"GENETIC CHARACTERIZATION OF BACHAUR CATTLE IN ITS NATIVE TRACT"

THESIS

SUBMITTED TO THE

RAJENDRA AGRICULTURAL UNIVERSITY

(FACULTY OF POST-GRADUATE STUDIES)

PUSA (SAMASTIPUR), BIHAR

In partial fulfilment of the requirements

FOR THE DEGREE OF

MASTER OF VETERINARY SCIENCE

(ANIMAL BREEDING AND GENETICS)

By

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2007

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CERTIFICATE-I

This is to certify that the thesis entitled **"Genetic Characterization of Bachaur Cattle in its native tract"** submitted in partial fulfillment of the requirements for the Degree of **Master of Veterinary Science (Animal Breeding & Genetics)** of the faculty of post-graduate studies, Rajendra Agricultural University, PUSA, Samastipur, Bihar is the record of bonafide research work carried out by **Dr. Subhash Chandra Prasad Singh, Registration No. M/ABG/18/2005-2006,** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that the assistance and help received during the course of this investigation and preparation of the thesis have been fully acknowledged.

29/5/07

(Siya Ram Singh)

Major Advisor &
Chairman of the Department

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CERTIFICATE-II

We the undersigned members of the Advisory Committee of **Dr. Subhash Chandra Prasad Singh, Registration No. M/ABG/18/2005-2006**, a candidate for the degree of **Master of Veterinary Science** with major in **Animal Breeding and Genetics** have gone through the manuscript of the thesis and agree that the thesis entitled **"Genetic Characterization of Bachaur cattle in its native tract"** may be submitted by **Dr. Subhash Chandra Prasad Singh** in partial fulfillment of the requirements for the degree.

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This is to certify that the thesis entitled **"Genetic Characterization of Bachaur cattle in its native tract"** submitted by **Dr. Subhash Chandra Prasad Singh, Registration No. M/ABG/18/2005-2006** in partial fulfillment of the requirements for the degree of **Master of Veterinary Science (Animal Breeding and Genetics)** of the faculty of post graduate studies, Rajendra Agricultural University, PUSA, Samastipur, Bihar was examined and approved on 05./01../ 2008.

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ACKNOWLEDGEMENT

Firstly I would like to thank my father and my mother and all the family members who gave me their blessings for completing my thesis work.

I am overwhelmed with rejoice to avail this rare opportunity to evince my profound sense of reverence and everlasting gratitude to ***Dr. S. R. Singh*** *, Chairman, PG Department Of Animal Breeding and Genetics Bihar Veterinary College Patna-14 for his kind efforts in getting the permission for my thesis work and his valuable guidance throughout the M. V. Sc. course. I express my heartiest thanks to* ***Dr. S. P. S. Ahlawat*** *Director, NBAGR and* ***Dr. R. K. Vijh*** *Principal Scientist & Incharge Technology and Business Promotion cell, NBAGR, Karnal for granting me permission to undertake my thesis work for partial fulfillment of degree of Master of Veterinary Science in Animal Genetics and Breeding.*

I feel immense Pleasure in expressing my deep sense of gratitude, appreciation, and heartfelt thanks to ***Dr. Rekha Sharma****, Scientist (SS), Core Lab NBAGR Karnal, My Co-advisor for her worthy guidance, meticulous supervision, constant encouragement, constructive criticism and help throughout the thesis work.*

I find no words to express my sense of sincere gratitude and indebtedness to ***Dr. A. K. Pandey****, Senior Scientist, Core Lab, NBAGR, Karnal for his invaluable guidance, sustained encouragement generous help and immense care given during the entire course of this study*

I am indebted and I owe my heartfelt thanks to all the members of my ***advisory committee*** *for their valuable suggestions throughout the M. V. Sc. Course. In addition I express my special thanks to* ***Mr. Yatendra Singh*** *(JRF, Core Lab) and* ***Mr. Vinod*** *(Lab Attendent, Core Lab) for providing me academic assistance and other infrastructural facilities to carry out the present study.*

I am extremely thankful to my friends ***Dr. Brishketu*** *and* ***Dr. Ramesh*** *for their extraordinary help during my stay in Karnal.*

I sincerely thank my wife ***Suniti Singh*** *and brother- in- law* ***Krishna Chandra*** *whose unbiased cooperation motivated me for the completion of this assignment.*

At last but not the least, I sincerely thank all the people in general and ***Dr. Pramod Prabhakar*** *in particular who helped me for completion of this work. Their critical input was an important factor that shaped its final content and organization. Not everyone is mentioned but none is forgotten.*

Needless to say all errors and omissions are mine.

29.05.07

Subhash Chandra Prasad Singh

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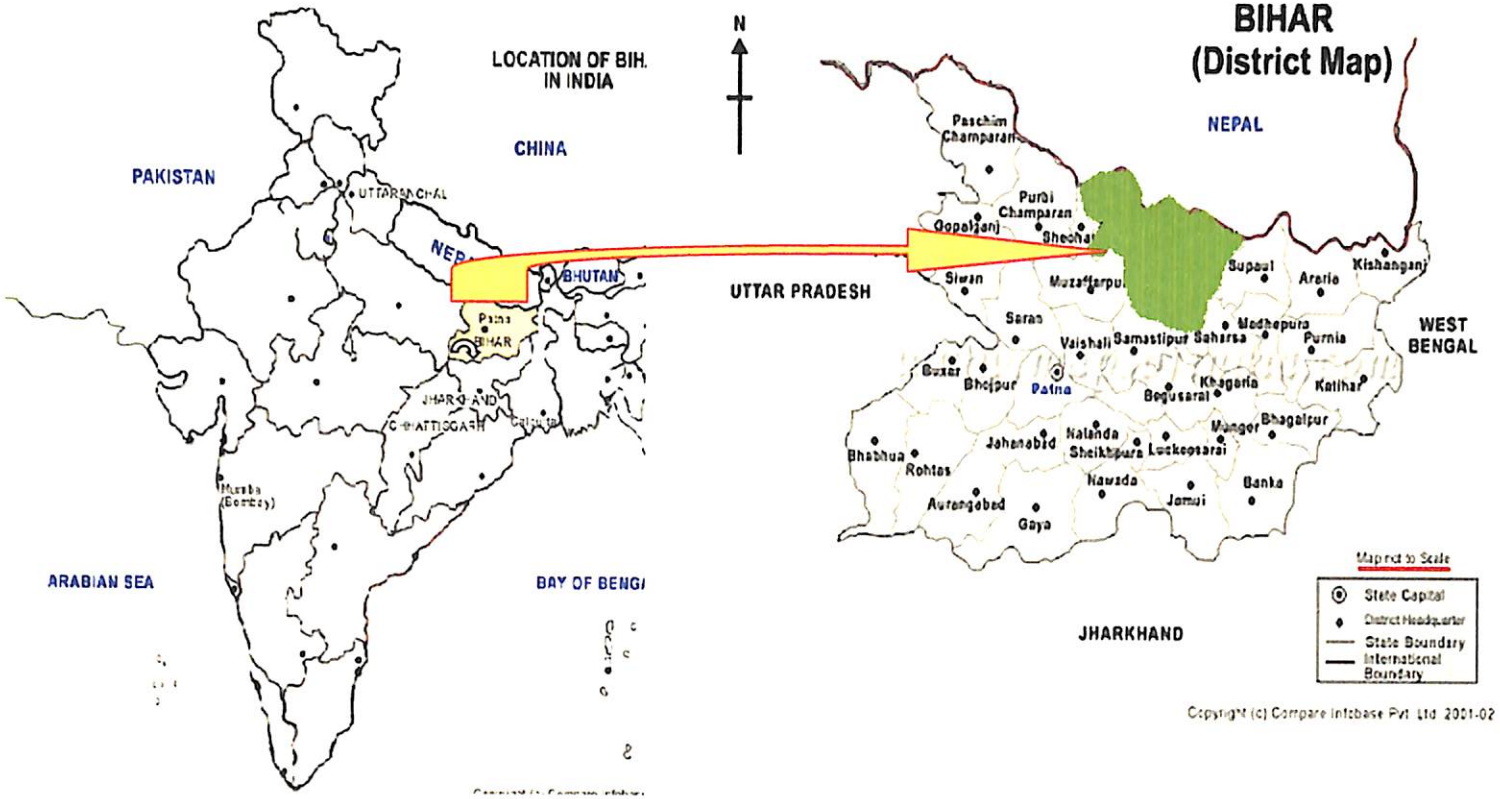
Abbreviations

AFLP	:	Amplified Fragment Length Polymorphism
$AgNO_3$	:	Silver Nitrate
AI	:	Artificial Insemination
APS	:	Ammonium per Sulphate
bp	:	Base Pairs
BV	:	Breeding Value
DNA	:	Deoxy Ribonucleic Acid
dNTP	:	Deoxy nucleotide tri Phosphates
EDTA	:	Ethylene Diamine Tetraacetic Acid
EtBr	:	Ethidium Bromide
FAO	:	Food and Agricultural Organization
F_{is}	:	Wright's Fixation Index
HWE	:	Hardy Weinberg Equillibrium
IAM	:	Infinite Allele Model
kb	:	kilo base
$KHCO_3$	:	Potassium bicarbonate
VNTR	:	Variable Number of Tandem Repeats
mt	:	Mitochondria
mg	:	Milligram
ml	:	Milliliter
min	:	Minutes
mM	:	Milli Molar
M	:	Molar
ng	:	Nanogram
Ne	:	Effective number of alleles
No	:	Observed number of alleles
nm	:	Nano metre

NaCl	:	Sodium Chloride
NH_4Cl	:	Ammonium Chloride
OD	:	Optical Density
PAGE	:	Polyacrylamide Gel Electrophoresis
PCR	:	Polymerase Chain Reaction
RFLP	:	Random Fragment Length Polymorphism
RAPD	:	Random Amplified Polymorphic DNA
RNA	:	Ribonucleic Acid
rpm	:	Rotation Per Minute
SDS	:	Sodium Dodecyl Sulphate
STR	:	Short Tandem Repeats
SSR	:	Simple Sequence Repeats
SMM	:	Stepwise Mutation Model
ss DNA	:	Single Stranded DNA
TPM	:	Two Phase Model
Taq	:	Taq Polymerase (from *Thermus aquaticus*)
T E	:	Tris EDTA
TBE	:	Tris Borate EDTA
TAE	:	Tris Acetate EDTA
TEMED	:	N N N'N' Tetramethyl Ethyl Diamine
Tris-HCl	:	Tris hydrochloride
µm	:	Micrometre
°C	:	Degree Celcius
µl	:	Micro Litre

INTRODUCTION

Fig. 1 Breeding Tract of Bachaur Cattle



India is one of the Mega-biodiversity centres in the world. It possesses more than 6% world's livestock diversity although its world's geographical share is only 2.3%. India ranks first in the cattle (185.18 millions) and Buffalo (97.92 millions) population, second in goats (124.36 millions), third in Sheep (61.47 millions) and fifth in Chicken (489.01 millions) population in the world (Livestock Census, 2003). In addition, India has good number of pigs (135.18 lakh), Horses (7.51 lakh), Mules (1.76 lakh), Donkey (6.50 lakh), Camel (6.32 lakh), Mithun (2.78 lakh), Yak (0.65 lakh).

India is an agrarian country where about 72% population lives in more than six lakh villages, of which more than 80% people directly or indirectly depend on agriculture and allied activities. Thus being complementary to the agriculture, Indian livestock resources play very important role in the national economy. Although share of GDP from agriculture as a whole has been declining over the years, the contribution of livestock in the GDP increased from less than 5% in 1980-81 to 5.4% in 2002-2003 at current prices.

Food and Agriculture Organisation(FAO) identifies 1568 cattle breeds distributed throughout the world (FAO, 2000) of which more than 55% are in the developing countries (FAO, 2000). As per Indian Council of Agricultural Research (ICAR) India is the breeding tract of 30 breeds of cattle (61 as per FAO); 15 breeds of buffaloes (19 as per FAO); 20 breeds of goats (29 as per FAO); 42 breeds of sheep (59 as per FAO); 3 breeds of pigs; 18 breeds of Poultry; 6 breeds of Horses (9 as per FAO); 3 breeds of Donkey and 6 breeds of Camel. Amongst cattle breeds, some of the breeds like Sahiwal, Tharparkar, Gir, Red-Sindhi are milch breeds; Hariana, Rathi, Deoni, Kankrej, Krishna-valley are dual purpose breeds and Bachaur, Amritmahal, Kenkatha, Kherigarh, Siri are draught purpose breeds. These breeds are famous for their utility in India as well as abroad. In addition to

described breeds about 80% indigenous livestock falls in non-descript category.

Such a mega-biodiversity in India has been the result of continuous evolutionary processes resulting in adaptation to the harsh climate, disease resistance to the various tropical diseases, better feed efficiency and production potential at low input in terms of feeds and fodders. The animal genetic resources and their diversity throughout the world including India are in a dramatic state of decline. The indiscriminate uses of cross breeding and grading up have been proved as the important cause of this decline. Recent techniques like Artificial Insemination (AI), Embryo Transfer Technology (ETT) have further aggravated the situation. Other causes are geographical reorganization; Purpose based farming system, mechanization of the agricultural works etc.

All these factors collectively resulted into the disappearance of a substantial number of uncharacterized local population of cattle alongwith the unique gene and gene combinations. It cannot be predicted that what might be needed in the future and what might be the consequences of the extinction of the livestock genetic diversity. Thus as a genetic security the conservation of native breed populations for their use in future is required.

The International Union for the Conservation of Nature and Natural Resources (IUCN) has defined the need for conservation as "The management for human use of the biosphere so that it may yield the greatest sustainable benefits to present generations while maintaining its potential to meet the needs and aspirations of future generations. Thus conservation is positive, embracing preservation, maintenance, sustainable utilization, restoration and enhancement of the natural environment". As a fundamental step in the process of conservation the phenotypic and genotypic characterization of breeds are done to know the

1. Overall magnitude of genetic diversity within the breed, phenotypically as well as at gene level.
2. Structure of relationship expressed as genetic distance amongst breeds.
3. Genetic uniqueness of the breed.
4. Degree of endangerment.
5. Cultural and historical value of the breeds.

As these conservation processes are very costly thus the prioritization of conservation for a breed should be very rational. Therefore, phenotypic characterization including livestock survey and preparation of breed descriptors as well as molecular genetic characterization are usually done. Molecular genetic characterization of a breed in a species is basically the measurement of genetic variability within and between the breeds. Array of mplecular markers are used for estimation of genetic diversity i. e. RFLP, AFLP, VNTRs-which includes minisatellites (15-60 bp repeats) and microsatellites (1-6bp repeats). Amongst all, microsatellites are now considered as the most powerful genetic markers for characterization and biodiversity evaluation.

Bachaur, a famous draught purpose cattle breed was concentrated in Bachaur Pargana (Madhubani, Sitamarhi and Darbhanga districts of Bihar). The breeding tract has shifted with time to northern part of Bihar bordering Nepal. The percentage of genetically viable population of Bachaur cattle in Madhubani, Sitamarhi and Darbhanga districts of Bihar were extrapolated to be 3.288%, 1.500% and 0.459% respectively, the overall value being 3.072%. Estimated breed population is approximately 11000 only (Singh, *et al.*, 2004). Bullocks of this breed are reared by the farmers, who are still dependent on traditional method of farming. Bullocks have excellent draft capacity. They can work for 6-7 hours continuously even in extremes of the weather.

Bachaur cattle are managed under extensive management. The animals are white to gray in colour with black muzzle, and eyelids. Switch of the tail is up to hock joint. Horns are small and straight. The average body length, height at withers and body girth are 125, 118 and 165cm in male and 120, 150 and 112 cm in females respectively. The average adult body weight is 270 and 243kg in male and female respectively. The age at first calving, calving interval and service period averaged 46 months, 400 days and 60 days respectively. The cows are poor milker producing an average of 2.2 kg of milk per day with average 5% fat and peak milk yield 3.63kg per day for average 180.08 days of lactation length. Although bullocks are excellent draft animal yet poor milk producing female of this breed is one of the main causes of the endangered status of this breed in addition to the dilution of breed by indiscriminate cross-breeding and mechanization of agricultural work.

In the present study molecular characterization of Bachaur breed of cattle has been done by using 22 FAO recommended highly polymorphic microsatellite markers with the following objectives.

1. To study the genetic diversity within Bachaur Cattle breed.
2. To asses whether the Bachaur Cattle breed has experienced any recent genetic bottleneck.

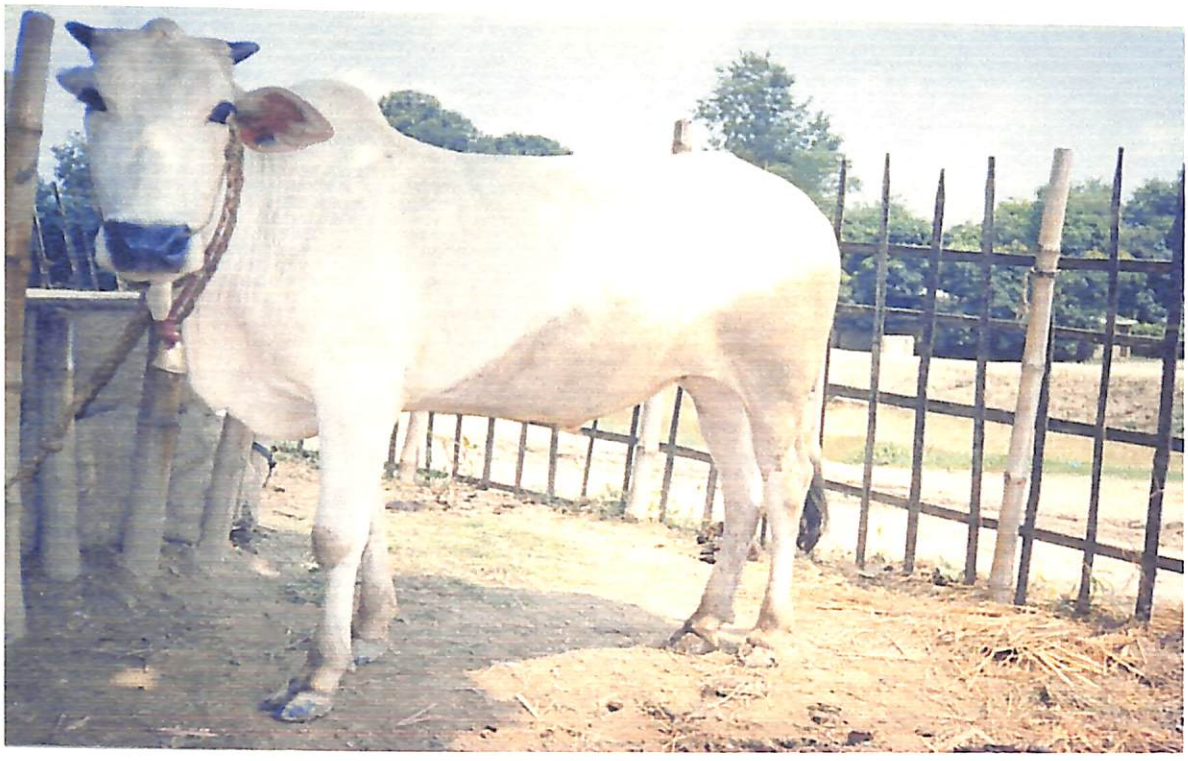


Fig. 2 A Typical Bachaur Bullock

Fig.3 A Typical Bachaur calf

Review Of Literature

2.1 Genetic Characterization.

Genetic characterization is the first step in breed conservation programme of the Animal Genetic Resources. It also has implications in future breeding strategies. It is the description of the genome to measure the genetic diversity and the uniqueness of the breed. As a first step to obtain an unbiased estimate of genetic variation a series of enzyme coding loci generally called Allozymes were studied (Lewontin and Hubby, 1966; Harris, 1966). These studies were based on the electrophoretic mobility of their products with the assumption that this assessment of variation would be independent of the functions of the genes.

Analysis of genomic DNA for the estimation of variation within and between the breeds of a species has been used extensively because of its authenticity and relative ease. The first work of its kind for estimation of differences at genomic DNA has its history in 1960s to 70s when organization of eukaryotic genome was studied (Britten and Kohne, 1968). The technique was **DNA-DNA hybridization**, which is based on the thermodynamic reannealing properties of heterologous single stranded DNA sequences. However, in recent years this technique has lost its ground due to theoretical and practical difficulties and has been replaced by more direct DNA sequence based approaches (Avise, 1994).

Restriction Fragment Length Polymorphism (RFLP) was the first screening technique to be used for detection of variation at DNA level. This technique was used in identifying both disease causing mutation and apparently neutral variants (Botstein *et al*, 1980; White *et al*, 1985; Donis-Keller *et al.*, 1987). In this technique polymorphism at the DNA sequence level can also be visualized as changes in the cleavage pattern of DNA fragments which have been treated with particular Restriction Endonuclease (RE).

Classical studies of the kinetics of DNA reassociation by Britten and Kohne (1968) showed that the genome of Eukaryotes can roughly be

divided in to four fractions such as **Fold back DNA, Single Copy DNA, Middle Repetitive DNA** and **Highly Repetitive DNA**. The middle repetitive DNA having hundreds to thousands base pairs repeated from dozen to thousand times in the genome and in the highly repetitive DNA few to hundreds of nucleotides are repeated thousands to millions time.

Repetitive fraction of the genome may be localized or dispersed among which localized repetitive DNA usually occurs as tandem array, are called Tandem Repetitive DNA (TR-DNA). TR-DNA has attracted much attention in the recent years because many TR-DNA loci are highly variables among individuals in a population. Thus they are very useful markers in biology and medicine. This line of research started with Wyman and White's (1980) observation of a locus highly polymorphic for restriction length variation and with the finding of Bell *et al.*, (1982) that the hypervariable locus near the insuline gene consisted of tandem repeats with multi allelic variation arising through variation in repeat copy number.

2.2 Molecular Marker

Genetic analysis of natural populations by the use of recent DNA technologies such as Polymerase Chain Reaction (Mullis *et al.*. 1986), Microarray technology and Fluorescence *in situ* hybridization (FISH) etc have made it possible to uncover a large number of genetic polymorphisms at the DNA sequence level. They have been used as markers for evaluation of the genetic basis for the observed phenotypic variability. The markers revealing variations at the DNA level are called molecular markers. A series of highly informative molecular markers have been recognized so far such as RFLP, AFLP, RAPD, Mt-DNA and VNTRs.

2.2.1 Restriction Fragment Length Polymorphism (RFLP)

It is a technique in which Restriction Endonuclease (RE) enzymes are used, which recognizes and cleaves specific sequences in DNA, yielding fragments of defined length. Beckmann *et al.*, (1986) screened DNA of Israeli Holstein-Friesian sires for RFLPs using labeled Growth Hormone, rat

muscle beta-actin and bovine chymosine DNA as probes, thereby illustrating the power of RFLPs in detecting genetic variability in the cattle populations. RFLP have successfully been used for characterization of gene of the bovine Mojor Histocompatibility Complex (MHC) (Singurdardottir *et al.*, 1988), growth hormone and prolactin genes in Holstein bulls (Cowan *et al*, 1989), ovine casein genes (Leveziel *et al.*, 1991) and type I interferon gene in cattle (Ryan and Womack, 1993). RFLP, although is specific yet it is laborious and time consuming. Thus with the discoveries of PCR based techniques this method was replaced by other decent methods.

2.2.2 Amplified Fragment Length Polymorphism (AFLP)

The AFLP technique is based on the selective PCR amplification of restriction fragments of genomic DNA ligated with oligonucleotide adapters. The oligonucleotide adapter and restriction site sequence both are used as target sites for primer annealing in the process of amplification of restriction fragments by PCR. The selective amplification is achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extension match the nucleotide flanking the restriction site. Typically 50-100 restriction fragments are amplified and detected on denaturing polyacrylamide gels. The AFLP technique provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity. They have been used in evaluating genetic diversity in Friesian cattle (Ajmone-Marsan *et a.l*, 1996) as well as in estimating genetic distances within and between cattle breeds (Ajmone-Marsan *et al.*, 2002)

2.2.3 Random Amplified Polymorphic DNA (RAPD)

This is a PCR based techniques. In this technique random DNA segments of genomic DNA are being amplified by using arbitrary primers thus called as RAPD and polymorphism generated can be used as genetic markers. RAPD have been used to detect genetic differences among cattle and sheep (Kantanen *et al.*, 1995) and plants (Sulaiman and Hasmain,

1996). Population specific RAPDs from pooled DNA samples of different populations were detected by Kemp and Teale (1994). Lougheed *et al.*, (2000) used RAPD markers along with microsatellites to compare the genetic variation among rattle snake population. Genetic variability within and between breeds among some Indian breed like Hariana, Red Sindhi, and Tharparker was estimated using RAPD fingerprinting (Bhattacharya *et al.*, 2004). Where as (Sharma *et al.*, 2004) used RAPD as molecular marker for molecular genetic characterization of Rathi and Tharparkar cattle breeds.

2.2.4 Mitochondrial DNA (Mt-DNA)

This marker has been used extensively as molecular marker to examine variation and evolutionary relationship among species (Avise, 1991). Animal Mt-DNA being circular contains 22 tRNAs, 2rRNAs and 13 protein coding genes and have the highly variable D-Loop region (Cann *et al.*, 1984). It shows unusual maternal inheritance and high rate of evolution than that of nuclear DNA (Brown *et al.*, 1979). This property of Mt-DNA makes it well suited for use in evolutionary studies (Stoneking and Soodyall, 1996)

Mt-DNA polymorphisms have been used to study the genetic variations between species (Sankoff *et al.*, 1992) within species (Okumura *et al.*, 1996) and within breeds (Bowling *et al.*, 1998). The D-loop polymorphism has been used to differenciate cow lineages (Ron *et al.*, 1992) horse breeds (Kim *et al.*, 1999 and Kavar, *et al.*, 1999), pig breeds (Takeda *et al.*, 1995) and water buffalo (Lan *et al.*,1998)

2.2.5 Variable Number of Tandam Repeats (VNTRs)

This is given by Nakamura *et al.*, (1987). These markers are characterized by a core sequence which consists of a number of identical repeated sequences. They can be divided into two categories based on the repeat length, these are Minisatellites and Microsatellites. The term Minisatellite was originally coined by Jeffrey *et al.*, (1991) to describe any tandem repeat region that is much shorter but has structural similarity to

classical satellite DNA. However it is used in a more restricted sense for a tandem array of repeats with a unit length 9 bp or longer (Shriver, 1993). They are frequently Hypervariable and its hypervariability has been exploited in developing the DNA Finger Printing Technique (Jeffrey, 1985). Now some synthetic minisatellite probes were constructed to develop finger prints of cattle breeds (Builtcamp *et al.*, 1991) and Horses (Ellegren *et al.*, 1992a).

2.2.5.1 Microsatellite

Microsatellite or Short Tandem Repeats (STRs) or Simple Sequence Repeat (SSR) or Simple Sequence Length Polymorphism (SSLP) or Sequence Tagged Microsatellite Repeat (STMR) are the well known DNA marker containing monotonous tandem repeats of 1-6 base pairs. It provides a vast store of polymorphic genetic marker (Tautz, 1989; Weber and May, 1989). The term microsatellite coined by Litt and Luty (1989) to characterize the tandemly repeated simple sequence motifs.

2.2.5.1.1 Properties and Distribution

Microsatellites have been used increasingly as the marker of choice for the genome analysis. It is used to measure genetic diversity within and between the breeds of animal genetic resources. It has several specific properties such as ubiquitous and even distribution throughout the genome with the frequencies as high as 1 in every 10 to 20 Kb (Edwards *et al.*, 1991). They are present on the Non-coding region of the genome and most of them are selectively neutral which make them compatible with the assumptions of most population genetic theories. They follow a typical Mendelian inheritance, which usually expressed in a co-dominant fashion. Microsatellites are often multi-allelic with mean heterozygosity of more than 70 percent. Microsatellites remain unaffected by the environmental factors and generally do not have pleiotropic effects on quantitative trait loci (QTL). Technically microsatellites are more desirable than the other markers as they can be successfully amplified by the Polymerase Chain

Reaction (PCR) followed by appropriate sizing by Polyacrylamide-Gel-Electrophoresis (PAGE). Finally microsatellites are highly polymorphic and have been found to be variable even in a population that has low level of allozyme and Mitichondrial variation (Estoup *et al.*, 1995a, 1995b, 1996; Paetkau and Strobeck, 1994).

2.2.5.1.2 Microsatellite evolution

Microsatellite alleles at a locus in a population change or mutate over time. It differs in the number of repeats without any change in the flanking regions sequence. Microsatellites mutation rates are approximately 10^{-3} to 10^{-4} (Dietrich *et al.*, 1992; Weissenbach *et al.*, 1992.)

2.2.5.1.2.1 Mechanism of Mutation

All the current hypotheses of size change (Mutation) at the VNTRs loci involve mispairing of repeats. They only differ in the timing of the DNA mispairing events i.e. Replication Mitosis and Meiosis. Thus there may be three mechanism of VNTR mutation.

A. Unequal Crossing-Over or Genetic Recombination during Meiosis

This is an inter-chromosomal phenomenon involving homologous chromosomes. Unequal crossing over causes an exchange of flanking markers. However Wolff *et al.* (1989) found that only 1 in 12 size change events at the D1S7 minisatellite locus had a recombination between two flanking markers.

B. Unequal Sister Chromatid Exchange (USCE)

This occurs during Mitosis and less frequently during Meiosis. It is an intra chromosomal phenomenon involving exchange between two copies of same chromosomes.

C. Slipped-Strand Mispairing (SSM) or Replication Slippage

It occurs during replication and appears to be the predominant mode of microsatellite mutation (Wolff *et al.*, 1989). Replication slippage is speculated to occur primarily during lagging strand synthesis (Schlotterer and Tautz, 1992)

2.2.5.1.2.2 Models of Microsatellite evolution

A. Infinite Allele Model (IAM)

According to this model every new mutation is assumed to give rise to a new electrophoretically distinguishable allele called Allelomorph. This model was given by Kimura and Crow (1964) and has proved very successful in explaining the observed allozyme variation (Nei, 1984) and up to some extent in explaining the observed frequencies of irregular composite microsatellite loci i. e. microsatellite made up of 2-3 core repeat units. (Estoup *et al.*, 1995a, 1995b; Shriver *et al.*, 1993)

B. Stepwise Mutation Model (SMM)

According to this model alleles can only mutate by the gain and loss of one repeat unit. SMM explains the condition where high amounts of homoplasy exist such as in case of microsatellite markers because most of the mutations seem to involve the gain or loss of a single repeat unit (Weber and Wong, 1993). This is important as homoplasy leads to the underestimation of the total amounts of variation and genetic distance, and to the overestimation of similarities of the populations.

C. Two-Phase Model (TPM)

This model has been developed recently to explain the variation observed at dinucleotide repeat microsatellite by Di Rienzo *et al.*, (1994). It is based on coalescence theory, that predict's the expected variance in the repeat number under different mutational processes and demographic histories. The TPM incorporates the mutational process of SMM, but allows for mutations of a larger magnitude to occur. The main difference in the model is that once a mutation has occurred it has a probability P to being a one step mutation and a probability 1-P of being a multi-step mutation.

It is clear that the evolution of microsatellites is a complex mutational process involving atleast two different mechanisms. The prevalence of each mechanism varies according to the structure of the repeat unit itself. Three to five bp repeats seems to evolve via the SMM while 1-2 bp repeats are

more likely via TPM. Further the prevalence of single step mutations decreases as the complexity of the repeat core increases. It has been suggested that microsatellites that have a more IAM like evolution (i. e. composite 4 repeats) should be best suited to study population, questions such as population sub division and genetic relationships (Estoup *et al.*, 1995a, 1995b) since they will contain the lowest level of Homoplasy.

2.2.5.1.3 Isolation of Microsatellite Markers

In order to construct the linkage maps, polymorphic markers need to be identified, isolated and characterized. Microsatellite (VNTR) is first detected from the entire genome and their unique flanking sequences are used to develop primers for amplification of the specific microsatellites by PCR. Broadly two strategies are used for the isolation of microsatellite markers.

A. Cosmid derived microsatellite markers

In this strategy, the genomic DNA (gDNA), after digestion with Restriction Endonuclease (RE) is cloned in to suitable vectors mostly cosmids thus forming a cosmid genomic library. The cosmids are then screened with lebelled $(CA)_n$ or $(GT)_n$ polynucleotide probe. The clones that hybridized with the probes are detected by autoradiography. The positive clones are isolated and insert (Microsatellite) that they harbour is sequenced and characterized. Appropriate primers are designed from the flanking region (Ellegren *et al.*, 1992; Toldo *et al.*, 1993; Steffen *et al.*, 1993; Moore *et al.*, 1994; Sunden *et al.*, 1993; Lang and Planty, 1994).

B. Microdissected chromosome derived microsatellite Markers

In this methodology a chromosome spread is obtained from a blood culture. The chromosome of interest is identified under a microscope. This chromosome is dissected using a micromanipulator. The microdissected chromosomal fragments are then used to construct genomic DNA library which is screened with radiolabelled (CA)n or (GT)n probes. Positive clones are isolated and subjected to PCR amplification. The PCR products are

sequenced and the sequences are checked for uniqueness to develop PCR primers (Zhao et al., 1999). A modification in this method involves the amplification of microdissected chromosomal fragments by PCR using degenerate oligonucleotide primers. To the amplified products biotinylated (CA)n probes are added. After denaturation and annealing the annealed DNA is added to Streptavidin paramagnetic particles and incubated to capture DNA fragments hybridized to biotinylated (CA)n probes. The bound DNA is eluted and amplified using appropriate primers. The amplified products are purified and sequenced to be used as markers (Dukhanina and Sverdlov, 1998; Sarkar *et al.*, 2001).

2.2.5.1.4 Applications of Microsatellite

Microsatellites have been established as an extraordinary molecular marker in the field of molecular biology research and have been proved to be usefull for a number of analyses. They have been initially utilized for genetic mapping (example Weissenbach *et al*, 1992; Nakamura *et al.*, 1987; Edward *et al.*, 1991; Silver, 1992) and studies of population structure (Flint *et al.*, 1989; Chakraborty and Jin, 1992; Bruford and Wayne, 1993) as well as extensively used for Linkage analyses in the association with susceptability genes (Friedl *et al.*, 1990). Microsatellite variation have been used to study the amount of recombinations between the closely related species (Gottelli *et al.*, 1994; Roy *et al.*, 1994), comparison of level of variation between species and populations and assessment of overall genetic variation (Gottelli *et al.*, 1994; Paetkau and Strobeck 1994; Taylor *et al.*, 1994). They can be used to estimate effective population size (Allen *et al.*, 1995) and to gain insight into the degree of population substructure including both the amount of migration between subpopulations (Allen *et al.* 1995; Gottelli *et al*, 1994) and genetic relationships among the various subpopulations (Bowcock *et al.*, 1994 Forbes *et al.*, 1995; Estoup *et al.*, 1996; Lade *et al.*, 1996). Presently microsatellites have been exploited to identify QTL for economically important traits. Ron *et al.*, 1994,using

microsatellite markers, identify one marker (D21S4) associated with significant effects on milk and protein yields. Using 159 microsatellite markers in 14 USH Holstein half sib families George *et al.*, 1995 demonstrated the presence of QTL for milk production on five chromosomes namely chromosome No. 1, 6, 9, 10 and 20. In another study significant association of microsatellites markers with somatic cell score (SCS, an indicator for susceptibility to mastitis), productive herd life, milk and meat production traits were established (Kucerova, *et al.*, 2004 and a; Haiguo, *et al.*, 2004). More recently using microsatellite markers Ashwell *et al.*, (1997) identified potential QTL for SCS, fat yield, fat percentage and protein yield and its percentage. Characterization of QTL for economically important traits using microsatellite markers will help in formulating more efficient breeding programme using MAS especially for bulls prior to progeny testing.

2.2.5.1.5 Problems associated with microsatellite markers

During the PCR amplification with microsatellite primers some technical problems are encountered which are as follows: -

A. Null Alleles:

Some alleles of microsatellite fail to amplify in the PCR because of mutation in the flanking region. These are termed as Null Alleles. It leads to serious underestimation of heterozygosity compared with that expected on the basis of Hardy-Weinberg-Equilibrium (Callen *et al.*, 1993). In a heterozygote of two different alleles, if allele fails to amplify due to failure of primer annealing then phenotype will appear as homozygote. This problem may overcomed by designing new primers.

B. Slippage :

Slippage of Taq Polymerase enzyme during PCR is one of the important problems associated with amplification of microsatellite. This leads to production of differently sized products. These are less intense and are referred as shadow banding. Further the Taq Polymerase has a tendency

to add an additional dATP at the 3' end of the amplified PCR products. These sometimes make the allele scoring difficult.

C. Homoplasy:

It can be defined as the co-occurrence of alleles that are identical by descent. If two alleles are inherited without any mutation from the same ancestral allele, they are identical by descent. But two alleles may have the same structure and even the same sequence but may not have been inherited from same ancestral allele, are called Identical by state. Thus Homoplasy helps in estimating the actual divergence between populations. Homoplasy due to different forward and reverse mutation may underestimate genetic divergence. Under the IAM of microsatellite evolution there is lowest level of Homoplasy.

2.3 Microsatellite based genetic characterization of cattle:

Although, the molecular genetic characterization based on molecular marker such as microsatellites, is a new technique, but extensive work particularly in case of cattle has been done in India and abroad.

2.3.1 Molecular genetic characterization of Exotic cattle

Schmid *et. al.*, (1999) studied the genetic diversity between and within swiss cattle breeds. The breeds were original Swiss Brown, Pure bred Smmental, Holstein, Herens and Evolenard. A set of 30 microsatellite markers was used in this study. All loci were found polymorphic in the five breeds with total allele number ranging from 2 in ILSTS 005 to 16 in TGLA 122. Great variations were found among breeds with respect to the total number of alleles at a given locus. The average expected heterozygosity within breeds ranged from 0.60 in Simmental to 0.69 in Holstein. Genetic distances between populations were measured with Cavalli-Sforza and Edwards' chord distance D_c. Two breeds Evolenard and Herens (matrix of D_c value was lowest. 0.029) were found closely related. All other distances were found two to three times larger.

Edwards *et al.*, (2000) established relationship between the endangered Pustertaler-Sprinzen and three related European cattle breeds viz. Pinzgauer, Vosges and Simmental by the analysis of data generated from 20 polymorphic microsatellite loci and finally concluded that the Pustertaler breed and the three breeds considered to be most closely associated, it cluster most closely with the Pinzgauer breed. The overall level of genetic variability in the Pustertaler breed is not significantly reduced compared with the three breed or indeed when compared to other European breeds. This may be expected due to known outcrossing with Pinzgauer and Simmental in the past.

Del Bo *et al.*, (2001) studied genetic diversity of seven autochthonous Italian cattle breeds viz. Aosta Black Pied (ABP), Aosta Red Pied (ARP), Aosta Chestnut (AC), Oropa Red Pied (ORP), Gray Alpine (GA), Rendena and Burlina, bred in the Alpine area to characterize their genetic structure and to study their phylogenetic origin. Two cattle breeds from Germany (German Brown and Holstein) and four from Switzerland (Simmental, Herens, Evolene and Brown Swiss) were also included in the study in order to determine the genetic diversity existing among Italian local breeds. They used seventeen internationally accepted panel of microsatellite markers. Microsatellites were highly polymorphic with a mean number of five alleles per locus. The mean PIC value within each breed ranged from 0.66 (Burlina) to 0.55 (Evolene and Herens). The average heterozygosity in each population for 17 loci ranged between 0.6 to 0.68. They found the strong genetic differentiation and high variability even in the endangered breeds. They used allele frequencies to estimate genetic distances and to draw a phyllogenetic tree. They found ASP and ABP as closest breed. The Holstein and AC were genetically most different. AostaValley breeds like Evolene and Herens constituted a tight cluster in the phylogenetic consensus tree. Ultimately the genetic differences among breed were found in accordance with thei geographical and historical origins.

Beja-Perreira *et al.*, (2003) characterized southwestern European bovine breeds with a set of 16 microsatellite and assessed historically as well as biogeographically. They analysed 889 unrelated individuals from fifteen representative Iberian breeds and three French breeds for 16 microsatellite loci. A total of 173 alleles were found across all loci with mean number of alleles (MNA) per locus 6.5. The unbiased estimates of gene diversity (expected heterozygosity, He) ranged from 0.611 to 0.709. They visualized the geographical distribution of the genetic differentiation between Iberian cattle breeds. Data from the principal components analysis (PCA) were used to construct synthetic maps. It revealed a large differentiation between Northern Iberian breeds rather than between more geographically distant breeds. A clear east-west gradiant was found that might be related with the model of demic diffusion of agriculture, but no strong evidence for an African genetic influence in the Iberian cattle breeds was analysed in this study.

Jordana *et al.*, (2003) studied the genetic structure and relationship among 18 Southwest European beef cattle breeds (10 from Spain 5 from Portugal and three from France). They used 16 DNA microsatellite data and F- Statistics for analysis. Level of apparent breed differentiation was found considerable and multilocus F_{ST} value indicated that around 6.8% of the total genetic variation could be explained by breed differences and remaining 93.2% by differences among individuals. They observed that a significant deficit of heterozygote of 7.6% ($P<0.001$) exists for each one of the analysed breeds except the Portuguese breeds Barrosa and Mirandesa and that of 13.9% ($P<0.001$) in the whole population. They suggested that, several factors were responsible for heterozygote deficiency such as the consanguinity produced by mating between the relatives, genetic hitchhiking effect (the locus can be under selection, being close to some morphological or productive trait of selective interest), presence of Null-alleles (non amplifying alleles) and finally presence of population sub

structure within the breed, which might lead to the Wahlund's effect. Finally they concluded that gene flow could have played an important role for genetic uniformity in population of narrow geographical vicinity and the genetic drift as the most important factors of genetic differentiation among the analysed population. Thus the apparent taxonomic distinctiveness of the breeds could be the result of a random drift, which can affect the genetic distances among populations.

Grzybowski and Prusak (2004) estimated genetic variation in nine European cattle breeds and also calculated the mean number of genes migrating in one generation (N_m) between population of nine cattle breeds maintained in Germany, Switzerland and Poland which ranged from 2.023 within the Polish cattle breeds to 1.214 within German population with average value 2.048 for whole population. Finally, result showed that the greatest exchange of genetic material took place between breeds from the same geographical regions. They concluded that the breed structure of the analyzed populations of European cattle is principally a reflection of geographic origin of individual breeds.

Metta *et al.*, (2004) used 5 di and 5 tri- nucleotide repeat loci, in 17 Ongole and 13 Deoni unrelated individuals to study the genetic variability among the breeds. The di-nucleotide repeats were found to be more polymorphic than (100%) that of 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 breeds respectively. The PIC value 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 among themselves with a F_{ST} value of 0.117 (P=0.01).

Steigleder *et al.*, (2004) estimated the genetic diversity of a Brazilian Creole cattle by analyzing data from the fourteen microsatellite loci in 73 animals. All the microsatellites were polymorphic with the PIC value 0.55

(BM3004) to 0.87 (TGLA227). The number of alleles per locus varied from 4 (BMS3004) to 13 (RM088). The observed heterozygosity varies from 0.51 to 0.99, higher than that of expected one which ranged from 0.59 to 0.89. The population genotype frequencies were not in Hardy-Weinberg equilibrium. The probability of identity between two animals was very small ranging from 0.03 to 0.21 the average value being almost zero (2×10^{-15}), which also indicate genetic diversity. Finally they concluded that although the breed was at is at the risk of extinction with its population not exceeding 500 animals but still had high degree of variability.

Cervini *et al.*, (2006) assessed the polymorphism of 10 microsatellites in Brazilizn Nellore cattle (*Bos indicus*) using a commercial multiplex system. Ninety-four alleles were detected from the 10 loci surveyed, yielding a mean value of 9.4 alleles per locus. All loci were not equally informative as revealed by allelic frequencies. A significant deficit of heterozygosity ($P<0.01$) was detected in the TGLA53, ETH10, ETH225, TGLA122 and INRA023 loci. The ETH3 locus did not show the heterozygote deficiency. Six loci deviated significantly from the Hardy-Weinberg equilibrium. The mean PIC value was 0.640 and the mean expected heterozygosity value was 0.679. This multiplex analysis could contribute toward pedigree information, adequate genetic improvements and breeding programs.

Rahimi, *et al.*, (2006) estimated genetic variation in Holstein young bulls of Iran AI station using molecular markers. A total of 119 individuals were genotyped at 13 microsatellite loci. The mean number of observed allele per microsatellie marker was 9.15 and average number of effective allele was less than the observed value (4.03). the average observed and expected heterozygosity values were 0.612 and 0.898 respectively. The mean PIC value was 0.694. Inbreeding was calculated as the difference between observed and expected heterozygosity. Observed homozygosity

was less than that of expected one, which reflects inbreeding of -3.7% indicating that there is genetic differences between bull-sire and bull-dams used to produce young bulls. The results obtained from this study demonstrated that the microsatellite DNA markers used in the present DNA typing are usefull and sufficient for individual identification and parentage verification without accurate pedigree information.

2.3.2 Molecular Genetic Characterization of Indian Zebu Cattle

Mukesh, *et al.*, (2004) assessed the genetic variation and established the relationship amongst the three Indian zebu cattle breeds, viz. Sahiwal (SC) Hariana (HC) and Deoni (DC) using bovine specific 20 microsatellite markers. The estimated mean allelic diversity was 5.2, 6.5 and 5.9 in SC, HC and DC, respectively. The average observed and expected heterozygosities for the population varied from 0.42 (SC) to 0.59 (DC), and from 0.61 (SC) to 0.70 (DC), respectively. Low value of genetic variability estimates was observed in SC when compared with the DC and HC, indicating some loss of variability in SC because of its relatively small population size. All the three cattle breeds showed a significant heterozygote deficit ranging from 17.2% in Deoni to 32.6% in Sahiwal. From global F-statistics a significant deficit of heterozygotes of 24.2% ($P<0.05$) was observed for each one of the analysed breeds whereas the total population had a 32.8% ($P<0.05$) deficit of heterozygotes. The F_{ST} estimate demonstrated that approximately 88.7% of the total genetic variation was because of the genetic differentiation within each breed. Pair wise breed differentiation, Nei's standard and DA genetic distance estimates revealed relatively close genetic similarity between HC and DC in comparison with SC. In the UPGMA based phylogenetic tree constructed from the genetic distances, HC and DC were grouped together in one cluster and SC in the other. The estimated time of divergence suggested a separation time of approximately 776 years between DC and HC, and a comparatively longer period (1296 years) between DC and SC.

Mukesh *et al.*, (2004) characterized Sahiwal cattle to know its population structure and within breed genetic variability. A total of 130 alleles were detected across 25 loci with mean of 5.20 alleles per locus. The observed and effective number of alleles ranged from 2-10 and 1.04 to 6.65 at locus ETH 3 and ILSTS 006 respectively. The range for observed and expected heterozygosity values across the 25 loci was 0.04 (ETH 3) to 0.86 (HEL 9) and 0.04 (ETH 152) to 0.85 (ILSTS 006) with mean value of 0.43 and 0.60 respectively. Polymorphism Information Content (PIC) values ranged from 0.03 (ETH 3) to 0.81 (ILSTS 006) with a mean of 0.54. They found substantial amount of genetic variability in Sahiwal that might be used in planning breeding strategies particularly in population of small sizes; furthermore, such variability could also be employed to detect genetic markers linked to quantitative trait loci (QTL).

Kumar, *et al.*, (2006) characterized the Hallikar cattle breed, native of Karnataka using 19 FAO recommended microsatellite marker. Number of alleles per locus ranged from three to nine with allele size ranging from 102 to 294 bp. These alleles were distributed in the frequency ranged between 0.306 and 0.8673 in the population. The mean observed and expected heterozygosities were 0.7515 ± 0.1734 and 0.7850 ± 0.1381 respectively. The high heterozygosity observed implies presence of higher genetic variability within Hallikar breed. The population was tested for Hardy-Weinberg equilibrium at 19 microsatellite loci (74 percent of the loci) were found to be at disequilibrium.

Pandey *et al.*, (2006) reported a genetic diversity of Kherigarh cattle, a utility daught-purposed breed of India by use of microsatellite marker recommended by the Food and Agriculture Organisation. A total of 131 alleles were distinguished by the 21 microsatellite markers used. All microsatellie loci were highly polymorphic with the mean allelic number 6.24 ± 1.7, ranging 4-10 per locus. The observed heterozygosity in the population ranged between 0.261 and 0.809 with the mean of 0.574 ± 0.131.

It indicated considerable genetic genetic variation in this population. This breed had not experienced a genetic bottleneck in the recent past.

Pandey *et al.*, (2006a) studied the genetic variability in Kenkatha cattle by using an array of 21 microsatellite markers. A total of 125 distinct alleles were identified in kenkatha cattle with the number of alleles 50.95±1.9 (ranging from 3 to 10 per locus). The observed heterozygosity in the population ranged between 0.250 and 0.826 with a mean of 0.540±0.171, signifying considerable genetic variation in this population. Population displayed heterozygote deficit in the tune of 21.4%. They found absence of genetic bottleneck in the population.

Sharma *et al.*, (2006) investigated microsatellite based within breed genetic variability in Gangatiri cattle, a dual purpose Indian cattle breed whose population is showing a declinig trend. 138 alleles were detected by the 22 microsatellite markers. All microsatellie loci were highly polymorphic with the mean allelic number 6.273±2.39. The observed heterozygosity in the population varied from 0.227 to 0.756 with the mean of 0.464±0.166. It indicates the less genetic variability within the breed. The population exhibited inbreeding (f=0.318) and heterozygote deficit. Bottleneck was absent. An immediate need for genetic management of the breed was found to be essential for its conservation and improvement.

Sharma *et al.*, (2006 a) studied the genetic variation of Ponwar cattle by the use of microsatellite markers. A total of 141 alleles were detected by 24 microsatellite loci. Number of alleles varied from 3-10 per locus with the mean allelic number 5.875±1.727. The observed heterozygosity in the population was 0.497±0.177, reflecting less genetic variation in this population. Population showed inbreeding (f=0.262) and heterozygote deficit. Genetic bottleneck was absent.

2.3.3 Association of Microsatellite Marker with QTL

Hai Guo *et al.*, (2004) reported the relationship of microsatellite DNA and beef performance trait of Yanbian Yellow cattle. They selected some microsatellite markers that are reported to be closely linked to the main performance traits of beef cattle. They found, the allele 209 of locus ETH10 was positively correlated with topline and shoulder whereas allele 213 had a positive correlation with pastern, rump and fineness, however allele 215 showed a negative correlation with topline and rump. For microsatellite locus IDVGA46, allele 249 had a positive correlation with the topline , fore limbs and hind limbs based on appearance evaluation, loin width with muscularity evaluation

Kim *et al.*, (2004) studied gDNA of 6 Hanwoo steers showing the differences in meat quality and quantity. Four bacteriophage genomic libraries containing the chromosomal DNA of Hanwoo steer used to isolate microsatellite for their possible use in the genetic selection. They demonstrated that 208 and 210 alleles of HW-YU-MS#3 microsatellite were closely related to the economic traits such as marbling score, daily gain in body weight, back fat thickness and Longissimus dorsi muscle area (Loin Eye Area) in Hanwoo cattle. HW-YU-MS#3 microsatellite was localized on bovine chromosome 17, on which QTL related to the regulation of the body fat content and muscle hypertrophy locus was previously known to exist. They finally suggested that these two alleles could be used as DNA marker related to economic traits for selection of hanwoo cattle in future.

Kucerova *et al.*, (2004) studied relation of microsatellite ETH10 to milk and meat production parameters in Czech Pied cattle. They found a significant association between ETH10 and milk fat content. Significant differences were observed between genotype group of ETH10 and weight at the beginning of fattening period and dressed carcass weight of bulls. These findings were an instigation to prove the effects of ETH10 on production parameters in lager population.

Kucerova *et al* (2004) studied the association between paternal microsatellite ETH10, heterozygous level of paternal microsatellites and milk production parameters in Czech Pied dairy cattle. They found highest and lowest milk fat content in the daughter whose sires had the ETH10 genotypes (217/222) 4.55% and the genotype (217/221), 4.09%, respectively. The highest value of all milk performances traits were recorded in cows whose sire had the ETH10 (217/223) genotype. The presence of the heterozygous level of paternal microsatellite was significant in relation to milk protein and milk fat content. Milk protein and fat contents were also 0.19 and 0.18% higher, respectively, in the daughter of sire with the lowest heterozygous level of microsatellite (60-70%) compared to those with the highest heterozygous level (90- 100%). However, the highest milk production (5195 Kg) was recorded in the group with the highest heterozygous level of microsatellite compared to those of the other groups (4947 and 4937).

Choi *et al.,* (2006) investigated microsatellite markers on bovine chromosome no. 1 and 14 for potential allelic association with carcass traits in Hanwoo (Korean Cattle). A total of 13 polymorphic microsatellite markers on bovine chromosomes 1 and 14 were used for alleleic association test with cacass characteristics in Hanwoo cattle. The number of individual marker alleles was varied from 4 to 11. For bovine chromosome 1, six significant allelic associations were found with carcass Breeding Value (BV) in the population. One significant allelic frequency differences with microsatellite marker BM711 were found for marbling score BV in Hanwoo populations. Two significant allelic frequency differences with microsatellite marker MCM 130 and BMS4049 were found for cold carcass BV and Two significant allelic frequency differences with microsatellite marker BMS4049 and BMS2263 were found for longissimus muscle area BV. Only one allelic frequency differences in the marker BMS2263 was found for back fat thickness BV. For bovine chromosome 14, five

significant allelic associations were also found with cacass BV. Allele 1 for RM 180 marker and allele 2 for BM4305 had significant association with longissimus muscle BV. Allele 1 of BL1029 and BMS2055 marker had significant association with marbling score BV. Allele 1 of BM4305 marker had significant association with back fat thickness BV.

Materials And Methods

3.1 Materials required

Chemicals, reagents, buffers and equipment used in the present study are listed in the Appendix. Most of the reagents and chemicals used in this study were of molecular biology grade.

3.2 Collection of Blood

Materials Required

- Sterile vacutainers with K_3EDTA
- Adaptor with needles
- Ice pack

Blood samples were acquired from 50 random and unrelated Bachaur cattle from the breeding tract (Madhubani, Darbhanga and Sitamarhi districts of Bihar) as per the guideline of MoDAD (Measurement of Domestic Animal Diversity) programme (FAO, 1995). To ensure unrelatedness of animals due to nonexistence of pedigreed account under field situations, animals were selected from distinct villages after interviewing the owners in detail. About 9-10 ml blood was collected aseptically by jugular vein into sterile vacutainers containing EDTA as anticoagulant. Blood was transported from field to the laboratory maintaining cold chain.

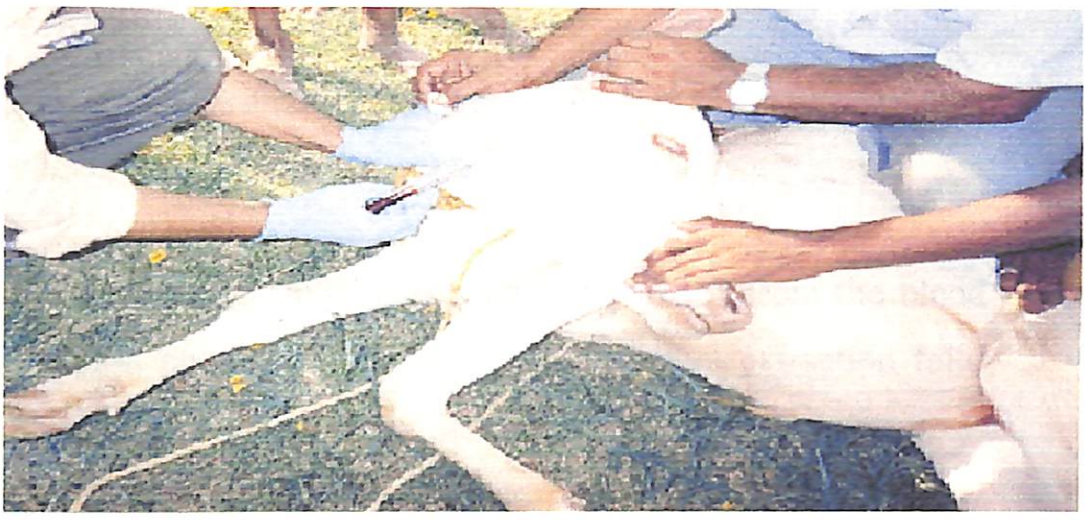


Fig 4. Collection of Blood Sample in a vacutainer from Jugular vein

3.3 Isolation of genomic DNA from the Blood.

Materials Required

- Equipments
  - Oakridge tubes (50 ml, three sets)
  - Glass culture tubes (30 ml, one set)
  - Eppendorf tubes (1.5 ml, one set)
  - Micropipette (5 ml) with autoclaved tips
  - Centrifuge machine
  - Vortex mixer
  - Weighing balance
- Reagents
  - Lysis buffer
  - Extraction buffer
  - TE buffer
  - SDS (20%)
  - Proteinase K (20 mg/ml)
  - Sodium acetate (3 M, pH 5.2)
  - Phenol (equilibrated with Tris at pH 8)
  - Chloroform
  - Isoamyl alcohol
  - Absolute alcohol
  - 70% alcohol
  - Ice flakes

Genomic DNA was isolated and purified from the blood cells using the standard phenol-chloroform-isoamyl alcohol extraction followed by ethanol precipitation described by Sambrook et al. (1989).

The detailed protocol is as under:

1. Two volume of ice cold lysis buffer (appendix) was added to whole blood in a 50 ml oakridge tube and mixed properly. The tube was kept on ice for

10 min., centrifuged at 10,000 rpm for 10 m. at 4 °C to pellet WBC. The supernatant was discarded carefully.

2. Above step was repeated until most RBCs were lysed and clear pellet of WBC was obtained (2-3 washes were sufficient to achieve clear WBC pellet).
3. Five ml Digestion buffer (appendix) was then added to the WBC pellet and mixed well by vortexing.
4. Sodium dodecyl sulphate (SDS, 20%) was added so that its final concentration become 0.5%, followed by addition of Proteinase K (Final conc. 100 µg/ml), mixed slowly followed by incubation at 56°C for overnight (or atleast for 6 hours) in a water bath.
5. After overnight incubation, equal volume of equilibrated phenol (pH~8.0) was added and Mixed slowly for 5-10 minutes by inverting the tubes to form uniform suspension. Centrifuge at 10,000 rpm for 10min at room temperature (25^0C).
6. Upper aquous phase (containing DNA) was collected in a separate oakridge tubes using sterilized Pasteur pipette.
7. Equal volume of phenol: chloroform: isoamyl alcohol (25: 24:1) was added and mixed well to form uniform suspension. The suspension was centrifuged at 10,000rpm for 10 min at 25°C.
8. Upper aqueous phase was saved without disturbing the interphase in a separate oakridge tubes using sterilized Pasteur pipette.
9. Equal volume of chloroform: isoamylalcohol (24:1) was added and mixed properly. Suspension was centrifuged at 10,000 rpm for 10 min at 25°C.
10. Upper aquous phase was separated in a glass culture tube and 1/10 volume of 3M-sodium acetate (pH 5.2) followed by 2 to 2.5 volume of chilled ethanol was added.
11. Mixed gently till DNA completely precipitates.

12. Precipitated DNA was spooled out in a sterilized eppendorf tube and washed twice by adding 1 ml 70% ethanol followed by centrifugation at 6,000 rpm for 6 minutes and then air dried.
13. Extracted and washed DNA pellet was dissolved in 300-500 µl of TE buffer (pH 8.0).
14. DNA dissolved in TE was incubated at 56°C for 15-20m for inactivation of DNAse and RNAse and genomic DNA stock was stored at -20^{0}C for future analysis.

3.4 Agarose gel electrophoresis to check the quality of isolated genomic DNA

Materials Required

- Equipments
  - Horizontal gel electrophoresis system
  - Power pack
  - UV Transilluminator
  - Gel casting plate
  - Comb
  - Microwave oven
  - Conical flask (250 ml)
  - Micropipette (2.5µl and 10µl) with autoclaved tips
- Reagents
  - 50x TAE buffer
  - Ethidium bromide
  - Loading dye
  - Agarose

Agarose gel electrophoresis is the method of separating and analyzing charged biomolecules 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. Larger sized DNA molecules face a large frictional force as they move through the gel and so have lower mobility as compared to the smaller sized DNA fragments that experience lesser force. A mixture of DNA molecules therefore separates into discrete bands during electrophoresis. Frozen stock of 50 genomic DNA samples of Bachaur cattle was diluted (1:10) with Milli Q water. Their quantity and quality was assessed by 0.6 % agarose gel electrophoresis using 1X TAE buffer. Based on the result of electrophoresis the selected samples were further diluted to a concentration of 50-100ng/µl. The detailed protocol is as under:

3.4.1 Dissolution of agarose

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

1. Two ml of 50x TAE was diluted to make 100ml in a 250ml Erlenmeyer flask to give final concentration 1x.
2. The required amount of agarose powder was weighed and added to the 1x TAE buffer.
3. The slurry was heated in a microwave oven until agarose was completely dissolved. Solution was gently swirled between heating cycles to release trapped air and re-suspend any agarose particles caught on the side of the flask.

4. The agarose solution was cooled until it reached a temperature of approx. 50-55°C (slightly hot to the hand). The flask was swirled occasionally to keep the contents at uniform temperature and prevent the agarose from gelling at the bottom of the flask. Ethidium bromide stock solution (10mg/ml in water) was added to the final concentration of 0.5 μg/ml.

3.4.2 Casting of Gel

1. The comb was placed at the desired position on the gel tray 0.5-1.0 mm above the surface of the gel plate so that the complete well is formed.
2. The warm agarose solution was poured into the mold until its thickness become 3mm to 5mm. Care should be taken to avoid the air bubbles under or between the teeth of comb. If bubbles form, they can be removed with the help of pointed end of pipette tips before the setting of gel.
3. After the gel was completely set (30-40 minutes at room temperature), comb was removed carefully by wriggling back and forth gently and then lifting up.

3.4.3 Gel loading

The gel along with gel tray was placed in the electrophoresis tank. fresh running buffer (1xTAE) was added to cover the gel up to height of about 1-2mm. The DNA samples were mixed with desired gel loading dye before loading into wells. The gel loading dye serves three purposes

(i) It increase the density of the samples

(ii) Ensuring that the DNA drops evenly into the wells

(iii) Adds color to the samples, thereby simplifying the loading process. Gel loading dye contains dyes that in an electric field move towards the anode at predictable rates.

Bromophenol blue migrates through agarose gels approximately 2.2 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 of 300bp in length, whereas xylene cyanol FF migrates at approximately the same rate as linear-double stranded DNA of 4kb in length, the relationships are not affected by the concentration of agarose provided concentration varies between 0.5% to 1.4%. After loading the DNA samples lid of the gel tank was closed, attached the electric leads and the power supply was turned on so that the DNA will migrate towards anode (red lead). A voltage of 40-80 V was applied (1 to 2.5 V/cm measured as a distance between the electrodes). 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. The gel was run until the Bromophenol blue and Xylene cyanol FF have migrated the appropriate distance through the gel.

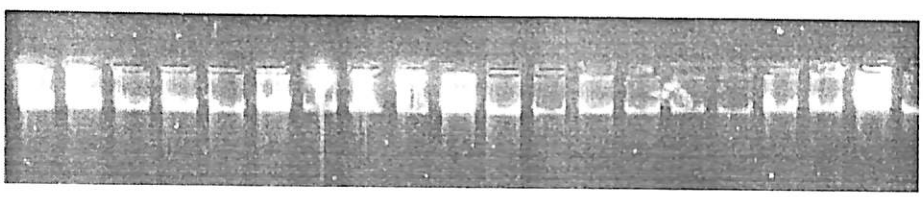


Fig. 5 Agarose gel electrophoresis of genomic DNA samples of Bachaur Cattle

3.5 PCR amplification of microsatellite markers

Materials reqired

- Equipments
  - Programmable thermal cycler
  - PCR tubes/plates (autoclaved)
  - Thermoseal
  - Micropipette
  - Eppendorf tubes
- Reagents
  - Genomic DNA

- 10x Taq polymerase buffer
- dNTPs (Deoxy-ribonucleotide mix)
- Primers (Reverse and Forward)
- Taq DNA Polymerase
- PCR grade water
- Ice flakes

Polymerase chain reaction is an *in-vitro* method for enzymatically synthesizing defined sequence of DNA. The reaction use two oligonucleotide primers that hybridize to opposite strands at the specific temperature 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.

Fig 6 A Typical Programmable Thermal Cycler (PTC-200)

3.5.1 Optimization of PCR condition

Various PCR parameter like concentration of genomic DNA template, dNTP, *Taq* polymerase, $MgCl_2$ and annealing temperature of primers were optimized to obtain a specific amplified product in significant quantity. PCR was carried out in a 25µl reaction volume which was kept constant for all reactions.

3.5.2 Microsatellite markers

In the present study, Twenty two hetrologous bovine microsatellite primer pair were used for diversity analysis of Bachaur Cattle population viz. CSSM66, HAUT24, ETH3, HEL5 CSRM060, HEL1, HEL9, ILSTS030, ILSTS033, ILSTS005, ILSTS011, ILSTS006, ILSTS054, ETH10, HAUT 27, ETH225, INRA035, INRA63, BM1818, BM1824, MM8 and MM12.

3.5.3 PCR Master Mix Preparation:

The master mix or cocktail was prepared for 50 reactions and aliquoted into each well of PCR plate containing genomic DNA sample. The PCR plate was marked properly depicting marker ID and sample ID.

Component	**Stock**	**Final concentration**
Genomic DNA	50-100 ng/ µl	100- 200 ng
PCR Buffer	10 X	1X
Forward Primer	1 µg/ µl	100 ng/ µl
Reverse primer	1 µg/ µl	100 ng/ µl
dNTP Mix	100 mM	10 mM
Taq Polymerase	5 U/µl	1 U/ µl
PCR grade water to make volume up to 25 µl		

3.5.4 Cycling Parameter in PCR

The PCR plate was briefly centrifuged for proper mixing of DNA and other reagents. Plate was then placed in the DNA thermal cycler. (PTC-200 thermocycler, MJ research, USA) The amplification protocol used as was follow:-

Denaturation	Step – 1	94°C for 2 min.
	Step – 2	94°C for 1 min.
Annealing	Step – 3	55°C for 1 min. or specific annealing temp.
Extension	Step – 4	72° C for 1 min.
	Step – 5	Go to step 2 for 30 times
	Step – 6	72°C for 10 min
Cooling	Step – 7	10°C for ever
	Step – 8	End

3.5.5 Methods for the detection of PCR product by Agarose gel electrophoresis

After completion of PCR programme, the PCR products were checked on 2% agarose for amplification. Samples were mixed with 5 µl loading dye and run under constant voltage (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 in the DNA. The amplified samples were identified and results were recorded for further analysis product on denaturing urea PAGE.

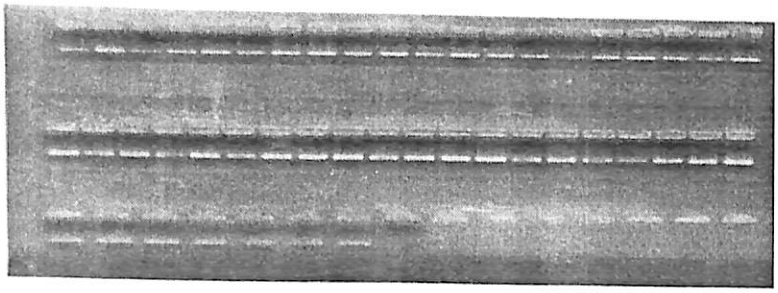


Fig. 7 Electrophoresis of amplified PCR products (ETH225) on 1.5 % agarose gel.

3.6 Resolution of PCR products on denaturing PAGE

Materials Required

- Equipments
  - Gel apparatus (IPC assembly, universal base, safety covers with cables, precision caster assembly
  - Power pack
  - Polycarbonate spacers (0.4 mm thick)
  - Shark tooth combs (0.4 mm thick, 49/72 wells)
  - Casting syringe with rubber tube
  - Washing syringe with needle
  - Temperature indicator
- Reagents
  - Page mix (6% denaturing)
  - APS
  - TEMED
  - Bind Silane
  - 10x TBE
  - 70% ethanol

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 can separate molecules of DNAs whose length differs by as little as 0.2%. The gel is polymerized in the presence of denaturing agent (urea) that suppresses base pairing in nucleic acid. Amplified PCR products were analyzed on 6% denaturing Polyacrylamide gels. Details of reagents used are given in appendix and the complete protocol followed for denaturing PAGE is given as under:

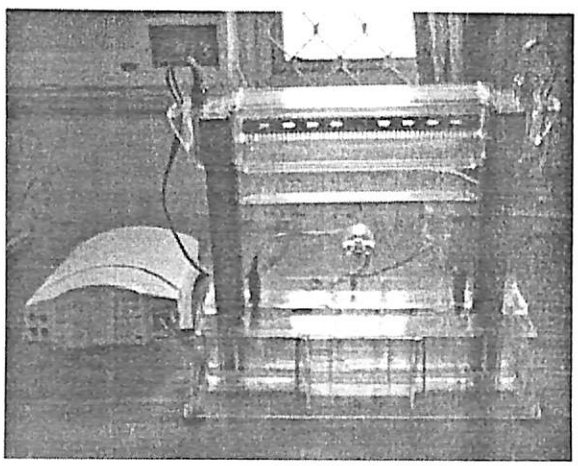


Fig 8. Vertical Sequencing gel electrophoresis apparatus used for analysis of PCR products on 6% denaturing PAGE gel.

3.6.1 Casting of gel

1. IPC and glass plates were cleaned with mild detergent and rinsed thoroughly with distilled water.
2. Both plates were rinsed with 70% ethanol and wiped to prevent any streaking on the plates.
3. Bind silane was applied at the edge of the glass plate and left for 10 minutes.

4. Spacers (0.4 mm) were placed in the side of the IPC and glass plate was kept on it.
5. Side clamps were fixed and comb was inserted between the glass plate and IPC so that the teeth of comb face upward.
6. The base was fitted to the whole assembly.
7 Gel mix was prepared by adding TEMED (40µl) and 10% APS(400µl) to the 6% urea denaturing page mix (80 ml) and mixed gently.
8 Gel mix was injected into the assembled glass plates using syringe.
9 The gel was allowed to polymerize at room temperature.

3.6.2 Electrophoresis of samples in PAGE gel

1. The comb of polymerized gel was removed and surface of the gel was washed thoroughly with tap water.
2. The assembly was placed in the lower buffer tank of the electrophoresis apparatus containing 1x TBE. Safety cover was fixed and temperature indicator tape was affixed on the plate. Now gel was pre-run at 75 Watt so that temperature of the plate goes up to 50°C.
3. The comb was refixed with teeth facing down and the wells were washed with the help of a syringe and needle by injecting TBE buffer. Loading dye was loaded in alternative wells to detect any leakage between the wells.
4. Denatured DNA samples having (heated at 95°C for 5 minutes) appropriate amount of gel loading dye (Bromophenol blue and xylene cyanol) was loaded into the wells using micropipette. For size determination 10 bp ladder (Invitrogen) was run in parallel as size standard.
5 Electrodes were connected to the power pack. Voltage was set at 80 volt and the gel was run until marker dyes had migrated the desired distance, as per expected allele size for each marker. Temperature of plate was kept maintained at 50°C through out the process.

3.6.3 Allele detection by Silver staining

materials required

- Equipments
  - Staining tray with its cover
  - Cellophane sheet
  - Gel cutter
  - Gel drying glass

- Reagents
  - Fixative and stop solution (10% acetic acid)
  - Staining solution (0.1% $AgNO_3$ with 2.0 ml formaldehyde,)
  - Developer solution (sodium carbonate 3% with formaldehyde 2.0 ml and 200 µl STS)
  - Formaldehyde solution (37-41%)
  - STS (1% w/v)
  - Distilled water

Silver staining protocol described by Bassam *et al.*, (1991) was used for detection of microsatellite allele in PAGE gels. This method can detect DNA even in the picogram range by the specific chemical reduction of silver ions. Silver binds to nucleic acid bases and is then selectively reduced by chemical reagents. The presence of formaldehyde during the silver staining improves both sensitivity and contrast. Formaldehyde selectively reduces silver ions to metallic silver. Thiosulphate helps in dissolving insoluble silver salts by complex formation removing silver ions from the gel surface. The presence of thiosulphate eliminates the formation of dark precipitate in the gel and developer solution. The protocol is as follows.

1. The gel was immersed in 1.5 liter of 10% acetic acid solution for 20-30 minutes at room temperature for fixing of DNA.
2. Washing of the gel was done thrice with distilled water by keeping gel plate in the tray containing about two liters of distilled water to remove acetic acid completely.
3. The gel was then stained using 2 liters of silver nitrate solution (0.1 % w/v) and 2.25 ml of 37-41% formaldehyde for 30 minutes at room temperature.
4. After staining gel was washed for 10 second with distilled water by keeping the gel plate in slanting position.
5. The stained gel was transferred to 1.5 liter chilled developer solution (45 gm of sodium carbonate, 2.25 ml of 37-41% formaldehyde, and 150 µl of 1% STS) and gel plate was agitated gradually.
6. Dark colour of DNA bands appeared after 3-5 minutes. Reaction was stopped with 10 % acetic acid solution.
7. Gel was cut and transferred on the cellophane sheet. It was preserved after air drying for scoring.

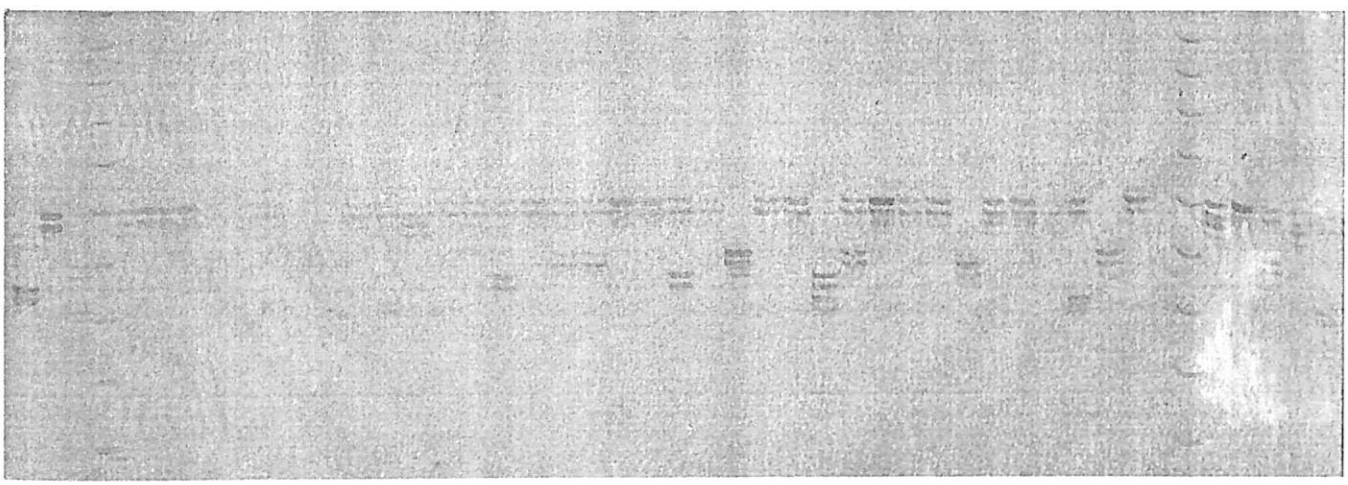


Fig. 9 Resolution of alleles at ETH225 locus in a silver stained PAGE gel

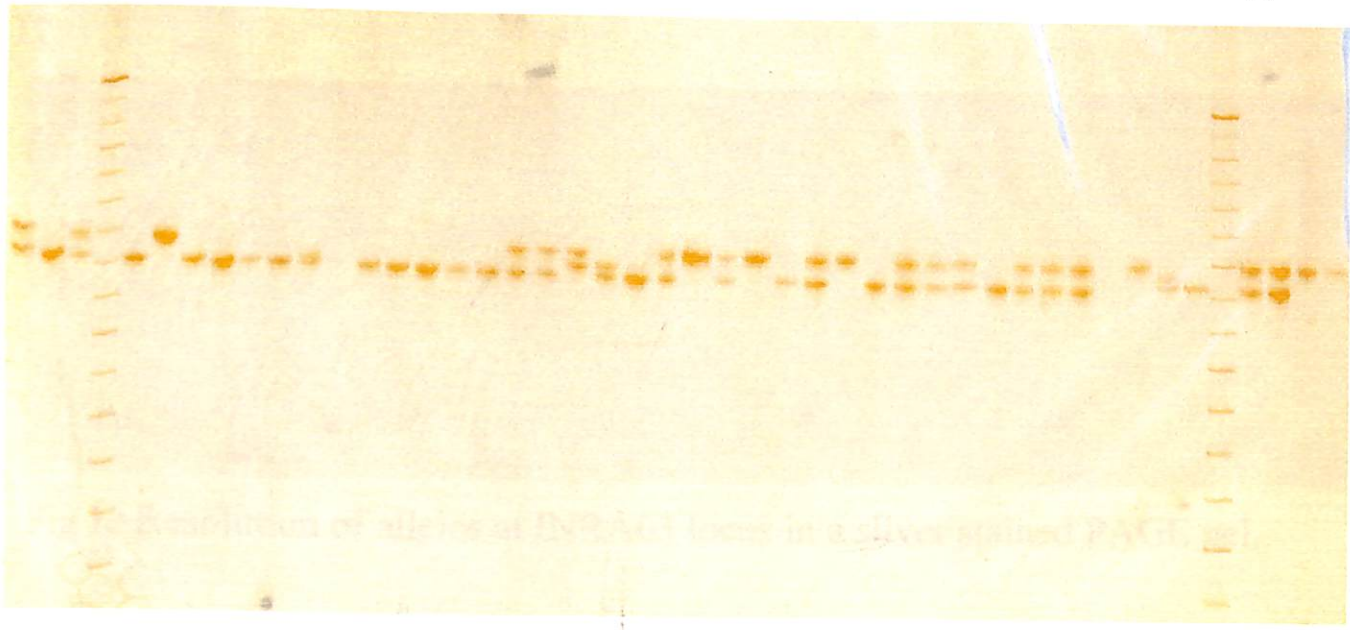


Fig. 10 Resolution of alleles at ILSTS011 locus in a silver stained PAGE gel.

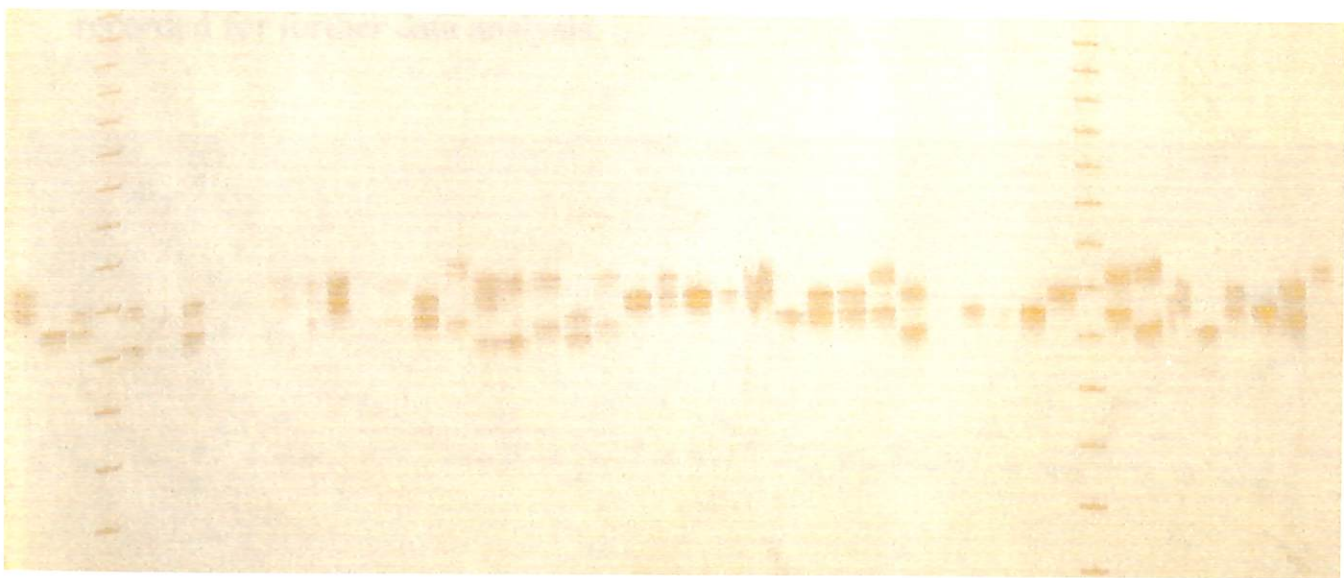


Fig. 11 Resolution of alleles at ILSTS005 locus in a silver stained PAGE gel.

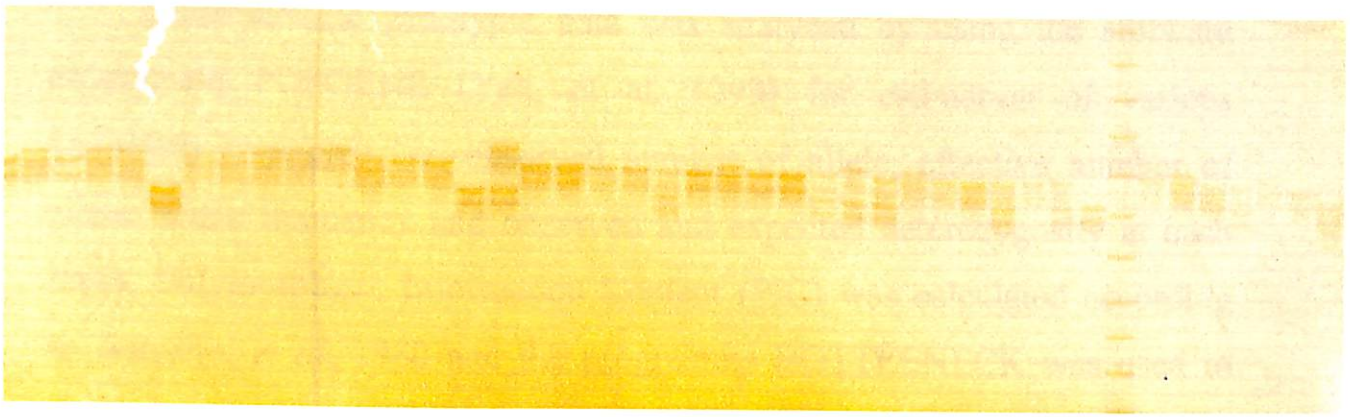


Fig 12 Resolution of alleles at INRA63 locus in a silver stained PAGE gel.

3.7 Alleles Scoring

For each microsatellite marker the microsatellite alleles were scored manually and denoted as A, B, C etc. Genotype of each individual were recorded for further data analysis.

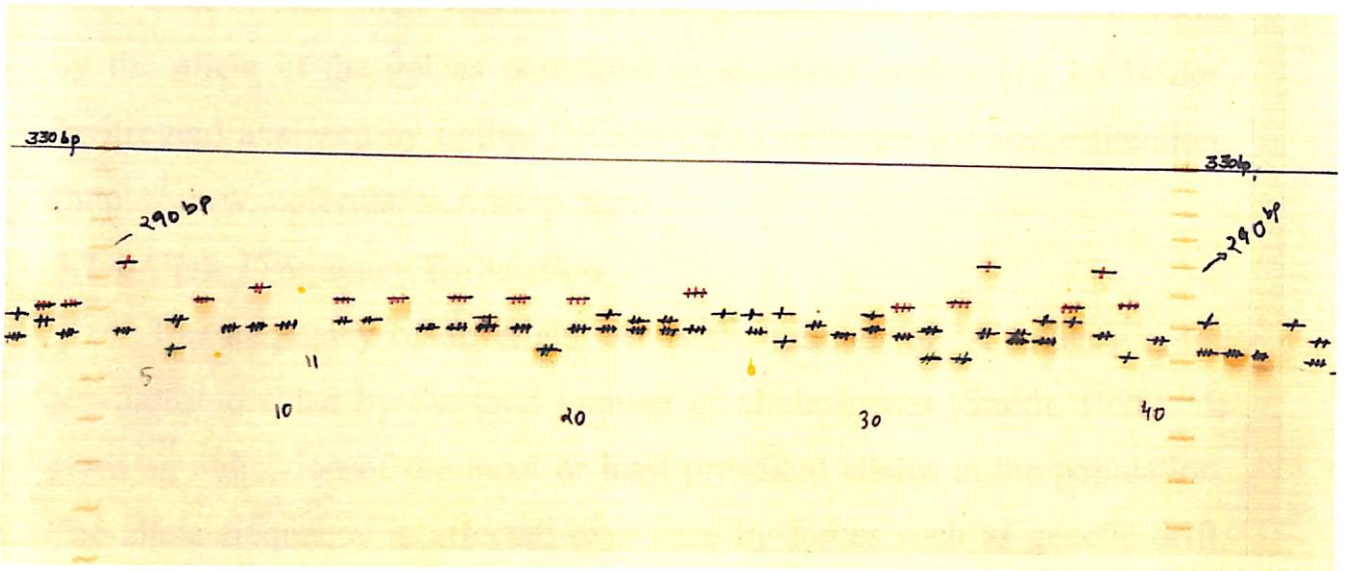


Fig. 13 Resolution of alleles at BM1818 locus in a silver stained PAGE gel (Scored).

3.7.1 Microsatellite Data analysis

Microsatellite genotypic data was analyzed by using the software programme POPGENE (Yeh, et al. 1999) for estimation of various variability measures viz. observed number of allele, effective number of allele, allele frequency and observed and expected heterozygosity at each locus. Polymorphism Information Content (PIC) was calculated according to Botstein, *et al.* 1980 and the programme BOTTLENECK was used to analyze population bottleneck in the investigated breed.

3.7.2 Allele number

Allele number is the total number of alleles for a given marker / locus in a population, which is counted with a non-zero frequency. A locus can have many different alleles. The allele number for each locus was determined manually from the silver stained gels.

3.7.3 Allele Size estimation

Size of each allele was determined by measuring the distance traveled by the allele in the gel as compared to standard marker (10 bp ladder, Invitrogen) analysed by online INCHWORM software for size estimation. (http:://www.molecularworkshop.com)

3.7.4 Allele Frequency Estimation

The frequency of an allele 'A' is the number of 'A' alleles in the population divided by the total number of alleles/genes (Smith, 1998). 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 etc.

3.7.5 Effective Allele Number (Kimura and Crow 1964)

The effective number of alleles (n_e) is the reciprocal of the sum of the square of allele frequencies.

$$n_e = 1 / \Sigma P_i^2$$

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

3.7.6 Gene diversity

Observed (H_o) and Expected heterozygosity or genetic diversity (H_e) on a locus, was calculated from the observed allele frequencies (Levene, 1949; Nei,1973) as executed in POPGEGE software (Yeh, et al. 1999). The formula used are

$$H_E = 1 - \sum P_i^2$$

Where Pi is the frequency of the i^{th} allele at a locus in a population.

The observed heterozygosities (Ho) were calculated as the actual percentage of heterozygosity occurring in sample population.

$$H_O = \frac{\text{Number of heterozygotes}}{\text{Total number of samples}} \times 100$$

3.7.7 Polymorphism Information Content (PIC)

PIC is a measure of informativeness of marker for genetic variability studies. The PIC value for each locus was calculated according to Botstein, *et al.*, 1980 using the given formula:

$$\mathbf{PIC} = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 p_j^2$$

where n= number of alleles

pi= allele frequency at the homozygous loci

p_i p_j= allele frequency at the heterozygous loci

3.7.8 Hardy Weinberg Equilibrium (HWE) :

A $\chi 2$-test of goodness of fit was carried out with the observed number and the expected number of alleles at each locus to check whether population was in Hardy-Weinberg equilibrium.

3.7.8.9 Linkage disequilibrium

Linkage disequilibrium among the microsatellite loci was analysed employing F-STAT version 2.9.3, an update version 1.2 (Goudet, 1995) for 22 microsatellite loci.

3.7.9 Bottleneck analysis

Temporary but significant reductions in population size are referred to as population bottlenecks. The effects can vary depending on both the size to which the population is reduced and the duration of the bottleneck (i.e., the number of generations). Limited population size can lead to a loss of genetic variation and subsequent loss of evolutionary potential. Bottleneck events in the population were tested by two methods. The first method consisted of three excess heterozygosity tests developed by Cornuet and Luikart (1996). A. Sign test, B. Standardized difference test, and C. Wilcoxon sign-rank test. The probability distribution was established using 1000 simulations under three models i. e. IAM, TPM and SMM. The second method was the graphical representation of the mode-shift indicator originally proposed by Luikart *et al.*, (1998).

Results And Discussion

All the 22 microsatellite loci (Table 1) which have been reported to be polymorphic in various *Bos taurus* and *Bos indicus* breeds (Schmid *et al.*, 1999; Edward *et al.*, 2000; Del Bo *et al.*, 2001; Jordana *et al.*, 2003; Metta *et al.*, 2004; Mukesh *et al.*, 2004; Sharma *et al.*, 2006) amplified successfully in the Bachaur cattle DNA samples. Specific banding patterns of different alleles were obtained from which individual genotypes were ascertained.

4.1 Linkage disequilibrium

It is the non-random association of alleles between two loci on a chromosome. Factors responsible for linkage disequilibrium might be physical linkage, epistatic selection, genetic hitchhiking and random genetic drift. In the present study five pairs of microsatellite loci were located on the five different chromosome viz. ILSTS011 and CSSM66 on chromosome no. 14, ILSTS005 and CSRM60 on 10, ILSTS30 and MM8 on 2, ILSTS54 and HEL05 on 21 and MM12 and ETH225 on the chromosome no. 9. Therefore, presence of linkage disequilibrium was checked especially for five pairs of loci but no linkage disequilibrium was found. Thus analysis was performed with all the 22 studied loci.

Table No. 1 Microsatellite markers, their primer sequence, location And specific annealing temperature.

Microsatellite markers	Primer Sequences (P_1, P_2)	Chromosome No.	A^T
ILSTS11	5'-GCTTGCTACATGGAAAGTGC-3', 5'-CTAAAATGCAGAGCCCTACC-3'	14	58
ILSTS05	5'-GGAAGCAATGAAATCTATAGCC-3', 5'-TGTTCTGTGAGTTTGTAAGC-3'	10	55
ILSTS30	5'-CTGCAGTTCTGCATATGTGG-3', 5'-CTTAGACAACAGGGGTTTGG-3'	2	55
ILSTS54	5'-GAGGATCTTGATTTTGATGTCC-3', 5'-AGGGCCACTATGGTACTTCC-3'	21	55
INRA35	5'-ATCCTTTGCAGCCTCCACATTG-3', 5'-TTGTGCTTTATGACACTATCCG-3'	16	55
INRA63	5'-ATTTGCACAAGCTAAATCTAACC-3', 5'-AAACCACAGAAATGCTTGGAAG-3'	18	55
MM8	5'-CCCAAGGACAGAAAAGACT-3', 5'-CTCAAGATAAGACCACACC-3'	2	55
MM12	5'-CAAGACAGGTGTTTCAATCT-3', 5'-ATCGACTCTGGGGATGATGT-3'	9	55
BM1818	5'-AGCTGGGAATATAACCAAAGG-3', 5'-AGTGCTTTCAAGGTCCATGC-3'	23	58
BM1824	5'-GAGCAAGGTGTTTTTCCAATC-3', 5'-CATTCTCCAACTGCTTCCTTG-3'	1	55
ETH225	5'-GATCACCTTGCCACTATTTCCT-3', 5'-ACATGACAGCCAGCTGCTACT-3'	9	57
HEL01	5'-CAACAGCTATTTAACAAGGA-3', 5'-AGGCTACAGTCCATGGGATT-3'	15	55
HEL05	5'-GCAGGATCACTTGTTAGGGA-3', 5'-AGACGTTAGTGTACATTAAC-3'	21	55
CSRM60	5'-AAGATGTGATCCAAGAGAGAGGCA-3', 5'-AGGACCAGATCGTGAAAGGCATAG-3'	10	55
ILSTS33	5'-TATTAGAGTGGCTCAGTGCC-3', 5'-ATGCAGACAGTTTTAGAGGG-3'	12	55
ETH3	5'-GAACCTGCCTCTCCTGCATTGG-3', 5'-ACTCTGCCTGTGGCCAAGTAGG-3'	19	64
ILSTS06	5'-TGTCTGTATTTCTGCTGTGG-3', 5'-ACACGGAAGCGATCTAAACG-3'	7	56
HAUT27	5'-TTTTATGTTCATTTTTTGACTGG-3', 5'-AACTGCTGAAATCTCCATCTTA-3'	26	55
CSSM66	5'-ACACAAATCCTTTCTGCCAGCTGA-3', 5'-AATTTAATGCACTGAGGAGCTTGG-3'	14	60
HEL09	5'-CCCATTCAGTCTTCAGAGGT-3', 5'-CACATCCATGTTCTCACCAC-3'	8	59
HAUT24	5'-CTCTCTGCCTTTGTCCCTGT-3', 5'-AATACACTTTAGGAGAAAAATA-3'	22	52
ETH10	5'-GTTCAGGACTGGCCCTGCTAACA-3', 5'-CCTCCAGCCCACTTTCTCTTCTC-3'	5	55

4.2 Allele numbers, and their Frequencies

A total of 132 alleles were detected in Bachaur cattle across the 22 microsatellite loci investigated. Table 2 shows the observed and effective numbers of alleles per locus. The observed number of alleles (N_o) per locus varied from 3 (ILSTS011) to 9 (INRA35) with the mean number of alleles (MNA) 6±1.633 per locus. The average number of alleles per locus in this study was less than that of Hariana breed, 6.5 (Mukesh *et al.*, 2004); Halliker breed, 6.368 (Kumar *et al.*, 2006); Gangatiri breed, 6.273 (Sharma *et al.*, 2006) and Kherigarh breed, 6.26 (Pandey *et al.*, 2006). However lower values of MNA than that of Bachaur was also reported in Sahiwal (5.2) and Deoni (5.9) by Mukesh *et al.* (2004); in Kenkatha (5.95) by Pandey *et al.* (2006) and in Ponwar Cattle (5.875) by Sharma *et al.* (2006a). The observed number of alleles for all the 22 microsatellite loci exceeded the effective number of alleles (N_e) which varied from 1.907 (ETH10) to 6.972 (HEL9) with a mean of 3.566±1.443. Table 3 shows the allelic frequencies of different alleles of all the studied microsatellite loci. Reasonable amount of polymorphism in Bachaur cattle is discernible from the allele frequency data. The more number of alleles at different loci increases the mean genetic diversity in population (Moioli, *et al.*, 2001). The level of variation depicted by number of alleles at each locus serves as a measure of genetic variability having direct effect on differentiation of breeds within a species (Glowtzki-Mullis *et al.*, 1995). Thus FAO has specified a minimum of four different alleles per locus is required for evaluation of genetic differences between breeds. By this criterion, all 22 microsatellite loci except ILSTS011 showed ample polymorphism for evaluating genetic variation within breed and exploring genetic differences between breeds.

Table No. 2 Observed (N_o) and Effective number (N_e) alongwith Polymorphism Information Content (PIC) of different alleles in Bachaur Cattle.

Locus	N_o	N_e	PIC
ILSTS11	3	2.152	0.491
ILSTS05	5	4.028	0.730
ILSTS30	4	2.272	0.534
ILSTS54	6	4.197	0.744
INRA35	9	6.565	0.839
INRA63	4	2.888	0.629
MM8	7	2.663	0.575
MM12	8	5.961	0.850
BM1818	8	4.666	0.772
BM1824	4	2.663	0.593
ETH225	5	2.035	0.494
HEL01	6	2.351	0.557
HEL05	8	3.112	0.663
CSRM60	6	2.585	0.597
ILSTS33	7	3.293	0.674
ETH3	6	3.806	0.717
ILSTS06	6	3.662	0.705
HAUT27	6	3.216	0.673
CSSM66	5	2.799	0.614
HEL09	8	6.972	0.848
HAUT24	7	4.663	0.772
ETH10	4	1.907	0.456
Mean	6	3.566	0.660
S. D.	1.633	1.443	0.117

Table 3. Allele frequencies at studied microsatellite loci in Bachaur cattle.

Microsatellite Loci	Alleles								
	A	B	C	D	E	F	G	H	I
ILSTS 011	0.3556	0.0667	0.5778						
ILSTS 005	0.2558	0.2791	0.3023	0.0698	0.0930				
ILSTS 030	0.0667	0.2333	0.6111	0.0889					
ILSTS 054	0.1585	0.0732	0.3659	0.2195	0.0244	0.1585			
INRA 035	0.0213	0.0851	0.1596	0.0426	0.2021	0.0426	0.0851	0.1489	0.2128
INRA 063	0.2128	0.5106	0.1064	0.1702					
MM 8	0.0326	0.0652	0.5761	0.1630	0.0761	0.0109	0.0761		
MM 12	0.0244	0.0244	0.1707	0.2439	0.0854	0.1829	0.1098	0.1585	
BM 1818	0.0333	0.0111	0.1778	0.1333	0.2000	0.3444	0.0556	0.0444	
BM 1824	0.0870	0.3152	0.5109	0.0870					
ETH 225	0.6778	0.0556	0.1444	0.0444	0.0778				
HEL 1	0.1154	0.0385	0.0192	0.6154	0.0385	0.1731			
HEL 5	0.0735	0.1912	0.0441	0.0294	0.0147	0.1029	0.5147	0.0294	
CSRM60	0.1310	0.0238	0.1190	0.0238	0.1190	0.5833			
ILSTS 033	0.0526	0.0263	0.4474	0.2895	0.0395	0.0263	0.1184		
ETH 3	0.0109	0.0978	0.3043	0.3696	0.1196	0.0978			
ILSTS 006	0.0854	0.0366	0.1585	0.3902	0.2927	0.0366			
HAUT 27	0.1029	0.1765	0.5000	0.1176	0.0588	0.0441			
CSSM 66	0.0222	0.1111	0.0667	0.3000	0.5000				
HEL 9	0.1000	0.1750	0.0875	0.1000	0.2250	0.1000	0.0750	0.1375	
HAUT 24	0.1707	0.0976	0.1341	0.3659	0.1341	0.0244	0.0732		
ETH 10	0.1413	0.0217	0.6957	0.1413					

4.3 Polymorphism Information Content (PIC)

Polymorphism Information Content (PIC) provides the information about the polymorphism of the microsatellite loci. It also determines whether the marker loci are suitable for population genetics study or not. Table 2 shows the value of PIC calculated for individual loci. Polymorphism Information Content values of all the studied microsatellite loci varied from 0.456 (ETH10) to 0.850 (MM12) with the mean value 0.660±0.117. Genetic marker showing PIC value higher than 0.5 are normally considered as highly informative, if PIC value ranges between 0.25 and 0.5 then the loci are considered as reasonably informative and loci having PIC value less than 0.25 are consiredered as slightly informative for the population genetic analysis (Botstein *et al.*, 1980). The high estimate of PIC substantiated the suitability of used set of DNA markers to applications such as parentage control, linkage-mapping programme in addition to genetic polymorphism studies (Rahimi *et al.*, 2006). Thus with the exception of ETH10 (0.456), ETH225 (0.494) and ILSTS011 (0.491) all loci were highly informative whereas these three loci were found to be reasonably informative. Similar results were found in the taurine and indicus breeds investigated earlier using microsatellite markers (Kumar *et al.*, 2003; Metta *et al.*, 2004; Sharma, *et al.*, 2006; Rahimi *et al.*, 2006).

4.4 Genetic variability within the Bachaur cattle

The estimated parameter pertaining to the Bachaur cattle viz. observed, expected, Nei's expected heterozygosity and heterozygote deficit (F_{IS}) at each of the 22 loci are presented in the Table 4. The observed heterozygosity within population varied from 0.065 (ETH10) to 0.875 (HEL9) with the mean 0.529±0.179. The observed heterozygosity was less than that of expected which ranged from 0.481 (ETH10)

Table No. 4 Observed Heterozygosity (H_o), Expected Heterozygosity (H_e) alongwith Nei's Expected Heterozygosity and Heterozygote Dificiency, f (F_{IS}).

Microsatellite Loci	Heterozygosity			
	Observed	Expected	Nei,s	Heterozygote Deficiency f (F_{IS})
ILSTS11	0.4667	0.5413	0.5353	0.1282
ILSTS05	0.6512	0.7606	0.7518	0.1338
ILSTS30	0.4000	0.5660	0.5598	0.2854
ILSTS54	0.5122	0.7712	0.7617	0.3276
INRA35	0.6170	0.8568	0.8477	0.2721
INRA63	0.4255	0.6607	0.6537	0.3490
MM8	0.4348	0.6314	0.6245	0.3038
MM12	0.7805	0.8425	0.8322	0.0622
BM1818	0.6667	0.7945	0.7857	0.1515
BM1824	0.4783	0.6314	0.6245	0.2342
ETH225	0.4444	0.5144	0.5086	0.1262
HEL01	0.6154	0.5860	0.5747	-0.0708
HEL05	0.2353	0.6888	0.6786	0.6533
CSRM60	0.6667	0.6205	0.6131	-0.0874
ILSTS33	0.5000	0.7056	0.6963	0.2819
ETH3	0.5652	0.7453	0.7372	0.2333
ILSTS06	0.7561	0.7359	0.7269	-0.0401
HAUT27	0.3824	0.6993	0.6890	0.4451
CSSM66	0.5556	0.6499	0.6427	0.1356
HEL09	0.8750	0.8674	0.8566	-0.0215
HAUT24	0.5610	0.7952	0.7855	0.2859
ETH10	0.0652	0.4809	0.4757	0.8629
Mean	0.5298	0.6884	0.6801	0.2296
S. D.	0.1799	0.1110	0.1097	

to 0.867 (HEL9) with the mean 0.688±0.111. The Nei's expected heterozygosity within the Bachaur population varied from 0.476 (ETH10) to 0.857 (HEL9) with overall mean of 0.680±0.110. Higher heterozygosity value than that of Bachaur cattle have been reported in many *Bos indicus* cattle such as in Deoni (0.59) by Mukesh *et al.* (2004); in Kherigarh (0.574) by Pandey *et al.* (2006). The observed heterozygosity value in Amrithmahal cattle was found to range from 0.654 to1.000 (Manjunatha Prabhu, 2004) and from 0.143 to 0.960 in Halliker cattle (Kumar *et al.*, 2006). Among *Bos Taurus* breeds Mateus *et al.* (2004) obtained average observed heterozygosity ranging from 0.553 to 0.743 in 10 native Portuguese cattle breeds, American Charolais and the Brazilian Caracu Rahimi *et al.* (2006) reported mean observed heterozygosity 0.765 in Holstein Young bulls of Iran AI station using microsatellite markers. Bachaur cattle thus had a reduced amount of genetic variation derived from its gene diversity as judged against the genetic variation recounted in several breeds investigated globally. However, lower value of observed heterozygosity than that of Bachaur breed, have also been reported in the Sahiwal (0.40) by Mukesh *et al.* (2004); Sharma *et al.* (2006a) reported observed heterozygosity to the tune of 0.497 in Ponwar and 0.4641 in Gangatiri cattle (Sharma *et al.*, 2006). Almost equal value of observed heterozygosity to the Bachaur cattle (0.529) was repoted in Hariana breed by Mukesh *et al.* (2004). Within population inbreeding estimate (F_{IS}) was significantly positive, as derived from table-wide randomizations ($P<0.05$). The F_{IS} estimate ranged between –0.874 (CSRM60) and 0.8629 (ETH10) with the average of 0.2296. Thus on an average a significant deficit (22.96%) of heterozygote exists in the Bachaur population. All the microsatellite markers with the exception of HEL9, ILSTS006, CSRM60 and HEL1 contributed to observed heterozygote shortage. Sharma *et al.* (2006) reported about the prevalence of negative correlation between observed heterozygosity (H_o) and Heterozygote deficit (F_{IS}) in Indian Zebu cattle. Data obtained in the present

study also followed a similar trend. Observed heterozygosity of Sahiwal, Gangatiri, Ponwar, Bachaur, Hariana, Kenkatha, Kherigarh and Deoni have been reported as 0.42, 0.46, 0.497, 0.529, 0.53, 0.54, 0.574 and 0.59 respectively whereas F_{IS} for these breeds were reported to be 0.32, 0.31, 0.262, 0.229, 0.21, 0.21, 0.188 and 0.17 respectively. Observed heterozygosity shows increasing trend with decline in F_{IS} value among the Indian zebu cattle. Number of factors might be responsible for the heterozygote deficit in a population. In the present study it might be due to Inbreeding, genetic hitchhiking, presence of null alleles, and presence of population substructure (Wahlund effect). In genetic hitchhiking, a neutral allele that has been statistically associated with the allele under selection at another locus might be carried along, or hitchhike because of selective advantage of associated allele. This association might be maintained for the period of time due to linkage and mating system. Ewens-Watterson neutrality test (Manly, 1985) indicated that heterozygote deficit or homozygosity in the present study might not be an outcome of selection as all the studied loci were found to be neutral except four viz. ILSTS005, INRA35, MM12 and HEL9. Sometimes, population may be divided into several heretogenous groups i.e. subpopulations. Some geographical or ecological barriers that limit the movement of animals between groups might separate subpopulations. If fairly large differences exist in allelic frequencies among these subsamples, and they are lumped together, there might be a net deficiency of heterozygotes and a corresponding excess of homozygote even in the presence of Hardy-Weinberg equilibrium within each subsamples (Wahlund, 1928). This phenomenon is called as Wahlund effect. Although, samples have been collected from two different places however, prospective Wahlund effect might not account significantly for the homozygosity as breeding tract of the Bachaur cattle had not been being separated by any geographical barriers in to separate pockets. Some alleles of microsatellite fail to amplify in the PCR because of mutation in the

flanking region. These are termed as Null Alleles. It leads to serious underestimation of heterozygosity compared with that expected on the basis of Hardy-Weinberg-Equilibrium (Callen *et al.*, 1993). Null alleles were also unlikely to be segregated at all the studied loci. Thus inbreeding or consanguinity, produced by mating between the relatives might be the major cause of heterozygosity deficit.

Bachaur is a draft purpose breed. Its bullocks are stardy and hard working in adverse condition also. Thus most of the males are used in carrying loads and agricultural operations by small frmers and these males are castrated at the age of one year leading to their genetic death. In addition to above, mechanization of agricultural works also reduced the number of breeding bulls (Singh, S. R., 2004). The only source of breeding bulls in the native tract had been the traditional system of bull donation in the cremation ceremony (Known as *BRIKHOTSARG SHRADH*). People release a male Bachaur calf after hot branding as mark of identification with the belief that this would ensure the peace of soul in the heaven. These male Bachaur calves would serve as breeding bull after attaining maturity. Bull donation is based on faith and belief in a family. In most of the cases these calves are released from same families regularly and serve as breeding bull. Thus there is inbreeding in this population owing to unplanned and unsystematic breeding strategies leading to mating between relatives.

4.5 Hardy Weinberg Equilibrium

Hardy Weinberg equilibrium test was used to predict whether the population is stable or not. The observed genotypes were compared with the expected genotypes in a χ^2 test of goodness of fit and likelihood ratio (G^2) test. The results of the test are presented in the Table 5. The population of Bachaur cattle was not in Hardy Weinberg equilibrium as 12 of 22 studied microsatellite loci produced highly significant $\chi 2$ value ($P<0.01$). The deviation of 54.55% loci from equilibrium may be due to several causes such as population subdivision, significant inbreeding, selection, genetic

drift or substantial inflow of genes from another population. Kumar *et al.* (2006) reported similar causes of deviation from Hardy Weinberg equilibrium for Hallikar population whereas Kim *et al.* (2002) repoted the lack of genetic equilibrium because of existence of null alleles, high mutation rate and size homoplasty of microsatellite loci, while investigating genetic diversity of Korean and Chinese goat breeds based on microsatellite markers. Cervini *et al*, (2006) found deviation of Brazilian Nellore cattle population from the Hardy Weinberg equilibrium due to heterozygote deficiency. In Mafriwal cattle, Selvi et al (2004) suggested that practice of using frozen semen of other breed for artificial insemination (AI) might be the reason for the deviation of investigated population from Hardy Weinberg equilibrium. In the present study observed heterozygosity of all the loci that has deviated from Hardy Weinberg equilibrium was less than that of their respective expected heterozygosity. Project report on survey and characterization of Bachaur cattle (Singh, S. R., 2004) reported very less number of breeding bulls in the breeding tract of Bachaur cattle. Therefore significant inbreeding might be the major cause of deviation of population from the Hardy Weinberg equilibrium in this case.

Table No. 5 Test for Hardy-Weinberg equilibrium

Locus	Hardy-Weinberg equilibrium		
	df	Chi square value	G^2
ILSTS11	3	5.3963^{NS}	3.327792
ILSTS05	10	12.1687^{NS}	12.750586
ILSTS30	6	20.2495^{**}	14.531767
ILSTS54	15	50.2366^{**}	45.445156
INRA35	36	73.6744^{**}	55.457333
INRA63	6	56.1978^{**}	58.501560
MM8	21	71.6043^{**}	53.244312
MM12	28	125.3281^{**}	57.546079
BM1818	28	36.7242^{NS}	26.653014
BM1824	6	10.8326^{NS}	9.213015
ETH225	10	12.7936^{NS}	11.255385
HEL01	15	8.6255^{NS}	10.639612
HEL05	28	209.6853^{**}	64.875874
CSRM60	15	18.2437^{NS}	20.278331
ILSTS33	21	40.1712^{**}	25.000837
ETH3	15	35.5048^{**}	43.566926
ILSTS06	15	19.6376^{NS}	17.989088
HAUT27	15	42.5750^{**}	32.438389
CSSM66	10	18.0007^{NS}	13.003909
HEL09	28	33.6176^{NS}	42.103697
HAUT24	21	40.3104^{**}	40.720127
ETH10	6	160.5211^{**}	63.521662

**= Highly Significant ($P<0.01$); NS= Non Significant

4.6 Ewens -Watterson Neutrality Test

Ewens-Watterson Test for Neutrality was performed for each of the 22 loci considered in the present study. The statistics has been presented in the Table 6. The observed sum of squared allele frequencies (observed F) i. e. homozygosities was compared with the 95% confidence intervals for the expected sum of squared allele frequencies (expected F). The 95% confidence interval and S. E. for observed F were calculated using 1000 simulated samples. In Bachaur cattle observed homozygosity at all the loci were found to be less than the corresponding expected homozygosity or mean homozygosity except loci MM8, ETH225, HEL1 and HEL5. However value of observed homozygosity at locus ILSTS005, INRA35, MM12 and HEL9 outlied the lower and upper limit of 95% confidence level. The Ewens Watterson Neutrality Test also indicates the selection acting on a locus. The expected homozygosity was calculated from the allelic frequencies with neutrality expectation and random mating assumption, i. e. only random genetic drift was responsible for changes in gene frequencies in the population in the absence of selection or migration. The value of F not significantly different from zero indicates that the only force acting on the population was random genetic drift and that there was no selection pressure acting on the population. If observed homozygosity is higher than expected homozygosity the locus is under going purifying selection i. e. homozygotes are favoured. When expected homozygosity is greater than observed homozygosity the locus is said to be under balancing selection i. e. heterozygotes are favoured in comparison to homozygotes. In this study most of the loci showed lower observed homozygosity than that of expected homozygosity. Therefore Bachaur cattle poputation was found to be under balancing selection (overdominant selection, frequency dependent selection etc.). Thus heterozygotes are being favoured in comparison to homozygotes in this population.

Table No. 6 The Ewens-Watterson Test for Neutrality in Bachaur cattle population.

Locus	Sample Size	Obs. F	Mean F	L 95	U 95
ILSTS11	90	0.4647	0.6725	0.3662	0.9563
ILSTS05	86	0.2482*	0.4723	0.2599	0.8250
ILSTS30	90	0.4402	0.5628	0.3040	0.8933
ILSTS54	82	0.2383	0.4086	0.2198	0.7552
INRA35	94	0.1523*	0.2945	0.1684	0.5514
INRA63	94	0.3463	0.5607	0.3094	0.8975
MM8	92	0.3755	0.3732	0.2023	0.7070
MM12	82	0.1678*	0.3225	0.1770	0.6029
BM1818	90	0.2143	0.3292	0.1817	0.6319
BM1824	92	0.3755	0.5708	0.3173	0.8956
ETH225	90	0.4914	0.4730	0.2573	0.8321
HEL01	52	0.4253	0.3769	0.2219	0.6908
HEL05	68	0.3214	0.3053	0.1747	0.5420
CSRM60	84	0.3869	0.4087	0.2384	0.7245
ILSTS33	76	0.3037	0.3525	0.1991	0.6371
ETH3	92	0.2628	0.4205	0.2351	0.7618
ILSTS06	82	0.2731	0.4087	0.2243	0.7168
HAUT27	68	0.3110	0.3995	0.2253	0.7327
CSSM66	90	0.3573	0.4855	0.2679	0.8523
HEL 09	80	0.1434*	0.3205	0.1850	0.5953
HAUT 24	82	0.2145	0.3627	0.1987	0.7133
ETH 10	92	0.5243	0.5577	0.3036	0.8956

F = Homozygosity; L95 (Lower), U95 (Upper) 95% confidence level

*= Deviation from Neutrality

All microsatellite loci except the loci ILSTS005, INRA35, MM12 and HEL9 were found to be neutral and unlinked to any selected trait. Similar results were obtained by Sharma *et al.* (2006 & 2006a) in Gangatiri and Ponwar cattle respectively. Arora, R. (2002) while investigating the buffalo population of Bhadwari and Tarai breeds also reported similar results.

4.7 Bottleneck analysis

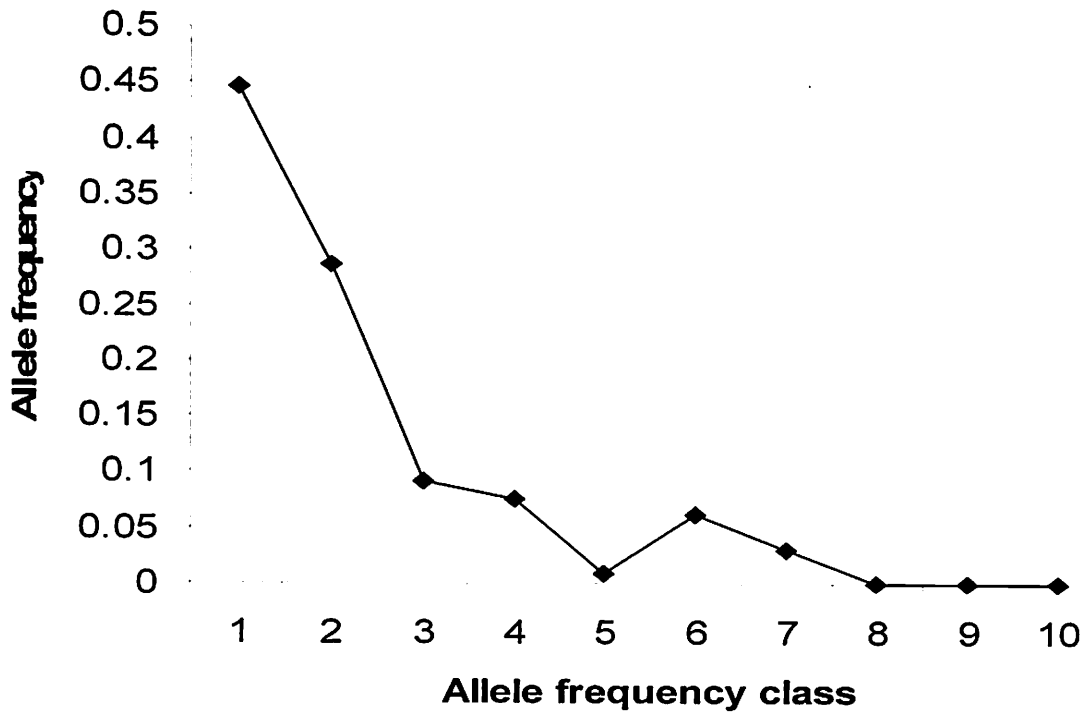
When a population experiences severe reduction in its size then it is said that the population has gone through bottleneck. A bottleneck population loses its genetic variability that may make the population susceptible to extinction because of the lack of the adaptive flexibility (Beardmore, 1983). When a bottleneck occurs in a population the effective population size is significantly reduced, it causes a correlative and progressive reduction in allele number and heterozygosity. Allelic diversity is reduced faster than the heterozygosity during a bottleneck (Nei *et al.*, 1975; Dennston, 1978). Thus bottleneck in population has been tested by determining the relationship between observed heterozygosity and expected number of alleles. Cornuet and Luikart (1997) developed tests to evaluate the differences between observed heterozygosity and heterozygosity expected from the observed number of alleles. The population thus showing heterozygosity excess would be considered as having experienced a recent genetic bottleneck. The data generated on 22 microsatellite loci for Bachaur cattle population was analysed using four tests viz. Sign Rank test, Standard Differences test, Wilcoxon's Rank test and a qualitative test of mode shift to find out if the population was in mutation drift equilibrium. Data have been shown in the Table 7.

Table No. 7 Bottle Neck Analysis Under Three models of Microsatellite evolution in Bachaur cattle population

Methods	Models	Sign test	Standardized test	Wilcoxon test
A. Frequency Methods	IAM	Hee = 12.96 Hd = 5 He = 17 P =0.05900	T2 = 2.434 P = 0.00747*	P(one tail for H deficiency) : 0.99766 P(one tail for H excess) : 0.00264* P(two tails for H excess or deficiency) : 0.00528
	TPM	Hee = 13.12 Hd = 8 He = 14 P = 0.43962	T2 = 0.366 P = 0.35732	P(one tail for H deficiency) : 0.71697 P(one tail for H excess) : 0.29396 P(two tails for H excess or deficiency) : 0.58793
	SMM	Hee = 13.05 Hd = 12 He = 10 P = 0.13457	T2 = -3.106 P = 0.00095*	P(one tail for H deficiency) : 0.08293 P(one tail for H excess) : 0.92201 P(two tails for H excess or deficiency) : 0.16586
B. Heterozygosity Method	IAM	Hee = 12.99 Hd = 7 He = 15 P = 0.25899	T2 = 1.635 P = 0.05099	P(one tail for H deficiency) : 0.97692 P(one tail for H excess) : 0.02504* P(two tails for H excess or deficiency) : 0.05007
	TPM	Hee = 13.04 Hd = 8 He = 14 P = 0.42582	T2 = -0.662 P = 0.25403	P(one tail for H deficiency) : 0.50000 P(one tail for H excess) : 0.51269 P(two tails for H excess or deficiency) : 1.00000
	SMM	Hee = 13 Hd = 14 He = 8 P = 0.02642*	T2 = -4.370 P = 0.00001*	P(one tail for H deficiency) : 0.00574 P(one tail for H excess) : 0.99484 P(two tails for H excess or deficiency) : 0.01147

Hee= Expected heterozygosity excess; Hd=Heterozygosity deficiency; He= Heterozygosity excess; P=Probability IAM=Infinite Allele Model; TPM=Two Phase Model; SMM= Stepwise Mutation Model; *Rejection of null hypothesis/Bottleneck

Fig. 14 L-shaped mode-shift graph showing lack of bottleneck in the Bachaur cattle population



The value of Heterozygosity excess (H_e) under IAM (17) and TPM (14) models of microsatellite evolution in frequency based method of sign test were greater than the expected heterozygosity excess (H_{ee}) which is 12.96 and 13.12 respectively, only H_{ee} value under SMM (13.05) was found to be greater than that of H_e value (10) under the same model. However, probability of accepting the null hypothesis under all the mode viz. IAM (0.059), TPM (0.439) and SMM (0.135) were found to be more than 0.05. In the heterozygosity based method of sign test similar results were obtained except for the SMM (P= 0.0264) where null hypothesis was rejected. But under IAM and TPM null hypothesis was expected as P value is greaterthan 0.05 in both the cases. Thus accepting the null hypothesis indicate that Bachaur cattle population was under the mutation drift equilibrium and has not experienced a recent bottleneck. The standardized differences test provided the T2 (Probability) statistics equal to 2.434 (0.00747), 0.366 (0.35732) and -3.106 (0.00095) for the IAM, TPM and SMM models respectively in frequency method. The probability value were less than the 0.05 for IAM and SMM model. Thus the hypothesis of population to be in mutation drift equilibrium was accepted under TPM only in allelic frequency based method whereas in the heterozygosity based method population was in mutation drift equilibrium under both the IAM and TPM (Table No. 7). In case of Wilcoxon test the probability value (one tail for heterozygosity excess) were 0.0026, 0.2939 and 0.9220 under IAM, TPM and SMM respectively in allelic frequency based method. In heterozygosity based method these values are 0.02504, 0.51269 and 0.99484 respectively. Thus null hypothesis can only be accepted in case of IAM model of microsatellite evolution in both the methods. Finally the qualitative or graphical method of Luikart and Cornuet (1996) was also utilized to visualize the allele frequency spectra. The microsatellite alleles were grouped in to 10 frequency classes which allow checking whether the

distribution followed the normal L-shaped form, where alleles with low frequencies (0.01-0.1) are the most abundant. The graph obtained showed a normal L-shaped distribution (Fig. No. 1). Taking results from all the four tests together, it is clear that serious demographic bottlenecks have not occurred in the Bachaur cattle population. Similar results had been reported in Gangatiri and Ponwar cattle by Sharma, *et al.* (2006 & 2006a), in Kherigarh and Kenkatha cattle by Pandey *et al.* (2006 and 2006a) respectively.

Summery And Conclusion

India is one of the Mega-biodiversity centres in the world. It possesses more than 6%world's livestock diversity. Majority of population depends upon the agricultural work and animal husbandry. As per the Indian Council of Agricultural Research India is the breeding tract of 30 cattle breeds whereas majority of the livestock falls under non-descript category

Bachaur is one of the famous draught purpose cattle breed in indiaits breeding tract has been the Bachaur Pargana (Madhubani, Sitamarhi and Darbhanga districts of Bihar). Now breeding tract has shifted with time to northern part of Bihar bordering Nepal. Although bullocks are excellent draft animal yet poor milk producing female of this breed is one of the main causes of the endangered status of this breed in addition to the dilution of breed by indiscriminate cross-breeding and mechanization of agricultural work. In the present study molecular genetic characterization of Bachaur breed of cattle has been done by using 22 FAO recommended highly polymorphic microsatellite markers with the following objectives.

1. To study the genetic diversity within Bachaur Cattle breed.
2. To asses whether the Bachaur Cattle breed has experienced any recent genetic bottleneck.

Blood samples were acquired from 50 randomly selected and unrelated Bachaur animals from the breeding tract. The blood samples were collected from thye jugular vein of the animal in vacutainer containing K3EDTA as anticoagulant. Genomic DNA was isolated and purified from the blood cells using the standard phenol-chloroform-isoamyl alcohol extraction followed by ethanol precipitation described by Sambrook et al. (1989). The quality and quantity of isolated genomic DNA was assessed by 0.6% AGE.

A set of 22 microsatellite markers recommended for cattle in FAOs DADIS MoDAD programme were utilized for generating microsatellite genotyping data in a panel of 47 animal. PCR was performed with 50-100

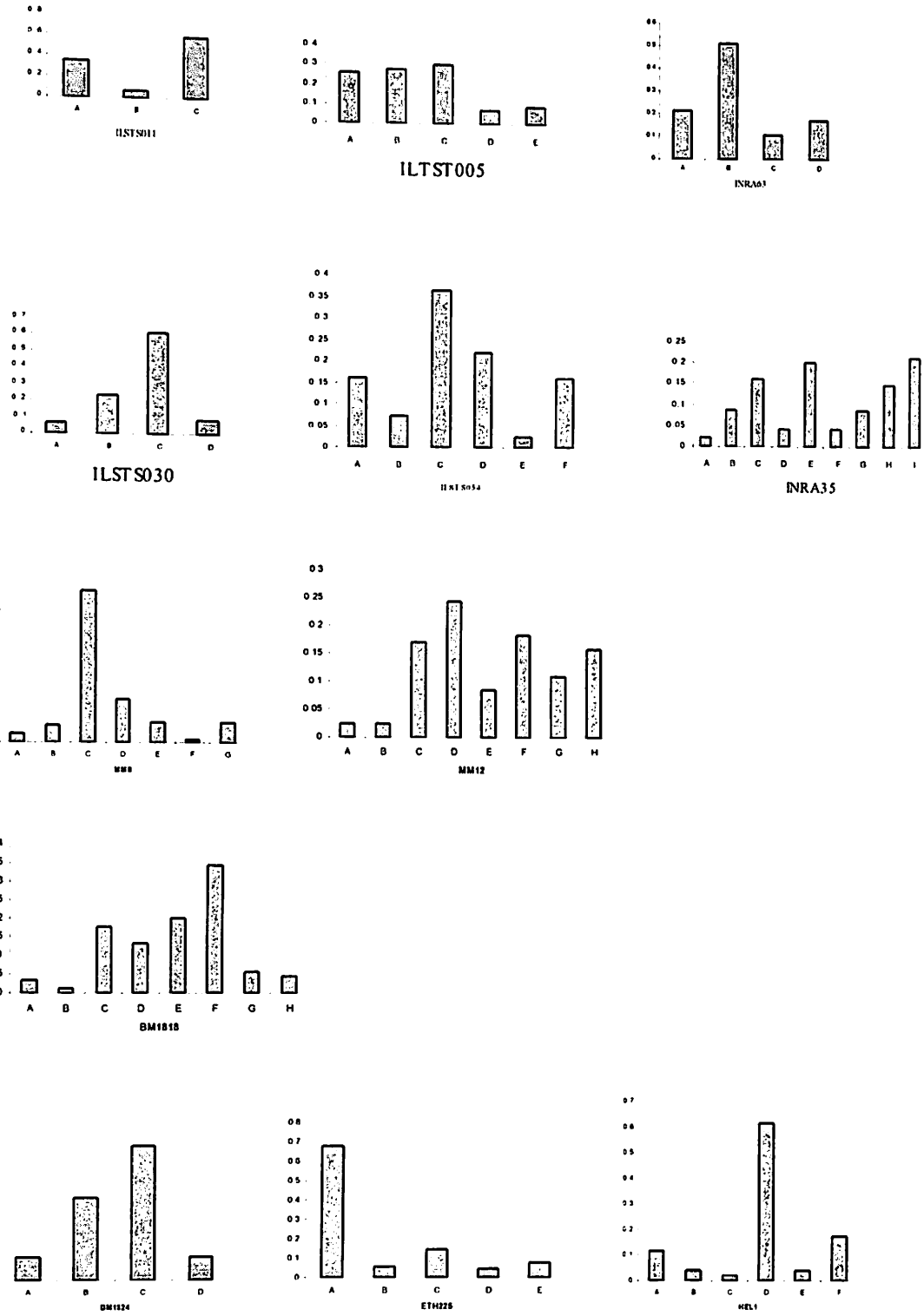
(ETH10) to 0.850 (MM12) with the mean (± s.d.) value 0.660±0.117. Thus with the exception of ETH10 (0.456), ETH225 (0.494) and ILSTS011 (0.491) all loci were highly informative whereas these four loci were found to be reasonably informative. The observed heterozygosity within population varied from 0.065 (ETH10) to 0.875 (HEL9) with the mean (±s.d.) 0.529±0.179. The observed heterozygosity was less than that of expected which ranged from 0.481 (ETH10) to 0.867 (HEL9) with the mean (±s.d.) 0.688±0.111. The Nei's expected heterozygosity within the Bachaur population varied from 0.476 (ETH10) to 0.857 (HEL9) with overall mean (±s.d.) of 0.680±0.110. A significant deficit (22.96%) of heterozygote exists in the Bachaur population. All the microsatellite markers with the exception of HEL9, ILSTS006, CSRM60 and HEL1 contributed to observed heterozygote shortage.

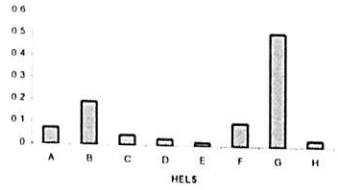
All the studied microsatellite loci except the loci ILSTS005, INRA35, MM12 and HEL9 were found to be neutral as judged by the Ewens-Neutrality test. It nullify the possible role of selection in the heterozygote deficit in the Bachaur population. the population of Bachaur cattle was not in Hardy Weinberg equilibrium as 12 of 22 studied microsatellite loci were producing highly significant $\chi 2$ value ($P<0.01$). significant inbreeding was found to be the major cause of deviation of population from the Hardy Weinberg equilibrium. Bottleneck analysis in the Bachaur cattle population has not experienced any recent bottleneck at least in recent past.

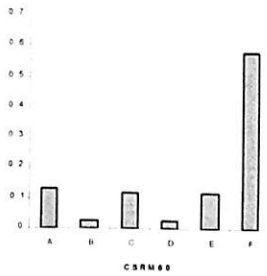
The present work contributes to the knowledge of existing genetic variation within the Bachaur cattle population in its native tract. The present study revealed the decreased variability in Bachaur cattle population in the breeding tract as prime concern. Therefore maintenance of the Bachaur cattle needs to be dealt properly. Conservation of genetic variation in Bachaur population should be considered by breeders, in the interest of long term future of the breed in the native tract. As a first step breed society needs to be created which will look after the complete maintenance and

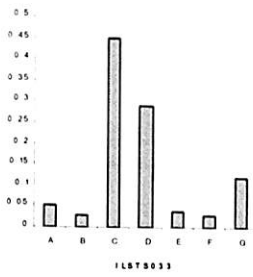
improvement of the breed. It will check the migration of pure breed from the breeding tract and availability of frozen semen of pure Bachaur bull to the farmers. Farmer's awareness programmes regarding the importance of conservation of animal genetic resources are also the need of the hour. Thereby, Bachaur cattle could be made economically sustainable in the transforming agricultural scenario of the country

Fig 15 Graphical representation of allele frequencies of microsatellite loci.




HEL5


CSRM60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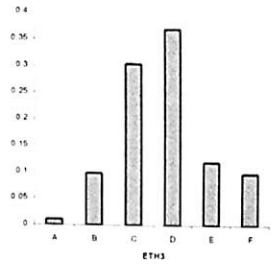
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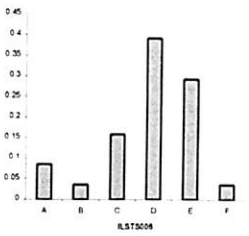
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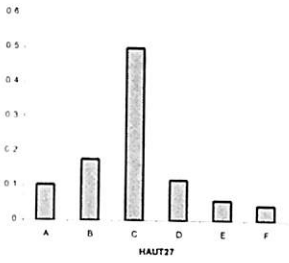
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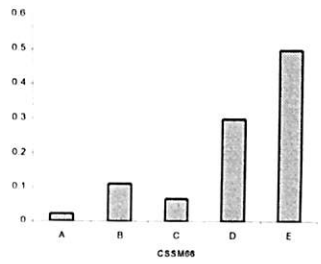
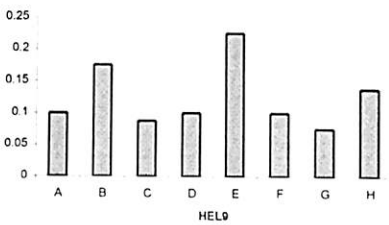
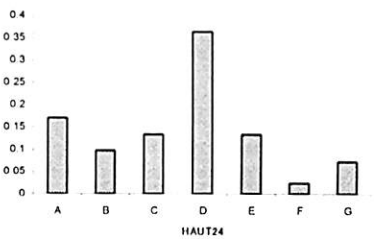

CSSM66


HEL9


HAUT24

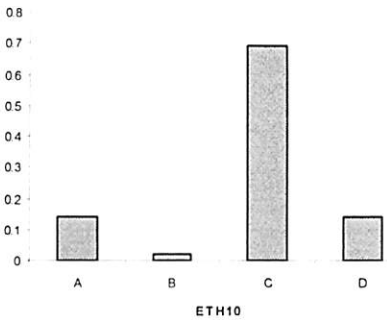

ETH10

Fig 16 Heterozygote deficiency (F_{IS}) in Bachaur cattle population across 22 microsatellite loci

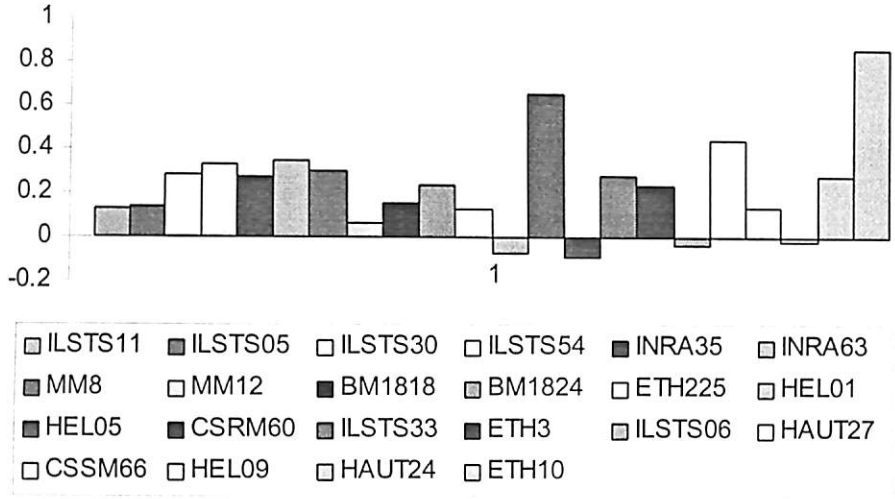
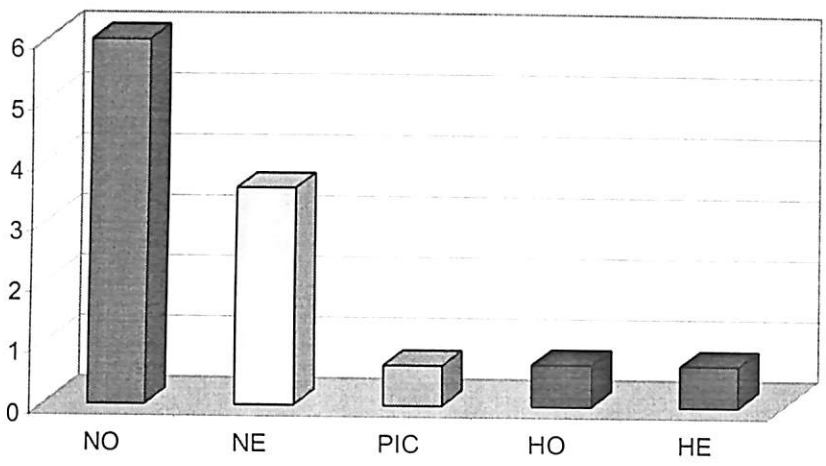


Fig. 17 Mean Diversity indices across 22 microsatellite loci in Bachaur cattle population



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APPENDIX 1

Sl. No.	Name of Chemicals/Reagents	Name of the Company
1.	Absolute Ethanol	S. D. Fine
2.	Acetic acid (glacial)	SRL
3.	Agarose M	Biogene, USA
4.	Acrylamide	SRL
5.	Ammonium Chloride	S. D. Fine
6.	Ammonium Per Sulphate	S. D. Fine
7.	Bisacrylamide	Biogene, USA
8.	Boric Acid	SRL
9.	Bromophenol Blue	Biogene, USA
10.	Chloroform	SRL
11.	DNTPs	Fermentas
12.	DNA ladder (10bp)	Invitrogen
13.	EDTA	S. D. Fine
14.	Formaldehyde (37-41%)	S. D. Fine
15.	Formamide	S. D. Fine
16.	8-hydroxyquinolene	Excelar
17.	Isoamylalcohol	SRL
18.	Magnesium Chloride	Ge Nei™
19.	Phenol	SRL
20.	Potassium bi carbonate	S. D. Fine
21.	Primers (P1, P2)	IDT and AuPrep-Oligo
22.	Proteinase K	USB, Amershan Life Sc.
23.	Silver nitrate	CDH
24.	Sodium acetate	S. D. Fine
25.	Sodium carbonate	S. D. Fine
26.	Sodium do decyl sulphate	S. D. Fine
27.	Sodium hydroxide	S. D. Fine
28.	Sodium thio sulphate	S. D. Fine
29.	Taq Polymerase	Ge Nei™
30.	Taq Poly. Buffer	Ge Nei™
31.	TEMED	Biogene, USA
32.	Tris buffer	S. D. Fine
33.	Urea	Biogene, USA
34.	Xylene cyanol	Sigma

APPENDIX 2

Lysis buffer (1000 ml)

NH_4Cl	: 8.3 g
$KHCO_3$	: 1.0 g
EDTA (0.5 M, pH 8)	: 200 µl

Extraction buffer (500 ml)

NaCl	: 2.19 g
EDTA (0.5 M, pH 8)	: 2.0 ml
Tris-HCl(1M, pH 8)	: 5.0 ml

TE buffer (100 ml)

Tris-HCl (1 M, pH 8.0)	: 1 ml
EDTA (0.5 M, pH 8.0)	: 0.2 ml

50x TAE Buffer (1000 ml)

Tris base	: 242 g
EDTA	: 37.2 g
Acetic acid	: 57.1 ml

10x TBE Buffer (1000 ml)

Tris base	: 108gm and
Boric acid	: 55gm
EDTA (0.5 M, pH 8.0)	: 40ml

6% urea polyacrylamide gel mix (1000 ml)

Urea	: 480gm
Acrylamide	: 57gm
Bisacrylamid	: 3 gm
10x TBE	: 100ml

Binding Silane (Working)

Bind Silane (stock)	: 3 µl
Ethanol (95 %)	: 1 ml
glacial acetic acid	: 5 µl

Silver Nitrate Solution (2000ml)

Silver nitrate	: 2g
Formadehyde(37%)	: 2ml
Distilled water up to	: 2000ml

Developer Solution (1500ml)

Sod. Carbonate: 30g
Formaldehyde (37%): 2.25ml
Sodium Thiosulphate (1%): 150 µl
Distilled water up to 1500ml

1M Tris HCl (pH 8)

121.2 g Tris base mixed in water. pH is adjusted with the conc. HCl to 8 and final volume made to 1000 ml. and sterilized by autoclaving.

0.5M EDTA (pH 8)

14.61 g EDTA or 18.61g Na2EDTA. 2 H2O mixed in distilled water. pH is adjusted by adding NaOH pelletes. Final volume made to 100 ml by adding distilled water. Sterilized by autoclaving.

3M Sodium acetate (pH 5.5)

24.6 g Sodium acetate, dissolved in 100ml distilled water. Adjust the pH with glacial acetic acid. Sterilized by autoclaving

SDS (20%)

20g of SDS mixed in 100 ml distilled water very gently. This solution is not to be sterilized.

Proteinase K

0.2g of proteinaseK mixed in 10ml of distilled water.

Ammonium per sulphate (10%)

1 g of APS is dissolved in the 10 ml of distilled water (it should be made fresh each time before use).

Ethidium bromide

0.2g Etbr mixed in 20ml distilled water and mixed well.

Sodium Thio-Sulphate solution (1%)

1g STS mixed in 100ml of distilled water

Loading Dye

Take 50ml resin in a beaker (250ml). add 150ml formamide keep it in fridge for 30 min. now stir it at 40c for 1 hour. Stop it and allow it to settledown. Separate out the formamide (approx. 50ml). now add 50mg bromophenol blue and 50 mg xylene cyanol and 1ml 0.5M EDTA in the separated phase. Stir it again for half an hour at room temperature. Fiter it. And aliquoted in eppendorf tube for use. (Resin can be used again :keep at RT for 3-4 days , add 100ml formamide).

APPENDIX 3

Sl. No.	Name of the Equipments	Company Name
1.	Centrifuge Machine a. HERMLE 2323K b. HERMLE Z233K-2	HERMLE Labortechnik
2.	Deep Freezer	Vesfrost
3.	Ice flaking machine	F100 Compact Icematic Spectrum associate
4.	PAGE assembly with Power Pac HV	BIORAD
5.	PCR Machine a. Peltier Thermal Cycler-200 b. i-Cycle	 M. J. Research BIORAD
6.	Refrigeretor	Whirlpool
7.	UV Transilluminator	UVP, USA
8.	Vacutainer	BD
9.	Vortex Machine	Spinix
10.	Water bath	Akashdeep
11.	Water Purification System	Milli-Q, UF PLUS Purification Pak
12.	Weighing balance	Sartorius