MOLECULAR CHARACTERIZATION OF SELECTED NATIVE SHEEP USING RAPD MARKERS

A Thesis Submitted

By

Shirin Pervin

Examination Roll No. MS 090710

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Dedicated to

My Beloved Parents

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ABSTRACT

Sheep is one of the important domestic animals in Bangladesh. Genetic improvement of this animal is of economic important, especially in reproductive performance and quality of meat, wool and milk. Genetic similarity and polymorphisms among three areas of sheep population was studied using Random Amplified Polymorphic DNA (RAPD) technique. The research work was conducted at Animal Cell Culture and Molecular Biotechnology Laboratory, Khulna University, Bangladesh in order to evaluate genetic variation among the sheep of different regions of Bangladesh. Twenty blood samples were collected from Bangladesh Livestock Research Institute Sheep Farm, Savar Dhaka, The quality and quantity of DNA were determined by agarose gel electrophoresis method and using spectrophometer. RAPD-PCR was employed to assess the genetic variability among the sheep population. PCR reaction were carried out using 10 random oligonucleotide primers among those three did produce distinctive reproducible band. Total 20 µl reaction volume was used for PCR reaction. Two microlitre DNA sample (2 ng per µl), 2 µl of 10x reaction buffer, 2 µl of Tag DNA Polymerase (1 unit per µl), 1 µl of oligonucliotide primer (100 pico mol per µl) and 11 µl of distilled water were use to make the reaction volume. RAPD patterns with a level of polymorphism (89.63%) was detected among sheep population. Genetic distance and Dendogram were measured using the software COMPONENT 2.0. Out of total 19 distinct bands, 17 were polymorphic. Tangail (LO95) shows comparatively higher genetic distance (1) with Naogaon (IB05), (IB16), (IB03) (IB10). Similarly, Naogaon (IB16) shows genetic distance (1) with Tangail (IJ02), Noakhali (OC23). Moreover, Noakhali (IC02) shows highest genetic dissimilarity with Naogoan (IB05).The results suggest that RAPD-PCR can be used successfully for analyzing genetic variation and similarity among different region of sheep. Although some phenotypic differences exist among some sheep that are genetically similar, this kind of variation may be from environmental effect.

DECLARATION

This thesis reports the original research work of author, except as otherwise stated. The material presented has not been submitted either in whole or in part for a degree from this or any other university. The findings of the research has not been/will not be published anywhere without the concurrence of the concerned supervisor/co-supervisor.

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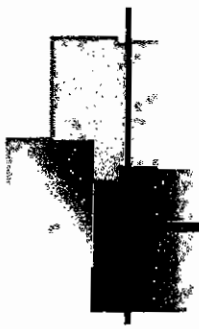
LIST OF ABBREVIARTION

%	:	Percent
µl	:	Micro liter
µM	:	Micro molar
^{0}C	:	Degree Celsius
A260	:	Spectrophotometer Absorbance Reading at 260 nm
A280	:	Spectrophotometer Absorbance Reading at 280 nm
AFLP	:	Amplified Fragment Length Polymorphism
Contd.	:	Continued
d H_2O	:	Distilled water
DNA	:	Deoxyribonucleic acid
dNTPS	:	Deoxy nucleotide triphosphates
DW	:	Distilled water
e.g.	:	Exempli gratia = for example
EDTA	:	Ethylene Diaminetetra Acetic Acid
et al.	:	Et alia = and others
EtBr	:	Ethidium Bromide
etc.	:	et. cet. Era = and the others
gm	:	Gram
HCl	:	Hydrochloric Acid
M	:	Molar
mg	:	Milligram
mg/l	:	Milligram perliter
ml.	:	Milliliter
mM	:	Milli Mole
N	:	Normal Solution
Na_2EDTA	:	Sodium salt of ferric ethylene diamine tetraacetate
NaCl	:	Sodium Chloride
NaOH	:	Sodium Hydroxide
OD	:	Optical Density



Chapter One

Introduction

INTRODUCTION

The domestic sheep (*Ovis aries*), the most common species of the sheep genus (*Ovis*), is a woolly ruminant quadruped which probably descends from the wild mouflon of South Asia and Southwest Asia. A wide symbology relates to sheep in ancient art, traditions and culture (Hasmat , 1968). Judaism uses many sheep references including the Passover lamb. Christianity uses sheep-related images, such as: Christ as the good shepherd; or as the sacrificed Lamb of God; the bishop's *Pastoral*; the lion lying down with the lamb. Greek Easter celebrations traditionally feature a meal of Paschal lamb. Sheep also have considerable importance in Arab culture; Eid ul-Ajha is a major annual festival in Islam in which a sheep is sacrificed (Gupta *et al.*, 1976).

The raising of sheep for wool, milk and meat became a major industry in Bangladesh and remains significant. However, particularly in some of the world poorest countries, they have additional important roles, including the provision of pelts, fertilizer and fuel providing a four-legged from financial investment point of view. They are also using resource unsuitable for others forms of agriculture. As a result, sheep and sheep shearing have become an important part of the folklore and cultural tradition of Bangladesh (FAO, 1971).

Sheep is one of the most important animals in our animal agriculture which provide meat and wool. According to 1983-84 Agriculture and Livestock census, there were 66,7189 thousand sheep in Bangladesh, which was increased to 169,0011 thousand in 1996. During these 12 years sheep population increased 2.53 times, with annual growth rate 5%. There are about 3.4 million of sheep per farm holding. The total sheep population of Bangladesh is 1.69 million (BBS, 2001).

Breeders of dual-purpose wool class sheep concentrate on fast growth, multiple births, ease of lambing and hardiness. An easy-care sheep is the Coopworth that has long wool and good lamb meat production qualities. Another dual-use breed is the Corriedale. Sometimes sheep are used for both purposes equally and cross-breeding is practiced to maximize both outputs. For example, Merino ewes providing wool may be crossed with Suffolk rams to produce lambs which are robust and suitable for the meat market (Faulkner, 1962).

Hair class sheep are the original class of sheep in the world, developed for meat and leather. They are prolific and highly resistant to disease and parasites. Hair class sheep are becoming more popular for their no-shear aspects. Raising sheep was and is important to farmers in many economies, given that sheep can give milk (and its derivative products, such as cheese), wool, sheep skin (used for making clothes, footwear, rugs, and other coverings) and meat (Bond, 1975). In the 21st century, sheep retain considerable importance in the economies of several countries. After China, the largest producers of sheep products are in the southern hemisphere. In the 21st century, in some situations, sheep can provide a return on investment of up to 400% of their cost annually including reproduction gains (Faulkner, 1962).

The Food and Agriculture Organization of the United Nations (FAO, 1971) estimates that at least one breed of traditional livestock becomes extinct every week and more than 30% of European livestock are currently estimated to be endangered. One of the major causes of extinction is the preference of one particular breed at one time and its spread at the expense of others. Breeds are also lost by an indiscriminate cross breeding. With every extinction event, it is most likely that unique characteristics (such as adaptation to local environmental conditions) are lost forever. Native Bengal sheep breeds might be harboring many more unique characteristics such as resistance to diseases and harsh environmental conditions, which can be employed in the future to improve the sheep breeds. Therefore, Bengal sheep may have higher priority in conservation than other sheep breeds.

The classification based on historical, anthropological and morphological evidence is not satisfactory for the purpose of conservation and utilization. Breed characterization requires knowledge of genetic variation that can be effectively measured within and between populations (Hetzel and Drinkwater, 1992). Genetic markers may provide useful information at different levels: population structure, levels of gene of gene flow, phylogenetic relationships, patterns of historical biogeography and the analysis of parentage and relatedness (Feral, 2002).

During the last few years, the great strides of molecular biology virtually gave access to the entire genome, but their complexity and high cost limited their use to precisely targeted projects in population biology. However, the polymerase chain reaction (PCR)

which induced a methodological revolution (Mulis and Faloona, 1987; Sakai *et al.*, 1988) has been applied for genetic variations studies.

Sheep-breeding work for fine wool, involving exotic fine-wool breeds and indigenous breeds (Gaddi, Nali, Chokla, Patanwadi, Nilgiri and Bonpala), yielded improvement in greasy wool production and fleece quality in the half-breds over the native breeds involved in crosses, but the improvement in greasy wool production was minimal where the native breed was already producing relatively high quantities of fleece (e.g. Nali and Chokla). Similarly, the improvement in fleece quality was minimal in breeds with already reasonably fine fleece, such as Nilgiri (Dass and Rajagopalan,1956).

DNA isolation methods involve the removal of the cell nuclear membranes, the separation of the DNA from all other cell components, and the protection of the DNA during the procedure. A variety of problems may be encountered during the isolation and purification of high molecular weight DNA from animal species. These include: (i) Partial or total DNA degradation due to the presence of endogenous nucleases. (ii) Co-isolation of highly viscous polysaccharides, which render the handling of samples difficult (Weising *et al.*, 1995).

The genetic diversity can be measured by various markers like polymorphisms in the gene product, such as, enzymes, blood groups, serum proteins or antigens; however, these markers do not reveal much polymorphism to differentiate the breeds/species to that extent. Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique has been used for estimation of the genetic variability among the breeds/species, but it has low reproducibility (Williams *et al*, 1990). RAPD markers are the randomly amplified target regions of less functional part of the genome that do not strongly respond to selection on the phenotypic level. Such amplified regions might accumulate more mutations, thereby offering a wider potential in assessing the intra/interbred genetic differentiations. The RAPD-PCR has been used to estimate the genetic variability among livestock species; sheep breeds (Erceg *et al.*,1999). Polymorphism at the DNA level is frequently used as genetic markers for herd studies in domestic animals. Genetic markers and the variation detected at these loci reflect the level of variation in the entire genome (Okumus and Mercan, 2007). The random amplified polymorphic DNA (RAPD) assay which uses short oligonucleotide primers of arbitrary sequence to amplify genomic DNA by PCR enables an approach for identifying genetic markers (Cushwa *el al.*, 1996). RAPD was utilized in breeds genetic studies in most

REVIEW OF LITERATURE

Evidence for the domestication of sheep dates around 7000 to 9000 BC in Iraq (Broad *et al.*, 1998). Domestic sheep are descended from the mouflon that is found from the mountains of Turkey to southern Iran. It has been found by DNA analysis to be one of two ancestors of domestic sheep (Krebs *et al.*, 2003).

The urial (*O. vignei*) is found from northeastern Iran to northwestern India. It has a higher number of chromosomes (58) than domestic sheep (54) which makes it an unlikely ancestor of the latter, but it interbreeds with the mouflon. The argali sheep (*O. ammon*) of inner Asia (Tibet, Himalayas, Altay Mountains, Tien-Shan and Pamir) has 56 chromosomes and the Siberian snow sheep (*Ovis nivicola*) has 52 chromosomes (Hiendleder *et al.*, 2002).

2.1. Bengal sheep

2.1.1 Origin and distribution

The sheep in Bangladesh are supposed to have been developed through natural selection since time immemorial (Talukder *et al.*, 2003). Indigenous sheep are sparsely distributed throughout the country. Higher concentration of sheep is found in the Barind area, Padma and Jamuna river basin area and Coastal area (Mukherjee, 2000). The ability to thrive in harsh environment even in the marshy char lands makes the indigenous sheep most suitable species of livestock in the coastal region of Bangladesh. They use to live on natural grass with almost no concentrate supplementation and are being raised by the landless and marginal farmers and the traders in the char lands. They are also well adapted in the semi arid areas. Sheep are most tolerant to disease in the humid and sub-humid tropics. They utilize low quality feedstuff, roughage pasture or farm wastes more efficiently than goats. Sheep produces lean meat as compared to cattle and goat. But the Bengal sheep has low reproductive rate as well as low milking efficiency. That's why they produce small number of lambs per annum; most of the lamb dies due to inappropriate quantity of their mother milk.

2.2 Categorization of sheep breeds

Probably the most useful way to categorize sheep breeds is by their primary purpose: meat, wool, or dairy. While most sheep breeds are dual-purpose (i.e. they produce both meat and wool) and some are even triple-purpose (dairy, meat, and wool), most sheep breeds excel in either the production of meat or wool, or dairy, seldom two or all three (Ikeda *et al.*, 1995).

Sheep breeds are often categorized as to whether they are more suitable as a ram or ewe in the breeding program. Ram or "sire" breeds should excel in growth and carcass (meat) characteristics whereas ewe or "dam" breeds should excel in fitness (e.g. longevity, parasite resistance) and reproductive traits (early puberty, prolificacy, milk production) (Hiendleder *et al.*, 2002).

Table 1: Classification of Sheep Breeds on the basis of utility

Meat (wool)	Meat (hair)	Wool	Dual-Purpose	Rare/Heritage
California Red	American Blackbelly	Variegated Mutant	Bluefaced Leicester	Black Welsh Mountain
Charollais	Barbado	Debouillet	Leicester Longwool	Cotswold
Dorset	Damara	Merino	Coopworth	Karakul
Hampshire	Dorper	Panama	Finnsheep	Leicester Longwool
Cheviot	Katahdin	Scottish Blackface	Polypay	Lincoln
Romanov	White/Dorpcroix		Romney	Navajo Churro
Texel				Santa Cruz

Source: (Patnayak and Mishra, 1979)

Table 2: Classification of Sheep Breeds by on the basis of fiber				
Fine	Medium	Long	Carpet	Hair (shedding)
Booroola Merino Debouillet Merino Rambouillet	Charollais Cheviot (Dorset Friesian Finnsheep	Border Leicester Coopworth Longwool Perendale	Cotswold Karakul Lincoln Scottish Blackface	Barbado Black Hawaiian Corsican Dorper Katahdin

Source: (Patnayak and Mishra, 1979)

2.3. Morphological characteristics

The indigenous sheep are small having an average live weight of 15 to 25 kg. Their body color is brown, gray, gray with black or white patches, face, ear and the feet mostly light black. Males have curly horns but females are polled. Tail is short and thin. Wool is coarse with high modulation (Mukherjee, 2000).

i) Size	Adult males	Adult females
Body weight (kg)	19.48 ± 0.13 *(21)*	19.76 ± 0.12
Body length (cm)	52.70 ± 0.23 *(21)*	52.31 ± 0.16
Height at withers (cm)	54.51 ± 0.41 *(21)*	54.46 ± 0.78
Chest girth (cm)	71.12 ± 0.61 *(21)*	75.03 ± 0.61

Source: (Patnayak and Mishra, 1979)

However, livestock diversity is shrinking rapidly. Among the domesticated populations, it is estimated that 1 to 2 breeds are lost every week.

The impact of these losses on the global or the local diversity remains undocumented. While it is already too late for many breeds in Europe, the situation is also particularly worrying in the developing world where rapid changes in production systems are leading to the replacement of breeds or best crossbreeding. It is an urgent need to document the diversity of our livestock genetic resources and to design strategies for their sustainable conservation.

The task is enormous. It has prompted the Food and Agricultural Organization (FAO) and other international organizations to develop domestic animal diversity information systems and databases (DAGRIS and DAD-IS 2.0). More recently, FAO has initiated a major country-driven documentation exercise, the 'State of the World's Animal of Animal Genetic Resources' (AnGR). Molecular markers provide different types of information such as individual genetic identification, parentage analysis, population diversity estimations, differentiation of populations, and calculation of genetic distances, genetic relationships, population genetic admixture estimation and inbreeding estimation.

2.5. Genetic Characterization of Sheep

The initial DNA extracts often contain a large amount of RNA, proteins and polysaccharides which may interfere with the extracted DNA and difficult to separate. Most proteins are removed by denaturation and precipitation from the extract using chloroform and/or phenol. RNAs on the other hand are normally removed by treatment of the extract with heat-treated RNase A. Polysaccharide-like contaminants are, however, more difficult to remove. They can inhibit the activity of certain DNA modifying enzymes and may also interfere in the quantification of nucleic acids by spectrophotometric methods (Wilkie *et al.*, 1993).

Typically, DNA, once extracted and purified, is stable when stored in TE, a Tris (hydroxymethylaminomethane) buffer at pH 8.0 containing 1 mM EDTA as a Mg^{2+} chelator (Mg^{2+} ions are required by DNAses-ubiquitous enzymes which degrade DNA). Storage of

DNA is usually at 4°C for short periods of time, or frozen when weeks, months, or years are involved (Clark and Christopher, 2000).

Pasha (2000) stated that after incubation at -20°C addition of cold ethanol (- 17°C to -28°C) a volume of 2.5 times higher than the mixture favors DNA to precipitate.

According to Puchooa, (2004), once the nucleic acids are collected, they are washed in the ash butler. The purity of genomic DNA is dependent on the number of washes. A three-time wash combined with a short-run centrifugation is sufficient for DNA purification and removal of endogenous nuclease or other proteins.

Ali, B.A. (2003) carried out a genetic analysis using RAPD markers for studying variation in four breeds of sheep (Baladi, Barki, Rahmani and Saffolk). Nineteen random primers were used to amplify DNA fragments in these breeds. RAPD patterns with a level of polymorphism were detected between breeds. Results showed closer proximity of Barki to Rahmani and Baladi (95.7 and 91.3%), respectively.

Tahmoorespu *et al.*, (1995) used 17 RAPD primers in some of Iranian sheep breeds to investigate RAPD marker development to distinguish genetic differences and similarity between and within some Iranian sheep breeds. By using these markers they had found that genetic diversity within and between sheep breeds are very low. They had suggested more studies with more primers are needed to finding RAPD markers that are informative within and between Iranian sheep breeds as sheep populations have high degree of homogeneity (Kantanen *et al.*, 1999).

Soysal *et al.*, (2005) studied the evolutionary relationship among three native and two crossbreed sheep breeds of Turkey. The study based on three microsatellite loci, the genetic diversity of the Kivircik, Awassi, Akkaraman breeds (native) of Turkey as well as two of their crossbreeds Türkgeldi and Konya Merino were studied comparatively. It was observed that their heterozygoties are all high (0.6673-0.7822) compared to previously studied breeds, as expected for populations close to the center of domestication. Neighbour Joining (NJ) tree

bands segregation in the nine F2 individuals.For RAPD analyses, the procedure described by Williams et al. (1990) with a little modification was used. 30ng of genomic DNA per sample were used. The samples were held at 95°C for 1 min and were then subjected to 40 PCR cycles of melting at 94°C for 10 min, annealing at 37°C for 18 s. extending at 72°C for 75 s using the Perkin Elmer GeneAmp PCR System 9600.The product were separated on 1.5% agarose gels Oligonucleotide primes for DNA chain elongation were designed after the published phytochrome sequence of cultivated rice (Kay *et al.*, 1989), so as to be complementary to exon sequences near junctions to the target introns. Oligonucleotides were synthesized with a DNA synthesizer and purified by electrophoresis on 20% polyacrylamide gel, followed by chromatography on Sep-Pak C18 columns (Waters). To amplify target sequences from total DNA, PCR amplification was carried out in a thermal cycler all follows: 1 minute denaturation at 94°C, 2 minute of primertemplate annealing at 55°C, and 3 minute of DNA chain elongation at 72°C. Reaction mixture was composed of 1 ng of total genomic DNA, 1ng each of 3' and 5' primers, 0.2mM each of dNTP, 1 OmM Tris-HCI, pH 8.3, 1.8mM $MgCl_2$, 1OmM KCl, 2.5 units of Taq DNA polymerase. One amplification reaction (40 cycles) usually yielded enough templates for several sequencing reactions (Sambrook *et al.*, 1989).

2.9 DNA Amplification Fingerprinting (DAF) and Random Amplified Polymorphic DNA (RAPD)

DAF and RAPD are amplification-based nucleic acid fingerprinting techniques (concurrent detection of multiple loci without assignment of a genotype) that use an *in vitro* enzymatic reaction to specifically amplify a multiplicity of target sites in one or more nucleic acid molecules (Micheli *et al.*, 1994). The RAPD technique was first employed by Williams *et al.* (1990) to examine human DNA samples from anonymous individuals. It uses random primers (Williams *et al.* 1990) and can be applied to any species without requiring any information about the nucleotide sequence. The amplification products from this analysis exhibit polymorphism and thus can be used as genetic markers. The presence of a RAPD band, however, does not allow distinction between hetero and homozygous states. The fragments are scored as dominant Mendelian elements, and the protocols are relatively simple. Chikkadevaiah and Nandini , (2005) established the phylogenetic relationships among sunflower hybrids genotypes and characterizes them based on both morphometric traits and

PCR based RAPD markers. At the molecular level, 25 scorable bands were produced with the 5 primers with the number of bands ranging from 3 for OPH-15 to 9 for OPG10. Of the 25 bands, 14 were polymorphic (58.11%). Mosges and Friedtu , (1994) described and compared the characterization of sunflower genotypes using three different marker systems. Sunflower lines and hybrids were analysed by three isozymes, oligonucleotides $(GACA)_4$, $(GATA)_4$, and $(GGAT)_4$ as hybridization probes, and 70 primers for PCR analysis. Polymorphisms were visible with each type of marker. However, the repeated probes, especially $(GATA)_4$, revealed the highest degree of variability and seem to be most appropriate for the generation of genotype-specific 'fingerprints'.

Kesseli *et al.*, (1994) showed the genetic linkage map of Latuca sativa (lettuce) using 53319 RAPD markers covering a map distance 1490-200 cm and the map distance of Helianthus annus (sunflower) did vary between 582-1380 cm and 123-235 RAPD using markers. Random amplified polymorphic DNA (RAPD) analysis was used to study the genetic diversity of six cultivars of potato. Amplification with three decamer random primers generated 35 RAPD markers of which 33 (94.29 %) was polymorphic. The proportion of polymorphic loci and the gene diversity estimates were 14.29% and 0.068, 28.57% and 0.075, 51.43% and 0.217 and 54.29% and 0.245 for Cardinal, Diament, Heera, Raja and TPS, respectively (Yasmin *et al.*, 2006). Randomly Amplified polymorphic DNA (RAPD) markers have rapidly gained popularity to detect polymorphisms among different germplasm of potato (Kayim *et al.* 1998, Forapani *et al.* 2001). The technique is based on the amplification of discrete regions of the genome by polymerase chain reaction (PCR) with short oligonucleotide primers of arbitary sequence (Welsh and McClelland., 1990; Williams *et al.*, 1990). Kosgey IS, *et al.*, (2006) reported that despite the large numbers and importance of adapted indigenous sheep and goats in the tropics, information on sustainable conventional breeding programmes for them is scarce and often unavailable. The aim is to better understand opportunities for genetic improvement of small ruminants by the resource-poor farmers in traditional smallholder and pastoral farming systems. Dismal performance of programmes involving breed substitution of exotics for indigenous breeds and crossbreeding with temperate breeds have stimulated a recent re-orientation of breeding programmes in tropical countries to utilize indigenous breeds, and most programmes are incipient. The

success rate of some breeding programmes involving native breeds is encouraging. Recently, the Food and Agriculture organization (FAO) of the United Nations proposed a global program for the management of genetic resources using molecular methodology for breed characterization (Bjornstad & Roed., 2001). This strategy strongly emphasized the use of molecular markers to assist the conservation and assessment of endangered breeds and to determine the genetic status of these breeds. With the advent of molecular techniques, it has become possible to characterize breeds by determining genetic relationships among animals based on differences in DNA (Karp *et al.*, 1996). The molecular DNA markers that have been used for breed characterization in both plants and animals include restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), microsatellites or simple sequence repeats, mitochondrial DNA (mtDNA), and randomly amplified polymorphic DNA (RAPD). Although they were the first DNA markers to be developed, RFLPs have continued to be useful in breed characterization of both plant and animal species

Welsh and McClelland (1990) reported that genetic modification technologies, developed initially in laboratory strains of selected bacteria and viruses, are essential tools for understanding the genomes of livestock. These tools allow researchers to isolate sequence and characterize any livestock gene; locate genes on chromosomes; follow the inheritance of any gene and/or chromosomal region in any pedigree; detect phenotypic variation due to, or associated with, variation in the DNA sequence of a gene and identify the genetic alteration causing this. Most of the many thousands of genes identified in livestock vary between individuals. Williams *et al.*, (1990) reported that a series of recent genetic studies has revealed the remarkably complex picture of domestication in both New World and Old World livestock. By comparing mitochondrial and nuclear DNA sequences of modern breeds with their potential wild and domestic ancestors, we have gained new insights into the timing and location of domestication events that produced the farm animals of today. The real surprise has been the high number of domestication events and the diverse locations in which they took place factors which could radically change our approach to conserving livestock biodiversity resources in the future.

Chapter Three

Materials and Methods

MATERIALS AND METHODS

This experiment was carried out at the Animal Cell Culture and Molecular Biotechnology Laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh. The samples of the experiment were collected from the sheep farm of Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka, Bangladesh. The different materials used and methodologies followed for the study has been described bellow.

3.1 Study materials

Twenty blood samples of sheep from Bangladesh Livestock Research Institute (BLRI) were collected for DNA isolation.

3.1.1 Morphological Characterization

The sheep's accession or tag no, sampling location and name of the breed are presented in Table 4.

Table 4: Sheep's accession or tag numbers, their source and identity

Sl. No.	Accession or Tag No.	Source	Identity
1	OC34	BLRI	Ewe
2	IB05	BLRI	Ram
3	LO95	BLRI	Ewe
4	IC02	BLRI	Ewe
5	IB16	BLRI	Ram
6	IJ18	BLRI	Ram
7	JO137	BLRI	Ram
8	IJ01	BLRI	Ram
9	J168	BLRI	Ram
10	IB17	BLRI	Ewe
11	IJ02	BLRI	Ram
12	IJ38	BLRI	Ewe
13	BI22	BLRI	Ram
14	OC03	BLRI	Ewe
15	LO93	BLRI	Ewe
16	IB01	BLRI	Ram
17	IJ06	BLRI	Ram
18	OC23	BLRI	Ewe
19	IB03	BLRI	Ram
20	IB10	BLRI	Ram

Tag No.0C23

Tag No.IJ38

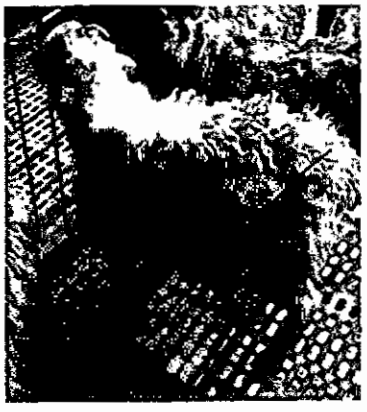
Tag No.LO95

Tag No.IJ18

Tag No.IB10

Tag No.IB01

Tag No.OC34



Tag No.JO137

Tag No.JI68

Figure: 1 Sheep from where the blood samples were collected

3.1.2 DNA isolation

3.1.3 Animal selection and blood sample collection

Blood samples of 20 animals belonging to 3 Bangladeshi sheep were randomly collected avoiding narrow genetic relationships among the sampled animals from sheep shades of BLRI goat and sheep farm for this study. One of these breeds, **Tangail** is the most consistent meat sheep breeds in Bangladesh and other two breeds are **Naogaon and Noakhali.**

Three ml blood was collected from each animal in 5 ml EDTA Vaccumtainer. The blood was gently mixed with EDTA (present in the vaccumtainer as an anticoagulant agent) and kept on ice to maintain low temperature in order to prevent cell lysis. Subsequently the blood samples were transported to the laboratory and stored at 0°C or -20°C until the isolation of genomic DNA.

DNA was extracted from blood by following the method of Bruce A. Roe, (1996), with some modifications. Quantity and quality of extracted DNA was analyzed by a T60 spectrophotometer at the wavelengths of 260nm and 280nm. The presence of DNA was confirmed by running the extracted DNA on a 2% agarose gel.

3.1.4 Preparation of Stock and Working Solutions

Components and concentration of stock solutions are presented in Appendix 1 to Appendix 3.

3.2 Quantitaion of Isolated DNA

Isolated genomic DNA may contain a large amount of RNA and pigments which usually cause over estimation of DNA concentration in a spectrophotometer. Thus, the DNA sample was evaluated both qualitatively (visual estimation- was it higher molecular weight or was there substantial shearing or degradation) and quantitatively (by spectrophotometer). Measurement of isolated DNA concentration was performed by comparing DNA with the standard DNA on agarose gel electrophoresis or by estimating the absorbance of DNA by spectrophotometer at 260 nm. Both the methods were carried out in this investigation.

3.2.1 50×TAE Buffer (p^H 8.3)

For 100 ml of 50 × TAE buffer, 24.2 g Trisma base (MW=121.1) was dissolved into 75 ml of sterile distilled water. Then 1.8609 g EDTA was added to the solution. Finally, 5.4 ml Glacial acetic acid was added in it. The final volume of the solution was adjusted to 100 ml.

3.2.2 Preparation of 2% Agarose Gel

For agarose gel electrophoresis, 80 ml 2% agarose gel was prepared. For casting the gel the following steps were maintained:

- Initially, 1.64 g agarose powder was weighed out and placed into a 250 ml conical flask.
- Then 500 µl of electrophoresis buffer (50 × TAE buffer) was added into the flask and 80 ml distilled water was added. The flask was enclosed with aluminum foil.
- The flask was then cooked into a microwave oven. Using a low to medium setting, the timer was set for 2 minutes. The oven was stopped and swirled the container gently to suspend the undesired agarose.
- When the agarose solution was cooled to about 50^0C, 5 µl from 10 mg/ml solution of ethidium bromide was added so that in the gel the concentration of ethidium bromide was maintained as 0.5 µg/ml
- The combs were setup for making holes for proper running of DNA sample.

3.2.3 Preparation of DNA Sample for Electrophoresis

- Five microlitre loading dye was added to 2 µl DNA sample and then mixed well using the same micropipette.

3.2.4 Documentation of the DNA Sample

- After electrophoresis, the gel was taken out carefully from the electrophoresis.
- The DNA was observed as band and photographed using a digital camera (a Gel Cam Polaroid Camera can also be used).

Staining of the gel by ethidium bromide was performed after electrophoresis. In this process the gel was immersed in the solution of ethidium bromide (which contains 0.5-1.0

μg/ml) at room temperature for 15 minutes. After staining, the gel was rinsed with distilled water and placed it on a UV transilluminator for viewing. The DNA fragments was visualized and photographed. If the background fluorescent was too strong, destaining of the gel was recommended by soaking it in distilled water for a few minutes or longer.

3.2.5 Measuring DNA Concentration and Quality by Spectrophotometer

Spectrophotometer is commonly used in laboratories for the measurement of DNA concentration and purity. The DNA concentration was obtained by multiplying the absorbance at 260 nm by a constant. The DNA purity was measured by dividing the absorbance at 260 nm by the absorbance at 280 nm.

The A260/A280 higher than 2.0 generally indicates RNA contamination. For A260/A280 ratio lower than 1.8, normally indicates protein or phenol contamination during extraction process. Good quality DNA should give A260/A280 ratio in the range of 1.8 – 2.0.

3.2.6 Preparation of the DNA Sample for Spectrophotometer

- The test samples were prepared by taking 20 μl of each DNA sample in the cuvette containing 1980 μl sterile distilled water. The samples were mixed well by using an adjustable micropipette.

3.2.7 Recording the absorbance readings

- At 260 nm, the absorbance readings were recorded by viewing the monitor of spectrophotometer.
- After recording the absorbance readings, the cuvette was rinsed out with sterile distilled water and tamped out on a paper wipe.

3.2.8 Calculation of the concentration of DNA

By using the absorbance readings at 260 nm, the original sample concentrations were determined according to the following formula:

$$\text{DNA concentration} = A260 \times \text{Dilution factor} \times \text{Conversion factor}$$

$$= A260 \times \frac{\text{Volume of distilled water (µl)}}{\text{Amount of the DNA sample (µl)}} \times 50$$

$$= (\mu g/ml)$$

$$= (ng/\mu l) \quad [\because \mu g/ml = ng/\mu l]$$

Here,

A260 = Spectrophotmetric absorbance reading at 260 nm of DNA sample.

Dilution factor = The ratio of volume of distilled water (µl) to amount of DNA sample (µl).

Conversion factor 50 = The 50 µg/ml of DNA contained in a solution which gives the Spectrophotmetric absorbance reading at 260 nm equal to 1.

3.3 PCR reaction:

3.3.1 Primer selection:

In this study 3 RAPD primers were screened out of 10 primers (Invitrogen) due to shortage of Taq DNA polymerase which has a vital role in amplification reaction. The primers are shown in table 4:

Table 5: List and sequences of Primers used in the experiment

Primer No.	Sequences	(G+C) Contents (%)	Annealing Temperature (^{0}C)
1	5′-AATSSGCTGG-3′	60	37
2	5′-TCAGCCACGT-3′	60	37
3	5′-TCTGCCATCC-3′	60	37
4	5′-GTTGCCACCC-3′	70	37
5	5′-TCTGTAGCCC-3′	70	37
6	5′-CTTATCAGGG-3′	50	37
7	5′-GGTGACGCAG-3′	70	3
8	5′-GAACGGACTC-3′	60	37
9	5′-TTCCCCCCAG-3′	70	37
10	5′-AGGCCACTCG-3′	70	37

3.3.2 PCR amplification:

The amplification conditions were based according to Williams *et al.*, (1990) with some modifications. PCR reactions were performed on each DNA sample in a 20 µ reaction mix containing 2 µl genomic DNA, 2 µl of 10x reaction buffer, 2 µl units of Taq DNA polymerase (5 times dilution by enzyme dilution buffer) 1 µl (100 picomol) of primer, , 11 µl of distilled water. DNA amplification was performed by thermal cycler (Eppendorf Mastercycler Gradient,). The PCR samples were overlaid with one drop of mineral oil. The amplification program included an initial denaturation step of 94°C for 3 minutes followed by 40 cycles of 94°C (denaturation), 1 minute; 37°C (annealing), 1 minute and 72°C (extention) for 2 minutes. The last cycle was followed by 10 minutes incubation at 72°C and hold at 8°C.

3.3.3 Gel electrophoresis of PCR amplified Product:

PCR products from each sample were confirmed by running 2 % agarose gel in 1x TAE buffer at 40V for 80 minutes. Loading dye (5 µl) was added to the PCR products and loaded in the wells. Two molecular weight markers DNA (ladder) were also loaded on one side of the gel. After electrophoresis, the gel was taken out carefully from the electrophoresis chamber, was washed with 7 µl ethidium bromide mixed distilled water (1OO ml) and placed on high performance ultraviolet light box (UV transilluminator) for

checking the DNA bands. The DNA was observed as band and photographed using a digital camera.

3.3.4 Data analysis:

RAPDs were scored according to the presence (1) or absence (0) of polymorphic DNA fragments. The scores obtained using all primers in the RAPD analysis were then combined to create a single data matrix. This was used for estimating polymorphic loci, Nei's (1973) gene diversity, gene frequency, genetic distance (D) and constructing a UPGMA (Unweight Pair Group Method of Arithmetic Means) dendogram among the populations using computer programe POPGENE (Version 1.31) (Yeh *et al.*, 1999).

Chapter Four

Results and Discussion

RESULTS AND DISCUSSION

4.1. DNA extraction and Quality determination

The method adopted here for DNA isolation was considered based on simple and easy available equipment and chemical without phenol for obtaining high quality and quantity of DNA for lateral use. Frozen bloods were used as sample for DNA extraction. This method was found suitable for the extraction of DNA. To determine the amount of isolated DNA, the visual estimation and spectrometric method were used. Visually the DNA was estimated by observing the DNA in the agarose gel electrophoreses comparing with known marker DNA. In spectrophotometric method amount of DNA was determined by taking absorbance reading at 260 nm.

Table 6: Determination of the quantity of DNA using spectrophotometer.

Sample No.	Tag no.	Absorbance reading at 260 nm	Quantity of DNA (ng/µl)
1	OC34	0.056	280
2	IB05	0.066	330
3	LO95	0.080	400
4	IC02	0.075	375
5	IB16	0.039	195
6	IJ18	0.056	280
7	JO137	0.068	340
8	IJ01	0.048	240
9	J168	0.083	415
10	IB17	0.075	375
11	IJ02	0.076	380
12	IJ38	0.085	425
13	BI22	0.055	275
14	OC03	0.083	415
15	LO93	0.065	325
16	IB01	0.078	390
17	IJ06	0.083	415
18	OC23	0.039	195
19	IB03	0.079	395
20	IB10	0.074	370

(Detail equation for finding the quantity of DNA was described in the chapter Materials and Methods under the sub-heading 3.2.8)

4.2. Primer Selection and RAPD Analysis

Three primers (Table 7) were selected that used in RAPD analysis of 20 sheep accessions amplified 20 different reproducible bands. three different primers generated various banding patterns ranging from 1 to 9 and the average bands per primer was 6.34 and of the 19 bands scored, 17 (89.63%) were found to be polymorphic and 2 bands (10.37%) were found to be monomorphic. Genetic diversity is of great significance for breeding programmes. Germplasms characterization is an important component of programmes on effective and efficient management/utilization of animal genetic resources. For successive genetic relationship morphological & molecular characterization are very effective. Morphological characterization was determined and showed genetic similarities and dissimilarities by collecting data and constructing the UPGMA dendrogram to estimate the genetic diversity of sheep phenotypically. This some effective population size could influence the structure of the variation. Determining the degree of variability with in an accession is necessary as a preliminary step for studying the genetic diversity among sheep breeds. In addition to the morphological characterization, a molecular screening is essential for determining the genetic variability. RAPD markers are the most effective method to determine the molecular characterization. A maximum of 17 polymorphic bands were obtained in PCR amplification from genomic DNA of the accessions used in present study, RAPD markers are randomly distributed throughout the genome, but most regions of the genome (90 %) are not expressed at the phenotypic level (Williams *et al.* 1990; Joyee *et al.* 1999; Dahlberg 2000). As a result, markers like RAPDs may accurately assay the degree of genetic change between two genomes, but they may not necessarily reflect the divergence in terms of changes in traits of agronomic importance. In addition, the environment markedly influences the expression of most of the phenotypic traits, while detection of molecular markers is not dependent from the environmental effects. In this experiment only 89.63% polymorphism are identified among the selected sheep breeds, which is reasonable when compared with the result of those experiment conducted by other researchers in different lab.

Table 7: The sequence of primers used and their monomorphic and polymorphic bands

Primer	Sequence of the primer	Mono-morphic bands	Polymorphic bands	Total bands	Poly-morphism
Primer 9	5-TTCCCCCCAG-3′	1	4	5	80 (%)
Primer 10	5-AGGCCACTCG-3′	1	8	9	88.89 (%)
Primer 6	5-CTTATCAGGG-3′	0	5	5	100 (%)
Total		2	17	19	
Average			5.67	6.34	89.63 (%)

Figure 2: RAPD profiles using primer 6. The number corresponds to the serial no. of the genotypes shown in Table 3. M is a Hind III size DNA marker

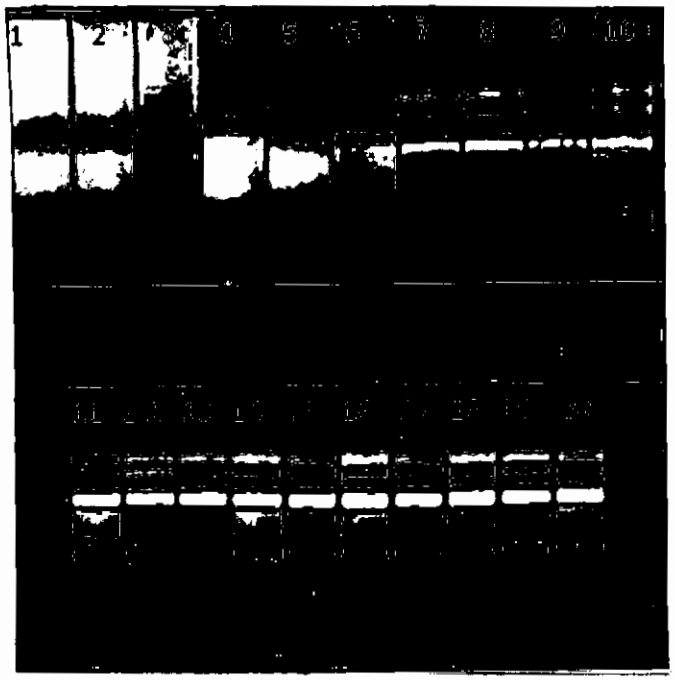


Figure 3: RAPD profiles using primer 9. The number corresponds to the serial no. of the genotypes shown in Table 3.

4.3. Genetic Distance

In this analysis, smaller numbers are associated with more genetically similar individuals, whereas larger numbers suggest genetically dissimilarity. There was a high level of genetic variation among the studied accessions of sheep as indicated by the proportion of polymorphic loci. Estimation of higher level of genetic variation in the sheep might be consistent with the fact that it is a highly polymorphic animal. The values of pair-wise comparisons of Nei's (1979) genetic distance between 20 sheep genotypes were computed from combined data sets for the 3 primers ranging from 0.1 to 1 (Table 8). The result is similar with Micheli *et al.*, 1994, Kosgey *et al,*. (2006), and Karp *et al.*, 1996. Tangail (LO95) shows comparatively higher genetic distance (1) with Naogaon (IB05), (IB16), (IB03) (IB10). Similarly, Naogaon (IB16) shows genetic distance (1) with Tangail (IJ02), Noakhali (OC23). Moreover, Noakhali

(IC02) shows highest genetic dissimilarity with Naogoan (IB05). There is also higher genetic distance found among the sheep of same region. Such as, Naogaon (IB16) shows genetic distance (1) with Naogaon (IB03) and (IB10). There are also some sheep that show higher genetic similarity. Such as, Naogoan (IB10) with Naogaon (IB03) and Tangail (IJ38) with Naogaon (B122). Sometimes it may be seen that, people moved from one region to another region with their livestock. It may be the reason for finding genetic similarity between the two sheep of same region or finding dissimilarity between two sheep of the same region. Moreover, in Bangladesh there is huge chance for discriminate breeding which is main reason for loosing genetic uniqueness.

Table 8: Determination of pair-wise genetic distance between experimental sheep populations using Nei's equation

Pop ID	Tan OC34	Nao IB05	Tan LO95	Noa IC02	Nao IB16	Tan IJ18	Nao JO137	Tan IJ01	Nao IJ68	Nao IB17	Tan IJ02	Tan IJ38	Nao B122	Noa OC03	Tan LO93	Nao IB01	Tan IJ06	Noa OC23	Nao IB03	Nao IB10
Tan OC34	-																			
Nao IB05	0.3334	-																		
Tan LO95	0.6142	1	-																	
Noa IC02	0.7778	1	0.5	-																
Nao IB16	0.7124	0.6	1	0.5	-															
Tan IJ18	0.7142	0.8334	0.6	0.3334	0.4	-														
Nao JO137	0.5716	0.8334	0.846	0.6	0.846	0.5384	-													
Tan IJ01	0.5716	0.8334	0.846	0.6	0.846	0.8822	0.1	-												
Nao IJ68	0.5716	0.8334	0.846	0.6	0.846	0.7646	0.2	0.1	-											
Nao IB17	0.5716	0.8334	0.6922	0.4666	0.846	0.6472	0.1	0.1	0.2	-										
Tan IJ02	0.818	0.7778	0.8	0.6666	1	0.7142	0.294	0.294	0.4118	0.1764	-									
Tan IJ38	0.7142	0.8334	0.6922	0.4666	0.846	0.6472	0.2	0.1	0.2	0.1	0.1764	-								
Nao B122	0.7142	0.8334	0.6922	0.4666	0.846	0.6472	0.2	0.1	0.2	0.1	0.1764	0	-							
Noa OC03	0.6667	0.75	0.6362	0.5384	0.818	0.7334	0.2222	0.1112	0.2222	0.1112	0.2	0.1112	0.1112	-						
Tan LO93	0.6362	0.7778	0.6	0.4	0.8	0.7142	0.1764	0.1764	0.294	0.1764	0.4284	0.1764	0.1764	0.0666	-					
Nao IB01	0.517	0.8334	0.6922	0.4666	0.846	0.6472	0.1	0.1	0.2	0.0	0.1764	0.1	0.1	0.1106	0.1764	-				
Tan IJ06	0.7334	0.846	0.7142	0.2726	0.8572	0.6666	0.238	0.1228	0.1428	0.1428	0.2222	0.0478	0.0478	0.2380	0.2222	0.1428	-			
Noa OC23	0.8334	0.76	0.818	0.6922	1	0.7334	0.3334	0.2222	0.3334	0.2222	0.0666	0.1112	0.1112	0.0	0.3334	0.2222	0.158	-		
Nao IB03	0.818	0.7778	1	0.8334	1	0.857	0.0156	0.294	0.294	0.4118	0.2858	0.4118	0.2924	0.3334	0.2856	0.4118	0.3334	0.2	-	
Nao IB10	0.818	0.778	1	0.8334	1	0.857	0.0156	0.294	0.294	0.4118	0.2858	0.294	0.2924	0.3334	0.2856	0.4118	0.4444	0.2	0	-

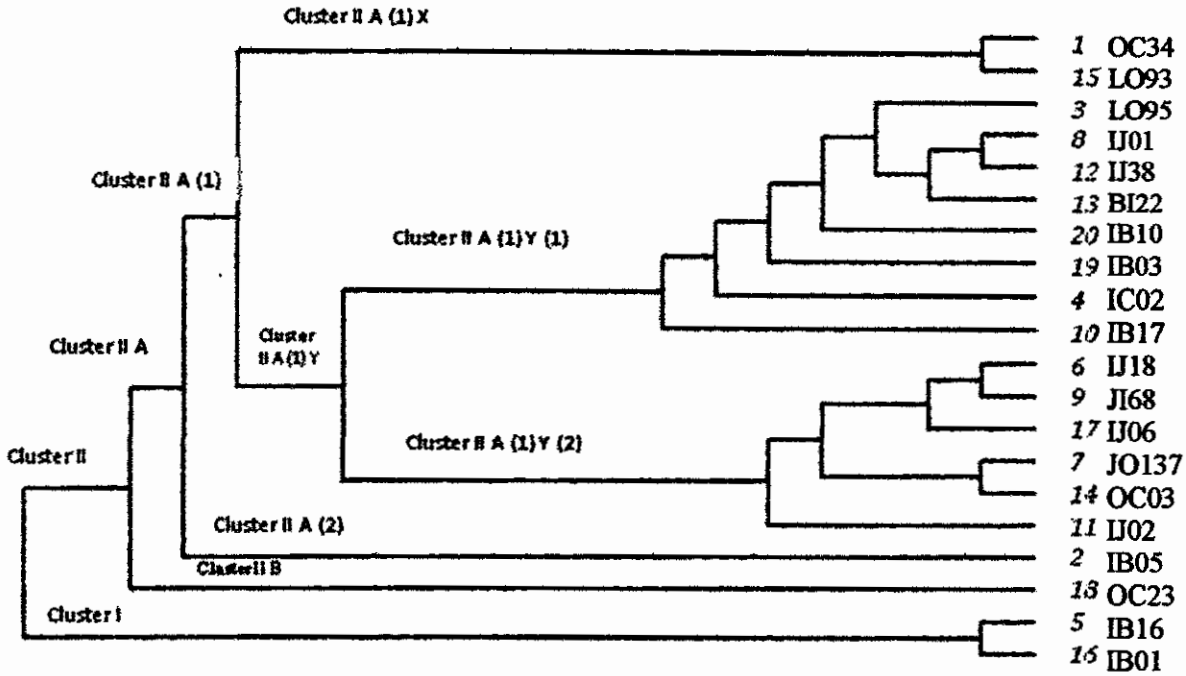


Fig 4: The unweighted pair group method with arithmetic mean (UPGMA) dendogram based on summarized data regarding differentiating among experimental sheep samples.

Table 9: Distribution of twenty sheep number under different clusters

Cluster no						**Total no. of accession in cluster**	**Accessions included in different clusters**
I						2	5,16
II	II A	II A(1)	IIA(1)	II A(1)X		2	1,15
				II A (1)Y	II A (1) Y (1)	8	3,8,12,13,20,19,4,10
					II A (1) Y (2)	6	6,9,17,7,14,11
		II A(2)				1	2
	IIB					1	18

4.4 Morphological Dendrogram Analysis

UPGMA dendrogram was constructed according to the morphological data. The dendrogram (Fig 4) shows that all the breeds were grouped into two major clusters (I) & (II.). Cluster II sub divided into II A and II B. Cluster II A (1) subdivided into II A (1) X and II A (1) Y. Cluster II A (1) Y sub divided into Cluster II A (1) Y (1) and IIA (1) Y (2). In cluster II A (1) Y (1) Sheep 3,8,12,13,20,19,4 and 10 are closely related but sheep 1 and 15 are separate. On the other hand cluster II A (1) Y (2), sheep 6,9,7,11,14 and 17 are closely related. Cluster I different from cluster II and cluster I included cattle 5 and 16. Sheep 2 and 18 are separate from all others.

Chapter Five

Summary and Conclusion

SUMMARY AND CONCLUSION

There was a high level of genetic variation among the studied accessions of sheep as indicated by the proportion of polymorphic loci. Estimation of higher level of genetic variation in the sheep might be consistent with the fact that it is a highly polymorphic animal. The values of pair-wise comparisons of Nei's (1979) genetic distance between 20 sheep genotypes were computed from combined data sets for the 3 primers ranging from 0.1 to 1. The result is similar with Micheli *et al.*, 1994, Kosgey *et al,*. (2006), and Karp *et al.*, 1996.

Genetic diversity is of great significance for breeding programmes. Germplasms characterization is an important component of programmes on effective and efficient management/utilization of animal genetic resources. For successive genetic relationship morphological & molecular characterization are very effective. Morphological characterization was determined and showed genetic similarities and dissimilarities by collecting data and constructing the UPGMA dendrogram to estimate the genetic diversity of sheep phenotypically.

In this study, three primers were used for RAPD analysis and they produce distinctive reproducible band. Total 19 bands were found from three primers among those 17 bands were polymorphic and 2 bands were monomorphic. Statistically, 89.63% band was found polymorphic. Although some phenotypic differences exist among some sheep that are genetically similar, that kind of variation may be from environmental effect. So, cross breeding between tow buffaloes that are genetically similar will be less productive. Although, Naogaon (IB16) and Naogaon (IB03), (IB10) have less phenotypic variation but cross breeding with each other will be effective, because of their high genetic variation. However, Naogaon (IB10) and Naogaon (IB03) possess phenotypic variation but because of their less genetic difference, crossbreeding will be less effective.

Chapter Six

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Chapter Seven

Appendices

APPENDICES

Appendix-1

COMPOSITION OF DNA EXTRACTION SOLUTION

1X Standard Saline Citrate (SSC); pH 7.03 (For 100ml)

NaCl: 0.875gm
Na-citrate: 0.441gm
Dissolved in 60ml distilled water, bring the final volume 100ml.

0.2M Na-acetate (For 100ml)
Na-acetate (anhydrous): 1.6406gm
Dissolved in 80ml distilled water, bring the final volume 100ml.

2M Na-acetate (For 100ml)
Na-acetate (anhydrous): 16.406gm
Dissolved in 80ml distilled water, bring the final volume 100ml.

10% SDS (For 100ml)
Sodium dodecoyle sulphate (SDS): 10gm
Dissolved in 80ml distilled water, adjust 100ml volume.

Proteinase K
Proteinase K 20mg in 1ml of distilled water.

Detergent
Detergent 20mg in 1ml of distilled water.

Phenol: Choroform: Isoamyl Alcohol (25:25:1)
TE Saturated Phenol: 25ml
Choroform: 25ml
Isoamyl Alcohol: 1ml
Total volume 51ml.

Choroform: Isoamyl Alcohol (25:1)
Choroform: 25ml
Isoamyl Alcohol: 1ml
Total volume 26ml.

TE (10:1) buffer (For 500ml)
1M Tris-HCl (pH7.6): 5ml
0.5M EDTA (pH8.0): 10ml
Adjust final volume 500ml with distilled water.

100% Cold Ethanol

70% Ethanol
Ethanol: 70ml
Distilled water: 30ml

50X TAE Buffer, pH 8.03 (For 100ml)
EDTA: 1.8609gm
Glacial acetic acid: 5.4ml
Tris-base: 24.2gm
Adjust 100 ml with distilled water.

1X TAE Buffer (For 400ml):
50× TAE buffer: 8ml
Distilled water: 392ml

14N HCl:
Concentrated HCl: 127.51 ml in 100 ml of distilled water.
Make the final volume 250 ml with distilled water.

1M NaOH (For 100ml):
NaOH pellets: 4 gm
Distilled water: 80ml
Make the final volume 100 ml with distilled water.

0.5 M EDTA; pH 8.0 (For 250ml)
EDTA: 46.525gm
Dissolved in 100ml distilled water
Adjust pH to 8.0 with 10M NaOH
Add distilled water to 250ml.

1M Tris-HCl; pH 7.6 (For 250ml):
Tris-base: 30.275gm
Dissolved in 100ml distilled water
Adjust pH to 7.6 with concentrated HCl
Make the final volume 250 ml with distilled water.

Appendix -2

Reagents used

Name of Reagent	Name of the Company
NaCl	AnalaR[R]
Na_2SO_4	Schnchardt,Germany
Tris-base	Hinweis
SDS	Hinweis
EDTA	Hinweis
Na-citrate[TM] Dihydrate	—
Agar	Invitrogen[TM] life technolgies
Gel loading dye	Invitrogen[TM] life technolgies

Appendix-3

EQUIPMENTS USED

Name of the equipment	Name of the Company
Autoclave machine	ALP
Centrifuge Machine	Eppendorf
Balance	ScalTec
Centrifuge tube	LABSCO
Drier	Eppendorf
DNA Concentrator	Centrivap Concentrator LABCONCO
EDTA Vacutainer	Becton Dickinson
Electrophoretic apparatus	Whatman Biometra
Incubator	———
Polaroid camera	———
Micropipette	Eppendorf
Steri Pack Syringe	Opso Saline
Tips	Eppendorf
UV Transilluminator	———
Vortex Mixer; Mode Vm-2000	———
Water bath	LABSCO

Appendix -4

PROTOCOL FOR DNA ISOLATION: Modification of Roe et al.

1. The liquid blood samples were obtained in EDTA Vaccumtainer tubes and were stored at -20°C freezer.
2. The frozen samples were thawed; 1.0 ml of 1X SSC buffer with 800 µl of blood was added in a 2 ml centrifuge tube and mixed well by vortexing. Those were centrifuged at 13000 g for 10 min.
3. 1ml of the supernatant was removed and discarded into disinfectant.
4. 1ml of 1X SSC buffer was added, vortexed, and centrifuged as above for 10 min and all of the supernatant was removed.
5. 400 µl of 0.2M sodium acetate was added to each pellet and vortexed briefly. Then 35 µl of 10% SDS and 15 µl of proteinase K was added, vortexed briefly and was incubated for 1.3 h at 55°C.
6. 500 µl of phenol: chloroform: isoamyal alcohol was added and vortexed for 30 sec. The sample was centrifuged for 20 min at 13000 g.
7. The aqueous layer was carefully removed to a new 1.5 ml microcentrifuge tube; 1 ml of ice cold 100% propanol was added, mixed and incubated for 10 min on ice.
8. It was centrifuged for 12 min at 13000 g. The supernatant was decanted and drained.
9. 1ml of 70% ethanol was added and centrifuged for 10 min. at 13000 g in a microcentrifuge.
10. The supernatant was decanted, and dryed the pellet in a Speedy-Vac for 10 minutes (or until dry).
11. The pellet was resuspended by adding 50 µl of TE (10:1) buffer. Then it was Incubated for 30 minutes at 55°C, vortexing periodically to dissolve the genomic DNA. The samples were store at -20^0 C.