ASSESSMENT OF GENETIC DIVERSITY IN INDIGENOUS SHEEP BREEDS OF RAJASTHAN USING MOLECULAR MARKERS

आण्विक मार्कर के उपयोग द्वारा राजस्थान की स्वदेशी भेड़ नस्लों में आनुवांशिक विविधता का मूल्यांकन

KRITIKA GAHLOT
M.Sc.

THESIS

DOCTOR OF PHILOSOPHY
(ANIMAL BIOTECHNOLOGY)

।पशुधनं नित्यं सर्वलोकोपकारकम्।

2017

Department of Veterinary Microbiology and Biotechnology
College of Veterinary and Animal Science, Bikaner
Rajasthan University of Veterinary and Animal Sciences
Bikaner – 334001

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THESIS

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

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(ANIMAL BIOTECHNOLOGY)

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By

KRITIKA GAHLOT
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2017

Rajasthan University of Veterinary and Animal Sciences, Bikaner
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ABBREVIATIONS

%	Percent
APS	Ammonium per sulphate
bp	base pair
dNTPs	deoxynucleoside triphosphates
EDTA	Ethylene diamine tetra acetic acid
FAO	Food and Agriculture Organisation
G	Gauge
g	gram (weight)
cm	centimetre
l	litre
µg	microgram
µl	microliter
mg	milligram
mM	milliMolar
M	Molar
ng	nanogram
OD	optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
pmole	picomole (s)
PIC	Polymorphic Information Content
rpm	rotations per minute
TBE	Tris Borate EDTA
TE	Tris EDTA
TEMED	N,N,N’,N’-tetramethylethylenediamine
U	Units
UV	Ultra Violet light
V	Volt
W	Watt
±	plus and minus
°C	Degree Celcius

A	Absorbance
F_{IS}	Coefficient of Inbreeding
H	Heterozygosity
H_o	Observed heterozygosity
H_e	Expected heterozygosity
MNA	Mean Number of Alleles
TBE	Tris Borate EDTA
Na	Observed Number of Allele
Ne	Effective Number of Allele
F	Fixation Index
PA	Private allele
RFLP	Restriction Fragment Length Polymorphism
RAPD	Randomly amplified polymorphic DNA
AFLP	Amplified Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
VNTR	Variable Number of Tandem Repeat
KAP	Keratin-Associated Proteins
DNA	Deoxyribonucleic acid
HWE	Hardy-Weinberg Equilibrium

1. INTRODUCTION

India has a diverse livestock population spread over large areas of having varied ecology and climate. Among the 15 species (non-carnivorous) domesticated in the world till date, India possesses 11 which include all major species of farm animals. In the recent past, majority of the registered livestock breeds of India has been characterized and documented. There are 144 registered indigenous breeds in the country, which include 37 for cattle, 13 for buffalo, 23 for goat, 39 for sheep, 6 for horses and ponies, 8 for camel, 2 for pig, 1 for donkey and 15 for chicken.

As the gene pool of sheep is quite diverse with 62.5 million (12.7% of the total livestock of India) sheep population ranks third in the world with 5.79% of the world sheep population (FAO, 2003). Sheep rearing is the major source of livelihood for small and marginal farmers and landless laborers in hilly areas, arid, semi-arid and east coastal region. According to the FAO World Watch List (2000), there are 60 breeds of sheep in India. This list involves both well-documented and lesser-known breeds, including some wild species. Although there are about 42 descript breeds of sheep in India, (NBAGR, 2015-16) and majority of sheep population (around 75%) does not belong to any of the defined breeds. The adaptability of these breeds to various regions depicts variation in the gene pool and this variation is the base for preservation of germplasm of sheep breeds. However, the genetic diversity of sheep breeds is decreasing due to the breed substitution/crossbreeding and this deserves severe consideration to discover various approaches to conserve it at the highest level. Rajasthan, ranks 3rd with a total of 11.24 million sheep population in India. There are eight major sheep breeds in Rajasthan namely Magra, Marwari, Chokla, Jaisalmeri, Malpura, Sonadi, Pugal and Nali are being reared.

1.1 Conservation for Sheep Breeds

At present, the sheep population comprises different numbers of breeds with respect to phenotypic characteristics. Some of these breeds such as Merino and Rambouillet have been chosen for the lean meat as well as fleece generation. These breeds can be considered as monetarily important breeds. However, there are some other breeds, which are not selected for one particular product, but rather they have predominance in survival under stressed environmental conditions. These are called relatively primitive breeds and Rajasthan local sheep breeds are an example of these types of breeds. As a general pattern in the world, economically significant breeds are threatening the primitive ones either through cross breeding with the economically important breeds; or through replacement of the primitive breeds as farmers stop raising the primitive ones. Therefore, high production parameters of monetarily significant breeds rely upon the current environmental conditions and good maintenance of the breeds. They developed in the fields far from the regions of domestication centers and should harbor low levels of diversity. Moreover, as being away from natural ecological conditions (since they are in a technology rich environment), possibly they lack local adaptations to environmental conditions. Even so, for the future of livestock supply, we need breeds with high genetic diversity, which have the power to accommodate environmental changes. Consequently, appropriate steps are required to prevent genetic erosion of the animal genetic resources and to salvage the heritage for future generations (FAO, 2006). To be more particular, in order to make the worldwide important animal food resources, sustainable, the genetic diversity must be maintained with respect to the development of local adaptations to the altering environmental conditions. FAO reported that in the next 20 years, 32% of sheep breeds are expected to go extinct. In a recent survey, it was contended that the breeds at the centers of domestication exhibiting high genetic diversity should have highest priority for conservation (Tapio *et al*., 2010). Conservation of primitive breeds requires considerable amount of economy. Data

on genetic variability among breeds may help in the improvement of breeding programs and is essential for preservation of hereditary assets and germplasm (Taberlet *et al*., 2011).

1.2 Microsatellite markers in relation to genetic diversity analysis

Advancements in the field of molecular biology provided new genetic DNA based markers (Microsatellite, RFLP, AFLP and SNP) to study the genetic diversity of animals and replaced allozymes for lessening the shortcomings; like non-neutrality and low allelic variation. Development of the Polymerase Chain Reaction (PCR) technique in 1983 by Kary Mullis, modernized the genetic diversity studies. With the help of this technique, the specific region of the DNA (locus) can be selectively amplified from a small amount of sample. A comparative study of the amplified region between the individuals of a population reveals a base for determination of genetic diversity within populations. It is be noted that there were regions of the genome composed of repeated units of 1-6 bp in length, with a typical copy number of 10-30. For example, "CCA" unit made out of three bases could be repeated 7-15 times in the chromosome extend of the individuals. This type of molecular marker known as microsatellites or short tandem repeats (STRs) that have alleles at a specific location (locus) can differ in the number of repeats and follows Mendelian inheritance. They are highly polymorphic and well distributed in the genome rather than protein loci, so they preferred in measuring the diversity of breeds. Subsequently, the diversity measure covers the genome yet they are typically not within the coding regions of genes. Therefore, microsatellite-based variations are neutral and variation in these neither loci influenced by artificial nor natural selection. Thus, microsatellite loci deliver unbiased knowledge about the genetic diversity of a genome (Jobling *et al*., 2004).

The mutation rates of microsatellites are evaluated approximately 10^{-3}-10^{-4} for each locus for every generation. This makes microsatellites valuable for considering evolution over brief time spans as it is for livestock populations

(hundreds or thousands of years), though nuclear base pair substitutions are more helpful for analyzing the evolution over a long time range that is millions of years. The profoundly polymorphic nature of microsatellites provides all essential information (Schlötterer, 2000; Goldstein and Shlötterer, 2000) in the area of genetic research for example, relationships among closely associated species and also within single species (Bowcock *et al*., 1994), paternity and kinship determination in forensic studies (Edward *et al*., 1992), in linkage analysis (Francisco *et al*., 1996; Mellersh *et al*., 1997) as well as in the reconstruction of phylogenies (Bowcock *et al*., 1994).

Consequently, microsatellites became a touchstone in determining the neutral genetic diversity in livestock; however it suffers some disadvantages like having the high risk of null alleles, interpretation difficulties (such as a subjective genotyping) and size homoplasy (Peter *et al*., 2007). Although microsatellite markers have high degree of polymorphism and codominance, making them extraordinarily informative for discrimination of breeds (Vaiman *et al*., 1996). It is widely used to assess the genetic diversity and population structure amongst the species for ex-situ & in-situ conservation procedures (Kantanen *et al*., 2000).The list of microsatellite loci covering the most informative ones formed and recommended by FAO and ISAG (International Society for Animal Genetics) (FAO, 2004).

Microsatellite markers are the most extensively used markers for diversity analysis in livestock breeds, as they combine high variability with co dominant inheritance and can be easily typed (Litt and Luty, 1989). Microsatellites have been widely used to characterize sheep breeds of several countries (Baumung *et al*., 2006; Peter *et al*., 2007; Cinkulov *et al*., 2008; Legaz *et al*., 2008; Dalvit *et al*., 2008). Microsatellite analysis of the Nali, Chokla (Sodhi *et al*., 2006), Marwari and Magra (Arora and Bhatia, 2006) breeds has been reported previously, but a comparative study of all four breeds with selected 18 microsatellite markers using a range of statistical methods has not yet been

described. Their unique characteristics and decreasing numbers make an analysis of their genetic diversity and relationships urgent. This study investigates the prevailing status of genetic diversity of the four sheep breeds namely Chokla, Nali, Marwari and Magra using microsatellite markers. The information generated will have a direct impact on the genetic management and conservation of breeds adapted to the arid region.

Besides the use of Microsatellite markers for characterization of sheep breeds, wool genes can also be used for identification of breeds as a molecular marker. Wool fiber is a highly organized structure, whose main histological components include the cuticle, the cortex (orthocortex, paracortex, and mesocortex), and the medulla (Plowman *et al*., 2009). Almost all of the fiber (90%) is composed of the cortex, which consists of keratin intermediate filament protein (KRT) embedded in a matrix of keratin-associated proteins (KAPs) (Kuczek and Rogers, 1987; Itenge-Mweza *et al*., 2007). KAPs cross-link the keratin intermediate filaments with inter-chain disulfide bonding, and form the wool fiber after keratinization (Gong *et al*., 2011). These proteins are the major protein constituents that make up the wool fiber.

These candidate genes used for identification of sequence variation in the sheep breeds, as they are highly polymorphic and showing variation between the breeds. A large amount of variation has observed in these proteins, and it is notable that such heterogeneity is still present despite of selective breeding to improve the quality of the wool fiber. Many studies have reported polymorphism in members of the keratin and KAP gene families. Furthermore, there have been some reports associating variation in the keratin and KAP loci with variation in fiber diameter, staple strength and wool color and brightness.

In the light of the above, objectives of the present study have chosen as follows:

1. **To select a suitable set of DNA markers for determining genetic diversity of different sheep breeds of Rajasthan.**
2. **To assess genetic variability within and between the sheep breeds using Molecular Markers.**
3. **To identify sequence variation of candidate wool genes in the sheep breeds of Rajasthan *.**

Note: * looking into the importance of sequence variation in wool trait genes, this objective has been included in this research work.

2. REVIEW OF LITERATURE

Domestic animals have played a central role in the welfare of human civilization. Sheep are most likely successor from the Asiatic mouflon (O. orientalis) in the Fertile Crescent (Zeder, 2008). Archaeological evidence of Iran proposed that selection of wooly sheep might have started around 6000 BC (Ensminger and Parker, 1986; Weaver, 2005). However, the earliest woven fleece fabric has just been dated to a few thousand years later (Smith *et al*., 1997). During the period of Bronze era, sheep with all the significant features of modern breeds were widespread throughout Western Asia (Ensminger and Parker, 1986). Environmental pressure as well as different agro-ecological zones shaped phenotypic variation that has left genetic footprints of the breeds (Lv *et al*., 2014). Molecular genetics, which also includes endogenous retrovirus and mitochondrial DNA, confirmed that the naval trade and colonization had a major impact on sheep movement in the Mediterranean areas (Pedrosa *et al*., 2007; Chessa *et al*., 2011). Molecular genetic studies combined with the accessible archaeological evidence suggested that domestic sheep with desired secondary characteristics, which commonly present in the modern breeds occurred first in South-West Asia and then spread successfully in Europe, Africa and the rest of Asia. Firstly, sheep utilized for meat and later on specialization for fleece and milk started. A great number of native breeds (above 1,400) have been shaped worldwide because of the fundamental evolutionary forces of mutation, selective breeding, adaptation, isolation and genetic drift brought on by human intervention together with the environmental effect. An adaptation of the breeds due to human and geographic impact is well studied (FAO, 2007). Furthermore, the exchange of hereditary material has increased significantly in the recent years. The Sheep are widely distributed species among all livestock. Therefore, the widely practiced breed combination resulted in about 443 well-documented composite sheep population worldwide in 68 nations (Shrestha, 2005).

2.1 Sheep genetic resource of India

Sheep comprises one of the most significant domestic animals species in India and plays a critical role in the livelihood of a large proportion of small and marginal farmers and landless laborers. These are well adjusted to the environment as well as disaster of numerous kinds, such as drought, famine and civil wars that frequently torment the continent. Their adaptive elements empower them to successfully cope with the stressful environment of marginal lands (Chenyambuga, 2002). According to the 2012 census, the country had 65.06 million sheep population ranking third in the world and has about 5% of the world population. About 4 million tons of mutton, 43.2 million kg of wool and around 40 million kg of skins produced annually in the country. According to the Food and Agriculture Organization (FAO, United Nations) and World Watch list (2000) there exist sixty breeds of sheep in India. The list includes both well-recognized and lesser-known breeds along with some wild species. Because of unpredictable crossbreeding, greater amount of intermixing and niche obliteration more than 50% of India's sheep breeds are presently under threat (Bhatia and Arora, 2005). The 42 breeds dispersed in various agro-climatic zones of the nation reflect the sheep diversity in India. Indian breeds have emerged through natural selection for adaptation to specific agro-ecological conditions such as northern temperate climate of the entire northern hilly region (as low as 40°C) to hot/sub-temperate humid climate of eastern region, semi-arid in the central peninsula and hot-humid along the coastal area of the southern peninsular region, and dry hot climate of Rajasthan desert (more than 45°C) of the northwestern-arid and semi-arid zone. Most of these Indian breeds are very well adapted to the harsh climate, long migration, tropical diseases, malnutrition and the shortage of drinking water. Unpredictable crossbreeding, uncontrolled intermixing and topographical revamping have prompted to danger and put to risk potentially critical ovine genetic material. Consequently, conservation of indigenous ovine germplasm has perceived as an increasing national concern.

2.2 Importance of domestic sheep diversity

The spectrum of genetic diversity existing among species, breeds and populations of all animal species, which have been domesticated and their immediate wild relatives has been well defined by Hammond (1993). It has developed during the millions of years of evolution to shape and balance out every species. Globally, there are around 40 livestock species (FAO, 1995), which have been tamed by humans over the last 12,000 years. During domestication, however, distinct and genetically unique breeds and strains have developed within each species as an aftereffect of human development and occupation. Variations among the breeds have formed by reproductive isolation, frequently enforced by the human through the pursuit of various breeding objectives and physical partition for a different period. Because of physical separation, each breed/strain has adjusted to the specific ecological condition to suit the nearby atmosphere and the necessities of the community. Therefore, in the conservation of tamed species, it is the variation between breeds in spite of the variation between species, which is of significant importance (Hammond, 1993; Barker, 1994).

Sheep biodiversity in India characterized by a high degree of endemism and variations in agro-climatic conditions of the different regions have led to the development of various breeds/strains that are well adapted to a specific set of environmental conditions. These breeds have generally named after their place of origin and some based on their prominent characteristics. A few breeds have evolved from the base populations created by crossing native and fine wool exotic breeds. Indigenous sheep contribute greatly to the agrarian economy, especially in areas where crop and dairy farming are not economical. However, intermixing of nearby breeds, an introduction of exotic breeds and change in the farming system has resulted in the decline in purebred population and in dilution of genetic merit. No efforts could make until ninety's for the conservation of sheep genetic resources in the country. Although, conservation of genetic resources in developing countries is far more complex, because, in a vast majority of the cases,

information about available genetic resources, their usefulness and methods of conservation are not clear. Determination of status and characterization of indigenous sheep genetic resources is essential for planning, domestic animal diversity conservation plans (DAD-IS, FAO). It is noteworthy that some sporadic attempts initiated in the past few years, however, have resulted in sketchy information on physical and economic characters of most of the breeds only from the organized farms/institutional herds (Bhatia and Arora, 2005).

The Indian Council of Agricultural Research (ICAR), India has established a National Bureau of Animal Genetic Resources (NBAGR) at Karnal to take up the description, evaluation and conservation of the livestock genetic resources and suggest strategies for their long-term conservation. Efforts made at NBAGR Karnal to determine the latest status by compiling information on characteristics of indigenous breeds of sheep through surveys on native tracts. Additional activities on molecular characterization of indigenous sheep breeds are also well under way at NBAGR (Sodhi *et al*., 2003) in view of worldwide recognition of the need for analysis of genetic structure and relationships of sheep populations/breeds to conserve ovine diversity (Arranz *et al*., 1998; Saitbekova *et al*., 2001; Sun *et al*., 2004). Conservation of sheep genetic resources, not only national but also an international issue, therefore, needs more attention to the present status of sheep biodiversity and outlining measures that are necessary if the goals of sheep diversity conservation and self-reliance are to be combined.

Genetic variation in the population occurred in the presence of different types of alleles and their frequencies across all individuals of a population considered together. Genetic variation within a population brought about by the change of allele frequencies over time to time because of natural selection, random genetic drift and gene flow. Genetic variation within a population is essential because it identified with heterozygosity, which known to improve fitness-related attributes (Allendorf and Leary, 1986). Variability among populations occurs through random processes (founder effects, demographic

bottlenecks, genetic drift and mutations), environmental conditions enforced by humans (Hartl and Clark, 1997). Variability between populations is also due to the adaptation of species to their local geographical conditions and it is crucial for the individuals having specific genes or gene combinations critical for sustainability in their native surroundings. This encourages better usage of sheep as well as other species for generation of food and agriculture to fulfill the present need and keeping up the possibility to meet the future possible changes. (Hammond, 1993; FAO, 1995).

It is important to consider genetic variability for the improvement of domestic animals to fulfill human needs, which mainly depends on both within and between breeds variation. Loss of genetic diversity decreases the ability of animals to respond to environmental change and will result in loss of genetic information potentially useful for breeding improvement (Hunter, 1996). Further, large differences between geographic regions, human needs for food and agriculture as well as production ability will affect and serves as the second purpose for conservation. As per Hammond (1994) right around seventy-five percent of the world agribusiness will stay at the low to medium input levels where animal production situations associated with stress for example feed shortage, drought and heat. Breeds in these situations are reservoirs of genetic information responsible for adjustments essential for production under adverse environment. Furthermore, the other purpose for conservation of livestock diversity is that sustainable crossbreeding plans require two suitable thoroughbred populations and sometimes more. The wide diversity of livestock breeds and strains accessible today is part of our cultural legacy and it merits protection.

2.3 Molecular techniques for the assessment of animal genetic diversity

2.3.1 Molecular techniques in the study of animal genetic diversity

Molecular markers are the most significant tools generally used by the researchers to describe, differentiate, or relate individuals within or between breeds or species. The advancement in DNA polymorphism techniques and later

data analysis has enormously increased the ability to comprehend the genetic relationship among species at the molecular level. DNA markers have utilized for the molecular characterization and genetic diversity studies in several species. The DNA polymorphism to be utilized generally for genome characterization and method was restriction fragment length polymorphism (RFLP) (Botstein *et al*., 1980) and it also have been used as markers in sheep genetics (Prayitno *et al*., 2011) for genetic improvement of sheep (EL-Hanafy and Salem, 2009) as well other animals (Soller and Beckmann, 1982). A variety of molecular techniques are presently being utilized in different research centers for the study of inter and intra-specific genetic variation at the DNA level. RFLP is also utilized for nuclear DNA (Jeffreys and Morton, 1987) and mitochondrial DNA (Hiendleder *et al*., 2002), minisatellites probing (Haberfeld *et al*., 1991), microsatellites PCR amplification (Pramod and Kumarasamy, 2015), random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams *et al*., 1990), amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995) and sequencing of mitochondrial DNA fragments (Hiendleder *et al*., 2007). These techniques produce varied information to resolve gene variation in taxonomic levels.

2.3.1.1 Restriction fragment length polymorphism (RFLP)

Polymorphism in DNA sequence frequently identified as a variation in length of DNA fragments generated by cutting the DNA with restriction enzyme (Botstein *et al*., 1980), such polymorphism is referred to as RFLP. In 1980, this technique has used to generate the genetic map of the human genome. RFLP is the result of variations in the sequence of nucleotides in the DNA of an individual animal. These differences are due to mutations in the genome occurring over time and are distinguished as variations in length of restriction fragments. In RFLP, DNA is digested with particular restriction enzymes that cut DNA at specific sites within a recognition sequence, usually about 4-6 bp long (Dowling *et al*., 1996). Since each enzyme cuts DNA at a specific recognition sequence, the whole digestion of a particular DNA generates an array of fragments. The variations in

fragment lengths are results of mutation, which either alter restriction sites or lead to insertions/deletions. Such polymorphism in a particular gene locus has utilized to recognize animal species, populations and individuals. The advantages are that RFLPs give profoundly reproducible band patterns and co-dominant markers consequently, heterozygotes are distinguishable. The limitations with RFLP markers are that, it is labor intensive, comparatively expensive, as a good supply of probes is required. The blotting and hybridization steps are tedious, and hard to automate. Likewise, the degree of detectable polymorphism might be restricted for intra-species comparison and adequate amounts (e.g. 10 μg per digestion) of good quality DNA are essential. Due to the last limitation, RFLPs are not relevant if very limited amount of source material or preserved tissues are accessible.

2.3.1.2 Mitochondrial DNA (mtDNA) analysis

Mitochondrial DNA (mtDNA) sequence differences among individuals or populations can measure indirectly through restriction site analysis (Upholt and David, 1977) or directly through DNA sequencing. The former technique is often less expensive and permits the rapid screening of larger samples rather than later one. Likewise, provides frequently more data as well. In restriction site analysis, isolated mtDNA processed with a restriction enzyme and the subsequent fragments separated by gel electrophoresis. The composite restriction fragment patterns obtained by subsequent digestion with almost 10-20 restriction enzymes. The pattern of restriction site loss or gain can be utilize to assess genetic differentiation and evolutionary history of populations or species (Avise, 1994). In sequencing, mtDNA genes are directly sequenced utilizing universal or species-specific primers that permit amplification of sequences as much as a few thousand base pairs long. Since various genes in the mtDNA genome highly developed at various rates, therefore, quickly advancing genes can be evaluate define the relationship of newly diverged populations as compared to gradually advancing genes that can be utilized to answer logical questions including distinctive species.

2.3.1.3 Random amplified polymorphic DNA (RAPD)

RAPD markers emerged in the early 1990s (Williams *et al*., 1990). It depends on mismatches in primer binding sites or insertion/deletion events and therefore results in the presence or absence of an amplified product from a single locus. Utilization of such primer in PCR amplification results in numerous distinct DNA band products. More specifically this technique employs random 10-base oligonucleotide as a primer to amplify distinct fragments of genomic DNA through PCR (Williams *et al*., 1990) i.e. rather than utilizing two specific primers as used in a conventional PCR. It amplifies DNA of any genome region that happens to flanked in inverse orientation within 5000 bp of each other. During the annealing step, the primer anneals to the template at two sites on the complementary strand of DNA template. If these priming sites are within an amplifiable range of each other, a distinct DNA product generated through the amplification. In RAPD, the amplification products separated on agarose gels in the presence of ethidium bromide and the resultant bands visualized under ultraviolet light (Welsh and McClelland, 1990; Williams *et al*., 1990, 1992). The presence or absence of bands can be scored and the data changed into similarity matrices for calculation of genetic distances (Gwakisa *et al*., 1994).

This method has unlocked a new area of genetic analysis as a result of its relative simplicity and speed. It is being used widely in sheep for numerous purposes such as breed characterization (Kantanen *et al*., 1995; Teale *et al*., 1995; Yadav et *al*., 2001), species identification, parentage testing, and pedigree analysis (Dodd *et al*., 2005), population studies (El Hentati *et al*., 2012), and detection of genetic variations (Kantanen *et al*., 1995). The advantages of RAPD are that there is no prerequisite for DNA probes or sequence information for the design of primers, the procedure is quick, basic and can be automated very small amount of DNA (e.g. 10 ng per reaction). In addition, this technique does not require any restriction enzyme and radioactivity, and the procedure is less expensive (Williams *et al*., 1993; Kantanen *et al*., 1995). Disadvantages of RAPD

are however numerous. First, RAPD methods are extremely delicate to the PCR reaction conditions, DNA quality and PCR temperature profiles (Karp *et al*., 1998). It is very important to keep up entirely consistent PCR conditions with a specific end goal to accomplish reproducible outcomes. Second, the markers are dominant, therefore heterozygotes cannot be identified. Third, in the absence of pedigree analysis, the identification of distinct bands in the multi-band profiles is not known and there can be an improbability in assigning markers to specific loci. This makes it hard to utilize RAPD in inter-population or inter specific evaluations (Dowling *et al*., 1996). Fourth, single band on the gel can sometimes be comprised of numerous co-migrating amplification products.

2.3.1.4 Amplified fragment length polymorphism (AFLP)

The AFLP is a more recent PCR-based technique (Vos *et al*., 1995), which is basically intermediate between RFLP and RAPD. In AFLP, the initial step includes restriction digestion of the genomic DNA and the oligonucleotide adapters are ligated to the ends of the digested fragments. The second step includes selective amplification of restriction fragments. Either a pre-selection step is accomplished utilizing magnetic beads followed by a round of selective PCR or two selective rounds of PCR amplification are required (Vos *et al*., 1995). In the final step, the amplified products are separated by polyacrylamide gel electrophoresis and can be visualized using radioactive or fluorescent labeling. Compared to RAPD, AFLP results are much more easily reproducible (Vos *et al*., 1995), faster, less labor intensive and provide more information (Powell *et al*., 1996). Due to of their extensive genome coverage, AFLP is particularly valuable for mapping. Besides, DNA fingerprinting and hereditary separations can be assessed between genotypes. However AFLPs share a significant number of the restrictions, with respect to band homologies and identities.

2.3.1.5 Variable number of tandem repeats (VNTRs)

In the genome of individuals, there are numerous regions composed of tandemly repetitive simple sequences. These tandem repeats are variable in numbers and are also called as a variable number of tandem repeats (VNTRs) Utilization of minisatellites and microsatellite are relying upon the size of the repeated sequence of base pairs. Jeffrey (1985) firstly coined the term minisatellites. Minisatellite region is described by randomly repeating oligonucleotide units varying from 10 to 60 bp in length (Haberfeld *et al*., 1991, Armour and Jeffreys, 1992). Although in microsatellite the repeated sequences are just 2 to 5 bp long (Tauz, 1989; Weber and May, 1989; Queller *et al*., 1993). These variations in both systems are the consequence of a change in copy number, the core mechanism of mutation and the differences in chromosomal location. Microsatellite loci are randomly dispersed and subject to replication slippage (Weber and May, 1989; Schlötterer and Tautz, 1992) while minisatellites loci have a tendency to be concentrated near telomeres and their length vary due to intra-molecular or intra-allelic recombination and gene conversion mechanisms (Jeffreys *et al*., 1994).

Minisatellites are examined by processing of DNA with restriction enzymes which do not cut within the tandem repeats followed by Southern blot transfer and hybridization with the help of synthetic oligonucleotide probes. Two methodologies can be applied to the study of minisatellites. In one method, the mixture of restriction fragments can be parted by size on an agarose gel in the presence of an electric field and visualized by hybridization with a radioactively labeled probe (Jeffreys *et al*., 1985). This result in a complex band patterns that are usually unique to an individual and widely known as DNA fingerprinting. DNA fingerprint has turned into an extremely powerful tool for testing parentage (Burke, 1989), relatedness of individuals and overall population variability (Wayne *et al*., 1991). The main advantage of this approach is that many probes can be applied across a wide range of plants and animals. An evident issue with

DNA fingerprinting is that it is a multilocus approach, which uncovers multiple fragments some of which segregating together. Such linkage disequilibrium between bands must be distinguished with the examination of the pedigrees. In addition, assigning particular fragments to a specific locus is difficult and thus, recognizing alleles and defining genotypes is unrealistic without pedigree analysis. Last but not least co-migrating fragments might not be homologous. This will lead to inflated band sharing coefficients between non-relatives (Lynch, 1988). In the second method, particular minisatellites loci are studied utilizing single locus probe rather than multilocus ones (Nakamura *et al*., 1987). The benefit of this technique is that alleles can be assigned to particular loci and genotypes can be distinguished. Instead, cloning of minisatellites loci is a comparatively complex process. This technique involves more efforts than multilocus fingerprinting and the variation at individual loci has a tendency to be systematically confined (Hanotte *et al*., 1991).

Microsatellite loci are normally analyzed one at a time via PCR (several loci can be examined simultaneously by multiplexing). The PCR amplification products can be visualized utilizing radioactive or fluorescence strategies. Under radioactive techniques, PCR-amplified microsatellites can be identified either by direct incorporation of a single labeled deoxynucleotide triphosphate (dNTP) like (α-32P) dCTP during thermal cycling (internal labeling) or a single 5’ (γ-32P) dATP end-labeled primer in the PCR mix (end labeling). The products are resolved on acrylamide gels, fixed, dried and autoradiographed. Under the fluorescence strategies, fluorescently labeled dNTPs are utilized for internal labeling in the PCR. Alternatively and more commonly one of the PCR primers will be fluorescently labeled. The PCR products, fluorescently labeled, are separated on a polyacrylamide gel and detected when excited to fluorescence by a laser and analyzed by computer software. Despite the fact that this is an expensive technique, this offers the extensive advantage in terms of size accuracy and enhances output. An advantage of microsatellite over minisatellites is their

smaller size and single loci can be amplified using PCR. Therefore, there is an incredible potential for considering allele frequencies at single hypervariable loci all populations. Additionally, codominant genotypes can be scored and correct allele sizes can be resolved. The strategy has extraordinary potential for speed and precision once the appropriate PCR primers are known and a lot of polymorphism can be resolved. The utilization of microsatellites over RFLPs and RAPDs is that their genetic basis of variability is readily apparent. Specific primers amplify a genomic region, including a well-defined repeat structure that is responsible for the observed variation. Compared to minisatellites, microsatellites are more appropriately spaced throughout the genome while minisatellites have a tendency to be discovered more frequently near the ends of chromosomes. Additionally, most minisatellites alleles are too long to be amplified effectively by PCR. Microsatellites usually are less than 400-500 bp long, comprising of 10- 30 copies of a repeat that is four bp in length. Thus, they are more amenable to PCR typing. The drawbacks of the microsatellite are that, the work essential to develop primers for each new species examined and that only a few allelic states are possible, subsequently, increasing the chance of parallel progression of a specific sequence repeat. However, with the increasing number of microsatellite maps for monetarily essential species and due to the fact that primers developed for one particular species can sometimes be applied across a wide range of related taxa (Moore *et al*., 1991). Cloning of microsatellites for the development of primers for new species will be superfluous in nearby future.

2.4 Application of Microsatellite markers in different areas

2.4.1 Microsatellite as genetic markers for population genetics studies

Microsatellites are generally called as Simple Tandem Repeats (STRs), are profoundly polymorphic (Weber and May, 1989), comprises of short sequence repeat motifs of tandemly repeated di, tri, tetra or penta-nucleotide sequences that occur in a large number of loci throughout the eukaryotic genome (Hamada *et al*., 1984, Tauz, 1989). The di-nucleotide repeats cytosine – adenine (CA) or Guanine

– thymine (GT) is most common in mammalian genomes (Meghen *et al*., 1994). Microsatellites with more than ten dinucleotide repeats have a tendency to be highly informative. Because of the high rate of mutation, the majority of microsatellite loci is very polymorphic in most mammalian species (Jeffreys *et al*., 1988; Weber, 1990). Alleles at microsatellite loci generally follow Hardy-Weinberg principle and segregate in a Mendelian fashion and these are particularly neutral. This makes them suitable with the hypothesis of the neutral theory of population genetics (Kimura and Crow, 1964). Microsatellite polymorphism has advantages over other techniques in that it is an extremely dependable, exceptionally exact repeatable technique that has the capability of being established everywhere throughout the world. The other potential advantage of microsatellite in population genetics is the fact that primers developed for a particular species can be sometimes applicable across a wide range of related taxa. For example, some primers developed for cattle can be used for sheep, goats, yak, etc. Microsatellite markers are presently the markers of choice for a wide range of molecular genetic studies such as establishing genetic linkage maps (Crawford *et al*., 1995), analysis of mating system and population structures (Bruford and Wayne, 1993; Queller *et al*., 1993; Schlötterer and Pemberton, 1994) and reconstruction of phylogenetic relationships among populations (Forbes *et al*., 1995; Takezaki and Nei, 1996; MacHugh *et al*., 1997). The most desirable characteristics of microsatellite loci are that they are found in large numbers and are relatively evenly distributed throughout mammalian genomes, co-dominantly inherited and easily scorable through polymerase chain reaction. Microsatellites are conserved among related species. Moreover, microsatellite variation is independent of age, sex and environmental changes and hence can be detected at the early stage of development.

The majority of microsatellites are found in non-coding regions of the genome. However, Morin *et al*. (1994) also reported the presence of microsatellite in protein coding region exhibiting regulatory role in gene expression and

trinucleotide repeats have been used for linkage analysis in association with disease susceptibility genes (Richards and Sutherland, 1994). Microsatellites have been found conserved between closely related species (Sun *et al*., 1996) allowing primer sets to be used across species (Kemp *et al*., 1995). They have been extensively used for linkage mapping in diverse organisms from human to mosquitoes (Weissenbach *et al*., 1992; Vaiman *et al*., 1996; Barendse *et al*., 1997) and their use has enabled the identification of quantitative trait loci in major livestock species (Raadsma *et al*., 2009). Microsatellites have proven useful in the analysis of paternity and kinship (Al-Atiyat *et al*, 2014) and statistical calculation of probability in sheep (Tomasco *et al*., 2002) and the population levels (Paetkau *et al*., 1995). Microsatellite variation has been used to study the amount of hybridization between closely related species (Diez-Tascón *et al*., 2000). Spatial distribution of alleles has been used to study local genes flow and population substructure (Gutiérrez-Espeleta *et al*., 2000). Effective population sizes and inbreeding have been estimated from microsatellite data (Rochus and Johansson, 2017; Al-Atiyat, 2016). Microsatellites have been increasingly used for the study of genetic variation between and within animal populations. They have been successfully applied in the study of genetic variation in livestock species such as poultry, sheep, goats, buffaloes and bovines (Haberfeld *et al*., 1991; Ganai and Yadav, 2001; Arora and Bhatia, 2004; Li and Valentini, 2004).

2.4.2 Microsatellite marker in detection of paternity

Paternity analysis studies have mostly used microsatellite markers for the reason that allozyme loci do not have enough variability to confirm parentage by exclusion. Al-Atiyat (2015) showed that polymorphic microsatellite markers are excellent and reliable tool for detecting paternity in Merino sheep population by comparing 28 microsatellite markers. Microsatellite markers provide accurate pedigree information and resolve the common problem of significant error in pedigree records in population. In order to assess paternity exclusion in Ghezel sheep, Saberivand *et al.*(2011) has adapted seven microsatellite loci. The study

suggests that the PE (Power of exclusion) is a most commonly used parameter in pedigree verification for microsatellite marker associated parentage studies. While Jakobava *et al*. (2002) obtained a high degree of excluding incorrect parentage using only five microsatellite markers. The importance of microsatellite null alleles in parentage and population genetic analyses of Kivircik sheep Yilmaz *et al.* (2016) were discussed using twenty microsatellite markers. Different combinations with multiplex microsatellite groups were successfully established to determine the power of paternity testing. Hence DNA-based paternity test using microsatellite markers improve selection accuracy and could be cost effective for parentage analysis.

2.4.3 Microsatellite marker in detecting genetic bottleneck

A demographic bottleneck happens when a vast population encounters a serious, transitory reduction in size due to environmental or demographic events. For instance, natural calamities, which happen at unpredictable interval such as drought, disease outbreak and war. These events may kill a specific percentage of a population. Thus, some genetic diversity is lost in the course of time. The extent of the loss in diversity relies on upon the span of the bottleneck and the development rate of the population afterward (Hunter, 1996). However, a genetic bottleneck can also occur in the absence of a demographic bottleneck when there are few breeders of one sex due to a skewed sex ratio or a polygynous (or polyandrous) breeding system. In many mammal species with a polygynous mating system, a few males could monopolize breeding and mate with many females, thus causing a genetic bottleneck in the absence of a demographic bottleneck (Luikart *et al*., 1997). Generally, a genetic bottleneck can create two issues (Carson, 1983): a loss of certain alleles or particularly rare alleles, if no individuals retaining those alleles, a reduction in the amount of dissimilarity occurred in genetically determined characteristics due to presence of fewer alleles and decline in heterozygosity. Bottlenecks have been appeared to lessen allozyme heterozygosity. Leberg (1992) recommended that exceptionally low levels of

allozyme heterozygosity in broad topographical overviews suggest the occurrence of one or more recent extreme bottlenecks. The general impact of bottlenecks is the decrease in the wellness of the individual in the population. Hence microsatellite markers are important tool to estimate recent genetic bottleneck in the sheep population by using three test viz. Sign rank test, Standardized differences test and Wilcoxon test where they are utilizing three models of mutation namely, Infinate allele model (IAM), Two phase model (TPM) and Stepwise mutation model (SMM). SMM is the most suitable model for microsatellite analysis revealed absence of significant heterozygote excess in Kilakarsal, Shahabadi and Ganjam sheep population (Radha *et al*., 2011; Pandey *et al*., 2009; Arora *et al*., 2010). The mode-shift indicator test was also utilized to detect potential bottlenecks. A graphical representation utilizing allelic class & proportion of alleles showed a normal "L" shaped distribution revealing absence of bottleneck in Magra, Bellary, Coimbatore, Kheri and Tibeten sheep population (Arora and Bhatia, 2006; Kumar *et al*.,2007; Kumarasamy *et al*., 2010; Arora and Bhatia, 2008; Sharma *et al*.,2016).

2.4.4 Microsatellite marker in Conservation and Phylogenetic studies

Conservation biology is a relatively new field of research that emerged in the seventies with the goal of preserving ecosystems, species and genes (Wilcox and Soule, 1980). Within this field, conservation genetics deals with the application of genetic concepts and tools to conservation problems. Characterization of breeds is necessary for the development of conservation programs, to determine which breeds should be conserve. Microsatellite markers have successfully used to determine genetic conservation of sheep breeds (Sharma *et al*., 2016; Crispim *et al*., 2013 and Taberlet *et al*., 2011). The relationship among breeds of species other than sheep have also been estimated, viz., dog (Pihkanen *et al*., 1996), goats (Saitbekova *et al*., 1999), horse (Bjornstad *et al*., 2000) and donkey (Jordana *et al*., 2001).

Microsatellite loci have successfully used to reconstruct phylogenetic relationships among populations. Phylogenetic trees are graphical representations of nodes (taxonomic units) and branches (pathways connecting nodes) that summarize the evolutionary relationships among organisms (Avise, 1994). Phylogenetic tree can be reconstructed using various methods as Distance based methods, UPGMA and Neighbor-joining method, Maximum parsimony method and Maximum likelihood method. The phylogenetic relationships in the sheep breeds of north western arid and saline marshy region have also been successfully assessed using different microsatellite markers set (Mukesh *et al*., 2006; Arora *et al*., 2011a). Thus, microsatellite DNA is very useful for clarifying the evolutionary relationship of closely related populations.

2.5 Genetic diversity parameters for statistical analysis

2.5.1 Mean number of allele (MNA) and Heterozygosity

Genetic diversity refers to the variation at the level of individual genes (polymorphism) which provides a mechanism for populations to adapt to their ever-changing environment. The more variation the better the chance that at least some of the individuals will have an allelic variant that is suited for the new environment, and will produce offspring with the variant that will in turn reproduce and continue the population into subsequent generations (http://genetics.nbii.gov/GeneticDiversity.html). The mean number of alleles (MNA) detected in each population and the expected heterozygosities are good indicators of the genetic polymorphism within the populations. The MNA is the average number of alleles observed in a population, while the expected heterozygosities are the proportion of heterozygote expected in a population. Numbers of alleles per locus per population obtained by direct counting.

Heterozygosity is an appropriate measure of genetic variability within a population when populations are expanding (Hanslik *et al*., 2000). It is the most widely used parameter to measure diversity within populations, defined by Nei

(1973) as the probability that two alleles chosen at random from the population are different.

The various values of mean number of alleles and heterozygosity successfully accessed in order to find genetic diversity within and between indigenous sheep breeds of India (Jyotsana *et al*., 2010; Sharma *et al*., 2010; Arora *et al*., 2011b; Radha *et al*., 2011; Yadav *et al*., 2011; Hepsibha *et al*., 2014; Surekha, 2015; Sharma *et al*., 2016). Generally, the MNA is dependent on the sample size because of the presence of unique alleles that occur at low frequencies in populations and because the number of observed alleles tend to increase with increases with population size (Nei, 1987). Therefore, the comparison of the MNA between samples of different sizes may not be meaningful unless the sample sizes are more or less the same (Nei, 1987).

2.5.2 Polymorphism Information Content (PIC)

Another measure of genetic diversity within breeds is Polymorphic Information content (PIC). The PIC is a parameter indicative of the degree of informativeness of a marker. The PIC value may range from zero to one. Loci with many alleles and a PIC value of one are most desirable. Following the criteria of Botstien *et al*., 1980, the investigated markers in indigenous sheep breeds of India were observed to be highly informative (PIC>0.5), reasonably informative (0.25<PIC<0.5) and slightly informative, less than 0.25 (Jyotsana *et al*., 2010; Sharma *et al*., 2010; Radha *et al*., 2011; Yadav *et al*., 2011; Hepsibha *et al*., 2014; Surekha, 2015; Sharma *et al*., 2016). Hence, the degree of informativeness of a marker reveals its usefulness in diversity analysis of a breed. A higher value of PIC means more alleles and greater polymorphism at that locus.

2.5.3 Hardy-Weinberg Equilibrium

The Hardy-Weinberg equilibrium is a law stating that in a large random mating population the gene and genotypic frequencies will remain constant from one generation to the next in the absence of disturbing factors such as mutation,

migration and selection. A population with this constant gene and genotypic frequencies said to be in Hardy-Weinberg Equilibrium (HWE). Many researchers were indulged in order to find whether the loci and the populations genotyped are in HWE and whether there are any significant deviations from the HWE in Indian sheep breeds as well as exotic sheep breeds (Girish *et al*., 2007; Prema *et al*., 2008a; Prema *et al*., 2008b; Kumarasamy *et al*., 2009; Pramod *et al*., 2009; Sharma *et al*., 2010; Radha *et al*., 2011; Ghazy *et al*., 2013; Hepsibha *et al*., 2014; Sassi-Zaidy *et al*., 2014; Surekha, 2015; Kavitha *et al*., 2015; Das *et al*., 2015; Salamon *et al*., 2014; Sharma *et al*., 2016). The natural processes of mutation, migration, non-random mating, genetic drift and both artificial and natural selection are factors that known to cause deviations from HWE. Ideal HWE populations do not actually occur in nature.

2.5.4 F-Statistics (F_{IS}/F_{IT}/F_{ST})

The populations of most, if not all, species show some levels of genetic structure, which may be due to a variety of non-mutually exclusive agents. Environmental barriers, historical processes and life histories (e.g. Mating system) may all, to some extent, shape the genetic structure of populations (e.g. Donnelly and Townson, 2000; Gerlach and Musolf, 2000; Palsson, 2000; Tiedemann *et al.*, 2000). The understanding of the genetic structure of a population or differentiation between populations is of interest to population geneticists because it reflects the number of alleles exchanged between populations, which influence the genetic composition of individuals within these populations (Balloux and Lugon-Moulin, 2002). The simplest parameters for assessing diversity among breeds using microsatellite data are the genetic differentiation or fixation indices. Population differentiation was successfully examined by fixation indices (F_{IS}/F_{IT}/F_{ST}) for each of the selected different sets of microsatellite markers in Indian sheep breeds as well as exotic sheep breeds (Sodhi *et al*., 2006; Girish *et al*., 2007; Bhatia and Arora, 2007; Peter *et al*., 2007; Ozerov *et al*., 2008; Arora and Bhatia, 2009; Jyotsana *et al*., 2010; Tapio *et al*., 2010; Sassi-Zaidy *et al*.,

2014; Mukesh *et al*., 2006). Where, F_{IS} calculates inbreeding in individuals relative to subpopulation, F_{IT} estimates reduction in heterozygosity of an individual in relation to total population and F_{ST} is a measure of genetic differentiation in the population using microsatellite markers.

2.5.5 Ewens Watterson neutrality test

One of the milestones of population genetics theory was the discovery of the Ewens sampling formula (Ewens, 1972). This formula provides an analytical expression for the sampling probability under the infinite allele model, whereby every mutation is of a new allelic type, for a sample obtained from a single population of constant size with no population structure. Using the Ewens's sampling formula, one of the most famous tests of neutrality, the Ewens–Watterson test (Watterson, 1977) was developed. In this test, the expected homozygosity, given the observed number of alleles, compared to the observed homozygosity. If the difference between the observed and expected homozygosity is larger than some critical value, the neutral null hypothesis can rejected. All the scrutinized loci observed to be neutral in Ewens Watterson neutrality test, suggesting that homozygosity in Shahabadi may not be as a consequence of selection (Pandey *et al*., 2009). Since 92% loci were detected neutral, selection as a cause of the decrease in observed heterozygosity was ruled out in the Tibetan sheep population. Thus, the difference between the observed and expected heterozygosity can attributed to the non-random mating among the individuals of the population (Sharma *et al*., 2016).

3.1 Materials

3.1.1 Chemicals and Reagents

- Acrylamide (Sisco Research Laboratories PVT. LTD)
- Agarose (molecular biology grade) (Bangalore, Genei)
- Ammonium per sulphate (SRL, Qualigens Fine Chemicals)
- Boric acid (molecular biology grade) (AnaleR, Glaxo Laboratories India LTD)
- Buffer Tablets (pH 4, 7, 9) (BDH Laboratories Reagents)
- DNA 100 bp marker ladder (Promega, USA and Bangalore, Genei)
- dNTP mix (Promega, USA)
- EDTA (Na_2EDTA) (ExcelaR, Qualigens Fine Chemicals)
- Ethanol (Jai Chemical and Pharma. Works)
- Ethidium bromide (SRL, Qualigens Fine Chemicals)
- Gel loading buffer (Bangalore, Genei)
- Magnesium Chloride 1.5mM (molecular biology grade) (Promega, USA)
- N’, N’, N’, N’ Bis-acrylamide (SRL, Qualigens Fine Chemicals)
- PCR assay buffer (Promega, USA)
- Microsatellite primers (GCC Biotech Pvt. Ltd.)
- Sodium chloride (MP Biomedicals, Inc.)
- Sodium hydroxide pellets (ExcelaR, Qualigens Fine Chemicals)
- Taq DNA polymerase enzyme (Promega, USA)
- TEMED (SRL, Qualigens Fine Chemicals)
- TRIS base (Bangalore, Genei)
- Tris-HCl (SQ, Qualigens Fine Chemicals)

3.1.2 Equipments

- Centrifuge machine (Thermo Fisher Scientific)
- Waterbath (Lab Companion.)
- Incubator (Lab Companion.)
- Deep freezer (Artiko)
- Spectrophotometer (Shimadzu, Japan)
- Horizontal agarose gel electrophoresis unit (Genei, Bangalore)
- Gel-documentation system (UVP)
- Eppendroff mastercycler gradient (Eppendroff)
- Electrophoresis unit (Hoffer SE600 series)
- UVP Doc-It®LS Image Acquisition Software version 6.3.3
- Micro pipette (Thermo Fisher Scientific)
- Autoclave (Lab Companion)
- Microwave oven (IFB)

3.1.3 Experimental Animals

This study focused on sheep of northwestern arid and semi-arid region of India, which includes Chokla, Magra, Marwdi and Nali. A total of 30 blood samples from each sheep breed were collected (Table 1). 120 individuals were analyzed. All the genetically unrelated animals randomly selected and the information collected after consulting pedigree records maintained and interviewing the owners in detail. Magra and Marwari blood samples collected from Bikaner district, villages near Bikaner and CSWRI, Bikaner. Chokla Blood samples collected from the bordering area of Nagaur and Bikaner district. Nali samples collected from Nali sheep farm and local sheep farm from Shekhawati.

The Magra sheep, also known as Bikaneri Chokla or Chakri and formerly known as the Bikaneri, is a breed of sheep that is found in the Bikaner, Nagaur, Jaisalmer and Churu districts of Rajasthan, India. However, purebreds are only found in the eastern and southern parts of the Bikaner district. The Magra sheep

known as lustrous carpet wool producing breed. The most important strain of Magra has flocks with extremely white and lustrous fleece, which found in a few villages around Bikaner.

Fig. 1 Magra sheep

Fig. 2 Marwari sheep

Marwari name originates from the home tract of the breed: Marwad. The breed resembles a black-headed Persian sheep, but is smaller in size and has good fleece. This breed found in Jodhpur, Jalore, Nagaur, Pali and Barmer districts, extending up to Ajmer and Udaipur districts of Rajasthan and the Jeoria region of Gujarat and are being improved through selection for fleece weight and carpet quality.

Chokla also has known as (Chapper, Shekhawati) Churu, Jhunhunu, Sikar & border areas of Bikaner, Jaipur & Nagaur districts of Rajasthan. This breed is Light to medium-sized; Face is generally devoid of wool and is reddish-brown/dark brown in color; Skin is pink; Ears are small to medium in length and tubular; Coat is dense; both sexes are polled. Although, Chokla is perhaps the finest carpet-wool breed, its wool being diverted to the worst sector due to scarcity in fine apparel wool in the country.

Fig. 3 Chokla sheep **Fig. 4 Nali sheep**

Nali, mainly found in Ganganagar, Churu & Jhunjhunu districts of Rajasthan and southern parts of Hissar & Rohtak districts of Haryana. It is a medium-sized; Face is light brown in color; Skin is pink; Ears are large and leafy; both sexes are polled; Tail is short to medium in length and thin; Fleece is white, coarse, dense and long-stapled; Forehead, legs and belly covered with wool; March clip is yellow but September clip is golden brown.

3.2 Blood collection

2 ml of blood was drawn from each animal intravenously from jugular vein using 18 G needle. Blood collected in 3ml commercial EDTA vials containing 8mg EDTA . Samples were transported to the laboratory on ice and stored at $4^{o}C$ until used.

Table 1: Sample collection of experimental animals

S.no.	Experimental Animal (Sheep breeds)	Number of blood samples
1	Chokla	30
2	Nali	30
3	Marwari	30
4	Magra	30
Total		120

3.3 DNA isolation

Genomic DNA was isolated using the QIAamp® DNA Mini Kit with slight modification (RPM of centrifuge has increased from mentioned in the protocol).

3.4 Quality and quantity of genomic DNA

The purity and concentration of the isolated genomic DNA were estimated using agarose gel electrophoresis and UV-absorption spectrophotometer respectively. The absorbance at 260 and 280 nm wavelength used to measure the optical density (OD) of the DNA samples. A ratio between 1.4 and 1.9 considered as a relatively pure DNA sample as it did not show any effect on PCR reaction (Sambrook and Russel, 2001).

Conc. of DNA (µg/ml) = OD 260 X dilution factor X 50

A working solution was prepared by diluting the samples in TE buffer (pH 8) or sterilized MiliQ water having approximately 30 ng/µl of DNA. Agarose gel electrophoresis (0.8 %) was carried out for confirming the quality of isolated genomic DNA (Sambrook and Russel, 2001). Appropriate amount of agarose was weighed and dissolved to make a final concentration of 0.8 % in 1X Tris-Borate EDTA (TBE) buffer. The agarose melted in a microwave oven (IFB). The solution allowed to cool sufficiently and ethidium bromide added at a concentration of 0.5 µg/ml of agarose gel. The gel tray sealed on either side by using adhesive tape and the comb placed in proper position. The heated agarose was poured into the gel tray carefully avoiding air bubbles. Once the gel was sufficiently solidified, the comb and the seal on either side were removed carefully. The gel tray kept in an electrophoresis tank (Bangalore, Genei) and 1X TBE buffer and poured to submerge the gel in the tank. The DNA samples were mixed with 1/6th volume of 6X gel loading buffer and loaded into the wells using a micro- pipette. The electrophoresis carried out at 85 volts at room temperature for about half an hour. Then the gel visualized under UV light and photographed

using UVP gel-doc system. Note for intact DNA fragments on the gel and avoid samples showing shearing.

3.5 Synthesis of Primers and DNA sequencing.

Published Microsatellite primers and designed wool genes primer pairs synthesized by GCC Biotech Pvt. Lt. India and DNA sequencing of the wool trait gene has been done by Xcelris Genomics, India.

3.6 Microsatellite markers

Microsatellite primer pair's custom synthesized and utilized in the study listed in following Table 2.

Table 2:Details of Microsatellite marker primers used in the study

Locus	Primer sequence	Allele Size (bp)	Chromosome	Annealing.Temp. (in°C)	Ref.
BM6526	F-CAT GCC AAA CAA TAT CCA GC R-TGA AGG TAG AGA GCA AGC AGC	146-172	26	60	Bishop *et al*. (1994)
BM757	F-TGG AAA CAA TGT AAA CCT GGG R- TTG AGC CAC CAA GGA ACC	178-198	9	55	Bishop *et al*. (1994)
BM8125	F- CTC TAT CTG TGG AAA AGG TGG G R- GGG GGT TAG ACT TCA ACA TAC G	107-135	17	55	Bishop *et al*. (1994)
BM827	F- GGG CTG GTC GTA TGC TGA G R- GTT GGA CTT GCT GAA GTG ACC	210-222	3	55	Bishop *et al*. (1994)
CSSM31	F- CCA AGT TTA GTA CTT GTA AGT AGA R- GAC TCT CTA GCA CTT TAT CTG TGT	130-170	23	55	Moore *et al*. (1994)
OarAE129	F- AAT CCA GTG TGT GAA AGA CTA ATC CAG R- GTA GAT CAA GAT ATA GAA TAT TTT TCA ACA CC	140-164	5	60	Penty *et al*. (1993)
OarCP34	F- GCT GAA CAA TGT GAT ATG TTC AGG R- GGG ACA ATA CTG TCT	110-128	3	63	Ede *et al*. (1995)

	TAG ATG CTG C				
OarFCB128	F- CAG CTG AGC AAC TAA GAC ATA CAT GCG R- ATT AAA GCA TCT TCT CTT TAT TTC CTC GC	108-134	2	63	Buchanan and Crawford (1993)
OarFCB48	F- GAG TTA TGT ACA AGG ATG ACA AGA GGC AC R- GAC TCT AGA GGA TCG CAA AGA ACC AG	138-166	17	55	Buchanan *et al* (1994)
OarHH35	F- AAT TGC ATT CAG TAT CTT TAA CAT CTG GC R- ATG AAA ATA TAA AGA GAA TGA ACC ACA CGG	128-160	4	63	Henry *et al* (1993)
OarHH41	F- TCC ACA GGC TTA AAT CTA TAT AGC AAC C R- CCA GCT AAA GAT AAA AGA TGA TGT GGG AG	96-120	6	63	Henry *et al* (1993)
OarHH64	F- CGT TCC CTC ACT ATG GAA AGT TAT ATA TGC R- CAC TCT ATT GTA AGA ATT TGA ATG AGA GC	116-132	4	55	Henry *et al* (1993)
OarJMP29	F- GTA TAC ACG TGG ACA CCG CTT TGT AC R- GAA GTG GCA AGA TTC AGA GGG GAA G	130-150	24	55	Penty *et al*. (unpublished data)
OarJMP8	F- CGG GAT GAT CTT CTG TCC AAA TAT GC R- CAT TTG CTT TGG CTT CAG AAC CAG AG	119-131	6	63	Penty *et al*. (unpublished data)
OarVH72	F- CTC TAG AGG ATC TGG AAT GCA AAG CTC R- GGC CTC TCA AGG GGC AAG AGC AGG	113-137	25	63	Pierson *et al*. (1993)
RM4	F- CAG CAA AAT ATC AGC AAA CCT R- CCA CCT GGG AAG GCC TTT A	135-143	15	55	Kossarek *et al*. (1993)
TGLA137	F- GTT GAC TTG TTA ATC ACT GAC AGC C R- CCT TAG ACA CAC GTG AAG TCC AC	119-161	5	55	Georges and Massey (1992)
TGLA377	F- GAC TGT CAT TAT CTT CCA GCG GAG R- GAT CTC TGG TTG AAA TGG CCA GCA G	86-122	2	55	Georges and Massey (1992)

All the primers as supplied by the manufacturer were initially added with 50µl of TE buffer (pH 8.0). After that, each primer reconstituted in sterilized DNase free MiliQ water to arrive at a final concentration of 10 p moles/µl.

3.7 PCR conditions

3.7.1 PCR reaction for Microsatellite marker amplification

PCR was carried out in a final reaction volume of 25µl. All PCR components were thawed and spinned for a few seconds prior to use. The volume of each component used according to Table no. 3.

Initially, the PCR conditions were standardized for annealing temperatures, $MgCl_2$ concentration and Taq DNA polymerase by performing a series of reactions with varying each of these PCR components. Annealing temperatures attempted in accordance with literature of each marker given (Crawford *et al*., 1995) but some markers required temperature optimization. Gradient PCR attempted for several markers to determine their exact annealing temperature. No significant change was observed by varying $MgCl_2$ concentration; hence, 1.5 mM concentration used for all amplifications. Taq DNA polymerase was used at 5U (Promega, USA) per reaction.

Table 3: Composition of master mix for PCR amplification

PCR Components	Volume added in each reaction
5X PCR assay buffer	5.00 µl
1.5 mM $MgCl_2$	3.00 µl
dNTP mix (10mM)	1.00 µl
Primer (F) (10 pmoles/µl)	1.00 µl
Primer (R) (10 pmoles/µl)	1.00 µl
Taq DNA polymerase (5 U/µl)	0.25 µl
DNase free MiliQ water	10.75 µl
Template DNA	3.00 µl
Total	25.0 µl

Table 4:GradientPCR reaction conditions

1.	Initial denaturation	94° C for 5 minutes.
2.	Cycle denaturation	94° C for 1 minute.
3.	Cycle annealing	52° C to 58° C for 1 minute.
4.	Cycle extension	72° C for 1 min
	Step 2-4 repeated for 29 Cycles	
5.	Final extension	72° C for 7 minutes.
	Hold	4° C

3.7.2 PCR reaction for wool traits genes

A mastermix for minimum of 10 samples was prepared. Mastermix was prepared for one additional sample to cover the pipetting error. The volume of each component used for wool genes according to Table no. 5.

Table 5:Composition of master mix for PCR amplification of wool traits genes

PCR Components	Volume added in each reaction
5X PCR assay buffer	5.00 µl
1.5 mM $MgCl_2$	3.00 µl
dNTP mix (10mM)	1.00 µl
Primer (F) (10 pmoles/µl)	.75 µl
Primer (R) (10 pmoles/µl)	.75 µl
Taq DNA polymerase (5 U/µl)	0.25 µl
DNase free MiliQ water	11.25 µl
Template DNA	3.00 µl
Total	25.0 µl

Table 6: Gradient PCR reaction conditions for wool traits genes

1.	Initial denaturation	94° C for 5 minutes.
2.	Cycle denaturation	94° C for 1 minute.
3.	Cycle annealing	58° C to 62° C for 1 minute.
4.	Cycle extension	72° C for 1 min
	Step 2-4 repeated for 28 Cycles	
5.	Final extension	72° C for 5 minutes.
	Hold	4° C

3.7.3 Agarose Gel Electrophoresis

To confirm PCR amplification, 5 µl of PCR product mixed with 1µl of 6X gel loading dye from each tube and 100 bp marker ladder were electrophoresed on 1-1.5% agarose gel (depending on the expected size of amplified product) containing ethidium bromide at a concentration of 0.5µg/ml at constant voltage 80 V for 45 minutes in 1X TBE. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

Table 7: Chemicals used for Agarose gel electrophoresis

(A) 10X TBE , pH 8.3	Tris Base-108g Boric Acid- 55g EDTA-40 ml pH (8.0) Used 1X TBE 1:4 dilution with DW
(B) Gel loading dye (6X)	0.25% Bromophenol Blue 40% sucrose(w/v) in 100DW
(C) Ethidium bromide	0.5µg/ml of DW

3.8 Microsatellite Typing

3.8.1 Polyacrylamide gel electrophoresis (PAGE)

The polymorphic typing of microsatellite marker was done using PAGE. For typing microsatellites, 8% native PAGE was run in Hoefer SE 600 series electrophoresis unit. The unit was assembled as given in instruction manual. The glass plates measuring 18 x 16 cm were cleaned thoroughly with mild detergent under tap water, rinsed with distill water and allowed to dry. If required wiped with ethanol to remove any grease spots and allowed to dry.

The plates were adjusted on the gel caster with 1.5mm spacers using screws and checked for any leakage using distilled water. 40 ml of 8% Polyacrylamide gel solution was prepared (after appropriate optimization) with following components (Sambrook and Russel, 2001).

Table 8: Gel preparation for PAGE (8%)

Acrylamide and N', N', N', N' Bis-acrylamide (29 : 1)	10.56ml
5X TBE	8 ml
Sterilized distilled water	21 ml
10% Ammonium persulfate	360 µl
TEMED	40 µl

The gel solution was gently poured in between the glass plates and comb was set. The gel was allowed to polymerize for 45 min without any disturbance. The comb has removed carefully, the wells washed with buffer or distilled water and the samples were loaded along with 100 bp ladder (Promega, USA). The gel clammed with a cam-operated system provided in the unit to upper buffer chamber and fills the lower chamber tank and upper buffer chamber with cold 1X TBE buffer. The electrodes connected appropriately to the electrophoresis power supply and the program was run at 80 V and 2 W. The gel run until the dye front of loading dye reached bottom of the gel. After the run was completed, the glass plates retrieved from the assembly and very carefully apart, those with the help of

scale provided in the unit. The gel still attached to one of the glass plate was carefully transferred to container having ethidium bromide solution and rocked gently to remove gel from glass plate. The gel was stained for about half an hour with gentle rocking. The gel analyzed under UV light and documented by UVP gel-doc system.

3.8.2 Visualization of gel and documentation

The gel was taken out from the tray. The bands of the gel visualized under UV light of gel documentation system. The product sizes were estimated with the help of 100 bp ladder as a standard marker. The types of bands and their genotypes were documented and used for further analysis.

3.9 Statistical analysis and Bioinformatics approach

Allele frequency, Observed number of alleles (Na), Effective number of alleles (Ne), Private alleles, Observed heterozygosity (H_{obs}) and Expected heterozygosity (H_{exp}) were calculated by GenAlEx 6.5 version (Peakall and Smouse 2006, 2012). Genetic variability among the breeds were calculated by Wright's F-statistics (Fis/Fit/Fst), (Nei's, 1972) Genetic distance, Genetic distance based phylogenetic tree were analysed by Neighbor-joining algorithm and Evens-Watterson Neutrality using population genetic analysis software POPGENE-1.32 version (32 bit) (Yeh *et al*., 1997). Hardy-Weinberg equilibrium (HWE) was estimated using GENEPOP 1.2 version (Raymond & Rousset, 1995). Fixation Index (Fis) was estimated using FSTAT version 2.9.3 (Goudet, 2001). Polymorphic Information content (PIC) for each locus estimated according to (Botstein *et al*., 1980). The bottleneck analysis was performed using Bottleneckv1.2.02 (Cornuet and Luikart, 1996).

3.9.1 Allele frequency

It is the proportion of a particular allele carried by individuals in a population and calculated by using formula

$$\text{Allele frequency} = \frac{Number\ of\ alleles\ at\ a\ locus\ in\ population}{Total\ number\ of\ alleles\ at\ that\ locus\ in\ population}$$

3.9.2 Mean number of alleles per locus (MNA)

It is the average number of alleles observed at a locus and estimated by formula

$$\text{Mean No. of alleles per locus} = \frac{Total\ Number\ of\ alleles\ in\ the\ population}{Total\ number\ of\ loci\ studied}$$

3.9.3 Effective number of alleles per locus(Ne)

The effective number of alleles, Ne was computed using the formula

$$Ne = 1/\sum \mathrm{Pi}^2$$

3.9.4 Heterozygosity

Observed heterozygosity and expected heterozygosity at each locus was calculated using following formula:

Ho = No. of Heterozygote/N, Where Ho = Observed Heterozygosity, N= number of samples

$$He = 1 - \sum_{n=1}^{n} pi^2$$

Where, He= expected heterozygosity, i.e. pi is the allele frequency of the ith allele.

Heterozygosity is also defined by genetic diversity within a population estimated using GenAlEx 6.5 version (Peakall and Smouse, 2006, 2012).

3.9.5 Fixation index

Fixation index (Inbreeding coefficient) is a statistical method to estimate inbreeding within a population on each locus, which was estimated using FSTAT version 2.9.3 (Goudet, 2001).

$$F = (He - Ho)/He$$

The values of inbreeding coefficient lie between -1 to +1, where positive values are a sign of inbreeding or null alleles, on the other hand negative values portray excess of heterozygosity.

3.9.6 F-Statistics

F-Statistics commonly used in population genetics, also called Wright's F-statistics (Wright, 1946, 1951, 1965). It is mainly linked with the inbreeding and genetic differentiation between subpopulations. Fixation indices measure the population differentiation or variation within and between populations. It contains three components F_{IS}, F_{IT} and F_{ST}. These indices will guide the breeder in determining the status of inbreeding in the population before commencing a breeding program.

3.9.7 F_{IS} (Within population variability)

F_{IS} measures the reduction of heterozygosity in an individual due to non-random mating within a breed/strain/population. It is the deviation of individual heterozygosity from the expected, based on allele frequencies in a random mating population. It varies from -1 to +1, but in nature it is always nearer to zero. Higher F_{IS} values indicate closer relationship among the individuals. It is similar to inbreeding coefficient or sometimes even referred as inbreeding coefficient.

$$f_{IS} = (\bar{H}e - \bar{H}o)/\bar{H}e$$

F_{IS} denotes the inbreeding coefficient of an individual and corresponds to subpopulation.

Where, $\bar{H}e$ = average of expected heterozygosity of subpopulation, $\bar{H}o$ = average of observed heterozygosity of subpopulation. "F_{IS}" calculates the drop in heterozygosity of an individual because of non-random mating within the subpopulation.

3.9.8 F_{IT} (Variation among individuals in a population)

F_{IT} is the measure of reduction in heterozygosity of an individual in relation to the total population. F_{IT} is considered as the most comprehensive measure of inbreeding as it takes into account both the effects of non-random mating (F_{IS}) and diversity among the populations/strains (F_{ST}). F_{IT} values near zero indicate no inbreeding in the population.

$$f_{IT} = (\bar{H}t - \bar{H}o)/\bar{H}t$$

Fit, denotes the inbreeding coefficient of an individual in relation to the total population.

Where, H_t= Total expected heterozygosity, $\bar{H}o$ = average of observed heterozygosity of subpopulation.

3.9.9 F_{ST} (Measure of population differentiation)

F_{ST} is the reduction in heterozygosity due to subdivision in the population. It measures the deviation of expected heterozygosity in sub populations from the expected, based on allele frequencies in random mating total population that includes all sub-populations. The F_{ST} ranges from 0 to 1 and is classified into low (<0.15), moderate (0.15 to 0.25) and high (>0.25) genetic differentiation (Wright, 1978). The genetic diversity was measured as coefficient of gene differentiation among the breeds. The lower F_{ST} values indicate higher relationship between the populations. It also referred as the coefficient of co-ancestry.

$$f_{ST} = (Ht - He)/Ht$$

Fst calculates genetic differentiation between subpopulation. The values of Fst lie between zeros to greater than zero; its value is zero for all subpopulation that are in Hardy-Weinberg equilibrium with the same allele frequencies.

3.9.10 Private alleles

Those alleles, which are exclusively, present in a single population amongst a wide compilation of populations (Szpiech and Rosenberg, 2011). Moreover, the study of private alleles is significant to find genetic distinctiveness among populations sampled.

$$\boldsymbol{\pi} = \sum_{i=1}^{m} \left[\mathbf{Pijg} \left(\prod_{j'=1}^{j} \boldsymbol{Qij'g} \right) \right]$$

Where, π = expected number of private alleles in a sample from a population, P_{ijg}= probability that a sample of g genes taken from the jth sample contains at least one copy of allele i; Q_{ijg} is the probability that samples of g gene taken from all of the other samples do not contain allele i (Kalinowski, 2004).

3.9.11 Polymorphic Information Content (PIC)

PIC is a quantitative measurement of markers informativeness, where informative means its usefulness to ascertain the markers proximity to a gene linkage analysis. High informativeness of markers is defined by polymorphism of marker or markers with many alleles (Hildebrand *et al*., 1992). It calculated by following formula.

$$\boldsymbol{PIC = 1 - \sum P_i^2 - \sum P_i^2 P_j^2}$$

$\mathbf{P_i}$ is the frequency of ith allele and $\mathbf{P_j}$ is frequency of jth allele of the population.

3.9.12 Genetic distance

The Nei's genetic distance and genetic identity (Nei, 1972) were also estimated in GenAlex 6.1 software. Phylogenetic consensus tree was constructed using the Unweighted Pair Group Method of Analysis (UPGMA) of clustering

were based on Nei's (1978) Genetic distance calculated by software POPGENE-1.32 version (32 bit).

3.9.13 Hardy – Weinberg Equilibrium

Hardy-Weinberg equilibrium explains that the both gene and genotype frequencies will be constant from generation to subsequent next generations (Labate, 2000). Hardy-Weinberg assumption is under following consideration: Diploid, sexual reproduction, Random mating, no selection, no mutation and no immigration (Mariette *et al*., 1999). Deviation from HWT indicates that one or some of mentioned factors make disequilibrium from this test. The chi - square test is useful for determining whether the allelic frequencies are in HW equilibrium. The breeder can assess the equilibrium status of the population with regard to the gene frequencies, which finally reflects the genotype frequencies. The statistical test follows this formula (Guo and Thompson, 1992):

$$HWT = \sum(\mathrm{Oi} - \mathrm{Ei})^2/\mathrm{Ei}$$

Where, HWT is Statistical test, Oi= Observed frequency, Ei= Expected frequency, df= Degree of freedom.

3.9.14 Genetic bottleneck analysis:

A bottleneck analysis was performed to assess whether the population decline had an impact on the maintenance of genetic variation within these breeds. The microsatellite data were subjected to a mode shift test, under the assumption of the two-phase model. The occurrence of a normal L-shaped curve revealed no loss of alleles in the investigated populations and hence the absence of a genetic bottleneck.

The Mode-shift indicator test was also utilized to detect potential bottlenecks, as the non-bottleneck populations that are near mutation-drift equilibrium are expected to have a large proportion of alleles with low frequency. This test discriminates many bottlenecked populations from stable populations

(Luikart, 1997; Luikart and Cornuet, 1997). A graphical representation utilizing allelic class and proportion of alleles showed a normal ‘L’shaped distribution. The L shaped curve indicated the abundance of low frequency alleles. This finding suggested the absence of any detectably large, recent genetic bottleneck (last 40–80 generations) in declining population, where the probability of low frequency allele's loss was very high.

3.10 KAP’S and KRT’S gene analysis in sheep

Genomic DNA was used for further amplification of wool keratin associate protein genes. The present study focused on four loci KRTAP1-4, KRTAP6-1, KAP1-1 and KRT1-2/K33amplified in Chokla, Nali, Marwadi and Magra sheep respectively to identify the sequence variation for each locus in four sheep breeds. The primers for KRTAP1-4 and KRTAP6-1 were designed using sheep reference genome (assembly Oar_V3.1/OviAris3) through Ampliseq designer and amplify 624 bp and 350 bp fragments, respectively, these were: upstream (5′-CGC CCA TAA CAA AAA CCC AAA TG-3′) and downstream (5′-GTGGATTTCCCACTTGCTCCA-3′) for KRTAP1-4 and upstream (5′-ACA TTG TTT TGT GTT GCT TAG TTG CT-3′) and downstream (5′-CCC ACA CTG AGA GCA TAT AAA AGG C-3′) for KRTAP6-1 locus. The primers used to amplify the KAP1-1 and K33 locus were sourced from Itenge-Mweza et al. (2007). They were as follows: upstream (5′-CAA CCC TCC TCT CAA CCC AAC TCC-3′) and downstream (5′-CGC TGC TAC CCA CCT GGC CAT A-3′) for KAP1-1, 311 bp fragment and upstream (5′-CAC AAC TCT GGC TTG GTG AAC TTG-3′) and downstream (5′-CTT AGC CAT ATC TCG GAT TCC CTC-3′) and defined a 480 bp region of the K33 locus. All primers were synthesized by GCC Biotech (India) Pvt. Ltd. India.

3.10.1 Sequencing and variation analysis in Indian sheep breeds

The sequence of PCR products of *KRTAP1-4*, *KRTAP6-1*, *KAP1-1* and *KRT1-2/K33*genes in Chokla, Nali, Marwadi and Magra sheep breeds were sequenced by Xcelris Genomics Pvt. Ltd., Gujrat, India. The sequences obtained were subjected to DNA to protein translation using online tool (http://insilico.ehu.es/translate/) and took longest ORF for the analysis, as we are interested only coding sequences (cds). After the getting all coding sequences, we did the nucleotide BLAST (Basic Local Alignment Search tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the similarity with the already submitted gene sequences. The sequences were also aligned using BioEdit software to study the variations in the nucleotide sequences.

The present study was undertaken with the objective to assess the genetic variability within the four sheep breeds of Rajasthan i.e. Magra, Chokla, Marwari and Nali. This study was conducted using 18 microsatellite markers and 30 animals per breed. Breed characterization requires basic knowledge of genetic variations that can be effectively measured within and between populations by using various statistical parameters viz. Allele frequency, Observed and effective number of alleles, Private alleles, Observed and expected heterozygosity, F-statistics, Hardy-Weinberg equilibrium, Polymorphic information content, Fixation index and Evens-Watterson Neutrality. Further, the genetic distance, phylogenetic relationship and genetic bottleneck analysis were also studied.

4.1 Genomic DNA purity and concentration

In the present study, all the DNA samples were observed to be of good quality and showed an intense band in the UV transilluninator and were later photographed in the Gel Documentation system.

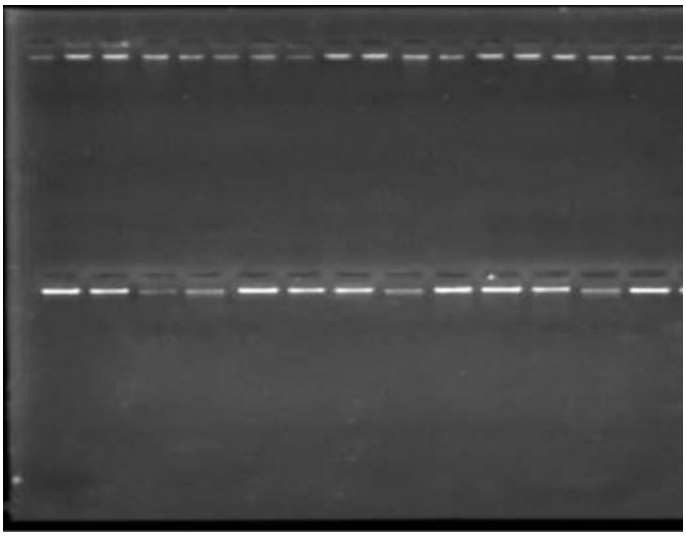

Fig. 5 DNA isolation

The concentration of DNA samples as read from UV spectrophotometer ranged for Magra, 0.4000-0.4500 µg/ml, 0.3600 – 0.4200 µg/ml for Marwari, 0.3516- 0.4300 µg/ml for Chokla and 0.3490- 0.3950 µg/ml for Nali. The representative image of isolated DNA has shown in figure 5.

4.2 Microsatellite marker Genotyping

Genotyping of the individual animal at various loci has done, based on the presence or absence of a particular allele and the genotypes of each animal at 18 different loci are detailed in Table 9a – 26a. Among the 18 loci studied few alleles did not show any amplification. The presence of two alleles of similar length (bp) at a locus was considered homozygous, while that with dissimilar length was considered heterozygous. The representative Gel images for the primer OarHH64 in Chokla, OarHH41 in Marwari, OarJMP8 in Nali and OarCP34 in Magra presented in following figures 6, 7, 8& 9.

4.3 Microsatellite Analysis

4.3.1 Allele frequency

The frequency of various alleles at different loci in the four sheep breeds is detailed in the Table (9b – 26b). The allele frequency ranged from 1.7 percent (BM8125, OarHH41, OarHH64, OarFCB48, TGLA137, OarCP34, OarAE129, CSSM31, BM6526, BM757, OarVH72, RM4) to 56.7% (185 bp at BM757) in Chokla, 1.7% (OarHH35, OarHH41, OarJMP8, OarFCB48, OarCP34, CSSM31, BM757, OarVH72) to 63.3% (100 bp at TGLA377) in Nali, 1.7% (OarHH35, OarFCB128, OarHH41, OarHH64, OarJMP8, OarFCB48, OarJMP29, BM757, OarVH72, RM4) to 53.3% (130 bp at OarHH35) in Marwari and 1.7% (BM8125, OarHH35, OarFCB128, OarHH41, OarHH64, OarFCB48, TGLA137, OarCP34, OarAE129, CSSM31, BM757, OarVH72, RM4) to 51.7% (135 bp at CSSM31) in Magra sheep. The allele frequencies in the present study were as low as 1.7% and as high as 63.30% at locus TGLA377 in Nali sheep breed (Table 9b – 26b).

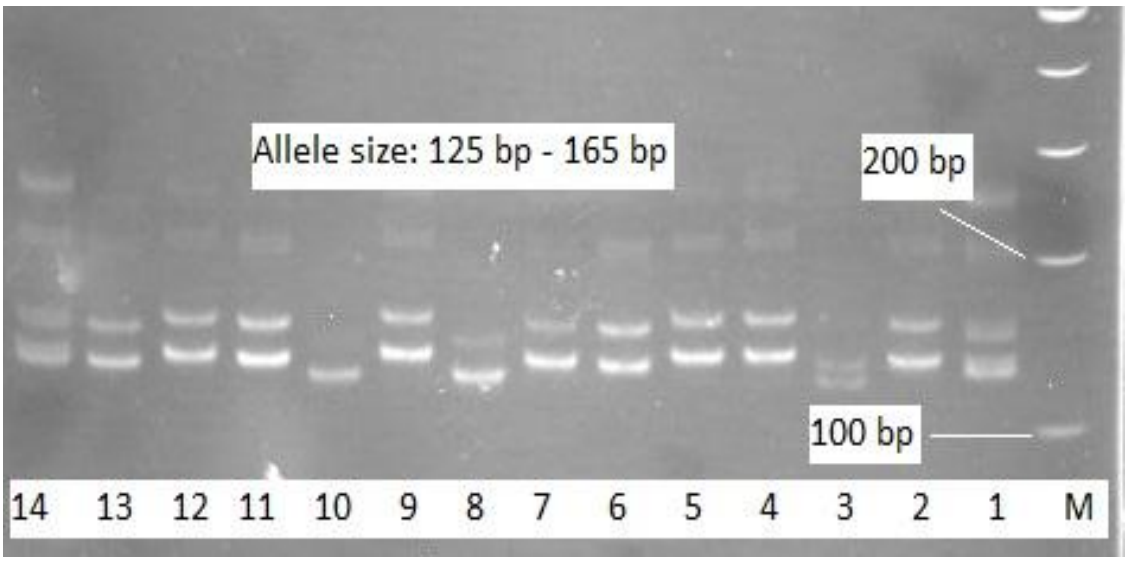

Fig.6: Polymorphism of microsatellite marker OarHH64 resolved on polyacrylamide gel (8%) for Chokla breed (1-14).

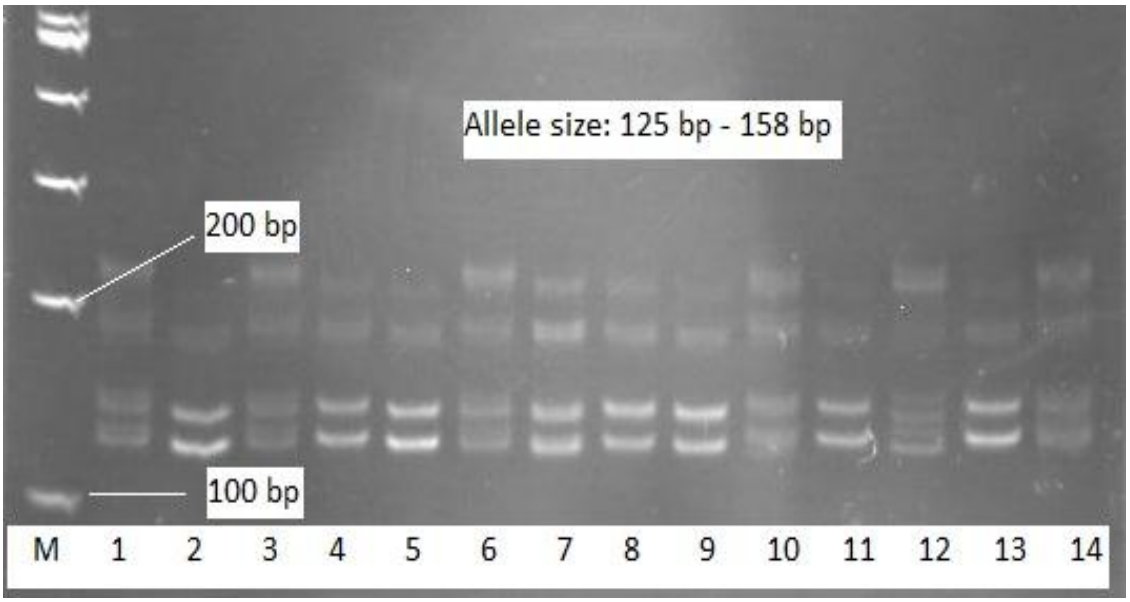

Fig.7: Polymorphism of microsatellite marker OarHH41 resolved on polyacrylamide gel (8%) for Marwari breed (1-14).

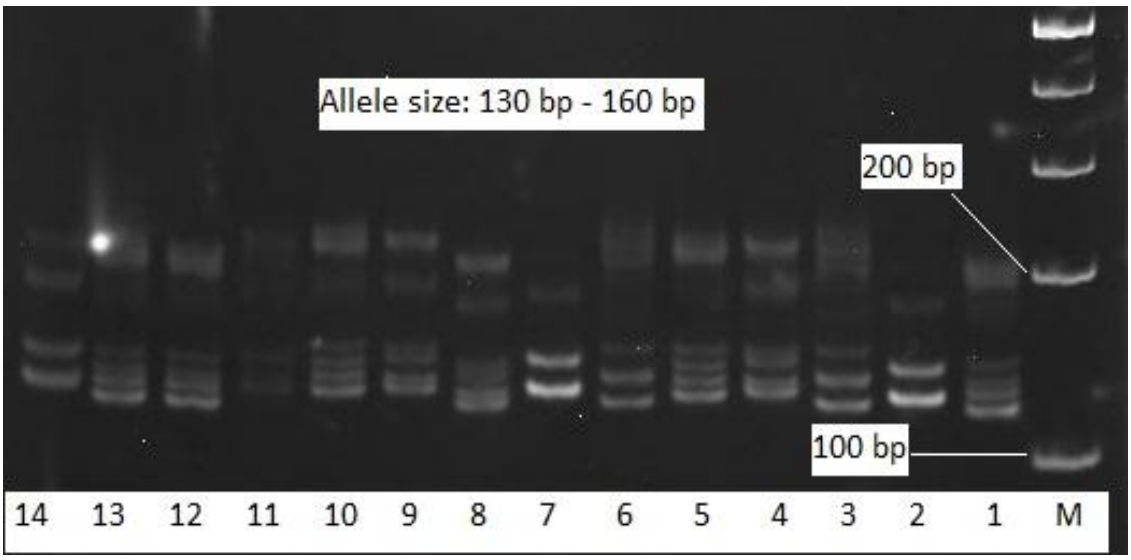

Fig.8: Polymorphism of microsatellite marker OarJMP8 resolved on polyacrylamide gel (8%) for Nali breed (1-14).

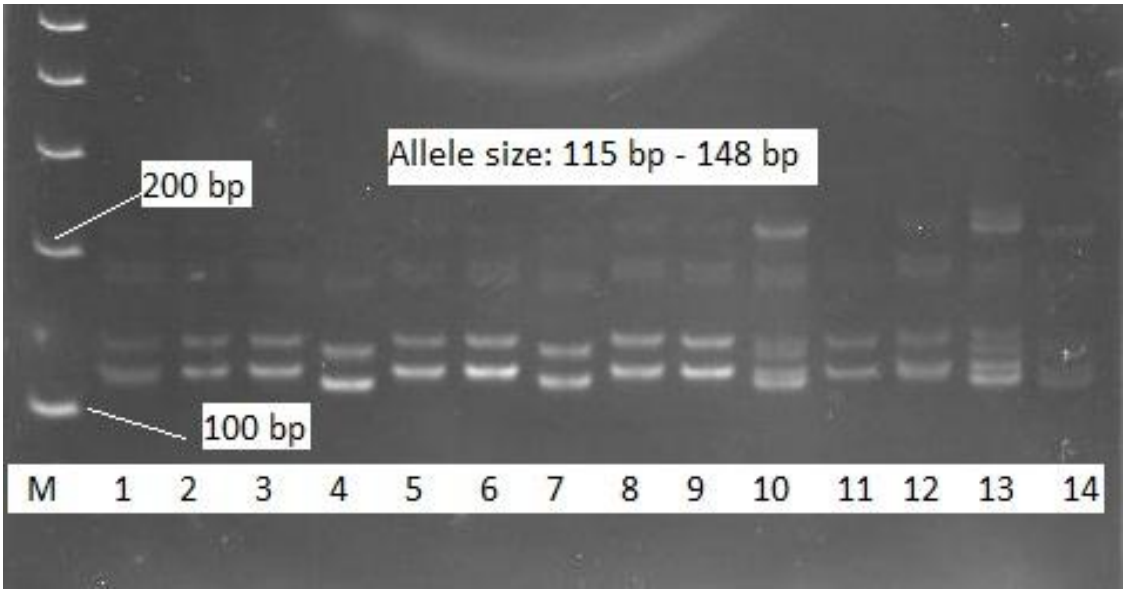

Fig.9: Polymorphism of microsatellite marker OarCP34 resolved on polyacrylamide gel (8%) for Magra breed (1-14).

The distribution of allele frequency in the present study is very discrete. Similar findings were also reported by El-Nahas *et al.* (2008) in Egyptian sheep breeds, Nanekarani *et al.* (2010) in Iranian sheep and Kumar *et al.* (2007) in Bellary sheep.

4.3.2 Alleles and their allelic pattern

A total of 224 alleles, ranging between 8 (TGLA377 and OarAE129) – 17 (OarFCB48) were found across 18 microsatellite loci (Table 33). The highest total number of alleles was detected in Marwari sheep (158) ranged between 4 (TGLA377) to 15 (OarFCB48). Whereas, Nali sheep was observed with the lowest number of alleles (133), ranged from 2 (TGLA377) to 10 (BM6526, OarVH72), number of alleles in Chokla and Magra were same with the value of 150, ranged from 4 (OarFCB128) to 13(OarFCB48) and 5 (CSSM31, TGLA377) to 13(OarVH72), respectively (Table 29 - 32). Whereas, many authors reported a lower number of alleles (Farid *et al.*, 2000; Girish *et al.*, 2007; Prema *et al.*, 2008a, 2008b; Nanekarani *et al.*, 2010; Das *et al*., 2015; Surekha *et al*., 2015; Wajid *et al*., 2014; Pandey *et al.* (2007, 2009); Sodhi *et al*., 2006; Mukesh *et al*., 2006 and Pariset *et al*., 2003). However, some authors reported higher number of alleles (Diez-Tascon *et al.*, 2000; Ozerov *et al.*, 2008; Jyotsana *et al*., 2010; Rodrigo *et al.*, 2010 ; Radha *et al.*, 2011; Peter *et al*., 2007; Sharma *et al*., 2010; Tapio *et al*., 2010; Arora *et al*., (2011a, 2011b, 2011c); Ghazy *et al*., 2013; Mukhongo *et al*., 2014; Sassi-Zaidy *et al*., 2014; Kavitha *et al*., 2015) than the present findings. Although, some authors reported the number of alleles in a similar magnitude with the present findings in Chokla, Nali, Marwari and Magra sheep (Sharma *et al*., 2016; Pramod *et al*., 2009; Bhatia and Arora, 2008; Sharma *et al*., 2006).

The allelic patterns across the studied 18 microsatellite loci are presented in Table 33. Overall in 4 breeds 16 markers were detected with greater than and equal to 10 alleles whereas two markers TGLA377 and OarAE129 were observed with 8 alleles. In the breed wise analysis, loci were found with higher than 10

alleles in Chokla, Marwadi and Magra breeds respectively 3 loci (BM6526, OarJMP29 and OarFCB48), 5 loci (OarFCB48, OarJMP8, OarJMP29, OarVH72 and RM4) and 3 loci (OarHH64, TGLA137 and OarVH72). However, remaining loci were found with the ranged between 4 to 10 alleles in respective breeds. A microsatellite preferably should have at least 4 alleles to be useful for the evaluation of genetic diversity as per the standard selection of microsatellite loci (Baker, 1994). Therefore, the selection of microsatellite markers with a variable number of alleles may reduce the risk of overestimating genetic variability, which might occur with the selective use of highly polymorphic loci.

The product size (bp) in four sheep breeds varied from 80 (TGLA377) to 265 (BM827) (Table 9b – 26b). The size of the most frequent alleles ranged from 95 to 225 bp in Chokla breed, 100 to 215 bp in Nali, 99 to 230 bp in Marwari and 100 to 220 bp in Magra (Table 9b– Table 26b).

The allele distribution and characteristics of different microsatellite loci are discussed for each locus separately in terms of product size, mean numbers of alleles, allele frequency, effective number of alleles and frequent alleles.

4.3.2.1Microsatellite BM8125

On the basis of variability in alleles, we found variable genotypes in different samples as mentioned in BM8125 locus, which are presented in Table 9a. On this locus, the allelic frequency ranged from 0.017 to 0.250, 0.033 to 0.267, 0.033 to 0.233 and 0.017 to 0.367 for Chokla, Nali, Marwari and Magra sheep respectively (Table 9b). A total of 11 alleles ranged from 105-154 bp were amplified in the four sheep breeds, where eight alleles were observed in Chokla and Nali, seven alleles were found in Marwadi and nine alleles were detected in Magra breed. The number of alleles recorded in the present study is similar to that reported by Ozerov *et al.* (2008) in Kazakh sheep (8 alleles), Kumar *et al.* (2007) in Bellary sheep (7 alleles) and Jyotsana *et al.*(2010) in three Gujarat sheep breeds (7 alleles). However, numbers of alleles (10 to 11) were reported by Rodrigo *et al.*

(2010) in Chilean breeds and Arora *et al.* (2011) into Rajasthan sheep breeds. Lesser number of alleles were also reported at the same locus by Arora and Bhatia, (2004) in Muzaffarnagari sheep (5), Girish *et al.* (2007) in Nilagiri sheep (5), Arora *et al.* (2008) in Jalauni sheep (6), Pramod *et al.* (2009) in Vembur sheep (6 alleles).

Table 9a: Genotypes of Chokla, Nali, Marwari and Magra sheep at BM8125 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at BM8125 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0105	0205	0305	0101
2.	0206	0306	0909	0101
3.	0306	0902	0104	0208
4.	0103	0303	0305	0308
5.	0407	0305	0202	0202
6.	0203	0303	0305	0308
7.	0408	0307	0405	0202
8.	0407	0206	0405	0904
9.	0307	0306	0404	0104
10.	0407	0306	0404	1107
11.	0206	0307	0405	0907
12.	0206	0306	0303	0908
13.	0307	0306	0405	0208
14.	0307	0303	0404	0405
15.	0303	0909	0205	0202
16.	0303	0104	0101	0202
17.	0103	0104	0101	0303
18.	0205	0104	0205	0308
19.	0306	0909	0101	0305
20.	0202	0104	1010	0304
21.	0105	0104	0205	0202
22.	0202	0104	0909	0308
23.	0105	0104	0105	0202
24.	0101	0205	0101	0202
25.	0101	0202	0101	0208
26.	0101	0205	0205	0202
27.	0101	0104	0205	0101
28.	0306	0306	0205	0202
29.	0306	0104	0101	0208
30.	0103	0306	0404	0308

The most repeated allele ranged between 125 bp in Magra and 140 bp in Marwari sheep breeds and the effective number of alleles was detected as 5.8, 6.2,

5.3 and 4.8 in Chokla, Nali, Marwari and Magra sheep breeds respectively. Published reports on effective number of alleles at the same locus depict a range of 1.85 to 3.37 (Girish *et al.*, 2007; Kumar *et al.*, 2007; Pramod *et al.*, 2009) in Albanian, Nilagiri, Bellary, Jalauni and Vembur sheep breeds.

Table 9b: Allele number, size and frequencies for BM8125 microsatellite locus

Allele No.	Allele size(bp)	Allele freq. of BM8125 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	120	0.233	0.150	0.233	0.116
2.	125	0.150	0.117	0.133	0.367
3.	130	0.250	0.267	0.083	0.150
4.	135	0.067	0.150	0.216	0.067
5.	140	0.067	0.067	0.233	0.033
6.	145	0.117	0.133	0.000	0.000
7.	150	0.100	0.033	0.000	0.033
8.	154	0.016	0.000	0.000	0.167
9.	115	0.000	0.083	0.069	0.050
10.	105	0.000	0.000	0.033	0.000
11.	110	0.000	0.000	0.000	0.017

4.3.2.2 Microsatellite OarFCB48

The genotypes for sheep breeds at OarFCB48 locus are presented in Table 10a. On this locus, the allelic frequency ranged from 0.017 to 0.167, 0.017 to 0.250, 0.017 to 0.167 and 0.017 to 0.383 in Chokla, Nali, Marwari and Magra respectively (Table 10b). A total of 17 alleles ranged from 135-198 bp were typed in the four sheep breeds, where thirteen alleles presented in Chokla, nine alleles detected in Nali, fifteen alleles were found in Marwari and seven alleles in the Magra sheep breed. The allele size of the most repeated allele on this locus was found as 150 bp in Magra, 165 bp in Marwari, 160 bp in Nali and 160 and 170 bp in Chokla presented in Table 10b.

The similar allele number was seen by Mukesh *et al*. (2006) (8) and Kumar *et al*. (2007) (9), Diez-Tascon *et al*. (2000) (14), Ozerov *et al*. (2008) (14),

Jyotsana *et al*. (2010) (13), and Arora *et al*. (2010) (13) and the lowest values were reported in studies made by Arora and Bhatia

Table 10a: Genotypes of Chokla, Nali, Marwari and Magra sheep at OarFCB48 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at FCB48 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0101	0202	0303	1104
2.	0101	0104	0101	0404
3.	0303	0104	1504	1104
4.	0210	0101	1504	0308
5.	0303	0206	1104	0303
6.	0303	0105	1515	0105
7.	0303	0303	1403	0101
8.	0303	0303	1403	0105
9.	0404	0505	1414	1111
10.	0509	0105	1303	1104
11.	0404	0206	1313	1105
12.	0505	0206	1301	0101
13.	0509	0101	1313	0105
14.	0509	0105	1212	0303
15.	0510	0207	0303	0303
16.	0505	0105	0305	1111
17.	0510	0404	0404	1104
18.	0713	0307	0404	1105
19.	0404	0409	0716	0101
20.	0610	0303	0408	1104
21.	0408	0202	0716	1105
22.	0612	0303	0505	1104
23.	0611	0307	0509	1105
24.	0606	0307	0917	0410
25.	0610	0307	0707	1111
26.	0606	0307	0608	1111
27.	0711	0304	0707	1105
28.	0707	0408	0505	1104
29.	0707	0307	0404	1104
30.	0510	0104	0505	1111

(2004) (6), Prema *et al*. (2008a) (5) and Pramod *et al*. (2009) (5). The allele size in the present study was similar to the findings obtained by the previous authors.

The effective number of alleles was observed as 8.6, 6.3, 10 and 4.2 in Chokla, Nali, Marwari and Magra sheep breed, respectively. The effective

number of alleles in other studies was seen as 5.62 by Arora and Bhatia (2004), 4.87 by Mukesh *et al*. (2006), 4.91 by Kumar *et al*. (2007), 4.43 by Prema *et al*. (2008a), 4.16 by Pramod *et al*. (2009), 6.68 by Jyotsana *et al*. (2010) and 4.22 by Radha *et al*. (2011). The most frequent allele was ranged between 150 to 170 bp (10b) in 4 sheep breed for the locus where as it was 132 bp in the study made by Hepshiba *et al*. (2014).

Table 10b: Allele number, size and frequencies for OarFCB48 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of OarFCB48 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	155	0.067	0.183	0.050	0.150
2.	158	0.017	0.133	0.000	0.000
3.	160	0.167	0.250	0.133	0.117
4.	165	0.117	0.133	0.167	0.183
5.	170	0.167	0.100	0.133	0.133
6.	172	0.131	0.050	0.017	0.000
7.	175	0.100	0.117	0.100	0.000
8.	180	0.017	0.017	0.033	0.017
9.	182	0.050	0.017	0.033	0.000
10.	185	0.100	0.000	0.000	0.017
11.	150	0.033	0.000	0.017	0.383
12.	135	0.017	0.000	0.033	0.000
13.	140	0.017	0.000	0.100	0.000
14.	145	0.000	0.000	0.067	0.000
15.	150	0.000	0.000	0.067	0.000
16.	190	0.000	0.000	0.033	0.000
17.	198	0.000	0.000	0.017	0.000

4.3.2.3 Microsatellite OarFCB128

The genotypes for sheep breeds at OarFCB128 locus are presented in Table 11a. On this locus the allelic frequency ranged from 0.033 to 0.467, 0.067 to 0.350, 0.017 to 0.450 and 0.017 to 0.283 in Chula, Nail, Marwari and Mara respectively (Table 11b). A total of 12 alleles ranged from 95-145 bp were amplified in the four sheep breeds, where four alleles were observed in Chokla, six alleles were present in Nail, nine alleles were found in Marwari and seven alleles were detected in the Mara sheep breed. The allele size of the most repeated

allele in OarFCB128 locus was found as 105 bp in Magra, 135 bp in Chokla 100 bp in Nali and 100 bp in Marwari sheep.

Table 11a: Genotypes of Chokla, Nali, Marwari and Magra sheep at OarFCB128 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at OarFCB128 Microsatellite loci in four sheep breeds			
	Chokla	Nali	Marwadi	Magra
1.	0103	0202	0203	0909
2.	0103	0502	0502	0606
3.	0103	0502	0502	0606
4.	0103	0508	0505	0909
5.	0101	0505	0502	0905
6.	0104	0707	1212	0202
7.	0103	0507	0512	0910
8.	0203	0202	0505	1010
9.	0203	0508	0704	1010
10.	0204	0505	0506	1010
11.	0203	0505	1212	0606
12.	0203	0101	0506	0505
13.	0203	0606	0505	0606
14.	0103	0606	1212	0910
15.	0103	0606	0512	0202
16.	0103	0606	1212	0909
17.	0103	0606	1212	0602
18.	0103	0505	0505	0606
19.	0103	0505	0704	0202
20.	0103	0505	0811	0910
21.	0103	0808	0502	0901
22.	0103	0606	0512	0910
23.	0103	0505	0502	0608
24.	0103	0606	0505	0902
25.	0101	0707	0505	0606
26.	0103	0202	0202	0909
27.	0303	0202	0505	1010
28.	0103	0101	0707	1010
29.	0303	0606	0505	1010
30.	0203	0505	0502	0910

The observation on the allele number was in accordance with the reports made by few authors as Girish *et al*. (2007) (4), Kumar *et al*. (2007) (2), Pramod *et al*. (2009) (5) and Hepshiba *et al*. (2014) (5), 9 alleles by Diez-Tascon *et al*. (2000).

However, the observed value was lower in the findings of Ozerov *et al*. (2008) and Jyotsana *et al*. (2010) who studied 11 alleles, Arora *et al*. (2010) who studied 18 alleles while Radha *et al*. (2011) studied 13 alleles respectively.The effective number of alleles ranged between 2.6 for Chokla to 4.4 for the Magra sheep breed. The values were in accordance with the earlier studies made by Girish *et al*. (2007) (3.11), Pramod *et al*. (2009) (4.27), Jyotsana *et al*. (2010) (4.08) and Hepshiba *et al*. (2014) (4.39). While, Arora *et al*. (2004) (5.62) showed in a greater extent.

Table 11b: Allele number, size and frequencies for OarFCB128 microsatellite locus.

Allele No.	Allele size (bp)	Allele Freq. of OarFCB128 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	118	0.383	0.067	0.000	0.017
2.	120	0.117	0.167	0.150	0.133
3.	135	0.467	0.000	0.017	0.000
4.	140	0.033	0.000	0.033	0.000
5.	100	0.000	0.350	0.450	0.050
6.	110	0.000	0.266	0.033	0.233
7.	125	0.000	0.083	0.066	0.000
8.	130	0.000	0.067	0.017	0.017
9.	95	0.000	0.000	0.000	0.267
10.	105	0.000	0.000	0.000	0.283
11.	145	0.000	0.000	0.017	0.000
12.	115	0.000	0.000	0.217	0.000

4.3.2.4 Microsatellite OarHH35

The genotypes forsheep breeds at OarHH35 locus are presented in Table 12a. On this locus the allelic frequency ranged from 0.033 to 0.350, 0.017 to 0.250, 0.017 to 0.533, and 0.017 to 0.233 in Chokla, Nali, Marwari and Magra respectively (Table 12b). A total of 11 alleles (115-155 bp) were typed in the four studied sheep breeds, where eight alleles were found in Chokla, nine alleles were detected in Nali, five alleles were observed in Marwari and nine alleles were found in the Magra sheep breed. The most repeated allele size was found as 135

bp in Magra, 130 bp in Marwari, 120 bp in Nali and 150 bp in Chokla presented in Table 12b.

Table 12a: Genotypes of Chokla, Nali, Marwari and Magra sheep at OarHH35 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at OarHH35Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0207	0206	0205	0202
2.	0207	0307	0202	0303
3.	0207	0206	0205	0202
4.	0206	0303	0505	0202
5.	0207	1010	0205	0202
6.	0307	0303	0202	0303
7.	0307	0202	0202	0101
8.	0307	0101	0202	0202
9.	0307	0202	0205	0207
10.	0307	1010	0202	0202
11.	0306	1005	0205	0202
12.	0508	1010	0211	0202
13.	0303	0106	0105	0307
14.	0508	1004	0205	0303
15.	0202	0205	0211	0303
16.	0207	0206	0205	0307
17.	0207	0105	0211	0303
18.	0206	0105	0211	0404
19.	0307	1005	0101	0507
20.	0307	1005	0211	1111
21.	0202	0206	0105	0606
22.	0101	1010	0211	0606
23.	0307	0105	0211	0505
24.	0307	0202	0211	0505
25.	0307	1005	1111	0507
26.	0307	0909	0211	0505
27.	0407	1005	0203	0507
28.	0407	0101	0202	1108
29.	0207	0105	0211	0202
30.	0307	1005	0202	0303

The previous reports at the same locus showed as 6 alleles by Arora and Bhatia (2004), Mukesh *et al*. (2006) found 8 alleles, 7 alleles by Girish *et al*. (2007), 10 alleles by Kumar *et al*. (2007) and Hepshiba *et al*. (2014), 6 alleles by

Pramod *et al*. (2009) and 11 alleles by Jyotsana *et al*. (2010), respectively. The size of the alleles ranged from 87-142 bp presented by Arora and Bhatia (2006), Mukesh *et al*. (2006), Girish *et al*. (2007), Kumar *et al*. (2007) and Hepshiba *et al*. (2014). The effective number of alleles for Chokla, Nali, Marwari and Magra sheep for this locus was estimated as 4.1, 5.9, 2.7 and 5.0 respectively, which were similar to those obtained in all other studies done for this locus. The most frequent allele ranged between 120 to 150 bp (Table 12b) in studying sheep breeds, whereas, it was 123 bp allele as reported by Hepshiba *et al*. (2014).

Table 12b: Allele number, size and frequencies for OarHH35 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of OarHH35 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	125	0.033	0.150	0.067	0.033
2.	130	0.218	0.183	0.533	0.317
3.	135	0.250	0.083	0.017	0.233
4.	138	0.033	0.017	0.000	0.033
5.	140	0.033	0.183	0.183	0.150
6.	148	0.050	0.084	0.000	0.067
7.	150	0.350	0.017	0.000	0.100
8.	155	0.033	0.000	0.000	0.017
9.	115	0.000	0.033	0.000	0.000
10.	120	0.000	0.250	0.000	0.000
11.	145	0.000	0.000	0.200	0.050

4.3.2.5 Microsatellite OarHH41

The genotypes for sheep breeds at OarHH41 locus are presented in Table 13a. On this locus the allelic frequency ranged from 0.017 to 0.267, 0.017 to 0.433, 0.017 to 0.267 and 0.017 to 0.417 in Chokla, Nali, Marwari and Magra respectively (Table 13b). A total of 12 alleles ranged from 125-165 bp were typed in the four studying sheep breeds, where seven alleles were found in Chokla, eight alleles were detected in Nali, ten alleles were observed in Marwari and nine alleles were present in the Magra sheep breed. The size of the most frequent allele in OarHH41 locus was found as 135 bp in Magra, 150 bp in Chokla, 130 bp in Nali and Marwari sheep presented in Table 13b.

Table 13a: Genotypes of Chokla, Nali, Marwari and Magra sheep at OarHH41 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at OarHH41 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0105	0101	0305	0101
2.	0303	0305	0105	0101
3.	0205	0202	0105	0306
4.	0303	0101	1005	0303
5.	0406	0101	0312	0303
6.	0406	0101	0212	0303
7.	0406	0205	0212	0104
8.	0406	0101	0408	0303
9.	0406	0101	0305	0303
10.	0406	0101	0406	0106
11.	0404	0101	0202	0106
12.	0406	0101	0202	0306
13.	0406	0101	0205	0303
14.	0507	0404	0202	0303
15.	0105	0101	0101	0101
16.	0105	0101	0104	0106
17.	0205	0305	0101	0306
18.	0101	0404	0112	0306
19.	0205	0305	1112	0101
20.	0305	0303	1111	0306
21.	0305	0404	1104	0409
22.	0202	0408	0112	0404
23.	0305	0306	1104	0308
24.	0405	0306	0101	0306
25.	0305	0409	0112	0205
26.	0305	0404	1111	1010
27.	0205	0409	0112	0303
28.	0205	0505	0101	0404
29.	0205	0306	0202	0303
30.	0406	0101	0105	0106

All the other studies for this locus varied between 4 to 9 alleles, while the size of the alleles was similar to the present findings. The effective number of alleles was 5.5, 3.9, 6.2 and 3.8 for Chokla, Nali, Marwari and Magra sheep breed. Perusal of the previous literature showed the value as 3.30 by Arora and Bhatia (2004), 3.70 by Arora and Bhatia (2006), 3.27 by Girish *et al*. (2007), 2.74 by Kumar *et al*. (2007), 5.34 by Arora *et al*. (2008), 2.76 by Prema *et al*. (2008a),

4.59 by Prema *et al*. (2008b), 4.834 by Pramod *et al*. (2009), 3.21 by Arora *et al*. (2010), 4.77 by Jyotsana *et al*. (2010), 3.46 by Radha *et al*. (2011) and 1.97 by Hepshiba *et al*. (2014). The most frequent allele ranged from 130 to 150 bp in all studied population when compared to 118 bp allele in the study made by Hepshiba *et al*. (2014).

Table 13b:Allele number, size and frequencies for OarHH41 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of HH41 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	130	0.083	0.433	0.267	0.216
2.	132	0.133	0.050	0.183	0.017
3.	135	0.150	0.134	0.050	0.416
4.	140	0.200	0.183	0.082	0.100
5.	150	0.267	0.100	0.117	0.017
6.	155	0.150	0.050	0.017	0.167
7.	165	0.017	0.000	0.000	0.000
8.	158	0.000	0.017	0.017	0.017
9.	160	0.000	0.033	0.000	0.017
10.	138	0.000	0.000	0.017	0.033
11.	125	0.000	0.000	0.117	0.000
12.	145	0.000	0.000	0.133	0.000

4.3.2.6 Microsatellite OarHH64

The genotypes for each population at OarHH64 locus are presented in Table 14a. On this locus the allelic frequency ranged from 0.017 to 0.200, 0.033 to 0.183, 0.017 to 0.267 and 0.017 to 0.183 in Chokla, Nali, Marwari and Magra respectively (Table 14b). A total of 13 alleles (115-175 bp) was typed in the four sheep breeds, where nine alleles were present in Chokla and Nali, eight alleles were found in Marwari and twelve alleles were detected in the Magra sheep breed. The allele size of the most frequent allele in OarHH64 locus was found as 135 and 145 bp in Magra, 140 bp in Marwari, 120 bp in Nali and 140 and 145 bp in Chokla presented in Table 14b.

Table 14a: Genotypes of Chokla, Nali, Marwari and Magra sheep at OarHH64 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at OarHH64 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0508	1010	0106	0303
2.	0508	0104	0408	0303
3.	0508	0104	0306	0409
4.	0509	0206	0202	0509
5.	0509	0303	0202	0505
6.	0505	0101	0406	0508
7.	0509	0202	0406	0202
8.	0509	1010	0406	0509
9.	0509	0505	0406	0303
10.	0408	0101	0202	0509
11.	0404	0505	0306	0613
12.	0408	1004	0306	0303
13.	0404	1010	0205	0404
14.	0404	1010	0306	0612
15.	0202	0303	0408	0105
16.	0306	0202	0408	0202
17.	0101	0406	0404	1010
18.	0408	0306	0408	0206
19.	0408	0508	0306	1010
20.	0306	0508	0408	1010
21.	0307	0404	0303	0105
22.	0101	0404	0509	1111
23.	0508	0505	0408	0105
24.	0101	0609	0408	0205
25.	0306	0609	0408	0105
26.	0408	0609	0202	1103
27.	0306	0609	0308	1010
28.	0303	0606	0409	1111
29.	0408	0609	0408	1010
30.	0508	1010	0202	0303

The studies made on the same locus by other authors was found as 7 alleles by Girish *et al*. (2007), 6 alleles by Arora *et al*. (2008), 5 alleles by Pramod *et al*. (2009) in Vembur, 5 alleles by Sharma *et al*. (2010) in Changthangi sheep, 3 alleles by Hepshiba *et al*. (2014) and 5 alleles by Radha *et al*. (2011). The allele size in the present study was in greater extent reported by other authors. The effective number of alleles was 6.6, 7.7, 5.3 and 7.8 in Chokla, Nali, Marwari and

Magra sheep breed respectively. The effective number of alleles in other findings was as shown 5.7 by Girish *et al*. (2007), 4.03 by Arora *et al*. (2008), 3.5 by Pramod *et al*. (2009) in Vembur, 1.6 by Sharma *et al*. (2010) in Changthangi sheep, 1.13 by Hepshiba *et al*. (2014) and 4.30 by Radha *et al*. (2011). The most frequent allele for this locus was ranged between 120 - 145 bp in studying population, which is similar to other reported works in the sheep population.

Table 14b: Allele number, size and frequencies for OarHH64 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of HH64 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	125	0.100	0.100	0.017	0.067
2.	130	0.033	0.083	0.183	0.100
3.	135	0.117	0.083	0.133	0.183
4.	140	0.200	0.133	0.267	0.050
5.	145	0.200	0.133	0.033	0.183
6.	155	0.067	0.168	0.167	0.050
7.	158	0.017	0.000	0.000	0.000
8.	160	0.183	0.033	0.167	0.017
9.	165	0.083	0.084	0.033	0.066
10.	120	0.000	0.183	0.000	0.167
11.	115	0.000	0.000	0.000	0.083
12.	172	0.000	0.000	0.000	0.017
13.	175	0.000	0.000	0.000	0.017

4.3.2.7 Microsatellite OarJMP8

The genotypes for each population at OarJMP8 locus are presented in Table 15a. On this locus the allelic frequency ranged from 0.050 to 0.283, 0.017 to 0.317, 0.017 to 0.250 and 0.017 to 0.300 in Chokla, Nali, Marwari and Magra respectively (Table 15b). A total of 14 alleles (115-160 bp) was typed in the four studying sheep breeds, where seven alleles were found in Chokla, nine alleles were detected in Nali, eleven alleles were observed in Marwari and ten alleles were present in the Magra sheep breed. The allele size of the most frequent allele in OarJMP8 locus was found as 130 bp in Magra, 135 bp in Chokla 140 bp in Nali and 130 bp in Marwari sheep were detected in OarJMP8, presented in Table 15b.

Table 15a: Genotypes of Chokla, Nali, Marwari and Magra sheep at OarJMP8 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at OarJMP8 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0206	0101	0206	0303
2.	0206	0206	0105	0407
3.	0205	0105	0313	0303
4.	0205	0303	0105	0404
5.	0202	0202	0313	0404
6.	0306	0205	0101	0404
7.	0303	0407	1408	0105
8.	0303	0101	0103	1101
9.	0404	0404	0509	1101
10.	0202	0404	0404	1111
11.	0404	0303	0413	0101
12.	0407	0101	0408	1111
13.	0407	0202	0413	1010
14.	0407	0404	0505	1205
15.	0202	0406	0114	1212
16.	0206	0404	0206	0101
17.	0307	0407	1204	1212
18.	0306	0404	0105	0106
19.	0306	0404	0105	0101
20.	0202	0609	0105	0206
21.	0303	0508	0305	0104
22.	0101	0406	0105	0303
23.	0306	0509	0105	0105
24.	0306	0407	0314	0101
25.	0206	0509	0114	0505
26.	0105	0609	0114	0101
27.	0206	0606	1204	0307
28.	0206	0606	1212	0303
29.	0206	0404	0114	0404
30.	0407	0606	0105	0101

The studies made on the same locus by other authors are 5 alleles by Girish *et al*. (2007), 7 alleles by Arora *et al*. (2008), 5 alleles by Pramod *et al*. (2009) in Vembur, 7 alleles by Sharma *et al*. (2010) in Changthangi sheep, 6 alleles by Hepshiba *et al*. (2014) and 6 alleles by Radha *et al*. (2011). The allele size in the present study was in greater extent reported by other authors. The effective number of alleles was 5.2, 5.6, 6.8 and 5.9 in Chokla, Nali, Marwari and

Magra sheep breed respectively. The effective number of alleles in other findings was shown as 4.2 by Girish *et al*. (2007), 4.83 by Arora *et al*. (2008), 3.3 by Pramod *et al*. (2009) in Vembur, 5.1 by Sharma *et al*. (2010) in Changthangi sheep, 4.4 by Hepshiba *et al*. (2014) and 3.39 by Radha *et al*. (2011). The most frequent allele for this locus was ranged between 130 - 140 bp (Table 15b) in studying population, which was varied in other studied population as shown 115-127 bp by Sharma *et al*. (2010), 131 – 149 bp by Radha *et al*. (2011), 117 – 129 bp by Hepshiba *et al*. (2014), 144 – 168 by Girish *et al*. (2007).

Table 15b: Allele number, size and frequencies for OarJMP8 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of OarJMP8 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	130	0.050	0.117	0.250	0.300
2.	135	0.283	0.100	0.033	0.017
3.	138	0.200	0.067	0.083	0.150
4.	140	0.134	0.317	0.117	0.168
5.	145	0.050	0.082	0.200	0.083
6.	150	0.200	0.183	0.033	0.033
7.	152	0.083	0.050	0.000	0.033
8.	158	0.000	0.017	0.033	0.000
9.	160	0.000	0.067	0.017	0.000
10.	115	0.000	0.000	0.000	0.033
11.	120	0.000	0.000	0.000	0.100
12.	125	0.000	0.000	0.067	0.083
13.	155	0.000	0.000	0.067	0.000
14.	142	0.000	0.000	0.100	0.000

4.3.2.8 Microsatellite OarJMP29

The genotypes for each population at OarJMP29 locus are presented in Table 16a. On this locus the allele frequency ranged from 0.033 to 0.183, 0.033 to 0.250, 0.033 to 0.267 and 0.050 to 0.333 in Chokla, Nali, Marwari and Magra respectively (Table 16b). The OarJMP29, which is a di-nucleotide repeat, (CA)21 amplified a total of 16 alleles (130-180 bp) in the four studied breeds, where eleven alleles were found in Chokla sheep, seven alleles were detected in Nali, twelve alleles were seen in Marwari and seven allele were observed in Magra

sheep breed. The allele size of the most frequent allele was found as 140 bp in Magra, 150 bp in Marwari, 148 and 150 bp in Nali and 140 and 160 bp in Chokla presented in Table 16b.

Table 16a: Genotypes of Chokla, Nali, Marwari and Magra sheep at OarJMP29 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at OarJMP29 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0408	0408	0306	0101
2.	0408	0508	1304	0303
3.	0509	1207	0101	0101
4.	0510	0407	0101	0101
5.	0509	0407	0101	1313
6.	0610	1212	0216	0303
7.	0610	0306	1515	0314
8.	0202	1207	1515	0314
9.	0510	0303	1515	0314
10.	0611	0407	1515	0206
11.	0404	0303	0104	0206
12.	0508	0404	0104	0206
13.	0510	0303	1515	0202
14.	0711	0404	1504	0206
15.	0101	1212	0404	0202
16.	0206	0303	0407	0207
17.	0206	1212	0408	0303
18.	0206	1212	0404	0101
19.	0206	1212	0202	0202
20.	0101	0303	0407	0303
21.	0206	1206	0407	0307
22.	0202	0505	0202	0202
23.	0101	0407	0408	0307
24.	0306	0404	0407	0214
25.	0104	0404	0408	1313
26.	0206	0303	0505	0202
27.	0101	0505	0303	1313
28.	0307	0505	1212	0202
29.	0206	1212	1515	1313
30.	0408	0404	0407	0202

The studies made on the same locus by other authors are as follows: 7 alleles by Mukesh *et al*., (2006) and Girish *et al*., (2007), 11 alleles by Jyotsana *et al*. (2010), 8 alleles by Arora *et al*., (2010) and 7 alleles by Radha *et al*., (2011).

The allele size in the present study was in the range reported by other authors. The effective number of alleles found as 7.9, 5.0, 6.3 and 4.8 in Chokla, Nali, Marwari and Magra sheep breed respectively. The effective number of alleles in other findings was shown as 3.56 by Arora *et al*. (2004), 4.02 by Mukesh *et al*. (2006), 5.40 by Girish *et al*. (2007), 3.15 by Kumar *et al*. (2007), 5.16 by Pramod *et al*. (2009), 2.93 by Hepshiba *et al*. (2014). The most frequent allele for this locus was ranged between 140 to 160 bp (Table 16b) in studying sheep breeds.

Table 16b: Allele number, size and frequencies for OarJMP29 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of OarJMP29 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	132	0.150	0.000	0.133	0.133
2.	140	0.183	0.000	0.083	0.333
3.	145	0.033	0.217	0.050	0.217
4.	150	0.100	0.250	0.267	0.000
5.	155	0.100	0.117	0.033	0.000
6.	160	0.183	0.033	0.017	0.067
7.	165	0.033	0.100	0.083	0.050
8.	170	0.068	0.033	0.050	0.000
9.	172	0.033	0.000	0.000	0.000
10.	175	0.084	0.000	0.000	0.000
11.	180	0.033	0.000	0.000	0.000
12.	148	0.000	0.250	0.033	0.000
13.	135	0.000	0.000	0.017	0.133
14.	162	0.000	0.000	0.000	0.067
15.	130	0.000	0.000	0.217	0.000
16.	158	0.000	0.000	0.017	0.000

4.3.2.9 Microsatellite TGLA137

The genotypes for each population at TGLA137 locus are presented in Table 17a. On this locus the allele frequency ranged from 0.017 to 0.200, 0.033 to 0.300, and 0.017 to 0.217 in Chokla, Nali, Marwari and Magra respectively (Table 17b). A total of 14 alleles (128-190 bp) was typed in the four studied breeds, where ten alleles were found in Chokla, nine alleles were seen in Nali and Marwari and twelve alleles were observed in the Magra sheep breed. The most frequent allele on this locuswas found as 150 bp in Chokla, 145 bp in Nali, 160 bp

in Marwari and 140 bp in Magra sheep, presented in Table 17b. The studies made on the same locus by other authors are reported as 6 alleles by Pramod *et al*. (2009) in Vembur and 7 alleles by Radha *et al*. (2011).

Table 17a: Genotypes of Chokla, Nali, Marwari and Magra sheep at TGLA137 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at TGLA137 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0207	0208	0606	0404
2.	0204	0303	0707	0308
3.	0202	0404	0303	0714
4.	0408	0609	0202	0606
5.	0204	0404	0202	0309
6.	0408	0505	0404	0308
7.	0303	0505	0407	0308
8.	0104	0303	0307	0714
9.	0303	0707	0707	0202
10.	0408	0404	0505	0909
11.	0408	0709	0505	0707
12.	0204	0308	0707	0808
13.	0202	0207	0407	0707
14.	0307	0207	0714	0909
15.	0303	0303	0606	0606
16.	0509	0303	0306	0707
17.	0308	0611	0303	0404
18.	0404	0307	0303	0404
19.	0303	0606	0404	0206
20.	0409	0606	0707	0411
21.	0609	0606	0408	0202
22.	0609	0711	0814	0207
23.	0910	0407	0508	0104
24.	0909	0303	0608	0202
25.	0808	0303	0808	0207
26.	0707	0711	0808	0202
27.	0707	0404	0808	1203
28.	0606	0303	0909	1303
29.	0707	0606	0707	0404
30.	0408	0303	0808	0202

The allele number in the present study is in greater extent comparatively reported by other authors. The effective number of alleles detected as 7.1, 5.7, 6.6 and 7.6 in Chokla, Nali, Marwari and Magra sheep breed respectively. The effective number of alleles in other findings shown as 5.3 by Pramod *et al*. (2009)

in Vembur and 2.7 by Radha *et al*. (2011). The most frequent allele for this locus was ranged between 140 - 160 bp (Table 17b) in studying sheep breeds, which was varied in other studied population shown as 135– 147 bp by Radha *et al*. (2011), 138 – 156 bp by Pramod *et al*. (2009).

Table 17b: Allele number, size and frequencies for TGLA137 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of TGLA137 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	135	0.017	0.000	0.000	0.033
2.	140	0.133	0.050	0.067	0.217
3.	145	0.167	0.300	0.133	0.100
4.	150	0.200	0.150	0.117	0.150
5.	152	0.017	0.067	0.083	0.000
6.	155	0.067	0.167	0.100	0.083
7.	160	0.133	0.150	0.233	0.167
8.	165	0.133	0.033	0.200	0.083
9.	170	0.116	0.033	0.033	0.083
10.	190	0.017	0.000	0.000	0.000
11.	172	0.000	0.050	0.000	0.017
12.	128	0.000	0.000	0.000	0.017
13.	130	0.000	0.000	0.000	0.017
14.	182	0.000	0.000	0.033	0.033

4.3.2.10 Microsatellite OarCP34

The genotypes for sheep breeds at OarCP34 locus are presented in Table 18a. On this locus the allele frequency ranged from 0.017 to 0.333, 0.017 to 0.250, 0.033 to 0.233 and 0.017 to 0.200 in Chokla, Nali, Marwari and Magra respectively (Table 18b). A total of 13 alleles (105-190 bp) was amplified in the four studied breeds where ten alleles were found in Chokla, eight alleles were detected in Nali and Marwadi and nice alleles were observed in the Magra sheep breed. The most repeated allele was found as 125 bp in Chokla, 115 bp in Nali, 120 bp in Marwari and 110 bp in Magra presented in Table 18b. The reports which showed lower values than the present study were seen as 7 alleles by Diez-Tascon*et al*. (2000), 6 alleles by Ozerov *et al*. (2008), 7 alleles by Arora and Bhatia (2004), 4 alleles by Mukesh *et al*. (2006), 6 alleles by Girish *et al*. (2007),

5 alleles by Pramod *et al*. (2009), 9 alleles by Jyotsana *et al*. (2010) and 4 alleles by Hepshiba *et al*. (2014).

Table 18a: Genotypes of Chokla, Nali, Marwari and Magra sheep at OarCP34 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at OarCP34 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0408	0406	0101	0303
2.	0303	0404	0202	0101
3.	0408	0407	0311	0202
4.	0503	0404	0202	0101
5.	0303	0211	0205	1212
6.	0303	0406	0211	0104
7.	0303	0506	0101	0105
8.	0409	0311	0211	0211
9.	0303	0202	0105	1212
10.	0404	0202	0205	0105
11.	0408	0101	1212	1212
12.	0408	0105	1313	0105
13.	0408	0211	1212	1212
14.	0306	0211	1313	1212
15.	0307	0202	0101	0306
16.	0306	0311	0101	0306
17.	0307	0205	0103	0202
18.	0202	0101	0311	0406
19.	0202	0306	0211	0406
20.	0408	0306	0311	0306
21.	0408	0211	0306	0202
22.	0306	0303	1212	0408
23.	0307	0406	0102	0408
24.	0202	0101	0211	0202
25.	0202	0411	0102	0404
26.	0202	0101	0311	0404
27.	0306	0101	0211	0303
28.	0110	0101	0306	0303
29.	0303	0202	0311	1212
30.	0202	0101	0211	0408

Whereas, the higher number of alleles (14) noticed on this locus by Kevorkian *et al*. (2010). The effective number of alleles obtained at the locus was 4.9, 5.9, 6.1 and 7.0 for Chokla, Nali, Marwari and Magra sheep, respectively. While other reported values for the same locus are 5.18 by Arora and Bhatia (2004), 3.67 by

Mukesh *et al*. (2006), 4.22 by Pramod *et al*. (2009), 3.78 by Radha *et al*. (2011) and 4.39 by Hepshiba *et al*. (2014). The size of the allele for the four sheep breeds ranged between 105 – 190 bp (Table 18b), which is higher than that reported by Arora and Bhatia (2006) (110-128), Mukesh *et al*. (2006) (114-128), Jyotsana *et al*. (2010) (107-123) and Radha *et al*. (2011) (111-129). The most repeated alleles ranged between 110 to 125 bp (Table 18b) in four sheep breeds studied where as it was 113 bp allele in Coimbatore sheep as studied by Hepshiba *et al*. (2014).

Table 18b: Allele number, size and frequencies for OarCP34 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of OarCP34 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	115	0.017	0.250	0.200	0.133
2.	120	0.200	0.217	0.233	0.150
3.	125	0.333	0.100	0.133	0.150
4.	130	0.167	0.150	0.000	0.167
5.	135	0.017	0.050	0.050	0.050
6.	145	0.067	0.100	0.033	0.083
7.	148	0.050	0.017	0.000	0.000
8.	150	0.117	0.000	0.000	0.050
9.	152	0.017	0.000	0.000	0.000
10.	190	0.017	0.000	0.000	0.000
11.	140	0.000	0.117	0.183	0.017
12.	110	0.000	0.000	0.100	0.200
13.	105	0.000	0.000	0.067	0.000

4.3.2.11 Microsatellite OarAE129

The genotypes for each population at OarAE129 locus are presented in Table 19a. On this locus the allelic frequency ranged from 0.017 to 0.367, 0.050 to 0.383, 0.050 to 0.267 and 0.017 to 0.400 in Chokla, Nali, Marwari and Magra respectively (Table 19b). A total of 8 alleles (150-180 bp) were typed in the four studied breeds where six alleles were observed in Chokla and Marwari, five alleles in Nali and seven alleles were found in Magra sheep breed. The most repeated allele was found as 155 bp in Chokla, 180 bp in Nali 175 bp in Marwari and 155 bp in Magra sheep was detected in OarAE129 marker presented in Table 19b.

Table 19a: Genotypes of Chokla, Nali, Marwari and Magra sheep at OarAE129 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at OarAE129 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0206	0303	0202	0303
2.	0206	0404	0202	0404
3.	0202	0406	0202	0406
4.	0406	0206	0202	0206
5.	0206	0206	0307	0206
6.	0202	0206	0207	0206
7.	0404	0305	0207	0305
8.	0202	0306	0407	0306
9.	0206	0306	0407	0202
10.	0206	0202	0404	0202
11.	0202	0303	0406	0202
12.	0202	0202	0406	0202
13.	0306	0404	0406	0307
14.	0506	0404	0404	0207
15.	0404	0505	0307	0207
16.	0406	0606	0307	0407
17.	0406	0606	0307	0206
18.	0406	0606	0202	0106
19.	0406	0606	0307	0101
20.	0101	0606	0303	0202
21.	0206	0206	0407	0106
22.	0106	0206	0407	0202
23.	0101	0206	0307	0101
24.	0202	0303	0307	0206
25.	0106	0303	0303	0406
26.	0202	0406	0407	0206
27.	0101	0406	0202	0206
28.	0206	0404	0808	0207
29.	0406	0606	0407	0206
30.	0206	0303	0307	0207

A wide range (3-18) of alleles has reported in different breeds by different workers. Farid *et al*. (2000) and Pramod *et al*. (2009) reported 8 alleles in Tan and Vembur breeds while 7 alleles were reported by Nanekarani *et al*. (2010). However, Arora *et al*. (2010) reported 18 alleles in six Rajasthan sheep breeds while smaller number of alleles ranging from 3 to 4 was reported by El-Nahas *et al*. (2008), Mukesh *et al*. (2006), Girish *et al*. (2007), Kumar *et al*. (2007), Prema

et al. (2008a), Arora *et al*. (2010) and Hepshiba *et al*. (2014). The allele size reported by earlier authors for this locus ranged from 138 -164 bp (Arora and Bhatia, 2004; Mukesh *et al*., 2006; Girish *et al*., 2007; Kumar *et al*., 2007; Pramod *et al*., 2009 and Radha *et al*., 2011). The effective number of alleles was 3.6, 3.8, 4.6 and 4.1 in Chokla, Nali, Marwari and Magra sheep breeds respectively. A perusal of published literature on this locus revealed the effective number of alleles ranging between 1.88 to 5.66 (Ahmed *et al*., 2014; Mukesh *et al*., 2006; Girish *et al*., 2007; Kumar *et al*., 2007; Pramod *et al*., 2009 and Hepshiba *et al*., 2014). The most frequent allele was ranged from 155 bp to 180 bp (Table 19b) in present studied sheep breeds, while Hepshiba *et al*. (2014) reported 142 bp as allele size in Coimbatore sheep which is lower than present studied population.

Table 19b:Allele number, size and frequencies for OarAE129 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of OarAE129 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	150	0.133	0.000	0.000	0.100
2.	155	0.367	0.167	0.233	0.400
3.	158	0.017	0.217	0.200	0.083
4.	160	0.167	0.183	0.217	0.083
5.	165	0.017	0.050	0.000	0.017
6.	180	0.300	0.383	0.050	0.217
7.	175	0.000	0.000	0.267	0.100
8.	170	0.000	0.000	0.033	0.000

4.3.2.12 Microsatellite BM827

The genotypes for each population at BM827 locus are presented in Table 20a. On this locus the allelic frequency ranged between 0.033 to 0.367, 0.033 to 0.250, 0.033 to 0.300 and 0.033 to 0.417 in Chokla, Nali, Marwari and Magra respectively (Table 20b).

Table 20a: Genotypes of Chokla, Nali, Marwari and Magra sheep at BM827 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at BM827Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0606	0206	0307	0101
2.	0606	0105	0206	0101
3.	0202	0808	0307	0202
4.	0103	0804	0307	0101
5.	0202	0202	0303	0804
6.	0202	0101	0307	0202
7.	0202	0805	0307	0104
8.	0202	0804	0303	0101
9.	0202	0105	0303	0808
10.	0303	0101	0303	0808
11.	0404	0805	0303	0104
12.	0404	0804	0409	0104
13.	0404	0804	0409	0101
14.	0404	0804	0404	0101
15.	0101	0205	0101	0101
16.	0202	0105	0206	0101
17.	0202	0808	0101	0810
18.	0202	0105	0101	0204
19.	0202	0202	0804	0110
20.	0707	0808	0808	0803
21.	0404	0105	0808	0803
22.	0606	0805	0804	0808
23.	0404	0205	0808	0803
24.	0404	0202	0804	0101
25.	0505	0101	0808	0808
26.	0505	0303	0804	0103
27.	0404	0306	0808	0803
28.	0505	0303	0101	0808
29.	0202	0804	0303	0101
30.	0404	0101	0307	0803

A total of 10 alleles (215-265 bp) were amplified in the four studied breeds, where seven alleles were detected in Chokla and Nali, eight alleles were found in Marwari and six alleles were observed in Magra sheep breed. The most repeated allele was found as 225 bp in Chokla, 215 bp in Nali, 230 bp in Marwari and 220 bp in Magra presented in Table 20b. The allele number in the present study was higher to the previous findings where 4 alleles shown by Pramod *et al*.

(2009) in Vembur while 5 alleles reported by Radha *et al*. (2011), Girish *et al*. (2007), Hepshiba *et al*. (2014) and Sharma *et al*. (2010) in Kilakarsal, Nilagiri and Changthangi sheep respectively. However similar findings were reported by Arora *et al*. (2008) in Jalauni sheep and sheep breed of southern and eastern of India. The allele size reported in other findings was 216 – 228 bp by Girish *et al*. (2007), 208 – 230 bp by Pramod *et al*. (2009), 214 – 224 bp by Sharma *et al*. (2010), 216 – 226 bp by Radha *et al*. (2011) and 216- 224 bp by Hepshiba *et al*. (2014). The effective number of alleles in the present study was 3.9, 5.6, 5.1 and 3.5 in Chokla, Nali, Marwari and Magra sheep breed, respectively which were varied in previous finding made on the same locus, ranged from 2.1 to 5.1 for different sheep breeds and the most frequent allele was ranged from 215 to 230 bp (Table 20b) in all studied population.

Table 20b:Allele number, size and frequencies for BM827 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of BM827 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	220	0.050	0.217	0.134	0.417
2.	225	0.367	0.150	0.033	0.083
3.	230	0.050	0.083	0.300	0.100
4.	240	0.300	0.100	0.134	0.083
5.	245	0.100	0.167	0.000	0.000
6.	250	0.100	0.033	0.033	0.000
7.	260	0.033	0.000	0.100	0.000
8.	215	0.000	0.250	0.233	0.284
9.	265	0.000	0.000	0.033	0.000
10.	235	0.000	0.000	0.000	0.033

4.3.2.13 Microsatellite CSSM31

The genotypes for each population at CSSM31 locus are presented in Table 21a. On this locus the allelic frequency ranged from 0.017 to 0.300, 0.017 to 0.417, 0.067 to 0.367 and 0.017 to 0.517 in Chokla, Nali, Marwari and Magra respectively (Table 21b).

Table 21a: Genotypes of Chokla, Nali, Marwari and Magra sheep at CSSM31 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at CSSM31 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0202	0306	1106	1111
2.	0105	0306	1111	0104
3.	0105	0606	1111	0104
4.	0105	0306	1111	0404
5.	0105	0303	1106	0111
6.	0105	0306	1106	0111
7.	0710	0606	1106	0111
8.	0202	0303	1106	0111
9.	0404	0306	1106	0101
10.	0404	0303	1111	0111
11.	0404	0606	0707	0303
12.	0105	0305	0404	0306
13.	0101	0305	1111	0103
14.	0509	0305	1111	0103
15.	0606	0306	0304	0103
16.	0606	0306	0303	0111
17.	0508	0710	0303	0101
18.	0105	0608	0303	0101
19.	0505	0306	0303	1111
20.	0505	0306	0306	0101
21.	0306	0306	0306	0103
22.	0710	0306	1106	0111
23.	0105	0306	1106	0101
24.	0710	0608	0404	0101
25.	0508	0608	0707	1111
26.	0306	0306	0404	0101
27.	0710	0306	0303	0111
28.	0306	0608	0303	0103
29.	0105	0306	1111	0111
30.	0505	0305	0303	0103

This (AC) 35 di-nucleotide microsatellite repeat produced a total of 11 alleles (135-185 bp) in the four studied breeds, where ten alleles were found in Chokla, six alleles were detected in Nali and five alleles were seen in Marwadi and Magra sheep breed. The most repeated allele was found as 155 bp in Chokla, 140 and 160 bp in Nali, 145 bp in Marwari and 135 bp in Magra sheep in CSSM31 marker as presented in Table 21b.

The number of alleles in the present study is lower when compared to the reports of 17 alleles in Kazakh sheep by Ozerov *et al*. (2008), 25 alleles in Chilean sheep by Rodrigo *et al*. (2000) and 24 alleles in sheep breeds from Rajasthan by Arora *et al*. (2010). Radha *et al*. (2011) also reported around 10 alleles based on studies on Magra, Jalauni, and Kilakarsal sheep which agree with Chokla breed in present study. Whereas only 5 alleles were reported in Nilagiri and Vembur sheep by Girish *et al*. (2007) and Pramod *et al*. (2009) which is also detected in Marwari and Magra sheep breed in the present study and only 2 alleles were reported in Coimbatore sheep by Hepshiba *et al*. (2014). The mean effective number of alleles was 6.0, 2.8, 3.8 and 2.8 in Chokla, Nali, Marwari and Magra sheep breeds respectively and the most frequent alleles were ranged from 140 bp to 160 bp (Table 21b) in Nali.

Table 21b: Allele number, size and frequencies for CSSM31 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of CSSM31 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	135	0.183	0.000	0.000	0.517
2.	138	0.067	0.000	0.000	0.000
3.	140	0.050	0.417	0.283	0.150
4.	150	0.100	0.000	0.117	0.067
5.	155	0.300	0.067	0.000	0.000
6.	160	0.117	0.417	0.167	0.017
7.	165	0.067	0.017	0.067	0.000
8.	175	0.033	0.067	0.000	0.000
9.	180	0.017	0.000	0.000	0.000
10.	185	0.067	0.017	0.000	0.000
11.	145	0.000	0.000	0.367	0.250

Mean effective number of alleles as observed from published literature ranged from 2 to 4.9 (4.9 in Magra sheep by Arora and Bhatia, 2006; 2.94 in Nilagiri sheep by Girish *et al*., 2007; 3.90 in Jalauni sheep by Arora *et al*., 2008; 4.158 in Vembur sheep by Pramod *et al*., 2009 and 2.00 in Coimbatore sheep by Hepshiba *et al*., 2014). The most frequent alleles at this locus was ranged from

140 bp to 160 bp which is more than Coimbatore sheep 128 & 146 as reported by Hepshiba *et al*. (2014).

4.3.2.14 Microsatellite BM6526

The genotypes for each population at BM6526 locus is presented in Table 22a. On this locus the allelic frequency ranged from 0.017 to 0.333, 0.017 to 0.417, 0.017 to 0.367 and 0.017 to 0.467 in Chokla, Nali, Marwari and Magra respectively (Table 22b). A total of 13 alleles (148-200 bp) were amplified in the four studied breeds where eleven alleles were found in Chokla, ten alleles were detected in Nali and Marwadi and seven alleles were observed in Magra sheep breed. The most repeated allele was found as 175 bp in Chokla, 165 bp in Nali, 170 bp in Marwari and 160 bp in Magra presented in Table 22b. Earlier reports on this locus indicated the presence of 11 alleles in Kazakh sheep (Ozerov *et al.*, 2008), 12 alleles in Egypt breeds (El-Nahas *et al.*, 2008), 4 alleles in Muzzafarnagri sheep (Arora and Bhatia, 2004), 6 alleles in Jalauni sheep (Arora *et al.*, 2008), 3 alleles in Madras Red sheep (Prema *et al.*, 2008a), 6 alleles in Vembur sheep (Pramod *et al.*,2009), 19 alleles in Rajasthan sheep (Arora *et al.*, 2010), 9 alleles in Kilakarsal sheep (Radha *et al.*, 2011) and 7 alleles in Coimbatore sheep (Hepshiba *et al.*, 2014).

The effective number of alleles was 5.660 for Chokla, 4.276 for Nali, 5.042 for Marwari and 3.209 for Magra sheep which were quite more than the values reported by Arora and Bhatia, (2004) as 2.571 in Muzzafarnagri, Prema *et al.*, (2008a) as 2.97 in Madras Red, where Arora *et al.*, (2008) as 4.42 in Jalauni, Pramod *et al.*, (2009) as 4.351 in Vembur, Hepshiba *et al.*, (2014) as 4.42 in Coimbatore sheep were reported more effective numbers of alleles than Chokla and Nali. However, Radha *et al.*, (2011) as 5.06 in Kilakarsal were detected more than Marwari sheep.

Table 22a:Genotypes of Chokla, Nali, Marwari and Magra sheep at BM6526 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at BM6526 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0202	1312	0307	1205
2.	0508	0207	1212	0202
3.	0509	0101	0508	1203
4.	0508	1303	0303	1212
5.	0508	0307	0310	0102
6.	0507	0205	0510	0112
7.	0101	0406	0303	1212
8.	0404	0406	0609	0101
9.	0303	0202	0505	1206
10.	0202	0307	0303	1206
11.	0404	0202	0510	1313
12.	0204	0202	0303	0101
13.	0408	0507	0610	0101
14.	0410	0303	0611	1212
15.	0508	0408	1212	1202
16.	0508	0307	0505	1212
17.	0505	0202	0507	0202
18.	0505	0407	0303	1202
19.	0510	0307	0307	1212
20.	0303	0202	0202	0202
21.	0303	0202	1212	1212
22.	0611	0407	0205	0203
23.	0404	0202	0303	1202
24.	0606	0404	0303	1202
25.	0505	0205	1203	0202
26.	0505	0202	0205	0203
27.	0611	0202	0311	1212
28.	0505	0202	0210	1212
29.	0303	0307	0303	1212
30.	0505	0202	0307	1202

Table 22b: Allele number, size and frequencies for BM6526 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of BM6526 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	155	0.033	0.033	0.000	0.133
2.	165	0.083	0.417	0.083	0.267
3.	170	0.133	0.133	0.367	0.050
4.	172	0.150	0.117	0.000	0.000
5.	175	0.333	0.050	0.167	0.017
6.	180	0.067	0.033	0.050	0.033
7.	185	0.017	0.150	0.066	0.000
8.	190	0.100	0.017	0.017	0.000
9.	192	0.017	0.000	0.017	0.000
10.	195	0.033	0.000	0.083	0.000
11.	200	0.033	0.000	0.033	0.000
12.	160	0.000	0.017	0.117	0.467
13.	148	0.000	0.033	0.000	0.033

4.3.2.15 Microsatellite BM757

The genotypes for each population at BM757 locus are presented in Table 23a. On this locus the allelic frequency ranged from 0.017 to 0.567, 0.017 to 0.567, 0.017 to 0.500 and 0.017 to 0.500 in Chokla, Nali, Marwari and Magra respectively (Table 23b). The BM757 a (GT)14 di-nucleotide repeat has amplified a total of 15 alleles (155-260 bp) in the four studied breeds, where six alleles were found in Chokla, four in Nali, nine were detected in Marwari and seven were found in Magra sheep breed. The most repeated allele was found as 185 bp in Chokla, 180 bp in Nali, 185 bp in Marwari and 160 bp in Magra sheep was detected in BM757 marker presented in Table 23b. The allele numbers obtained in Marwari and Magra sheep in the present study are in accordance with reports of Diez-Tascon *et al.* (2000), Ozerov *et al.* (2008), Pramod *et al.* (2009) and Jyotsana *et al.* (2010), who reported from 7 to 9 alleles. However, lesser number of alleles (4-6) for the same locus was reported by Arora and Bhatia, (2006, 2008), Girish *et al.* (2007), Kumar *et al.* (2007) and Prema *et al.* (2008a) in Magra, Marwari, Sonadi, Bellary and Mecheri sheep, respectively. Whereas,

Arora *et al.* (2011a) reported 14 alleles, which is higher in number at this locus in six sheep breeds of Rajasthan.

Table 23a: Genotypes of Chokla, Nali, Marwari and Magra sheep at BM757 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at BM757Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0204	0101	0202	1515
2.	0101	0202	0810	1515
3.	0101	0101	0204	1313
4.	0101	0404	0204	1313
5.	0101	0101	0204	1313
6.	0204	0101	0204	1313
7.	0202	0101	0204	1212
8.	0206	0202	0204	1313
9.	0204	0202	0204	1212
10.	0203	0101	0203	1201
11.	0202	0202	0207	1203
12.	0202	0101	0204	1212
13.	0202	0202	0203	1212
14.	0202	0202	0203	1212
15.	0204	0202	0211	1515
16.	0204	0101	0203	1515
17.	0204	0101	0205	1313
18.	0204	0202	0204	1301
19.	0204	0101	0204	1313
20.	0202	0101	0208	1313
21.	0204	0101	0210	1313
22.	0204	0202	0204	1302
23.	0205	0203	0211	1314
24.	0202	0202	0210	1313
25.	0204	0101	0204	1313
26.	0204	0101	0209	1315
27.	0204	0101	0202	1314
28.	0204	0101	0707	1315
29.	0202	0202	0204	1313
30.	0204	0101	0203	1212

The allele size in the present study is in line with that obtained by Kumar *et al.* (2007) in Bellary (225-241 bp), however a lower size of alleles than present study were reported by Prema *et al.* (2008a) in Mecheri (176-206 bp), and Arora and Bhatia, (2009) in Jalauni, Marwari and Sonadi (178-200 bp). The effective

number of alleles ranged from 2.1 in Nali sheep to 3.2 in Marwari sheep. Published literature revealed a range of 2.6 to 5.0 alleles at this locus (Girish *et al.*, 2007; Kumar *et al.*, 2007; Prema *et al.*, 2008a; Pramod *et al.*, 2009). The most frequent alleles at this locus ranged from 160 bp (Magra) to 185 bp (Chokla, Marwari) (Table 8.8). These are in line with the allele of 180 bp length reported by Hepshiba *et al.* (2014) in Coimbatore sheep.

Table 23b: Allele number, size and frequencies for BM757 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of BM757 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	180	0.133	0.567	0.000	0.033
2.	185	0.567	0.383	0.500	0.017
3.	210	0.017	0.017	0.083	0.017
4.	220	0.250	0.033	0.217	0.000
5.	225	0.017	0.000	0.017	0.000
6.	260	0.017	0.000	0.000	0.000
7.	198	0.000	0.000	0.050	0.000
8.	200	0.000	0.000	0.033	0.000
9.	205	0.000	0.000	0.017	0.000
10.	230	0.000	0.000	0.050	0.000
11.	240	0.000	0.000	0.033	0.000
12.	155	0.000	0.000	0.000	0.233
13.	160	0.000	0.000	0.000	0.500
14.	170	0.000	0.000	0.000	0.033
15.	175	0.000	0.000	0.000	0.167

4.3.2.16 Microsatellite TGLA377

The genotypes for each population at TGLA377 locus are presented in Table 24a. On this locus the allelic frequency was ranged from 0.033 to 0.367, 0.367 to 0.633, 0.033 to 0.417 and 0.067 to 0.300 in Chokla, Nali, Marwari and Magra respectively (Table 24b). A total of 8 alleles (80-110 bp) were amplified in the four studied sheep breeds where six alleles were found in Chokla, two in Nali, four were observed in Marwari and five were seen in Magra sheep breed. The most repeated allele was found as 95 bp in Chokla, 100 bp in Nali and Magra and 99 bp in Marwari presented in Table 24b.

Table 24a: Genotypes of Chokla, Nali, Marwari and Magra sheep at TGLA377 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at TGLA377 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0404	0505	0104	0404
2.	0505	0505	0104	0505
3.	0303	0505	0104	0505
4.	0606	0305	0104	0606
5.	0505	0305	0104	0707
6.	0606	0305	0104	0707
7.	0202	0305	0104	0707
8.	0303	0305	0104	0707
9.	0606	0305	0104	0303
10.	0606	0505	0104	0505
11.	0606	0505	0108	0606
12.	0606	0505	0303	0303
13.	0606	0305	0108	0606
14.	0606	0305	0303	0707
15.	0404	0505	0104	0505
16.	0505	0305	0303	0404
17.	0404	0305	0404	0404
18.	0303	0305	0104	0505
19.	0303	0305	0404	0404
20.	0303	0305	0404	0505
21.	0303	0305	0404	0606
22.	0101	0305	0104	0505
23.	0303	0305	0103	0505
24.	0101	0305	0103	0606
25.	0303	0305	0303	0707
26.	0101	0305	0104	0707
27.	0303	0305	0303	0606
28.	0303	0305	0303	0404
29.	0606	0505	0104	0707
30.	0303	0305	0404	0505

The allele size ranged from 80 – 110 bp (Table 24b), whereas the other studied breed shown as 76 – 100 bp by Pramod *et al*. (2009), 94 – 126 bp by Radha *et al*. (2011). The studies made on the same locus by other authors are 6 alleles by Pramod *et al*. (2009) in Vembur and 11 alleles by Radha *et al*. (2011). The allele number in the present study was in lesser extent reported by other authors. The effective number of alleles was 3.9, 1.8, 3.0 and 4.2 in Chokla, Nali,

Marwari and Magra sheep breed respectively. The effective number of alleles in other findings was as shown 2.3 by Pramod *et al*. (2009) in Vembur and 4.3 by Radha *et al*. (2011). The most frequent allele for this locus was ranged between 95 - 100 bp (Table 24b) in studied population.

Table 24b:Allele number, size and frequencies for TGLA377 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of TGLA377 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	80	0.100	0.000	0.317	0.000
2.	90	0.033	0.000	0.000	0.000
3.	95	0.367	0.367	0.233	0.067
4.	99	0.100	0.000	0.417	0.167
5.	100	0.100	0.633	0.000	0.300
6.	105	0.300	0.000	0.000	0.200
7.	110	0.000	0.000	0.000	0.267
8.	98	0.000	0.000	0.033	0.000

4.3.2.17 Microsatellite OarVH72

The genotypes for each population at OarVH72 locus are presented in Table 25a. On this locus the allelic frequency was ranged from 0.017 to 0.500, 0.017 to 0.233, 0.017 to 0.217 and 0.017 to 0.200 in Chokla, Nali, Marwari and Magra respectively (Table 25b). A total of 14 alleles (128-170 bp) were amplified in the four studied breeds where eight alleles were found in Chokla, ten alleles in Nali, eleven alleles were observed in Marwari and thirteen alleles were detected in Magra sheep breed. The most repeated allele was found as 135 bp in Chokla, 150 bp in Nali, 135 bp in Marwari and 135 bp in Magra sheep. The allele number in the present study was higher to the previous findings made as 6 alleles by Ahmed *et al*. (2014) in Kail sheep, 5 alleles by Arora and Bhatia, (2004) in Muzzafarnagri, 5 alleles by Girish *et al*. (2007) in Nilagiri, 6 alleles by Kumar *et al*. (2007) in Bellary and 5 alleles by Pramod *et al*. (2009) in Vembur. However, similar findings were reported by Arora *et al*. (2010) in Rajasthan sheep (10) and Radha *et al*. (2011) in Kilakarsal (8). The allele size reported in other findings was 113-137 bp by Arora and Bhatia, (2006), 128-140 by Girish *et al*. (2007),

124-142 bp by Kumar *et al*. (2007), 124-134 by Pramod *et al*. (2009), 121-151 bp by Jyotsana *et al*. (2010), 126-140 bp by Radha *et al*. (2011) and 126-134 bp by Hepshiba *et al*. (2014).

Table 25a: Genotypes of Chokla, Nali, Marwari and Magra sheep at OarVH72 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at OarVH72 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	0303	0202	1011	1010
2.	0202	0111	0913	0909
3.	0303	0111	1004	1010
4.	0207	1011	0104	0202
5.	0207	1011	0104	0202
6.	0101	1011	0104	1010
7.	0207	0909	1013	0207
8.	0101	0909	1013	0211
9.	0207	0101	1003	0202
10.	0202	0909	1013	0202
11.	0202	0909	1013	0305
12.	0202	1005	1010	0514
13.	0202	0905	0905	0305
14.	0202	1010	0903	1313
15.	0205	0101	1013	1004
16.	0207	0211	0106	1010
17.	0208	0202	0202	0304
18.	0207	0307	0205	0404
19.	0207	0311	0206	1111
20.	0104	0211	0202	0505
21.	0308	0211	0207	0505
22.	0207	0101	0207	0506
23.	0206	0211	0305	0404
24.	0101	0311	0206	0511
25.	0101	0211	0204	1307
26.	0101	0311	0306	1111
27.	0202	0307	0207	1112
28.	0202	0408	0207	1108
29.	0202	0909	1013	0202
30.	0207	1011	0207	1010

The effective number of alleles in the present study was 3.1, 6.5, 7.8 and 7.5 for Chokla, Nali, Marwari and Magra sheep which were similar to the findings of the previous literature made on the same locus on different sheep breeds and

the most frequent allele was ranged from 135 to 150 bp (Table 25b) in all studied population and it was 128 bp in Coimbatore sheep as shown by Hepshiba *et al*. (2014).

Table 25b: Allele number, size and frequencies for OarVH72 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of OarVH72 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	132	0.183	0.133	0.067	0.000
2.	135	0.500	0.150	0.217	0.200
3.	138	0.083	0.083	0.067	0.050
4.	142	0.017	0.017	0.083	0.100
5.	145	0.017	0.033	0.050	0.150
6.	152	0.017	0.000	0.067	0.017
7.	155	0.150	0.033	0.083	0.033
8.	160	0.033	0.017	0.000	0.017
9.	128	0.000	0.183	0.050	0.033
10.	130	0.000	0.117	0.183	0.183
11.	150	0.000	0.233	0.017	0.133
12.	170	0.000	0.000	0.000	0.017
13.	148	0.000	0.000	0.117	0.050
14.	165	0.000	0.000	0.000	0.017

4.3.2.18 Microsatellite RM4

The genotypes for each population at RM4 locus are presented in Table 26a. On this locus the allelic frequency was ranged from 0.017 to 0.317, 0.050 to 0.317, 0.017 to 0.200 and 0.017 to 0.300 in Chokla, Nali, Marwari and Magra respectively (Table 26b). A total of 12 alleles (145-180 bp) were amplified in the four studied breeds, where nine alleles were found in Chokla and Magra, seven alleles were detected in Nali and eleven alleles were observed in Marwari sheep breed. The most repeated allele was found as 150 bp in Chokla, 158 bp in Nali, 155 and 158 bp in Marwari and 150 bp in Magra presented in Table 26b. The studies made on the same locus by other authors are 4 alleles by Girish *et al*. (2007), 6 alleles by Pramod *et al*. (2009) in Vembur; the allele size in the present study was in greater extent reported by other authors.

Table 26a: Genotypes of Chokla, Nali, Marwari and Magra sheep at RM4 microsatellite locus represented by allele numbers.

Sample No.	Genotypes at RM4 Microsatellite loci in four sheep breed			
	Chokla	Nali	Marwadi	Magra
1.	1010	0202	0307	1010
2.	1010	0205	0202	1010
3.	0808	0205	0303	0808
4.	1005	0205	0206	1005
5.	0811	0205	1010	0811
6.	1010	0303	0307	1010
7.	1005	0303	0307	1005
8.	0207	0303	1112	0207
9.	0105	0306	0407	0105
10.	0902	0407	0407	0902
11.	1005	0306	0407	1005
12.	1011	0407	1107	1011
13.	1005	0407	0202	1005
14.	0202	0406	0202	0202
15.	0808	0101	0205	0808
16.	1005	0205	0911	1005
17.	0808	0306	0303	0808
18.	0909	0105	0909	0909
19.	0909	0306	0306	0909
20.	0911	0305	0303	0911
21.	1005	0303	0205	1005
22.	0911	0306	0206	0911
23.	0811	0202	0205	0811
24.	1011	0306	1010	1011
25.	1004	0306	0207	1004
26.	0904	0407	0909	0904
27.	0808	0206	0101	0808
28.	1010	0307	0101	1010
29.	1010	0205	0407	1005
30.	0909	0303	0303	0808

The effective number of alleles was 5.3, 5.2, 7.6 and 5.5 in Chokla, Nali, Marwari and Magra sheep breed respectively. The effective number of alleles in other findings was as shown 2.8 by Girish *et al*. (2007), 3.8 by Pramod *et al*. (2009) in Vembur. The most frequent allele for this locus was ranged between 150 - 158 bp (Table 26b) in studied sheep population which was similar to other reported works in sheep population.

Table 26b: Allele number, size and frequencies for RM4 microsatellite locus

Allele No.	Allele size (bp)	Allele freq. of RM4 Microsatellite locus			
		Chokla	Nali	Marwadi	Magra
1.	152	0.017	0.050	0.067	0.017
2.	155	0.067	0.183	0.200	0.067
3.	158	0.000	0.317	0.200	0.000
4.	160	0.033	0.083	0.067	0.033
5.	170	0.117	0.133	0.050	0.133
6.	172	0.000	0.150	0.050	0.000
7.	175	0.017	0.083	0.150	0.017
8.	145	0.167	0.000	0.000	0.200
9.	148	0.167	0.000	0.083	0.133
10.	150	0.317	0.000	0.067	0.300
11.	165	0.100	0.000	0.050	0.100
12.	180	0.000	0.000	0.017	0.000

4.3.3 Mean number of alleles in sheep breeds

Overall, mean number of allele was found as 12.4 across all the 18 microsatellite loci (Table 33). The breedwise, mean number of alleles were found high as 8.778±0.65 in Marwari and followed by Chokla 8.333±0.530, Magra 8.333±0.530 and Nali 7.389±0.506 sheep, respectively (Table 27). The effective number of allele (Ne) at each locus was less than the observed number of allele. Overall in all breeds the effective number of alleles (Ne) ranged from 3.6 (BM757) to 9.7 (OarJMP29) with a mean value of 6.9±1.5707 (Table 33). The breedwise mean effective number of alleles in four sheep breeds were found high in Marwadi, 5.554±0.450 and followed by Chokla, 5.177±0.409, Magra, 4.992±0.382 and Nali, 4.958±0.375 sheep breed, respectively (Table 27).

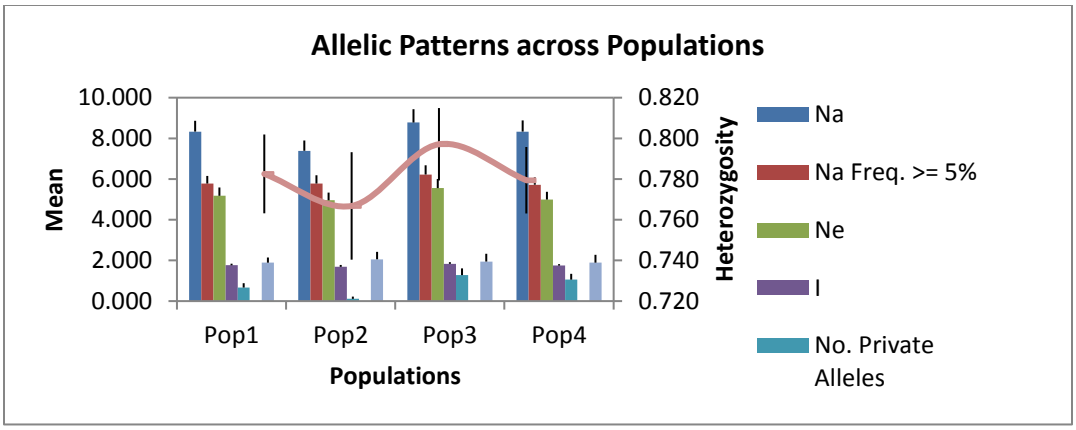

Fig. 10Allelic patterns across all breeds of sheep

Table 27: Mean and SE value of Na, Ne, Ho, He and F for sheep breeds

Genetic diversity	Chokla (Pop1)	Nali (Pop2)	Marwari (Pop3)	Magra (pop4)
Na	8.333±0.530	7.389±0.506	8.778±0.655	8.333±0.548
Ne	5.177±0.409	4.958±0.375	5.554±0.450	4.992±0.382
Ho	0.576±0.057	0.507±0.050	0.602±0.041	0.422±0.038
He	0.783±0.019	0.767±0.026	0.797±0.018	0.779±0.016
F	0.256±0.079	0.325±0.083	0.235±0.057	0.453±0.052

Allelic diversity (mean number of observed alleles per locus) lower than that observed in Chokla, Nali, Marwari and Magra sheep has been reported in several Indian sheep breeds viz. 7.34 in Sarda sheep (Pariset *et al*., 2003), 5.04 in Muzzafarnagri sheep (Arora and Bhatia, 2004), 5.7 in Magra sheep (Arora and Bhatia, 2006), 6.27 in Nali sheep, 5.63 in Chokla sheep and 5.63 in Garole sheep (Mukesh *et al*., 2006), 5.52 in Nali sheep and 5.32 in Chokla sheep (Sodhi *et al*., 2006), 6.24 in Marwari and 5.88 in Sonadi sheep, 6.7 in Bellari sheep (Kumar *et al*., 2007), 4.0 in Bonpala sheep (Pandey *et al*., 2007), 5.5 in Magra, 6.1 in Marwari and 5.6 in Sonadi sheep (Arora and Bhatia, 2008), 5.3 in Kheri sheep (Bhatia and Arora, 2008), 5.92 in Jaluani sheep (Arora and Bhatia, 2008), 5.00 in Mecheri sheep (Prema *et al*., 2008a), 5.85 in Jalauni, 6.25 in Marwari and 5.75 in

Sonadi sheep (Arora and Bhatia, 2009). However, the mean number of alleles in present studying sheep breeds were detected lower, than other previously studied sheep breeds viz. 12 in Kazak sheep (Ozerov *et al*., 2008), 9.8 in Madras Red sheep (Selvam *et al*., 2009), 16.2 in Sinai, 15.3 in Rahmani and 15.8 in Ossimi sheep (Ghazy *et al*., 2013), 15.88 in Barbarian Sheep (Sassi-Zaidy *et al*., 2014). The average number of alleles in present studying breeds were comparable with the values observed as 7.40 in Hassan sheep, 8.25 in Patanwadi, 9.05 in Marwari and 8.00 and Dumba sheep (Jyotsana *et al*., 2010), 8.760 in Changthangi sheep (Sharma *et al*., 2010), 8.64 in Munjal sheep (Yadav *et al*., 2011), 6.67 in Okiramatian and 9.33 in Kapiti sheep (Mukhongo *et al*., 2014), 8.12 in Tiruchy Black sheep (Kavitha *et al*., 2015).

Therefore, sufficient genetic variability exists in studied sheep breeds (Chokla, Nali, Marwari and Magra), while the highest genetic variability detected in Marwari sheep and lowest genetic variability found in Magra sheep breed in terms of mean number of alleles and heterozygosity.

4.3.4 Population specific alleles or Private alleles

The list of locus-wise population specific alleles and their frequencies are presented in Table 28. A total of 55 population specific alleles were observed in all four sheep breeds, while in breed wise study the specific alleles were found as 12 in Chokla, 2 in Nali, 22 in Marwari and 19 in Magra sheep breed, respectively. The locus BM757 recorded as highest number of specific allele (5) in Marwari, 4 alleles in Magra at BM757 locus, 3 alleles in Chokla at OarJMP29 locus and 2 alleles in Nali at OarHH35 locus. However,the frequency of population specific alleles was 2 (21.7%) and 1 (13.3%) in Marwari, 2 (26.7%), 1 (28.3%), 1 (50%), 1 (23.3%) and 1 (16.7%) in Magra sheep and 1 (25%) in Nali sheep. The alleles with a frequency above 10 percent, may be more reliable and can be used to identify the population.

Table 28: Population specific alleles or Private alleles (PA) in sheep breeds

Locus	PA in Cohkla/Freq.	PA in Nali/Freq.	PA in Marwari/Freq.	PA in Magra/Freq.
BM8125				11(0.017)
OarFCB48			14(0.067),15(0.067),16(0.033),17(0.017)	
OarFCB128			11(0.017),**12(0.217)**	**9(0.267),10(0.283)**
OarHH35		9(0.033),**10(0.250)**		
OarHH41	7(0.017)		11(0.0117),**12(0.133)**	
OarHH64	7(0.017)			11(0.083),12(0.017), 13(0.017)
OarJMP8			13(0.067),14(0.100)	10(0.033),11(0.100)
OarJMP29	9(0.033),10(0.083),11(0.033)		**15(0.217),**16(0.017)	14(0.067)
TGLA137	10(0.017)			12(0.017),13(0.017)
OarCP34	9(0.017),10(0.017)		13(0.067)	
OarAE129			8(0.033)	
BM827			9(0.033)	10(0.033)
CSSM31	2(0.067),9(0.017)			
BM6526				
BM757	6(0.017)		7(0.050),8(0.033),9(0.017),10(0.050),11(0.033)	**12(0.233),13(0.500),** 14(0.033),**15(0.167)**
TGLA377	2(0.033)		8(0.033)	**7(0.267)**
OarVH72				12(0.017),14(0.017)
RM4			12(0.017)	
Total	12	2	22	19

4.3.5 Polymorphic Information Content

The mean polymorphic information content (PIC) is an ideal index to measure the polymorphism of alleles. The PIC value of more than 0.5 indicates high polymorphism, 0.25 to 0.50 a moderate and less than 0.25, a low polymorphism.

Table29: Observed number of allele (Na), Effective number of allele (Ne), Observed heterozygosity (Ho), Expected heterozygosity (He), Fixation index (F) for Chokla sheep breed.

Locus	Na	Ne	Ho	He	F	PIC
BM8125	8.000	5.806	0.733	0.828	0.114	0.806
OarFCB48	13.000	8.612	0.467	0.884	0.472	0.857
OarFCB128	4.000	2.635	0.867	0.621	-0.397	0.546
OarHH35	8.000	4.186	0.867	0.761	-0.139	0.725
OarHH41	7.000	5.521	0.833	0.819	-0.018	0.794
OarHH64	9.000	6.667	0.700	0.850	0.176	0.832
OarJMP8	7.000	5.263	0.667	0.810	0.177	0.784
OarJMP29	11.000	7.965	0.767	0.874	0.123	0.853
TGLA137	10.000	7.143	0.567	0.860	0.341	0.844
OarCP34	10.000	4.986	0.567	0.799	0.291	0.774
OarAE129	6.000	3.696	0.600	0.729	0.177	0.684
BM827	7.000	3.991	0.033	0.749	0.956	0.713
CSSM31	10.000	6.081	0.633	0.836	0.242	0.818
BM6526	11.000	5.660	0.433	0.823	0.474	0.803
BM757	6.000	2.486	0.600	0.598	-0.004	0.543
TGLA377	6.000	3.913	0.000	0.744	1.000	0.706
OarVH72	8.000	3.175	0.467	0.685	0.319	0.650
RM4	9.000	5.389	0.567	0.814	0.304	0.792
Mean±SE	8.333±0.530	5.177±0.409	0.576±0.057	0.783±0.019	0.256±0.079	0.751

Overall, loci were highly polymorphic with a 100% value of polymorphism in all the four sheep breeds. The polymorphic information content (PIC) value in 4 sheep breeds was also found high (>0.5). The value of the PIC ranged between 0.6799 (BM757) and 0.8634 (OarHH64) presented in Table33. The breed-wise, highest mean PIC value was detected in Marwari sheep (0.763) ranging between 0.589 (OarHH35) to 0.856 (OarVH72 and RM4) (Table 31), while the mean PIC value in other studying sheep breeds were followed by Chokla (0.751) ranged between 0.543 (BM757) to 0.857 (OarFCB48), Magra (0.7480) ranged between 0.593 (CSSM31) to 0.854 (OarHH64) and Nali (0.7317) ranged between 0.356 (TGLA377) to 0.853 (OarHH64), respectively Table 29, 32 and 30. The values indicate that all the markers considered for this study are highly informative and able to characterize studying sheep breeds population.

Published literature revealed a wide range of PIC values among Indian sheep breeds. The PIC values ranged from 0.301 to 0.863 in Tibetan sheep with the mean value of 0.632 (Sharma *et al*., 2016), 0.533 to 0.808 in Muzzafarnagri sheep (Arora and Bhatia, 2004), 0.210 to 0.831 in Nali and 0.346 to 0.768 in Chokla sheep (Sodhi *et al.,* 2006), 0.458 to 0.827 in Nilagiri sheep (Girish *et al.,* 2007), 0.240 to 0.820 in Jalauni sheep (Arora *et al.,* 2008), 0.079 to 0.806 in Kheri sheep (Bhatia and Arora, 2008), 0.454 to 0.785 in Madras Red sheep (Prema *et al.,* 2008b) and from 0.371 to 0.836 in Vembur sheep (Pramod *et al.,* 2009). Pandey *et al.* (2007) and Radha *et al.* (2011) reported the average PIC values of 0.60 and 0.831 in Shahabadi and Kilakarsal sheep breeds, respectively.

The mean PIC values in the present study evaluated in Chokla, Nali, Marwari and Magra sheep were found higher than the PIC values already wrote about Chokla and Nali sheep (Mukesh *et al*., 2006; Sodhi *et al*., 2006), Tibetan sheep (Sharma *et al.,* 2016), Shahabadi sheep (Pandey *et al.,* 2007), Jalauni sheep (Arora *et al.,* 2008) and Vembur (Pramod *et al.,* 2009), while Radha *et al.* (2011) shown higher value of PIC than the present study in Kilakarsal sheep.

Table 30:Observed number of allele (Na), Effective number of allele (Ne), Observed heterozygosity (Ho), Expected heterozygosity (He), Fixation index (F) for Nali sheep breed.

Locus	Na	Ne	Ho	He	F	PIC
BM8125	8.000	6.250	0.800	0.840	0.048	0.8209
OarFCB48	9.000	6.316	0.667	0.842	0.208	0.8228
OarFCB128	6.000	4.215	0.167	0.763	0.782	0.729
OarHH35	9.000	5.960	0.600	0.832	0.279	0.8093
OarHH41	8.000	3.913	0.333	0.744	0.552	0.7146
OarHH64	9.000	7.759	0.433	0.871	0.503	0.8537
OarJMP8	9.000	5.678	0.433	0.824	0.474	0.8062
OarJMP29	7.000	5.056	0.333	0.802	0.584	0.7732
TGLA137	9.000	5.732	0.367	0.826	0.556	0.8049
OarCP34	8.000	5.941	0.533	0.832	0.359	0.812
OarAE129	5.000	3.879	0.400	0.742	0.461	0.7016
BM827	7.000	5.625	0.600	0.822	0.270	0.7979
CSSM31	6.000	2.804	0.800	0.643	-0.244	0.5795
BM6526	10.000	4.276	0.533	0.766	0.304	0.7423
BM757	4.000	2.130	0.033	0.531	0.937	0.4348
TGLA377	2.000	1.867	0.733	0.464	-0.579	0.3567
OarVH72	10.000	6.593	0.633	0.848	0.253	0.8289
RM4	7.000	5.248	0.733	0.809	0.094	0.7834
Mean±SE	7.389±0.506	4.958±0.375	0.507±0.050	0.767±0.026	0.325±0.083	0.7317

The PIC values in this study were found greater than 0.5, indicating that these microsatellite markers can be effectively used for molecular characterization,

population assignment (MacHugh *et al*., 1997) as well as genome mapping studies in addition to genetic diversity analysis.

4.4 Genetic Diversity

Genetic diversity measures the genetic variation among the populations in terms of estimates of heterozygosity.

4.4.1 Heterozygosity

The heterozygosity estimates at individual loci are presented in Table 29 - 32. The heterozygosity is the unit of measurement for population diversity and variation. In the present study the population revealed a higher degree of polymorphism and genetic variation with an average of expected gene diversity (H_{exp}), ranged from 0.727 (BM757) to 0.902 (OarJMP29) with an overall average value of 0.853 (Table 16). Observed heterozygosity (H_{obs}) across 18 loci ranged between 0.360 (BM827) and 0.659 (OarHH35 and BM8125) with the mean value of 0.546 (Table 33).

Breedwise, the highest average value of H_{obs}(H_{exp}) were detected as 0.602 (0.797) in Marwari sheep, where it is represented as 0.576 (0.783) in Chokla sheep, Nali breed showed the value 0.507 (0.767) and the lowest heterozygosity value detected in Magra sheep breed, 0.422 (0.779), respectively (Table 31, 29, 30 and 32). Amongst four sheep breeds observed heterozygosity was ranged between 0.300 (TGLA137) to 0.900 (BM757, OarVH72) in Marwari, 0.0 (TGLA377) to 0.867 (OarHH35, OarFCB128) in Chokla, 0.033 (BM757) to 0.800 (CSSM31, BM8125) in Nali and 0.00 (TGLA377) to 0.667 (OarAE129) in Magra sheep breed, respectively (Table 31, 29, 30 and 32). Similarly, expected Heterozygosity (He) ranged between 0.637 (OarHH35) to 0.900 (OarFCB48) in Marwari, 0.598 (BM757) to 0.884 (OarFCB48) in Chokla, 0.464 (TGLA377) to 0.800 (CSSM31, BM8125) in Nali and 0.643 (CSSM31) to 0.873 (OarHH64) in Magra sheep breed.

The observed and expected heterozygosity H_{obs}(H_{exp}) value in published literature was shown lower than the present study as 0.47 (0.64), 0.47 (0.65) and 0.44 (0.59) for Chokla, Nali and Garole respectively by Mukesh *et al*., 2006; 0.505 (0.686) in Marwari, 0.591 (0.713) in Chokla, 0.672 (0.766) in Magra, 0.645 (0.728) in Nali, 0.665 (0.697) in Jaisalmeri and 0.647 (0.731) in Pugal sheep by Arora *et al*. (2011); expected heterozygosity 0.651 in Nali and 0.657 in Chokla sheep observed by Sodhi *et al*. 2006; 0.597 (0.694) in Magra sheep reported by Arora & Bhatia (2006); 0.473 (0.672) in Tibetan sheep by Sharma *et al*. (2016); 0.618 (0.725) in Kilakarsal sheep by Radha *et al*. (2011).

While the values were detected high and shown as 0.989 (0.831) in Karakul sheep by Nanekarani *et al*. (2010); 0.5905 (0.860) in Barki, 0.5471 (0.811) in Ossimi, 0.615 (0.855) in Rahmani by El-Nahas *et al*. (2008); 0.680 (0.799) in Pomarancina sheep, 0.659 (0.761) Garfagina sheep, 0.658 (0.783) in Zerasca, 0.662 (0.778) in Massese, 0.741 (0.805) in Appenninica sheep by Bozzi *et al*. (2009). However, the values in similar magnitude as shown 0.6991 (0.716) in Changthangi by Sharma *et al*. (2010); 0.6255 (0.6297) in Coimbatore sheep by Hepsibha *et al*. (2014) and 0.761 (0.721) in Nilgiri sheep by Girish *et al*. (2007).

The present study revealed that expected heterozygosity (H_{exp}) was higher than observed heterozygosity (H_{obs}). Various factors such as inbreeding, genetic hitchhiking, null alleles and occurrence of population substructure (Wahlund effect) have been reported as the reasons for the heterozygote deficiency in population (Nei 1987).

Table 31: Observed number of allele (Na), Effective number of allele (Ne), Observed heterozygosity (Ho), Expected heterozygosity (He), Fixation index (F) for Marwari sheep breed.

Locus	Na	Ne	Ho	He	F	PIC
BM8125	7.000	5.373	0.500	0.814	0.386	0.786
OarFCB48	15.000	10.000	0.467	0.900	0.481	0.8006
OarFCB128	9.000	3.579	0.500	0.721	0.306	0.6879
OarHH35	5.000	2.757	0.667	0.637	-0.046	0.5898
OarHH41	10.000	6.250	0.667	0.840	0.206	0.8232
OarHH64	8.000	5.538	0.767	0.819	0.064	0.7947
OarJMP8	11.000	6.870	0.867	0.854	-0.014	0.8222
OarJMP29	12.000	6.338	0.467	0.842	0.446	0.8049
TGLA137	9.000	6.691	0.300	0.851	0.647	0.8317
OarCP34	8.000	6.102	0.633	0.836	0.243	0.8138
OarAE129	6.000	4.627	0.633	0.784	0.192	0.7488
BM827	8.000	5.172	0.467	0.807	0.421	0.7776
CSSM31	5.000	3.838	0.367	0.739	0.504	0.6987
BM6526	10.000	5.042	0.533	0.802	0.335	0.783
BM757	9.000	3.209	0.900	0.688	-0.308	0.6559
TGLA377	4.000	3.035	0.633	0.671	0.056	0.6048
OarVH72	11.000	7.895	0.900	0.873	-0.031	0.8563
RM4	11.000	7.660	0.567	0.869	0.348	0.856
Mean±SE	8.778±0.655	5.554±0.450	0.602±0.041	0.797±0.018	0.235±0.057	0.763

4.4.2 F-Statistics

The fixation indices measure the genetic divergence of sub-populations within the total population. The F – statistic describes the amount of inbreeding like effects within sub-population (F_{IS}), among the sub-populations (F_{ST}) and in the total population (F_{IT}).

The Mean F_{IS}, F_{IT}, and F_{ST} values of all sheep populations were 0.325±0.024, 0.386±0.024 and 0.090±0.013 respectively (Table 34). The overall estimates were significantly ($P<0.05$) different from zero. F_{IS} statistics, ranged from 0.161 (CSSM31) to 0.520 (TGLA137) presented in Table 34. All loci contributed significantly to the within population heterozygote deficit. Further, within population inbreeding estimates (F_{is}) indicated a deficit of heterozygotes as shown 23.5% in Marwari, 25.6% in Chokla, 32.5% in Nali and 45.3% in Magra, respectively Table 31, 29, 30 and 32. The highest numbers of loci exhibiting deficiency in heterozygosity were observed in the Magra sheep breed, while the number of such loci was lowest in the Marwari sheep could be credited to less genetic segregation between the subpopulation of this breed.

The mean F_{IS} values obtained in the present study were higher than the values obtained by Ahmed *et al.* (2014) in Kail sheep (0.052), Prema *et al.* (2008b) in Madras Red sheep (0.048), Sharma *et al*. (2010) in Changthangi sheep (0.047) Pramod *et al.* (2009) in Vembur (0.2954) and Radha *et al.* (2011) in Kilakarsal (0.147).

The F_{IS} value for Chokla (0.256) and Marwari (0.235) in the present study was decreased from previously reported studies on Chokla sheep (0.286 and 0.299) by Mukesh *et al*. (2006) and Sodhi *et al*. (2005). Whereas other study made on chokla sheep had shown less F_{IS} value (0.176) than present study. However, in Nali and Magra sheep the F_{IS} value shown as 0.325 and 0.453 respectively, increased from the previous reported studies (Arora and Bhatia *et al*., 2011; Sodhi *et al*., 2006) on same breed.

Table 32:Observed number of alleles (Na), Effective number of alleles (Ne), Observed heterozygosity (Ho), Expected heterozygosity (He), Fixation index (F) for Magra sheep breed.

Locus	Na	Ne	Ho	He	F	PIC
BM8125	9.000	4.813	0.567	0.792	0.285	0.7701
OarFCB48	7.000	4.255	0.600	0.765	0.216	0.734
OarFCB128	7.000	4.412	0.333	0.773	0.569	0.7371
OarHH35	9.000	5.085	0.233	0.803	0.710	0.7782
OarHH41	9.000	3.838	0.467	0.739	0.369	0.708
OarHH64	12.000	7.895	0.467	0.873	0.466	0.8549
OarJMP8	10.000	5.960	0.333	0.832	0.599	0.8117
OarJMP29	7.000	4.878	0.367	0.795	0.539	0.7684
TGLA137	12.000	7.627	0.400	0.869	0.540	0.8518
OarCP34	9.000	7.004	0.433	0.857	0.494	0.8406
OarAE129	7.000	4.147	0.667	0.759	0.122	0.7288
BM827	6.000	3.586	0.433	0.721	0.399	0.6782
CSSM31	5.000	2.804	0.600	0.643	0.067	0.5933
BM6526	7.000	3.209	0.433	0.688	0.370	0.6436
BM757	7.000	2.985	0.267	0.665	0.599	0.6191
TGLA377	5.000	4.286	0.000	0.767	1.000	0.7293
OarVH72	13.000	7.563	0.400	0.868	0.539	0.8222
RM4	9.000	5.505	0.600	0.818	0.267	0.796
Mean±SE	8.333±0.548	4.992±0.382	0.422±0.038	0.779±0.016	0.453±0.052	0.7480

A positive F_{IS} value was observed for all the breeds investigated in the present study, suggesting a lack of heterozygotes in the four breeds. This deficit of heterozygotes may be attributed to the Wahlund effect (population

substructure) due to pooling samples (within the breed) from different breeding flocks (i.e., different places in the same area).

Table 33:Overall genetic variation in all studied sheep breeds

Locus	Na	Ne	Ho	He	PIC
BM8125	11	6.8	0.6598	0.8579	0.8533
OarFCB48	17	9.3	0.5361	0.8982	0.8568
OarFCB128	12	6.7	0.4948	0.8565	0.8341
OarHH35	11	5.7	0.6598	0.8310	0.7967
OarHH41	12	6.3	0.5876	0.8471	0.82
OarHH64	13	8.0	0.6186	0.8807	0.8634
OarJMP8	14	7.7	0.6289	0.8755	0.8534
OarJMP29	16	9.7	0.4948	0.9022	0.8498
TGLA137	14	7.3	0.4330	0.8684	0.8482
OarCP34	13	7.2	0.5567	0.8660	0.8283
OarAE129	8	5.1	0.5567	0.8112	0.7794
BM827	10	6.8	0.3608	0.8587	0.8367
CSSM31	11	6.5	0.6082	0.8528	0.8223
BM6526	13	7.4	0.5052	0.8703	0.8356
BM757	15	3.6	0.4742	0.7275	0.6799
TGLA377	8	4.8	0.4227	0.7962	0.7618
OarVH72	14	6.8	0.6289	0.8586	0.7995
RM4	12	9.1	0.6082	0.8957	0.8596
Mean±SE	12.4±2.3901	6.9±1.5707	0.5464±0.0871	0.8530±0.0417	0.821

The high genetic homogeneity or lack of heterozygotes in these Indian sheep breeds might also be ascribed to inbreeding (overall positive F_{IS} value).

Hence, concerted genetic management efforts are suggested in the investigated sheep breeds, especially Magra, to avert the adverse effects of inbreeding.

Table 34: F-statistic for overall sheep breeds at each locus

Locus	Fis	Fit	Fst
BM8125	0.206	0.243	0.047
OarFCB48	0.351	0.385	0.052
OarFCB128	0.351	0.466	0.177
OarHH35	0.220	0.289	0.089
OarHH41	0.268	0.320	0.071
OarHH64	0.307	0.334	0.039
OarJMP8	0.307	0.345	0.055
OarJMP29	0.417	0.463	0.080
TGLA137	0.520	0.532	0.024
OarCP34	0.348	0.377	0.045
OarAE129	0.237	0.284	0.062
BM827	0.505	0.548	0.086
CSSM31	0.161	0.291	0.155
BM6526	0.372	0.443	0.113
BM757	0.275	0.433	0.218
TGLA377	0.484	0.576	0.180
OarVH72	0.267	0.311	0.060
RM4	0.255	0.309	0.073
Mean±SE	0.325±0.024	0.386±0.024	0.090±0.013

The level of genetic differentiation among the sheep breeds measured in terms of F_{ST}. The value of F_{ST} was 9%, which was comparatively lower in this study. The genetic differentiation value (Fst) per locus varied from 0.024

(TGLA137) to 0.218 (BM757) with an average of 0.090±0.013 across all the loci (Table 34). Across different breed pairs, maximum differentiation was observed in Nali-Magra pair with the value 0.069, while Nali-Marwari (0.055) was the least differentiated pair. The within breed genetic variation are 91%, more than between breed genetic variation. We can use this interpretation for improving genetic material and conservation of sheep population of India.

The mean F_{ST} value obtained in the present study was found as 0.090, which is higher than the values obtained by Arora and Bhatia, (2011) in six Indian sheep breeds (0.061), Sodhi *et al*. (2006) evaluated in Chokla and Nali sheep breeds (0.083). While, the highest values reported by Arora *et al*. (2011) in southern and eastern sheep breeds (0.132), Mukesh *et al*. (2006) examined in Chokla, Nali and Garole sheep breeds (0.183). This low level of differentiation among the sheep of the arid region could be due to continuous intermixing and crossbreeding during migration, to improve production traits.

The mean F_{IT} value reported in the present study was 0.386, which is higher than the values obtained by Arora and Bhatia, (2011) in six Indian sheep breeds (0.202), Sodhi *et al*. (2006) in Nali and Chokla sheep breed (0.370), Arora *et al*. (2011) in southern and eastern sheep breeds (0.175) of India. However, the higher values were obtained by Mukesh *et al*. (2006) in Chokla, Nali and Garole sheep breeds (0.410).

4.5 Genetic distance &Dendogram

The Nei's Genetic distance analysis amongst the four sheep population showed the smallest distance between Nali and Marwari (0.504) and maximum distance between Marwari and Magra (0.702) presented in Table 35. This observation was also supported by Phylogenetic tree analysis by using the UPGMA method which showed two clusters where Chokla, Nali, Marwari were clustered in one group which is accordance with previously reported studies on Chokla and Nali by Mukesh *et al*. (2006), in this study the Garole animals were

clustered exclusively in one group, whereas grouping of both Nali and Chokla animals together supported their close genetic proximity (Fig 11). Another study made on these breeds revealed that low genetic distance values (D_S = 0.229 and D_A = 0.168) in Chokla and Nali supported high genetic similarity between these two breeds by Sodhi *et al*. (2006).

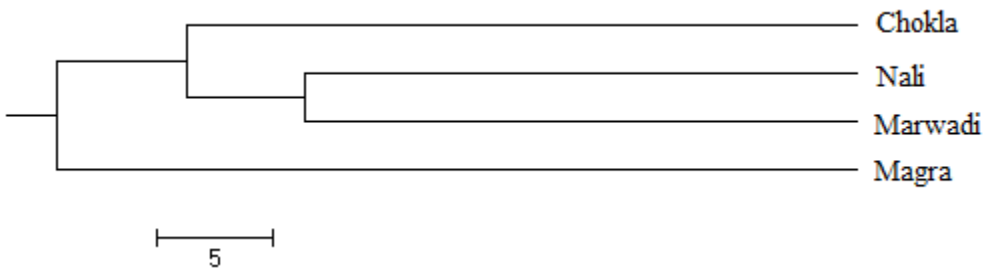

Fig.11: Phylogenetic analysis of four cattle breeds Chokla, Nali, Marwari and Magra population were based on Nei's (1978) Genetic distance software POPGENE- 1.32 version (32 bit).

Table35: Pairwise Population Matrix of Nei Genetic Distance

Chokla	**Nali**	**Marwari**	**Magra**	
0.000				**Chokla**
0.604	0.000			**Nali**
0.606	**0.504**	0.000		**Marwari**
0.685	0.680	**0.702**	0.000	**Magra**

The genetic distance between the Nali and Marwari breeds was observed to be lower in our study than in earlier report (Arora and Bhatia, 2011). The present investigation of four breeds of the arid region together provides a clearer picture of the current status of their genetic relationships.

4.6 Hardy Weinberg Equilibrium

The populations were tested for departure from Hardy-Weinberg equilibrium frequencies for all the loci. The Chi-square values with their respective degree of freedom for different loci utilized for genotyping in the present study are given in the Table 36, 37, 38 & 39. In this study several loci departed from the equilibrium frequency in the four populations studied.

Table 36: Chi-square test for Hardy Weinberg equilibrium in Chokla sheep

Pop	Locus	DF	ChiSquare	Significance
Pop1	BM8125	28	63.395	***
Pop1	OarFCB48	78	154.496	***
Pop1	OarFCB128	6	18.316	Equilibrium
Pop1	OarHH35	28	114.773	***
Pop1	OarHH41	21	66.538	***
Pop1	OarHH64	36	136.367	***
Pop1	OarJMP8	21	66.178	***
Pop1	OarJMP29	55	129.231	***
Pop1	TGLA137	45	81.348	***
Pop1	OarCP34	45	144.000	***
Pop1	OarAE129	15	33.854	Equilibrium
Pop1	BM827	21	153.333	***
Pop1	CSSM31	45	164.381	***
Pop1	BM6526	55	136.611	***
Pop1	BM757	15	38.408	***
Pop1	TGLA377	15	150.000	***
Pop1	OarVH72	28	59.248	***
Pop1	RM4	36	71.806	***

Pop1 = Chokla, Significance level at ***p<0.001

Table 37: Chi-square test for Hardy Weinberg equilibrium in Nali sheep

Pop	Locus	DF	ChiSquare	Significance
Pop2	BM8125	28	109.345	***
Pop2	OarFCB48	36	75.533	***
Pop2	OarFCB128	15	97.886	***
Pop2	OarHH35	36	100.657	***
Pop2	OarHH41	28	87.856	***
Pop2	OarHH64	36	125.934	***
Pop2	OarJMP8	36	105.288	***
Pop2	OarJMP29	21	74.063	***
Pop2	TGLA137	36	106.978	***
Pop2	OarCP34	28	63.508	***
Pop2	OarAE129	10	43.786	***
Pop2	BM827	21	69.833	***
Pop2	CSSM31	15	74.256	***
Pop2	BM6526	45	117.352	***
Pop2	BM757	6	60.057	***
Pop2	TGLA377	1	10.055	Equilibrium
Pop2	OarVH72	45	142.614	***
Pop2	RM4	21	81.215	***

Pop2= Nali, Significance level at ***$p<0.001$

The results of the X^2 test of goodness of fit (Table 36, 37, 38 & 39) revealed that the population was in Hardy-Weinberg Equilibrium proportions for 2 microsatellite loci (OarFCB128 and OarAE129) in Chokla sheep, 1 loci (TGLA377) in Nali sheep, 4 loci (OarHH35, OarHH41, BM6526 and BM757) in Marwari sheep and 3 loci (CSSM31, OarAE129 and BM757) in Magra sheep. The remaining 16 loci in Chokla, 17 loci in Nali, 14 loci in Marwari and 15 loci in

Magra showed a significant departure from HWE. Similar departures from HWE were also reported in various breeds of sheep shown by Radha *et al*. (2010), Mukesh *et al*. (2006), Surekha *et al*. (2015), Sassi-Zaidy *et al*. (2014).

Table 38:Chi-square test for Hardy Weinberg equilibrium in Marwari sheep

Pop	Locus	DF	ChiSquare	Significance
Pop3	BM8125	21	102.987	***
Pop3	OarFCB48	105	201.946	***
Pop3	OarFCB128	36	118.806	***
Pop3	OarHH35	10	16.374	Equilibrium
Pop3	OarHH41	45	74.170	Equilibrium
Pop3	OarHH64	28	78.614	***
Pop3	OarJMP8	55	120.544	***
Pop3	OarJMP29	66	174.759	***
Pop3	TGLA137	36	129.269	***
Pop3	OarCP34	28	101.288	***
Pop3	OarAE129	15	73.517	***
Pop3	BM827	28	134.086	***
Pop3	CSSM31	10	73.906	***
Pop3	BM6526	45	79.444	Equilibrium
Pop3	BM757	36	42.200	Equilibrium
Pop3	TGLA377	6	32.480	***
Pop3	OarVH72	55	93.971	***
Pop3	RM4	55	151.811	***

Pop3= Marwadi, Significance level at ***$p<0.001$

The reports on exotic sheep breeds revealed similar departure of the populations from HWE at various microsatellite loci (Diez-Tascon *et al*., 2000;

Tomasco *et al*., 2002; Alvarez *et al*., 2009; Ivankovic *et al*., 2005; Jandurova *et al*., 2005; Calvo *et al*., 2006, Das *et al*., 2015).

Table 39: Chi-square test for Hardy Weinberg equilibrium in Magra sheep

Pop	Locus	DF	ChiSquare	Significance
Pop4	BM8125	36	96.278	***
Pop4	OarFCB48	21	56.893	***
Pop4	OarFCB128	21	60.502	***
Pop4	OarHH35	36	169.016	***
Pop4	OarHH41	36	129.440	***
Pop4	OarHH64	66	161.531	***
Pop4	OarJMP8	45	139.193	***
Pop4	OarJMP29	21	86.349	***
Pop4	TGLA137	66	156.891	***
Pop4	OarCP34	36	114.054	***
Pop4	OarAE129	21	43.100	Equilibrium
Pop4	BM827	15	44.277	***
Pop4	CSSM31	10	16.696	Equilibrium
Pop4	BM6526	21	53.001	***
Pop4	BM757	21	44.669	Equilibrium
Pop4	TGLA377	10	120.000	***
Pop4	OarVH72	78	141.142	***
Pop4	RM4	36	72.523	***

Pop4= Magra, Significance level at ***$p<0.001$

On the other hand, some of the reports showed that the sheep populations studied were in HWE for the respective microsatellite loci (Gutierrez-Espelata *et al*., 2000; Soysal *et al*., 2005; Sharma *et al*., 2010; Salamon *et al*., 2014).

The present study showed a significant departure from HWE, which might be due to both the systematic (selection, migration and mutation) and dispersive (genetic drift and inbreeding) forces operating in the population. This deviation from HWE may also be attributed to the presence of unobserved null alleles (non amplifying alleles).

4.7 Genetic Bottleneck Analysis

A decrease in population number may impact the effective population size andthe maintenance of genetic variation within a population (Cornuet and Luikart, 1996). Since Marwari, Chokla, Nali, and Magra sheep are declining, a bottleneck analysis was performed to assess whether the population decline had an impact on the maintenance of genetic variation within these breeds. The microsatellite data were subjected to a mode shift test, under the assumption of the two-phase model. The occurrence of a normal L-shaped curve in all four breeds (Fig. 12) revealed no loss of alleles in the investigated populations and hence the absence of a genetic bottleneck. Similar observations on bottleneck analysis were obtained by Kavitha *et al*., (2015) in Tiruchy blacksheep, Sharma *et al*., (2016) in Tibetan sheep, Arora *et al*., (2010) in Ganjam sheep, Radha *et al*., (2011) in Kirakarsal sheep, Kumar *et al*., (2007) in Bellary sheep, Arora and Bhatia, (2006).

The result of bottleneck analysis revealed that all the breeds studied are non-bottlenecked, i.e., it has not undergone any recent reduction in the effective population size and remained at mutation-drift equilibrium. In the present study, no mode-shift was detected in the frequency distribution of alleles and normal L-shaped curve was observed.

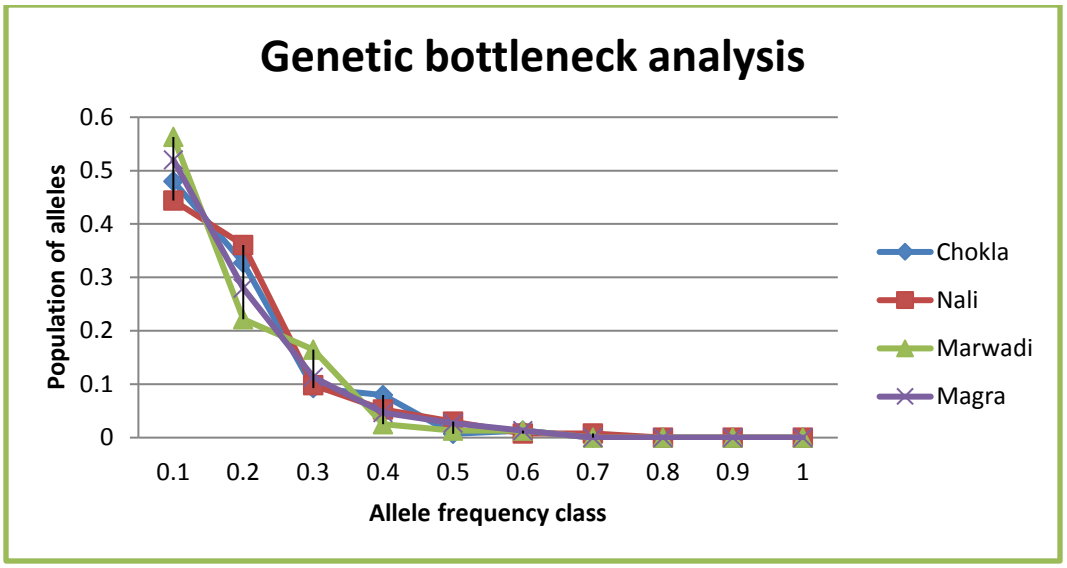

Fig. 12: Mode shift "L" curve for all studied breeds of sheep

4.8 Ewens–Watterson Test

Ewens–Watterson Test for Neutrality revealed that all the microsatellite except 6 markers BM8125, OarHH41, OarHH64, OarJMP8, OarJMP29 and TGLA137 in Chokla, 4 markers BM8125, OarHH64, OarCP34, BM827 in Nali, 6 markers BM8125, TGLA137, OarCP34, OarAE129, OarVH72, RM4 in Marwari and 1 marker CSSM31 in Magra (observed F values lie outside of the upper and lower limits of 95% confidence region of the expected F values) were neutral. Since 66.6% loci in Chokla, 77.7% in Nali, 66.6% in Marwari and 94.4% of Magra breed were neutral, selection as a cause of the decrease in observed heterozygosity was ruled out. Similar observations were made by Sharma *et al*., 2016 in Tibetan sheep revealed that all the microsatellite except OarHH47 and INRA63 were neutral. Thus the difference between the observed and expected heterozygosity can be attributed to the non-random mating among the individuals of the population were sampled, where Magra breed showed a high value of neutrality comparatively to other breeds. The deficite in heterozygosity was also reflected in the positive F_{IS} (Inbreeding estimates) value (0.453±0.052), which ranged from 0.067 to 1.000.

4.9 Sequence analysis results for KAP's and KRT's genes

4.9.1 Sequence analysis of KAP1-1 gene (formerly known as B2A)

Four samples of different sheep breeds were obtained and designated as 1, 2, 3 and 4 for Magra, Chokla, Nali and Marwari breeds, respectively. The PCR amplification was successfully performed and amplified product was 311 bp for KAP1-1 gene (Fig.13). Nucleotide sequences obtained from sequencing were subjected to DNA to protein translation in order to find coding sequences (cds). We found a variable length of cds (234, 237, 246 and 168) in four breeds of Rajasthan. The coding sequences for Magra, Chokla and Nali shared 100% homology with the (KAP1.1) gene in Chinese merino sheep (Gene bank accession no. HQ110109) and KAP1.1 beta allele (Gene bank accession no. AY835604) of Itenge-mweza *et al*. (2007). Where (KAP1-1) partial cds of Marwadi differed from the HQ110109 & AY835604 and shared 96% identity. Further to identify variation in KAP1-1 gene sequence in Magra, Chokla, Nali and Marwadi sheep were aligned using BioEdit software. Sufficient variability was observed in Marwari sheep in comparison to other breeds at 30 to 90 base pair position with different nucleotides to other breeds. The substitution of nucleotide also occurred in 104,106, 139 and 141 positions with T-G, G-C, A-G and C-T substitution respectively, in Marwari sheep (Fig 14.).

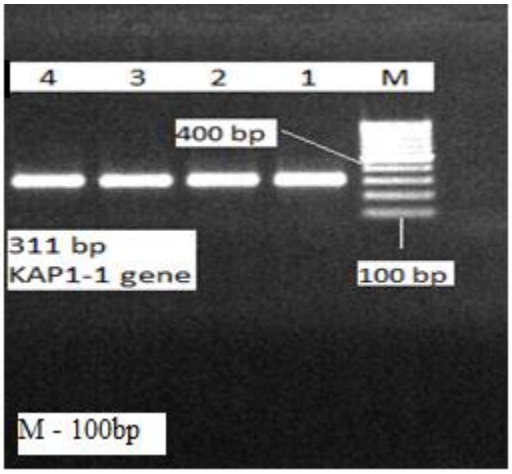

Fig. 13: PCR amplification of KAP1-1 gene in four sheep breeds.

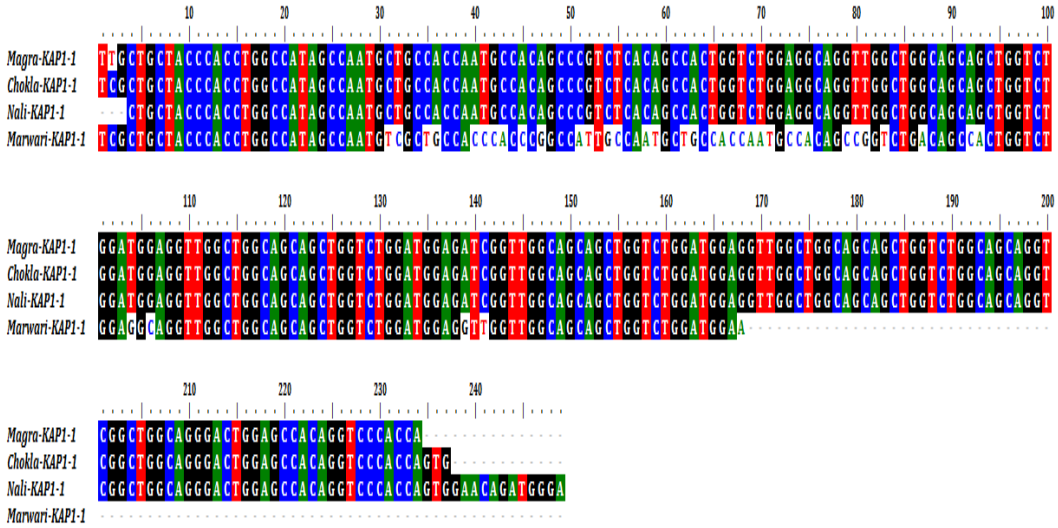

Fig. 14: Multiple sequence alignment of KAP1-1 gene between the breeds.

4.9.2 Sequence analysis of KRT1-2 or K33 gene

Four samples of different sheep breeds were obtained and designated as 1, 2, 3 and 4 for Magra, Chokla, Nali and Marwari breeds, respectively. The PCR amplification was successfully performed and amplified product was 480 bp for KRT1-2 gene (Fig.15) respectively. After the sequencing, sequences were obtained and subjected to identify coding part of the sequence. The length of the cds was found 187 bp in all respective breeds. The coding sequence of Magra-KRT1-2 and Chokla-KRT1-2 shared 99% homology with (KRT1.2) keratin intermediate filament type I (Gene bank accession no. KF718800) and (KRT1.2) gene type I protein variant 2 in Chokla sheep. Where sequence of KRT1.2 in Nali sheep shared 99% homology with (KRT1.2) protein variant 1 in Chokla sheep (Gene bank accession no. KJ524448), with keratin microfibril type I gene (Gene bank accession no. M23912) and KRT1.2-D allele (Gene bank accession no. AY835601) of Itenge-mweza *et al*. (2007). Variability was observed in Nali sheep other than studying breeds on this locus. The substitution occurred in the position of 63 (T-C), 95 (C-T), 144 (C-T) and 187 (T-C) in the gene sequence of

Nali sheep in reference to other breeds. However, deletion occurred in Magra, Nali and Marwadi at 55th position, while in Chokla, it is 64 (Fig. 16).

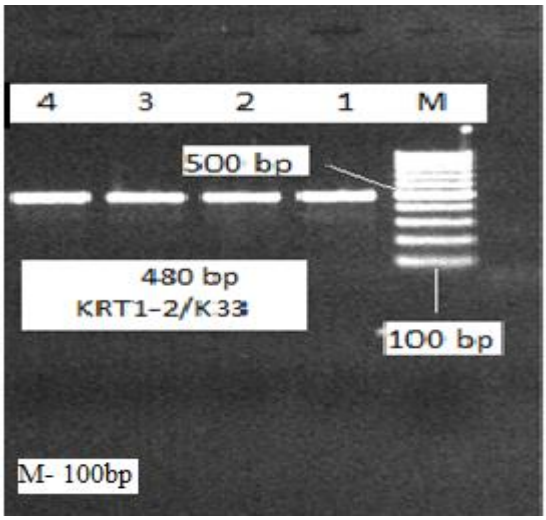

Fig. 15: PCR amplification of KRT1-2/K33 gene in four sheep breeds.

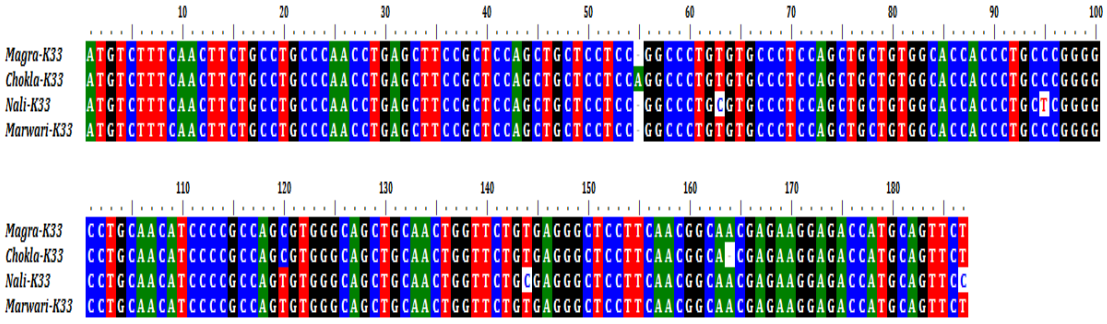

Fig. 16: Multiple sequence alignment of KRT1-2 gene between the breeds.

4.9.3 Sequence analysis of KRTAP1-4 gene

Four samples of different sheep breeds were obtained and designated as 1, 2, 3 and 4 for Magra, Chokla, Nali and Marwari breeds, respectively. The PCR amplification was successfully performed and amplified product was 624 bp for KRTAP1-4 gene (Fig.17). After the sequencing, sequences were obtained and subjected to identify coding part of the sequence. The length of the cds was found 417 bp in all respective breeds. The coding sequence of Magra-KRTAP1-4,

Chokla-KRTAP1-4, Nali-KRTAP1-4 and Marwari-KRTAP1-4 shared 99% homology with (KRTAP1-4)-E, B, A, D, I, G, F and C alleles (Gene bank accession no.GQ507745, GQ507742, GQ507741, GQ507744, GQ507749, GQ507747, GQ507746 and GQ507743) of Gong *et al*. (2010). The less nucleotide variation was observed among the breeds, while the substitution occurred at 25th position (T-C) in magra and at 293thposition (A-T) in Nali sheep (Fig 18.).

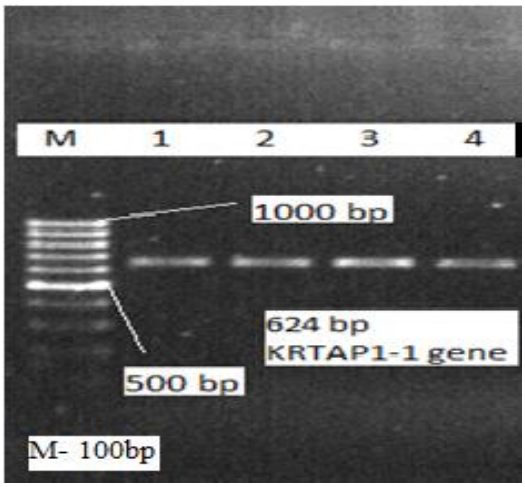

Fig. 17: PCR amplification of KRTAP1-4 gene in four sheep breeds.

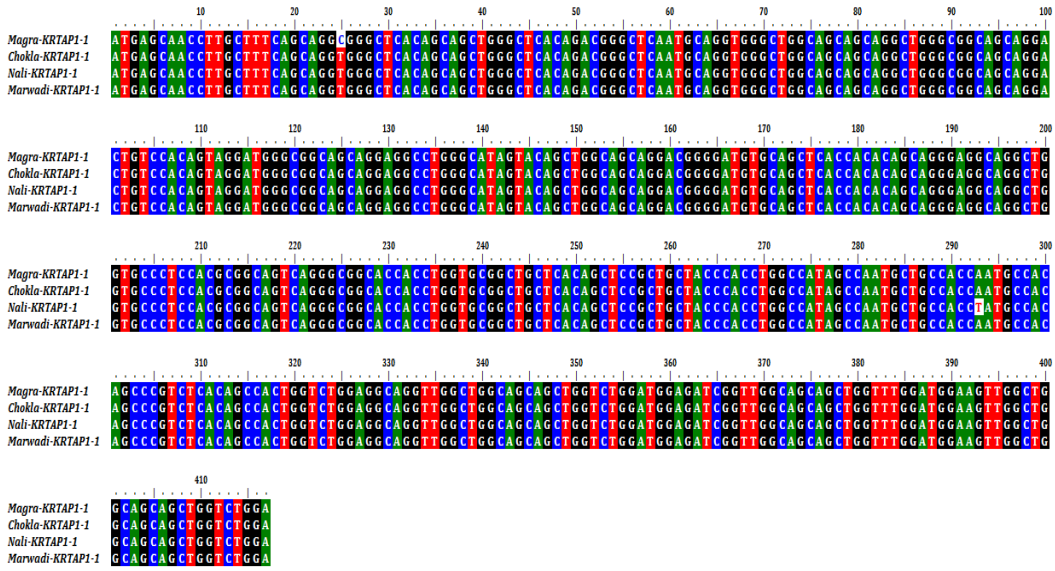

Fig.18: Multiple sequence alignment of KRTAP1-4 gene between the breeds.

4.9.4. Sequence analysis of KRTAP6-1 gene

Four samples of different sheep breeds were obtained and designated as 1, 2, 3 and 4 for Magra, Chokla, Nali and Marwari breeds, respectively. The PCR amplification was successfully performed and amplified product was 935 bp for KRTAP6-1 gene (Fig. 19) respectively. After the sequencing, sequences were obtained and subjected to identify coding part of the sequence. The length of the cds was found variable as 291, 309, 300 and 285 bp respectively. The coding sequence of Magra-KRTAP6-1, Chokla-KRTAP6-1, Nali-KRTAP6-1 and Marwari-KRTAP6-1 shared 100% homology with (KAP6) E-1 allele, KRTAP6-1, KA6-1-D allele, KAP6-B-1, KAP6-E and KAP6-D allele (Gene bank accession no. JQ043168, NM001193399, GU319875, JQ043167, JQ043159, JQ043158, respectively). Nucleotide sequences showing similarity between the breeds, while Nali sheep showing nucleotide subsitution at position 211 (G-T), 173 (G-C), 224 (G-A), 230 (T-C), 239 (G-A), 248 (A-C), 254 (G-T). Chokla breed showing substitution at 12th position (T-C) of the sequence (Fig. 20) .

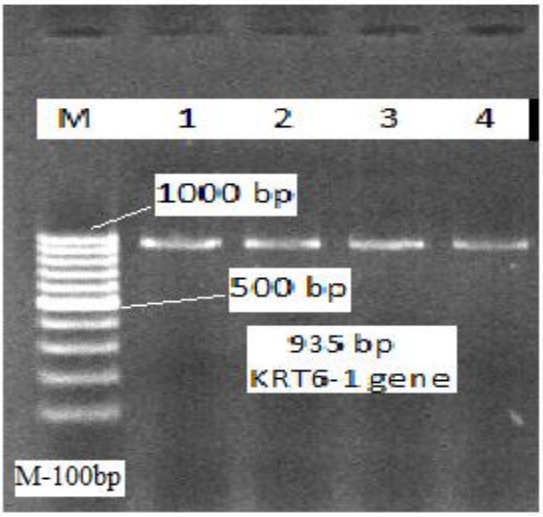

Fig. 19: PCR amplification of KRTAP6-1 gene in four sheep breeds.

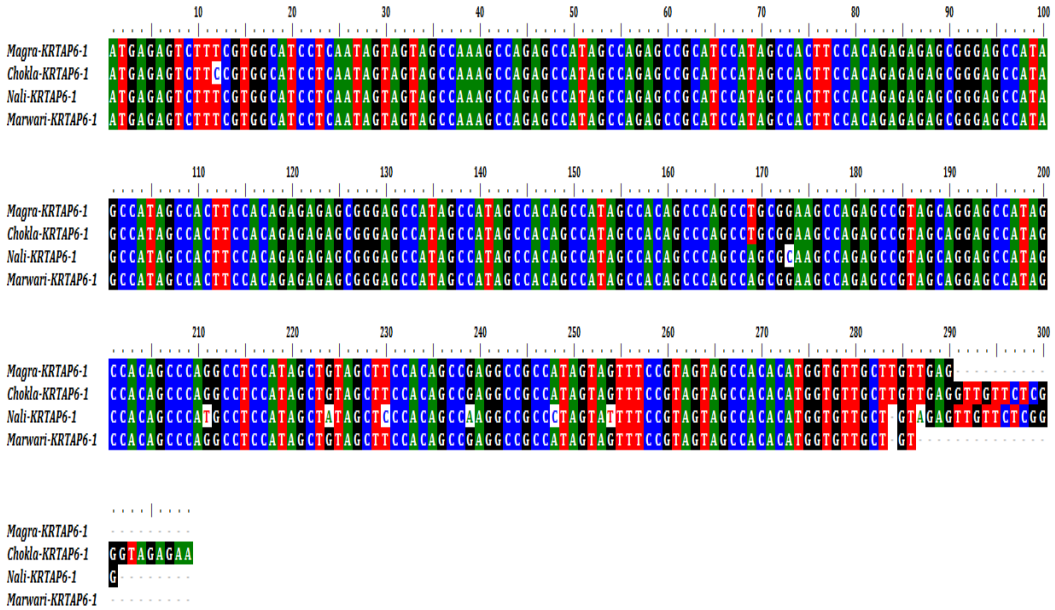

Fig. 20: Multiple sequence alignment of KRTAP6-1 gene between the breeds.

Sheep breeds of Rajasthan i.e. Magra, Chokla, Marwari and Nali were studied using 18 microsatellite markers. 30 animals per breed were taken for the study. The Genomic DNA extracted from whole blood. The concentration of DNA samples measured with the help of UV spectrophotometer for respective breeds.

PCR products of the microsatellites were analyzed by running on 8% PAGE to identify DNA polymorphism. All the eighteen microsatellite loci used in the study were polymorphic with a reasonable informativeness. A total of 224 alleles, ranging from 8-17 (Mean= 12.4) were found across 18 microsatellite loci. The most polymorphic marker was OarFCB48 with a total of 17 alleles and least polymorphic loci were TGLA377 and OarAE129 with 8 alleles each.

The effective number of alleles ranged between 2.486 to 8.612 in Chokla, 1.867 to 7.759 in Nali, 2.757 to 10.000 in Marwari and 2.804 to 7.895 in Magra with overall mean effective number of alleles 5.177 for Chokla, 4.958 for Nali, 5.554 for Marwari and 4.992 for Magra sheep. The allele frequency ranged between 1.7% to 56.7% in Chokla, 1.7% to 63.3% in Nali, 1.7% to 53.3% in Marwari and 1.7% to 51.7% in Magra sheep. The allele frequency were found highestfor 5th number of allele (100 bp) at locus TGLA377 in Nali sheep over the four sheep breeds.

A total of 12 out of 150, 2 out of 133, 22 out of 158 and 19 out of 150 alleles found to be population specific for Chokla, Nail, Marwari and Magra sheep respectively. The locus OarJMP29 recorded the highest number of specific alleles (3) in Chokla, OarHH35 recorded the highest number of specific alleles (2) in Nali, BM757 recorded the highest number of specific alleles (5) in Marwari and BM757 recorded the highest number of specific alleles (4) in Magra. However, the frequency of most of these population-specific alleles was very low and only 2 (21.7%), and 1 (13.3%) in Marwari, 2 (26.7%), 1 (28.3%), 1 (50%), 1

(23.3%), 1 (16.7%) in Magra and 1 (25%) in Nali, alleles have the frequency of more than 10 percent.

Overall loci were highly polymorphic with a 100% value of polymorphism. The mean PIC value 0.751 ranged from 0.543 (BM757) to 0.857 (OarFCB48) in Chokla, 0.7317 ranged between 0.356 (TGLA377) to 0.853 (OarHH64) in Nali, 0.7480 found ranged between 0.593 (CSSM31) to 0.854 (OarHH64) in Magra sheep. The highest mean PIC value detected in Marwari sheep was 0.763, ranging between 0.589 (OarHH35) to 0.856 (OarVH72 and RM4).

The mean expected heterozygosity among the breeds was estimated as 0.783 (Chokla), 0.767 (Nali), 0.797 (Marwari) and 0.779 (Magra). The expected heterozygosity ranged from 0.884 (OarFCB48) to 0.598 (BM757) in Chokla, 0.464 (TGLA377) to 0.871 (OarHH64) in Nali, 0.637 (OarHH35) to 0.900 (OarFCB48) in Marwari, 0.643 (CSSM31) to 0.873 (OarHH64) in Magra sheep. The mean observed heterozygosity ranged from 0.000 (TGLA377) to 0.867 (OarHH35, OarFCB128) in Chokla, 0.033 (BM757) to 0.800 (CSSM31, BM8125) in Nali, 0.300 (TGLA137) to 0.900 (BM757, OarVH72) in Marwari, 0.000 (TGLA377) to 0.667 OarAE129) in Magra sheep.

The overall mean inbreeding coefficient was 0.256 in Chokla, 0.325 in Nali, 0.235 in Marwari and 0.453 in Magra sheep. All the loci showed positive, mild to moderate inbreeding in all the sheep breeds studied. The mean values of F_{IS}, F_{IT} and F_{ST} were found as 0.325, 0.386 and 0.090, respectively. The F_{IS} values ranged from 0.161 (CSSM31) to 0.520 (TGLA137), F_{ST} values ranged from 0.243 for the locus BM8125 to 0.576 for the locus TGLA377 and F_{IT} values ranged from 0.024 for the locus TGLA137 to 0.218 for the locus BM757 among sheep breeds studied.

The Genetic distance analysis amongst the four sheep population showed that the least distance found between Nali and Marwari (0.504) and maximum distance found between Marwari and Magra (0.702). The phylogenetic studies among four sheep breeds showed two clusters, where Chokla, Nali and Marwari were clustered in one group and Nali-Marwari further subdivided in one cluster of sheep breeds. On the other hand, Magra did not cluster with other sheep population with 100% bootstrapping.

The results of bottleneck analysis revealed that the studied sheep breeds are non-bottlenecked, i.e., it has not undergone any recent reduction in the effective population size and remained at mutation-drift equilibrium. In the present study, no mode-shift was detected in the frequency distribution of alleles and a normal L-shaped form was observed.

Ewens–Watterson Test for Neutrality revealed that 66.6% loci in Chokla, 77.7% in Nali, 66.6% in Marwari and 94.4% in Magra breed were neutral. Therefore, selection as a cause of the decrease in observed heterozygosity was ruled out. Thus, the difference between the observed and expected heterozygosity can be attributed to the non-random mating among the individuals of the population sampled, where Magra breed showed the high value of neutrality comparatively to other breeds. This was also reflected in the positive F_{IS} (Inbreeding estimates) value (0.453±0.052), which ranged from 0.067 to 1.000.

Further the sequence analysis of *KRTAP1-4, KRTAP6-1, KAP1-1* and *KRT1-2/K33* gene for wool in Chokla, Nali, Marwadi and Magra sheep breeds showed nucleotide variations at different positions in their gene sequences. Homology was able to identify the close similarity of gene sequences obtained from Indian sheep with other reported sequences in BLAST search.

The present study of genetic diversity in four sheep breed population of the Northwestern arid region of Rajasthan was successfully estimated using 18 microsatellite markers. All the loci were highly polymorphic with 100% value of polymorphism observed in all four sheep breeds. Further, in breed wise study data has shown high genetic variability is present in Marwadi sheep population. The high PIC value indicating informativeness of markers to characterize sheep population and able to detect abundant genetic diversity in sheep breeds. In spite of high genetic variability the differences between mean observed and expected heterozygosity suggested a tendency of markers towards heterozygote deficiency same as reflected by positive inbreeding estimation (F_{is}) value. Further, low level of differentiation (F_{ST}) in sheep population could be accredited continuous intermixing and crossbreeding during migration to improve production traits. Where, the genetic distance and phylogenetic analysis would be useful to clarify their recent origin and relationships within them. It is easy to conclude that microsatellite markers are a powerful genetic tool in estimation of genetic variation within and among closely related populations.

In this study, data has also revealed the cause of heterozygote deficiency due to non-random mating among the individuals of the population evaluated by Ewens-watterson test. The maximum percent of neutrality detected in Magra sheep, which is the indication of greater risk to loss of genetic diversity in this breed. Subsequently, low observed heterozygosity and a significant deficit of heterozygote in all four sheep breeds, suggesting immediate efforts must be focused on its management and conservation programs to improve these breeds.

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ASSESSMENT OF GENETIC DIVERSITY IN INDIGENOUS SHEEP BREEDS OF RAJASTHAN USING MOLECULAR MARKERS

Ph.D. Thesis

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Abstract

This study illustrates the genetic diversity and relationships within and among four indigenous sheep breeds viz; Chokla, Nali, Marwari and Magra of the northwestern arid region of Rajasthan. The study was based on the use of eighteen microsatellite markers suggested by FAO (ISAG) for the estimation of genetic diversity in sheep. All the microsatellite markers were polymorphic and a total of 224 alleles, ranging from 8 – 17 (Mean=12.4) were found across 18 microsatellite loci. OarFCB48 was found to be highly polymorphic with a total of 17 alleles and least polymorphic loci were TGLA377 and OarAE129 with 8 alleles observed. The average observed heterozygosity was less than the expected heterozygosity. The observed and expected heterozygosity values ranged from 0.3608 (BM827) to 0.6598 (OarHH35, BM8125) with an overall mean of 0.5464 ± 0.0871 and from 0.7275 (BM757) to 0.9022 (OarJMP29) with an overall mean 0.8530±0.0417, respectively. The overall polymorphic information content (PIC) value in 4 breeds of sheep was also found high (>0.5) and ranged between 0.6799 (BM757) and 0.8634 (OarHH64). The estimate of heterozygote deficiency varied from 0.161 (CSSM31) to 0.520 (TGLA137) with a mean positive value of 0.325±0.024, suggesting a deficit of heterozygotes in all four breeds. The Mode shift analysis revealed a normal "L" shaped curve indicating all sheep breeds are non-bottlenecked. The geneftic differentiation estimate (F^{ST} = 9%) suggesting low levels of differentiation between the breeds. Ewens–Watterson Test for Neutrality revealed that 66.6% loci in Chokla, 77.7% in Nali, 66.6% in Marwari and 94.4% in Magra breed were neutral and attributed to the non-random mating among the individuals of the population. The Genetic distance among the breeds revealed a close relationship between the Nali and Marwari (0.504) and maximum distance was found between Marwari and Magra (0.702) sheep. Phylogenetic analysis was based on the UPGMA method further supported close genetic relationship between Nali-Marwari breeds as they clustered in one group, on the other hand Chokla and Magra showed clear distinctness and clustered in another group of sheep breeds.

आण्विक मार्कर के उपयोग द्वारा राजस्थान की स्वदेशी भेड़ नस्लों में आनुवांशिक विविधता का मूल्यांकन
विद्यावाचस्पति शोध ग्रन्थ

पशु सूक्ष्मजीव विज्ञान एंव जैवप्रोद्योगिकी विभाग,
पशु चिकित्सा एंव पशु विज्ञान महाविद्यालय,
राजस्थान पशु चिकित्सा और पशु विज्ञान विश्वविद्यालय
बीकानेर (334001) राजस्थान

शोध कर्ता : **कृतिका गहलोत**
मुख्य उपादेष्टा : **प्रोफेसर सुधीर कुमार कश्यप**

अनुक्षेपण

इस अध्ययन में राजस्थान के उत्तर–पश्चिम शुष्क क्षेत्र की चार स्वदेशी भेड़ नस्लों जैसे चोकला, नाली, मारवाड़ी और मगरा के बीच आनुवांशिक विविधता और उनके संबंधों को दर्शाया गया है। यह अध्ययन FAO (ISAG) के द्वारा सुझाए गए अठारह माइक्रोसेटेलाइट मार्करों के उपयोग से भेड़ नस्लों के आनुवांशिक विविधता के आंकलन पर आधारित है। सभी 18 माइक्रोसेटेलाइट मार्कर बहुरूपीय थे और चारों नस्लों में कुल मिलाकर 224 एलील्स मिले, जिनकी सीमा (रेंज) 8–17 (मीन = 12.4) पायी गयी। सबसे बहुरूपी मार्कर OarFCB48 पाया गया जिसमें कुल मिलाकर 17 एलिल्स मिले और सबसे कम बहुरूपी लोसाई TGLA377 एवं OarAE129 पाये गये, जिनमें 8 एलील्स मिले। पायी गयी औसत विषमयुग्मजता (0.5464 ± 0.0871) अपेक्षित विषमयुग्मजता (0.8530 ± 0.0417) से कम थी जिनका मान क्रमशः 0.3608 (BM827) से 0.6598 (OarHH35, BM8125) और 0.7275 (BM757) से 0.9022 (OarJMP29) के मध्य पाया गया। भेड़ की चार नस्लों में समग्र पोलीमोर्फिक इनफार्मेशन कंटेट (PIC) का मान भी उच्च (>0.5) प्राप्त हुआ, जो कि 0.6799 (BM757) से 0.8634 (OarHH64) के मध्य था। विषमयुग्मको की कमी का अनुमान 0.325 ± 0.024 के औसत सकारात्मक मूल्य के साथ 0.161 (CSSM31) से 0.520 (TGLA137) तक पाया गया, जिसमें सभी चार नस्लों में विषमयुग्मों की कमी का सुझाव है। मोड शिफ्ट विश्लेषण में एक सामान्य "L" आकार के वक्र को दर्शाया गया है जो कि सभी भेड़ नस्लों में गैर–बोटलनैक का संकेत देता है। नस्लों के बीच विभिन्नता के निम्न स्तर का सुझाव करते हुए आनुवांशिक विभेदन (F^{ST}) का अनुमान 9% प्राप्त हुआ। तटस्थता के लिए ईवेन्स–वॉट्ससन टेस्ट ने बताया कि चोकला में 66.6%, नाली में 77.7%, मारवाड़ी में 66.6% और मगरा में 94.4% तटस्थ मिले जो कि नस्लों के बीच गैर–यादृच्छिक संभोग के लिए जिम्मेदार है। नस्लों के बीच आनुवांशिक दूरी ने नाली और मारवाड़ी (0.504) के मध्य घनिष्ठ संबंध दर्शाया एवं मारवाड़ी और मगरा (0.702) के बीच अधिकतम दूरी पायी गई। फाइलोजेनेटिक विश्लेषण जो कि UPGMA पद्धति पर आधारित है, जिसने नाली–मारवाड़ी नस्लों के बीच निकट आनुवांशिक संबंधों का समर्थन किया है क्योंकि वे एक समूह में पाये गये, दूसरी ओर चोकला और मगरा ने भेड़ की नस्लों के दूसरे समूह में स्पष्ट भिन्नता को दर्शाया गया हैं।

APPENDIX

Phosphate buffer saline (1 %)

Solution A :	Sodium diphosphate	1.4 gm
	Distilled water	1000 ml
Solution B :	Sodium dihydrogen orthophosphate	1.4 gm
	Distilled water	1000 ml

An amount of 84.1 ml of solution A and 15.9 ml of solution B were mixed and 8.5g sodium chloride was added. The volume was made to 1000 ml with distilled water and autoclave it at 15 lbs (121oC) for 15 min.

Agarose solution (0.8% and 1.5-2%)

To prepare 0.8% agarose solution for genomic DNA analysis 0.8 gm of molecular grade agarose powder was dissolved in 100 ml of 1X TBE buffer.

To prepare 1.5-2% agarose solution for PCR products analysis 1.5 and 2 gm respectively of molecular grade agarose powder was dissolved in 100 ml 1X TBE buffer.

Ammonium per sulphate (10%)

To prepare 10% ammonium per sulphate solution 0.5 gm of molecular grade APS was dissolved in 5 ml of distilled water and stored at 4° C. It is advisable to prepare APS solution afresh every time acrylamide gel is prepared.

Buffers for pH meter

Buffer tablets of pH 4, 7 and 9 were crushed in a clean pestle-mortar and dissolved in 100 ml of sterilized distilled water.

EDTA (0.5 M), pH- 8.0

To 800 ml of distilled water 186.1 g of disodium ethylene diaminetetra acetate.$2H_2O$ was added and shaked vigorously on a magnetic stirrer for several hours. The pH was adjusted to 8.0 with 1.0 N NaOH, dispensed into aliquots and sterilized by autoclaving.

Ethanol (70%)

In 70 ml of 100% ethanol, add 30 ml of distilled water.

Ethidium bromide

1 g of ethidium bromide was added to 100 ml of distilled water and stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The container was wrapped in aluminum foil or the 10 mg/ml solution was transferred to a dark bottle and stored at room temperature.

Saturated sodium chloride solution (6 M)

For 100 ml of 6 M solution, 35.06 g of NaCl was dissolved in 80 ml of distilled water. The volume was made up to to 100 ml, filtered and stored at room temperature.

20% Sodium dodecyl Sulphate (SDS) solution

20 g of SDS powder was dissolved initially in 50 ml of distilled water and then stirred on magnetic stirrer at high speed. Finally, the volume of the solution was made upto 100 ml. The solution was filtered and kept at room temperature.

Tris Borate EDTA (TBE) buffer, pH 8.3

5X Stock solution:

54g Tris Base

27.5g Boric Acid and

20mL 0.5m EDTA (pH 8).

Distilled water was added to above and volume was made up to 1000 ml.

A working solution of 1X TBE was prepared by adding 200 ml of stock solution to 800 ml of distilled water.

Tris EDTA (TE) buffer, pH 8

Tris solution (0.05 M):

4.44 g/l Tris HCl

2.65 g/l Tris base

The above were dissolved in 1000 ml of distilled water.

Tris solution (0.05) 20 ml

EDTA (0.05 M) (pH) 200µl

Distilled water was added to above to make up the volume 100 ml.