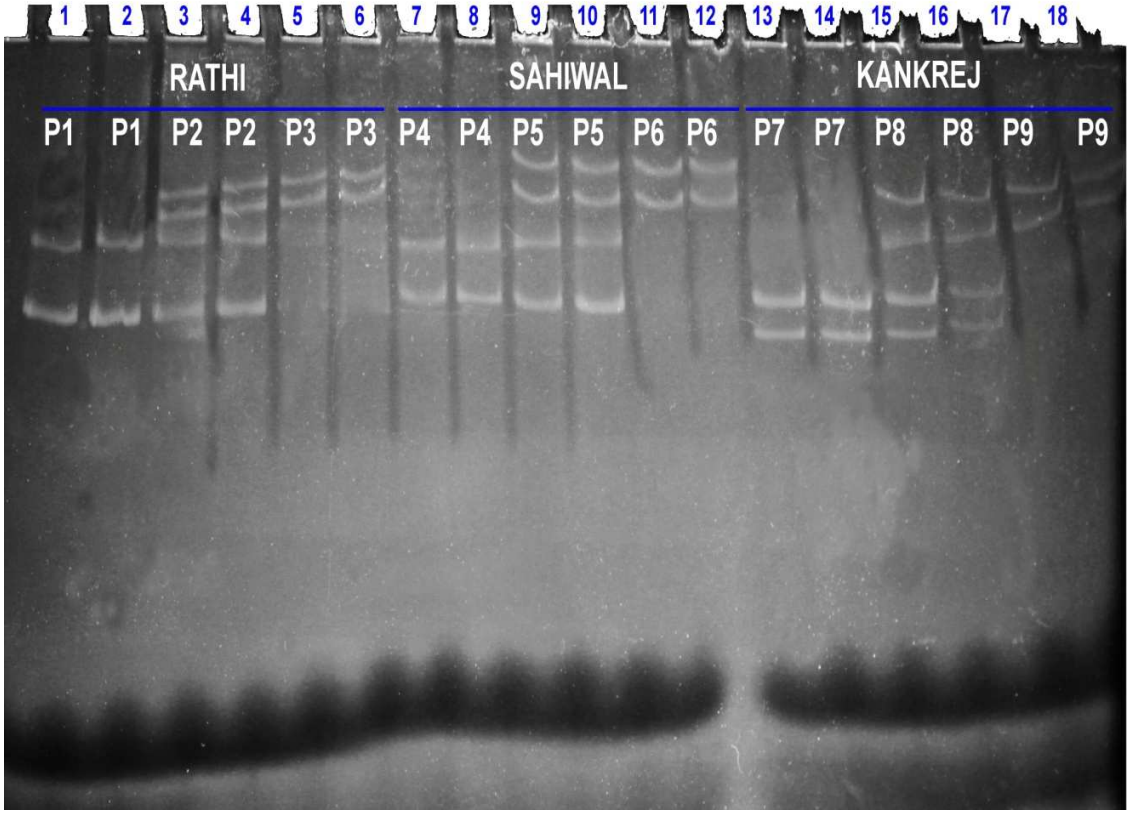
MW: Molecular weight marker, Lane 1- 7: PCR Amplicons of 294 bp

Fig. 4.9 Restriction fragment analysis of exon-4 of *PRL* gene



Lane 1: Molecular weight marker, Lane 2-3: genotype *GG*, Lane 4-5: genotype *AA*, Lane 6-7: genotype *AG*

Fig. 4.10 SSCP analysis of exon-4 of *PRL* gene



Lane 1-6: SSCP patterns in Rathi breed

Lane 7-12: SSCP patterns in Sahiwal breed

Lane 13-18: SSCP patterns in Kankrej breed

Fig. 4.11 Multiple sequence alignment of exon-4 of *PRL* gene

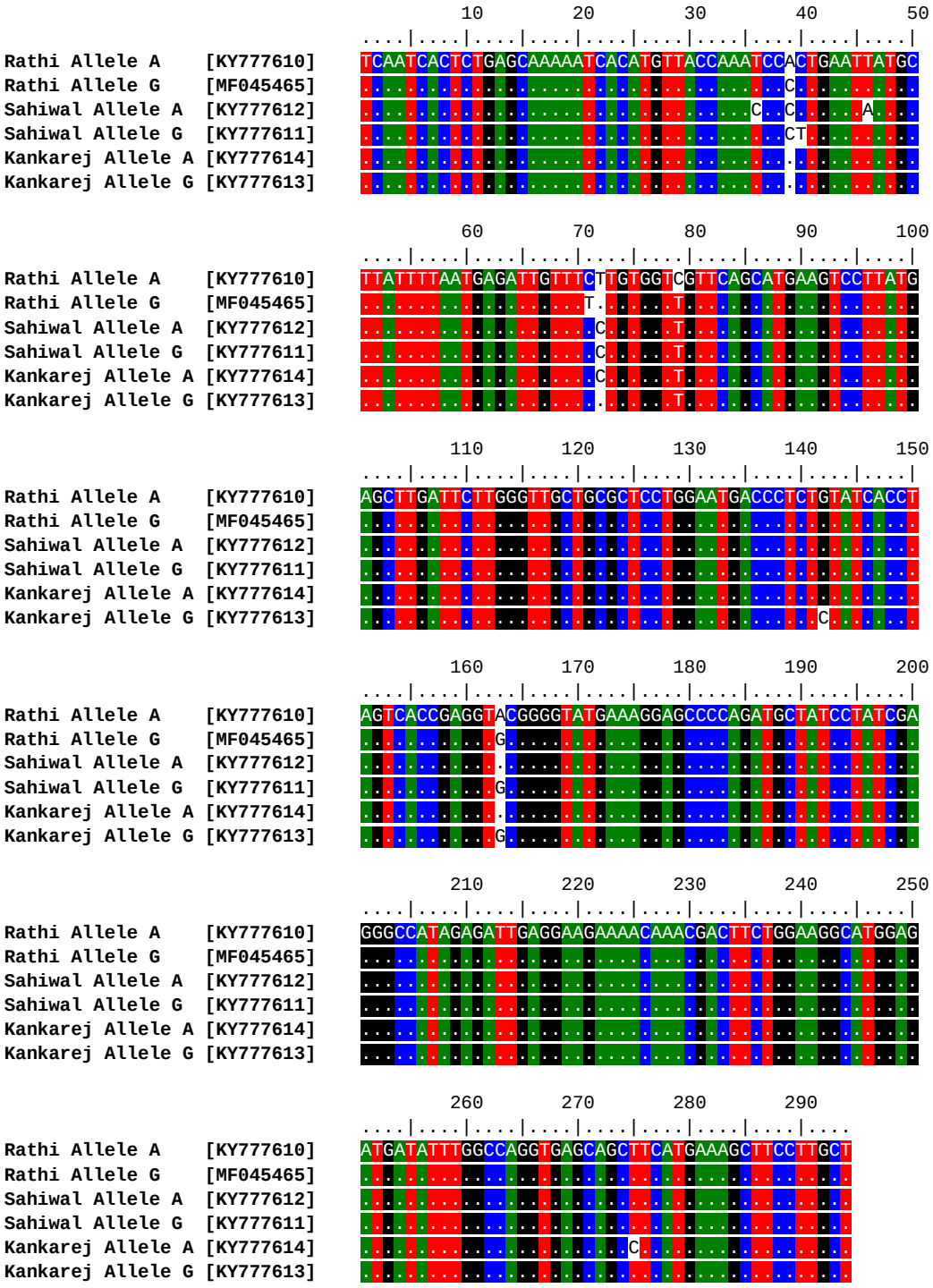


Fig 4.12 Haplotypes of exon-4 of *PRL* gene based on nucleotide positions and gene frequency estimates of different *Rsa* I genotypes

PRL Exon-4 region	Base position	Base substitution*					
Intron-4	275	T	T	T	T	T	C
Exon-4	163	A	A	A	G	G	G
Exon-4	142	G	G	C	G	G	G
Intron-3	79	C	T	T	T	T	T
Intron-3	72	T	C	T	T	C	C
Intron-3	71	C	C	C	T	C	C
Intron-3	46	T	T	T	T	A	T
Intron-3	40	C	T	C	C	C	C
Intron-3	39	A	C	A	C	C	A
Intron-3	36	T	T	T	T	C	T

Breeds	A1	A2	A3	G1	G2	G3	n	Gene frequency of *Rsa*I allele (0.0 0.2 0.4 0.6 0.8 1.0)
Rathi							75	
Sahiwal							75	
Kankrej							75	

*****Based on sequence in GenBank, accession number AF426315

Fig. 4.13 NJ Phylogenetic tree of exon-4 of *PRL* gene

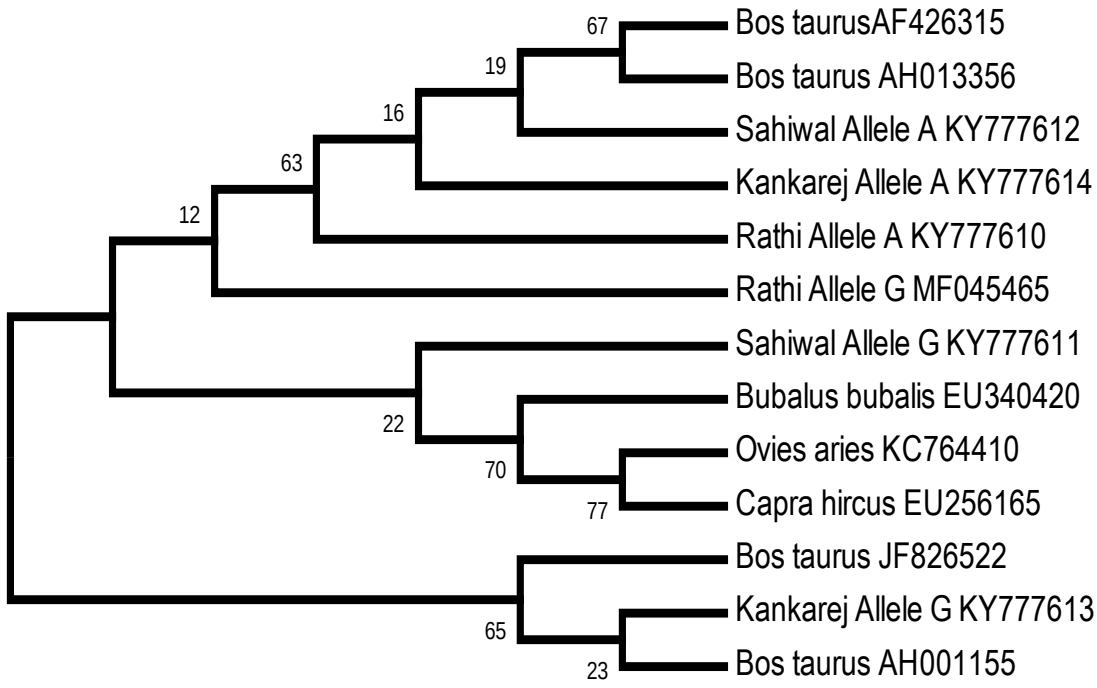
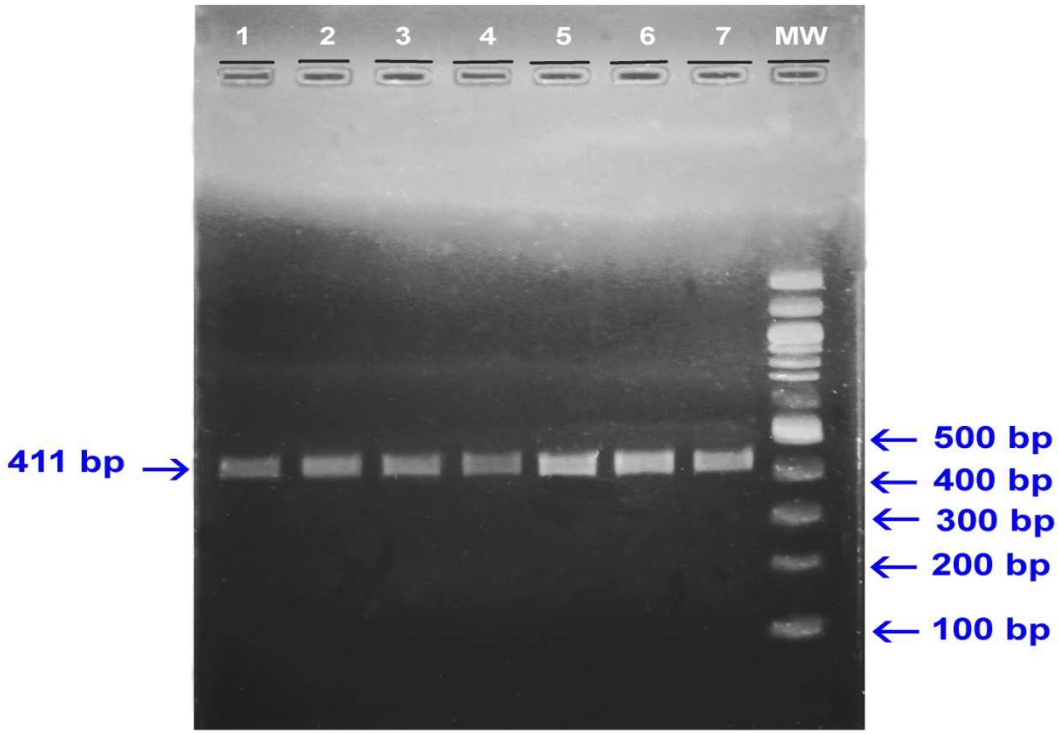
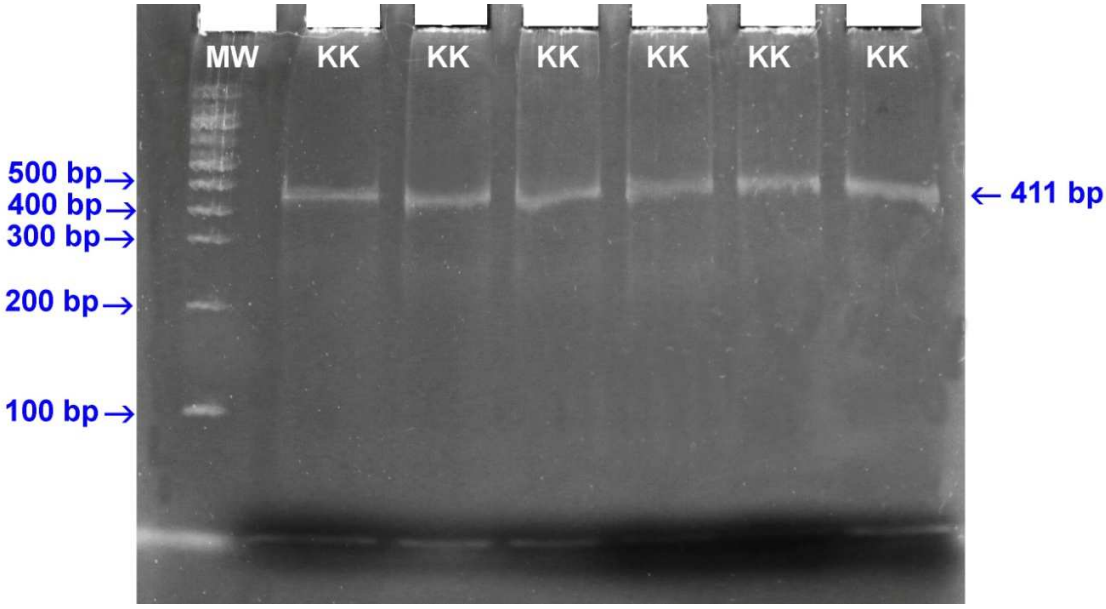


Fig. 4.14 PCR amplicons of exon-8 of *DGAT1* gene



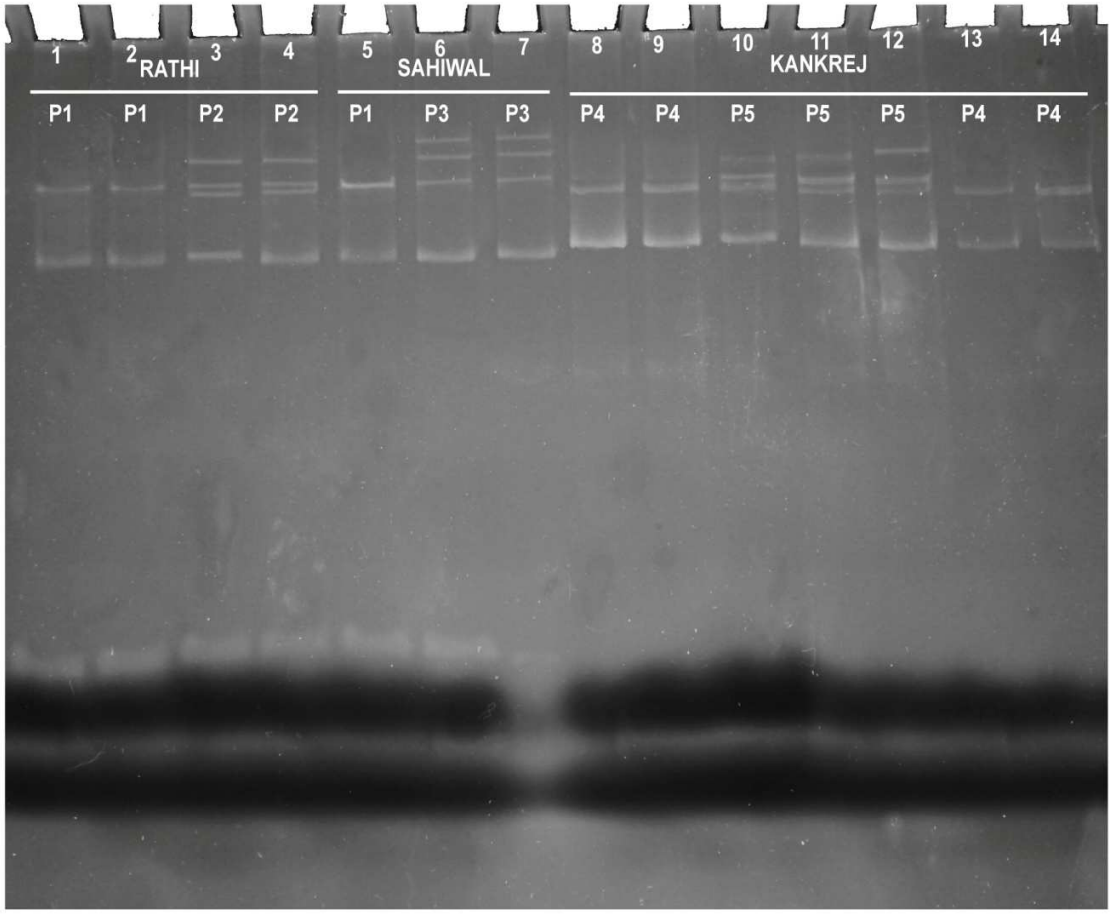
Lane 1- 7: PCR Amplicons of 411 bp, MW: Molecular weight marker

Fig. 4.15 Restriction fragment analysis of exon-8 of *DGAT1* gene



Lane 1: Molecular weight marker, Lane 2-7: genotype *KK*

Fig. 4.16 SSCP analysis of lysine variants of *DGAT1* gene



Lane 1-2:	SSCP pattern P1 (*K1K1*) in Rathi breed
Lane 3-4:	SSCP pattern P2 (*K1K2*) in Rathi breed
Lane 5:	SSCP pattern P1 (*K1K1*) in Sahiwal breed
Lane 6-7:	SSCP pattern P3 (*K1K3*) in Sahiwal breed
Lane 8,9,13,14:	SSCP pattern P4 (*K2K2*) in Kankrej breed
Lane 10,11,12:	SSCP pattern P5 (*K2K4*) in Kankrej breed

Fig. 4.17 Multiple sequence alignment of *DGAT* 1 gene

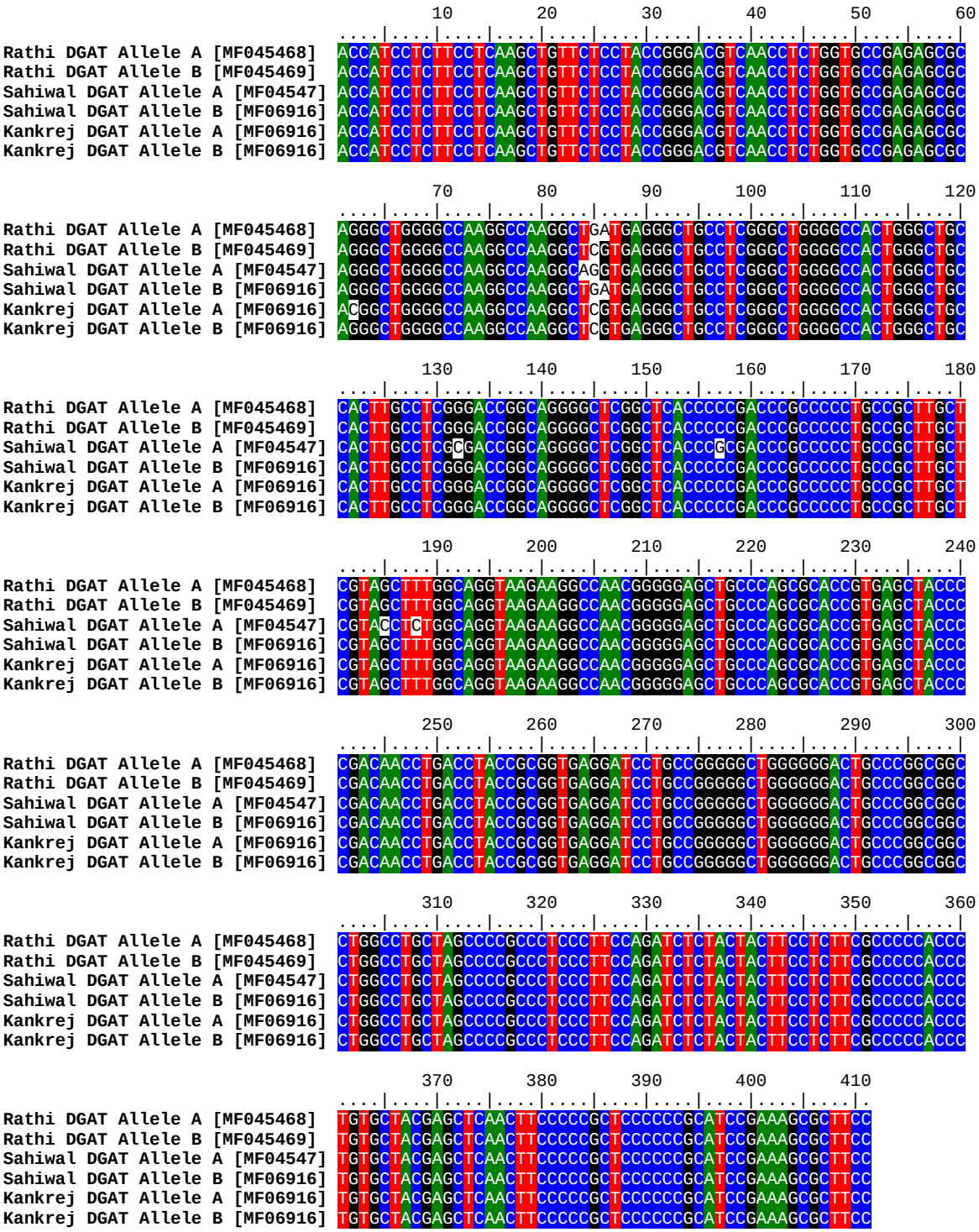


Fig 4.18 Haplotypes of lysine variants of *DGAT1* gene based on nucleotide positions and gene frequency estimates of different *Eae* I genotypes

DGAT1 region	Base position	Base substitution*			
Exon-8	201	A	A	A	A
Exon-8	200	A	A	A	A
Exon-8	188	T	T	C	T
Intron-7	185	G	G	C	G
Intron-7	157	C	C	G	C
Intron-7	132	G	G	C	G
Intron-7	86	A	G	G	G
Exon-7	85	G	C	G	C
Exon-7	84	T	T	A	T
Exon-7	62	G	G	G	C

Breeds	K1	K2	K3	K4	n	Gene frequency of lysine variants (0.2 / 0.4 / 0.6 / 0.8 / 1.0)
Rathi	■	■			75	
Sahiwal	■		■		75	
Kankrej		■		■	75	

*Based on sequence in GenBank, accession number AJ 318490

Fig. 4.19 NJ Phylogenetic tree of *DGAT1* gene