GENETIC CHARACTERIZATION OF HILL CATTLE OF HIMACHAL PRADESH USING MOLECULAR MARKERS

THESIS

BY

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Submitted to

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CERTIFICATE-I

This is to certify that the thesis entitled "**Genetic Characterization of Hill Cattle of Himachal Pradesh using Molecular Markers**" submitted in partial fulfilment of the requirements for the award of the degree of **Master of Veterinary Science** in the subject of **Animal Breeding, Genetics & Biostatistics** of Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Mr. Kailash Kumar Mahajan**, son of Shri Rattan Lal Mahajan under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation has been fully acknowledged.

Place: Palampur
Dated: 6-05-2008

Dr. O. P. KAILA
Chairman,
Advisory Committee

CERTIFICATE-II

This is to certify that the thesis entitled **"Genetic Characterization of Hill Cattle of Himachal Pradesh using Molecular Markers"** submitted by **Mr. Kailash Kumar Mahajan,** son of Shri Rattan Lal Mahajan to the Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, (H.P.), India, in partial fulfilment of the requirements for the award of the degree of **Master of Veterinary Sciences** in the subject of **Animal Breeding, Genetics and Biostatistics** has been approved by the Advisory committee after an oral examination of the student in collaboration with an External Examiner.

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(Kailash Kumar Mahajan)

Place: Palampur

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

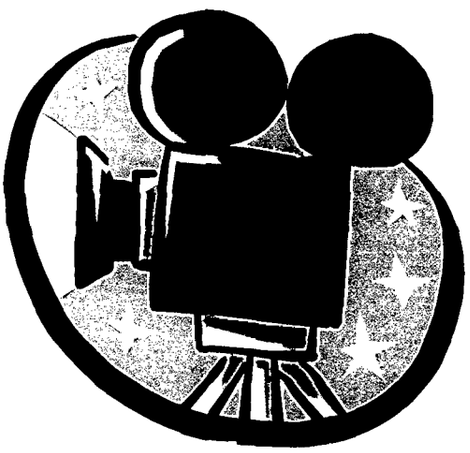
LIST OF PLATES

LIST OF ABBREVIATIONS

Abbreviation	Meaning
A	Absorbance
AFLP	Amplified fragment length polymorphism
$AgNO_3$	Silver nitrate
AnGR	Animal Genetic Resources
APS	Ammonium Per sulphate
DAD-is	Domestic Animal diversity Information System
dATP	Deoxy adenosine triphosphate
dCTP	Deoxy cytosine triphosphate
dGTP	Deoxy guanosine triphosphate
dNTPs	Deoxy nucleotides
Ds	Standard genetic distance
dTTP	Deoxy thymidine triphosphate
EtBr	Ethidium Bromide
FAO	Food And Agriculture Organization
He	Expected heterozygosity
Ho	Observed heterozygosity
IPC	Integrated Plate Chamber
Kb	Kilo base pairs
$MgCl_2$	Magnesium chloride
MoDAD	Measurement Of Domestic Animal Diversity
NATP	National Agriculture Technology Program
ng	Nanogram
NBAGR	National Bureau Of Animal Genetics Resources
PAGE	Poly Acrylamide Gel Electrophoresis
PIC	Polymorphism Information Content
PCR	Polymerase chain reaction

pg	Pico gram
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SDS	Sodium Dodecylsulphate
SSR	Simple Sequence Repeats
STR	Short Tandem Repeats
STS	Sodium Thiosulphate
Taq Poly	Taq Polymerase
TAE	Tris Acetate Ethylene Diamine Acetic Acid
TBE	Tris Borate Ethylene Diamine Acetic Acid
TEMED	N,N,N'N'-tetra methylene diamine
Tm	Melting Temperature
µl	Microlitre
µg	Microgram
UV	Ultra Violet
VNTR	Variable Number Of Tandem Repeats
X	Genomic chromosome number

Chapter I



Introduction

HILL CATTLE OF HIMACHAL PRADESH

Plate 1: Photograph showing adult hill cattle of Himachal Pradesh.

CHAPTER I

INTRODUCTION

The Indian subcontinent is bestowed with rich flora and fauna by virtue of which India ranked 6th largest mega biodiversity of the world. Livestock resource as important part of biodiversity play major role in Indian agricultural economy. There are 30 recognized breeds of cattle, 10 breeds of buffaloes, 42 breeds of sheep, 20 of goats, 8 of camel, 6 of horses and 18 breeds of poultry in our country. Besides them major population of cattle and other livestock comprised of nondescript types, that could not be classified into distinct breeds so far. India has 219.64 million cattle population which is 16.21% of world cattle population (FAO, 2001).

Mechanization, unrecognized efforts, unplanned and indiscriminate breeding among native stocks as well as human bias in favour of certain breeds are directly or indirectly have lead to the dilution of germplasm of indigenous cattle. Hence, there is an urgent need to prevent the rapid erosion of animal genetic resources and the diversity amongst them. This is true for the breeds in other developing and underdeveloped countries also, where many of them have been lost or are in danger of extinction. Many of them will be lost without even having been adequately characterized or studied. Around 16% of them have become extinct and 15 % are considered as rare (FAO, 2000). In India, native cattle breeds that are less economical under current market conditions are being interbred or replaced by improved exotic breeds. The conservation of different breeds is important if they have genetically unique characteristics (Hall and Bradley, 1995). Genetic conservation programmes are, however, costly to implement and it is not possible to conserve all genetic variation in all populations. An essential step is the evaluation of genetic resources and the selection of appropriate populations for conservation. A strategy

is therefore required that will maintain the widest possible level of genetic variation across the species (FAO, 1996). The multipurpose native and traditional breeds are often well adapted to home tract conditions, climate, diseases and nutritional environment. Such breeds may also be better adapted to locally produced forage, or be more resistant to geographically localized pathogens and parasites. Conservation of such genetic groups is crucial not only from their adaptability, disease resistance but also to preserve the local ecology. Surprisingly, the satisfactory national and international efforts for conservation of wild animals are ongoing but serious efforts have not been made for conservation of domestic animals, which normally affects environment in more significant terms than the wild ones.

FAO has taken lead in coordinating the development of an important research activity under the characterization programme. Measurement of Domestic Animal Diversity (MoDAD) programme of FAO is dedicated to conservation and characterization of livestock. In India, National Bureau of Animal Genetic Resources (NBAGR), Karnal is the national centre with mandate of conservation of Indian breeds of livestock.

Generally cattle are the most prominent (47.5%) followed by goats (15.9%) buffaloes (12.3%) and sheep (10.4%) in western and central Himalayas. Himachal Pradesh a North-western hilly state has 5.04 millions of livestock population. The cattle contribute about 43.53% of the total and about 30 % of cattle population is graded up with the exotic germ plasm. The hill cattle although are low in production but these animals are tough, fit for hilly terrains, adapted to cold and harsh climate, disease resistant, thriving on poor pastures and nutrients.

Protein polymorphisms were the first markers used in livestock. However the level of polymorphism observed in proteins is often low which has reduced the general applicability of protein typing in diversity studies. With the development of PCR and sequencing technologies, associated with automatic and/or semi automatic large scale screening system, DNA-based polymorphism based markers are the markers of choice for molecular-based surveys of genetic variation.

Genetic characterization through the use of molecular markers associated to powerful statistical approaches is providing new avenues for making choices for conservation and rational management of animal genetic resources. These include biochemical polymorphisms, RFLP, RAPDs, mtDNA, SSR or Microsatellites and SNPs. Among these, the microsatellite markers have become the markers of choice for population studies due to their dense distribution in the genome, high variability co-dominant inheritance and relative ease of detection. The Food and Agricultural Organization (FAO) of the United Nations has proposed a global programme for the management of genetic resources using molecular methodology for breed characterization (Bjornstad and Roed, 2001). This strategy places a strong emphasis on the use of molecular markers to assist the conservation and assessment of endangered breeds and to determine the genetic status of these breeds. Molecular markers have been widely used to access the variability, since they provide information on every region of the genome, regardless of the level of gene expression.

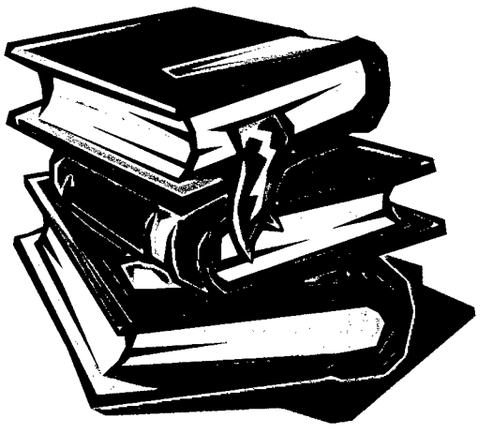
The Himalayan economy is basically agro-pastoral and dependent on livestock. Out of 449 million domestic animals in the country nearly 9% are in the Himalayan region. The land holdings being very small and livestock supplement in the income and sustain

livelihood of the farming communities of the hills. In general, within the framework of mixed farming the livestock provide milk, meat, wool, hide, skin, motive power for agricultural operations, transport and manure etc. and are of immense economical value to the farmers.

In spite of these qualities the hill cattle have not so for been given any importance to find out genetic similarity or diversity among these animals. Keeping in view the importance of these animals and to find out genetic similarity and variability in the animal at different locations, study was undertaken with the following objectives.

1. Genetic characterization of the hill cattle of Himachal Pradesh using molecular markers.
2. To study the genetic diversity of the hill cattle available at different locations of Himachal Pradesh.

Chapter II



Review of Literature

CHAPTER II

REVIEW OF LITERATURE

During the last forty years, biochemical assays provide an important tool to uncover genetic variation at molecular level. Now it has become clear that, these can provide rich insights in to the genetic structure and evolutionary history of the organisms. Earliest pioneering study on ABO blood group system revealed that humans show marked variation regarding this trait. Studies of multilocus protein or allozyme variation, with minor modifications and refinements, became the standard tool for investigations of biochemical genetic variation for the next twenty years. The first technique for estimation of differences at the actual genomic DNA level was developed in the 1960's, initially to study the organization of eukaryotic genomes (Britten and Kohne, 1968), and the technique was subsequently applied to questions of molecular evolution and systematics. This technique known as DNA•DNA hybridization is based on the thermodynamic reannealing properties of heterologus single stranded DNA sequences. However, in recent years the technique has lost ground due to theoretical and practical difficulties and has been superseded by more direct DNA sequence-based approaches (Avise, 1994).

Molecular biology was revolutionized by the discovery of bacterial restriction endonucleases, those cleaves duplex DNA at specific oligonucleotide sequences (Meselson and Yuan, 1968). Several hundreds of these enzymes have now been characterized and they proved to be very useful tools for molecular evaluation and population genetics. In conjunction with the technique of Southern hybridization (Southern, 1975), they have provided a very powerful method to assay genetic variation at the DNA level. Polymorphisms at the DNA sequence level can be visualized as changes in the cleavage patterns of DNA fragments, which have been treated with particular restriction enzymes.

These polymorphisms are usually termed Restriction Fragment Length Polymorphisms (RFLPs) (Botstein, et al., 1980).

The advent of the polymerase chain reaction (PCR) by Mullis, et al., (1986) dramatically changed this equation and facilitated direct economically feasible DNA sequence determination from a large number of individual organisms. During the 1980's the attention of genomic scientists was focused on a new class of hyper variable regions of DNA which were first revealed using Southern blot analyses of repetitive elements in the human genome, known as minisatellites (Jeffreys, et al., 1985). The DNA probes originally employed by Jeffreys and his co workers hybridize to conserved core sequences (10-15 bp in length). These were scattered as numerous arrays in the human genome as part of a system of dispersed tandem repeats, and are referred to as Variable Number of Tandem Repeat (VNTR) loci. Each repeat unit is about 16-64 bp in length.

Changes in the repeat copy number arising from high rates of unequal crossing-over during meiosis give rise to a hypervariable pattern of increases and decreases in the lengths of particular arrays. The methodology surrounding this technique (particularly when applied to human forensics) was termed as DNA fingerprinting. Although hyper variable minisatellites arrays were uncovered in many animal and plant taxonomy, with a few notable exceptions, they have never really been successfully applied to population genetic problems. The complexity of the gel profiles, which contain perhaps 20 or more scorable bands, the high mutation rate to new length variants and the difficulty in obtaining consistent results, has generally precluded the wide application of this technique to evolutionary genetics. PCR-based methodologies have been developed which allow amplification of single VNTR arrays from the genome. However, this type of approach has

been superseded by the discovery of a new class of genetic marker, which can be assayed using PCR. This class of genetic marker was developed in the late 1980's and is commonly known as the Microsatellite (Litt and Luty, 1989; Tautz, 1989; Weber, 1993).

Another type of genetic analysis, which has been used for population genetic surveys in recent years, is the Random Amplified Polymorphic DNA (RAPD) approach. This was first described by Williams, et al. (1990) and involves PCR amplification of few random anonymous genomic sequences using short primers (10bp) of arbitrary sequences to interpret polymorphism in genomic DNA. The method typically generates polymorphisms with dominance-recessive characteristics. The technique suffers from many of the drawbacks of DNA fingerprinting, particularly the issue of reproducibility (Hedrick, 1992). So far, the RAPD technique has remained popular within the plant population genetics community but has failed to gain ground in a wider context. There has been a range of other PCR-based techniques developed during the 1990's for analysis of genomic DNA variation. These techniques include methods such as single strand conformation polymorphisms (SSCPs) and amplified fragment length polymorphisms (AFLPs). However, these methods have not been widely applied in evolutionary studies.

In recent years, microsatellite has become choice of markers among the types of genetic markers used for studying population genetic relationships of closely related individuals or populations. The popularity gained by these markers is mainly attributed to their ability of uncovering large number of alleles at a single locus, even with in a population. The use of microsatellites has become a well recognized technique for DNA profile identification. These are often highly polymorphic, non destructive and they are widely dispersed in

eukaryotic genome. This makes it possible to distinguish between closely related individuals at the DNA level.

Microsatellite markers

Microsatellites or simple tandem repeats (STRs) are ubiquitously inherited repeat regions in eukaryotic genomes. The term microsatellite was introduced by Litt and Luty (1989) to characterize the tandemly repeated simple sequence motifs, one to six nucleotides (mono, di, tri, tetra, penta and, hexanucleotides tandem repeats) in length. These elements have been shown to display length variation, which is stably inherited in a Mendelian fashion. The most thoroughly studied type to-date has been the (dC-dA) n• (dG-dT) n repeats which form the majority of dinucleotide repeats in mammalian genomes.

A typical (dC-dA) n dinucleotide microsatellite region

CGTTACGGAT**CACACACACACACACACACACACA**CCTGATCAAGTAT

GCAATGCCTA**GTGTGTGTGTGTGTGTGTGTGTGT**GGACTAGTTCATA

They occur at a frequency of one microsatellite per 10Kb DNA and numbering to atotal of 50,000 - 100,000 in the mammalian genome. Their short length makes them amenable to amplification by PCR and subsequent separation by polyacrylamide gels with the resolution of alleles differing by as little as single base pair. As such they have become the mainstay of international efforts to produce linkage maps of various mammalian genomes and have also proved to be invaluable tools for the identification of genetic lesions associated with inherited human disease. In addition, mutation at certain types of trinucleotide repeat loci has been shown to be responsible for some of the more important human inherited disorders.

Evolution of microsatellites

To "evolve" simply means to change. Microsatellite alleles change (mutate) over time. Microsatellite alleles differ in the number of repeats. For example, one allele may have 7 repeats of a CT motif, and another allele may have 8 repeats. In a population, there may exist many alleles at a single locus, with each allele having a different length. An individual who is homozygous for a locus will have the same number of repeats on both chromosomes, whereas a heterozygous individual will have different numbers of repeats on the two chromosomes. The regions surrounding the microsatellite locus, called the flanking regions, may still have the same sequence. This is important because the flanking regions can therefore be used as PCR primers when amplifying microsatellite loci, and can be conserved across genera or sometimes-even families. Below, the two lines represent the sequences on two homologous chromosomes in a diploid organism.

Homozygous: (Both strands have 7 CT repeats)

CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCT**ATCGGTACTACGTGG

CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCT**ATCGGTACTACGTGG

5' flanking region microsatellite locus 3' flanking region

Heterozygous: (One strand has 7 repeats, and the other has 8 repeats)

...CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCT**ATCGGTACTACGTGG...

...CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCTCT**ATCGGTACTACGTGG..

Microsatellite alleles

Mutation! Interestingly, it is estimated that microsatellites mutate 100 to 10,000 times as fast as base pair substitutions. This makes microsatellites useful for studying evolution over short time spans (hundreds or thousands of years), whereas base pair substitutions are more useful for studying evolution over long time spans (millions of years). There are two hypotheses that explain how microsatellites mutate 1) unequal crossing over in meiosis and 2) strand-slippage replication. Of these, strand-slippage replication appears to be the predominant mode at microsatellites. Strand-slippage is speculated to occur primarily during lagging strand synthesis. For example, it may involve the slippage of the newly synthesized DNA strand upon dissociation of a polymerase complex. This slippage creates a transient bulge, which upon DNA repair would be either removed, or lead to the elongation of the repeat. Alternatively, the formation of a transient bulge in the template strand may lead to the shortening of the repeat (Ellegren, 2000).

Models of microsatellite mutations

Theoretical mutation models have been derived to explain the evolutionary processes of microsatellites from which genetic distances and population differentiation are estimated. Kimura and Crow (1964) gave the Infinite Allele Model (IAM), according to this model a mutation can involve any number of tandem repeats and always results in a new allele state not previously existing in the population. But this model does not confer with the slipped strand impairing mechanism responsible for microsatellite length variation. This mechanism leads to small changes in the repeat numbers and alleles may mutate towards allele states that are already present in the population. In order to explain the discrepancies in the mutational processes, the Step-wise Mutation Model (SMM) was

introduced in the 1970s. The model assumes that the entire sequence of allelic states can be expressed as integers and mutation results in a change in one repeat unit either by insertion or deletion (Kimura and Ohta, 1978). The SMM has been applied to microsatellite allele frequencies by (Valdes, et al., 1993) and the two models have been tested by (Estoup, et al., 1995). In addition to this model, (DiRienzo, et al., 1994) described the Two Phase Model (TPM), where a limited proportion of mutations involve several repeats.

Microsatellites-genetic markers for the future

There are several advantages of utilizing Microsatellites over the other markers, which make them desirable. Microsatellite loci are found in large numbers and are relatively evenly spaced throughout the genome. Further, most of these loci are selectively neutral which makes them compatible with the assumptions of most population genetic theory.These follow a typical Mendelian inheritance, which usually expresses in a co-dominant fashion, and are often multiallelic giving mean heterozygosity of more than 70 per cent. They remain unaffected by the environmental factors, and generally do not have pleiotropic effects on quantitative trait loci (QTL). Microsatellite marker analysis methodologies are DNA based and this brings advantages that are both attractive as well as amenable. For example: (i) the DNA samples can not only be isolated very conveniently from blood of live individuals but can also be isolated from tissues like sperm, hair follicle, and even from archival preparations, (ii) the DNA samples can be stored for longer periods and can readily be exchanged between the laboratories, and (iii) the analysis of DNA can be carried out at an early age or even at the embryonic stage, irrespective of the sex.Technically microsatellites are more desirable loci because they can be analyzed via

the Polymerase Chain Reaction (PCR). The alleles can be unambiguously sized on. Polyacrylamide gels.PCR analysis of small fragments also allows the analysis of degraded samples in which the mean fragment size of the genomic DNA has been severely reduced through environmental insult. Finally, microsatellites have been found to be variable even in populations, which have low levels of allozyme and mitochondrial variation.

Estimation of Genetic Diversity Using Microsatellite Markers

A reliable approach to measure the genetic distance is to estimate the difference in the frequencies of different genetic variants (alleles) at a number of marker loci. Breeds which share the same alleles at similar frequencies are genetically closely related, whereas those having the same alleles at different frequencies (or different allele's altogether) are further apart. Distances or relationships between breeds can then be summarized using a phylogenetic tree or by multidimensional scaling. Variation within a breed can also be estimated by examining the number and frequency of alleles.

Studies on East African breeds showed a considerable amount of within-breed variation based on allele numbers and heterozygosity values, suggesting that the populations studied were fairly out bred. Only the allele frequencies in the Kenya Boran showed a significant deviation from the Hardy-Weinberg equilibrium (Maule, 1990).
The characterization of five bovine dinucleotide repeats $(TG)_n$ were studied by Vaiman *et al.* (1992). During the screening procedure, they found that one-third of the clones had essentially the same flanking sequences.

The bovine and ovine microsatellite sequences were extracted by Moore *et al.* (1992) from the EMBL and GENEBANK databases. When analyzed for numbers of alleles

and degree of heterozygosity in the CSIRO cattle reference families, number of alleles ranged from 1 to 14 with 15.8 to 100% heterozygosity. Six of the 13 bovine microsatellite markers were polymorphic in sheep. Similarly two of the four ovine microsatellites were polymorphic in cattle. These data defined 11 bovine and 8 ovine microsatellite systems, which were associated with known genes and were thus useful for comparative mapping studies.

Brezinsky *et al.* (1993) reported a set of five new bovine microsatellite polymorphisms based on (CA) n repeats. They found that microsatellites are highly polymorphic and thus represent valuable markers for genome mapping.

Goudarzi *et al.* (1993) studied French cattle breeds to develop a valuable system for multiplexing of the markers and reported the product sizes of various microsatellites.

The HEL-5 microsatellite locus was characterized by Kaukinen and Varvio (1993). The numbers of alleles reported were seven with range (147-171 bp). Mean and total heterozygosity were reported to be 0.736 and 0.790, respectively.

Kemp *et al.* (1993) reported a set of six bovine microsatellite polymorphisms based on (CA) n repeats. They were highly polymorphic and thus represented valuable markers for the genome mapping. Four of the six were polymorphic in sheep and two were in goat.

Vaiman *et al.* (1994) studied a set of 99 cattle microsatellites for characterization, syntenymapping and polymorphism in which 136 cattle microsatellite clones were isolated from 10 cosmid and 126 plasmid libraries and sequenced. Oligonucleotide primers (117 pairs) were synthesized, and a PCR product of expected size was obtained for 88 microsatellite sequences (75%). Microsatellite polymorphism was checked on at least 30 unrelated animals of different breeds. Almost all the autosomal and X chromosome

microsatellites displayed polymorphism, with the number of alleles varying between two and 44. They assumed that these microsatellites could be very helpful in the construction of a primary public linkage map of the bovine genome, with an aim of finding markers for economic trait loci in cattle.

The genetic variability within and between cattle breeds was investigated by Ciampolini *et al.*(1995) using polymorphisms of 17 microsatellites in 220 unrelated animals belonging to four Italian beef cattle breeds (Chianina, Marchigiana, Romagnola, and Piemontese). Variations of allelic frequencies were examined to characterize the breeds and their relationships. Wahlund coefficients, PIC values, and Haldane exact test for Hardy-Weinberg proportions were calculated. The results showed that the Hardy-Weinberg equilibrium was not always maintained. Moreover, a method, based on the consideration of a multilocus genotype of each animal, was set up to measure the genetic similarity between animals or within groups of animals. All the results showed that, whereas Chianina occupied an intermediate position and Piemontese was the most distinct of all four breeds, Marchigiana and Romagnola displayed the strongest similarity. The microsatellite multilocus genotype was particularly efficient in evaluating the between- and within-breeds genetic similarities and for subgrouping genetically more homogeneous animals.

A panel of 81 new polymorphic bovine, ovine and caprine microsatellite markers was screened by Kemp and Hishida (1995) to analyze mean polymorphic information content which was determined as 0.66 using 97 markers in 20 cattle. Thirty-nine of the markers were polymorphic in sheep and 32 were polymorphic in goat. This study identified a set of 18 robust markers that were polymorphic in all three species and that

covered 14 bovine chromosomes. These formed a group of markers suited to genetic distance analysis and parentage control in cattle, sheep and goat.

Arranz *et al.* (1996) studied the genetic variation at five microsatellite loci in four breeds of cattle (Avilena-Negra Iberica, Morucha, Sayaguesa and Brown Swiss). Value of observed alleles, genetic diversity, PIC and effective allelic number indicated that the microsatellite showing the lowest variability was ILSTS 005 among the five microsatellites.

Moazami-Goudarzi *et al.* (1997) studied to determine the genetic variation between 10 cattle breeds using 17 microsatellite loci and 13 biochemical markers. Microsatellite loci were amplified in 31- 50 unrelated individuals from 10 cattle breeds: Charolais, Limousin, Breton Black Pied, Parthenais, Montbéliard, Vosgien, Maine-Anjou, Normande, Jersey and Holstein. Neighbor-joining trees were calculated from genetic distance estimates. The robustness of tree topology was obtained by bootstrap resampling of loci. A total of 210 alleles of the 17 microsatellites were detected in the study and average heterozygosities ranged from 0·53 in the Jersey breed to 0·66 in the Parthenais breed.

Gortari *et al.* (1997) reported the evaluation of 1036 bovine microsatellite primer pairs for their suitability as linkage markers in sheep. Approximately 58% (605/1036) of bovine primer pairs amplified a locus in sheep. Sixty-seven per cent (409/605) of amplified loci were detected as polymorphic. Marker heterozygosity, allele number and range of allele sizes were significantly lower in sheep than cattle. However, median fragment size was similar. These data suggested that high-resolution comparative linkage maps between closely related species can be constructed relatively efficiently.

Kappes *et al.* (1997) reported a bovine linkage map constructed with 1236 polymorphic DNA markers and 14 erythrocyte antigens and serum proteins. The map contained 627 new markers and 623 previously linked markers, providing a basis for integrating the four published bovine maps.

A total of 208 alleles were detected across the 18 autosomal microsatellite loci studied. Mean number of alleles ranged from 4.3 in the N'Dama to 7.7 in the Kenya Boran. No significant difference was found between the number of alleles in the two sanga breeds (Danakil and Abigar) and the African zebu. Allele frequencies of 8 out 18 loci in the Kenya Boran deviated from Hardy-Weinberg equilibrium (Okomo, 1997).

The polymorphism of 23 microsatellites in the four main cattle breeds in Belgium (Holstein Friesian, Belgian Blue, Belgian Red Pied and East Flemish) was analyzed by Peelman *et al.* (1998). Heterozygosity, PIC, the effective number of alleles, exclusion probability and the probability of genotypic identity for two random individuals were calculated for all microsatellites and all breeds. The Belgian Blue breed was generally a little less polymorphic in comparison with the other three breeds. Estimates of the genetic distances between these breeds confirmed the widely accepted proposition that the Belgian Blue was the most genetically distinct of these breeds. Exclusion probabilities in parentage control cases are >0·9999 in all four breeds when all 23 microsatellites are used and >0·98 with only the two most polymorphic multiplexes.

The genotype data from 20 microsatellites typed in 253 animals was analyzed by MacHugh *et al.* (1998) to assess the genetic structure of seven European pedigree cattle breeds. Estimation of genetic subdivision using classical drift-based measures shows that the average proportion of genetic variation among breeds varies between 10 and 11% of

the total, depending on the estimator used. The phylogenetic tree displayed a remarkable degree of breed clustering and reflected an extensive underlying kinship structure, particularly for the Swiss Simmental breed and four breeds originating from the British Isles. Condensation of allele frequencies and individual genotypic compositions using principal component analysis was also used to investigate genetic structure among breeds and individual animals. Correct breed designation could be inferred with accuracies approaching 100% using data from a panel of 10 microsatellite loci.

A set of 33 cattle microsatellite primer pairs was tested with the DNA of American bison from a captive population in Belgium by Mommens *et al.* (1998) and evaluated for usefulness in parentage testing. Among the polymorphic markers, the number of alleles ranged from two to nine. Heterozygosity, PIC and probability of exclusion (PE) values were low by comparison with those obtained with the same markers in cattle. An internationally accepted set of nine microsatellites gave cumulative PE values of 0·98 and 0·97.

Slate *et al.* (1998) tested 174 bovine microsatellite primer pairs for use in a primitive breed of sheep and two species of deer. Of 173 markers, 127 (73·4%) gave a product in Soay sheep (Ovis aries) of which 54 (42·5%) were polymorphic. One hundred and twenty-nine of 174 (74·1%) markers gave a product in red deer (Cervus elaphus) of which 72 (55·8%) were polymorphic. In sika deer (*Cervus nippon*) 126 of 171 (73·7%) microsatellite primers gave a product with 47 (37·3%) polymorphic. The study showed a high proportion of bovine microsatellite loci were conserved across artiodactyl species.

The distribution and evolutionary pattern of the conserved microsatellite repeat sequences (CA) n, (TGG) 6 and (GGAT) was reported by Mattapallil and Ali (1999) to

determine the divergence time and phylogenic position. The result showed a high level of heterozygosity among the buffalo, cattle, sheep and goat. Result also suggested that with respect to these repeat loci, the water buffalo genome shared a common ancestry with sheep and goat after the divergence of subfamily Bovinae from the Bovidae.

The applicability of bovine autosomal microsatellite markers for population genetic studies was reported by Hooft *et al.* (1999) in African buffalo. A total of 168 microsatellite markers were tested for PCR amplification on a test panel of seven African buffalo. Amplification was observed for 139 markers (83%), and 101 markers were studied further with 91 (90%) being polymorphic. The mean number of alleles per marker was 5·0 ±0·2 and the mean heterozygosity per marker was 0·61 ±0·03. Considering the overall high level of polymorphism, it was concluded that most bovine microsatellite markers are applicable in African buffalo.

Martín-Burriel *et al.* (1999) studied six native Spanish cattle breeds using 30 microsatellite markers. Allele frequencies were calculated and used for the characterization of the breeds and the study of their genetic relationships. Different genetic distance measures were calculated and used for dendogram construction. The closest populations were those representing Asturian breeds, the most divergent being Menorquina and Fighting Bull. The latter also showed the lowest diversity values (mean number of alleles per locus and heterozygosity). Genetic distances obtained between the other populations under analysis were similar to those reported for different European cattle breeds.

Genetic diversity in eight Swiss goat breeds (Creole breed from the Caribbean and samples of Ibex and Bezoar goat) was estimated by Saitbekova *et al.* (1999) using PCR amplification of 20 bovine microsatellites on 20-40 unrelated animals per breed. A total of

352 animals were tested. The average heterozygosity within population was higher in domestic goat (0·51-0·58) than in Ibex (0·17) and Bezoar goat (0·19). Twenty-seven per cent of the genetic diversity in the total population could be attributed to differences between the populations. However, with the exclusion of Ibex from the total population, this proportion dropped to 17%. Principal component analysis showed that all Swiss goat breeds were closely related, whereas the Creole breed, Ibex and Bezoar goat were clearly distinct from all eight Swiss breeds.

The phylogenetic relationships in the tribe Bovini was derived by Ritz *et al.* (2000) from polymorphisms at 20 bovine microsatellite loci. This study comprised 17 representative populations: eight *Bos taurus*, two Bos indicus, one Poëphagus, one Bibos, one Bison, three Bubalus and one Syncerus. Phylogenetic analyses using 2 chord (DC) distances revealed substantial divergence among species. Neighbor-joining trees with both distance measures showed only minor differences. Bos Taurus and Bos indicus grouped first, followed by Bos frontalis (Mithun) and Bos grunniens (Yak), Bison bison branched off next and Bubalus bubalis and Syncerus caffer emerged as the two most divergent species from the Bos clade.

The microsatellites HEL-5, HEL-9, INRA-063, and BM2113 were used to analyze genetic similarities and differences of geographically isolated Criollo cattle herds in Mexico by Russell *et al.* (2000). Allele frequencies and genotypic deviations from Hardy Weinberg equilibrium were tested using the GENEPOP program. Eleven alleles were generated at HEL-5 for the populations sampled (149 to 169 bp). Amplification with HEL-9 produced 12 alleles (145, 149 to 169 bp) and showed common high frequency alleles at 149, 157, and 159 bp for animals from all regions. For INRA-063 there were five alleles

with 182 and 184 bp in low frequency. For BM2113 there were 10 alleles in the Criollo cattle (125 to 143 bp), with an equal distribution of frequencies for all alleles. Cattle from the Temoris region were genetically most distant from Criollo cattle of the other five regions.

Hanslik *et al.* (2000) used genotypic data from 39 microsatellite loci typed in 211 animals to assess the genetic differentiation between European and American Holstein Friesian cattle populations. Gene diversities were similar in all five Holstein Friesian populations surveyed, ranging from 0.43 to 0.48. A tree of individuals based on the proportion of shared alleles indicated a clear distinction between European and American Holstein Friesian populations. Similarly, genetic differentiation between populations, as measured by FST, was highly significant.

The potential of microsatellites as a cattle breed marker was analyzed by Benyamin *et al.* (2000) in four cattle breeds namely Bali (six heads), Simmental, Limousin, and Brangus (each breed consists of three heads) using eight primers. The primer sequences were based on the information from International Cattle Diversity Projects. The result showed that primer HEL-9 could be used to find specific allele in Bali cattle. Furthermore, primer ILSTS-005 could be used to find specific allele in Brangus cattle. The total numbers of alleles found were one to three with the average of 1.78 for Bali, 1.56 for Simmental and 1.22 for Brangus and Limousin.

Fifteen bovine microsatellites were evaluated by Schnabel *et al.* (2000) for use in parentage testing in 725 bison from 14 public populations. The number of alleles per locus ranged from five to 16 in bison and from five to 13 in cattle. On average, expected heterozygosity, PIC and probability of exclusion values were slightly lower in bison than

in cattle. A core set of 12 loci was further refined to produce a set of multiplexed markers suitable for routine parentage testing.

Edwards *et al.* (2000a) described the assessment of a panel of four Y-specific microsatellite markers for polymorphism in a range of cattle and related species. Three of the microsatellite loci (INRA124, INRA189 and BM861) displayed putative taurine- and zebu-specific alleles which can be useful indicators of male-mediated gene flow in hybrid populations.

The genetic relationships between the endangered German Pustertaler-Sprinzen cattle breed and the Pinzgauer, Vosges and Simmental breeds - was estimated by Edwards *et al.* (2000b). Within-breed diversity of the four breeds was also assessed. Twenty microsatellite markers were amplified in 27-50 unrelated individuals from populations of each breed. Within-breed variation was estimated from average heterozygosity values and mean number of alleles. Breed relationships were evaluated by genetic distance and a neighbour-joining tree was calculated from these estimates. From both the average heterozygosity values and mean number of alleles calculated, the Pustertaler breed appeared to be genetically at par with the other breeds analyzed. The breed tree showed an 85% support for the Pustertaler-Pinzgauer grouping.

Genetic variation, genetic distance and time of divergence within and among Jamnapari, Barbari and Sirohi Indian breeds of goat was described by Ganai and Yadav (2001) using 16 heterologous cattle microsatellite markers. The mean number of alleles and mean allele size per microsatellite marker in goats were 5.37 ±0.78 and 143.9 ±33.75 bp respectively while the average values of heterozygosity and PIC values were 0.54 ±0.2 and 0.48 ±0.20, respectively. Five out of the eight genetic distance methods were highly

correlated, revealing a closer relationship between Jamnapari and Barbari goats. A phylogenetic tree constructed from inter-individual distances revealed that the individuals clustered according to the breed to which they belonged.

Genetic diversity among Canadienne, Brown Swiss, Holstein, and Jersey cattle was estimated by Hansen *et al.* (2002) from relationships determined by genotyping 20 distantly related animals in each breed for 15 microsatellites located on separate chromosomes. The Canadienne, Holstein, and Jersey cattle had an average of six alleles per loci compared with five alleles for Brown Swiss. Furthermore, a number of potentially breed-specific alleles were identified. The allele size variance among breeds was similar, but varied considerably among loci. All of the loci studied were equally heterozygous, as in Brown Swiss, Canadienne, and Holstein cattle (0.68-0.69) whereas Jersey cattle showed lower heterozygosity (0.59). The within-breed estimates of genetic distance were greater than zero and found to be significant. The genetic distance between Canadienne (0.156) and Holstein (0.156), Brown Swiss (0.243), and Jersey (0.235) was negligible, suggesting the close relationship. Brown Swiss and Holstein (0.211) cattle also demonstrated a close relationship. In contrast, the Jersey breed was genetically distant from the Brown Swiss (0.427) and Holstein cattle (0.320).

The genetic relationships and genetic variability among six native French cattle breeds (Abondance, Tarentaise, Villard de Lans, Montbeliarde, Limousin, and Charolais) and one foreign breed (Holstein) were studied by Maudet *et al.* (2002) using 23 microsatellite markers. These breeds were also compared with four Swiss breeds genotyped in a previously published study. Interestingly, the French alpine breeds have smaller population sizes but showed higher genetic variability than the larger Holstein

breed. Neighbor-joining trees and principal components analysis showed that alpine breeds tend to cluster together. Abondance and Tarentaise breeds were closely related, whereas the Holstein was highly differentiated from all breeds analyzed.

One hundred and eight microsatellite primer pairs, originally identified from cattle, were evaluated by Navani *et al.* (2002) for their applicability in buffalo. Eighty-one primer pairs (75%) amplified discrete products, and of these, 61 pairs (56%) gave polymorphic band patterns on a panel of 25 buffaloes. The mean number of alleles per polymorphic marker was 4.50 ±0.20, and the mean heterozygosity per polymorphic marker was 0.66 ±0.02.

Genetic variability and population structure of north-east Asian cattle was reported by Kim *et al.* (2002) using 13 microsatellite loci for a total of 200 individuals including Korean, Chinese, Japanese Black and European Holstein cattle. Observed and expected heterozygosity, two estimators (FST and GST) of gene differentiation, and Nei's DA distance were evaluated. Based on expected mean heterozygosity, the lowest genetic diversity was exhibited in Japanese Black cattle (0.471), and the highest in Chinese cattle (0.744). Korean cattle revealed a relatively high degree of genetic diversity (0.728). Average proportion of genetic variation because of interpopulation subdivision among north-east Asian cattle varied between 10.9 and 9.9 %, depending on the estimator used. N-J tree based on Nei's DA genetic distance showed that Korean and Chinese cattle were closely related; whereas Japanese Black cattle were clearly distinct from the other two populations, forming a north-east Asian out group.

Machado *et al.* (2003) studied genetic diversity of four cattle breeds using microsatellite markers. Four cattle breeds (Gyr, Nellore, Guzerat and Holstein) with 18

samples each were analyzed. Holstein breed was the most distinct from the other breeds. The closest genetic distance was 0.25 between Guzerat and Nellore. A total of 64 alleles in all four breeds were detected using nine microsatellite primers. The average number of alleles per locus was 7.11 ±3.21. The most informative locus was BMS1237 with 53% of observed heterozygosity and the least informative locus was BMS3004 with 12% only. The average heterozygosity detected for the nine loci were 35% and the expected value for Hardy-Weinberg equilibrium was 53%. This low heterozygosity suggested a high endogamy level among the animals sampled within each breed.

Arora *et al.* (2004), using 22 heterologus bovine microsatellite markers have analyzed and Tarai buffalo populations. They found average number of alleles across all loci to be 4.7 with low genetic distance existing between two populations. Their data indicates suitability of use of bovine microsatellite markers for analysis of buffalo genetic diversity.

Metta *et al.* (2004) By using 5 di- and 5 tri-nucleotide repeat loci, 17 Ongole and 13 Deoni unrelated individuals were studied. Of the ten loci, eight revealed polymorphism in both the breeds. The di-nucleotide repeat loci were found to be more polymorphic (100%) than tri-nucleotide repeat loci (60%). A total of 39 polymorphic alleles were obtained at 4.5 alleles per locus in Ongole and 4.1 in Deoni. The average expected heterozygosity was 0.46 (±0.1) and 0.50 (±0.1) in Ongole and Deoni breeds, respectively. The PIC values of the polymorphic loci ranged from 0.15 to 0.79 in Ongole and 0.13 to 0.80 in Deoni breeds. Six Ongole specific and three Deoni specific alleles were identified. The two breeds showed a moderate genetic relationship between themselves with a *F*ST value of 0.117 ($P = 0.01$).

Genotype data from 30 microsatellites were used by Mateus *et al.* (2004) to assess genetic diversity and relationships among 10 native Portuguese cattle breeds, American Charolais and the Brazilian Caracu. Hardy–Weinberg equilibrium was observed for all loci/population combinations except for five loci in Brava de Lide and one locus in Alentejana that exhibited heterozygote deficiency. Estimates of average observed and expected heterozygosities, total number of alleles (TNA) per breed and mean number of alleles (MNA) per locus/population were obtained. A total of 390 alleles were detected. TNA among Iberian cattle ranged from 170 to 237 and MNA ranged from 5.67 to 8.07. The highest observed heterozygosities were found in the Caracu, Maronesa, Garvonesa and Arouquesa and the lowest in Brava de Lide and Mirandesa. Estimation of population subdivision using Wright's FST index showed that the average proportion of genetic variation explained by breed differences was 9%. Neighbour- joining phylogenetic trees based on DA distances showed that the genetic relationships of present-day Portuguese native breeds are consistent with historical origins in the Brown Concave (Arouquesa, Mirandesa, Marinhoa) and Red Convex (Mertolenga, Alentejana, Garvonesa, Minhota) evolutionary groups. The Iberian Black Orthoide group, represented by Brava de Lide and Maronesa, and the Barrosa breed appeared to be more closely related to the Brown Concave group but may represent a separate lineage. The Caracu breed was not found to be closely associated with any of the native Portuguese breeds.

Freeman *et al.* (2005) studied two-step regression approach in cattle using three different sets of microsatellite data, to combine population genetics estimates of diversity and admixture. This regression based method is independent of the loci genotyped but requires common breeds in the data sets. They have shown that combining microsatellite

data sets can provide new insights on the origin and geographical distribution of genetic diversity and admixture in cattle, which will facilitate global management of this livestock species.

Work carried out by Sodhi *et al.* (2005) revealed that the two *Bos indicus* breeds sharing the common breeding tracts to be genetically differentiated enough as separate breeds by using microsatellite polymorphism.

A. E. O. Malau-aduli *et al.* (2005) studied a confirmatory scan for the regions of bovine chromosome 1 segregating the quantitative trait loci (QTL) influencing birth weight, weaning weight, yearling weight, and pre weaning and post weaning average daily gains and was performed by genotyping half-sib progeny of four Japanese Black sires using microsatellite DNA markers. Data were analyzed by generating an F -statistic every 1 cM on a linkage map by the regression of phenotype on the probabilities of inheriting an allele from the sire after adjusting for the fixed effects of sire, sex, parity and season of birth as well as age as a covariate. Permutation tests at chromosome-wide significance thresholds were carried out over 10,000 iterations. A significant QTL for birth weight at 114 cM was detected in the sire 2 family. This identification of a birth weight QTL in Japanese Black cattle may be useful for the implementation of marker-assisted selection.

Kumar *et al.* (2006), studied on Genetic variation and relationships among eight Indian riverine buffalo breeds and the neighbor-joining tree constructed from chord distances, multidimensional scaling (MDS) display of F(st) values and Bayesian clustering approach consistently identified the Toda, Jaffarabadi, and Pandharpuri breeds as one lineage each, and the Bhadawari, Nagpuri, Surati, Mehsana and Murrah breeds as

admixture. The results of these studies will be useful for development of rational breeding and conservation strategies for Indian buffaloes.

Comparative analysis of genetic structures of five cattle breeds has been carried out using polymorphism of two different types of molecular-genetic markers--20 structural genes and 128 DNA loci (the DNA fragments flanked by inverted microsatellite loci repetitions--ISSR-PCR method) by Gorodnaia and Glazko_(2006). Part of polymorphic loci and the average value of the polymorphic information contents (PIC) of ISSR-PCR markers have appeared much higher in commercial breeds with high effective number in comparison with Ukrainian breeds.

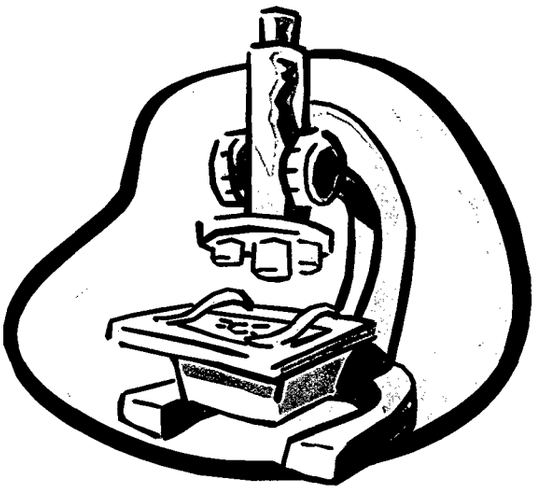
Li *et al.* (2006) studied, single nucleotide polymorphisms (SNPs) in growth hormone 1 (GH1), insulin-like growth factor 1 (IGF1) and leptin (LEP), all candidates for production traits in cattle, were characterized in North Eurasian cattle breeds. Allele frequencies of IGF1 exhibited significant ($P < 0.05$) deviation from neutral expectation and therefore, might be associated with divergence in North Eurasian cattle because of genetic selection. Allele frequencies and lower heterozygosity of LEP may indicate a recent introduction of an alternative allele in this geographic region. Locus FST estimates were highest for IGF1 (0.151, r ¼ 0.042) and lowest for GH (0.062, r ¼ 0.020). They suggest a slightly higher population differentiation across the candidate genes (FST ¼ 0.108) than across microsatellites (FST ¼ 0.095), possibly because of selection and stochastic effects. Twenty-seven domesticated yellow cattle breeds of China and three introduced cattle breeds were analyzed by means of 30 microsatellite markers by Zhang et al. (2007) to determine the level of genetic variation within and among populations as well as the population structure. In all, 480 microsatellite alleles were observed across the 30 breeds

with the mean number of alleles per locus of 9.093 for native breeds and 6.885 for the three introduced breeds.

MacNeil *et al.* (2007) assessed genetic differentiation of cattle isolated on Chirikof Island from several breeds commonly used for commercial production in North America including breeds popularly believed to have contributed to the Chirikof Island population. A set of 34 microsatellite loci was used to genotype Angus, Charolais, Hereford, Highland, Limousin, Red Angus, Salers, Shorthorn, Simmental, Tarentaise and Texas Longhorn cattle sampled from North America and the Chirikof Island population. Resulting FST statistics for these loci ranged from 0.06 to 0.22 and on average, 14% of total genetic variation was between breeds. Population structure was modeled as a bifurcating tree or genetic network, Chirikof Island cattle appeared to be unique and strongly differentiated relative to the other breeds that were sampled. Bayesian clustering for multiple-locus assignment to genetic groups indicated low levels of admixture in the Chirikof Island population.

Brenneman *et al.* (2007) studied the genetic diversity among breeds under evaluation for tropical adaptability traits that affect the performance of beef cattle at the Sub tropical Agricultural Research Station (STARS) near Brooksville, FL, USA. Twenty-six microsatellite loci were used to estimate parameters of genetic diversity among the breeds. American Brahman, Angus, Senepol and Romosinuano; the latter was comprised of two distinct bloodlines (Costa Rican and Venezuelan). Genotypes of 47 animals from each of these STARS herds were analyzed for genetic diversity and genetic distance. Gene diversity ranged between 0.64 (Costa Rican line of Romosinuano) and 0.75 (American Brahman).

Chapter III



Material and Methods

CHAPTER III

MATERIAL AND METHODS

The indigenous cattle in Himachal Pradesh are described as non-descript type because of absence of any scientific study. These cattle populations in the lower area got contaminated with Sahiwal and Hariana breeds. However in the mid and higher hills they are some what homogenous. These cattle are characterized genetically using microsatellite markers. Microsatellite derived Genetic characterization of Hill cattle involves the following steps:

- Selection of experimental animals.
- Collection of blood samples.
- Isolation of genomic DNA from Hill Cattle blood sample.
- Quantitation and quality analysis of the isolated DNA.
- PCR amplification of genomic DNA at 14 microsatellite loci.
- Checking PCR product on Agarose gel electrophoresis.
- Resolution/separation of PCR amplified product on denaturing Urea PAGE.
- Allele visualization using Silver staining and scoring the alleles.
- Analysis of microsatellite data.

Animals and Collection of blood samples

Material for the present study comprised of blood samples collected from randomly selected unrelated animals from Shimla, Kinnaur, Solan, Sirmaur districts of Himachal Pradesh. Blood samples were acquired from 50 randomly selected unrelated animals from the breeding tracts as per the guidelines of MoDAD (Measurement of Domestic Animal Diversity) programme (FAO, 1995). To ensure unrelated ness of animals due to nonexistence of pedigreed account under field situations, animals were selected from

distinct villages after interviewing the owners' in detail. Blood samples (8-10ml) were collected in vacutainers containing ethylene diamine tetra acetic acid (EDTA) as anticoagulant. Once the required number of blood samples was collected, they were brought to the laboratory in ice and immediately shifted to -20°c for the further storage.

Extraction of genomic DNA

Genomic DNA is the basic material for the molecular genetic characterization of livestock species. Success of such studies largely depends on the quality of DNA isolated. Quality of genomic DNA means that it should be of high molecular weight (>100-150 kb) and free from any impurities like RNA, protein, organic solvent or salt. Any biological material such as blood, semen, hair, follicles, tissue can be the source of contamination in genomic DNA. The most preferred is the peripheral blood from the live animal as good quality DNA can most easily be obtained from this source. Generally, isolation of genomic DNA from mammalian samples involves four major steps:

1. Lysis of cells using a detergent such as SDS.
2. Digestion of proteins, released from cell lysis with Proteinase K.
3. Extraction of DNA with phenol. and,
4. Precipitation of DNA with alcohol.

Requirements

(a) Materials:

- Crushed ice.
- Plastic (Oakridge) tubes (autoclaved) and its stand.
- Glass culture tubes (autoclaved).
- Weighing balance.

- Micropipettes.
- Yellow, blue and white tips.
- Marker pen.
- Gloves.

(b) Stock solution:

- NH_4CI (1 M).
- $KHC0_3$ (1 M).
- EDTA (0.5 M).
- NaCI (1 M).
- Tris-Cl (1 M), pH 8.0.
- Proteinase K (20 mg/ml stock).
- SDS (20%).
- Phenol (Equilibrated with Tris pH 7-8).
- Phenol: Chloroform: Isoamyl alcohol (IAA) (25:24: 1).
- Chloroform: IAA (24: 1).
- Sodium Acetate (3 M, pH 5.2).
- Ethanol.
- TE Buffer (pH 8.0).
- 70% Ethanol.

Protocol

(a) Lysis cycle:

1. Frozen Blood samples (8-10ml) were thawed in water bath at room temp. (Approx. 25°C to 30°C) and transferred to the Oakridge tubes.

2. To the whole blood added 2 volumes of chilled lysis buffer (1 X) (Annexure I).
3. Mixed thoroughly by vortexing and kept on ice for 8-10 minutes to allow the lysis of RBC.
4. Centrifuged at 10,000 rpm at 4°C for 10 min.
5. The red colored supernatant containing the lysed RBC was discarded and the above steps were repeated three times until the pellet became white in color.

(b) Proteinase-K digestion:

1. 5 ml of extraction buffer was added (Annexure I) and mixed well.
2. SDS (final Conc. 0.5%) and Proteinase -K (final Conc. 100µg /ml) were added.
3. Solution was mixed gently and tubes were incubated at 56°C overnight.

(c) Phenol extraction:

1. After incubation equal volume of Tris saturated phenol (pH 8.0) was added.
2. Mixture was mixed gently by repeated inversions of the tubes for 5-10 min.
3. Content was centrifuged at 12,000 rpm at 25°C for 10 minutes.
4. Upper aqueous phase (containing DNA) was transferred into the fresh Oakridge tube.
5. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added.
6. Centrifugation was followed as in previous steps.
7. Upper phase was carefully collected in the fresh Oakridge tube.
8. Equal volume of chloroform: isoamylalcohol (24: 1) was added.
9. Content was mixed well for 5-10 minutes and centrifuged as in previous step.
10. Upper phase was collected in the fresh borosil glass tube.

(d) DNA precipitation and washing:

1. To this aqueous phase, $1/10^{th}$ volume of 3M sodium acetate and double volume of chilled ethanol was added.
2. Content was mixed slightly by inversions and precipitated DNA was obtained (visible as white stringy strands).
3. DNA was spooled out and transferred to an eppendorf tube.
4. The precipitated DNA was washed twice with 70% ethanol and air dried at room temp.
5. DNA pellet was dissolved in TE buffer (pH 8.0) and kept at 65°c for 20 min.
6. DNA was stored at -20°c for future use.

Quantitation and Quality analysis of the isolated DNA

Two methods are widely used to measure the amount of nucleic acid in a preparation. If the sample is pure (without significant amount of contaminants such as proteins, phenol, agarose or other nucleic acids), spectrophotometric measurement of the amount of ultra violet irradiation absorbed by the bases is simple and accurate. If the amount of DNA or RNA is very small or if the sample contains significant quantities of impurities, the amount of nucleic acid can be estimated from comparison of the intensity of fluorescence emitted by ethidium bromide of unknown and standard DNA samples by running the mini gel.

Material required

(a) Equipments:

Spectrophotometer.

Quartz cuvette.

Microwave oven/heater.

UV transilluminator.

Gel documentation unit.

(b) Solutions:

- Agarose (0.6%).
- Ethidium bromide (10 mg/ml).
- 50 x TAE (242 gm tris base, 57% Glacial acetic acid, 100 ml 0.5 M EDTA (pH-8)

  Loading buffer (0.25% bromophenol blue, 40% glycerol in water).

Spectrophotometric determination

For quantitating the amount of DNA, optical density (O.D.) was measured at wave lengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of one corresponds to approximately 50μg/ml for double stranded oligonucleotides. The standard conversion of spectrophotometric readings i.e. ODS to nucleic acid concentration are given below:-

Spectrophotometric conversions

1A°260 Unit of double stranded DNA = 50μg/ml

1A°260 Unit of single stranded DNA = 33μ g/ml

1A°260 Unit of single stranded RNA = 40μg/ml

The ratio between the readings at 260 nm and 280 nm (OD260/OD 280) provides an estimation of purity of the nucleic acid. Pure preparations of DNA have an OD 260/OD 280 values ranging from 1.8 and 2.0, respectively. If there is contamination with protein or phenol, the OD260/OD280 will be significantly less than the values given above; the accurate quantification of the amount of nucleic acid will not be possible.

Procedure

(a) DNA (50µl) was diluted in 1,950µl of distilled water and mixed well.

(b) The sample cell was filled with TE, making sure not to leave bubbles, which detract the light beam and introduce errors.

(c) Similarly, sample cell was filled with diluted DNA. Cross contaminating sample and reference during pipetting must be avoided.

(d) The spectrophotometer was adjusted sequentially to measure the OD at 260 and 280 nm.

(e) The concentration of DNA sample was estimated using the following formula:

$$\text{Quantity of DNA in } \mu g/\mu l = \frac{\text{OD (260 nm) x dilution factor x 50}\mu g/ml}{1000}$$

Determination by Agarose gel electrophoresis

Frozen stock of Hill Cattle genomic DNA samples were diluted 1:10 and their quantity and quality was assessed by running them in 0.6 % agarose gel using 1X TAE buffer. After checking, the selected samples were diluted to a concentration of 50-100ng/µl for further analysis. Using agarose gel electrophoresis we can separate and analyze charged bio molecules like DNA, RNA and proteins. The use of gels such as starch, polyacrylamide and agarose as supporting media provides enhanced resolution particularly for nucleic acids and proteins. The location of DNA within the gel can be easily detected by staining with ethidium bromide and very small quantities of DNA (1-10ng) can be detected by this method. Agarose is used for the detection of large and double stranded DNA. Naturally occurring DNA molecules usually have very high molecular weight; they cannot penetrate into the pores of polyacrylamide gel. These gels can be poured in a

variety of shapes, size and porosity and can be run in different configuration. The choice for the shape, size and porosity of the gel depends upon the size of the DNA fragment to be separated. Small sized DNA fragment pass through smaller pores in the gel and the larger fragments through larger pores. Larger sized DNA pores face more frictional force as they move through the gel and so have lower mobility as compared to the smaller sized DNA fragments that experienced less frictional force. A mixture of DNA molecules therefore separates into distinct bands during electrophoresis.

The agarose melts at about 90^0 C and solidify at about 40^0 C. Agarose gels can be casted in casting trays of different size. Placing combs of different size in the casting tray, before pouring of gel, forms the wells. DNA samples are loaded into the wells in the gels. Diluted genomic DNA samples (1:10 of stock) were loaded on to 0.6% agarose gel for checking quality and quantity of DNA samples.

The protocol of this experiment involves:

Dissolving agarose

Two types of running buffers are commonly used for DNA gel electrophoresis in agarose: Tris-acetate EDTA (TAE) and Tris-borate EDTA (TBE). It is convenient to prepare the running buffer as 50x stock solutions and dilute as necessary immediately before the electrophoresis. We used TAE buffer for this study.

1. 2ml of 50x TAE was diluted to 100ml in a 250ml Erlenmeyer flask.
2. The required amount of agarose powder was weighed and added to the buffer solution.
3. The slurry was heated in microwave oven until agarose dissolved. In microwave oven slurry was heated for 3min in 1min increments, the solution gently swirled

between heating cycles to release trapped air. To this re-suspend any agarose particles caught on the side of the flask.

4. Agarose solution was cooled until it reached a temperature of approx. 50-55^{0}C (slightly hot to the hand). Flask was occasionally swirled, to keep the contents at uniform temperature. These prevent the agarose from gelling at the bottom of the flask and also prevent the formation of bubbles. Ethidium bromide solution was taken from a stock solution of 10mg/ml in water and a final concentration of 0.5 µg/ml was made. Agarose was cooled to 50^{0}C and ethidium bromide was added to it. The flask was gently swirled to disperse the dye.

Gel casting

1. The outside edges of the open ends of the plastic tray supplied with the electrophoresis apparatus was carefully taped using a very sticky tape, e.g. strapping tape or electrical tape.
2. Gel tray (mold) was placed on horizontal surface.
3. The comb was placed at the desired position on the gel tray (0.5-1.0mm) above the plate so that the complete well is formed/ the teeth should not touch the gel bed.
4. It was checked by pushing a folded paper towel beneath the teeth. Two or even three combs can be placed parallel to each other for analysis.
5. Warm agarose solution was poured into the mold. Any leakage at the taped edges was analyzed.
6. Bubbles formed, in gel were removed with the help of pointed end of pipette tips before the gel had set.

7. After the gel was completely set (30-40 minutes at room temperature), comb was removed by wriggling back and forth gently and then lifting up.

Loading the gel

The gel was mounted in the electrophoresis tank top of the gel was flooded with fresh running buffer (1xTAE) to cover the gel to a depth of about 1-2mm. Samples of DNA were mixed with the desired gel-loading buffer with the help of a pipette. DNA samples were carefully loaded under the buffer in the required number of wells.

The wells of gel cast from the basic recipe typically have a volume of 25-35µl and contain a 10-15µl DNA sample. A piece of dark paper was placed beneath the gel tank to make the wells more visible.

The gel loading buffers serves the following purposes:

- They increase the density of the samples, ensuring that the DNA drops evenly into the wells.
- They add color to the samples, thereby simplifying the loading process.
- They contain dyes that in an electric field move towards the anode at predictable rates.

Bromophenol blue migrates through agarose gels approximately 2 fold faster than xylene cyanol FF independent of agarose concentration. Bromophenol blue migrates through the agarose gel run in 0.5x TBE at approximately the same rate as linear double stranded DNA 300bp in length, whereas xylene cyanol FF migrates at approximately the same rate as linear-double stranded DNA 4kb in length, the relationships are not affected by the concentration of agarose in the gel over the range of 0.5% to 1.4% the maximum

amount of DNA that can be applied to a slot depends on the number of fragments in the samples and their sizes.

The lid of the gel tank was closed after loading the DNA samples and the power is switched on so that the DNA migrates towards anode (red lead). Apply a voltage of 50 volts for 60 minutes at room temperature. If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis) and, within a few minutes, the Bromophenol blue should migrate from the well into the body of the gel. On completion of electrophoresis, the gel was visualized under UV transilluminator (bio-rad).

PCR amplification of microsatellite markers

Polymerase chain reaction is an in-vitro method for enzymatically synthesizing defined sequence of DNA. The reaction uses two oligonucleotide primers that hybridize to opposite strands and flank the target DNA sequence to be amplified. A heat stable DNA polymerase such as Taq polymerase catalyses the elongation of the primers. Template denaturation, primer annealing, and extension of the annealed primers by the polymerase results in exponential accumulation of a specific DNA fragment. The primer extension products synthesized in a given cycle can serve as the template in the next cycle.

Materials

PCR machine, PCR tubes (autoclaved), micropipette, ice flakes and purified genomic DNA.

Master Mix

1. 10x Taq polymerase buffer.
2. dNTPs.

3. Primers (Reverse & Forward).
4. Taq DNA polymerase.
5. PCR grade water (autoclaved dd water).

Primers

The FAO recommended 14; primers were used for cattle genetic diversity analysis and have been selected as part of 25 hetrologous primers identified in laboratory for cattle genetic diversity analysis.

Protocol

Using fresh clean & autoclaved tips, the following reactants were added to an absolutely clean eppendorf tube (1.5ml) placed on ice.

Genomic DNA (50-100 ng)	-	2.00 µl
Taq Polymerase (3 unit/ µl)	-	0.60 µl
Primer (forward 5 pmol)	-	1.00 µl
Primer (reversed 5 pmol)	-	1.00 µl
dNTPs (10 mM)	-	0.5 µl
10 x buffer	-	2.5 µl
PCR water	-	adjusted by calculation
Total	-	25 µl

After adding all the ingredients the cocktail was mixed gently. 23 µl of cocktail was briefly centrifuged and immediately dispensed into PCR tubes containing 2.0 µl DNA (50-

100ng). Finally tubes were placed in the thermocycler. The PCR protocol was set same for all the primers except annealing temperature.

PCR Protocol

1.	Initial Denaturation	Step - 1	94°C for 10 min.
2.	Denaturation	Step - 2	94°C for 15 sec.
3.	Annealing	Step - 3	55-58°C for 20 second
4.	Extension	Step - 4	72° C for 20 sec
5.	No. of cycles	Step - 5	39 times
6.	Final Extension	Step - 6	72°C for 10 min
7.	Cooling	Step - 7	4°C for ever

Components of the reaction mixture

1. **Template DNA:** Usually the amount of template DNA is in the range of 0.01- 1 ng for plasmid or phage DNA and 0.1- 1 µg for genomic DNA, for a total reaction mixture of 50µl. Higher amounts of template DNA usually increase the yield of non specific PCR products but if the fidelity of synthesis is crucial, maximal allowable template DNA quantities together with a limited number of PCR cycles should be used to increase the percentage of "correct" PCR products. Nearly all routine methods are suitable for template DNA purification. Although even trace amounts of agents used in DNA purification procedures (phenol, EDTA, proteinase K etc.) strongly inhibit Taq DNA polymerase. Ethanol precipitation of DNA and repetitive treatment of DNA pellets with 70% ethanol is usually effective in removing traces of contaminants from the DNA samples.

2. **Primers: Guidelines for primers selection:**

- PCR primers are usually 15-30 nucleotides in length. Longer primers provide higher specificity.
- The GC content should be 40-60%. The G and C nucleotides should be distributed uniformly throughout of the primer. More than three G or C nucleotides at 3- end of the primer should be avoided, as non specific priming may occur.
- The primers should not be self - complementary or complementary to any other primer in the reaction mixture, in order to avoid primer-dimer and hairpin formation.
- The melting temperature of flanking primers should not differ by more than 5°C. So the GC content and length must be chosen accordingly.
- All possible sites of complementary between primers and the templates DNA should be noted.
- If primers are degenerated, at least 3 conservative nucleotides must be located at the primer 3-end.

3. **Estimation of the melting and annealing temperatures of primer:**

If the primers are shorter than 25 nucleotides, the approx. melting temperature (Tm) is calculated using the following:

$Tm = 4 (G + C) + 2 (A + T)$

G, C, A, T – are the number of respective nucleotides in the primers

Annealing temperature should be approximately 5°C lower than the melting temperature. If the primer is longer than 25 nucleotides, the melting temperature

should be calculated using specialized computer programs where the interactions of adjacent bases, the influences of salt concentration etc. are evaluated.

4. **$MgCl_2$ concentration:**

Since Mg^{++} ions form complex with dNTPs, primers and DNA templates, the optimal concentration of $MgCl_2$ has to be selected for each experiment. To few Mg^{++} ions results in a low yield of PCR product, and too many increase the yield of non specific products and promote misincorporation. Lower Mg^{++} concentrations are desirable when fidelity of DNA synthesis is critical. The recommended range of $MgCl_2$ concentration is 1-4 mM, under the standard reaction condition specified. In the experiment, at a final dNTP concentration of O.2mM, $MgCl_2$ concentration ranges 1.5+0.25mM (in Taq buffer with KCI) and of 2.0+0.5mM (in Taq buffer with $(NH_4)2S0_4$)) are suitable in most cases. If the DNA samples contain EDTA or other chelaters, the $MgCl_2$ concentration in the reaction mixture should be raised proportionally.

5. **dNTPs:**

It is very important to have equal concentration of each dNTP (dATP, dCTP, dGTP & dTTP). The concentration of each dNTPs in the reaction mixture is usually 200mM. As in accuracy in the concentration of even a single dNTP dramatically increase the miss incorporation level. When maximum fidelity of the PCR process is crucial, the final dNTPs concentration should be 1050 µl, since the fidelity of DNA synthesis is maximal in this concentration range. In addition, the concentration of $MgCl_2$ should be selected empirically, starting from 0.1mM and increasing in 0.lmM steps, until a sufficient yield of PCR product is obtained.

6. **Taq DNA polymerase:**

   Usually 1-1.5µl of taq DNA polymerase is used in 50 µl of reaction mix. Higher Taq DNA polymerase concentration may cause synthesis of nonspecific products. However, if inhibitors are present in the reaction mix (e.g. if the template DNA used is not highly purified), higher amounts of taq DNA polymerase (2-3 µl) may be necessary to obtain a better yield of amplification products.

7. **Reaction Overlay:**

   If necessary the reaction mixture can be overlaid with mineral oil or paraffin (melting temperature 50°C-60°C) of special PCR grade. One half of the total reaction volume is usually sufficient.

8. **Cyclic Condition:**

   Amplification parameters depend greatly on template, primer and amplification operators used.

Agarose gel electrophoresis of PCR products

After completion of the PCR programme, the PCR products were checked on 1.5% agarose for the amplification. Before loading into the wells, gel-loading dye (Xylene-cyanol, Bromophenol blue in glycerol) was added to the sample. After loading the gel, the samples were run under constant voltage conditions (100 V) till the two dyes get separated. Amplified products appeared as sharp orange color bands under UV light due to the intercalation of ethidium bromide. The amplified samples were identified and results were recorded for further analysis on denaturing urea PAGE gel to record the alleles.

Resolution of PCR products on Urea-PAGE:

Polyacrylamide gels are chemically cross-linked gels formed by the polymerization

of acrylamide with a cross-linking agent N, N'methylene bis acrylamide. The polymerization is initiated by free radical formation usually carried out with ammonium per sulphate as the initiator and TEMED as a catalyst. Resolving power of polyacrylamide gels is very high as it can separate a molecule of DNA whose length differs by as little as 0.2%. The gel is polymerized in presence of denaturing agent (urea) that suppresses base pairing in nucleic acid. Amplified PCR products were analyzed on the 6% denaturing Polyacrylamide gels.

Requirements

(a)Equipments:

- Gel apparatus *(M/* S Bio Rad)
- Inner Glass Plate
- Outer Glass Plate
- Universal base
- Safety covers with cables
- Comb, syringe
- Power supply (3000 v capacity)
- Polyvinyl spacers (0.4mm thick)

Chemicals

- Acrylamide
- Bis acrylamide
- Urea
- Ammonium per sulphate
- TEMED

- Page Mix (Annexure 1)
- Bind saline
- Ethanol
- 10 x TBE: Dissolved 108 g Tris base and 55 g boric acid in 900 m deionized water. Added 40 ml 0.5m EDTA (pH 8.0) and final volume was made to 1 litre. Stored at room temperature or 4°C. Stock solution can be stored for about 1month at room temperature in a crew capped glass bottles.

Protocol

Casting of gel:

1. Glass plates and spacers were cleaned with soap and rinsed thoroughly with distilled water. Plates were allowed to dry on the filter paper.
2. Both plates were rinsed with 70% ethanol and wiped to prevent any streaking on the plates.
3. Bind saline (10µl) was applied at the edge of the glass plate and left for 10 minutes.
4. Spacers (0.4 mm) were placed between the two glass plates which were fixed tightly with the help of clamps.
5. 400µl of APS and 40µl of TEMED were added to 70-80 ml of PAGE mix and above solution was drawn into the barrel of 120ml syringe.
6. Gel mix was poured into the assembled glass plates using syringe. Flat side of comb (0.4 mm) was inserted before the polymerization of the gel.
7. The gel was allowed to polymerize for about one hour.

Electrophoresis of samples in Urea-PAGE gel

1. The comb of polymerized gel was removed and surface of the gel was washed with 1X

TBE buffer.

2. The assembly was placed in the lower buffer tank of the electrophoresis apparatus containing 1x TBE. Safety cover was fixed and the gel was pre-run for 30 minutes 80 W.
3. The comb was fixed with teeth side down and the wells were washed with TBE buffer. Loading dye was loaded in alternative wells to detect any leakage between the wells.
4. Denatured DNA samples (heated at 95°C for 5 minutes) were mixed with appropriate amount of gel loading dye (Bromophenol blue and xylene cyanol) and loaded (3-5µl) into the wells using micropipette. For size determination 10 bp ladder (Invitrogen) was loaded.
5. Electrodes were connected to the power pack. Voltage was set and the gel was run as per the size of the PCR products.
6. After completion of the electrophoresis, gel was laid on bench top and the side arms were removed.
7. Plates were slowly separated taking care that gel would got stuck to the bottom plate.
8. Using silver staining method, position of DNA band in the polyacrylamide gel was detected.

Silver staining for allelic differentiation

Highly sensitive detection of nucleic acid in the nano gram range has been achieved by the specific chemical reduction of silver ions. Bassam *et al.,* (1991) had used a method of silver staining nucleic acids in which formaldehyde selectively reduces silver ions to metallic silver under alkaline conditions. The presence of formaldehyde in the silver stain improves both sensitivity and contrast. Reduction of silver by formaldehyde is

concentration dependant. Higher concentration of formaldehyde increases sensitivity but also increases back ground staining. Higher formaldehyde level produces darker bands as opposed to the light brown bands obtained at lower concentration. Sulphate dissolve insoluble silver salts by complex formation, removing silver ions from the gel surface, which in turn minimize non- specific staining. A concentration of 4 mM thiosulphate is sufficient enough to reduce non-specific back ground staining without noticeably affecting DNA image development. Higher than this concentration have no noticeable benefit and can reduce the sensitivity of DNA detection. The presence of thiosulphate eliminates the formation of a dark precipitate in the gel and developer solution.

The staining procedures consist of three steps:

(a) Fixing the DNA bands on the gel by acetic acid.

(b) Incubation of the gel in the silver nitrate solution

(c) Developing the DNA bands with the help of developer.

Requirements

(a) Reagents:

- Fixative and stop solution (10% acetic acid).
- Staining solution (silver nitrate with formaldehyde).
- Developing solution (sodium carbonate with the formaldehyde).
- Distilled water.

(b) Materials:

- Photographic tray.
- Orbital shaker.
- Beakers.

- Cylinders.
- Cellophane sheets.
- Glass plates.
- Gel cutter.

Protocol:

1. The gel was immersed in 2.0 litre (10%) acetic acid for 20 minutes so that DNA bands in the gel got fixed. The fixation helps to prevent the diffusion of the DNA bands.
2. The gel was rinsed thrice with distilled water (2 min. each).
3. The gel was stained by incubating it in 1.5 liter of silver nitrate solution and 2.25 ml. of 37% formaldehyde at room temperature for 30 minutes under constant gentle shaking.
4. The gel was washed briefly for 10 sec. in distilled water.
5. Quickly the gel was transferred to chilled 1.5 litre developing solution.
6. The dark colour of DNA bands appeared in 3-5 min.
7. Immediately, reaction was stopped with 10% acetic acid.
8. The required portion of the gel was cut and air dried with the help of cellophane sheets.
9. The stored gels were kept properly for subsequent microsatellite data analysis and interpretation of the gel.

Scoring of silver stained PAGE gels for microsatellite alleles

1. The gels were superimposed with transparency.
2. Bands and ladder were marked with thin OHP marker pens. Mark upper most part of each band and follow this criterion uniformly in the gel.
3. Use different colors for different alleles.
4. In a fine resolved gel the problem of duplex is common. In this homozygote and

heterozygote had 2 and 4 bands respectively. This happens because each allele had two mutual complementary strands having equal size but due to sequence difference they show differential migration.

5. When 3 bands were visible with middles one thicker concept of"1+2+1" was applied as it was a heterozygote.
6. Electrophoresis distance travelled by each allele was measured from its respective wells using dial caliper.
7. Each allele was labeled as A, B, C, D, ...
8. Travelled distances of each allele were feeded in online inchworm software and sizes of the alleles were calculated.
9. The allelic data was feeded in excel sheet, and is used in various input files of different population analysis software.

Statistical Analysis of Microsatellite data

Allele Number: Alleles are a set of alternative forms of the same gene occupying the same relative position or locus on homologous chromosomes. Allele number is the total number of alleles for a given marker. The allele number for each locus was determined manually from the silver stained gels.

Allele Frequency / Gene Frequency:

The frequency of an allele' A' is the number of' A' alleles in the population divided by the total number of alleles/genes. It gives an indication of the most or least prevalent alleles in the population. The allele frequency is affected over time by forces such as genetic drift, mutation and migration.

Effective Allele Number (Kimura and Crow, 1964):

The effective number of alleles (ne) was calculated from markers using

$n_e = I / \Sigma P_i^2$

Where P_i is the frequency of the i^{th} allele.

Heterozygosity:

Heterozygosity is the state of possessing different alleles at a given locus in regard to a given character. It is a measure of genetic variation in a population. The population heterozygosity at a locus was given by the formula:

$H = I - \Sigma P_i^2$

Where Σ stands for summation over all alleles (Nei, 1973) and *Pi* is the frequency of the i^{th} allele at a locus in a population. The average heterozygosity per locus *(H)* is defined as the mean of *H* over all structural loci in the genome.

However, the unbiased estimate of the expected heterozygosity at a locus is (if $N <$ 50, *N* is sample size).

$$HE = \frac{2N}{2N - 1}\left[1 - \Sigma pi\right]$$

Software for microsatellite data analysis:

POPGENE is a user-friendly Microsoft window-based computer package for the analysis of genetic variation among and within natural populations using co-dominant and dominant markers and quantitative traits. It performs most type of data analysis encountered in population genetics and related fields. It was used to compute summary statistics (e.g., allele frequency, gene diversity, genetic distance, F-statistics, multilocus structure, etc.) for single-populations.

Chapter IV

Results

CHAPTER IV

RESULTS

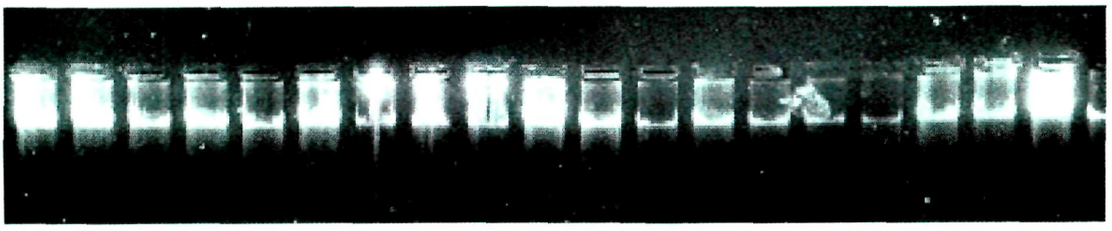
For characterization and estimation of genetic diversity in Hill cattle using microsatellite markers FAO/Dadis listed cattle microsatellite loci were selected for this study. Based on their amplifications on test samples they showed their distribution across cattle genome and location on different chromosomes. These loci are presented in Table 1. Total, 14 microsatellite primers were selected for the estimation of genetic diversity in Hill cattle population of Himachal Pradesh.

Table 1: Primer sequence for different microsatellites used for estimation of genetic diversity among Hill cattle of Himachal Pradesh.

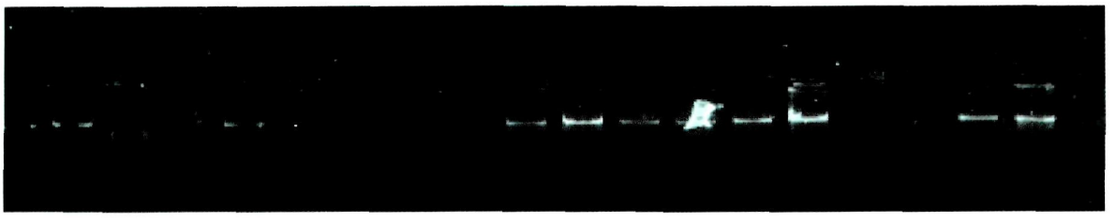
S.N0	Locus Name	Sequence	No of bp
1.	MM-8	cccaaggacagaaaagact	19
		ctcaagataagaccacacc	19
2.	ETH-152	tactcgtagggcaggctgcctg	22
		gagacctcagggttggtgatcag	23
3.	INRA-005	caatctgcatgaagtataaatat	23
		cttcaggcataccctacacc	20
4.	INRA-23	gagtagagctacaagataaacttc	24
		taactacagggtgttagatgaactca	26
5.	BM-1824	gagcaaggtgtttttccaatc	21
		cattctccaactgcttccttg	21
6.	ETH-3	gaacctgcctctcctgcattgg	22
		actctgcctgtggccaagtagg	22
7.	HAUT-27	ttttatgttcatttttgactgg	23
		aactgctgaaatctccatctta	22
8.	ETH-225	gatcaccttgccactatttcct	22
		acatgacagccagctgctact	21
9.	CSRM-60	aagatgtgatccaagagagaggca	24

		aggaccagatcgtgaaaggcatag	24
10.	ETH-185	tgcatggacagagcagcctggc	22
		gcaccccaacgaaagctcccag	22
11.	HEL-13	taaggacttgagataaggag	20
		ccatctacctccatcttaac	20
12.	ILST-030	ctgcagttctgcatatgtgg	20
		cttagacaacaggggtttgg	20
13.	CSSM-66	acacaaatcctttctgccagctga	24
		aatttaatgcactgaggagcttgg	24
14.	ILST-033	tattagagtggctcagtgcc	20
		atgcagacagttttagaggg	20

The DNA was isolated from 50 unrelated blood samples collected at random from different animals. The standard protocol was followed to isolate DNA with some modifications in the laboratory. The concentrated stock of DNA samples was checked on 0.6% agarose gel and all the fourteen microsatellites were PCR amplified and resolved on 1.5% agarose gel and was visualized using ethidium bromide staining. The Figures showing concentrated stock, diluted stock and PCR amplified products are presented in Figure 1 and Figure 2 respectively.



Concentrated stock



Diluted stock

Figure 1: Figure depicting the concentrated stock and diluted stock of genomic DNA.

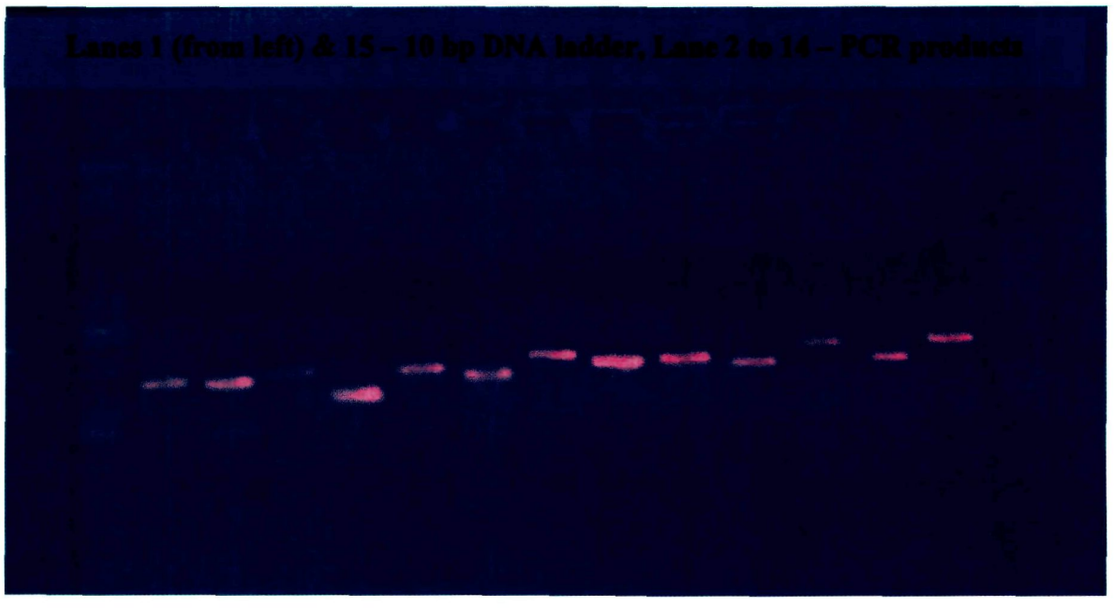


Figure 2: UV illumination of PCR products run on agarose gel electrophoresis.

PCR was attempted on 50 samples for each microsatellite. However, it could not be amplified on all samples and even few samples were weakly amplified. Uniform amplification required optimization with respect to DNA quality, $MgCl_2$ concentration, annealing temperature, dNTP concentration and taq polymerase volume. DNA quantity was optimized by including a DNA volume of 2.00µl in PCR master mix. Optimization was particularly required for the stored DNA samples with poor quality. Many samples responded to optimization and their product could be improved. The samples which could not be amplified in spite of optimization were excluded from analysis.

Optimization was also carried out for $MgCl_2$ concentration pending upon the demand of the microsatellite. The quantity of $MgCl_2$ was increased from initial 1.5mM to 1.5+0.25 mM in taq buffer with KCL. Later on the annealing temperature was also optimized with in the range of 55-58°C, to minimize the multiple bands and increase the specificity. Initially the dNTP included at the rate of 200µM was furter reduced to 150µM concentration. Similarly, taq polymerase was initially attempted with 1 unit was later reduced to 0.5 units in the reaction mixture.

Microsatellite Analysis

1. Microsatellite MM-8:

Microsatellite MM-8 contains 19 bp for forward and reverse primer sequences. On microsatellite analysis four alleles size (153-169bp) were typed in Hill cattle. The observed, effective numbers of alleles and Shannon information index were found to be 4.00, 3.0600 and 1.1683. The values are presented in Table 2.

Table 2: Summary of genetic variation statistics for all loci.

S. No.	Locus	Sample Size	No. of alleles	na*	ne*	I*
1.	MM8	92	4	4.0000	3.0600	1.1683
2.	ETH152	80	3	3.0000	1.1945	0.3491
3.	1NRA05	84	4	4.0000	2.6328	1.0938
4.	INRA23	78	5	5.0000	2.8725	1.2376
5.	BM1824	92	7	7.0000	5.8534	1.8382
6.	ETH3	94	2	2.0000	1.2094	0.3156
7.	HAUT27	94	8	8.0000	4.3356	1.7046
8.	ETH225	92	3	3.0000	2.1234	0.8297
9.	CSRM60	68	2	6.0000	2.6124	1.2147
10.	ETH185	92	4	4.0000	3.0556	1.2224
11.	HEL13	88	5	5.0000	3.2240	1.3386
12.	ILST30	60	6	6.0000	2.9605	1.3679
13.	CSSM66	84	4	4.0000	2.9229	1.1921
14.	ILST33	80	5	5.0000	2.9385	1.2869
Mean		84	4.4285	4.7143	2.9283	1.1543
St. Dev.			1.7415	1.6375	1.1588	0.4248

* na = Observed number of alleles, * ne = Effective number of alleles [Kimura and Crow (1964)], * I = Shannon's Information index [Lewontin (1972)]

The allele frequencies for this locus ranged from 0.0217(allele 4) to 0.3913(allele 3). The allele frequencies are presented in Table 3 and shown graphically in Figure 3. For this microsatellite locus the genotypic frequencies ranged from 0.0217 to 0.3696, which are in Table 4. The population studied was not found in Hardy- Weinberg equilibrium. The observed and expected heterozygosity values were 0.5435 and 0.6806, respectively (Table 5). The Fis value for this locus ranged from -0.3731(allele a) to 1.0000(allele d), (Table 6). The upper and lower neutrality values for this locus were found to be 0.8956(U95) and 0.3093(L95), are presented in Table 7.

Table 3: Allelic frequencies of microsatellite MM-8 in Hill cattle of Himachal Pradesh

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	169	0.2717
2.	2	160	0.3152
3.	3	156	0.3913
4.	4	153	0.0217

Table 4: Genotypes and genotypic frequencies of microsatellite MM-8 in Hill cattle of Himachal Pradesh.

S. No.	Genotype	No.	Freq.
1.	(A,A)	0	0
2.	(B,A)	17	0.3696
3.	(B,B)	6	0.1304
4.	(C,A)	8	0.1739
5.	(C,B)	0	0

6.	(C,C)	14	0.3043
7.	(D,A)	0	0
8.	(D,B)	0	0
9.	(D,C)	0	0
10.	(D,D)	1	0.0217
Total		46	1
Chi Square	124.1257 ^{S}		
Degree of freedom	6		

2. Microsatellite ETH-152:

Microsatellite ETH-152 contains 22 bp for forward and 23 bp for reverse primer sequences. On microsatellite analysis three alleles size (183-193bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be 3.0000, 1.1945, and 0.3491. The values are presented in Table 2. The allele frequencies for this locus ranged from 0.0250(for allele 1) to 0.9125(for allele 2). The allele frequencies are presented in Table 8 and shown graphically in Figure 4.

Table 8: Allelic frequencies of microsatellite ETH-152 in Hill cattle of Himachal Pradesh.

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	193	0.0250
2.	2	190	0.9125
3.	3	183	0.0625

Table 5: Microsatellite Locus Names, number of alleles, size range and heterozygousity levels in Hill cattle of Himachal Pradesh.

S.N0	Locus Name	No of Alleles	Size Range	Obs.Het.	Ex. Het.	Nei	Avg. Het.
1.	MM-8	4	153-169	0.5435	0.6806	0.6732	0.6732
2.	ETH-152	3	183-193	0.1250	0.1649	0.1628	0.1628
3.	INRA-005	4	133-164	0.2381	0.6277	0.6202	0.6202
4.	INRA-23	5	208-212	0.4359	0.6603	0.6519	0.6519
5.	BM-1824	7	152-194	0.3913	0.8383	0.8292	0.8292
6.	ETH-3	2	170-178	0.1915	0.1750	0.1732	0.1732
7.	HAUT-27	8	140-168	0.4894	0.7776	0.7694	0.7694
8.	ETH-225	3	139-142	0.0652	0.5349	0.5291	0.5291
9.	CSRM-60	6	192-210	0.3529	0.6264	0.6172	0.6172
10.	ETH-185	4	183-198	0.9565	0.6801	0.6727	0.6727
11.	HEL-13	5	219-234	0.5682	0.6978	0.6898	0.6898
12.	ILST-030	6	141-157	0.6667	0.6734	0.6622	0.6622
13.	CSSM-66	4	156-162	0.7857	0.6658	0.6579	0.6579
14.	ILST-033	5	150-176	0.2250	0.6680	0.6597	0.6597
Mean		4.4285		0.4311	0.6051	0.5977	0.5977
St.Dev		1.7415		0.2579	0.1969	0.1946	0.1946

Obs. Het – Observed heterozygosity ; Ex. Het – Expected heterozygosity; Nei– Nei's expected heterozygosity; Avg Het.– Average heterozygosity

Table 6: Wright's fixation index (Fis) as a measure of heterozygote deficiency or excess.

SI no.	Allele \ Locus	Allele A	Allele B	Allele C	Allele D	Allele E	Allele F	Allele G	Allele H	Total
1.	MM8	-0.3731	0.1440	0.6349	1.0000	****	****	****	****	0.1927
2.	ETH152	-0.0256	0.5303	-0.0667	****	-.0127	****	****	****	0.2322
3.	INRA005	0.8078	0.1711	-0.0500	0.7035	****	****	****	****	0.6161
4.	INRA023	0.5895	0.0565	0.2571	0.2778	-0.0130	****	****	****	0.3313
5.	BM1824	0.7237	0.8619	0.3213	0.7262	0.6305	-0.1795	1.0000	****	0.5281
6.	ETH3	-0.1059	-0.1059	****	****	****	****	****	****	-0.1059
7.	HAUT27	0.4778	0.4268	0.2797	0.4919	0.6179	-0.0562	-0.0330	-0.0217	0.3639
8.	ETH 225	0.9560	0.8644	0.4773	****	****	****	****	****	0.8767
9.	CSRM 60	0.2408	-0.0303	-0.0149	1.0000	0.4035	0.6402	****	****	0.4282
10.	ETH 185	-0.2105	0.2269	-0.7159	-0.4375	****	****	****	****	-0.4218
11.	HEL 13	0.1251	0.4211	0.0174	-0.0602	0.6423	****	****	****	0.1763
12.	ILST 30	0.7818	-0.0714	-0.3043	-0.0011	-0.0526	-0.0526	****	****	-0.0067
13.	CSSM 66	0.1711	-0.1200	-0.0144	-0.5556	****	****	****	****	-0.1943
14.	ILST 33	1.0000	1.0000	0.5497	-0.0390	-0.0811	****	****	****	0.6589

Table 7: The Ewens-Watterson Test for Neutrality.

Sl. no	Locus	n	k	Obs. F	Min F	Max F	Mean*	SE*	L95*	U95*
1.	MM8	92	4	0.3268	0.2500	0.9369	0.5619	0.0283	0.3093	0.8956
2.	ETH152	80	3	0.8372	0.3333	0.9513	0.6572	0.0310	0.3650	0.9509
3.	1NRA05	84	4	0.3798	0.2500	0.9311	0.5581	0.0282	0.3030	0.8861
4.	INRA23	78	5	0.3481	0.2000	0.9027	0.4691	0.0245	0.2485	0.8310
5.	BM1824	92	7	0.1708	0.1429	0.8781	0.3695	0.0161	0.2011	0.6916
6.	ETH3	94	2	0.8268	0.5000	0.9789	0.8059	0.0286	0.5020	0.9789
7.	HAUT27	94	8	0.2306	0.1250	0.8622	0.3242	0.0123	0.1845	0.6290
8.	ETH225	92	3	0.4709	0.3333	0.9575	0.6611	0.0323	0.3726	0.9572
9.	CSRM60	68	6	0.3828	0.1667	0.8638	0.4029	0.0189	0.2232	0.7569
10.	ETH185	92	4	0.3273	0.2500	0.9369	0.5643	0.0305	0.3126	0.9156
11.	HEL13	88	5	0.3102	0.2000	0.9132	0.4830	0.0253	0.2655	0.8301
12.	ILST30	60	6	0.3378	0.1667	0.8472	0.3933	0.0191	0.2156	0.7550
13.	CSSM66	84	4	0.3421	0.2500	0.9311	0.5647	0.0280	0.3138	0.8861
14.	ILST33	80	5	0.3403	0.2000	0.9050	0.4652	0.0218	0.2553	0.8134

For this microsatellite locus the genotypic frequencies ranged from 0.0500 to 0.8750, (Table 9). The population studied was not found in Hardy- Weinberg equilibrium. Table 5 shows the observed and expected heterozygosity values as 0.1250 and 0.1649, respectively. The Fis value for this locus ranged from -0.0256(allele a) to 0.5303(allele b), shown in Table 6. The upper and lower neutrality values for this locus were found to be 0.9509(U95) and 0.3650(L95), (Table7).

Table 9: Genotypes and genotypic frequencies of microsatellite ETH-152 in Hill cattle of Himachal Pradesh.

S. No.	Genotype	No.	Freq.
1.	(A,A)	0	0
2.	(B,A)	0	0
3.	(B,B)	35	0.875
4.	(C,A)	2	0.05
5.	(C,B)	3	0.075
6.	(C,C)	0	0
Total		40	1
Chi Square	30.3725 S		
Degree of freedom	3		

No. = Number of Animals; Freq. = Genotypic frequency; S = significant

3. Microsatellite INRA-005:

Microsatellite INRA-005 contains 23 bp for forward and 20 bp for reverse primer sequences. On microsatellite analysis four alleles size (133-164 bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be 4.00, 2.6328, and 1.0938. The values are presented in Table 2. The allele frequencies for this locus ranged from 0.0476(for allele 3) to 0.4524(for allele 1). The allele frequencies are presented in Table 10 and shown graphically in Figure 5.

Table 10: Allelic frequencies of microsatellite INRA-005 in Hill cattle of Himachal Pradesh

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	164	0.4524
2.	2	154	0.0952
3.	3	135	0.0476
4.	4	133	0.4048

For this microsatellite locus the genotypic frequencies were observed in the range 0.0238 to 0.4048, which are shown in Table 11. The population studied was not found in Hardy- Weinberg equilibrium. The observed and expected heterozygosity values were 0.2381and 0.6277, respectively presented in Table 5. The Fis value for this locus ranged from -0.0500(allele c) to 0.8078(allele a), (Table 6). The upper and lower neutrality values for this locus were found to be 0.8861(U95) and 0.3030(L95), (Table7).

Table 11: Genotypes and genotypic frequencies of microsatellite INRA-005 in Hill cattle of Himachal Pradesh.

S. No.	Genotype	No.	Freq.
1.	(A,A)	17	0.4048
2.	(B,A)	0	0
3.	(B,B)	1	0.0238
4.	(C,A)	4	0.0952
5.	(C,B)	0	0
6.	(C,C)	0	0
7.	(D,A)	0	0
8.	(D,B)	6	0.1429
9.	(D,C)	0	0
10.	(D,D)	14	0.3333
Total		42	1
Chi Square	43.8055 ^{S}		
Degree of freedom	6		

No. = Number of Animals; Freq. = Genotypic frequency; S = significant

4. Microsatellite INRA-023:

Microsatellite INRA-005 contains 24 bp for forward and 26 bp for reverse primer sequences. On microsatellite analysis five alleles size (208-212 bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be

9.	(D,C)	1	0.0256
10.	(D,D)	1	0.0256
11.	(E,A)	0	0
12.	(E,B)	0	0
13.	(E,C)	0	0
14.	(E,D)	1	0.0256
15.	(E,E)	0	0
Total		39	1
Chi Square	34.4009 ^{S}		
Degree of freedom	10		

No. = Number of Animals; Freq. = Genotypic frequency; S = significant

For this microsatellite locus the genotypic frequencies ranged from 0.0256 to 0.4103, (Table 13). The population studied was not found in Hardy- Weinberg equilibrium. The observed and expected heterozygosity values were 0.4359 and 0.6603, respectively presented in Table 5. The Fis value for this locus ranged from -0.0130(allele e) to 0.5895(allele a), shown in Table 6. The upper and lower neutrality values for this locus were found to be 0.8310(U95) and 0.2485(L95), (Table7).

5. Microsatellite BM-1824:

Microsatellite BM-1824 contains 21 bp for forward and 21 bp for reverse primer sequences. On microsatellite analysis seven alleles size (152-194 bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be 7.00, 5.8534, and 1.8382. The values are presented in Table 2. The allele frequencies for this locus ranged from 0.0435 (for allele 7) to 0.2283 (for allele 3). The allele frequencies are

presented in Table 14 and shown graphically in Figure 7. For this microsatellite locus the genotypic frequencies were observed in the range 0.0217 to 0.1957, which are shown in Table 15. The population studied was not found in Hardy- Weinberg equilibrium. The observed and expected heterozygosity values were 0.3913 and 0.8383, respectively presented in Table 5. The Fis value for this locus ranged from -0.1795(allele f) to 1.0000(allele g), shown in Table 6. The upper and lower neutrality values for this locus were found to be 0.6916(U95) and 0.2011(L95), presented in Table7.

Table 14: Allelic frequencies of microsatellite BM-1824 in Hill cattle of Himachal Pradesh.

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	194	0.1957
2.	2	191	0.1957
3.	3	187	0.2283
4.	4	182	0.0870
5.	5	173	0.0978
6.	6	162	0.1522
7.	7	152	0.0435

REPRESENTATIVE GELS SHOWING ALLELES FOR VARIOUS MICROSATELLITE MARKERS.

Plate 2: BM-1824 microsatellite alleles resolved on 6% Urea- PAGE (denaturing) and visualized by silver staining.

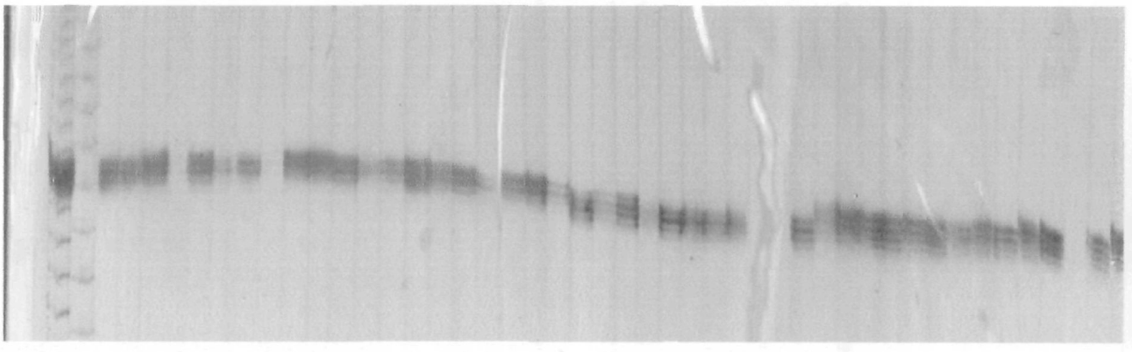


Plate 3: CSSM-66 microsatellite alleles resolved on 6% Urea- PAGE (denaturing) and visualized by silver staining.

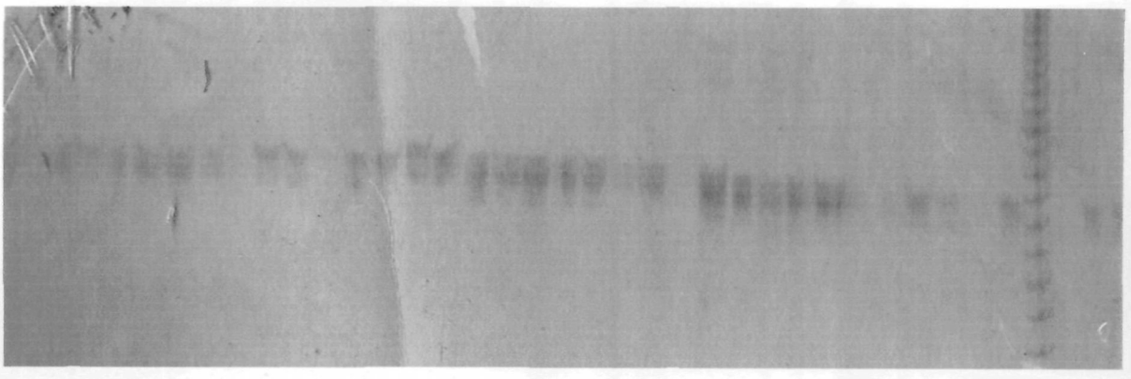


Plate 4: ETH-152 microsatellite alleles resolved on 6% Urea- PAGE (denaturing) and visualized by silver staining.

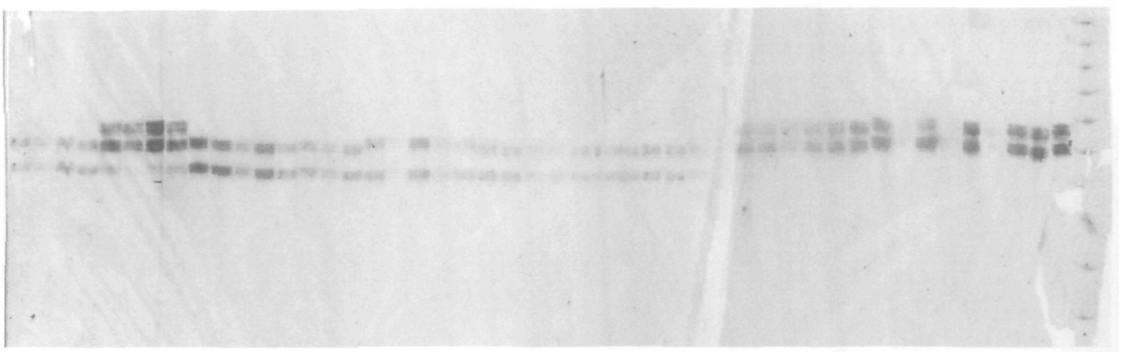


Plate 5: ETH-185 microsatellite alleles resolved on 6% Urea- PAGE (denaturing) and visualized by silver staining.

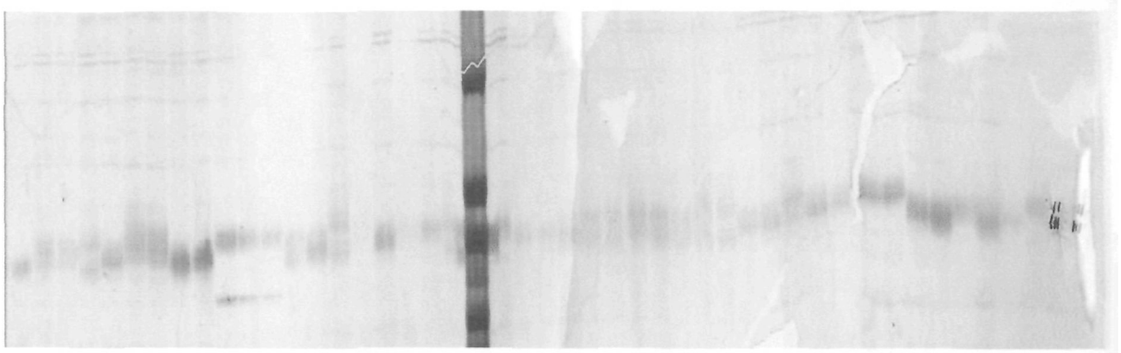


Plate 6: HEL-13 microsatellite alleles resolved on 6% Urea- PAGE (denaturing) and visualized by silver staining.

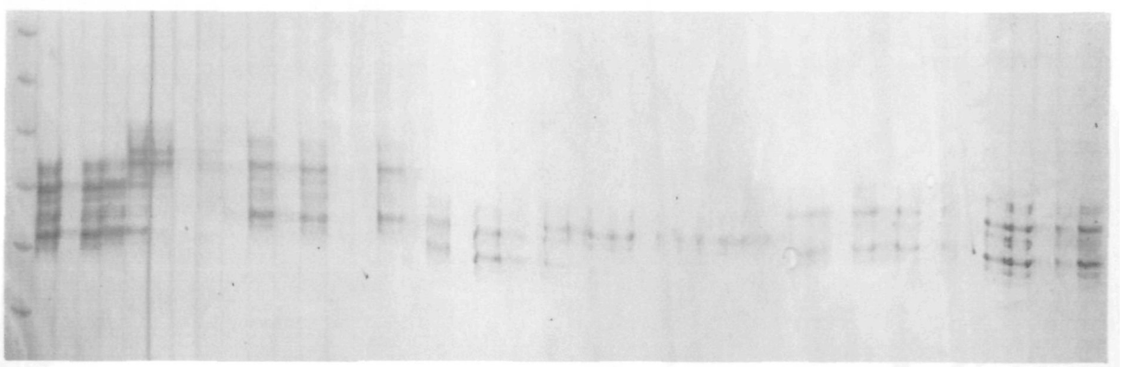


Plate 7: ILST-30 microsatellite alleles resolved on 6% Urea- PAGE (denaturing) and visualized by silver staining.

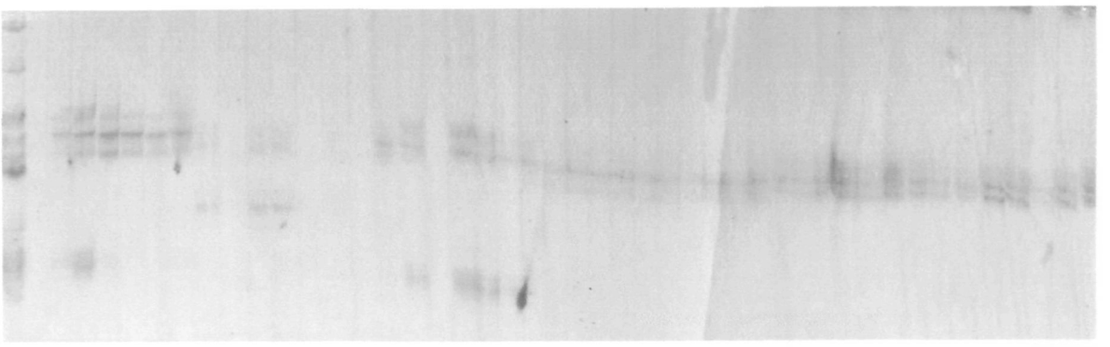


Plate 8: ILST-33 microsatellite alleles resolved on 6% Urea- PAGE (denaturing) and visualized by silver staining.

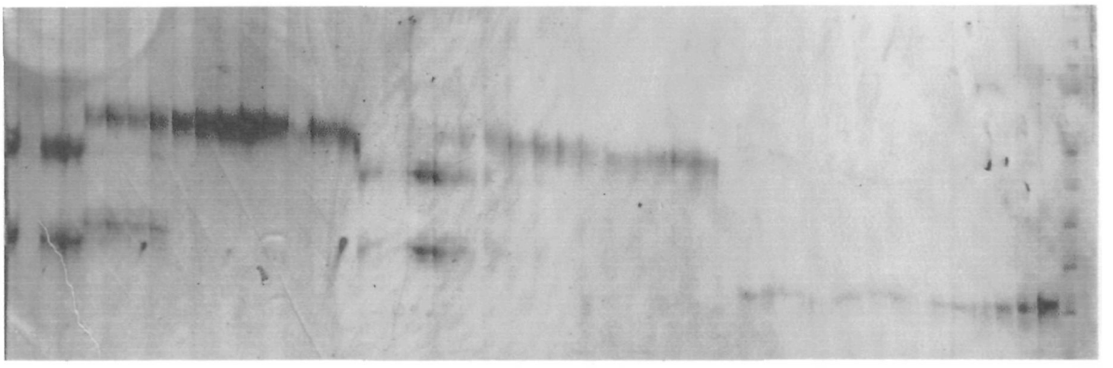


Plate 9: INRA-005 microsatellite alleles resolved on 6% Urea- PAGE (denaturing) and visualized by silver staining.

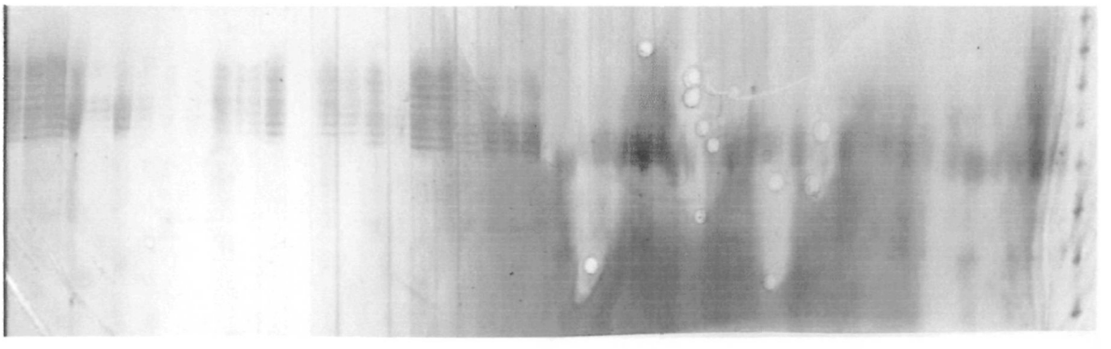


Plate 10: MM-8 microsatellite alleles resolved on 6% Urea- PAGE (denaturing) and visualized by silver staining.

Table 15: Genotypes and genotypic frequencies of microsatellite BM-1824 in Hill cattle.

S. No.	Genotype	No.	Freq.
1.	(A,A)	7	0.1522
2.	(B,A)	0	0
3.	(B,B)	8	0.1739
4.	(C,A)	0	0
5.	(C,B)	0	0
6.	(C,C)	5	0.1087
7.	(D,A)	0	0
8.	(D,B)	0	0
9.	(D,C)	1	0.0217
10.	(D,D)	3	0.0652
11.	(E,A)	1	0.0217
12.	(E,B)	0	0
13.	(E,C)	1	0.0217
14.	(E,D)	1	0.0217
15.	(E,E)	3	0.0652
16.	(F,A)	3	0.0652
17.	(F,B)	2	0.0435
18.	(F,C)	9	0.1957
19.	(F,D)	0	0
20.	(F,E)	0	0
21.	(F,F)	0	0
22.	(G,A)	0	0
23.	(G,B)	0	0
24.	(G,C)	0	0
25.	(G,D)	0	0
26.	(G,E)	0	0
27.	(G,F)	0	0
28.	(G,G)	2	0.0435
Total		**46**	**1**
Chi Square	**177.3238** [S]		
Degree of freedom	**21**		

6. Microsatellite ETH-3:

Microsatellite ETH-3 contains 22 bp for forward and 22 bp for reverse primer sequences. On microsatellite analysis two alleles size (170-178 bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be 2.00, 1.2094, and 0.3156. The values are presented in Table 2. The allele frequencies for this locus ranged from 0.0957 (for allele 1) to 0.9043 (for allele 2). The allele frequencies are presented in Table 16 and shown graphically in Figure 8. For this microsatellite locus the genotypic frequencies were observed in the range 0.1915 to 0.8085, which are shown in Table 17. The population studied was not found in Hardy- Weinberg equilibrium.

Table 16: Allelic frequencies of microsatellite ETH-3 in Hill cattle of Himachal Pradesh.

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	178	0.0957
2.	2	170	0.9043

The observed and expected heterozygosity values were 0.1915 and 0.1750, respectively presented in Table 5. The Fis value for this locus came out to be -0.1059, shown in Table 6. The upper and lower neutrality values for this locus were found to be 0.9789(U95) and 0.5020(L95), presented in Table7.

Table 17: Genotypes and genotypic frequencies of microsatellite ETH-3 in Hill cattle of Himachal Pradesh.

S. No.	Genotype	No.	Freq.
1.	(A,A)	0	0
2.	(B,A)	9	0.1915
3.	(B,B)	38	0.8085
Total		47	1
Chi Square	177.3238 ^{NS}		
Degree of freedom	21		

No. = Number of Animals; Freq. = Genotypic frequency; NS = Non-Significant

7. Microsatellite HAUT-27:

Microsatellite HAUT-27 contains 23 bp for forward and 22 bp for reverse primer sequences. On microsatellite analysis eight alleles size (140-168 bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be 8.00, 4.3356, and 1.7046. The values are presented in Table 2. The allele frequencies for this locus ranged from 0.0213 (for allele 8) to 0.3830 (for allele 3). The allele frequencies are presented in Table 18 and shown graphically in Figure 9. For this microsatellite locus the genotypic frequencies were observed in the range 0.0213 to 0.2128, as shown in Table 19. The population studied was not found in Hardy- Weinberg equilibrium. The observed and expected heterozygosity values were 0.4894 and 0.7776, respectively presented in Table 5.

Table 18: Allelic frequencies of microsatellite HAUT-27 in Hill cattle of Himachal Pradesh.

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	168	0.0426
2.	2	164	0.1277
3.	3	162	0.3830
4.	4	159	0.2128
5.	5	155	0.1277
6.	6	148	0.0532
7.	7	143	0.0319
8.	8	140	0.0213

The Fis value for this locus came in the range -0.0217(allele h) to 0.6179(allele e), shown in Table 6. The upper and lower neutrality values for this locus were found to be 0.6290(U95) and 0.1845(L95), presented in Table7.

Table 19: Genotypes and genotypic frequencies of microsatellite HAUT-27 in Hill cattle of Himachal Pradesh.

S. No.	Genotype	No.	Freq.
1.	(A,A)	1	0.0213
2.	(B,A)	0	0
3.	(B,B)	3	0.0638
4.	(C,A)	1	0.0213
5.	(C,B)	1	0.0213

6.	(C,C)	10	0.2128
7.	(D,A)	1	0.0213
8.	(D,B)	2	0.0426
9.	(D,C)	5	0.1064
10.	(D,D)	6	0.1277
11.	(E,A)	0	0
12.	(E,B)	1	0.0213
13.	(E,C)	3	0.0638
14.	(E,D)	0	0
15.	(E,E)	4	0.0851
16.	(F,A)	0	0
17.	(F,B)	1	0.0213
18.	(F,C)	3	0.0638
19.	(F,D)	0	0
20.	(F,E)	0	0
21.	(F,F)	0	0
22.	(G,A)	0	0
23.	(G,B)	1	0.0213
24.	(G,C)	2	0.0426
25.	(G,D)	0	0
26.	(G,E)	0	0
27.	(G,F)	0	0

28.	(G,G)	0	0
29.	(H,A)	0	0
30.	(H,B)	0	0
31.	(H,C)	1	0.0213
32.	(H,D)	0	0
33.	(H,E)	0	0
34.	(H,F)	1	0.0213
35.	(H,G)	0	0
36.	(H,H)	0	0
Total		47	1
Chi Square	68.3158 ^{S}		
Degree of freedom	28		

No. = Number of Animals; Freq. = Genotypic frequency; S = significant

8. Microsatellite ETH-225:

Microsatellite ETH-225 contains 22 bp for forward and 21 bp for reverse primer sequences. On microsatellite analysis three alleles size (139-142 bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be 3.00, 2.1234, and 0.8297. The values are presented in Table 2. The allele frequencies for this locus ranged from 0.0435 (for allele 3) to 0.5543 (for allele 1). The allele frequencies are presented in Table 20 and shown graphically in Figure 10. For this microsatellite locus the genotypic frequencies were observed in the range 0.0217 to 0.5435, (Table 21).

Table 20: Allelic frequencies of microsatellite ETH-225 in Hill cattle of Himachal Pradesh

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	142	0.5543
2.	2	140	0.4022
3.	3	139	0.0435

The population studied was not found in Hardy- Weinberg equilibrium. The observed and expected heterozygosity values were 0.0652 and 0.5349, respectively presented in Table 5. The Fis value for this locus came in the range 0.4773(allele c) to 0.9560(allele a), as in Table 6. The upper and lower neutrality values for this locus were found to be 0.9572(U95) and 0.3726(L95), presented in Table7.

Table 21: Genotypes and genotypic frequencies of microsatellite ETH-225 in Hill cattle of Himachal Pradesh.

S. No.	Genotype	No.	Freq.
1.	(A,A)	25	0.5435
2.	(B,A)	1	0.0217
3.	(B,B)	17	0.3696
4.	(C,A)	0	0
5.	(C,B)	2	0.0435
6.	(C,C)	1	0.0217
Total		46	1
Chi Square	55.7701 S		
Degree of freedom	3		

No. = Number of Animals; Freq. = Genotypic frequency; S = significant

9. Microsatellite CSRM-60:

Microsatellite CSRM-60 contains 24 bp for forward and 24 bp for reverse primer sequences. On microsatellite analysis six alleles size (192-210 bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be 6.0000, 2.6124, and 1.2147. The values are presented in Table 2. The allele frequencies for this locus ranged from 0.0147 (for allele 3) to 0.5588 (for allele 5). The allele frequencies are presented in Table 22 and shown graphically in Figure 11.

Table 22: Allelic frequencies of microsatellite CSRM-60 in Hill cattle of Himachal Pradesh.

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	210	0.1618
2.	2	205	0.0294
3.	3	202	0.0147
4.	4	200	0.0294
5.	5	198	0.5588
6.	6	192	0.2059

For this microsatellite locus the genotypic frequencies were observed in the range 0.0294 to 0.4118, which are shown in Table 23. The population studied was not found in Hardy- Weinberg equilibrium. The observed and expected heterozygosity values were 0.3529 and 0.6264, respectively presented in Table 5. The Fis value for this locus came in the range -0.0149(allele c) to 1.0000 (allele d), shown in Table 6. The upper and lower neutrality values for this locus were found to be 0.7569(U95) and 0.2232(L95), presented in Table7.

Table 23: Genotypes and genotypic frequencies of microsatellite CSRM-60.

S. No.	Genotype	No.	Freq.
1.	(A,A)	2	0.0588
2.	(B,A)	0	0
3.	(B,B)	0	0
4.	(C,A)	0	0
5.	(C,B)	1	0.0294
6.	(C,C)	0	0
7.	(D,A)	0	0
8.	(D,B)	0	0
9.	(D,C)	0	0
10.	(D,D)	1	0.0294
11.	(E,A)	6	0.1765
12.	(E,B)	1	0.0294
13.	(E,C)	0	0
14.	(E,D)	0	0
15.	(E,E)	14	0.4118
16.	(F,A)	1	0.0294
17.	(F,B)	0	0
18.	(F,C)	0	0
19.	(F,D)	0	0
20.	(F,E)	3	0.0882
21.	(F,F)	5	0.1471
Total		34	1
Chi Square	116.6797 ^{S}		
Degree of freedom	15		

No. = Number of Animals; Freq. = Genotypic frequency; S = significant

10. Microsatellite ETH-185:

Microsatellite ETH-185 contains 22 bp for forward and 22 bp for reverse primer sequences. On microsatellite analysis four alleles size (183-198 bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be 4.00, 3.0556, and 1.2224. The values are presented in Table 2. The allele frequencies for this locus ranged from 0.0761(for allele 2) to 0.4457 (for allele 3). The allele frequencies are presented in Table 24 and shown graphically in Figure 12. For this microsatellite locus the genotypic frequencies were observed in the range 0.0217 to 0.5, which are shown in Table 25. The population studied was not found in Hardy- Weinberg equilibrium. The observed and expected heterozygosity values were 0.9565 and 0.6801, respectively presented in Table 5. The Fis value for this locus came in the range -0.2105(allele a) to 0.2269 (allele b), as presented in Table 6. The upper and lower neutrality values for this locus were found to be 0.9156(U95) and 0.3126(L95), presented in Table7.

Table 24: Allelic frequencies of microsatellite ETH-185 in Hill cattle of Himachal Pradesh.

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	198	0.1739
2.	2	195	0.0761
3.	3	191	0.4457
4.	4	183	0.3043

Table 25: Genotypes and genotypic frequencies of microsatellite ETH-185 in Hill cattle of Himachal Pradesh.

S. No.	Genotype	No.	Freq.
1.	(A,A)	0	0
2.	(B,A)	0	0
3.	(B,B)	1	0.0217
4.	(C,A)	16	0.3478
5.	(C,B)	0	0
6.	(C,C)	1	0.0217
7.	(D,A)	0	0
8.	(D,B)	5	0.1087
9.	(D,C)	23	0.5
10.	(D,D)	0	0
Total		46	1
Chi Square	47.4967 ^{S}		
Degree of freedom	6		

No. = Number of Animals; Freq. = Genotypic frequency; S = significant

11. Microsatellite HEL-13:

Microsatellite HEL-13 contains 20 bp for forward and 20 bp for reverse primer sequences. On microsatellite analysis five alleles size (219-234 bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be 5.00, 3.2240, and 1.3386. The values are presented in Table 2. The allele frequencies for this

locus ranged from 0.0568(for allele 4) to 0.4432 (for allele 1). The allele frequencies are presented in Table 26 and shown graphically in Figure 13. For this microsatellite locus the genotypic frequencies were observed in the range 0.0227 to 0.2727, which are shown in Table 27. The population studied was not found in Hardy- Weinberg equilibrium.

Table 26: Allelic frequencies of microsatellite HEL-13 in Hill cattle of Himachal Pradesh.

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	234	0.4432
2.	2	228	0.1364
3.	3	225	0.2955
4.	4	222	0.0568
5.	5	219	0.0682

The observed and expected heterozygosity values were 0.5682 and 0.6978, respectively presented in Table 5. The Fis value for this locus came in the range -0.0602(allele d) to 0.6423 (allele e), shown in Table 6. The upper and lower neutrality values for this locus were found to be 0.8301(U95) and 0.2655(L95), presented in Table7.

Table 27: Genotypes and genotypic frequencies of microsatellite HEL-13 in Hill cattle of Himachal Pradesh.

S. No.	Genotype	No.	Freq.
1.	(A,A)	10	0.2273
2.	(B,A)	3	0.0682
3.	(B,B)	3	0.0682
4.	(C,A)	12	0.2727
5.	(C,B)	3	0.0682
6.	(C,C)	4	0.0909
7.	(D,A)	3	0.0682
8.	(D,B)	0	0
9.	(D,C)	2	0.0455
10.	(D,D)	0	0
11.	(E,A)	1	0.0227
12.	(E,B)	0	0
13.	(E,C)	1	0.0227
14.	(E,D)	0	0
15.	(E,E)	2	0.0455
Total		44	1
Chi Square	31.2471^{S}		
Degree of freedom	10		

No. = Number of Animals; Freq. = Genotypic frequency; S = significant

12. Microsatellite ILST-030:

Microsatellite ILST-030 contains 20 bp for forward and 20 bp for reverse primer sequences. On microsatellite analysis six alleles size (141-157 bp) were typed in Hill cattle.

Table 28: Allelic frequencies of microsatellite ILST-30 in Hill cattle of Himachal Pradesh

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	157	0.0833
2.	2	154	0.0667
3.	3	151	0.2333
4.	4	146	0.5167
5.	5	143	0.0500
6.	6	141	0.0500

The observed, effective numbers of alleles and shanon information index were found to be 6.00, 2.9605, and 1.3679 (Table 2). The allele frequencies for this locus ranged from 0.0500(for allele 6) to 0.5167 (for allele 4). The allele frequencies are presented in Table 28 and shown graphically in Figure 14. For this microsatellite locus the genotypic frequencies were observed in the range 0.0333 to 0.2667, which are shown in Table 29. The population studied was not found in Hardy- Weinberg equilibrium. The observed and expected heterozygosity values were 0.6667 and 0.6734, respectively presented in Table 5. The Fis value for this locus came in the range -0.0011(allele d) to 0.7818 (allele a), shown in Table 6. The upper and lower neutrality values for this locus were found to be 0.7550(U95) and 0.2156(L95), as shown in Table7.

Table 29: Genotypes and genotypic frequencies of microsatellite ILST-30.

S. No.	Genotype	No.	Freq.
1.	(A,A)	2	0.0667
2.	(B,A)	0	0
3.	(B,B)	0	0
4.	(C,A)	1	0.0333
5.	(C,B)	1	0.0333
6.	(C,C)	0	0
7.	(D,A)	0	0
8.	(D,B)	3	0.1
9.	(D,C)	9	0.3
10.	(D,D)	8	0.2667
11.	(E,A)	0	0
12.	(E,B)	0	0
13.	(E,C)	3	0.1
14.	(E,D)	0	0
15.	(E,E)	0	0
16.	(F,A)	0	0
17.	(F,B)	0	0
18.	(F,C)	0	0
19.	(F,D)	3	0.1
20.	(F,E)	0	0
21.	(F,F)	0	0
Total		30	1
Chi Square	37.2631 S		
Degree of freedom	15		

No. = Number of Animals; Freq. = Genotypic frequency; S = significant

13. Microsatellite CSSM-66:

Microsatellite CSSM_66 contains 24 bp for forward and 24 bp for reverse primer sequences. On microsatellite analysis four alleles size (156-162 bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be 4.00, 2.9229, and 1.1921. The values are presented in Table 2. The allele frequencies for this locus ranged from 0.0952(for allele 1) to 0.4405 (for allele 3). The allele frequencies are presented in Table 30 and shown graphically in Figure 15. For this microsatellite locus the genotypic frequencies were observed in the range 0.0238 to 0.4762, which are (Table 31). The population studied was not found in Hardy- Weinberg equilibrium. The observed and expected heterozygosity values were 0.7857 and 0.6658, respectively presented in Table 5. The Fis value for this locus came in the range -0.0144(allele c) to 0.1711 (allele a), shown in Table 6. The upper and lower neutrality values for this locus were found to be 0.8861(U95) and 0.3138(L95), presented in Table7.

Table 30: Allelic frequencies of microsatellite CSSM-66 in Hill cattle of Himachal Pradesh.

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	162	0.0952
2.	2	161	0.1071
3.	3	159	0.4405
4.	4	156	0.3571

Table 31: Genotypes and genotypic frequencies of microsatellite CSSM-66 in Hill cattle of Himachal Pradesh.

S. No.	Genotype	No.	Freq.
1.	(A,A)	1	0.0238
2.	(B,A)	2	0.0476
3.	(B,B)	0	0
4.	(C,A)	0	0
5.	(C,B)	1	0.0238
6.	(C,C)	8	0.1905
7.	(D,A)	4	0.0952
8.	(D,B)	6	0.1429
9.	(D,C)	20	0.4762
10.	(D,D)	0	0
Total		42	1
Chi Square	20.3015 ^{S}		
Degree of freedom	6		

No. = Number of Animals; Freq. = Genotypic frequency; S = significant

14. Microsatellite ILST-033:

Microsatellite ILST-033 contains 20 bp for forward and 20 bp for reverse primer sequences. On microsatellite analysis five alleles size (150-176 bp) were typed in Hill cattle. The observed, effective numbers of alleles and shanon information index were found to be 5.00, 2.9385, and 1.2869. The values are presented in Table 2. The allele frequencies for this locus ranged from 0.0375(for allele 4) to 0.2000 (for allele 1). The

allele frequencies are presented in Table 32 and shown graphically in Figure 16. For this microsatellite locus the genotypic frequencies were observed in the range 0.075 to 0.4, which are shown in Table 33. The population studied was not found in Hardy- Weinberg equilibrium. The observed and expected heterozygosity values were 0.2250 and 0.6680, respectively presented in Table 5. The Fis value for this locus came in the range -0.0390(allele d) to 1.0000 (allele a), shown in Table 6. The upper and lower neutrality values for this locus were found to be 0.8134(U95) and 0.2553(L95), presented in table 7.

Table 32: Allelic frequencies of microsatellite ILST-33 in Hill cattle of Himachal Pradesh.

S. No.	Allele Number	Allele size(bp)	Allelic frequency
1.	1	176	0.2000
2.	2	175	0.1750
3.	3	172	0.5125
4.	4	161	0.0375
5.	5	150	0.0750

Table 33: Genotypes and genotypic frequencies of microsatellite ILST-33 in Hill cattle of Himachal Pradesh.

S. No.	Genotype	No.	Freq.
1.	(A,A)	8	0.2
2.	(B,A)	0	0
3.	(B,B)	7	0.175
4.	(C,A)	0	0
5.	(C,B)	0	0
6.	(C,C)	16	0.4
7.	(D,A)	0	0
8.	(D,B)	0	0
9.	(D,C)	3	0.075
10.	(D,D)	0	0
11.	(E,A)	0	0
12.	(E,B)	0	0
13.	(E,C)	6	0.15
14.	(E,D)	0	0
15.	(E,E)	0	0
Total		40	1
Chi Square	86.6767^{S}		
Degree of freedom	10		

No. = Number of Animals; Freq. = Genotypic frequency; S = significant

Allele diversity

The average observed number of alleles varied from 2.00 (ETH-3) to 8.00 (HAUT-27) with an average value of 4.4285±1.7425. The average effective number of alleles per locus varied from 1.2094(ETH-3) to 5.8534 (BM-1824), with an average number of effective alleles (2.9283±1.588). This data of alleles presented at these loci is informative of genetic diversity analysis of Hill cattle population of Himachal Pradesh.

The locus HAUT-27 showed the maximum number of 8 alleles, closely followed by BM-1824 with 7 alleles. At HAUT-27 locus, lowest frequency of H allele was 0.0213 and highest (0.3830) of C allele. This shows the alleles occurring at these frequencies is also not because of chance, however, the effect of selection or mutation in the long run can not be ruled out. This means that these alleles do exist in the random mating population in the cattle of this region and thus explains the genetic diversity existing with population. The lowest frequency of 0.0128 (INRA-023) was also not due to any chance factor but it also exists in the random breeding population. All the alleles at the different loci are informative for genetic diversity analysis.

Heterozygosity level in cattle population

The average observed heterozygosity was 0.4311±0.2579 at different loci. The highest level of observed heterozygosity was 0.9565 (ETH-185) and lowest as 0.0652 (ETH-225). The expected heterozygosity was highest (0.8383) at BM-1824 locus and lowest (0.1574) at ETH-3 locus. The difference in average observed and expected heterozygosity was markedly higher.

Heterozygosity deficiency

The Wright fixation index (Fis) as a measure of heterozygote deficiency data are presented in the Table 6 for different microsatellite loci. The Wright fixation index was highest 0.6161 at INRA- 05 and lowest -0.0067(ILST-30). On an average, the heterozygous deficiency was higher at different loci, which indicates that the heterozygosity is being lost at different loci. This explains that this could be due to higher level of inbreeding in the population.

Neutrality of markers

The data presented in Table-7, indicates the majority of loci had U95 values were in the range of 0.8134(ILST-33) to 0.9789(ETH-03), except HAUT-27 (0.6290) BM-1824 (0.6916), ILST30 (0.7550) and CSRM-60 (0.7569). Their values at L95 were in the lower range from 0.1845 (HAUT-27) to 0.3138 (CSSM-66) except ETH-3 having L95 of 0.5020. This data shows that 13 out of 14 primers had desired neutrality and these markers explain adequate usefulness for genetic diversity analysis in Hill cattle population of Himachal Pradesh.

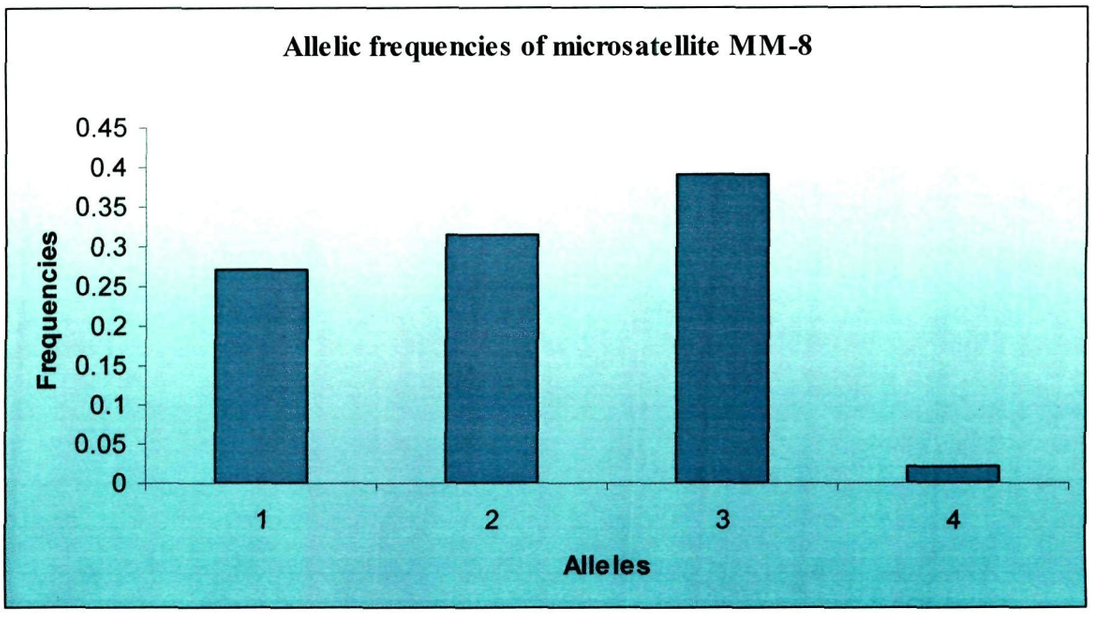


Figure 3: Allelic frequencies of microsatellite MM-8 in Hill cattle of Himachal Pradesh.

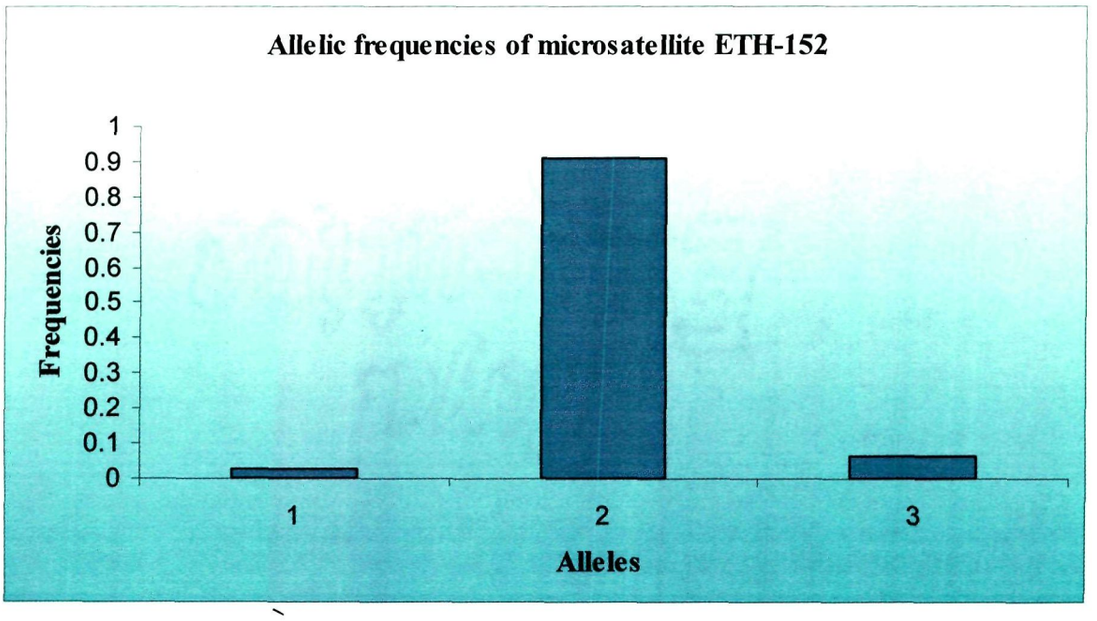


Figure 4: Allelic frequencies of microsatellite ETH-152 in Hill cattle of Himachal Pradesh.

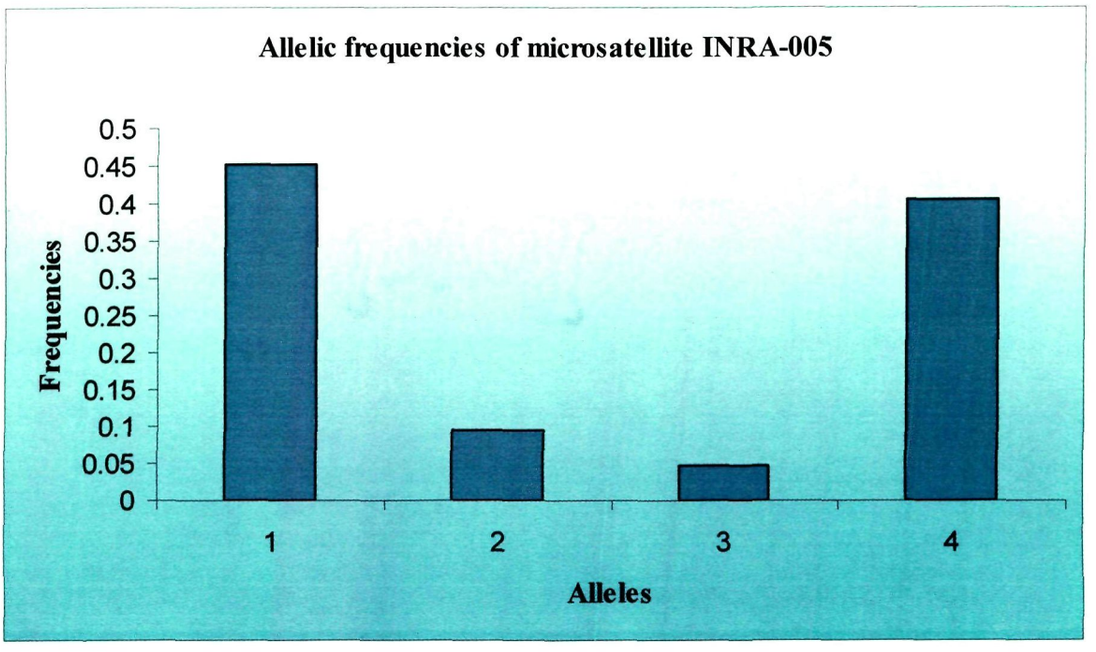


Figure 5: Allelic frequencies of microsatellite INRA-005 in Hill cattle of Himachal Pradesh.

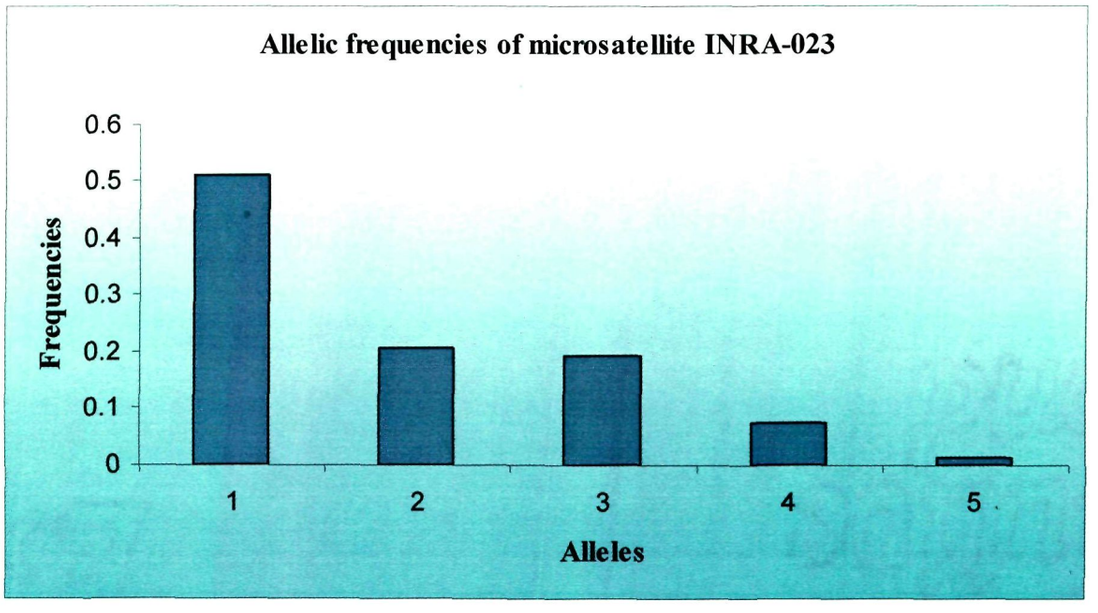


Figure 6: Allelic frequencies of microsatellite INRA-023 in Hill cattle of Himachal Pradesh.

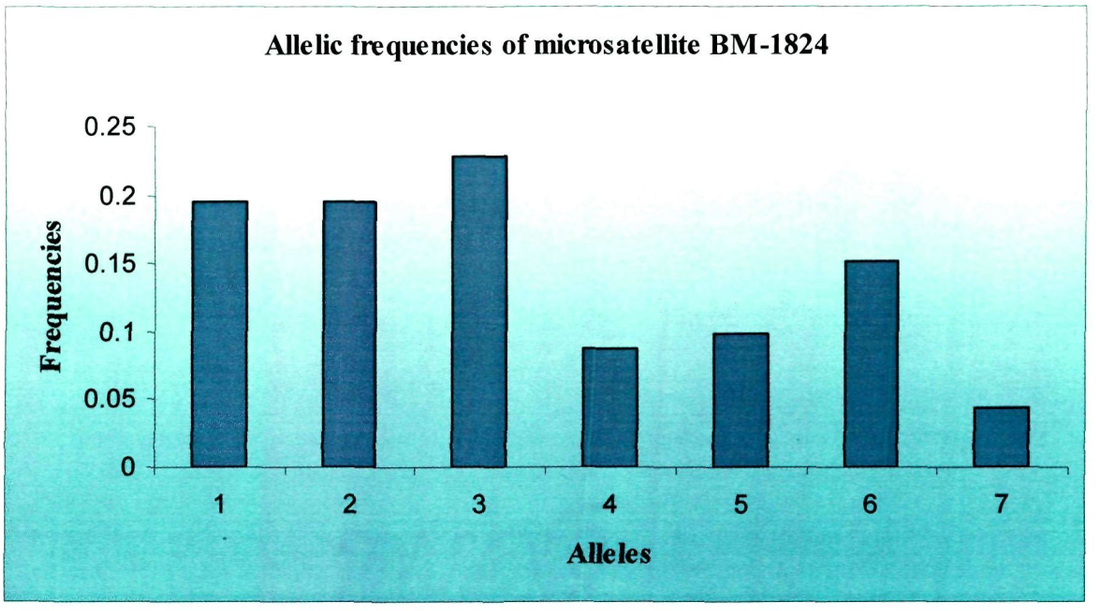


Figure 7: Allelic frequencies of microsatellite BM-1824 in Hill cattle of Himachal Pradesh.

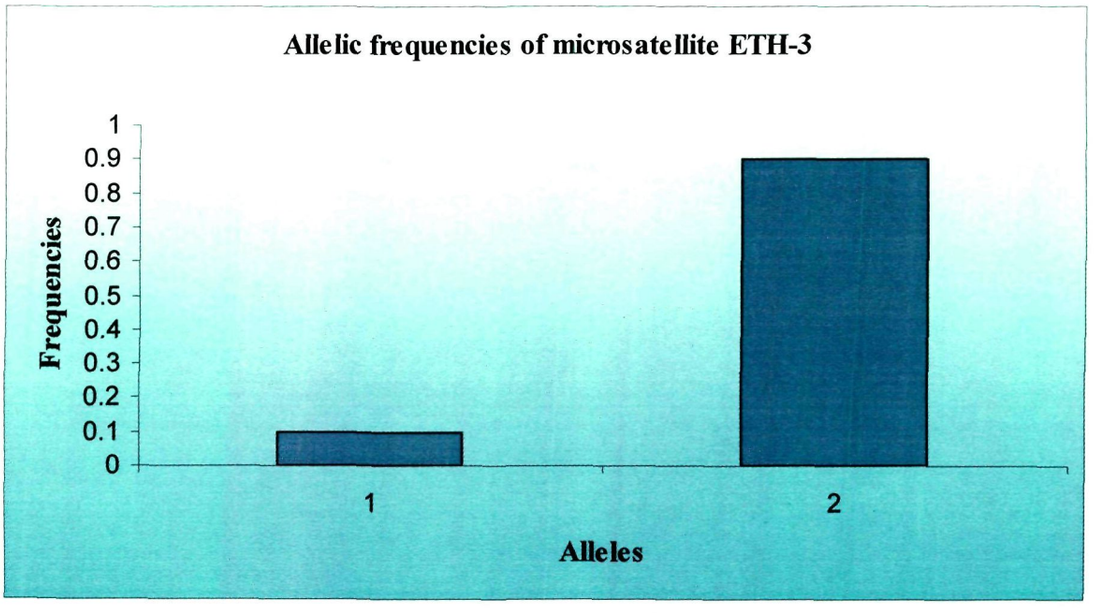


Figure 8: Allelic frequencies of microsatellite ETH-3 in Hill cattle of Himachal Pradesh.

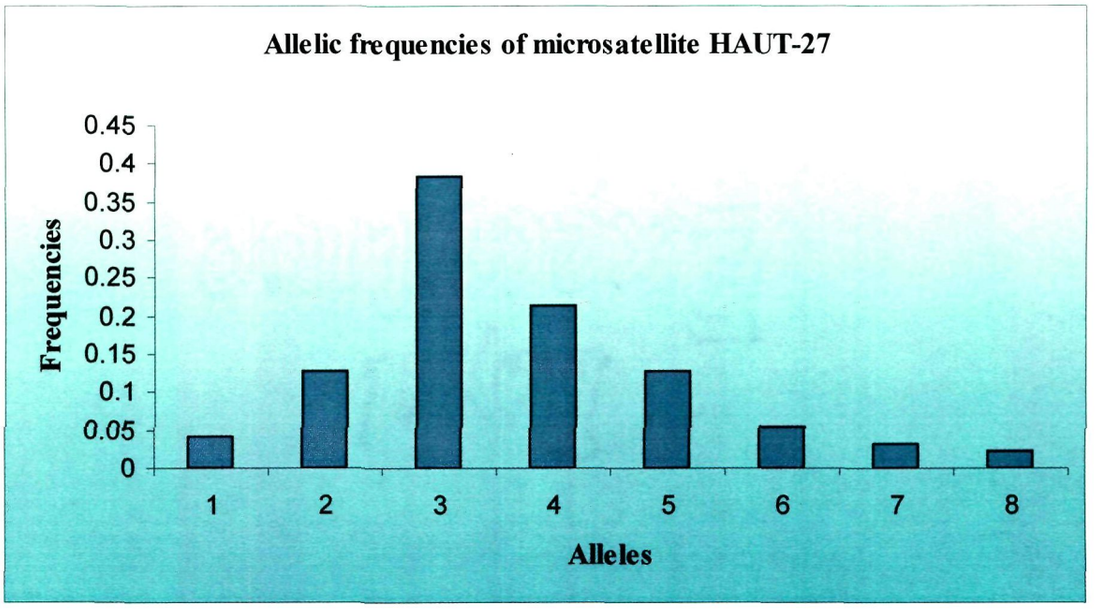


Figure 9: Allelic frequencies of microsatellite HAUT-27 in Hill cattle of Himachal Pradesh.

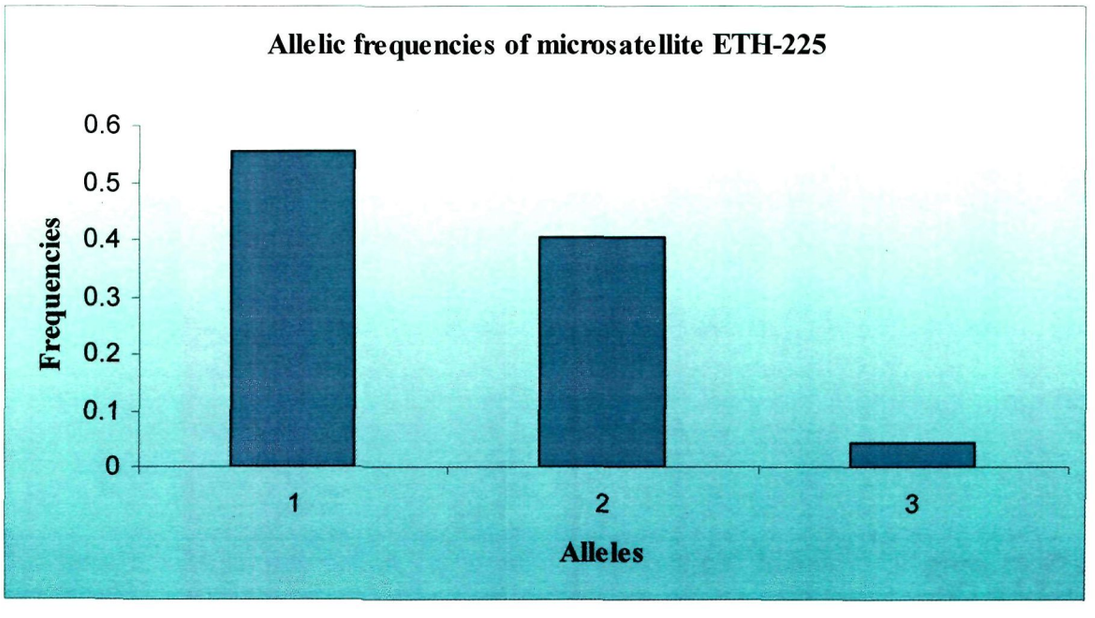


Figure 10: Allelic frequencies of microsatellite ETH-225 in Hill cattle of Himachal Pradesh.

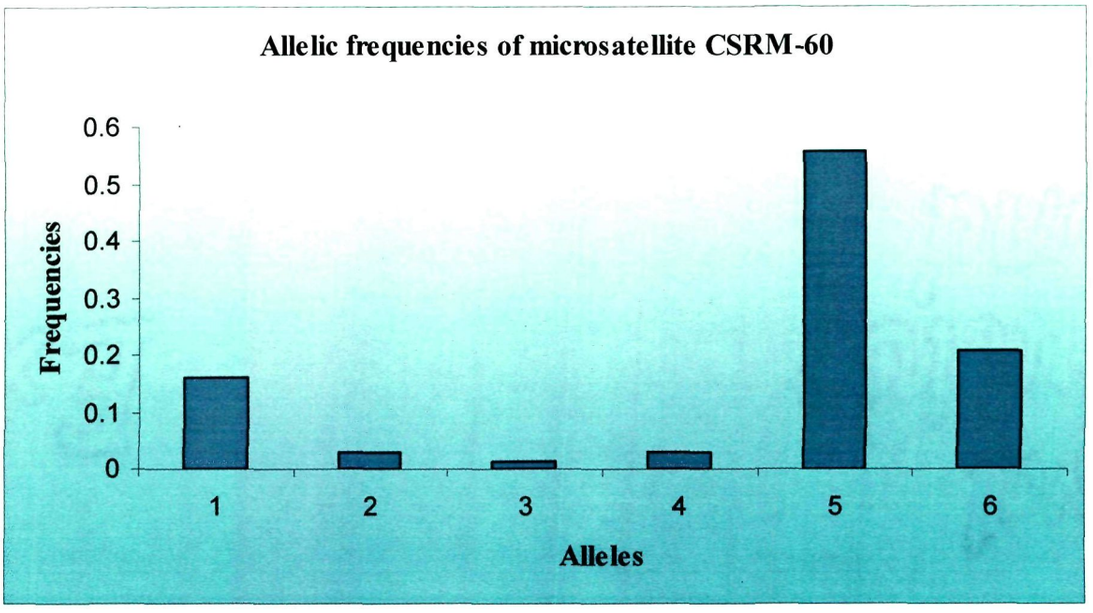


Figure 11: Allelic frequencies of microsatellite CSRM-60 in Hill cattle of Himachal Pradesh.

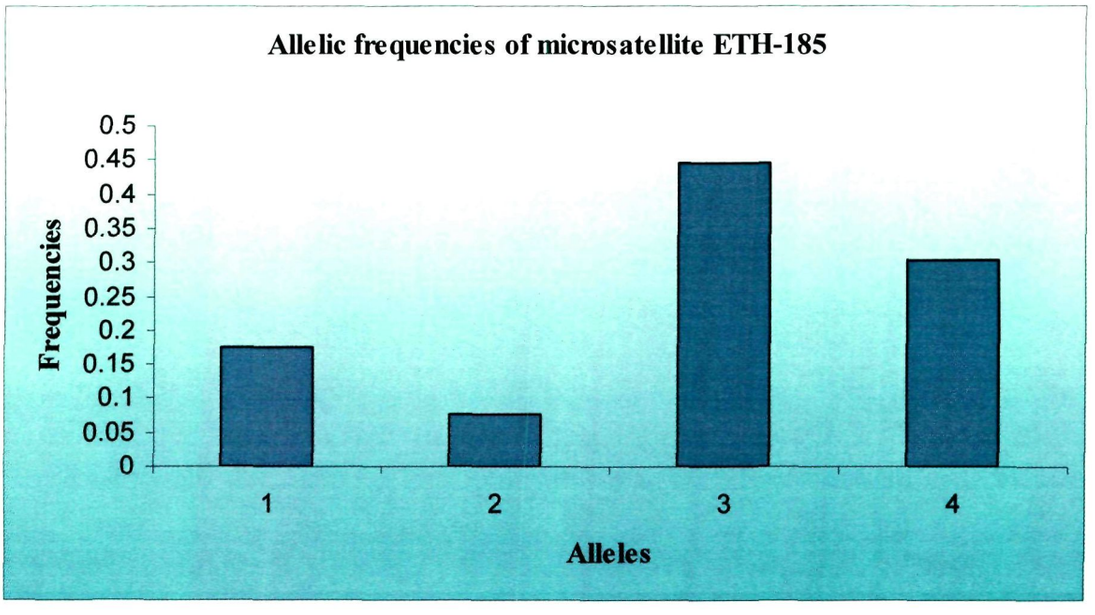


Figure 12: Allelic frequencies of microsatellite ETH-185 in Hill cattle of Himachal Pradesh.

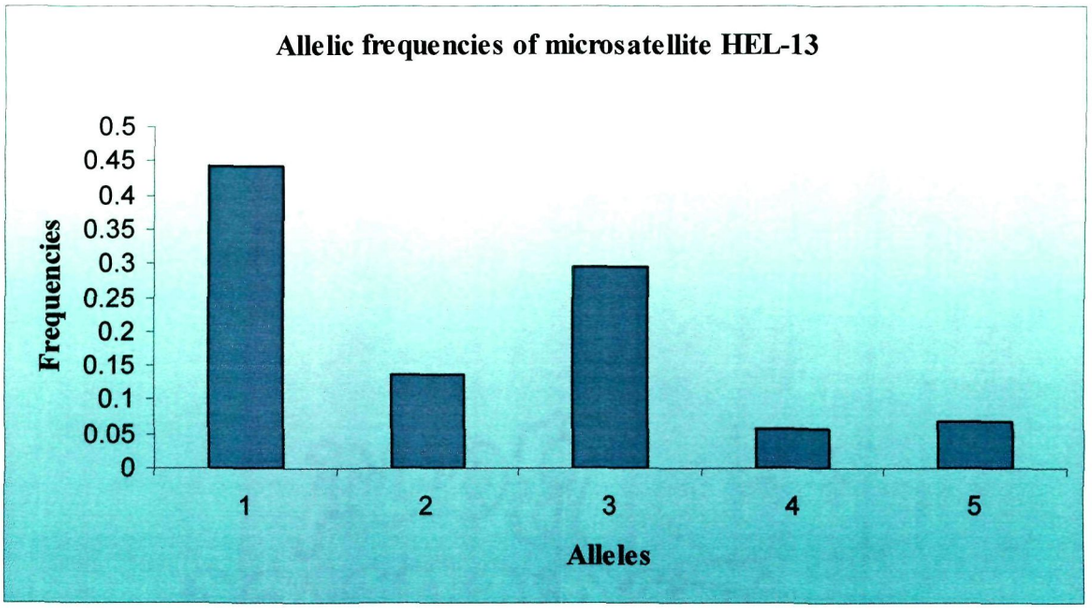


Figure 13: Allelic frequencies of microsatellite HEL-13 in Hill cattle of Himachal Pradesh.

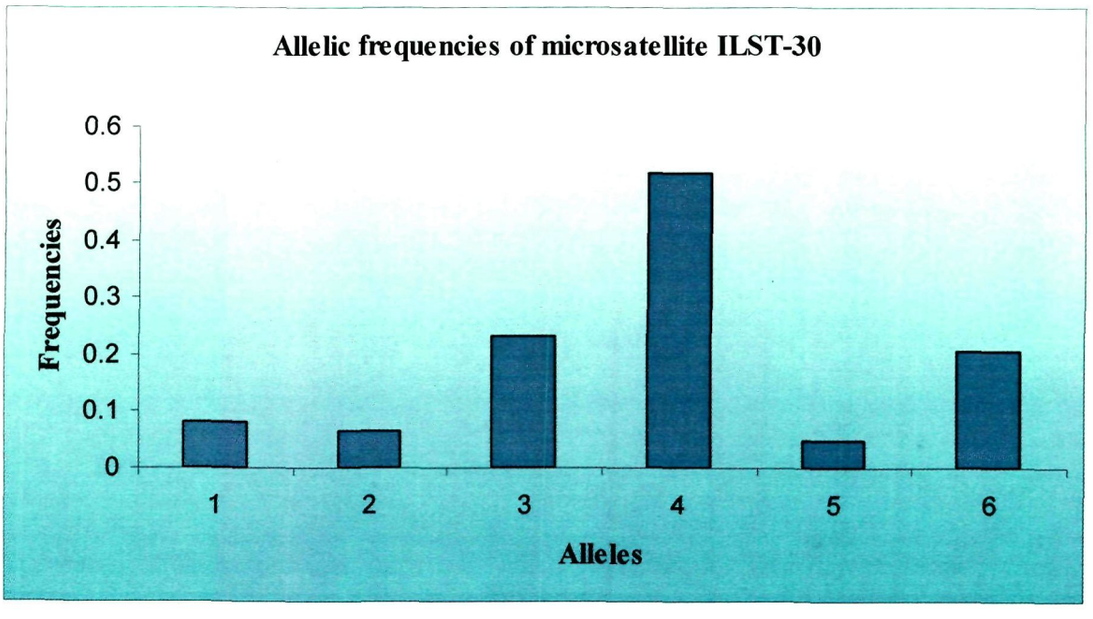


Figure 14: Allelic frequencies of microsatellite ILST-30 in Hill cattle of Himachal Pradesh.

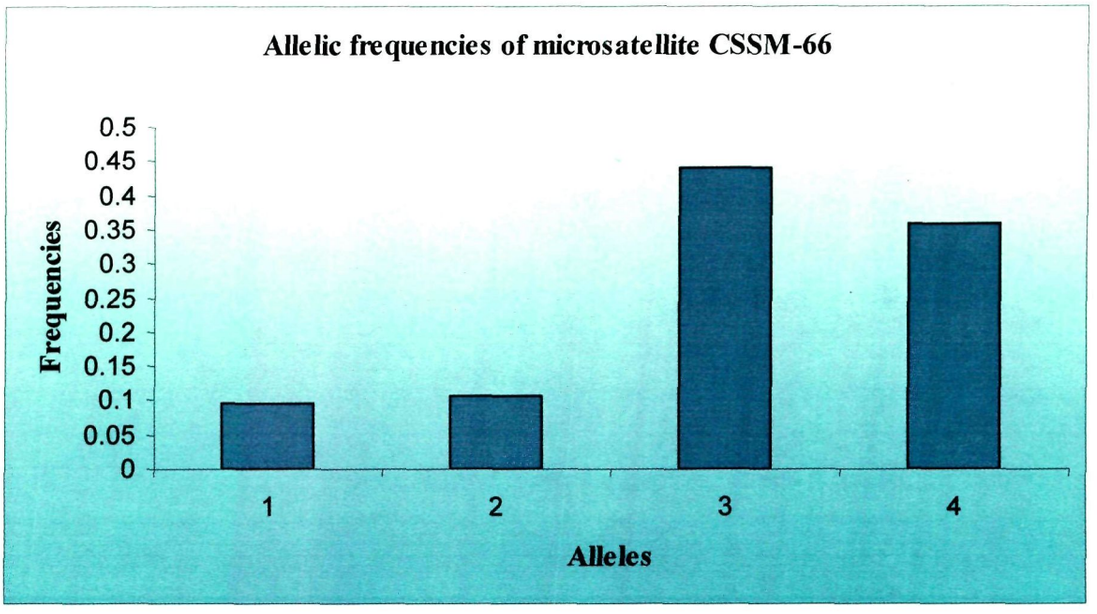


Figure 15: Allelic frequencies of microsatellite CSSM-66 in Hill cattle of Himachal Pradesh.

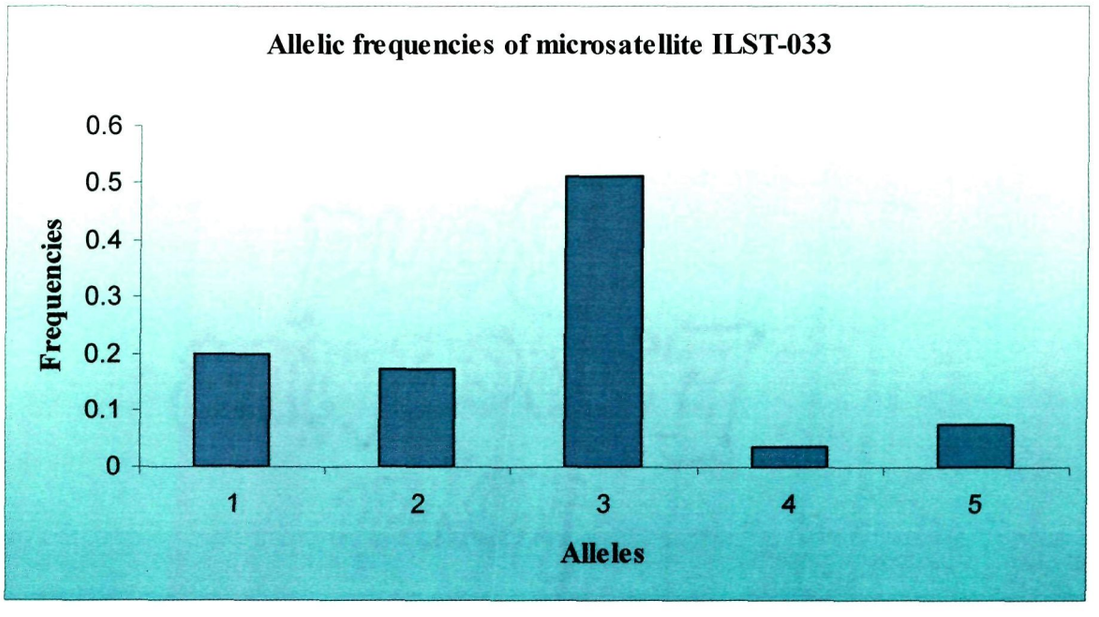


Figure 16: Allelic frequencies of microsatellite ILST-33 in Hill cattle of Himachal Pradesh.

Chapter V

Discussion

CHAPTER V

DISCUSSION

Microsatellites or simple tandem repeats (STRs) were chosen for characterizing Hill cattle of himachal Pradesh as they are ubiquitously inherited repeat regions in eukaryotic genomes and are stably inherited in a Mendelian fashion. Their short length made them amenable to amplification by PCR and subsequent separation by polyacrylamide gels with the resolution of alleles differing by as little as single base pair. In addition, mutation at certain types of trinucleotide repeat loci has been shown to be responsible for some of the more important human inherited disorders. Some important qualities that underline the practical utility of microsatellites as molecular markers are:

1. They are variable and exhibit a high level of allelic variation.
2. They are co dominantly inherited.
3. All co dominantly inherited alleles in an individual are visible, which is not the case for dominant markers, such as blood groups.
4. They are very versatile in their application can be used to detect genetic variability and population structure differentiation among populations, phylogeny, paternity testing and evaluation of recent genetic and demographic history, such as population bottleneck.
5. They are easily analyzed and occur regularly throughout the genome, making them especially suitable for genetic analysis.

All the 14 microsatellite loci which are documented to be polymorphic in various bovine breeds (MacHugh et. al., 1997, Edwards, 2000, Kim et. al., 2002, Dorji et. al., 2003, Jordana et. al., 2003, Metta et. al., 2004, Mukesh et. al., 2004) amplified effectively and generated specific banding patterns from which individual genotypes were assessed.

In the study of livestock breeds where the primary focus is on estimating breed relationships, it is assumed that the markers are neutral to selection and populations are in equilibrium under drift and migration (Barker et al. 1999). Therefore, our understanding of genetic structure of Hill cattle breed for estimation of breed relationships will benefit from analysis of microsatellite markers and population structure of investigated breed by estimation of diversity variables. Several genetic variability measures namely number of alleles, heterozygosity levels and values of gene diversity for each marker and mean diversity indices (H) for the investigated breed calculated from allele frequency data assuming the population in Hardy- Weinberg equilibrium. We therefore studied these parameters using FAO-Dadis cattle primers which have shown adequate polymorphism in other breeds studied earlier by Sodhi et al., 2005.

The Ewens- Watterson test for neutrality is considered as an index of neutrality of markers from selection process operative in the population. Our data indicated that observed F value of all microsatellite loci except ETH-03 lied within the lower and upper boundaries of the 95% confidence region for expected F (Table 7). These results proved the neutrality of tested microsatellite loci, suggesting as no failure due to any kind of selection imposed on these loci. Therefore, these microsatellite markers supported their usefulness to establish genetic structure of Hill cattle population of himachal Pradesh similar to other analysis of genetic relationships among Indian cattle breeds as reported by Sodhi et al. 2005. All the 14 microsatellite markers were effective for the evaluation of polymorphism in alleles. The microsatellite loci showed high level of genetic variability as exhibited by wide range of alleles which varied from 2 (ETH-3) to 8 (HAUT-27) across different loci. Genetic variation was determined by Moazami-Goudarzi *et al.* (1997)

between 10 cattle breeds by using 17 microsatellite loci. A total of 210 alleles of the 17 microsatellites were detected in the study.

High mutation rate accounts for the large variability in microsatellites. Unequal recombination exchange between homologous chromosomes during meiosis and polymerase slippage mechanism that tend to produce non-identical copies of repeated DNA sequences seem to contribute to high microsatellite mutation rate. The level of variation depicted by number of alleles at each locus in the present study was similar to earlier reports in cattle, dog and sheep breeds (Buchanan *et al.* 1994; Alter *et al.* 2001 and Glowtzki *et al.* 1995). The allelic diversity serves as a measure of genetic variability having direct impact on differentiation of breeds within a species. Comparison of effective number of alleles (that represents the number of equally frequent variant) with observed alleles at each locus give information about the predominance of certain alleles in each locus. The numbers of alleles forms the basis of all other diversity indices and genetic distance measurements, and are thus extensively used for construction of phylogenetic trees. In a study conducted by Okomo (1997) a total of 208 alleles were detected across the 18 autosomal microsatellite loci. Mean number of alleles ranged from 4.3 in the N'Dama to 7.7 in the Kenya Boran. No significant difference was found between the number of alleles in the two Sanga breeds (Danakil and Abigar) and the African zebu. A total of 168 microsatellite markers were tested by Hooft *et al.* (1999) for PCR amplification on a test panel of seven African buffalo. Amplification was observed for 139 markers (83 %) from which 101 markers were studied further with 91 (90%) being polymorphic. The mean number of alleles per marker was 5·0 ±0·2 and the mean heterozygosity per marker was 0·61±0·03. Considering the overall high level of polymorphism, it was concluded that most

bovine microsatellite markers are applicable in African buffalo. Genetic diversity among Canadienne, Brown Swiss, Holstein and Jersey cattle was estimated by Hansen *et al.*(2002) from relationships determined by genotyping 20 distantly related animals in each breed for 15 microsatellites located on separate chromosomes. The allele size variance among breeds was similar, but varied considerably among loci. All of the loci studied were equally heterozygous, as in Brown Swiss, Canadienne, and Holstein cattle (0.68-0.69) whereas Jersey cattle showed lower heterozygosity (0.59).

Allele frequency distribution at the 14 analyzed loci varied between 0.0128 (INRA-23) and 0.9125 (ETH-152). Similar to present studies considerable variation in the distribution of allele frequencies between loci has also been been reported by Sodhi *et al,* 2005. It has previously been demonstrated that a set of alleles carried by an organism for a panel of microsatellites can be used to identify the source population. Using the allele frequency spectra Buchanan *et al.* (1994) were able to assign a breed designation to simulated genotypes of sheep populations with an accuracy approaching 100% in most cases. Low frequency of most common alleles (95%) at each investigated locus further supported polymorphic nature of the used microsatellites and their utility in measurement of diversity indices based genetic polymorphism studies. Non amplifying alleles due to mutation at PCR primer sites have been frequently reported particularly when the markers are transferred between species. In our study, the clear deficiency of heterozygotes observed at the loci ETH-3 and ETH-152 (expected unbiased heterozygosity 0.1750 and 0.1649 respectively) in Hill cattle suggested that this may possibly be the situation in some of these cases. Other parameters are also indicative of the genetic variation included gene diversity. One hundred and eight microsatellite primer pairs, originally identified from

cattle, were evaluated by Navani *et al.* (2002) for their applicability in buffalo. Eighty-one primer pairs (75%) amplified discrete products, and of these, 61 pairs (56%) gave polymorphic band patterns on a panel of 25 buffaloes. The mean number of alleles per polymorphic marker was 4.50±0.20, and the mean heterozygosity per polymorphic marker was 0.66 ±0.02. Machado *et al.* (2003) in their recent studies observed a total of 64 alleles in all 4 breeds were obtained with 9 primers used and each breed showed an average of 53% of the total number of alleles. The average number of alleles per locus was 7.11±3.2. The most informative locus was BMS1237 (53% of direct count heterozygosity) and the least informative one was BMS 3004 (12%). Two loci were found to be very little informative. BMS3004 showed very little heterozygosity (5%) for Gyr, Guzerat and Holstein breeds and BL4 showed 5% heterozygosity in Gyr, 0% in Nellore and 11% in Guzerat. BMS1237, on the other hand, showed very high heterozygosity for Gyr (33%), Nellore (53%), Guzerat (41%) and Holstein (82%).

In order to maintain genetic conservation programmes with the objective criteria, the general genetic variability of investigated breed by estimation of average diversity measures has to be taken into account. Medium mean number of alleles per locus (4.4285) displayed sufficient genetic variation in Hill cattle population of Himachal Pradesh however; lesser variation with in each locus suggests that the experimental population could be under mutation drift equilibrium. The number of alleles in the present study further suggested utility of used microsatellites in construction of higher resolution linkage maps of cattle genome of indigenous known and lesser known breeds.

Heterozygosity is an appropriate measure of genetic variability within a population when populations are expanding. Therefore heterozygosity values were used as an estimate

for variability of surveyed cattle population. Average observed heterozygosity level at all loci was 0.4311 and significantly lower than mean expected heterozygosity values 0.6051 for Nei's estimates. The low heterozygosity values reflected the presence of lower number of polymorphic loci in Hill cattle population. This lower level of heterozygosity may be because of lesser number of breeding bulls in the region and some degree of inbreeding in the population cannot be ruled out. These observations on molecular data supports our observation of field data recording in this district where it was general expression of farmers that cows from one village are served by one or two bulls in the main breeding season by natural mating. This implies a higher amount of genetic variability that can be exploited even in populations of small sizes and employed in planning breeding strategies. However, lower values of heterozygosity reflect a considerable loss of biodiversity as a consequence of decline in numbers and inbreeding. The significant differences between observed and expected heterozygosities suggested that there is some degree of inbreeding in the population. In the context of setting priorities for conservation, Barker (1999) reported that breed with lowest average heterozygosity should be preferred in choosing breeds that otherwise have equal priority.

Fifteen bovine microsatellites were evaluated by Schnabel *et al.* (2000) for use in parentage testing in 725 bison from 14 public populations. The number of alleles per locus ranged from five to 16 in bison and from five to 13 in cattle. On an average, expected heterozygosity was slightly lower in bison than in cattle. A core set of 12 loci was further refined to produce a set of multiplexed markers suitable for routine parentage testing.

As less levels of genetic heterogeneity was further reflected within Hill cattle by average gene diversity of 1.0761 (Saitbekova *et al.1999)* and mean PIC value of 0.513

(Alter *et al.200l).* The high estimates of PIC greater than 0.50 further substantiated the suitability of used set of markers to applications such as genetic distance measurements, linkage mapping programmes, parentage control (Kemp *et ai.1995).* Similar tendencies of the variables viz., mean number of alleles and mean heterozygosity estimates observed in the present finding were also reported by Yang *et al.* (1999) in Chinese breeds.

Estimated means of Wright's fixation index (Fis) for Hill cattle population 0.315 revealed a moderate level of inbreeding, which may be explained by the absence of any directional selection. These results are in accordance with that of previously reported for Greek population by Maule *et at.* 1990. They further observed from the patterns of within population genetic variation at marker loci and suggested that it is possible to deduce demographic factors important to the conservation of domestic animal diversity. Breeds can be observed to have lost within population genetic variation as a result of low effective population sizes and increased inbreeding.

The microsatellites HEL-5, HEL-9, INRA-063, and BM2113 were used to analyze genetic similarities and differences of geographically isolated Criollo cattle herds in Mexico by Russell *et al.*(2000). Allele frequencies and genotypic deviations from Hardy-Weinberg equilibrium were tested using the GENEPOP program. Eleven alleles were generated at HEL-5 for the populations sampled (149 to 169 bp). Amplification with HEL-9 produced 12 alleles (145, 149 to 169 bp) and showed common high frequency alleles at 149, 157, and 159 bp for animals from all regions. For INRA-063 there were five alleles with 182 and 184 bp in low frequency. For BM2113 there were 10 alleles in the Criollo cattle (125 to 143 bp), with an equal distribution of frequencies for all alleles. Selvi et al (2004) investigated molecular characterization of Mafriwal dairy cattle of Malaysia. The

study showed the allele frequency for the polymorphic loci ranged from 0.01-0.31 and genotype frequencies ranged from 0.03 to 0.33. The mean overall heterozygosity was 0.79. The breed under their investigation showed high genetic variability despite being a nucleus herd.

From this study, it appears that lesser degree of genetic diversity and reduced heterozygosity, despite larger population of hill cattle in Himachal Pradesh is probably because of high level of inbreeding. The increased level of inbreeding could be due to lesser number of breeding bulls because of non-immigration and small number of animals in population, which was supported by our field observations. The exchange of the males from adjacent places may solve the problem.

Chapter VI

Summary

CHAPTER VI

SUMMARY

India possesses a vast genetic diversity in livestock. The genetic variability within and between breeds has recently been explored using molecular markers. Microsatellite loci are the markers of choice for genetic exploration.

The present research work was carried out to characterize hill cattle and to study genetic diversity of hill cattle using microsatellite markers. A minimum of 50 blood samples were collected at random from their respective breeding tracts and brought to the lab on ice. DNA was extracted from blood as per John's method with modifications and dissolved in Tris-EDTA buffer. Quality check and quantification was done by UV Spectrophotometry and electrophoresis on 0.6% agarose gel. The DNA concentration was determined and samples were diluted 10-30 times (approx. 30 ng/µl) with MiliQ water.

Initially sixteen microsatellite loci (MM-8, ETH-152, INRA-005, INRA-23, BM-1824, ETH-03, HAUT-27, ETH-225, CSRM-60, ETH-185, HEL-13, ILST-030, CSSM-66, ILST-033, CSRM-60, & ILST-06) were selected from the available list of 30 microsatellites suggested by FAO/DADIS for estimation of genetic diversity in cattle. The microsatellite loci were amplified from genomic DNA samples by PCR using locus specific primers by standard PCR protocol. The PCR protocol was same for all the primers except annealing temperature comprised of initial denaturation at 94°C for 10 minutes followed by 39 cycles of denaturation at 94 °C for 15 seconds, annealing at 55-58 °C for 20 seconds and extension at 72 °C for 20 seconds and final extension at 72 °C for 10 minutes. PCR amplification was confirmed on 1.5 % agarose gel containing ethidium bromide.

The amplified products visualized as a single compact band of expected size under UV light were documented by gel documentation system. The PCR products for different

microsatellite loci were resolved on 6% denaturing (urea) polyacrylamide gels along with 10 bp DNA ladder at 80 W (1600-2500 V). Microsatellite alleles were visualized by silver staining. Genotyping of each sample was done and allelic size was determined.

Fourteen microsatellite markers (MM-8, ETH-152, INRA-005, INRA-23, BM-1824, ETH-03, HAUT-27, ETH-225, CSRM-60, ETH-185, HEL-13, ILST-030, CSSM-66, & ILST-033) which gave amplification in the cattle breed were included in the analysis. The observed alleles, effective number of alleles, gene frequency, observed heterozygosity, expected heterozygosity parameters were estimated through PopGene Programme.

In hill cattle, the mean observed and effective numbers of alleles were found to be 4.7143±1.6375 and 2.9283±1.1588 respectively across all loci. Total 66 alleles were observed in hill cattle with maximum alleles (8) contributed by locus HAUT-27 and the least alleles (2) by ETH-3. The average heterozygosity was observed to be 0.4311±0.2579 across all loci. The observed and expected heterozygosity (0.9565 and 0.8383 respectively) were highest for ETH-185 and BM-1824 locus and least (0.0652 and 0.1649 respectively) for ETH-225 and ETH-152 locus.

Except ETH-03, all other microsatellites (MM-8, ETH-152, INRA-005, INRA-23, BM-1824, HAUT-27, ETH-225, CSRM-60, ETH-185, HEL-13, ILST-030, CSSM-66, & ILST-033) had shown high levels of variability. The screened loci were not found to be in accordance with Hardy- Weinberg equilibrium proportions for several microsatellite frequencies.

Following conclusions could be drawn from the results obtained

1. 13 out of 14 primers had desired neutrality and these markers explain adequate usefulness for genetic diversity analysis in Hill cattle population of Himachal Pradesh.
2. The results show the occurrence of alleles at various frequencies is not because of chance.
3. The effect of selection or mutation in the long run can not be ruled out and the alleles exist in the random mating population in the cattle of this region explaining the genetic diversity existing within population.
4. On an average, the heterozygous deficiency was higher at different loci, which indicates that the heterozygosity is being lost at different loci. This could be due to higher level of inbreeding in the population.
5. Lesser degree of genetic diversity, reduced heterozygosity, despite larger population of hill cattle in Himachal Pradesh is probably because of high level of inbreeding.
6. The increased level of inbreeding could be due to lesser number of breeding bulls in the region, which was supported by our field observations.
7. There is a need to put more bulls for natural breeding or semen from more number of elite bulls be made available to cover the breeding cows in the region.
8. The study can be extended to include large many microsatellites in a large sample size to further validate the results.

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APPENDICES

APPENDIX-I

SOLUTION AND BUFFER

Extraction/ digestion (SE) buffer:

Tris (pH 8.0)	10mM
NaCl	400mM
EDTA (pH 8.0)	2mM

Tris EDTA:

Tris.CI (pH 8.0)	10mM
EDTA	1mM

0.5 M EDTA:

Dissolve 18.91gms of EDTA in 80 ml distilled water by stirring the solution vigorously. Adjust the pH to 8.0 using 10M NaOH. Make the final volume to 100 ml.

10% APS:

Take 1gm of Ammonium persulphate powder; dissolve in 10 ml distilled water.

10%SDS:

Dissolve 1gm of Sodium dodecyl sulphate in 10 ml of sterile distilled water in sterile glass vial and keep the solution at room temperature.

Phenol equilibration (Tris saturation):

Melt 500 ml of phenol at 68°C for one hour. Add 8-hydroxy quinoline at a final concentration of 0.1 %. Saturate phenol with equal volume (500 ml) 1 M Tris (pH 8.0) until pH of phenol reaches 7.8. Store phenol under 100 ml 0.1 M Tris (pH 8.0) containing 0.2% beta mercaptoethanol in a brown colored bottle and store at 4°C.

RBC lysis buffer:

NH_4Cl	155 ml
$KHC0_3$	10 ml
EDTA	2 ml

Volume made up 1000 ml by distilled water, sterilized by autoclaving.

Proteinase K:

Dissolve 20 mg of proteinase K in 1 ml of double distilled water. Make 400 µl aliquots per vial and store at -20°C. Make up to a final volume of 100 ml, sterilize by autoclaving.

Ethanol 70%:

Ethanol 99.9%	70 ml
Distilled water	30 ml

TE buffer:

Tris (1 M, pH 8.0)	1.00 ml
EDTA (0.5 M, pH 8)	200 µl

Final volume made up to 100 ml sterilized by autoclaving and store at room temperature.

Agarose gel:

	0.6%	**1.5%**
Agarose	0.60 gm	1.50 gm
TAE (IX)	100 ml	100 ml

Microwave for 3 min., add 4.5 µl EtBr.

Ethidium Bromide (10 mg/ml):

EtBr	0.2 gm (200 mg)

Add 20 ml of distilled water. Stirr it on a magnetic stirrer for several hours to ensure that the dye dissolves completely. Wrap the container with aluminum foil and store at 4°C.

Page Mix:

Acrylamide powder	57 gm
Bis-acrylamide	3 gm
Urea	480 gm
TBE	100 ml

Make up total volume 1000 ml with distilled water.

Page gel casting:

Page mix	80 ml
APS	400 µl
TEMED	40 µl

Preparation of 6X gel loading dye:

Bromophenol blue	0.25% (w/v)
Xylene Cyanol FF	0.25% (w/v)
Glycerol in water	30 % (v/v)

Store at 4°C.

TBE buffer (10%):

Tris base	108 gm
Boric acid	55 gm

EDTA (0.5 M, pH 8.0)	40 ml

Add double distilled water to 1000 ml, filter and autoclave before use. Store at room temperature.

TAE buffer (50X):

Tris base	242 gm
Na_2 EDTA.$2H_20$	37.2 gm
Glacial acetic acid	57.1 ml

Make up final volume 1000 ml with deionized water. Store at room temperature or 4°C.

Preparation of ladder (working):

Take 2 µl of *invitrogen ladder* and dilute it 10times with dye (add 18 µl dye).

Fixative (10 X) solution:

Take 200 ml of acetic acid and dilute it 10X with distilled water or add 1800 ml distilled water.

Silver staining solution:

Silver nitrate ($AgNO_3$)	2 gm
Distilled water	2000 ml
Formaldehyde	2250 µl

Developing solution:

Sodium Carbonate	60-80 gm
Distilled water	1500 ml
Sodium thiosulphate (STS)	200 µl
Formaldehyde	2250 µl

Mix content well and store at -20°C for half hour before using.

Bind Saline (working):

Take bind saline (stock)	3 µl
95% ethanol	1 ml
Glacial acetic acid	5 µl

1 % Sodium thiosulphate:

STS	1 gm
Distilled water	100 ml

Preparation of dNTP's'(working) for PCR:

Take 10 µl of each dNTP's in a micro tube and 600 µl of autoclaved water. Now centrifuge it for a few second for proper mixing.

APPENDIX-II

INSTRUMENTS

Name	Company
Autoclave	Yarco, India
Centrifuge	HERMLEZ-233MK2, SIGMA 4K 15, USA
Distilled Water Unit	Millipore
Electronic Balance	Adair dutt AD-180
Filter Paper(0.22 µl)	Millipore
Horizontal Electrophoresis Assembly	BIO-RAD, Pharmacia Biotech.
Horizontal Agarose Cast Assembly	BIO-RAD
Micropipette	Eppendorf
Microwave Oven	Ken star
pH Meter	Thermo electron Corporation
Thermal Cycler	BIO-RAD, MJ Research, USA
UV Transilluminator	UVP (Ultra violet product), LKB Broma
Vacuum Pump	Millipore
Vertical PAGE Assembly	BIO-RAD Seq, Gen, USA