STUDIES ON DNA POLYMORPHISM IN BUFFALOES USING MICROSATELLITE MARKERS

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BY
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DEDICATED

TO

MY BELOVED PARENTS

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By

AMIT KUMAR PANDEY

**A Thesis submitted to the National
Dairy Research Institute (Deemed University), Karnal
In partial fulfilment of the requirements
for the Degree of**

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IN
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Approved by

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10th June, 1999

CERTIFICATE

This is to certify that the thesis entitled **"STUIDES ON DNA POLYMORPHISM IN BUFFALOES USING MICROSATELLITE MARKERS"** submitted by **AMIT KUMAR PANDEY**in partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE** in **ANIMAL BIOTECHNOLOGY** of the **NATIONAL DAIRY RESEARCH INSTITUTE (Deemed University)**, Karnal (Haryana), INDIA, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

BRYadav.

(B.R. YADAV)
MAJOR ADVISOR & CHAIRMAN
(GUIDE)

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Amit Kumar Pandey

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LIST OF ABBREVIATIONS

A : Adenine
AP-PCR : Arbitrarily Primed PCR
APS : Ammonium Persulphate
bp : Base pair
C : Cytosine
DAF : DNA Amplification Fingerprinting
DNTP : Deoxy Nucleotide Triphosphate
DFP : DNA Fingerprinting
DNA : Deoxyribonucleic acid
ddNPT : Dideoxy Nucleotide Triphosphate
DTT : Dithio Thritol
FAO : Food and Agriculture Organization
FEC : Faecal egg count
FISH : Fluorescent *in situ* hybridization
G : Guanine
GTI : Gel temperature Indicator
IPC : Integrated Plate Chamber
MAS : Marker assisted selection
MHC : Major Histocompatibility Complex
NDRI : National Dairy Research Institute
PAA : Polyacrylamide
PCR : Polymerase chain reaction
PIC : Polymorphism Information content
QTL : Quantitative trait locus
RAPD : Random amplified polymorphic DNA
RE : Restriction endonucleases
RFLP : Restriction fragment length polymorphism
SNF : Solids not fat
Str : Small tandem repeats
T : Thymine
T_A^{Opt} : Optimum annealing temperature
TBE : Tris Borate EDTA
TEMED : N,N, N', N'' Tetramethyl Diamine
UK : United Kingdom
USA : United States of America
USB : United State Biologicals
VNTR : Variable number of tandem repeats

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

India has diverse and very rich genetic resources and the privilege of having best breeds of buffalo. Indian buffalo (*Bubalus bubalis*) with a population of about 80.1 million and account for nearly 52.7 percent of the world buffalo population of 152 million. Although buffalo population is about 29 percent of the total Bovine population (Cattle and Buffalo), buffalo contributes more than 50 percent of the milk produced by the two bovine species and, therefore, plays a pivotal role in meeting the requirement of milk for human consumption (FAO yearbook, 1996). Buffalo possesses several other outstanding qualities such as higher fat and SNF in its milk, it has long lactation length and production life with high efficiency in milk production and the most important being, it's a triple purpose animal (producer of milk, meat and draft power).

India has some of the best breeds of buffalo in the world. These buffalo are well adapted to Indian conditions. The different breeds are morphologically quite distinct, however it is very difficult to differentiate them by karyotyping or biochemical tests. Cross breeding is quite common among different breeds and there is significant decline in pure breed buffaloes. Moreover, as a result of slaughter programme there is continuous erosion of the centuries old highly adaptive germplasms. Hence, there is an urgent need to characterize and evaluate the indigenous animal genetic resources.

The aim of animal breeding has always been to improve and to exploit the genetic potential of animal for the welfare of the mankind. The basis for improvements is hereditary variation in both quantitative and qualitative traits within and among populations. Variability, the raw material for selection, was earlier measured at gross anatomical or morphological and biochemical levels, known as classical method of variability estimation. The classical methods have reached a level of uniformity i.e., the selection limit, and further improvement is not possible due to exhaustion of the variability (Al-Murani, 1972). This is because of the fact that variability in the classical markers is limited, and many of the traits are sex limited, age dependent and also significantly influenced by the environment. Further, these markers are useful only in the measurement of variability of those functional genes which significantly express phenotypically. Owing to the low variability in the classical marker systems there is an urgent need for research at the molecular level using modern genetic tools. There is also a need to develop the marker system for pedigree and paternity testing to see the descent of the animals and accordingly plan the mating systems.

There exists a significant homology in chromosomal banding structures and coding sequences of DNA between cattle, sheep, goat and buffalo. Genome conservation has also been found extended to microsatellite loci between different species of animals although less conserved than coding sequences. The heterologous PCR primers in related mammalian groups are used quite commonly. The conserved microsatellite loci can be used to construct the comparative linkage maps and to study the polymorphism between and within species. Cattle being a cosmopolitan species, world-wide work is in progress on the analysis of its genome by various approaches including microsatellite markers. A lot of resources and manpower is required for the development of primers for microsatellite markers. Therefore the use of heterologous primer pairs in less studied animal species like buffalo can save time and other resources. Keeping this in view the present study was undertaken with the following objectives:

1. To standardize the protocol of *in vitro* amplification of microsatellite loci by PCR and resolution of PCR products by denaturing sequencing gel electrophoresis.
2. To study the DNA polymorphism in buffaloes revealed by bovine microsatellite primers.

CHAPTER 2

REVIEW
OF
LITERATURE

2. REVIEW OF LITERATURE

Genetic polymorphism is the simultaneous occurrence of two or more genetically determined variant forms of a particular characteristic at a frequency where the rarest could not be maintained by recurrent mutation alone.

Polymorphism can be studied at various levels

- Anatomical or morphological level
- Biochemical level
- Molecular level

Polymorphism at the phenotypic level is studied by comparing several anatomical or morphological features e.g., pigmentation, body size, variation in certain body features conformity etc. Polymorphism at this level is quite low and is usually difficult to study within breeds of the same species and particularly the traits that are age and sex dependent. Moreover environmental factors also influence the expression of some of the traits.

The basis of studying polymorphism at biochemical level is the result end of 'central dogma' that is "the genes are perpetuated as sequences of nucleic acid, but function by being expressed in the form of proteins". So any change at the gene sequence level is expressed as a change in the amino acid sequences of the protein. This change at the amino acid level can produce two or more forms of the same protein which can be differentiated by either electrophoretic or by several immunogenetic methods. The electrophoretic markers include the proteins and allozyme polymorphism in different body fluids and immunogenetic markers include blood group antigenic systems, lymphocyte antigenic system (MHC).

Allozyme electrophoresis is a potential method for detecting and estimating genetic variation. Allozymes are protein variants of a genetic locus and behave in a Mendelian fashion. These are interpretable as simple allelic products of a gene. However most of the variation present in the genome can not be detected by this method due to several reasons:

- About three percent of the genome constitutes the coding sequences.
- Redundancy in the genetic code,
- Replacement of every amino acid does not lead to a charge difference that governs the mobility of the protein variant in gel,
- Many enzymes are age and sex dependent and are significantly influenced by environment,

The progress in molecular genetics has given rise to a new class of markers called molecular markers or genomic markers. The molecular markers have several advantages over the classical markers. The limitations of classical markers include low degree of polymorphism, sex and age dependence, influence of environment and dominance effect. The advantages of molecular markers are many viz. they reveal almost unlimited polymorphism at the DNA sequence level that can potentially be exploited as genetic markers. These markers are numerous and distributed ubiquitously throughout the genome, follow typical Mendelian inheritance, unaffected by environmental factor and do not have pleiotropic effect. These markers reveal variation in both the coding and non-coding sequence regions.

2.1 POLYMORPHISM AT DNA SEQUENCE LEVEL

In terms of DNA polymorphism a trait is encoded by a piece of DNA with two or more alleles (sequence variants), of which at least two occur at a frequency of more than one percent in a given population. DNA polymorphism occurs due to changes in the base sequence of DNA caused by events such as point mutations, base substitution, deletions, insertion and their rearrangements. This polymorphism is further sustained by the process of meiotic recombination, ensuring that no offspring has a genome made up of a simple collection of entire grandparental chromosomes. DNA polymorphism study helps in understanding the extent of genetic variability in a population.

The characteristics of an organism are specified by its genetic information represented as a precise nucleic acid sequence. The sum total of this sequence information present in its cell is called genome. Eukaryotic genome comprises of both coding as well as non-coding sequences. The coding sequences that control the essential functions do not vary much between individuals. However, function of non-coding sequence is still obscure.

The eukaryotic genome is broadly classified as unique and repetitive DNA sequences. Unique sequences are those having approximately one single copy per haploid genome and are mostly protein coding genes (Rosbash *et al.*, 1974). Repetitive DNA sequences are further classified into moderately repetitive and highly repetitive sequences. The repetitive sequences are mostly in non-coding region of DNA. These non-coding sequences include intragenic DNA consisting of psuedogenes, introns, leaders, trailer and extragenic DNA. The extragenic DNA consists of unique low number DNA, moderate copy number DNA and high copy number DNA. The high copy numbers are further classified into satellite, minisatellite and microsatellite depending upon the size of the nucleotide repeat unit. They are dispersed in the genome in various forms or arranged in tandem repeats. The tandemly repeated sequences are especially liable to undergo misalignments during chromosome pairing and thus the size of tandem clusters tend to be highly polymorphic with wide variation between individuals. This difference in the size of the tandem cluster causes the allelic variation.

2.2 METHODS OF ANALYSIS OF DNA POLYMORPHISM

In the last two decades many methods have been developed for visualization of DNA sequences variation in the genome of animals. The main methods include:

1. Restriction fragment length polymorphism (RFLP)
2. Randomly amplified polymorphic DNA (RAPD)
3. Minisatellite sequence polymorphism
4. Microsatellite sequence polymorphism

2.2.1 Restriction Fragment Length Polymorphism (RFLP)

Polymorphism at the DNA sequence level is often detected as a variation in length of fragments produced by cutting DNA with restriction enzymes. Botstein *et al.*, (1980), referred such polymorphism as restriction fragment length polymorphism (RFLP). In this method a DNA molecule when digested with a particular restriction endonuclease (RE) results in a set of reproducible DNA fragments of well defined lengths. These fragments are then separated by gel electrophoresis and subsequently transferred onto solid support like nylon membrane (Southern, 1975). The fragments of interest are then visualized by hybridization with an appropriately labeled DNA sequence used as probe. Mutation in genomic DNA viz., substitution, deletions, insertions or inversions may create, eliminate or translocate the recognition site of RE and thereby affect the length of resultant restriction fragments and may thus bring about a observable polymorphism between different species or populations (Botstein *et al.*, 1980). Restriction endonucleases with six base pair recognition sequence are usually used in combination with agarose gel electrophoresis.

Studies of mitochondrial DNA variation using RFLP have shown genetic difference between Riverine and Swamp Buffaloes (Amano *et al.*, 1994; Tanakar *et al.*, 1995). Similarly genetic diversity between these two breeds of buffaloes have also been carried out studying the mitochondrial DNA D-loop and cytochrome-b sequence variation (Lau *et al.*, 1998).

The studies on RFLPs have some limitations: The mean heterozygosity of eukaryotic DNA is low, approximately. 0.001 per base pair (Cooper and Schmidtke, 1984) and even if any restriction endonuclease could detect a few RFLPs at a given locus the detected RFLP's are usually dimorphic (presence or absence of a restriction cleavage site) consequently it would be uninformative in pedigree analysis whenever critical individuals are homozygous. The use of RFLPs is further limited by the use of mostly single locus (or species-specificity) probes.

2.2.2 Randomly Amplified Polymorphic DNA (RAPD)

The discovery of the technique of in vitro amplification of DNA using polymerase chain reaction (PCR) by Kary B. Mullis

(Mullis and Faloona, 1987) has revolutionized the way genomic organization can be studied. This technique has also practically made possible the study any region of genome. The PCR based methods have a lot of advantages over the traditional methods (e.g., Southern blotting) viz: they are highly sensitive and fast, require much less quantity of DNA samples, allow automation of the entire technique and thus are less labour intensive.

The PCR based RAPD analysis is very simple. In this technique a very small amount (in nanograms) of genomic DNA is subjected to PCR using short synthetic oligonucleotides of random sequence. The principle behind RAPD is that the genome contains several priming sites close to one another (i.e., within amplifiable distance) that are in an inverted orientation. The technique essentially scans a genome for these small inverted repeats and amplifies DNA segments of variable length. The amplification products are then resolved on agarose gel by electrophoresis resulting in DNA fingerprinting type of banding pattern. Since the primers used are arbitrary nucleotide sequences these markers are named as randomly amplified polymorphic DNA (Williams *et al.*, 1990). Two different modifications of the RAPD markers have been described viz., DNA amplification fingerprinting (DAF) using short random primers of 5-8 bases (Caetano-Anolles *et al.*, 1991) and arbitrarily primed PCR (AP-PCR) using slightly longer primers (such as universal M13) (Welsh and McClelland, 1990, Welsh *et al.*, 1991). Some of the advantages of this technique are:

i) Easy to carry out.

ii) Obviates the need of time consuming and cumbersome procedures.

iii) Requires a very small amount of DNA.

iv) Does not require any radiolabelled nucleotides.

v) Does not require any prior knowledge of nucleotides.

vi) Any organism can be mapped with same set of primers.

vii) Primer can be directed against a repeated motif sequence (Welsh and McClelland, 1992).

In contrast there are also certain limitations and factors which influence the RAPD fingerprinting. The size of primer determines the degree of the specificity. Amplification of fewer numbers of bands with shorter primer has been observed (Mac Pherson *et al.*, 1993). The main limitations of RAPD fingerprinting are the lack of specificity as well as its sensitivity to reaction conditions. A slight change in the conditions may affect the reproducibility of amplification products (Hadrys *et al.*, 1992; MacPherson *et al.*, 1993; Schierwater and Ender, 1993; Ellsworth *et al.*, 1993; Micheli *et al.*, 1993; Bielawski *et al.* 1995). Other limitations include: co-migration of a amplified product of similar size but of different locus (Hadrys *et al.*, 1992; Peinado *et al.*, 1992); amplification of some ambiguous and not easily scorable fragments (William *et al.*, 1990) due to non-specific priming or heteroduplex formation between related amplification products or other secondary structure artifacts.

2.2.3 DNA Polymorphism Analysis using Minisatellite Markers

The genetic complexity inherent in repetitive DNA families can be used to provide individual specific genetic markers. Minisatellites (Jeffreys *et al.*, 1985a,b.), also known as variable number of tandem repeats (VNTRs) (Nakamura *et al.*, 1987), comprise arrays of short sequence units (11-60 bp) which are tandemly repeated in the genome at different loci. The resulting length variation is detected with restriction enzymes, which do not cleave the repeat unit and a multilocus probe under reduced stringency conditions. This enzyme and single multilocus probe combination can simultaneously detect many independent polymorphic loci, producing a highly complex banding pattern specific to individual.

The probes being used for DNA polymorphism studies are available from at least 3 different sources viz., cloned cDNA and genomic DNA, synthetic oligonucleotides and PCR generated probes. Nakamura *et al.*, (1987) used synthetic oligonucleotides corresponding to consensus sequences of tandem repeats present in the introns of several genes for screening and isolation of VNTRs. Jefferys probes were isolated from intron adjoining to human myoglobin (Jefferys *et al.*, 1985). These probes can cross hybridize to produce DNA fingerprints in many species like mammals (Jefferys and Morton, 1987), birds (Burke and Bruford, 1987) fishes (Baker *et al.*, 1991) and invertebrates (Coffroth *et al.*, 1992). The property of certain VNTRs to cross hybridize across species has been exploited in isolation of new VNTRs, an approach known as probe walking (Washio *et al.*, 1989). Sequences of minisatellite are also found in viral genomes. One of the commonly used probe of viral origin is a GC rich probe consisting 15 bp sequence (GAGGTGGXGGTCT) found within protein III region of single standard filamentous bacteriophages MB (Vassart *et al.*, 1987). This probe produces polymorphic fingerprinting in several species of animal, plants and microorganisms (Ryskov *et al.*, 1988; Crawford *et al.*, 1991).

Minisatellite based DNA fingerprinting (DFP) has a wide range of applications viz. Individual identification (Jefferys *et al.*, 1985a); parentage determination (Jefferys *et al.*, 1985; Geldermann *et al.*, 1986; Bovenhus and Van Arendock, 1991); population studies involving estimation of level of individual and population homozygosity, relatedness between individual, estimation of within and between population variation (Dallas, 1988; Kuhnlein *et al.*, 1989; Dolf *et al.*, 1992); linkage analysis for finding linked markers to economically relevant mono or polygenic entities offers the possibility of marker assisted selection (Soller and Beckmann, 1982; Smith and Simpson, 1986; Georges *et al.*, 1990; Lanneluc, 1992).

2.2.4 Microsatellite Sequence Polymorphism

Simple sequence repeat (Str) (Tautz and Renz, 1984) or microsatellites (Litt and Luty, 1989) are short tandemly repeated sequences with repeat units of 1-6 nucleotides which can be conveniently amplified by the polymerase chain reaction (PCR). These are densely and evenly distributed through out the eukaryotic genome, averaging between 5000 to 100,000 in mammals. These sequence motifs were first recognized in early 1970s, e.g., $(TAGG)_n$ in satellite DNA of hermit crab (Skinner *et al.*, 1974). Further investigation of Str sequences for example, on sex-specific satellite DNA isolated from W chromosomes of the snake Elaphe radiata (Singh *et al.*, 1980), revealed longer stretches of GATA/GACA motifs. Since then a large number of str sequences have been identified in the genomes of a number of eukaryotic species including man (Tautz, 1989), mouse (Love *et al.*, 1990), pig (Winterio *et al.*, 1992) and horse (Ellegren *et al.*, 1992). Dinucleotide repeats, particularly CA/GT repeats are very abundant (Stallings *et al.*, 1991) and have great potential as genetic markers (Todd *et al.*, 1991). Many of the microsatellites that have been reported are polymorphic, the variation in length of different alleles being shown to arise from the variation in the number of repeat units. (Oudet *et al.*, 1991).

Initially Ali *et al.* (1986) used synthetic oligonucleotides probes $(GATA)_n$ / $(GACA)_n$ specific to Str sequences and established oligonucleotide fingerprinting technique. Scheffer *et al.* (1988) improved the technique of oligonucleotides fingerprinting in man using a variety of additional synthetic oligonucleotide probes consisting of repeat units of 2-4 bp. Since then, a wide variety of synthetic oligonucleotide probes have been used to reveal the mutlilocus-multi-allelic fingerprinting in man, animals and plants.

The analysis of microsatellite markers is usually done by PCR technique. The microsatellite islets contained as stretch of sequence of unique DNA are amplified by PCR using a pair of flanking oligonucleotides as primers (Beckmann and Soller, 1990). The amplified products on gel electrophoresis reveal high polymorphism at specific loci that could be measured in terms of percent heterozygosity and polymorphism information content (PIC) (Fries, 1993). The microsatellite primers can also serve as highly locus specific markers.

The short length of microsatellites makes them amenable to amplification by PCR and subsequent separation by polyacrylamide gel with the resolution of alleles by as little as single base pair. Many microsatellite markers have been found to be highly polymorphic and this variability is derived from variation in the number of repeat units in different alleles. Technically, microsatellites are easy to isolate and PCR based typing of alleles can be readily automated. They permit a highly precise dissection of the genetic structure of population or breed (Buchnan *et al.*, 1994; Blovin *et al.*, 1996; Goldstein *et al.*, 1996).

2.3 APPLICATIONS OF MICROSATELLITE MARKERS

DNA polymorphism studies using microsatellite markers have got much wider applications as compared to conventional dimorphic RFLPs and minisatellite markers. These include individual identification, parentage determination, breed, line, or strain characterization and population genetic studies. Besides, these markers also can be used for linkage analysis of economically important traits, disease resistance and gene mapping.

2.3.1 Individual Identification and Parentage Determination

Identification and parentage determination are very important in livestock species. Identity testing is useful in artificial insemination programmes. Particularly for checking the semen of prized bulls. Parentage determination is helpful in progeny testing programmes and paternity related disputes (reviewed by Shashikanth, 1999).

Buitkamp *et el.* (1991) conducted extensive studies on DNA fingerprinting obtained by 11 different synthetic probes in cattle. Among these only three probes $(GTG)_5$, $(GT)_8$ and $(GGAT)_4$ gave polymorphic banding pattern and found to be useful for individual identification. Ellegren *et al.* (1992) used $(TG)_n$ probe for hybridization with equine DNA digested with *Hinf*I enzyme. The probability that two unrelated individual would have the same DNA fingerprint was estimated to range between 1.0 x 10^{-4} to 7 x 10^{-6} within four breeds of horses selected for the study. Plante *et al.* (1992) screened 31 pairs of cattle twins by DNA fingerprinting out of which 30 pairs of twins exhibited identical fingerprints.

Ron *et al.* (1996) used 12 microsatellite loci to test the paternity of 173 cows and 102 bulls and found out that 5.2 per cent of cows and 2.9 per cent of bulls had misidentified paternity. Heyen *et al.* (1997) undertook parentage study by using 22 microsatellite primer pairs on 17 bovine autosomes in 1022 Holstein cattle and 311 beef cattle belonging to five breeds. Mommens *et al.* (1998) used a set of 33 microsatellite primer pairs on American bison from a captive population in Belgium and evaluated for usefulness in parentage testing.

2.3.2 Population studies

Genetic structure of population holds the key to the selection strategies which in turn are very important for the genetic improvement of the livestock species. The population studies involve individual and population homozygosity, estimation of relatedness between individual, estimation of within and between population variation etc. Buitkamp *et al.* (1991) studied 3 cattle breeds, viz., German Friesian, Red Pied and Simmental using $(GTG)_5$ oligonucleotide probe. The degree of variability differed notably between the breeds. Arranz *et al.* (1996) compared 14 protein markers and 5 microsatellite loci in differentiation of 5 population of cattle. The genetic variation at microsatellite loci was greater than protein loci. Genetic distance estimates based on microsatellite variation were 4-12 fold greater than those from protein markers.

Moazami *et al.* (1997) conducted studies to determine the genetic variation between 10 cattle breeds by using 17 microsatellite loci and 13 biochemical markers. A total of 210 alleles of the 17 microsatellites were detected in this study and average heterozygosities ranged form 0.53 in the Jersey breed to 0.66 in the Parthenais breed.

Peelman *et al.* (1998) analysed genetic variability of 23 bovine microsatellite markers in the four main cattle breeds in Belgium (Holstein-Friesian, Belgian-Blue, Belgian-Red Pied and East-Flemish). Estimates of genetic distances between these breeds confirmed the widely accepted proposition that the Belgium Blue is the most genetically distinct of these breeds.

2.3.3 Linkage Analysis

The findings of many worker have shown linkage of molecular markers to economically important traits and offered the possibility of marker assisted selection (MAS) in animal breeding, which is expected to improve genetic response by influencing both accuracy and time of selection (Soller and Beckmann, 1982, Smith and Simpson, 1986).

Georges *et al.*, (1993) tested the hypothesis that the genetic disorder, progressive degenerative myeloence-phalopathy (Weaver disease) in cattle has been shown to be associated with increased milk yield. To test these hypothesis, an extensive linkage study was made of a bovine pedigree segregating for the weaver condition, using 82 multisite haplotypes, 38 VNTR markers and 233 bovine dinucleotide microsatellite and synteny mapping with cattle – rodent somatic cells. A microsatellite locus (TGLA 116) closely linked to the weaver gene was identified.

Crawford *et al.* (1997) crossed two lines of Romney Sheep selected for high and low faecal egg counts (FEC) to produce Rams with a high chance of being heterozygous at a QTL for disease resistance. These Rams were then further mated and five half-sib pedigrees were generated. These were then exposed to pasture infected with *Trichostrongylus colubriformis* on two occasions. The FEC was measured over five days at the end of each challenge and sires and progeny were genotyped using ten microsatellite markers. They found an association between log transformed FEC and the marker MAF 64 in family 3 (P = 0.05).

Ashwell *et al.* (1997) typed 16 microsatellite markers in 7 large half-sib US Holstein cattle families. Potential QTL were identified for somatic cell score, fat yield, fat percentage, protein yield and protein percentage. Three markers (BM-203, BM-4505 and BM-2078) reported associated with significant effects for more than one trait. The results further suggested the presence of a QTL for milk yield and protein yield on chromosome 21.

2.4 POLYMORPHISM REVEALED USING MICROSATELLITE PRIMERS OF HETEROLOGOUS ORIGIN

One of the main objectives of the present study was to use heterologous microsatellite primers to reveal polymorphism that is cattle primers on buffalo genome. In contrast to coding sequences, microsatellites are less conserved between different species. However, the use of heterologous PCR primers in related mammalian groups is possible. Cross species utilization of microsatellite loci not only saves time and effort in the laboratory, but also enables the construction of comparative maps between related species. Comparative maps provide information on conservation of synteny between species and give an estimate of genome coverage of a map (O'Brien *et al.*, 1993). According to O'Brien *et al.* (1993) evolutionary conserved, gene encoding loci be termed Type-I loci and that less conserved, anonymous DNA markers such as microsatellites termed Type-II loci.

2.4.1 Studies using Heterologous Microsatellite primers: Other than buffalo

Survey of literature showed the use of cattle microsatellite primers across the species. It has been reported that around 60 percent primers amplify a product in both sheep (Moore *et al.* 1991; Crawford *et al.* 1995; deGortari *et al.* `1997) and goat (Pepin *et al.*, 1995).

The first sheep genetic map contained only 86 microsatellite cloned in sheep compared to 126 taken from the bovine map (Crawford *et al.* 1995). Kemp *et el.* (1995) reported that 48 percent of the bovine microsatellite primer pairs were polymorphic in sheep and 39 percent were polymorphic in goats, and 22 per cent markers were polymorphic in all 3 species.

Gortari *et al.* (1997) reported that approx. 58 percent (605/1036) of bovine primer pairs amplified a locus in sheep. Sixty-seven percent (409/605) amplified loci were detected as polymorphic. The allele number and range of allele sizes were significantly lower in sheep than cattle.

Slate *et al.* (1998) tested 174 bovine microsatellite primer pairs for use in a primitive breed of sheep and two species of deer. They found that 73.4 percent (127/173) gave a product in Soay sheep (*Ovis aries*) of which 42.5 percent (54/127) were polymorphic. In Red deer (*Cervus elaphus*) 74.1 percent (129/174) were conserved of which 55.8 per cent (72.129) were polymorphic. In Sika deer (*Cervus nippon*) 73.7 per cent (126/171) were conserved of which 7.3 percent (47/126) were polymorphic.

Ganai *et al.* (1999) used 16 bovine primers in three breeds of goats (*Capra hircus*). All the primers amplified products. However, marker heterozygosity, allele number and range of allele sizes of all the loci were significantly lower in goats then cattle sampled in the study.

2.4.2 Studies using Heterologous Microsatellite primers: Buffalo

In the recent past many studies have been reported in sheep goat, and other species where bovine microsatellite have been used and have revealed polymorphism. However, very few studies have been conducted in buffalo. Moore *et al.* (1995) reported that a set of 80 bovine DNA derived microsatellite primers amplified loci in both Swamp and Riverine types of water buffaloes (*Bubalus bubalis*). The primers were first chosen on basis of their robustness (yield and reproducibility) in amplification and tested on large number of Swamp and Riverine type of water buffaloes to determine the allele number and sizes and percent heterozygosity. They found that the number of alleles generally correlated to levels of heterozygosity with exception at locus CSS MO 45. Heterozygosity levels were higher in buffalo in which the largest number of alleles was observed except at few loci.

Iannuzzi *et al.*, (1997a) mapped three bovine cosmid derived microsatellites (IDV-GA49, IDVA-7 and IDVGA-47) previously assigned to cattle synteny groups U1, U7, and U21, respectively, to riverine buffalo (*Bubalus bubalis*) chromosomes 3p22 (IDVGA-47, U 21), 5q21 (IDVGA-49, U1) and 5p19 (IDVGA-7, U-7) using sequential FISH and R-banding techniques. In an another study Iannuzzi *et al.* (1997b) mapped six bovine cosmid derived microsatellites previously assigned to riverine buffalo by FISH. Sequential FISH and R-banding allowed the precise identification of chromosomes and localization of probe-signal on chromosome bands. They for the first time assigned indirectly six bovine syntenic groups to riverine buffalo chromosomes, thereby extending its physical map.

Simonsen *et al.* (1998) studied the population structure of African buffalo (*Syncerus caffer*). They found a high level of genetic variability within the species and its even distribution among populations.

Yadav *et al.* (1998) reported the use of 22 bovine primers on the genomic DNA of 36 animals of Murrah breed of buffalo (*Bubalus bubalis*). All the primers amplified the products, however, 14 primers revealed polymorphism. The number of alleles varied from 2 to 5 for different microsatellite markers.

CHAPTER 3

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. ANIMAL

A total of 22 Murrah and 9 Nili-Ravi buffaloes (*Bubalus bubalis*) comprised the material for this study. The list of all these animals and their pedigree is presented in Appendix-I.

3.2. DNA SAMPLES

Genomic DNA was isolated from blood/semen following proteinase-K digestion and Phenol-chloroform extraction method. The DNA samples were part of already available stock in the laboratory. For each breed DNA sample from two sire families (comprised of one sire, five dams and five offspring i.e., one from each dam) were taken.

3.3. BOVINE MICROSATELLITE PRIMER PAIRS

In the present study the primer pairs of heterologous species were used. Bovine microsatellite primer pairs were kindly provided by Dr. Craig Beattie and Dr. L.B. Schook, University of Minnesota, St. Paul, USA. A total of 54 primer pairs were screened. The list of screen primer pairs is presented in Appendix-II.

3.4. CHOICE OF PRIMER PAIRS

Each primer pair was first tested using four random samples of buffaloes. The primer pairs which were found to be conserved (i.e., gave amplification of the respective microsatellite locus in buffalo) and also were polymorphic in buffalo were further studied using large number of DNA samples.

3.5. IN VITRO AMPLIFICATION OF MICROSATELLITE LOCI USING POLYMERASE CHAIN REACTION

In vitro amplification of the microsatellite loci in buffalo using Bovine microsatellite primer pairs was carried out by polymerase chain reaction (PCR).

3.5.1. Optimization of PCR parameters

The PCR parameters, viz., concentrations of genomic DNA and $MgCl_2$, and annealing temperature (T_A^{opt}) were optimize to obtain a specific amplified product in sufficient quantity. The reaction volume was kept constant at 10µl.

3.5.2. Preparation of PCR mix

After optimizing the PCR conditions for each primer pair master mix was prepared inside a laminar flow hood in a DNA free atmosphere and on ice.

In a microfuge tube following components were added as follows:

Components	Stock	Volume	Concentration
PCR buffer (Promega Cat No.M – 190A)	10 X	0.4 µl	1X
$MgCl_2$ (Promega Cat. No. A-351)	25 mM	-	Optimized
Forward primer	-	0.25 µl	As per standard protocol
Reverse primer		0.25µl	As per standard protocol
Hot mix		1.25µl	For 10µl
Taq DNA Polymerase (Bangalore, Genei)	3U/µl)		0.75µl per reaction
Autoclaved mili Q grade water		Upto 4µl	To make volume

3.5.3. Protocol of PCR amplification

1. In a 200 µl microamp tube (Perkin Elmer Cat. No. N-801-0533) 6µl of genomic DNA @ 2.5 ng/µl was taken and subsequently 4µl of PCR mix was added.
2. PCR amplification was carried out in a Thermal Cycler (Gene Amp 9600, Perkin Elmer) following the optimized conditions as given in Table below.
3. After completion of PCR, 9 µl of tracking dye was added into each tube to stop the reaction. The tubes were than kept at -20°C till further use.

Amplification was carried out as per the steps given below:

Step	Temperature	Time	No. of cycle
I. Initial Denaturation	95 °C	2 min.	One
II. Cycle			
Denaturation	94 °C	30 sec.	35
Annealing	T_A^{Opt}	30 sec.	
Extension	72 °C	30 sec.	
III. Final extension	72 °C	10 min.	One
IV Soaking	4 °C	For 4-12h	

3.6. PROTOCOL FOR M13 SEQUENCING LADDER

Sequenase Version 2.0 DNA Sequencing Kit (USB, Product. No. US-70770) was used for this purpose.

Single stranded DNA and labelling mixture containing dNTPs were separately diluted in two different 1.5 ml microfuge tubes.

3.6.1.1. Annealing reaction

1. The single stranded DNA was mixed with 5X reaction buffer and primer.
2. The mixture was heated to 65^{0}C for 4 min. and gradually cooled to 35^{0}C over 30 min. period using a water bath.

3.6.1.2. Preparation of termination mixture tubes

Four separate 1.5ml microfuge were labelled as G, A, T, C to each tube respective ddNTPs were added and then the tubes were incubated at 37^{0}C

3.6.1.3. Sequenase dilution

The enzyme sequenase was diluted with the enzyme dilution buffer.

3.6.1.4. Labelling reaction

In cold annealed DNA mixture from step 1, DTT 0.1M, dilute labelling mix, α-^{33}P dCTP and dilute sequenase were mixed together and incubated at 20^{0}C for 5 min.

3.6.1.5. Termination reaction

Equal quantities of above labelling mixture was then transferred into each G, A, T, C tubes of step 3 and again incubated at 37^{0}C for 5 min.

3.6.1.6. Stop reaction

Stop buffer was added to each tube to stop the above reaction.

3.7. DENATURING SEQUENCING GEL ELECTROPHORESIS

The amplified product was separated by denaturing sequencing gel electrophoresis using Sequi-GEL-GT electrophoresis apparatus (BioRad, UK, Cat. No. 165-3860 to 165-3863). The detail procedure is described below:

3.7.1. Cleaning and siliconizing plates

The glass plates were cleaned thoroughly to ensure "bubble free" gel:

1. Both the glass plates (outer and bounded) were cleaned with laboratory detergent and washed thoroughly with warm tap water. Finally the glass plates were rinsed with deionized water and allowed to dry in room conditions.

2. The clean and dry glass plates were wiped with absolute alcohol to remove any traces of grease.

3. The 'bounded' glass plate was siliconized with 5-6 drops of siliconizing solution Sigmacote (Sigma, Cat. No. SL-2). The glass plate was coated completely and evenly by spreading the reagent with a piece of muslin cloth, using a motion from the top to the bottom of the plate.

4. Prior to the assembling the plates, each glass plate was finally wiped to dryness with a small amount of absolute alcohol.

3.7.2. Assembling the glass plates sandwitch

The glass plates and other components were assembled following the instructions of the manufacturer. The spacers were cleaned thoroughly and coated with high vacuum grease (Himedia, Cat. No. RM-406) to avoid leaking. The glass plates were handled with gloved hands and care was taken to avoid any fingerprints on glass plates.

3.7.3. Casting the gel

1. Preparation of gel solution: Following constituents were mixed to prepare the working polyacrylamide (PAA) gel solution.

Components	**Volume/ Quality**	**Final Concentration**
Urea	25.2g	7M
10 X TBE Buffer	12.00ml	1 X
40% Acrylamide/Bis acrylamide (19:1)	21ml	7%
Distilled water	upto 120 ml	

The gel solution was then filtered through 0.45μ filter and degassed for 30 min. at room temperature under vacuum.

2. The bottom of the assembled glass plates was sealed using acrylamide plug. The sealing gel solution was prepared as follows:

Polyacrylamide working solution	20ml
25% Ammonium Persulphate (APS)	100 µl
TEMED	100 µl

After mixing the solution was poured on the strip of 3MM filter paper (Whatman Cat. No.3030917) kept on casting tray. The glass plate sandwitched was placed immediately on casting tray and sealing was allowed for 2-5 minutes.

3. The running gel solution was prepared by mixing 100 ml of working PAA solution, 100µl of 25% APS and 100 µl TEMED. The solution was mixed by swirling. Using a 50 ml syringe the gel solution was poured slowly from top between two plates of sequencing apparatus. Care was taken to prevent formation of air bubbles. After pouring the gel assembly was laid nearly flat (5^0 inclination or less) on the working bench.

4. The flat side of the comb (Shark-teeth comb) was inserted properly (not more than 5 mm inside edge of the short glass plate). The comb was held at this position with bulldog metal clamps. The gel was left overnight at room conditions for polymerization.

3.7.4. Setting up for operation

1. The Integrated Plate Chamber (IPC) assembly containing the polymerized gel was placed into the Universal Base. The Gel Temperature Indicator (GTI) was stuck on to the outer plate, near the centre of the gel.

2. The lower buffer chamber was filled with about 300-350 ml of 1X TBE buffer, similarly upper tank was filled with the same buffer.

3. The gel was then pre-run at normal operating voltage (i.e. 1500 to 1900) for at least 20 min. or till the temperature of the gel reached around 45^{0}C.

3.7.5. Loading the gel

1. The amplified samples were heated to 95^{0}C for 5 min. for denaturation of the DNA and immediately quenched on ice.

2. The power supply was turned off, the comb was removed and the groove was thoroughly rinsed with the help of a Pasteur pipette to remove the salt.

3. The comb with the teeth facing the gel was inserted till the teeth of the comb just entered the gel surface.

4. Eight microlitres of each PCR product was loaded slowly in each well (between teeth of the comb). The lanes were numbered for identification of each sample or animal.

5. M13 sequencing ladder (G, A, T, C) was loaded along the side of samples after 8-10 lanes as size marker.

3.7.6. Running the gel

1. After making sure that both the safety covers were in place the voltage was turned on and raised to required voltage level slowly.

2. The gel was allowed to run for 3.5 to 5 hrs. at 1700-1900 volts and 60-80 watt. Care was taken that temperature of the gel was around 50^{0}C.

3.8. GEL DRYING AND AUTORADIOGRAPHY

1. On completion of electrophoresis time power was turned off, the glass plate assembly was taken out and upper buffer was discarded. The gel assembly was dismantled by removing side-bars and was placed on the table with bonded plate upper side. Comb was removed and the bounded plate was slowly lifted leaving the gel on the other glass plate.

2. The dry filter paper sheet of 3 mm Whatman was laid on the gel and pressed with a cotton swab flat so that the gel stuck to the paper.

3. The gel was picked up carefully by lifting the filter paper from one end.

4. The gel was than covered with saran wrap.

5. The extra edges of the filter paper were trimmed of so that the gel could get fitted into the gel dryer.

6. The gel was then dried under vacuum at 80^0C for 45 min.

7. The dried gel was then aligned with an X-ray film inside a cassette fitted with intensifying screen. The cassette was stored for 2-4 days at room temperature to transfer the radioactive signal from gel to X-ray film.

8. X-ray film was developed following standard procedure.

3.9. ANALYSIS OF AUTORADIOGRAPH

During electrophoresis PCR products were size marked by running the M13 sequencing ladder. Analysis was done after scoring of alleles and genotyping. Scoring was done manually by comparing the band size from the standard M 13 ladder.

3.9.1. Scoring of microsatellite loci

The conservation of the bovine microsatellite primer pairs was observed and recorded. The conserved primer pairs were further observed for polymorphism. Scoring of the alleles was done for each primer revealing polymorphism.

3.9.2. Estimation of the Allele frequency

The allelic frequency was calculated using the formula:

$$P_i = K_i/N$$

Where,

P_i = Allele frequency

K_i = No. of observations of allele 'i'

N = Total No. of observations

3.9.3. Estimation of Heterozygosity

The heterozygosity was calculated using the following formula (Nei *et al.*, 1974):

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

Where,

H = Heterozygosity

P_i = Frequency of allele 'i'

3.9.4. Estimation of Polymorphism Information Content (PIC)

The PIC was estimated by using the following formula (Botstein *et al.*, 1980):

$$PIC = \sum P_{i,j}\,(1-0.5P_{i,j})$$

Where,

$P_{I,j}$ = 2 $P_I.P_j$

P_i = Frequency of Allele 'i'

P_j = Frequency of allele 'j'

CHAPTER 4

RESULTS
&
DISCUSSION

4. RESULTS AND DISCUSSION

The present investigation was undertaken to amplify and analyze buffalo genome with cattle microsatellite primers. The results obtained in the study are presented under different sections:

4.1. OPTIMIZATION OF PCR PARAMETERS

The PCR parameters, viz., concentration of genomic DNA, $MgCl_2$, annealing temperature (T_A^{opt}) were optimized by taking DNA from two animals each of Murrah and Nili-Ravi buffaloes. PCR was performed taking different concentration of genomic DNA keeping the concentration Of $MgCl_2$ and annealing temperature constant. It was found that a genomic DNA concentration of 2.5 μg/μl was optimum for the amplification of all the loci in both the breeds. The $MgCl_2$ concentration was found optimum at 1.5 mM which was same as in cattle. The annealing temperature was also found similar as in cattle and was specific for each primer.

DISCUSSION

Polymerase chain reaction (PCR) is a method for amplifying specific DNA sequences in *in vitro* conditions. PCR makes possible the genetic analysis of tiny samples of DNA in a relatively simple process. Short length and ubiquitous distribution makes microsatellite typing very useful and easy through PCR. In this type of studies genomic DNA is amplified with microsatellite markers by PCR using the forward and reverse primers synthesized by highly conserved flanking sequences. This approach and methodology has been followed in various livestock species, cattle, (Moazami *et al.*, 1997), sheep deGortari *et al*, 1997), goat

(Pepin *et al.*, 1995; Ganai, 1999), buffalo (Moore *et al.*, 1995). The present investigation was a similar approach as in other species and was in confirmation of earlier reports.

4.2. CONSERVATION OF BOVINE MICROSATELLITE IN BUFFALO AND THEIR INFORMATIVENESS

The cattle microsatellite primers were used in buffaloes with a view to find their conservation and power of polymorphism for genetic analysis. The observation as described under:

4.2.1. Conservation

A total of 54 microsatellite primer pairs of bovine origin were screened in buffalo genomic DNA (Table-1). The primer pairs were first tested in individual trial using four random samples of DNA buffalo and a control of cattle DNA. It was found that out of 54 primers screened 38 gave amplification products in buffalo and in controls (cattle) all the primers gave amplification. The observations showed that 70.4% of cattle primer pairs were conserved in case of buffaloes.

DISCUSSION

One of the important properties of microsatellite primers is that the flanking sequences are quite conserved across related species. Cattle being a cosmopolitan animal a lot of research work is going on its genome and various markers have been developed which are being used as heterologous primers by other research workers in related species such as in sheep (deGortari *et al.*, 1997), in goat (Ganai, 1999) in buffaloes (Yadav *et al.*, 1998), in deer (Slate *et al.*, 1998). In the present study bovine microsatellite primer pairs were used to study polymorphism in buffalo. In earlier reports on buffaloes, using the different set of primers Moore *et al.*, (1992) got a conservation percentage of 70 percent, Yadav *et al.*, 1998 found that out of 22 loci all were conserved in buffalo. The less conservation percentage in the present study might have been due to error in amplification during PCR or the absence of the particular cattle locus in buffalo.

4.2.2. Polymorphism

The conserved microsatellite markers were then further studied using 22 Murrah and 9 Nili-Ravi samples of DNA. The status of the primers is given in Table-1. The conserved microsatellite were either monomorphic (non-informative) (Fig-10) or polymorphic (informative) (Fig-2 & 3). Out of 38 screened primer pairs, 23 were polymorphic (42.6%) and 15 were monomorphic (27.8%). Out of the conserved primers 60.5 % (23/38) were informative.

DISCUSSION

The cattle loci found to be conserved in buffalo genome were either monomorphic or polymorphic. The polymorphism was considered by the presence of more than one band at that particular locus (Fig-5). Moore *et al.* (1995) reported 57% polymorphic loci in buffalo using bovine primers. Yadav *et al.* (1998) found that 64% of bovine primers were polymorphic in buffalo genome. In the present study 42.6% bovine loci were polymorphic. A few primers viz., BM-7160, BM-7228, BMS-817 which were monomorphic in sheep (deGortari *et al.*, 1997) were found to be polymorphic in buffalo (Fig-3).

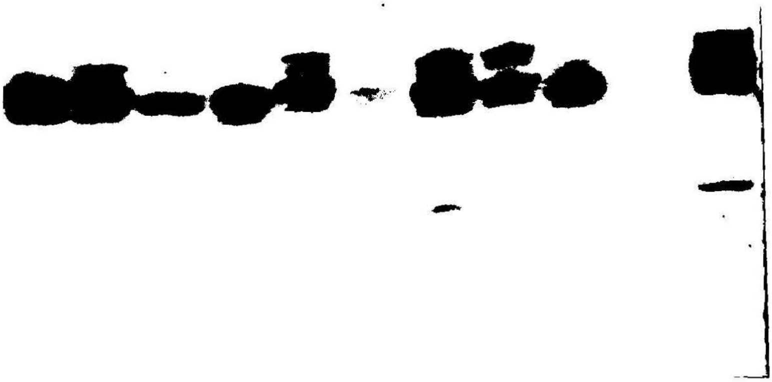

Fig. 2. An autoradiograph showing the resolution of the PCR products by denaturing PAGE at the microsatellite locus MB-068 in buffalo. This locus is less polymorphic, with most of the animals monomorphic and a few dimorphic.

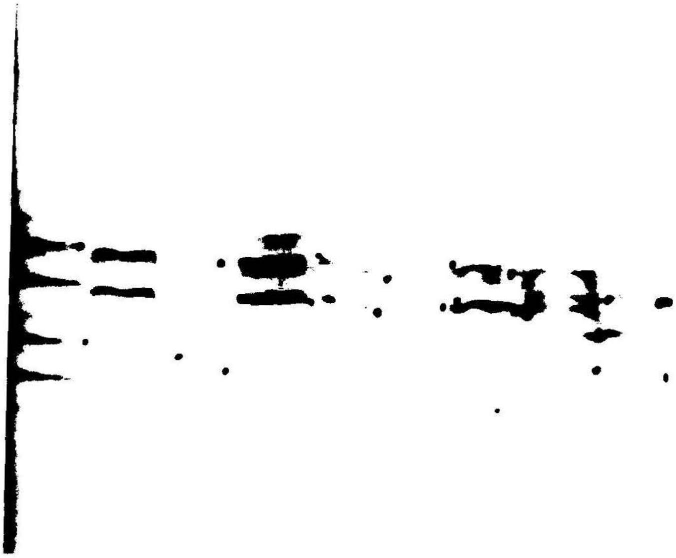

Fig. 3. An autoradiograph showing the resolution of the PCR products by denaturing PAGE at the microsatellite locus BM-7160 showing di-nucleotide repeat polymorphism in Murrah breed of buffalo

Fig. 5. An autoradiograph of a denaturing polyacrylamide gel showing di-nucleotide repeat polymorphism using the bovine microsatellite primer pairs specific to the marker BM-4509. The first 11 lanes constitute a half-sib family of Murrah breed showing Mendalian inheritance of the alleles; and the next nine lanes constitute the random samples of the Nili-Ravi breed of buffalo.

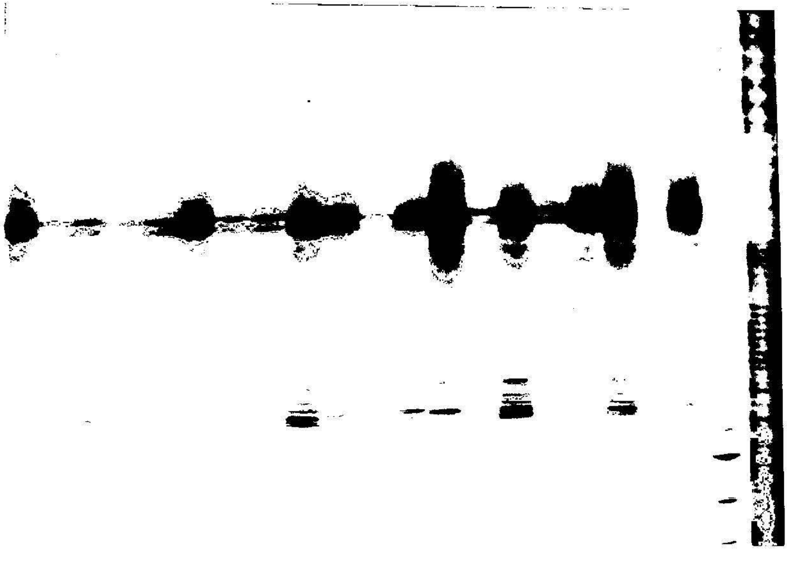

Fig. 10. An autoradiograph of a denaturing polyacrylamide gel showing a monomorphic locus BM-1834 in Murrah and Nili-Ravi buffaloes.

TABLE - 1 STATUS OF THE BOVINE LOCUS SCREENED IN BUFFALOES

Sl. No.	Locus	Annealing Temperature ^{0}C)	Allele Size in cattle (bp)	Status
1.	BM-1508	56	105-115	M
2.	BM-1834	58	162-164	M
3.	BM-1857	54	103-137	M
4.	BM-1862	58	191-224	P
5.	BM-2901	58	115-130	P
6.	BM-3010	54	118-140	M
7.	BM-3507	58	159-187	P
8.	BM-4006	56	97-119	M
9.	BM-4102	58	157-169	P
10.	BM-4307	58	187-203	NA
11.	BM-4509	58	119-133	P
12.	BM-5004	58	120-154	P
13.	BM-6026	56	148-168	NA
14.	BM-6436	58	198-208	NA
15.	BM-6458	54	129-145	M
16.	BM-7160	58	135-189	P
17.	BM-7228	56	137-155	P
18.	BM-7237	58	94-102	NA
19.	BM-8116	54	123-139	NA
20.	BM-8225	56	135-145	M
21.	BM-8230	60	103-108	M
22.	BMC-2208	58	171-177	M
23.	BMC-2228	56	139-147	NA
24.	BMC-5221	58	169-171	P
25.	BMS-1319	56	118-140	NA
26.	BMS-1385	58	85-95	M
27.	BMS-1788	58	89-107	P

Contd.../-

Contd…Table – 1.

Sl. No.	Locus	Annealing Temperature ^{0}C)	Allele Size in cattle (bp)	Status
28.	BMS-332	58	127-149	P
29.	BMS-462	58	114-126	P
30.	BMS-466	58	101-121	P
31.	BMS-468	60	128-134	M
32.	BMS-499	58	102-128	NA
33.	BMS-511	58	110-130	NA
34.	BMS-585	58	124-130	P
35.	BMS-651	58	109-135	P
36.	BMS-778	58	135-163	P
37.	BMS-817	58	112-130	P
38.	BMS-820	56	106-116	P
39.	BMS-835	58	133-145	P
40.	BMS-862	58	130-140	NA
41.	BMS-963	58	134-154	P
42.	BR-6027	60	124-136	NA
43.	INRA-136	56	182-184	NA
44.	MB-019-CY	58	188-224	NA
45.	MB-020	58	173-183	NA
46.	MB-045	56	181-185	NA
47.	MB-056-ILS	54	181-185	M
48.	MB-063-PIA	58	194-219	M
49.	MB-068	58	147-167	P
50.	MB-076	60	172-190	M
51.	MB-077	58	157-252	P
52.	MB-085	58	185-205	NA
53.	MB-110	58	124-150	M
54.	MB-116	58	91-103	P

M = Monomorphic P = Polymorphic NA= Not Amplified

4.3. ALLELE SIZE RANGE AND NUMBER OF ALLELE AT DIFFERENT MICROSATELLITE LOCI IN BUFFALO

The microsatellite primer revealing polymorphisms were scored manually by comparing the band size with the standard M13 ladder. Allele size and number were scored and comparison was made between the allele range in cattle and buffaloes. The detail of allele range of cattle and buffaloes is given in Table-2. The range of allele size observed in buffalo is almost the same as that of the cattle (Fig-7). However, figures. 1 and 9, depicting different allele sizes of buffalo at locus BMS-332 is 121-131bp and BMS-820 is 104-110, respectively and is below the range of allele size in cattle which is 127-149 base pairs and 106–160 base pairs respectively (Stone *et al.*, 1995). The figures 4 and 5 depict the buffalo allele size at locus BM-651 as 123-139 bp and locus BM-4508 as134-142 bp, these allele sizes exceed the range of allele sizes in cattle at both the loci i.e., BM-651as109-135 bp and BM-4509 as 119-133 bp (Stone *et al.*, 1995). Comparison of the allele range between the two breeds i.e., Nili-Ravi and Murrah is quite similar (Table-2). The number of alleles was calculated for each locus in both the breeds is given in Table-3. The allele number in case of Murrah ranged from 2 (BM-044; Fig.-8) to 6 (BMS-651, Fig-4). The allele number in case of Nili-Ravi ranged from 2 (MB-077, Fig. -8) to 5 (BMS-585, Fig.-6; BM-7228; BMS-820, Fig-9). It was observed that the number of allele (2) was very low at the locus MB-077 (Fig-8)

Discussion

The allele size in sheep at locus BM-7228 has been reported (deGortari *et al.*, 1997) low (113 bp) in comparison to cattle (137-155). In the present study it was found that the allele size

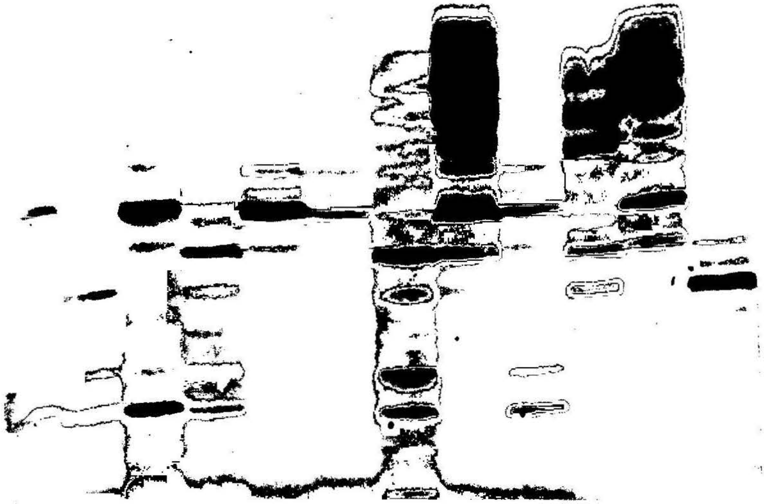

Fig.1. An autoradiograph of a denaturing polyacrylamide gel showing di-nucleotide repeat polymorphism in Murrah breed of buffalo using the bovine microsatellite primer pairs specific to the marker BMS-332. The four different alleles at this locus are 121, 123, 125, 131

Fig. 4. An autoradiograph of a denaturing polyacrylamide gel showing di-nucleotide repeat polymorphism in Murrah breed of buffalo using the bovine microsatellite primer pairs specific to the marker BMS-651. The first two lanes constitute the M13 size marker.

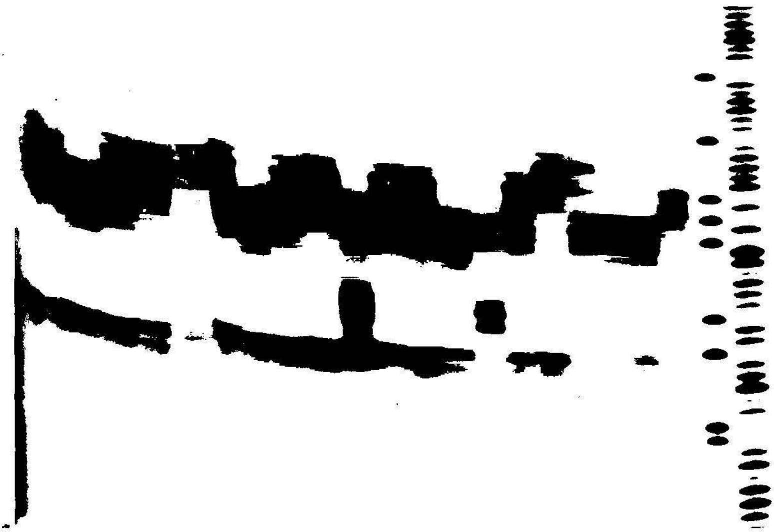

Fig. 6. An autoradiograph of a denaturing polyacrylamide gel showing di-nucleotide repeat polymorphism in Murrah (1-11 lanes) and Nili-Ravi (12-20 lanes) using the bovine microsatellite primer pairs specific to the marker BMS-585. The last two lanes constitute the M13 size marker.

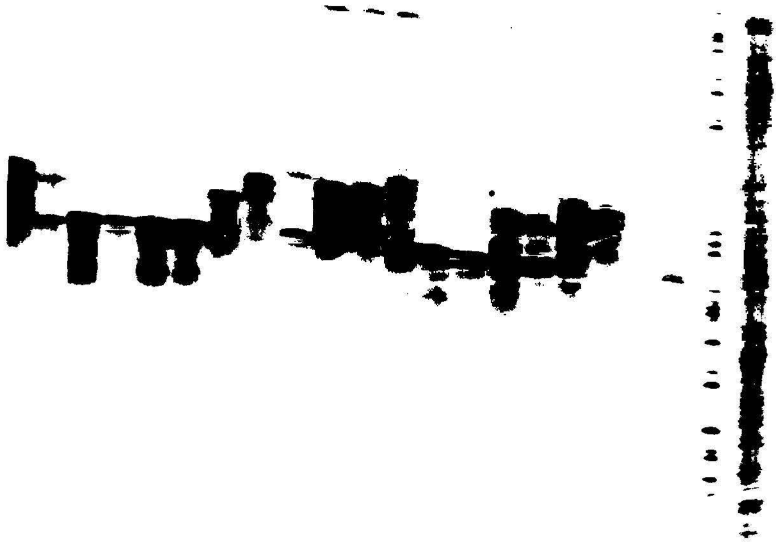

Fig. 7. An autoradiograph of a denaturing polyacrylamide gel showing di-nucleotide repeat polymorphism in Murrah (1-11 lanes) and Nili-Ravi (12-20 lanes) using the bovine microsatellite primer pairs specific to the marker BM-1862. The last two lanes constitute the M13 size marker.

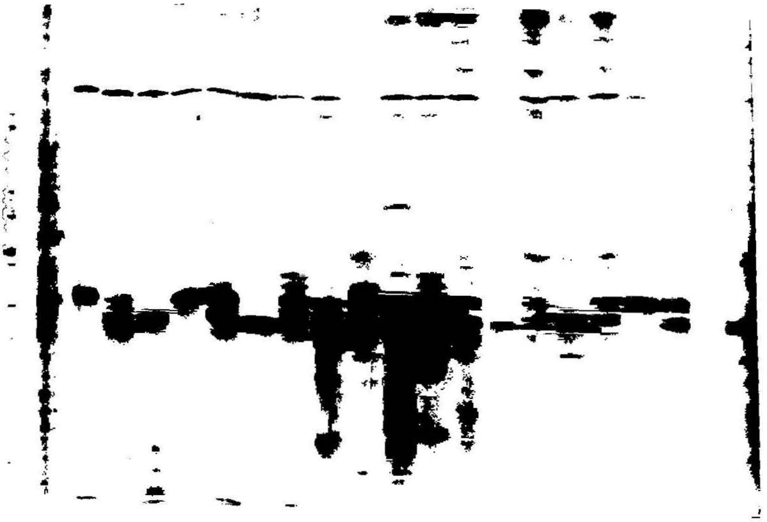

Fig. 8. An autoradiograph of a denaturing polyacrylamide gel showing di-nucleotide repeat polymorphism in Murrah (1-11 lanes) and Nili-Ravi (12-20 lanes) using the bovine microsatellite primer pairs specific to the marker MB-077.

Fig. 9. An autoradiograph of a denaturing polyacrylamide gel showing di-nucleotide repeat polymorphism in Murrah (1-11 lanes) and Nili-Ravi (12-20 lanes) using the bovine microsatellite primer pairs specific to the marker BMS-820.

TABLE - 2. COMPARISON OF THE RANGE OF ALLELE SIZE IN CATTLE AND BUFFALO

Primers	Allele size range in cattle (bp)*	Allele size range in buffaloes	
		Murrah	Nili -Ravi
1. BMS 585	124–130	114 – 126	120 – 128
2. BM 4509	119–133	134 – 142	134 – 140
3. BM 7160	135–189	181 – 187	181 –185
4. BMS 817	112–130	112 – 120	114 –118
5. BMS 963	134–154	146 – 154	146 –152
6. BM 1862	191–224	196 – 208	196 –208
7. MB 077	157–252	234 – 242	234 –242
8. BM 7228	137–155	132 – 144	124 –138
9. BMS 820	106–160	104 – 110	100 –110
10. BMS 332	127–149	121– 131	NS
11. BMC 5221	169–171	160 –166	NS
12. BMS 651	109–135	123 –139	NS
13. BMS 835	133–145	127 – 137	NS

* Stone *et al*., 1995

NS = Not studied

range in case of buffalo (132-144) was within the allele size range of cattle (Stone *et al.*, 1995). However at locus BM-5221 and BM- 835 deGortari *et al.* (1997) found that the allele size range in sheep was very high as 181-217 bp and 155–177bp in comparison to cattle as 169–171bp and 133-145 bp respectively. In the present study it was found that the allele size range in case of buffalo for the locus BM-5221 is 160-166 bp and for BMS-835 is 127-137 bp and was well within the allele size of cattle (Stone *et al.*, 1995).

The number of allele in case of sheep at the same loci as found by deGortari *et al.*, (1997) was very less ranging from 1 to 3 except for loci BMC-5221 (7) BMS-332 (8) and BMS-835 (8). In buffalo it was found to be ranging between 2-6.

4.4. HETEROZYGOSITY AND POLYMORPHISM INFORMATION CONTENT

The heterozygosity for different microsatellite markers calculated from allele frequency ranged from 0.47 (BMS-835) to 0.77 (BMS-651) in Murrah and 0.49 (MB-077) to 0.76 (BMS-817) in Nili-Ravi. Lowest heterozygosity was observed for the locus MB-077 (Fig-8) with only 2 alleles. The average heterozygosity over different loci in Murrah and Nili-Ravi are 0.67 and 0.69 respectively (Table-3). Frequency distribution of alleles and polymorphism information content of various microsatellite loci in Murrah (Table-4), Nili-Ravi (Table-5) were calculated. The pooled allele frequency and PIC at different loci in Murrah and Nili-Ravi was also calculated and is given in Table-6. The range of PIC varied from 0.375 at the locus MB-077 to 0.734 at locus BMS-651 (Fig-4) for Murrah. In case of Nili-Ravi it varied from 0.358 at locus MB-077 to 0.754 at locus BMS-585 (Fig-6). The pooled PIC for both Murrah and Nili-Ravi ranged from 0.371 at locus MB-077 to 0.746 at locus BMS-585 (Fig-6).

TABLE - 3. NUMBER OF ALLELES AND HETEROZYGOSITY AT DIFFERENT LOCI IN MURRAH AND NILI-RAVI BUFFALOES

Primers	Murrah		Nili-Ravi	
	No. of alleles	Heterozygosity	No. of alleles	Heterozygosity
1. BMS 585	4	0.76	5	0.75
2. BM 4509	5	0.72	4	0.73
3. BM 7160	4	0.67	3	0.67
4. BMS 817	5	0.74	3	0.76
5. BMS 963	5	0.71	4	0.71
6. BM 1862	4	0.68	4	0.66
7. MB 077	2	0.49	2	0.49
8. BM 7228	5	0.72	5	0.72
9. BMS 820	4	0.7	5	0.7
10. BM 7160	3	0.67	3	0.65
11. BMS 585	5	0.72	5	0.74
12. BMS 332	4	0.69	NS	NS
13. BMC 5221	3	0.51	NS	NS
14. BMS 651	6	0.77	NS	NS
15. BMS 835	3	0.47	NS	NS
Average Hetrozygosity		0.67		0.69

NS = Not studied

TABLE - 4. FREQUENCY DISTRIBUTION OF ALLELES AND PIC AT VARIOUS MICROSATELLITE LOCI IN MURRAH BUFFALOES.

Locus	Alleles	n	Frequency	PIC
BMS 585	114	1	0.0227	0.732
	118	1	0.00227	
	120	2	0.045	
	122	14	0.318	
	124	7	0.159	
	126	12	0.272	
	128	7	0.159	
BM 4509	134	6	0.272	0.675
	136	3	0.136	
	138	9	0.409	
	140	2	0.090	
	142	2	0.090	
BM 7160	181	6	0.272	0.651
	183	8	0.363	
	185	6	0.272	
	187	2	0.090	
BMS 817	112	4	0.181	0.717
	114	4	0.181	
	116	4	0.181	
	118	8	0.363	
	120	2	0.090	
BMS 963	146	2	0.090	0.670
	148	8	0.363	
	150	4	0.181	
	152	7	0.318	
	154	1	0.045	

Contd…../-

Contd….. Table 4.

BM 1862	196	11	0.550	0.571
	202	2	0.100	
	204	1	0.050	
	206	5	0.250	
	208	1	0.050	
MB 077	234	10	0.5	0.375
	242	10	0.5	
BM 7228	132	4	0.181	0.661
	136	3	0.136	
	138	10	0.454	
	140	1	0.045	
	144	4	0.181	
BMS 820	104	9	0.401	0.491
	106	2	0.090	
	108	2	0.040	
	110	9	0.409	
BMC 5221	160	2	0.090	0.530
	162	1	0.045	
	164	8	0.363	
	166	11	0.500	
BMS 651	123	1	0.045	0.734
	125	4	0.181	
	127	4	0.181	
	129	8	0.363	
	131	2	0.090	
	139	3	0.136	
BMS 332	121	9	0.409	0.640
	123	5	0.227	
	125	2	0.090	
	131	6	0.272	
BMS 835	127	5	0.227	0.419
	133	15	0.687	
	137	2	0.090	

TABLE - 5. FREQUENCY DISTRIBUTION OF ALLELES AND PIC AT VARIOUS MICROSATELLITE LOCI IN NILI-RAVI BUFFALOES

Locus	Alleles	N	Frequency	PIC
BMS 585	120	4	0.222	0.754
	122	4	0.222	
	124	4	0.222	
	126	4	0.222	
	128	2	0.111	
BM 4509	134	2	0.125	0.622
	136	2	0.125	
	138	7	0.437	
	140	5	0.312	
BM 7160	181	5	0.312	0.842
	183	8	0.500	
	185	3	0.187	
BMS 817	114	8	0.500	0.484
	116	7	0.437	
	118	1	0.062	
BMS 963	146	1	0.062	0.580
	148	7	0.437	
	150	6	0.375	
	152	2	0.125	
BM 1862	196	7	0.437	0.620
	198	1	0.062	
	206	4	0.250	
	208	4	0.250	
MB 077	234	10	0.625	0.358
	242	6	0.375	
BM 7228	124	1	0.062	0.653
	128	1	0.062	
	132	4	0.250	
	136	3	0.187	
	138	7	0.437	
BMS 820	100	1	0.062	0.687
	104	2	0.125	
	106	4	0.250	
	108	2	0.125	
	110	7	0.437	

TABLE - 6. POOLED ALLELE FREQUENCY AND PIC AT DIFFERENT LOCI IN MURRAH AND NILI-RAVI BUFFALOES.

Locus	Alleles	N	Frequency	PIC
BMS 585	114	1	0.016	0.746
	118	1	0.016	
	120	5	0.083	
	122	17	0.283	
	124	11	0.183	
	126	16	0.266	
	128	9	0.150	
BM 7228	124	1	0.026	0.675
	128	1	0.026	
	132	8	0.210	
	136	6	0.157	
	138	17	0.447	
	140	1	0.026	
	144	4	0.105	
BM 1862	196	18	0.500	0.611
	198	1	0.027	
	202	2	0.055	
	204	1	0.027	
	206	9	0.250	
	208	5	0.138	
BMS 651	123	1	0.045	0.647
	125	4	0.181	
	127	4	0.181	
	129	8	0.363	
	131	2	0.090	
	139	3	0.136	

Contd…./

Contd....Table - 6.

BMS 963	146	3	0.078	0.691
	148	15	0.394	
	150	10	0.263	
	152	9	0.263	
	154	1	0.026	
BMS 817	112	4	0.105	0.698
	114	12	0.315	
	116	11	0.289	
	118	9	0.236	
	120	2	0.052	
BMS 820	100	1	0.026	0.649
	104	11	0.289	
	106	6	0.157	
	108	4	0.105	
	110	16	0.421	
BM 4509	134	8	0.210	0.405
	136	5	0.131	
	138	16	0.157	
	140	7	0.184	
	142	2	0.052	
BM 7160	181	11	0.280	0.597
	183	16	0.420	
	185	9	0.230	
	187	2	0.052	
BMC 5221	160	2	0.090	0.530
	162	1	0.045	
	164	8	0.363	
	166	11	0.500	
BMS 332	121	9	0.409	0.640
	123	5	0.227	
	125	2	0.090	
	131	6	0.272	
BMS 835	127	5	0.227	0.416
	133	15	0.681	
	137	2	0.090	
MB 077	234	20	0.555	0.371
	242	16	0.444	

DISCUSSION

Frequencies of alleles and genotypes are the simplest descriptors of the genetic variation. For multilocus multiallelic data, the other useful measures of genetic diversity are population heterozygosity and polymorphism information content (PIC). PIC is a measure of the degree of polymorphism at the marker locus (Botstein *et al.*, 1980). This quantity is used in the context of gene mapping and genetic diagnosis. PIC of a locus is thus the probability of detection of the linkage of a marker locus to an index locus. The PIC values estimated for different marker loci clearly indicate their usefulness in the genetic mapping as well as finding of linkages with the disease/trait of interest under consideration.

Population heterozygosity indicates the mean percentage of individuals heterozygous per locus. Heterozygosity is calculated directly from the observed diploid genotypes or from the observed frequencies of the alleles assuming the population is in Hardy-Weinberg equilibrium. The heterozygosity is proportional to the number of alleles and their frequencies and is higher at loci with evenly distributed alleles.

The results indicated that genetic variability measured by using microsatellite markers is very high, to the extent of 77%. The animals were sampled from two breeds which are under pressure selection since long. Theory of selection suggests that the variability within the population decreases due to selection for many generation. However, using molecular markers like microsatellite, present investigation indicate that there is ample variation within the two breeds and the same can be exploited for further improvement of the animals through Marker Assisted Selection (MAS).

CHAPTER 5

SUMMARY & CONCLUSIONS

5. SUMMARY AND CONCLUSIONS

5.1. INTRODUCTION

The aim of animal breeding has always been to improve and exploit the genetic potential of livestock for the welfare of the mankind. The basis for improvement is hereditary variation and for the evaluation of this variability many methods are available. However the morphological and biochemical markers have some drawbacks like low level of polymorphism and significant influence of the environment on their expression. Besides, some of this markers are sex limited and age dependent. Recent developments in molecular biology have led to the discovery of a number of markers, which reveal polymorphism at DNA sequence level, viz., RFLPs, Minisatellite, RAPD and Microsatellite. The present study was undertaken to test the feasibility and unravel the polymorphism in buffalo by microsatellite marker due to their exceptional variability, relative ease of storing and ubiquitous distributions in the genome. These microsatellite markers have also been found to be conserved across relative species.

5.2. MATERIALS AND METHOD

5.2.1. Animal and DNA samples

A total of 31 (22 Murrah and 9 Nili-Ravi) buffaloes comprised the material for this study. The DNA samples were part of already available stock in the laboratory.

5.2.2. *In vitro* Amplification of Microsatellite Loci using Polymerase Chain Reaction (PCR)

In vitro amplification of the Microsatellite loci in buffaloes using bovine Microsatellite primer pairs was carried out by Polymerase Chain

Reaction. The PCR parameters viz., Concentration of genomic DNA, $MgCl_2$ and Annealing temperature (T_A^{opt}) were optimized.

5.2.3. Choice of bovine Microsatellite primer pairs

In the present study heterologous (cattle) primer pairs were used in buffalo genome. A total of 54 primer pairs were screened by testing in four random samples. The conserved and polymorphic primer pairs were further studied using DNA samples from sire families of two breeds.

5.2.4. Denaturing Sequencing Gel Electrophoresis

The amplified product was resolved on denaturing sequencing of gel electrophoresis using sequi-GEL-GT electrophoresis apparatus on 7 per cent Acrylimide gel having a urea concentration of 7 M. The amplified samples were denatured at 95 ° c for 5 minutes and carefully loaded after prerunning the gel for atleast 20 minutes at normal operating voltage (1500-1900 volts). The gel was allowed to run for 3.5 to 5 hours at 1500 to 1900 volts and 60 to 80 Watts.

5.2.5. Gel Drying and Autoradiography

The gel was picked up carefully by lifting on the filter paper from one end and covered with saran wrap. The gel was then dried under vacuum at 80°c for 45 minutes. The dried gel was then exposed to X-ray film inside a cassette fitted with intensifying screen. After storing the cassette for 2 to 4 days at room temperature, X-ray film was developed by standard protocol.

5.2.6. Analysis of Autoradiograph

Analysis of Autoradiographs was done after scoring of alleles and genotyping. Scoring was done manually by comparing the band size from the standard M13 ladder.

5.3. RESULTS AND DISCUSSION

5.3.1. Optimization of PCR parameters

Optimizations of PCR parameters reveal that $MgCl_2$ concentration and the annealing temperatures for each primer pairs were same as in cattle. The genomic DNA and concentration of 2 ng/μl was found to be optimum.

5.3.2. Conservation of Bovine Microsatellite in Buffalo and their informativeness

It was found that out of 54 primers screened 38 gave amplification products in buffalo i.e. 70.4 per cent of cattle primer pairs were conserved in case of buffaloes. Amongst these conserved Microsatellite 42.6 per cent was polymorphic and 28.7 per cent was monomorphic. Out of the conserved primers 60.5 per cent were informative.

5.3.3. Allele size range and number of allele at different Microsatellite loci in buffalo

The range of allele size observed in buffalo was almost the same as that of cattle except at a few loci where the range either exceeds or falls below the allele size range of cattle. The number of allele was calculated for each locus in both the breeds. The allele number ranged from 2 to 6 and 2 to 5 in Murrah and Nili-Ravi respectively.

5.3.4. Heterozygocity and Polymorphism Information Content (PIC)

The Heterozygocity and PIC of both the breeds were calculated. The heterozygocity of different Microsatellite markers calculated from allele frequency ranged from 0.47 to 0.77 in Murrah and 0.49 to 0.79 in Nili-Ravi. The average heterozygocity over different loci in Murrah and Nili-Ravi are 0.67 and 0.69 respectively. The range of PIC varied from 0.375 to

0.734 for Murrah and 0.358 to 0.754 for Nili-Ravi. The pooled PIC for both Murrah and Nili-Ravi ranged from 0.371 to 0.746.

CONCLUSION

The present investigation was undertaken to study the extent of conservation and polymorphism of bovine Microsatellite markers in buffaloes. Based on this study following conclusions were made.

- The PCR parameters viz., $MgCl_2$ concentration and annealing temperature were same for cattle and buffaloes. The genomic DNA concentration was optimum at 15 ng for a reaction volume of 10μl.
- The conservation of about 70 per cent of cattle primers in buffaloes indicates a significant homology in genome of cattle and buffalo.
- There was no significant difference in the allele size, range and number between species except for a few loci.
- High PIC value indicates that the markers used in the study were highly informative and can be used for genetic mapping as well as for finding of linkages with the disease/trait of interest under consideration.
- The variability measured by using Microsatellite markers were quite high and can be used for selection of breeding animals.
- The results also suggests that heterologous PCR primers can be used in less studied and related mammalian groups by which one can save a lot of resources

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APPENDIX

APPENDIX – I

LIST OF THE ANIMALS AND THEIR PEDIGREE

Murrah		Nili-Ravi	
Family I	**Family II**	**Family I**	**Family II**
3125 (S)	3567 (S)	N-3 (S)	N-1007 (S)
3273 (D)	3379 (D)	N-718 (D)	N-87 (D)
3983 (O)	4188 (O)	N-9 (O)	N-1021 (O)
3317 (D)	0237 (D)	N-1004 (D)	N-86 (D)
3944 (O)	4252 (O)	N-25 (O)	N-1020 (O)
3259 (D)	3597 (D)	N-1016 (D)	N-117 (D)
3947 (O)	4155 (O)	N-976 (O)	N-1015 (O)
3152 (D)	3491 (D)	N-1011 (D)	N-66 (D)
3908 (O)	4161 (O)	N-974 (O)	N-1027 (O)
	3581 (D)		
	4162 (O)		

S = Sire ; D = Dam ; O = Offspring

APPENDIX – II

LIST OF MICROSATELLITE MARKERS USED *

Sl. No.	**Microsatellite markers**	**Sl. No.**	**Microsatellite markers**
1.	BM-1508	28.	BMS-332
2.	BM-1834	29.	BMS-462
3.	BM-1857	30.	BMS-466
4.	BM-1862	31.	BMS-468
5.	BM-2901	32.	BMS-499
6.	BM-3010	33.	BMS-511
7.	BM-3507	34.	BMS-585
8.	BM-4006	35.	BMS-651
9.	BM-4102	36.	BMS-778
10.	BM-4307	37.	BMS-817
11.	BM-4509	38.	BMS-820
12.	BM-5004	39.	BMS-835
13.	BM-6026	40.	BMS-862
14.	BM-6436	41.	BMS-963
15.	BM-6458	42.	BR-6027
16.	BM-7160	43.	INRA-136
17.	BM-7228	44.	MB-019-CY
18.	BM-7237	45.	MB-020
19.	BM-8116	46.	MB-045
20.	BM-8225	47.	MB-056-ILS
21.	BM-8230	48.	MB-063-PIA
22.	BMC-2208	49.	MB-068
23.	BMC-2228	50.	MB-076
24.	BMC-5221	51.	MB-077
25.	BMS-1319	52.	MB-085
26.	BMS-1385	53.	MB-110
27.	BMS-1788	54.	MB-116

* Supplied by Dr. L.B. Schook (developed by Dr. Craig Beattie)

APPENDIX –III

1. 40 % Acrylamide/Bisacrylamide Solution (19:1)

Acrylamide (Bio rad. Cat. No. : 161 – 0107)	95 g
Bis acrylamide (Bio rad. Cat. No. : 161 – 0201)	5 g
Distilled water	upto 250 ml

- Prepared by adding approx. 125 ml of water to acrylamide . Add bis-acrylamide and mix untill disolved. Adjust volume to 250 ml
- Filter through 0.45 μ filter
- Store tightly in an amber colored bottle.

Caution : Acrylamide is neurotixic

2. 25% Ammonium Persulphate (APS)

APS	0.125 g
Dist. water	500 μl

- Dissolve the above two
- Make fresh and store at –20^{0}C

3. Denaturing polyacrylamide gel solution (7% Acrylamide)

Urea	210 g (7 M)
40% Acrylamide/Bisacrylamide	87.5 ml (7%)
Dist. water	Upto 500 ml

- Filter the above solution through 0.45 μ filter
- Store at 4^{0}C after degasing at room temperature for 15 min.

4. dNTP mix for PCR (for 40 μl)

dATP	12 μl
dTTP	12 μl
dGTP	12 μl
dCTP	3 μl
Dist. water	1 μl

contd…./-

contd….Appendix-III

5. Hot dNTP mix (for 204 μl)

Dist. water	200 μl
dNTPs mix	2 μl
α ^{33}P dCTP (10μCi/μl)	2 μl

6. TBE buffer (10 X)

Tris base	108 g
Boric acid	55 g
Na_2 EDTA.$2H_2O$	9.3 g
Dist. water	upto 1000 ml

- Filter and autoclave

7. Tracking dye (10 X stock solution)

(i) Stock Solution (10 X)

Formamide	9.8 ml
0.5 M EDTA (pH : 8)	200μl
Xylene cyanol	25 mg
Bromophenol Blue	25 mg

(ii) Working solution

Stock solution (10 X)	1 ml
Formamide	9 ml
0.5 M EDTA	180 μl

8. 0.5 M EDTA (pH : 8.0)

EDTA	18.6 g
NaOH pellets	2 g
Dist. water	80 ml

Mix vigorously on a magnetic stirer.

Adjsut pH with 10 N NaOH

Autoclave

VERIFIED
Manjeet Singh
Signature