MOLECULAR AND CYTOGENETIC CHARACTERIZATION OF SOME INDIAN SHEEP BREEDS USING RAPD-PCR AND CHROMOSOMAL BANDING TECHNIQUES

Thesis

SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE
OF

Master of Veterinary Science

IN

ANIMAL GENETICS AND BREEDING

BY

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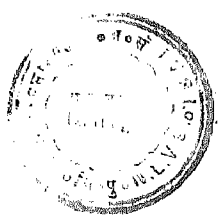
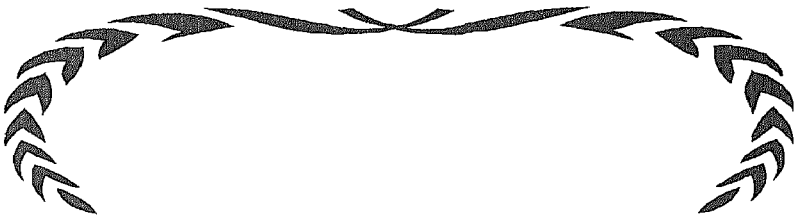
Roll No 3670

TO
(DEEMED UNIVERSITY)
INDIAN VETERINARY RESEARCH INSTITUTE
IZATNAGAR - 243 122 (U. P.), INDIA

1998





Dedicated to.....

my

beloved

Mother

Dr. PUSHPENDRA KUMAR
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Dated : 27th July, 1998

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Certified that the research work embodied in this thesis entitled **"MOLECULAR AND CYTOGENETIC CHARACTERIZATION OF SOME INDIAN SHEEP BREEDS USING RAPD-PCR AND CHROMOSOMAL BANDING TECHNIQUES"** submitted by **Dr. K. Ganesh Kumar** Roll No. **3670**, for the award of **Master of Veterinary Science** in the dicipline of **Animal Genetics and Breeding** Indian Veterinary Research Institute, is the original work carried out by the candidate himself under my supervision and guidance.

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[Pushpendra Kumar]
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We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate which is being presented by him for the award of **Master of Veterinary Science** degree of this Institute.

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Master of Veterinary Science,** degree of Indian Veterinary Research Institute.

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ACKNOWLEDGEMENT

I am deeply indebted to Dr.Pushpendra Kumar, Chairman Advisory committee, for his adroit guidance, erudite suggestion, assiduous annotate, constructive criticism and being constant inspiration throughout my career of this study.

It is paramount to give awe and respect to Dr. Sanjeev Kumar, member of Advisory Committee, for his laudable succous who accoutred me with cognizance and facilities for my work. Its my benision to get preeminent help from him.

Its my Benediction to have Dr. S.P.S. Ahlawat, Dr. R.K.Singh, Dr. A.K. Tiwari and Shri G.S. Bhist as members of Advisory Committee. I am grateful for their help in my work.

I want to record my thanks to Dr. Kaushik and Dr. Patel (CAZRI), Rajasthan, The Registrar, Director and Head, Livestock Research Station, Kattupakkam, (TNVASU), Chennai and The Incharge, Sheep and Goat Farm, IVRI for their kind permission for collection of blood samples.

Reverrent thanks to Director, IVRI, J.D. (Acad.) for providing me with necessary facilities. It is also hitime for thanking ICAR for providing me with the scholarship necessary for my study in the form of ICAR-JRF.

Regards to Dr. T.P. Kannan for his laudable support and help both in my research work and personal life and I am happy to extol Drs. Hari, Vijay Ananth, Ganesh, Sivaraman, Lesli, Suresh Kannan and Ramakrishnan for their plauditory support.

I am grateful to Mr. Dharmendra and Bhupendra of Noble Computer Center for their service and cooperation during typing.

Finally, I want to thank all of my friends who spend their valuable time for finishing this study at a stipulated time.

K. Ganesh Kumar

ABBREVIATION

bp	-	basepair
cm	-	centimeter
conc.	-	concentration
^0C	-	degree centigrade
DNA	-	Deoxyribonucleic Acid
dATP	-	deoxy adenosine triphosphate
dCTP	-	deoxy cytosine triphosphate
dGTP	-	deoxy guanosine triphosphate
dTTP	-	deoxy thymine triphosphate
dNTP	-	deoxy nucleoside triphosphate
EDTA	-	Ethylene diamine tetra acetic acid
Fig.	-	Figure
hr	-	hour
Kb	-	Kilobases
MAS	-	marker assisted selection
mg	-	milligram
ml	-	millilitre
mM	-	millimolar

min.	-	minutes
µg	-	microgram
µl	-	microlitre
µM	-	micromolar
µg	-	nanogram
nm	-	nanometer
O.D	-	optical density
PBS	-	*Phosphate buffered saline*
PCR	-	Polymerase chain reaction
pH	-	hydrogen ion concentration
RAPD-PCR	-	randomly amplified polymorphic DNA -polymerase chain reaction
RNA	-	Ribonucleic Acid
rpm	-	revolutions per minute
sec.	-	seconds
Tm	-	Temperature minimum
TAE	-	Tris acetate EDTA
TBE	-	Tris borate EDTA
U	-	units
UV	-	ultraviolet
v	-	*volume*
V	-	volts
w	-	weight

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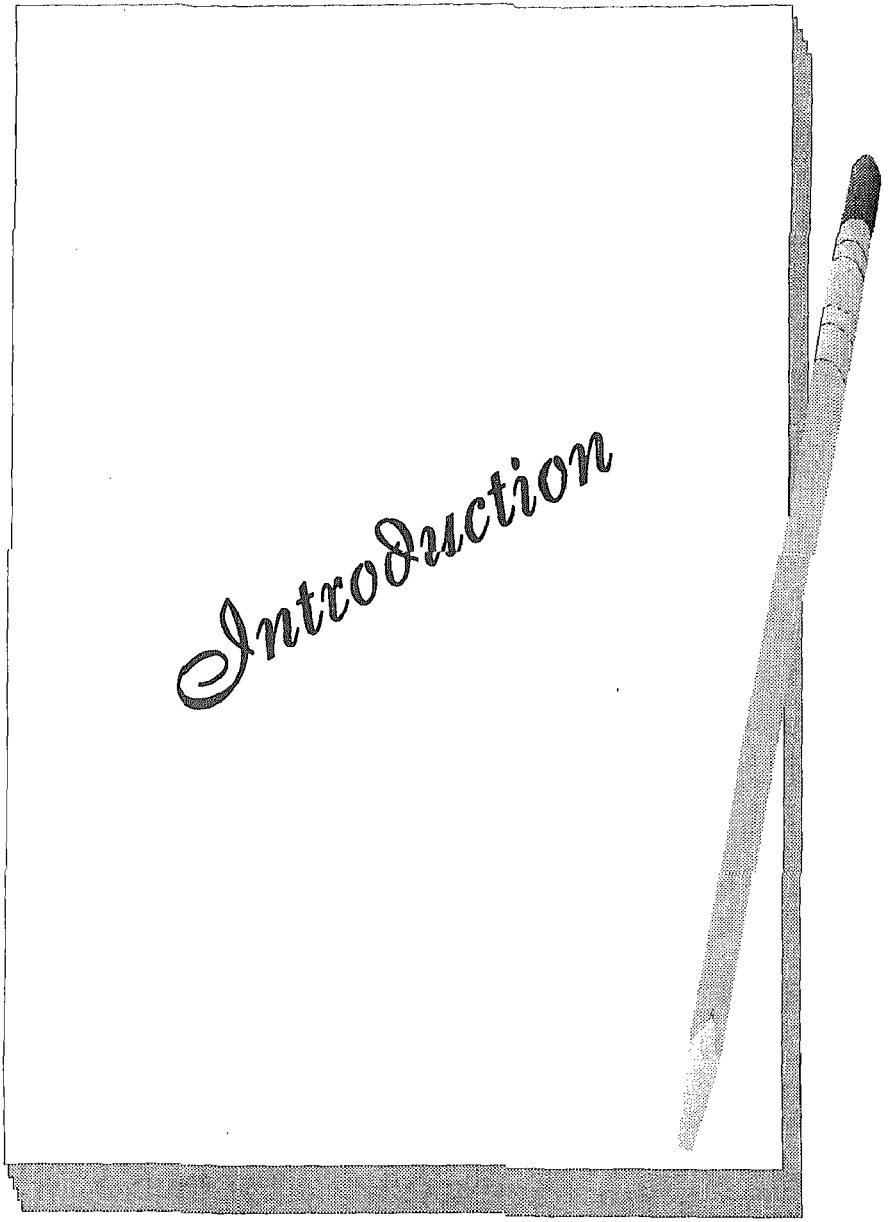
CONTENTS

Introduction



INTRODUCTION

Livestock raising in India is as ancient as Aryan civilization. Although the exact date of breed development effort is not possible, the development of breeds in different regions took place some 7000 years ago. Among the livestock species, sheep plays a major role in both meat and wool production. At present India possesses 44.80 millions of 40 different recognised breeds of sheep i.e. sixth largest in the world and the total meat and wool production from these animals is 4.34 million tonnes & 0.35 million tonnes, respectively (FAO, 1994). As India is a country with widely varying agro-climatic conditions, the breeding of native sheep breeds over years by sheer intuition made them to thrive better under harsh climatic conditions and resistant to many pathogens and non specific diseases.

Unfortunately the genetic variability of these species is reducing through breed substitution. Genetic erosion and extinction threaten an increasing number of these animals, the major consquence being loss of global genetic diversity and many breeds have degraded very fast even some are in the stage of being extinct. Therefore it is necessary for any conservation programme to develope an efficient strategy for preservation, using genetic markers which characterise distinct populations.

Several new technologies are being used to produce continued genetic change in the sheep breeds. These technologies are catogorised broadly as (i) Improved modeling, selection and evaluation methods. *(ii)* New developments in immunogentics and *(iii)* New developments in cytogenetics and molecular genetics.

Among these the selection methods based on the phenotypic expression of an animal is influenced by the genotype x environment interaction, therefore it is not possible to determine accurately the genotype on the basis of phenotype with respect to economic traits (Falconer, 1989).

The biochemical approaches like protein polymorphism have not yielded appreciable results in most species due to the availibility of few polymorphic markers (Baker and Manwell, 1980). Only 5-10% of the entire genome is expressed in terms of proteins etc and the rest 90-95% remains unexpressed. Hence studying and quantifying genetic variation mainly by biometrical and biochemical studies cannot reveal much information regarding the actual and entire genome.

The cytogenetic investigations have found numerous practical applications. The karyotyping is necessary for better understanding of inter relationship and evolutionary trend among allied and related species and breeds. A number of diseases and syndromes in animals have been conclusively associated with structural and numerical anomalies of the chromosomes. An indepth anlaysis of chromosomes in breeding stock can prevent the transmission of these karyological anomalies.

The knowledge from the use of molecular biology almost surely will allow unforeseen applications to improve the livestock species. The primary use of this knowledge will be to understand gene effects and interactions that are ultimately expressed as phenotypes. These phenotypes may relate to meat/wool production, disease resistance and more efficient production.

The introduction of recombinant DNA techniques and the advent of allozyme electrophoresis and RFLP analysis to produce DNA fingerprinting pattern made it possible to detect many polymorphisms in most organisms at the protein or DNA level. Finding DNA polymorphisms is simpler than association of DNA variation to traits of economic importance (Freeman and Linderberg, 1993). This DNA polymorphic approach can be employed to detect population specific alleles, to measure the amount of genetic diversity among each species, to evaluate the change in variation in species overtime and can be used in marker assisted selection programmes, parentage analysis, species or breed identification and population genetic studies.

□□□..2

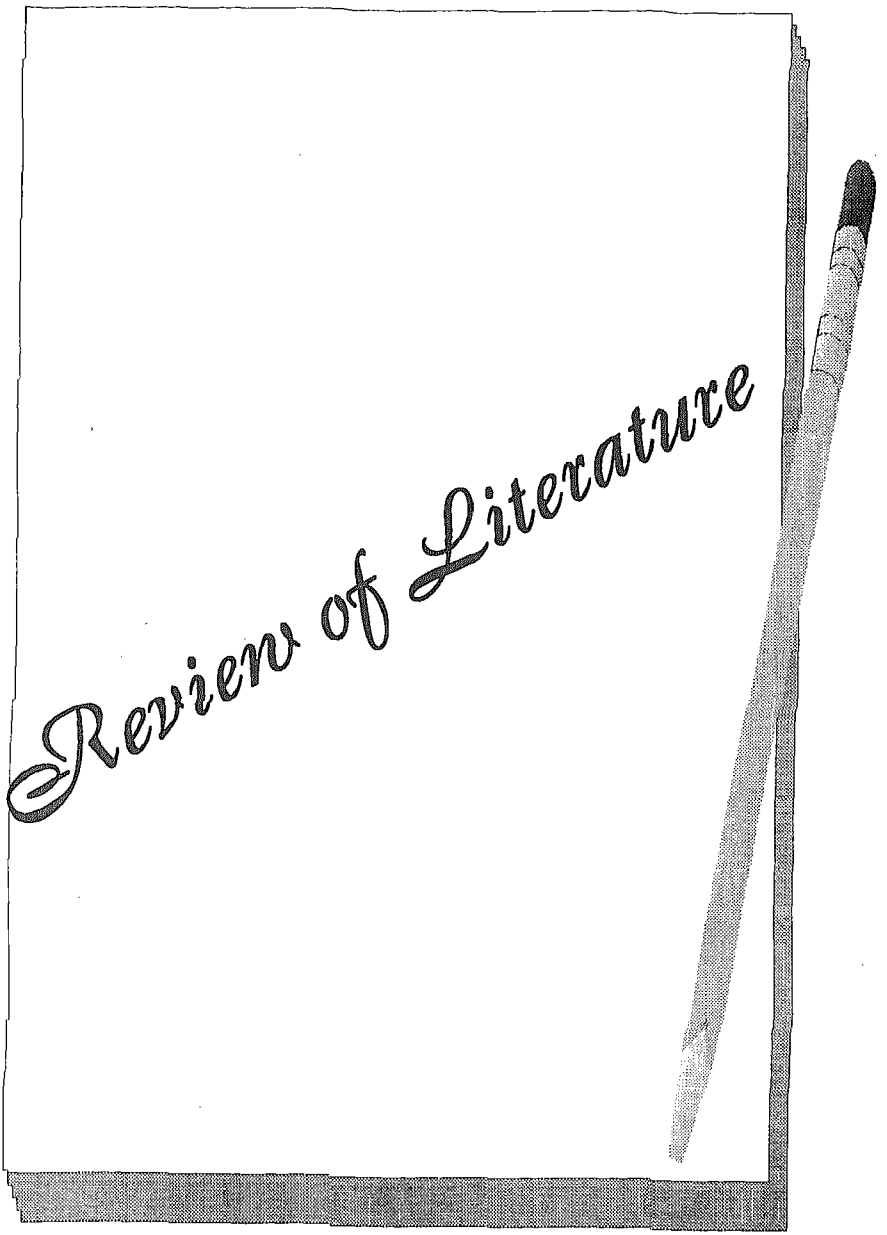
Although the modern techniques for studying genetic polymorphisms have provided many new vistas of research, they do have some limitation. For instance RFLP analysis requires fairly large amounts of genomic DNA and cloned probes that may be specific to an organism or group. So the scientific community is looking for alternative strategies to identify genetic markers which characterise some distinct populations.

A recent technique termed as randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) capable of detecting DNA polymorphism is based on random amplification of DNA segments by PCR with short oligonucleotide primers and annealing at low temperature (Williams *et al.*, 1990). This technique, has been successfully applied in genetic studies of various animal and plant species (Welsh & McClelland 1990; Baird *et al.*, 1992; Bailey *et al.*, 1994; Rothuizen *et al.*, 1994; Plotsky *et al.*, 1995 and Ezer *et al.*, 1996) as well as for the characterization of bovine and ovine populations (Bardin *et al.*, 1992; Kemp and Teale, 1994; Gwakisa *et al.*, 1994; Cushwa *et al.*, 1994 and Rao *et al.*, 1996). The ease with which many RAPD polymorphic loci can be generated without prior sequence information and the direct reading of results from agarose gel make the RAPD technique ideal for screening genetic differences in large outbred populations.

The application of the chromosomal banding as well as the RAPD-PCR technique in the genetical study of Indian sheep breeds is very limited to the best of our knowledge. Hence to exploit the potential of such powerful techniques to unravell the genetic make up of indigenous sheep breeds and to investigate the differences residing in the genetic material, the present study will be carried out with the following objectives.

OBJECTIVES :

1. To study the variation in RAPD fingerprint for differentiation of various indigenous sheep breeds and to identify breed specific RAPD markers, if any.
2. To determine band frequencies of various RAPD finger prints and to study the intra and inter breed similarities and dissimilarities using RAPD polymorphism.
3. To study the chromosomal profile and banding pattern of these animals.


Review of Literature

REVIEW OF LITERATURE

POLYMERASE CHAIN REACTION

About 20 yrs. ago the recombinant DNA technology based on the replication of DNA of plasmids was introduced as a tool for the biological sciences. This method requires long time to replicate a particular DNA sequence. But in 1984, Karry mullis developed a DNA amplification technique based on an *in vitro* rather than an *in vivo* process known as the polymerase chain reaction, this method can produce large amounts of a specific DNA fragment in a simple enzymatic reaction from a complex DNA template in a matter of hours rather than the weeks or months the traditional cloning requires. The method utilizes a DNA polymerase enzyme and two oligonucleotide primers to synthesize a specific DNA fragment from a single strandard DNA template sequence (Arnheim and Erlich, 1992). The PCR has several advantages over other amplification techniques in its simplicity, being less time consuming, being automated, economical of DNA (only picograms or nanograms of DNA) thus allowing large number of samples to be rapidly typed with little labour.

Optimization of PCR reaction conditions for RAPD-PCR.

In a standard PCR reaction, a pair of strand specific oligonucleotide primers (typically 18 to 25 bases each) are used. The sequence of the primers is based on the DNA sequence information from the regions flanking the target sequence. The primers anneal to these specific sites on the denatured template during annealing phase of the PCR reaction and define the specific region that will be amplified. In the RAPD assay, a single oligonucleotide primer of arbitrary sequence (typically 10 bases) is used for the PCR reaction. The primer design is usually constrained by three factors : G+C percentage (50-70%), no palindromes greater than 6 bases, and no

complementarity at the 3 end (Williams et al., 1993). During the annealing phase of the RAPD-PCR reactions the arbitrary primer will anneal to sequence - complementary sites on the denatured DNA template. If two of the primers anneal to the template on opposite stands, in the appropriate orientation and within amplifiable distance (i.e. approximately 2500 bases or less), then a discrete fragment will be amplified. Each RAPD primer is potentially capable of amplifying a number of fragments (1 to 10 or more) from different loci during the same PCR reaction (Waugh and Powell, 1992).

PCR reagent concentrations, thermal cycler temperature profiles, and gel electrophoresis conditions are typically developed for each application.

(i) Template DNA

One of the most important variable is the concentration of genomic DNA (Williams et al., 1993). Because different DNA extraction methods produce DNA of widely different purity, it may be necessary to optimize the amount of DNA used in the RAPD assay to achieve reproducibility and a strong singal. A large amount of template DNA had a pronounced effect : with 100 or 200 ng there was less product, and with 200 ng there was no amplification at all in some reactions. With the lowest amounts of template the amplification seemed slightly less efficient, but the complete pattern was always produced (Rothwizen *et al.*, 1994). But, Comincini et al., reported that the RAPD-PCR protocol was not appear to be strongly dependent on the quality/quantity of target DNA (Comincini *et al.*, 1996). This fact makes the RAPD extremely flexible, applecable without modification to a large variety of organisms, using both purified DNAs and crude extractions. One conclusion may be that the type of genome does not determine the optimal conditions.

Use of pooled DNA

A potential problem with the application of RAPD to genetic studies of heterogenous populations is that a large number of individuals need to be examined. Using by using mixed DNA samples this problem can be solved (Michelmore et al., 1991). When genomic DNA from two individuals of the same species are mixed in different ratios most polymorphic bands are amplfied in proportion to the amount of their respective genomic DNA template. These bands may be amplified in proportion because they present equally good matches to the primer at their respective

genomic target sites. Some polymorphic bands, however, may be poorly amplified and are detected only when their respective genome is present in several fold excess over a competing genome of the same species (Michelmore *et al.*, 1992). The mixing experiment suggest that the outcome of an amplification reaction is determined in part by a competition for priming sites in the genome and genome of high complexity should have more target sites with better complementarity to a primer, as compared, to a genome of low complexity (Williams *et al.*, 1993). DNA segments that complete poorly in the amplification reaction are expected to be unreliable as genetic markers. Zhang et al., suggested the use of mixed DNA samples for assessing genetic distance among lines of chickens (Zhang *et al.*, 1995).

(ii) Primers

Primer concentrations between about 40 and 50ng/50μl reaction mixture are optimal. At lower concentrations it becomes difficult to detect amplification products in a stained agarose gel, and at higher concentrations smearing of the bands become evident. No effect of primer concentration on the relative intensities of bands has been noted (Williams et al., 1993).

To support DNA amplification under standard conditions, a 10-base oligonucleotide primer should contain at least 4 G+C bases. For primers containing 5 G+C bases, a length of 9 bases his the minimum that will support efficient amplification as detected by staining with ethidium bromide. Oligonucleotides shorter than 9 bases may be used, but smaller amounts of amplified products are obtained and staining methods of greater sensitivity are required to detect the products (Williams et al., 1993). A G+C content of 60% had been found to produce the largest number of well resolved bands (Nadeau *et al.*, 1992).

Use of paired primers

William et al., 1993 used a set of 7 primers individually and in all possible 21 pairwise combinations to amplify DNA segments from soyabean. Each primer when used individually amplified an average of 5.3 bands, whereas each pair of primers amplified only 4.4 bands. Of the average 4.4 bands seen with paired primers, half (2.2 bands) could be identified as 'old' bands amplified by

one of the individual primers comprising a pair, and half (2.2) were 'new' bands dependent on both primers. From this experiment the authors concluded that the primers should be used individually to maximize the number of unique bands generated per reaction; and they should be used in pairs to maximize the number of unique bands obtained per primer.

Magnesium ion concentration

The magnessium ion concentration typically affects the relative intensity of amplified bands. As the $MgCl_2$ concentration increases, some DNA segments are amplified more efficiently while others are amplified less efficiently. Williams *et al.*, suggest the use of 1.5mM excess of magnessium over the total nucleotide concentration (Williams *et al.*, 1993) and optimal concentration of 2.5 mM was used by Rothuizen *et al.* (Rothuizen *et al.*, 1994).

(iii) dNTPs and Taq DNA polymerase

A deoxynucleotide triphosphate concentration of 100μl for each of the four bases is adequate for generating RAPDs. At lower concentrations the intensity of stained bands in the gel becomes progressively weaker. No effect of deoxynucleotide triphosphate concentration on the relative intensities of the amplified bands has been noted.

The outcome of the amplification reaction is determined not only by the primer sequence employed, but also by the DNA polymerase. The recommended concentration of T. aquatious Ampli taq is 20 units/ml. Although this works well for the genomic DNA for most species, it may be worthwhile to optimize this parameter for some applications. For example, a concentration between 15 to 20 units/ml is generally adequate for amplifying sheep DNA.

Amplification programme

The most important variable in RAPD procedure is the annealing temperature (Welsh and McClelland 1990). Rothnizen *et al.*, suggest a annealing temperature of 35°C for optimal results. The amplification was less efficient at 30°C and 40°C: less DNA was produced and the number of products was reduced. At 45°C there was no amplification at all (Rothuizen et al., 1994) Williams *et al.* suggest a annealing temperature of 36°C for one minute.

Agarose gel electrophoresis

Williams et al. suggested that the RAPD band pattern can be detected by using the DNA samples in a 1.4% (w/v) agarose gel. Ezer *et al.*, reported that the colling of agarose gel to 36°C-39°C with stirring before allowing the agarose to become motionless constantly produces a flat frelt of well resolved bands. If poured at 50°C recommended as the maximum temp. for the can units, bands were invariably wavy, smeared, and unresolved (Ezer *et al.*, 1996). A high voltage during the 2 min follows by a low voltage for long time seems to be produce well resolvable band (Schaffer, 1981)

PRINCIPLES OF RAPD ASSAY

Nature of polymorphism

The molecular nature of the polymorphism and detected in the RAPD assay has not been described. This would require DNA sequencing of several genomic primer-binding sites from a number of polymorphic parents. It has been suggested that the RAPD polymorphism result from sequence differences in one or both of the priming sites, which prevents primer annealing. Amplification is probably initialed at many sites, which may often be imperfectly complementary to the primer, and which form short inverted repeats seperated by upto several thousand nucleotides. On theroetical grounds, such sites would be expected to appear in a genome of random nucleotide sequence at a frequency that is related to genome complexity. Experimental observations indicate that these sites are distributed throughout a genome. The amplified sequences between the sites belong to all abundance classes, from low copy to highly repetitive. Williams *et al.*, 1993 suggest that some RAPD bands could result from self priming events due to a hairpin loop formation at the 3' terminus of the first amplified stand (Williams *et al.*, 1993). Such products would amplify as large inverted respeats.

RAPD polymorphism can also result from mutations (e.g. deletions, insertions, and inversions) that either affect the presence and/or orientation of the primer binding sites, alter the size of the region between these sites, or prevent amplification (Williams *et al.*, 1990). Bowditch

et al. (1993) suggested that polymorphism may also result if secondary structures from around the primer annealing sites during the relatively low annealing temperatures. These secondary structures could interfere with primer annealing and/or extension by the DNA polymerase. Mutations in these regions could either stabilize (or) destabilize the formation of the secondary strcutures and result in RAPD polymorphisms. Therefore mutations in regions other than the primer annealing sites may be detected by the RAPD assay. RAPD polymorphisms are typically detected as the presence or absence of particular fragments after gel electrophoresis. However, other types of polymorphisms (e.g. length and brightness) have been identified (Williams *et al.*, 1990; Echt et al., 1992; Hunt and Pag, 1992; Williams *et al.*, 1993; Harvat and Medrano, 1994). The brightness polymorphisms may be a result of amplification of a tandem repeat bands that is polymorphic for copy number or differential amplification due to sequence differences in the priming site(s) (Cactano-Anolles *et al.*, 1991; Hunt and Page, 1992).

Primer-template complimentarily

By nature of the theory of single primer PCR, RAPD polymorphism must originate from regions of the genome that contain inverted repeats that are within an amplifiable distance. Williams *et al.* (1993) used the following equation to predict the number of amplified RAPD fragments :

$$b = (2000 \times 4^{-2n})C$$

Where b is the expected number of occurances (i.e. expected number of amplified RAPD fragments per reaction), 2000 bp is the distance between two copies of the annealed RAPD primer (i.e. 500 to 2500 bp), n is the length (bases) of the arbitrary oligo nucleotide primer, and C represents the genome complexity (i.e. the total length of different sequences in the genome).

Assuming full complementarily between the primer target hybred of $n = 10$ bp, then we would expect to see $b = 3.64$ fragments per primer from a typical mammalian genome ($C = 2 \times 10^9$; Lewin, 1994). Published reports of the number of scorable fragments per primer for

mammalian species range between 3 to 18 (baboons and humans; Rieky *et al.,* 1992), 5 to 16 (dogs; Rothuizen and Van Wolferen, 1994), 3 to 12 (Cattle; Gwakisa *et al.,* 1994 and 1 to 12 sheep; Cushwa *et al.,* 1995). In order for a primer to amplify 10 fragments, the average number of complementary bases in the primer-target hybrid (n) is 9.64, using the above equation. Therefore, full primer - template complementarly is most likely not required for amplification. Additional evidence to support this conclusion is provided by the number of RAPD fragments amplified from bacterial species with smaller genomes (Williams et al., 1993). The availability of better target sites may account for the occurance of 5 fragments in the above genome instead of 0.007 (b = 0.007). On the basis of this observation, full primer target complementarity could not be necessary (n = 7.6 bp).

Genome distribution

The distribution of RAPD markers throughout the genome is an important consideration when evaluating the usefulness of these markers. There are many published reports verifying the distribution of RAPD markers in the genome of plants, fish and mouse. Cushwa *et al.* (1995) studied the sheep genome using RAPD and found that there are 45 markers which were assigned to specific sheep chromosomes forty of the RAPD markers were distributed among 17 of the 26 autosomes and the remaining five markers were assigned to the sex chromosomes. Levin *et al.* (1993) reported that 13 RAPD markers mapped to the chicken z chromosomes were widely distributed over the chromosome and that a RAPD marker would likely be linked to any trait loci located on this chromosome.

Assay reproducibility

The consistent reproducibility of any genetic marker is an important characteristic. One of the most important factors determining the applicability of RAPD for molecular genetic studies is the reproducibility of the reaction products. A number of studies have investigated the reproducibility of RAPD markers. Factors such as template DNA concentration, ethanol precipitable contaminants, $MgCl_2$ concentration, primer GC content and concentration, and thermal cycler efficiency have been shown to influence amplification (Nadeau *et al.,* 1992; Rieky *et al.,* 1992;

Scott *et al.*, 1992; Serikawa *et al.*, 1992; Bowditch *et al.*, 1993; Muralidharan and Wakeland, 1993; Penner *et al.*, 1993; Wolff *et al.*, 1993; Micheli *et al.*, 1994; Park and Kohel, 1994). Although the RAPD assay is more sensitive to reaction conditions than the standard PCR, the reproducibility of assay results has been reported by many authors. In general, the RAPD assay protocols must be optimized for each application and these protocols must be stringently followed to ensure assay reproducibility.

The type and source of the DNA polymerase used to generate certain RAPD polymorphisms is an important consideration when attempting to duplicate results between laboratories. Meunier and Grimont (1993) reported that variability in commerical preparations of Taq DNA polymerase was a major source of variation in RAPD products. This was also confirmed by Cushwa et al. 1996 and they suggests that the type and source of DNA polymerase must be specified to other laboratories attempting to duplicate RAPD markers.

Applications of RAPD for improvement of livestock species

RAPD related research in mice preceded any reported studies involving other domestic animal species. These studies provided evidence for the reproducibility of the RAPD assay and clearly demonstrated its usefulness for distinguishing inbred strains of mice and identifying and mapping RAPD genetic markers. In spite of the positive results reported in these studies, use of the RAPD assay for genetic analyses of other domestic animal species has been fairly limited in comparison to published reports involving plant microorganism, and insect species. However, a variety of recently published studies have utilized the RAPD assay for genetic analyses of domestic animal species, including cattle sheep and chickens.

Identification of breed or species specific markers and estimation of genetic divergence using RAPD

Several studies have utilized the RAPD assay to screen pools of DNA, created by combining individuals of a similar breed or species, and identify breed or species specific markers. Bailey and Lear (1994) screened pools of DNA from thoroughbred and Arabian horses to identify markers that would distinguish between the two breeds. One marker was identified that was

present in 31 analyzed Arabian horses but was absent in 20 screened thorough bred horses. Suggested uses for this markers included detecting crossbreeding and estimating genetic divergence between breeds.

Gwakisa *et al.* (1994) found RAPD markers in Zebu cattle breeds that were relatively breed specific. They identified two RAPD primers that produced fingerprints which distinguished between DNA pools of three Zebu (Bos indicus) cattle breeds. One particular band (*ILO 1127* primed PCR) was present in 61% of one breed, in only 6% of another and not at all in a third. Data from the fingerprints were used to estimate genetic homogenity and divergence between the breeds. The authors suggest that these data will be useful in breed conservation efforts. In a related study Kemp and Teale (1994) created DNA pools of two cattle subspecies (Bos indicus and Bos tanrus to identify distinguishing RAPD markers. A RAPD marker was identified that was present in all B. indicus animals and absent in all the *B. taurus* animals. The authors suggest that these markers will be useful in detection of cross breeding between these sub species, which is desirable in order to conserve the West African *B. taurus* breeds natural resistance to trypanosome infection, and for studying evolutionary relationships.

Lee and Chang (1994) utilized a single RAPD primer to generate species-specific fingerprints that permitted distinction between nine different species (bovine, chicken, dog, duck, goat, human, pig, rabbit and rat). The authors concluded that the RAPD assay provided a relatively simple, fast and sensitive species identification fingerprinting method that would be useful in analyzing crime scene evidence and identifying products from endangered species.

Zhang *et al.* (1995) reports that the RAPD assay using mixed DNA samples could be used effectively for assessing genetic distance among lines of chickens. Cargill *et al.* (1995) utilized the pooled-DNA screening approach to identify a sheep versus goat-specific RAPD marker, which was subsquently converted to a SCAR marker. The SCAR marker will be used in subsquent studies to detect chimerism in interspecific sheep-goat hemopoietic chimeras. This type of chimeric animal, which has circulating blood cells of both sheep and goat, is a useful experimental model for studies of cell lineage, interspecies pregnancy and immunological tolerance (Cargill *et al.*, 1995).

Kantanen *et al.* (1995) detected a high degree of presence of polymorphic RAPD markers in Finnish sheep breeds and based on the similarity indices the authors concluded that the sheep populations showed a higher degree of homogenity than the cattle breeds. Teale *et al.* (1995) used a single short oligonucleotide primer ILO 1065 which is typically produced with male *Bos indicus* template DNA. The RAPD product was not seen among the amplified products of male *Bos taurus* template DNA or female template DNAs derived from animals of a variety of breeds, both *Bos indicus* and *Bos taurus*. The authors suggest that the ILO1065 primed RAPD offers a powerful and simple means to detect introgression of Zebu genes into *Bos taurus* cattle populations.

In a similar study Sergio *et al.* (1996) applied this technique for the study of systematic relationship among the Cervidae family and reported that the RAPD markers are in Hardy-Weinberg equilibrium within and between loci (Gilbert *et al.*, Lynch, 1990) and suggest a seperation of *C. capreolus* from both the Cervinae and Odocoleinae. And they conclude that the RAPD provides an efficient and sensitive method to estimate the status of organisms of controversial systematics (Sergio *et al.*, 1996).

Rao *et al.* (1996) quantified the genetic divergence among the four species of the Artiodactyla family Bovidae using Jaccard's similarity coefficients and showed closer proximity of buffalo to cattle, which confirm its genetic closeness with cattle than sheep and goat. As these species exibited species specific RAPD profiles the authors suggest that they can be identified unambiguosly in the event of disputes using RAPD analysis. Using Wagner parasimony analysis the authors report that the cattle along with buffalo may belong to Bovidae and may have originated from the same ancestral parent and the rate of divergence between subfamilies seem to be uniformly maintained on single evolutionary line.

Detection of polymorphisms

Although the exact nature of RAPD polymorphism is not known, the most likely causes are nucleotide changes in DNA sequence at a primer binding site, or structural aberrations (Williams

et al. 1990). The RAPD assay has been used in several studies to investigate the success of identifying RAPD polymorphisms. Rothuizen and Van Wolferen (1994) optimized RAPD assay conditions for canine genome analysis, and reported that an average of 14 per cent of the RAPD products were polymorphic from dogs of 16 different breeds and these RAPD markers were inherited in a Mendelian manner in dogs. The authors concluded that the RAPD assay reprsented a useful approach for generating genetic markers that could be used to screen for linkages to canine inherited diseases.

Plotsky *et al.* (1995) utilized the RAPD assay to assess genetic variability within and between 13 highly inbred lines of chickens. The assay results confirmed the high degree of inbreeding in the lines and RAPD were judged to be an effective method for identifying genetic polymorphisms that were line-specific and breed specific.

Cushwa and Medrano (1994) used the RAPD assay to screen the parents of one of the Ag Research IMF sheep reference pedigrees with 130 primers. A total of 96 polymorphisms were identified between the sire and dam, and 56% (54/96) of these polymorphisms visibly segregated in the 12 full-sib progeny (i.e. an estimate of heterozygosity at the RAPD loci). These results demonstrated the usefulness of the RAPD assay to identify segregating polymorphisms in the IMF pedigrees.

Ezer *et al.* (1996) studied the genome of beagles with AP-PCR and found that the rate of polymorphisms was an average of 10%. The authors concluded that the canine species has a high background of polymorhism and that a relatively restricted set of gene loci are responsible for the production of significant phenotypic change resulting in distinct breeds.

Genetic mapping

When RAPD marker is detected as a DNA segment amplified from one parent in a genetic cross but not from the other parent, the marker can be followed in the segregating progeny and can be assigned to a locus in a genetic map. More over RAPDs can provide DNA markers in genomic regions that are not accessible to RFLP analysis due to the presence of respective DNA sequences. Two studies in domestic animal species have utilized the RAPD assay

to identify and genetically map RAPD markers. In chickens RAPDs have been portionlarly useful in assigning markers to the Z chronomosome (Levin *et al.*, 1993) and to develop the autosomal map (Levin *et al.*, 1994). Of 21 markers currently assigned to the chicken Z chromosome 16 are RAPD markers. A total of 68 RAPDs have been integrated into the chicken genome map and this represents approximately 15% of the total number of mapped markers (Burt *etal.*, 1995). Cushwa *et al.* (1995) identified 52 parental RAPD polymorphisms that visible segregated in the progeny of atleast three of nine Ag Research IMFpedigrees, and forty five of these polymorphisms showed significant linkage (i.e.odds of at least 1000 in favor of linkage) to at least one other previously contributed marker representing approximately 10% of the mapped markers (Cockett and Medrano, 1996).

Identification of sex specific markers

It has been reported in birds (Griffiths and Tiwari, 1993a; Levin *et al.*, 1993), cattle (Antoniu *et al.*, 1994; Gwakisa *et al.*, 1994), mice (Wardell *et al.*, 1993) and sheep (Cushwa and *Medrano, 1994), the RAPD assay is useful for identifying* sex specific markers. These markers will be useful for verifying the sex of individual DNA samples, but they could also have other important uses, such as embryo sex identification Gutierrez *et al.* (1997) developed an accurate method for determining the sex of ovine embryos using PCR primers developed from an ovine-specific y-chromosome random amplified polymorphic DNA marker (*Ucd 043*). The assay was 100% accurate in confirming the sex of the individuals as it detected the ovine male specific fragment in dilution containing as little as 10 pg of male DNA in 50 ng of female DNA (Gutierrez *et al.*, 1997). This experiment represents an application for a genetic marker (*Ucd043*) identified using the RAPD assay. This RAPD marker was confirmed to be y-chromosome linked (Cushwa *et al.* 1996), was converted to a SCAR marker, was physically mapped to y-chromosome by *in situ* hybridization (Cushwa *et al.*, 1996), and has now been utilized for sex diagnosis of sheep. The addtional advantage of using this SCAR marker for embryo sexing, compared to more traditionally used markers such as *Zfy/Zfx* (Aasen and Medrano, 1990) and *sry* (Griffiths and Tiwari, 1993b), is that it is sheep specific.

Cytogenetic Works

In India the chromosomes of sheep were studied for the first time by Benzamin and Bhat, (1978). Subsequently the chromosomes of Munjal, Nali and Malpura breeds of sheep were reported by Bhatia and Shanker (1989) and Gupta and Gupta 1991 and 1994. The diploid count in these breeds were found to be 54. The karyotype comprised of 3 pairs of metacentric and 23 pairs of acrocentric autosomes. The X chromosome was largest of the acrocentric numbers and Y was the small by armed chromosome. The chromosomal complement of Avikalin (Rambouillet xMalpura) was also found to be same as reported in other breeds of sheep (Gupta and Gupta, 1991).

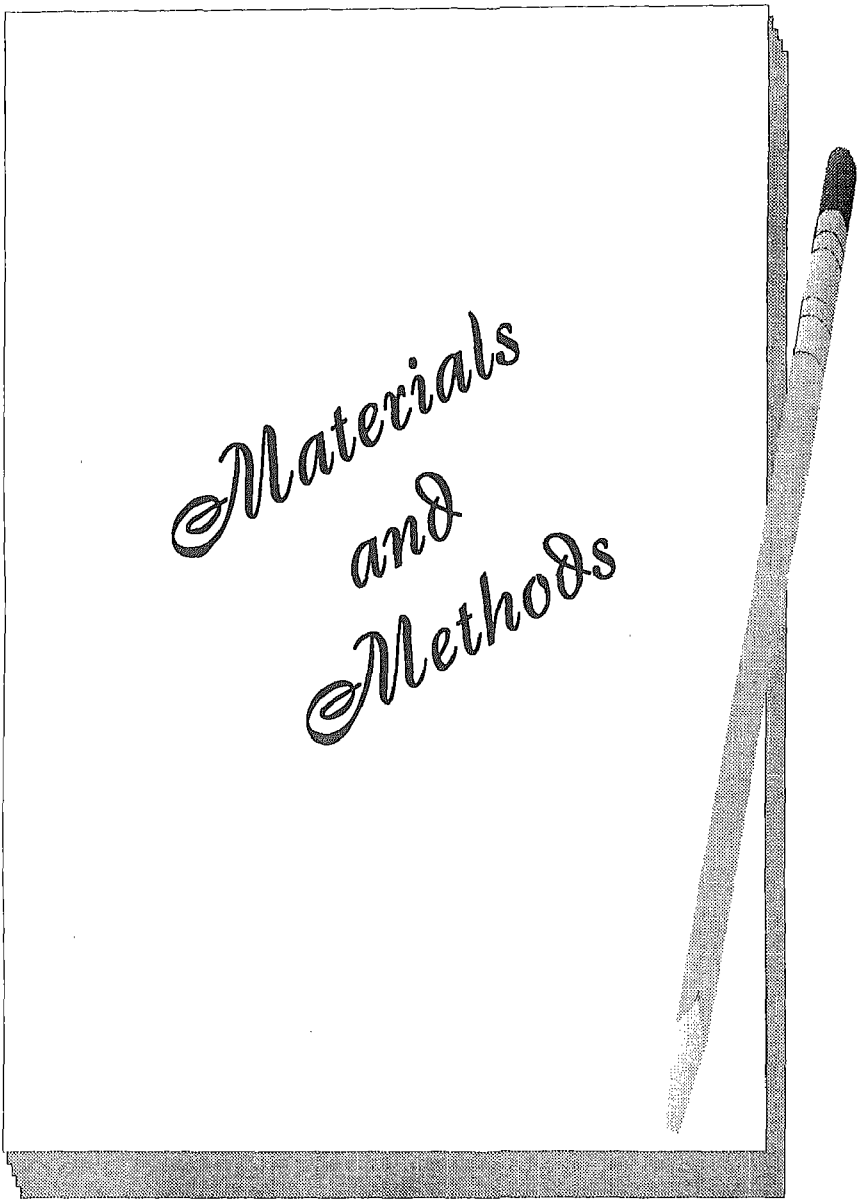
Gupta and Gupta, (1991) reported the relative length of chromosomes of Avikalin, Malpura and Rambouillet breeds and found significant variation for the chromosome number 4, 6, 9, 13, 15, 16, 17, 18, 26 ($P<0.01$) and 20, 21, 22, 23, 24 and 25 ($P<0.01$).

Many authors have indicated that identification of homologous chromosomes can be obtained by employing G-banding technique (Hegeltom and Gutavsson, 1974; Eldridge, 1975). An international conference was held at reading in 1976 for standardisation of banded karyotypes helped in identifying unequivocally many chromosomes involved in many translocations. Buckland and Evans (1978) studied the G-band karyotype of 12 species of Bovidae and revealed considerable degree of chromosome arm homology throughout the group. Toll and Halnan (1976) showed similarities in the G-banded karyotpes of Australian swamp buffalo ($2n = 48$), goat ($2n = 60$), cattle ($2n = 60$) and sheep ($2n = 54$).

C-banding which localises the constitutive heterochromatin regions in metaphase chromosome can be produced by various techniques (Arrighi and Hsu, 1971). For the first time Sumner (1972) developed the BSG technique for C-banding in livestock species (Sumner, 1972).

Bhatia and Shanker, (1991) reported C-banding in Nali breed of sheep and found all the 23 pairs of acrocentric autosomes exibited distinct densely stained C-bands in their centromeric regions. However, 3 pairs of metacentric autosomes revealed comfortably a smaller block of C-bands in majority of such chromosomes. The X-chromosome failed to show symptomatic C-bands. The Barbara sheep and the eland herd had virtually no demonstrable C-band (Buckland and Evans, 1978).

Materials and Methods



MATERIALS AND METHODS

MATERIALS

3.1 Experimental animals

Ten animals of each breed of indigenous sheep viz., Marwari, Mandya, Madras red and Muzaffarnagari were used for the present investigation.

3.1.1 Marwari

These are medium sized animals with black colour face. Ears are small and tubular. Both sexes are polled. The home tract of this breed is in and around the areas of Jodhpur. Blood was collected from this breed maintained at Central Arid Zone Research Institute (CAZRI), Jodhpur, Rajasthan.

3.1.2. Mandya

Mandya is relatively small animal. Ears are long, leafy and drooping. Both sexes are polled. Blood from this breed was collected from the clinics, Madras veterinary college, Chennai.

3.1.3 Madras red

Body colour of this breed is predominantly brown, the intensity varying from light tan to dark brown. Rams have strong corrugated and twisted horns; the ewes are polled. Blood from these animals were collected from livestock research station, Kattupakkam, TNVASU, Chennai.

3.1.4 Muzaffarnagari

These are medium to large animals, with slightly convex face. Both sexes are polled. Tail is extremely long and reaches fetlock. Fleece is white, coarse and open. The animals maintained at sheep and goat farm, IVRI, Izatnagar were used for blood collection.

3.1.5 Chemicals/equipments/miscellaneous items

The list of these items have been given in the annexure-I.

3.2 METHODS

3.2.1 Cytogenetic Work

Blood Collection and Transportation

Normally 5-6 ml. blood sample was collected from the jugular vein of each animal in a 10 ml. capacity vaccutainer tube (Becton and Dickinson, USA), containing 143 USP units of sodium heparin as an anticoagulant. Blood was gently mixed with heparin by tilting the vacutainer tube. The sample tubes were brought to the laboratory in a double walled thermocol ice box containing ice in it. The samples were transfered to refrigerator and kept there till cultures were set up.

Lymphocyte Cultures

Chromosome preparations were made by using the short-term whole blood lymphocyte culture technique with modifications suggested by Yadav and Balakrishnan (1985). The technique is described briefly below :

Preparation of glassware

Necessary glassware and other equipments such as cultute tubes, millipore filter assembly, filteration flask, conical flasks and beakers etc. were thorouhly cleaned with teepol detergent solution and dried at 150°C for over night.

The millipore filter assembly and glassware to be used in preparing media and culturing lymphocytes were wrapped in aluminium foil and sterilized in an autoclave for 15 minutes at 15 lbs/inch hot steam pressure.

Preparation of culture medium

A conical flask containing 500 ml. glass distilled water was autoclaved and was transfered into the Laminar flow hood. After cooling of the water the proportionate amount of media constituents were added into the flask as following :

TC 199 medium	(Hi - media)	10.9 gm/litre
Pokeweed mitogen	(Sigma)	2.5 mg/litre
Penicillin - G	(Sigma)	12 mg/litre
Streptomycin sulfate	(Sigma)	20 mg/litre

The constituents were dissolved comletely by gentle swirling of the flask. Sterile sodium bicarbonate (4.40 gm/100 ml. of glass distilled water) was added drop by drop till the pH was 7.2 as judged by light pink colour of the medium.

The medium was filtered through 0.22 micro filter (millipore) with the help of a vacuum pump. After filteration of the medium, 100 ml. aseptically prepared cattle serum was added into the medium flask and mixed gently.

The completed medium was distributed into screw capped culture tubes in the aliquotes of 4.5 ml. each. The cap was fixed air-tight after flaming the cap and mouth of the culture tube. The culture medium was ready for use and it was either used fresh or stored in a frozen state (-20°C) *till needed.*

Setting up of Cultures

From each of the blood samples, an aliquote of 0.5 ml. was added to the culture tube containing 4.5 ml. medium, and the caps of these tubes were replaced tightly after flaming the cap and the mouth of the tube. The cultures were incubated in a B.O.D. incubator at 37.5°C for 72 hours. Cultures were visually checked in the mornings and evenings for colour and sedimentation *of cells.*

Harvesting of Cultures

One hour prior to harvesting time, two drops (3.2 μg/culture) of colchicine solution was added to each culture. After one hour, contents of each culture tube were transfered to 15 ml. capacity centrifuge tubes and centrifuged at 1500 rpm for 15 minutes. Supernatant was discarded and the cell pellet was subjected to hypotonic treatment with 10-15 ml. of 0.075M Potassium chloride for 6-8 minutes (7 minutes yielded optimal results). Hypotonic treatment was terminated by adding one ml. of freshly prepared and chilled fixative (3:1; Methanol: Acetic acid) to each centrifuge tube and mixing thoroughly using separate pasteur Pipettes, Contents were again centrifuged for 10 minutes at 1000 rpm. Supernatant was again discarded and cell pellet was resuspended in 5 ml. of fixative. Process of addition of fixative and centrifugation was repeated thrice so as to get a clear whitish pellet in the centrifuge tube.

After the last washing the supernatant was removed by Pasteur pipette leaving about 0.5-1.5 ml. fixative over the cell pellet. The cells were suspended in the fixative by gentle mixing with Pasteur pipettes. Three drops of this cell suspension were dropped on clean, moist and labelled slides. Slides were either air dried or flame dried as explained in next section. Remaining cell suspension was stored at -20°C for use in future.

Preparation of Slides

By Air drying method

Three drops of cell suspension were dropped on the slide by Pasteur pipette from a height of about two feet. The slide was then allowed to dry naturally in the room air. After the methanol dried, the remaining droplets of acetic acid were just flipped away. The slides were kept in incubator for 3-10 days before processing them for banding.

By flame drying method

As usual three drops of cell suspension were dropped from a height of two feet on each slide. The slide was touched to the flame of a spirit lamp. Slides were kept in the incubator for a few hours and stained with Giemsa for conventional studies.

Conventional staining of Slides

Fresh working solution of Giemsa stain was prepared in a conical falsk by taking 94 ml. glass distilled water, 4 ml. phosphate buffer (pH 6.8) and 2 ml. Giemsa solution. Slides were stained for 30 minutes, rinsed thoroughly in distilled water, extra moisture removed by putting the slides in the folds of a filter paper, and then kept in an incubator for 2-4 hours for drying before mounting. Slides were mounted with D.P.X. and were kept in incubator for 24-48 hours for drying.

C-Banding

The BSG (Barium hydroxide/Saline/Giemsa) technique reported by Sumner (1972) was used for C-banding. The slides were incubated in 0.2 N HCl for 1 hours at room temperature and rinsed with distilled water. Subsequently, the slides were incubated in 1% $Ba(OH)_2$ solution for 6-8 minutes at 56°C in a hot waterbath, rinsed thoroughly in 3 successive changes of glass distilled water at 56°C to remove traces of $Ba(OH)_2$ and incubated again for 2 hours in 2 x SSC (double salt of sodium chloride and sodium citrate) solution in a hot waterbath at 60°C and stained for 1 hour in 2% Giemsa solution at pH 6.8.

G-Banding

The technique described by Seabright (1971) was followed for G-banding. The slides were kept in a couplin jar containing 0.01% trypsin (Difco, USA) solution in a waterbath at 37°C. After 50-90 seconds the slides were taken out and immediately dipped in 2% Giemsa solution pH 6.8 for 60-120 seconds. The slides were rinsed thoroughly with distilled water and the extra water was soaked by filter paper folds. The slides were dried by keeping in an incubator at 37°C for 2 hours.

NOR Banding

Freshly prepared slides were used for nucleolus organiser region (NORs) banding. The method reported by Howell and Black (1980) with slite modification was used. For selective staining of NROs, 2 drops of the colloidal developer (2% gelatin) and 4 drops of aqueous silver (50% $AgNO_3$) were pipetted onto the surface of a microscope slide containing chromosome preparation. The solutions were mixed and covered with cover glass. The slide was placed into the surface of slide warmer at 70°C for 2 min. to get the silver staining mixrue yellow. The slide

was then removed and the cover glass and staining mixture was rinsed off using deionized water. The slide was then blotted dried, mounted and screened under microscope for examiantion of NORs.

Screening of slides and photography

Normally 50-60 well spread metaphase plates of each animal were examined under microscope. Microphotographs were taken with Leitz Orthomat Camera on 35 mm ORWO film of 100 ASA.

Preparation of Karyotype

The chromosomes were enlarged from 35 mm negative to a 20 cm x 30 cm size print. The karyotypes were prepared as per standard procedure (Pushpendra Kumar, 1987).

4.0 Molecular Genetic Work

4.1 Collection of blood and genomic DNA isolation

4.1.1. Collection and storage of blood samples

Approximately 20 ml of venous blood was collected in vials containing EDTA as anticoagulant. Blood was transferred immediately to the lab in an icebucket. However, if blood is frozen before extraction equal volume of lysis buffer I (0mM Tris-HCl pH 8.0, 5mM EDTA, 50mM NaCl) was added and stored at -20°C.

4.1.2 Isolation of DNA

DNA isolation was done as per the standard protocol described by Sambrook *et al.* (1987).

1. The blood samples were taken out from the freezer and incubated in a water bath set at 56°C for 10 minutes.
2. Blood samples were centrifuged at 4500 rpm for 20 minutes, and the red colured supernatant containing the lysed RBC was discarded, with care so that the pellet of WBC was not disturbed.

3. Four volumes of lysis buffer II (75mM NaCl, 2mM EDTA, pH 8.0) was added and homogenized thoroughly.

4. Once the buffy coat was completely suspended 10% SDS @ 200μl per 10 ml blood was added and mixed properly by inverting the tube several times. Finally proteinase-k at a concentration of 100-150μg/ml was added and incubated at 40°C for 5 hours or overnight.

5. After completion of incubation with the incubated lysate equal volume of Tris-saturated phenol (pH 8.0) was added and mixed thoroughly by inverting the tubes several times, and centrifuged for 15 minutes at 3500 rpm.

6. Supernatant was collected and equal volumes of phenol : chloroform : isoamyl alcohol (25:24:1) was added. After thoroughly mixing the content was centrifuged at 3500 rpm for 15 minutes and the upper aqueous phase was transferred to a fresh tube.

7. Equal volume of chloroform : isoamyl alcohol (24:1) was added and mixed thoroughly following centrifugation the upper phase was collected in a fresh tube.

8. With the aquous phase equal volume of ethyl alcohol was added to precipitate the DNA.

9. DNA was washed thrice with 70% ethanol and once with absolute alcohol to remove excess salt.

10. DNA was dissolved in appropriate volumes of sterile distilled water.

4.1.3. Quantitation of DNA

1. 5μl of DNA sample was dissolved in 2995μl of distilled water and spectrophotometric reading was taken at OD_{260} and OD_{280} against distilled water (3000μl) as a blank.

2. OD ratio was calculated by OD_{260}/OD_{280}. If the ratio is below 1.7 and above 2, the sample was re-extracted with phenol for the second time.

3. Amount of DNA was calculated as, amount of DNA (μg/ml) = OD_{260} x 50 x dilution factor

4. *Though quality of DNA with respect to the presence of impurities in the DNA* sample can be determined spectrophotometrically as disscussed above in step 2 and 3, molecular weight of the isolated DNA (ideally it should be of *>100-150 kb) was determined by horizontal agarose gel electrophoresis.*

4.2 Randomly Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR Product Analysis

RAPD-PCR is based on random amplification of DNA segments by PCR with short oligonucleotide primers using Taq DNA, dNTPs in suitable buffer with standard PCR programmes and electrophoresis of amplified products.

4.2.1 Primers

Six arbitrary short oligonucleotide primers (10-mer) with a GC content of 60-90% were used in this study based on the earlier reports of revealing the breed specific RAPD patterns. The detail of the primer sequence is given below.

Primer	Sequence	GC%/
OPM-2	5'-ACAACGCCTC-3'	60
OPM-5	5'-GGGAACGTGT-3'	60
K-4	5'-GCATGCGATC-3'	60
ILO-14	5'-GCCGTCCGAG-3'	80
ILO-15	5'-GGGACGTCTC-3'	70
ILO-17	5'-CCGCGCCGGT-3'	90

4.2.2 Reaction mixture

The PCR reaction mixture tube (50μl) contained 0.75U Taq DNA polymerase, buffer (500mM kcl, 100mM Tris HCl, 1.5mM $MgCl_2$, 1% Triton x 100) supplied by the enzyme manufacturer

(Bangalore Genei, India),m 100μM of each dNTP; 1.5 mM $MgCl_2$; 40ng of random primer and 50-100ng of template DNA.

4.2.3 PCR Programme

PCR reactions were carried out in a thermal cycler (PTC-200, MJ Research, USA) by using the programme described by Cushwa *et al.* (1996) with slight modification.

i) Initial denaturation of 94°C (4 min)

ii) (a) Denaturation at 94°C (1 min)

(b) Primer annealing at 36°C (1 min)

(c) Primer extension at 72°C (2 min) steps (a) to (c) were repeated for 45 times.

iii) Final extension at 72°C (10 min)

The reaction mixture for RAPD-PCR amplification was prepared in 0.2 ml thin walled PCR tubes. The master mix containing 5μl Taq DNA polymerase buffer 0.5μl (100μM) each of dNTPs, 4μl (40ng) of primer and 0.25μl of Taq DNA polymerase for each tube was prepared for the required number tubes and equally distributed to all tubes after thorough mixing. All these procedure was carried out in 4°C. Then the amount of distilled water to make the volume to 50μl was calculated and added. Finally 1μl of DNA (approximately 50ng) was added in each tube seperately and the tubes were centrifuged for a ten seconds. Then the tubes were keptfor amplification in the thermal cycler. After the final extension step approximately 8μl of 6 x loading dye (Bromophenol Blue)was added in each tube and stored at -20°C until further use.

4.2.4. Agarose gel electrophoresis

The amplified products were analysed by running the products in a ethidium bromide stained horizontal agrose gel electrophoresis system at 2-4 v/c.m using 1.5% w/v agarose gel stained with ethidium bromide. Photograph was taken under UV transilluminator.

4.3. Size estimation of RAPD products

Standard Molecular size marker (λ DNA/EcoRF, HindIII digest) was used for size estimation

of RAPD products. They were non parellel to the amplified products were estimated by their comparison with standard molecular size markers using a computer programme (Schaffer and Sedenoff, 1981).

4.4. Recording of data and statistical analysis

Only distinct and prominent bands were scored for estiamtion of band frequencsis (BF), Band sharing frequencies (BSF) and also for breed specific patterns. The bands were scored as either one or zero for their presence or absence, respectively. Statistical analysis of the data so generated was carried out as detailed below.

4.4.1 Band frequency (BF)

Band frequencies of RAPD fingerprints were determined as the ratio of number of animals (n) carrying a particular band to the total number of animals (N) screened per breed,.

$BF = n/N$

4.4.2 BAND SHARING FREQUENCY (BSF)

The BSF within as well as between lines (Bab) were calculated as per Smith *et al.* (1996) using following formula

$$B_{ab} = 2.b_{ab} / (b_a + b_b)$$

where B_{ab} = Band sharing frequency

b_a = Number of bands present in a $\sum_{i=1}^{c}$

b_b = Number of bands present in b $\sum_{i=1}^{c}$

b_{ab} = Number of bands present in both a and b

4.4.3 Mean average percentage difference (MAPD)

Mean average percentage difference (MAPD) according to Gilbert *et al.,* 1990 was calculated as mentioned below :

(i) Percentage difference (PD) = [(Nab)/(Na+Nb)] x100

(ii) Average percentage difference (APD) = $1/C \Sigma PDi$

(iii) Mean average percentage difference (MAPD) = $1/R \Sigma APDi$

where, Nab is the number of fragments that differs between two individuals for a single primer. Na and Nb are number of fragments resolved by a and b individuals. C is the number of interbreed pairwise comparison, R is the number of random primers used.

4.4.4. GENETIC DISTANCE (Dxy)

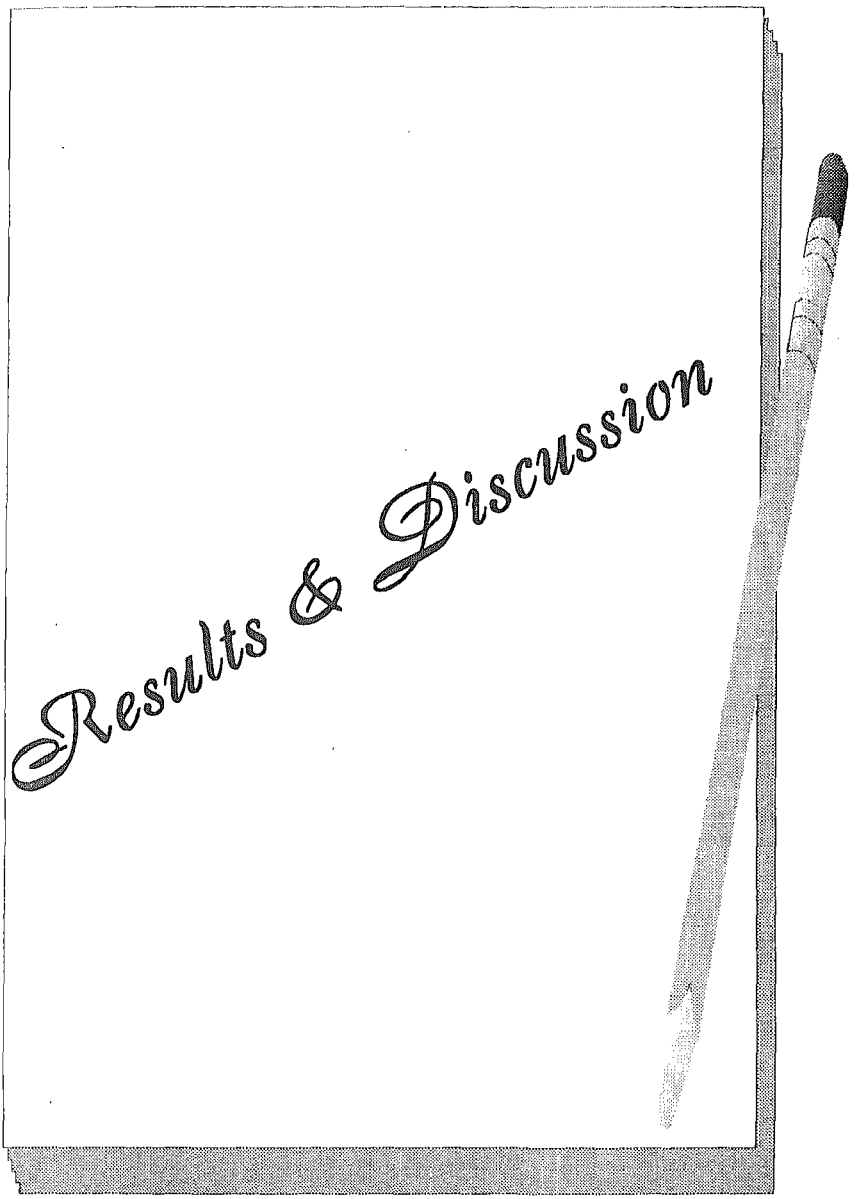
The BSF estimates both within as well as between lines were used to determine the Genetic distance (Dxy) between populations as per Smith *et al.* (1996) by using following formula

$D_{xy} = -\ln (B_{xy} / \sqrt{B_x . B_y})$

where B_{xy} = BSF between population x and y

B_x = Within line BSF in population x

B_y = Within line BSF in population y


Results & Discussion

RESULTS AND DISCUSSION

In the present investigation the RAPD-PCR analysis has been conducted to develope RAPD patterns for various indigenous sheep breeds viz. Marwari, Mandya, Madras red and Muzaffarnagari to identity breed specific RAPD markers, to determine band frequency (BF) of various RAPD bands, to evaluate the intra and inter breed similarities and dissimilarities if any, between RAPD pattern. The cytogenetic investigation was carried out with the objective of *studying chromosomal profile and banding pattern of these animals.*

5.1. Molecular genetic work

5.1.1 Genomic DNA isolation

Blood samples were collected in 30ml vials containing EDTA as an anticoagulant and the extracted DNA was dissolved in autoclaved triple glass distilled water and kept for 5-7 days for complete dissolution. The TE buffer (10 mM Tris, 1mM EDTA) was not used as solvent because the EDTA is a chelating agent for magnessium ions which are necessary for Taq DNA polymerase activity (Innis *et al.* 1990). The quality of the DNA was checked by running the samples in 0.8% w/v agarose gel and the samples showing smearing/streaking were discarded because the smearing/ streaking indicated shearing/degradation of DNA. The degraded DNA as template in PCR reduces the resultant product, because terminal deoxynucleotidal transferase activity of Taq DNA polymerase may inhibit 3 annealing (Golenberg *et al.,* 1996), hence not used in the present study.

5.1.2 RAPD-PCR optimization

The technique employed in the present study i.e. RAPD-PCR, is highly sensitive for reaction conditions and the efficiency of this technique can be increased by optimization of the

reaction conditions and the efficiency of this technique can be increased by optimization of the PCR conditions (Yu and Pauls, 1992). In the present study the optimization of RAPD-PCR was carried out by varying the units of Taq DNA Pol. and Mg^{2+} ion concentration as was done by Cushwa *et al.* (1996). The Mg^{2+} ion concentration of 1.5 mM was found optimum for primers OPM-2, OMP-5, ILO-15 and ILO17 and 2mM for k-4 and ILO14. The taq DNA plymerase concentration of 0.75U/50μl reaction mixture was found to be effective with all primers. Present study suggested that the concentration of Mg^{2+} ion should be optimized whenever a new primer is used for the first time with the sheep genome. Similar observations were reported by Williams *et al.* (1993) and Sambrook *et al.* (1989).

RAPD Finger Prints

Only distinct, reproducible and scorable amplified products were considered for analysis of Band frequency (BF), Band sharing frequency (BSF) and Genetic Distance among breeds. Before carrying out the detailed individuals' RAPD-PCR analysis, the intial screening was done for each of the 6 perimers to ensure satisfactory amplification patterns and polymorphism. The size of the clearly resolvable amplified products ranged from 0.3 to 1.9 kb. The products beyond this range were not considered for anlysis owing to their poor resolution. The observed range of products could be due to insufficiency of the extension reaction under the PCR conditions at higher molecular sizes and limitation of the resolving power of agarose gels at lower sizes (Bowditch *et al.*, 1993).

The analysis revealed that OPM-2 and OPM-5 primer amplified all the 4 breeds satisfactorily but yielded almost a monomorphic pattern (Fig. 6). However, other primers revelaed polymorphic patterns. The average number of scorable bands ranges from 6 to 15 in different sheep breeds. These are summarised in Table 1.

The average number of scorable bands with primer OPM-2 in different sheep breeds varied from 11 to 17. The molecular sizes of amplified products ranged from 340 to 1450 bp. Product of 605 pb was found to be present in all the individuals of all the breeds (Fig. 3). But this primer yielded some typical polymorphic bands at a size of approximately 1045 bp. This band may be predicted as giving polymorphic pattern with the primer.

Table 1. The average number of amplified fragments scored from randomly amplified polymorphic DNA of 6 primers in four sheep breeds.

Primer	Marwari	Mandya	Madras Red	Muzaffarnagari
OPM-2	13.50±0.50	13.75±0.49	12.00±0.71	15.25±0.63
OPM-5	6.00±0.00	6.00±0.00	6.00±0.00	7.00±0.41
K-4	11.75±0.48	11.25±0.25	11.50±0.29	11.00±0.00
ILO-14	8.00±0.00	6.50±0.24	6.50±0.50	6.00±0.00
ILO-15	7.5±0.29	8.75±0.25	7.75±0.25	9.00±0.00
ILO-17	10.75±0.75	13.25±0.49	12.75±1.44	13.50±1.04

The mean number of amplified products varied from 11 to 13 with primer k-4. This primer gave more bands with mandya and marwari breed. The yielded product of this primer varied from 585 bp to 2009 bp (Fig. 4). A typical polymorphic band was obtained at a size of 670 bp. This band was distinct in the marwari and mandya breeds only. Comparatively more number of polymorphic bands were obtained with this primer than the other primers.

The primer ILO17 amplfied approximately 9 to 16 bands (Fig. 1). Polymorphic bands were obtained at sizes of approximately 1338 bp, 679 bp and 615 bp. This primer was found to be giving some polymorphic pattern in all the four breeds, like k-4 primer. Further screening with more number of individuals are needed before coming to a conclusion that this may be a breed specific primer.

Approximately 6 to 9 bands were obtained from both the ILO 14 and ILO15 primers. Both these primers were found to be mostly monomorphic in their pattern. But ILO 14 was found to be giving one polymorphic band only with the mandya and madras red sheep at the size of 632 (Fig. 7) ILO15 developed an polymorphic patterns at sizes of 516 bp and 550 bp (Fig. 2). Further analysis is needed before coming to a conclusion that these primers can be used for identifying breed specific markers.

OPM-5 primer amplified approximately 6 to 8 bands. More number of bands were seen in Muzaffarnagari sheep than others. But most of the bands were found to be monomorphic with this primer. Two typical monomorphic bands were obtained at a size of approximately 750 bp (Fig. 6).

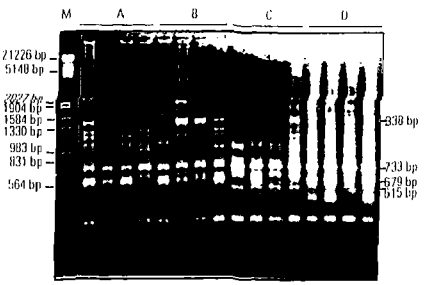


Fig 1 RAPD fragments of sheep generated with IIO-17 primer.
M Molecular size marker (λ DNA/EcoRI-HindIII Double digest)
A Marwari, B Mandya, C Madras red, D Murrafarnagri

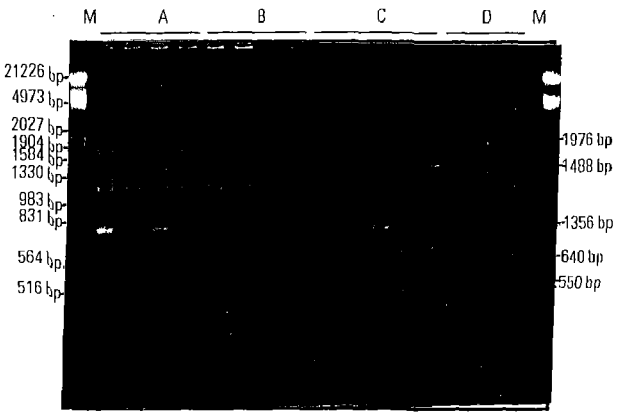


Fig. 2 : *RAPD fragments of sheep generated with ILO-15 primer.*
M – Molecular size marker (λ DNA/EcoRI-HindIII Double digest)
A – Marwari, B – Mandya, C– Madras red, D– Muzzafarnagri

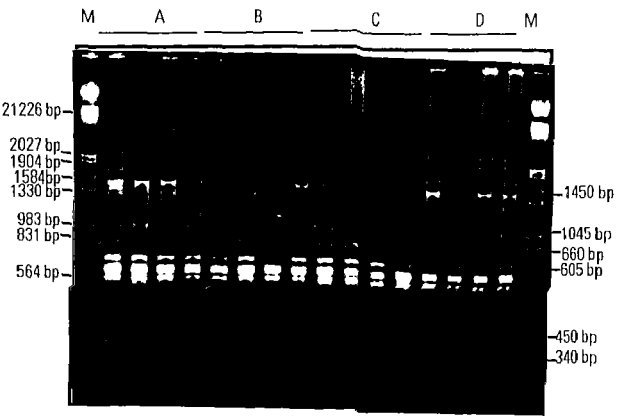


Fig. 3 : RAPD fragments of sheep generated with OPM-2 primer.
M – Molecular size marker (λ DNA/EcoRI-HindIII Double digest)
A – Marwari, B – Mandya, C– Madras red, D– Muzzafarnagri

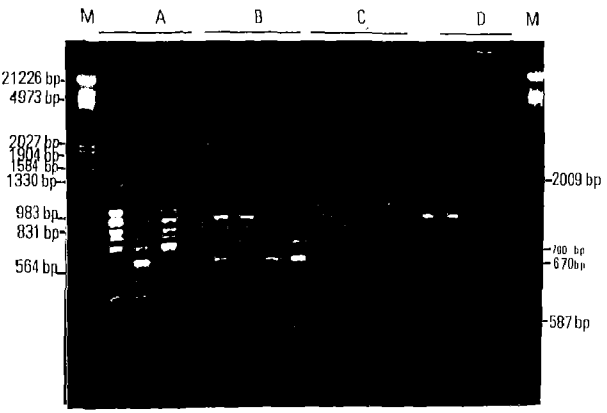


Fig. 4 : RAPD fragments of sheep generated with K-4 primer.
M – Molecular size marker (λ DNA/EcoRI-HindIII Double digest)
A – Marwari, B – Mandya, C– Madras red, D– Muzzafarnagri

5.1.4. RAPD analysis of pooled DNA samples

The DNA mix was prepared for each breed by mixing 6 samples in equal amount from all the breeds. The amplification pattern of pooled DNA samples with the primers ILO17, OPM-2, OPM-5 and k-4 were presented in fig 5. The number of amplified products was low as compared to individual amplification pattern and varied from 6 to 12 only. The observation of reduced number of bands in pooled DNA samples as compared to individual amplification was similar to the reports of Michelmore *et al.* (1992). Variation in the intensities of band was observed in comparison with individual DNA samples. This may be because of that the particular band may be present in equally good matches to the primer at their respective genomic target sites and because of high complex genome with better complementarity to the primer.

Band frequency

The frequency of all amplified products seen as bands were estimated the molecular weight of bands which were differing by ±5% were considered as single band and the frequencies were estimated for all the six primers. The band frequency of different RAPD-PCR products are given in table 2. Primers OPM-2 and ILO-17 gave large number of scorable bands in the present analysis as compared to other primers. The primers ILO-17, OPM-2 and k-4 gave almost equal band frequency at sizes ranging from 2000 bp to 600bp.

Band sharing frequency

The band sharing frequencies (BSF) were calculated by using a computer programme prepared by Shri G.S. Bisht, I/c Computer Centre, IVRI, Izatnagar which was based on the report of Lynch (1990) and Smith *et al.* (1996). BSF gives an estimation of number of bands (amplified products) shared by different breeds. The within and between breed band sharing frequencies were estimated by considering only the distinctly scorable amplified products.

Within breed band sharing frequency

The primer-wise within breed band sharing frequencies have been tabulated in table 3.

The within breed band sharing frequency ranged from 0.6585 to 1 (ILO-17). Higher band for all the primers screened except ILO-17. The primer ILO-17 showed high variability of BSF values

Table2. Frequencies (BF) of various amplified products of sheep breeds with each of the 6 primers.

A. With primer ILO-14

Mol. Wt. of Bands	Marwari	Mandya	Madras Red	Muzaffarnagari
2340	1.00	0.00	0.00	0.00
1935	1.00	0.00	0.00	0.00
1470	1.00	1.00	1.00	1.00
1250	1.00	1.00	1.00	1.00
1095	1.00	1.00	1.00	1.00
865	1.00	1.00	1.00	1.00
695	1.00	1.00	1.00	1.00
630	0.00	0.50	0.25	0.00
570	0.00	0.00	0.25	0.00
520	1.00	1.00	1.00	1.00
B.	With Primer ILO-17			
3715	0.00	0.50	0.00	0.00
2570	0.50	0.50	0.25	0.00
2445	0.75	0.50	0.50	0.75
2285	0.75	0.75	0.50	0.00
2110	0.00	0.25	0.00	0.25
1875	0.00	0.25	0.25	0.75
1775	0.00	0.50	0.00	0.00
1550	0.00	0.00	0.25	0.75
1445	0.75	0.75	0.75	0.25
1270	1.00	0.75	0.50	0.75
1190	1.00	1.00	1.00	1.00
1040	0.00	1.00	0.50	0.75
770	0.00	0.25	0.00	1.00
730	1.00	1.00	0.50	1.00
700	1.00	1.00	1.00	1.00
680	0.00	0.0	0.50	0.00
615	1.00	1.00	1.00	0.75
550	0.75	1.00	0.25	0.75
500	0.00	0.25	0.00	0.75

C With Primer ILO-15

Mol. Wt. of Bands	Marwari	Mandya	Madras Red	Muzaffarnagri
2540	1.00	1.00	1.00	1.00
1975	0.25	1.00	0.00	0.50
1480	0.0	0.0	0.0	0.50
1355	1.0	1.0	1.0	1.0
1160	1.0	1.0	1.0	1.0
1050	0.0	0.0	1.0	1.0
905	1.0	1.0	1.0	1.0
805	1.0	1.0	1.0	1.0
640	0.5	0.0	0.0	0.0
625	1.0	1.0	1.0	1.0
550	0.0	1.0	1.0	1.0
515	1.0	1.0	0.0	0.0
D	With Primer OPM-2			
2580	1.0	0.5	0.25	0.75
1710	1.0	1.0	0.5	1.0
1465	1.0	1.0	1.0	1.0
1450	0.25	0.25	0.0	0.25
1290	0.75	0.75	0.25	1.0
1045	0.25	0.75	0.25	0.0
1000	0.25	0.0	0.25	0.25
940	1.0	1.0	1.0	1.0
820	1.0	1.0	1.0	1.0
705	1.0	1.0	1.0	1.0
660	1.0	1.0	1.0	1.0
605	1.0	1.0	1.0	1.0
540	1.0	1.0	1.0	1.0
500	1.0	1.0	1.0	1.0
460	0.0	0.75	0.0	0.0
450	1.0	1.0	1.0	1.0
420	1.0	1.0	1.0	1.0
395	0.0	0.0	0.25	0.0
350	0.0	0.25	0.25	1.0
340	0.0	0.0	0.0	1.0

E	With Primer K-4			
Mol. Wt. of Bands	Marwari	Mandya	Madras Red	Muzaffarnagri
2010	1.0	0.5	0.0	1.0
1360	1.0	0.5	1.0	1.0
1295	1.0	1.0	1.0	1.0
1035	1.0	1.0	1.0	1.0
930	1.0	1.0	1.0	1.0
860	1.0	1.0	1.0	1.0
808	1.0	1.0	1.0	1.0
760	1.0	1.0	1.0	1.0
700	0.25	0.0	1.0	1.0
670	0.25	0.75	0.0	0.0
660	0.25	0.0	0.50	0.0
590	1.0	1.0	1.0	0.0
540	1.0	1.0	1.0	1.0
480	1.0	1.0	1.0	1.0
F	With Primer OPM-5			
1245	1.0	0.0	0.0	0.75
810	1.0	1.0	1.0	1.0
750	1.0	1.0	1.0	1.0
645	0.0	1.0	1.0	1.0
530	0.0	1.0	1.0	1.0
495	1.0	0.0	0.0	0.0
440	1.0	1.0	1.0	1.0
405	1.0	1.0	1.0	1.0
365	0.0	0.0	0.0	0.25

sharing frequency was seen within Marwari breed (0.9452) comparatively lower BSF were found for 1-6 primer ILO 17 irrespective of the breed, where as higher BSF values were observed for the primer OPM-5 (0.9283 to 1).

Between breed band sharing frequency

The between breed band sharing frequency estimates for all the six primers used in the study are presented in table 4.

Band sharing frequency is an indicator of relativeness between breeds (Nei and Li, 1979). The estimates for all the breeds analysed in this study showed close genetic resemblance

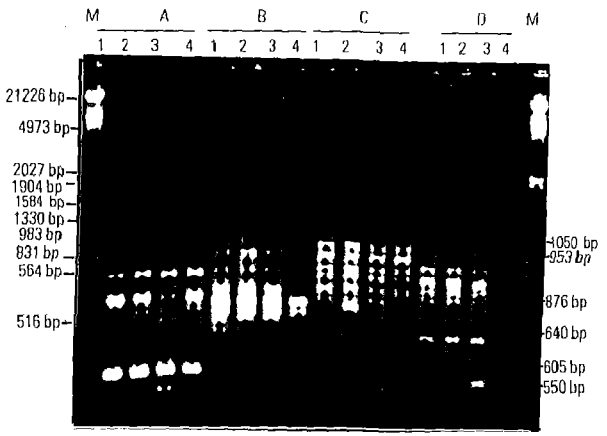


Fig. 5 : RAPD fragments of sheep generated with pooled DNA samples.
M – Molecular size marker (λ DNA/EcoRI-HindIII Double digest)
A – ILO-17, B – OPM-2, C– OPM-5, D– K-4 primers
1– Marwari, 2– Mandya, 3– Madras red, 4 –Muzzafarnagri

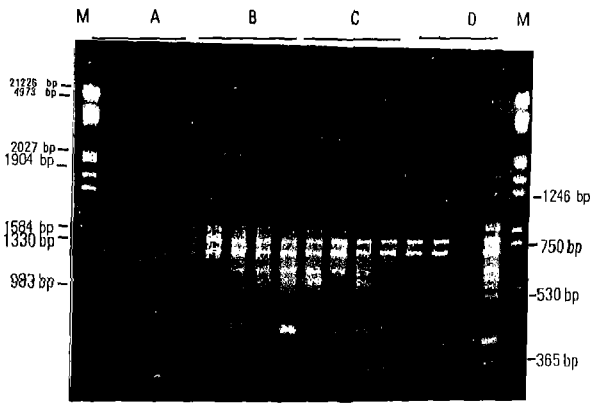


Fig. 6 : RAPD fragments of sheep generated with OPM-5 primer.
M – Molecular size marker (λ DNA/EcoRI-HindIII Double digest)
A– Marwari, B– Mandya, C– Madras red, D –Muzzafarnagri

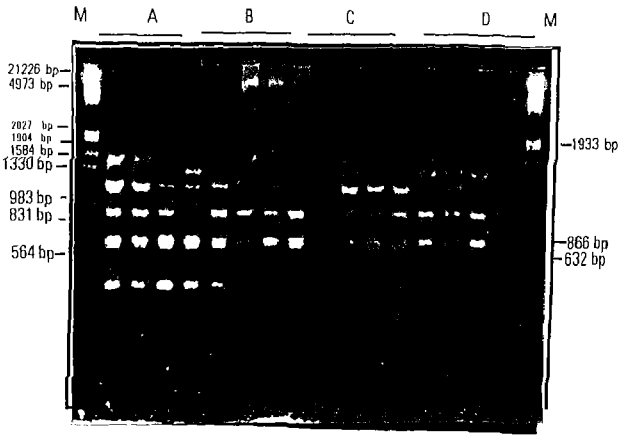


Fig. 7 : RAPD fragments of sheep generated with ILO-14 primer.
M – Molecular size marker (λ DNA/EcoRI-HindIII Double digest)
A– Marwari, B– Mandya, C– Madras red, D –Muzzafarnagri

ranging from 0.5412 to 0.7612 among different breed combinations. By this primer the Muzaffarnagari and Madras red breeds showed very less genetic closeness. However, the average BSF values for all the primers for this breed combination reveals BSF value of 0.8494. This indicates that the ILO-17 primer can be used for further polymorphic studies in sheep breeds.

Table 3. Average within breed band sharing frequencies (BSF) in four breed using each of the six primers

Primer	Marwari	Mandya	Madras Red	Muzaffarnagari
OPM-2	0.9267	0.8848	0.8491	0.9314
OPM-5	1.0000	1.0000	1.0000	0.9283
K-4	0.9377	0.9480	0.9710	1.0000
ILO-14	1.0000	0.9487	0.9286	1.0000
ILO-15	0.9250	1.000	1.000	0.9259
ILO-17	0.8820	0.7573	0.7145	0.6585

While screenign the Mandya and Madras red breeds with OPM-5 primer the BSF value was 1. with reference to this primer these two breeds seems to be genetically similar. In addition, the average BSF was the highest for this breed combination for all the primers (0.852).

Mean Average Percentage Difference (MAPD)

Interbreed dissimilarities were calculated on RAPD fingerprints obtained with six primers. In each case 16 interbreed pairwise comparisons of individual animals were made. The data in table 4 show that this value varied for each primer, and for each paired breed comparison. MAPDvalues estimated for different breed combination taken in this study ranges from 4.90 to 9.44%. However, higher MAPD values (10.60% to 50%) were reported for different Zebu cattle breeds varied at different countries by Gwakisa *et al.* (1993).

But forthe present investigation the animals were taken from the same country. This may be the reason for the lower MAPD values and higher geneticresemblence among presently investigated animals.

Table 4. Band sharing frequencies (BSF) average percentage differences (APD) and mean average percentage differences (MAPD) between breeds

	Marwari - Mandya		Marwari - Madras Red		Marwari - Muzarfarnagari		Mandya - Madras Red		Mandya- Muzaffemagari		Madras Red - Muzaffarnangari	
Primers	BSF	APD	BST	APD	BSF	APD	BSF	APD	BSF	APD	BSF	APD
OMP-2	0.9048	3.1421	0.8669	7.6160	0.8717	6.4791	0.8581	8.0718	0.8558	5.5573	0.8195	12.2196
OPM-5	0.6667	0.0000	0.6667	0.0000	0.7299	11.3551	1.0000	0.0000	0.9258	11.3551	0.9255	11.3551
K-4	0.9301	3.1712	0.8921	3.1721	0.9011	3.1703	0.8799	2.1739	0.8538	1.0869	0.8893	2.1739
ILO-14	0.8286	10.4764	0.8304	10.7143	0.8571	14.2857	0.9450	5.5036	0.9615	3.8460	0.9643	3.5714
ILO-15	0.8658	8.0913	0.7625	3.3333	0.7316	9.1912	0.8235	6.0692	0.8333	1.4706	0.9412	7.5368
ILO-17	0.7612	11.2766	0.6383	12.4705	0.5417	12.1880	0.6055	9.1019	0.5693	6.0905	0.5562	10.3858
Average B.S.F.	0.8262		0.7760		0.7722		0.852		0.833		0.8494	
MAPD		6.0263 ±1.8657		6.2177 ±1.9793		9.4449 ±1.6617		5.1534 ±1.4201		4.9011 ±1.5376		7.8738 ±1.7169

Genetic Distance

The genetic distance among four sheep breeds taken in this study were estimated using band sharing frequencies, within as well as between breeds as described by Smith *et al.* (1996). The estimates of genetic distance based on all the six primers have been tabulated in table 5.

Table 5. Genetic distance (D) estimates, four sheep breeds

Broods	Marwari	Mandya	Madras Red	Muzaffarnagari
Marwari	–	0.1263	0.1825	0.1906
Mandya	–	–	0.0820	0.1003
Madras Red	–	–	–	0.0761
Muzaffarnagari	–	–	–	–

The genetic distance estimates among four breeds ranges from 0.0761 (Madras red and Muzaffarnagari) to 0.1906 (Marwari and Muzaffarnagari). The estimate reveal that Madras red and Muzaffarnagari breeds were the most closely related breeds (D = 0.0761) than any other. Marwari breed was found to be most distant from rest of the three breeds. However, Mandya and Madras red were found to be more closely related (D = 0.0820). There were no report on RAPD polymorphism study on these four sheep breeds in the available literature, hence the findings could not be compared. However, this study has generated the basic data in these sheep and these observations may be treated as the base line information on RAPD polymorphism, which could be further explained in subsquent studies.

5.2 Cytogenetic work

Metaphase chromosome preparations were made by short term lymphocyte culture for the blood samples of 10-12 animals of both the sexes of Marwari, Mandya, Madras red and Muzaffarnagari, breeds of sheep. Atleast 50 complete metaphase plates were examined from each animal. While screening the somatic chromosome complement, apparently well spread and

unbroken metaphase plates were chosen for study. Representative karyotypes were prepared for each breed.

5.2.1 Chromosomes of breeds of sheep

a. Description of the karyotype

The diploid number of chromosomes revealed at somatic metaphase plates in all the breeds of sheep studied was 54 which is similar to other reported breeds of sheep viz. Muzaffarnagari (Benjamin and Bhat, 1978), malpura (Gupta and Gupta, 1991), Nali and munjal (Bhatia and Shankar, 1989, 1994). In contrast, wild sheep population which comprised snow sheep (2n=52), fouflon sheep (2n=54), arkhar/argali sheep (2n=56) and urial sheep (2n=58) exhibited chromosome polymorphism (Bunch, 1978). Nadler *et al.* (1971, 73) hypothesized that chromosome reduction occurred during the process of domestication. The male chromosome compliment was 54 XY (Fig. 8) while that of female was 54 XX (Fig 10).

The karyotype comprised of 3 pairs of submeta/metacentric autosomes, 23 pairs of acrocentric autosomes and a pair of sex chromosomes. The X chromosome was largest acrocentric chromosome supporting the observations of Bunch and Foote (1976). The Y chromosome appeared as very small submeta/metacentric (Fig. 9 and 11). The morphological attributes of autosomes and sex chromosomes were similar to those reported earlier in Malpura (Gupta and Gupta, 1991) and Nali (Bhatia and Shankar, 1989, 1994).

The conventional karyotypes were prepared for all four breeds to see whether any apparent morphological changes exist between the breeds. No morphological changes could be observed in the karyotypes of all the breeds. Therefore, representative karyotype of sheep (Marwari) (Fig. 9 and 11) is presented. The conventional karyotype was uniform in various breeds as reported by Gupta and Gupta. 1991).

b. Chromosomal banding

Howell and Blacks (1980) colloidal silver method gave highly reproducible results of NOR banding. The NORs were found on the telomers of chromosome pairs of 1, 2, 3, 4 and 25 (Fig.

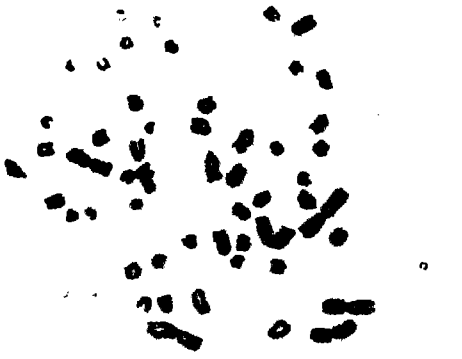


Fig 8 A metaphase plate of Marwari male

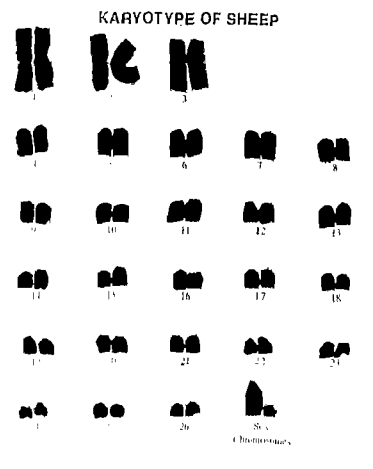


Fig. 9 A karyotype of Marwari male

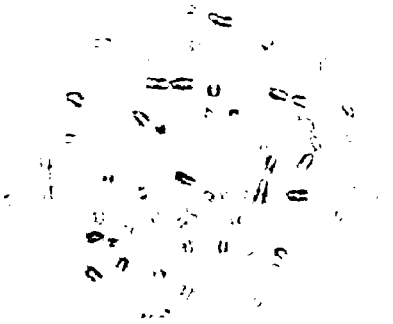


Fig. 10 A metaphase of Marwari female

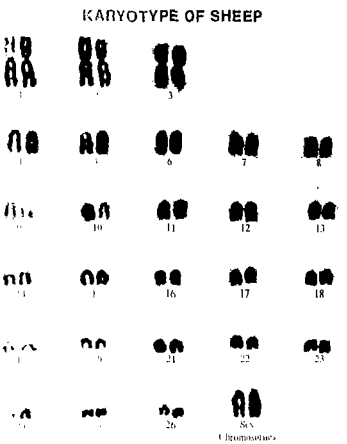


Fig. 11 A karyotype of Marwari female

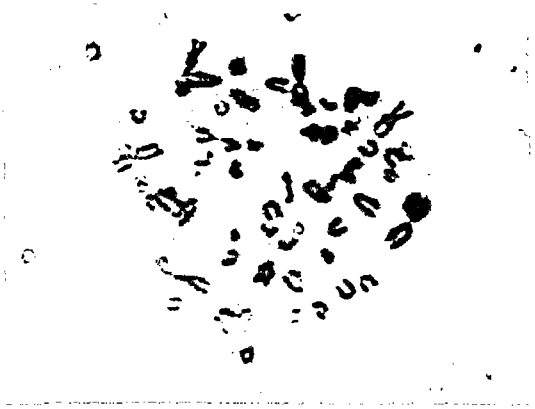


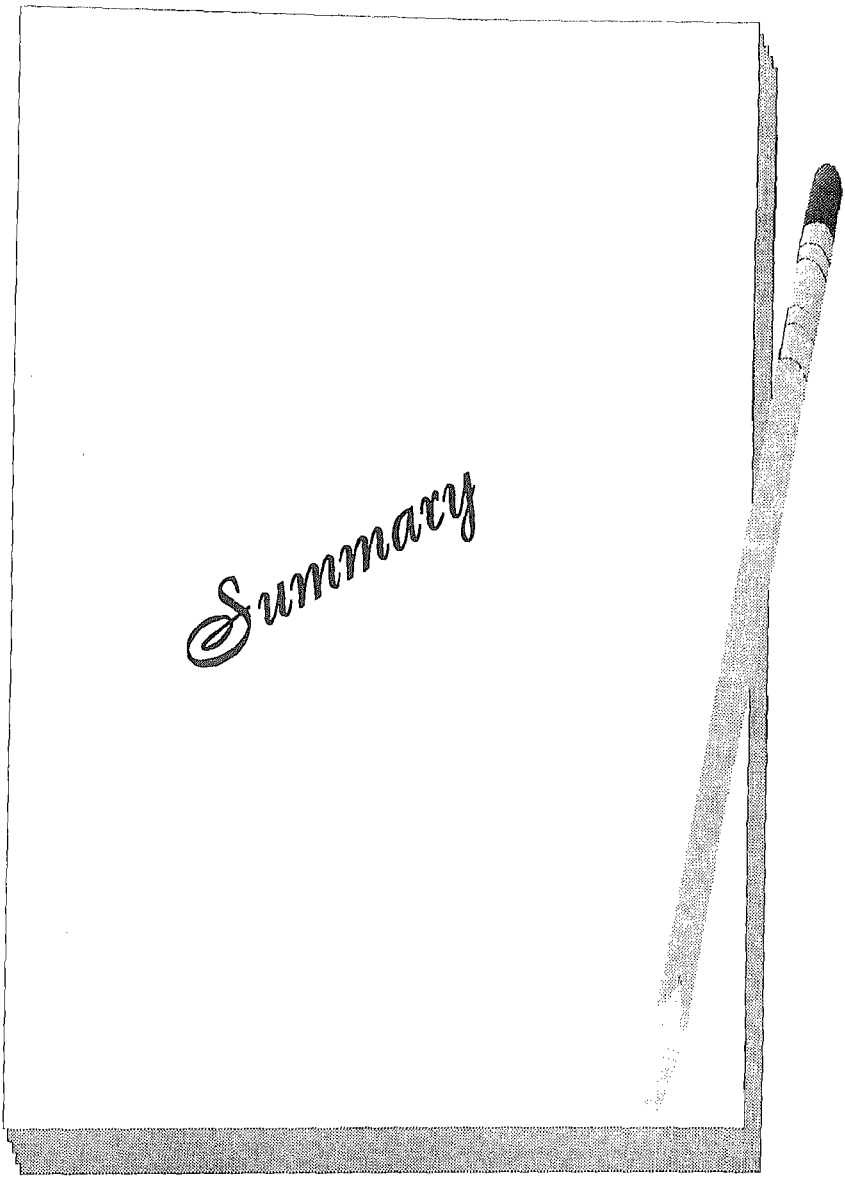
Fig. 12 A metaphase plate of NOR banding of Marwari female

Fig. 13 A metaphase plate of G banding of Marwari female

12 and 13). The per cent of NOR positive cells found in various breeds of sheep were as : Muzaffarnagari, 53.70; Marwari, 58, Madras red, 57.70 and Mandya 60.2.

The NOR pattern reported in these breeds of sheep were almost same as reported by Bhatia and Shankar (1992-93).

C-banding revealed distint darkly stained centrochromatin in 23 pairs of acrocentric autosomes and very light band in three submeta/metacentric autosomes. The X and Y chromosomes were C-band negative. The G-banding revealed specific dark and light bands in the metaphase chromsomes. The C and G-band patterns were grossly similar in all the breeds under study. Bhatia and Shankar (1992-93) also reported all most same C and G band patterns in munjal breed of sheep. Only preliminary studies on C and G bandings were conducted.


Summary

SUMMARY

Livestock raising in India is as ancient as Aryan civilization. Among the livestock species sheep plays a major role in both meat and wool production. Report on molecular and cytogenetic characterization of sheep breeds is hardly available in literature, hence the present study was planned for RAPD-PCR analysis and chromosomal profile of sheep breeds.

The investigation was carried out on 4 different sheep breeds (Marwari, Mandya, Madras red, Muzaffarnagari) with objectives to develope RAPD patterns to identify breed specific RAPD markers if any, to determine band frequencies of various RAPD fingerprints, to evaluate similarities and dissimilarities within and between sheep breeds using RAPD polymorphism, to evaluate genetic distance among various sheep breeds and to study chromosomal profile and banding pattern of these animals.

Genomic DNA was isolated from 20ml of venous blood by phenol extraction method concentration and purity was checked by taking OD values at 260 and 280 nm the concentration was calculated by formula, concentration (μg/ml) = OD_{260} x 50 x dilution factor. In general the concentration of DNA ranged from 260 to 1200 μg/ml and OD ratio (260/280) ranged from 1.7 to 2. Quality of DNA was checked by running DNA samples in 0.8% agarose gel and only intact (without smearing/shearing) samples were considered for further analysis.

A total of 6 different primers (OPM-2, OPM-5, K-4, ILO-14, ILO-15, ILO-17) having GC content of 60 to 90% were used for present study. Optimization was carried out for all the primers by varying different components of PCR. The concentration of Taq DNA pol. and Mg^{2+} ions were found to be most crucial factors for reproducibility of banding pattern. Primers OPM-2, OPM-5,

ILO-15, ILO-17 were amplified with 0.75U Taq DNA pol. and 1.5 ml $MgCl_2$, whereas, for other two primers, K-4 and ILO-14 0.75U Taq DNA pol. and 2mM $MgCl_2$ were found to be optimum. Mixed DNA samples of various breeds were also screened by using ILO-17, OPM-2, OPM-5 and K-4 primers for generating breed specific RAPD fingerprints.

The average number of scorable bands with primers OPM-2, OPM-5, K-4, ILO-14, ILO-15 and ILO-17 were found to be present in the range of 6 to 17 with molecular sizes of amplified products ranged from 340 to 2009 bp in different breeds. More number of polymorphic amplified products were revealed by ILO-17 K-4 and OPM-2 where as in other primers (ILO-14, ILO-15 and OPM-2), very few number of polymorphic amplified products were found.

Within breed BSF were found to be higher than between breed BSF indicating the genetic closeness within the breeds studied. Between breed BSF was ranging from 0.7722 to 0.8520. The between breed BSF was more between Mandya and Madras red, less between Marwari and Muzaffarnagari.

The mean average percentage difference (MAPD) for different breed combination was also estimated and it was found in the range between 4.90 to 9.44%. This shows higher resemblence between the sheep breeds taken for this study.

The genetic distance estimates among 6 breeds ranged from 0.0761 (Madras red and Muzaffarnagari) to 0.1906 (Marwari and Muzaffarnagari). The estimates revealed that the Madras red and Muzaffarnagari breeds are more closely related (D = 0.0761).

Cytogenetic studies were conducted on 4 breeds of sheep viz. Marwari, Mandya, Madras red and Muzaffarnagari. The diploid chromosome number in all the breeds was found to be 54 with 54 XY for male and 54 XX for female. The karyotype comprised of 3 pairs of submeta/metacentric autosomes, 23 pairs of acrocentric autosomes and a pair of sex chromosomes. The X chromosome was largest acrocentric while Y was small submetacentric. The NORs were found on the telomeric ends of chromosome pairs of 1, 2, 3, 4 and 5. C-bands were found on 23 pairs of acrocentric autosomes and very light bands in three meta/submetacentric autosomes. The banding revealed specific dark and light bands in metaphase chromosomes.

MINI ABSTRACT

The Marwari, Mandya, Madras red and Muzaffarnagari sheep breeds were analysed by RAPD-PCR technique and chromosomal study. The genomic DNA was isolated from 20ml of venous blood and purified by phenol chloroform method. PCR analysis was conducted using OPM-2, OPM-5, K-4, ILO-14, KLO-15 and ILO-17 primers band frequency (BF), band sharing frequency (BSF), genetic distance and mean average percentage differences were calculated from the data generated by RAPD analysis. The mean number of scorable bands ranged from 6 to 17 and their sizes ranged from 340 to 2009 bp. The primer ILO-17 yielded typical polymorphic pattern with all the breeds.

The mean average percentage difference (MAPD) for different breed combination was ranged between 4.90 to 9.44%. Diploid chromosome number in all the breeds of sheep studied was found to be 54 with 54 XY for male and 54 XX for female. C, G and NOR banding patterns were also approximately same in all the breeds.

लघु सारांश

मारवाड़ी, मान्ड्या, मद्रास रेड व मुजफ्फर नगरी, नस्लों की भेड़ों के लगभग दस–दस जानवरों को उनके आर. ए. पी. डी. चिन्हकों व गुणसूत्रों के अध्ययन के लिए चुना गया । ताजे व हिमीकृत रक्त प्रतिदर्शों से दो विभिन्न विधियों द्वारा जीनोमिक डी. एन. ए. निकाला गया तथा फीनोल क्लोरोफार्म विधि द्वारा परिष्कृत किया गया । पी. सी. आर. विश्लेशण में अनियमित प्राइमर (ओ. पी. एम–2, ओ. पी. एम–5, के–4, आई. एल. ओ.–14, 15 एवं 17) जिसमें 'जी' + 'सी' न्यूक्लियोटाइड की मात्रा 60–90 प्रतिशत थी, का प्रयोग किया गया ।

आर. ए. पी. डी. तकनीक द्वारा प्राप्त किये गये आकड़ों से बैंड आवृति (बी. एफ.) बैण्ड शेयरिंग आवृति (बी. सी. एफ.) औसत प्रतिशत अन्तर (ए. पी. डी.) अनुवंशिक समानता इन्डैक्स (आई) और अनुवंशिक दूरी (डी) इत्यादि की गणना की गई । आंकलन करने योग्य बैण्डस का औसत 6 से 17 तथा उनका आकार 0.34 से 2.009 किलोबेस पाया गया । आई. एल. ओ.–17 प्रायमर द्वारा विभिन्नतापूर्ण प्रारूप पाये गये । एम. ए. पी. डी. का रेंज 4.90 से 9.44 प्रतिशत था ।

सभी प्रजातियों में गुणसूत्रों का टू. एन. नम्बर 54 पाया गया तथा गुणासूत्रों का आकार भी एस जैसा ही था । सी. जी. व एन. ओ. आर. बैंडिंग भी सभी प्रजतियों में मोटे तौर पर एक जैसी ही पायी गई ।

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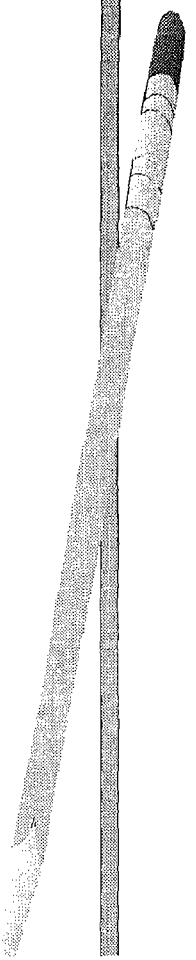

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□□□.....

ANNEXURE

Buffers and Solutions

PBS : Phosphate buffered saline (pH 7.2-7.4)

NaCl	-	8 gm
KCl	-	2 gm
Na_2HPo_4	-	1.44 gm
KH_2Po_4	-	0.24 gm

Dissolve in 800 ml of Auto.D.W., adjust pH to 7.4 with HCl and make volume to 1000 ml. Sterilise by Autoclave. Store at 4°C

Lysis Buffer : (pH 8.0)

2M Tris HCl (pH 8.0)	-	2.5 ml
0.4M EDTA (pH 8.0)	-	6.25 ml
2M NaCl	-	5 ml

Auto.D.W. make upto 100 ml. Store at 4°C

10% SLS : Sodium lauryl sulphate

Sodium lauryl sulphate	-	10 gm

Auto.D.W.		100 ml

Store at room temperature. Heat at 60°C before use

5X TBE

Tris base	-	54 gm
Boric acid	-	27.5 gm
0.4M EDTA (pH 8.0)	-	25 ml

Auto.D.W. upto 1000 ml

Sterilise by autoclave. Store at room temperature

50X TAE

Tris base	-	24.2 gm
Glacial acetic acid	-	5.71 ml
0.4M EDTA (pH 8.0)	-	25 ml

Auto.D.W. upto 100 ml

Sterilise by autoclave. Store at room temperature

6X Gel Loading Dye (Sambrook *et al.*, 1989)

Bromophenol blue	-	0.25 %
Sucrose	-	40 % (W/V)

Store at 4°C

20,000X Ethidium Bromide : 10 mg/ml

Ethidium bromide	-	10 mg
Auto.D.W.	-	1 ml

Wrap in a aluminium foil (Photosensitive)

Store in a dark place at room temperature.

Tris-Saturated Phenol preperation

- ☐ Melt phenol at 68°C by keeping in waterbath
- ☐ Measure the required volume, Add 8-hydroxy quinoline to a final concentation of 0.1% (It is an antioxident, gives yellow colour to phenol)
- ☐ Extract phenol several times with equal volume of 1M Tris(pH8.0)
- ☐ *Then with 0.1M Tris, until the pH of the aqueous phase is more than 7.6*
- ☐ Add 0.2% B-mercaptoethanol
- ☐ Mix thoroughly and store in amber colored bottle at 4°C

Chloroform-Isoamyl alcohol preperation (24:1)

Chloroform	-	24 ml
Isoamyl alcohol	-	1 ml

Mix thoroughly and store at 4°C

Phenol-Chloroform-Isoamyl alcohol preperation (25:24:1)

Tris saturated phenol	-	25 ml
Chloroform-Isoamyl alcohol	-	25 ml

Mix thoroughly and store at 4°C

1X TE (Tris:EDTA :: 10:1)

2M Tris	-	125 ul
0.4M EDTA	-	62.5 ul

Auto.D.W. upto 25 ml

2M Tris

Tris	-	242.2 gm

Dissolve in 800 ml Auto.D.W., adjust pH to 8.0 by HCl, make volume to 1000 ml. Sterilise by autoclave and store at 4°C

0.4M EDTA (pH 8.0)

EDTA - 14.88 gm

Dissolve in 50 ml Auto.D.W. by keeping it on magnetic stirrer for 1-2 hrs, then adjust the pH to 8.0 by HCl. Make volume to 1000 ml, sterilise by autoclave, store at 4°C.

CHEMICALS

Agarose

Boric acid

Bromophenol blue

Chloroform

EDTA

Ether

Ethidium bromide

Glacial acetic acid

Heparine

HCl

8-hydroxy quinoline

Isoamyl alcohol

Mercaptoethanol

Phenol

Sodium chloride

Sodium lauryl sulphate

Sucrose

Tris

Enzymes and Biologicals

Taq DNA polymerase enzyme

Taq DNA polymerase - 3 units/µl

Store at -20°C

10X Taq buffer

Tris-HCl (pH 8.8) - 100 mM

KCl - 500 mM

$MgCl_2$ - 15 mM

Triton X-100 - 1 %

Store at -20°C

Proteinase K : (5 mg/ml)

Proteinase K - 5 mg

Auto.D.W. - 1 ml

Store at -20°C

Primers :

Stock : 100 ug dissolved in 100 µl

Working : Add 4 µl from stock in 198 µl of Auto.D.W.

From this use 4 µl (40 ng) per PCR mixture.

dNTP solution (pH 7.0)

dATP	-	10 mM
dCTP	-	10 mM
dGTP	-	10 mM
dTTP	-	10 mM

Store at -20°C

Equipments

Anamed digital balance

Autoclave

Digital pH meter

High speed refrigerated centifuge

Hot air oven

Incubator

Magnetic stirrer

Microcentrifuge

Microwave oven

Shaking waterbath

UV-VIS-Spectrophotometry

Submarine gel electrophorosis

Table top centrifuge R/8C

Thermocycler PTC-200 DNA Engine

UV transilluminator

Vortexer

Glasswares and Plasticwares.

Routine glasswares like flask, measuring cylinder, beakers, test tubes, pippetts, etc.. were used.

Plastic wares like blue and yellow tips, adjustable micropippettes Tarson P 10 , P 20, P100, P 200 and P1000, 1.5 ml eppendroff tubes, thin walled 0.5 ml PCR tubes (Axygen, USA), rubber bulb, latex gloves , etc.. were used.

Molecular Weight Marker fragment Sizes (bp)

λ Hind III/Eco RI digest - 13 fragments

21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983831, 564 and 125

Heating the digest at 65°C for 5 min. and rapid cooling on ice ensures clear visualisation of 3530 bp fragment.

□□□.....

CURRICULUM VITAE

The author Dr. K. Ganesh Kumar, S/o Mr. S. Karuppaswamy was born on 20th January, 1973 in Tuticorin district, Tamilnadu. He passed his High School and Higher Secondary from State Board with Distinction, in the year 1988 and 1990, respectively. He completed his B.V.Sc. Degree from Madras Veterinary College (Tamilnadu Veterinary and Animal Sciences University). During his B.V.Sc. curriculum, he had participated in many sports and games activities of University. Then he had joined for post graduation at IVRI, Izatnagar (U.P.) with specialization in Animal Genetics and Breeding by competing in an All India Competitive Enterance Examination held by ICAR. He has been a recipient of ICAR-JRF and obtained an OGPA of 8.085 out of 10 during his master programme.

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