Molecular characterization of selective indigenous, cross-bred and exotic cattle using RAPD markers

A thesis submitted
by

Dewan Ariful Islam
Examination Roll No. MS-080707
Session 2007-08

Submitted To

Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University in partial fulfillment of the requirements for the degree Master of Science (MS) in Biotechnology and Genetic Engineering.

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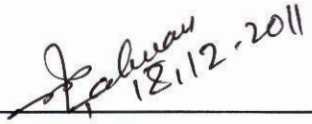

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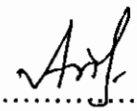
September, **2011**

DECLARATION

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 The findings of the research has not been/will not be published anywhere without the concurrence of the concerned supervisor/co-supervisor and the author.



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Dewan Ariful Islam
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Dedicated to

My Parents and Grand Parents who have brought me up

Acknowledgement

Thanks and all praises to almighty Allah, Who has given me the ability to perform this thesis work successfully with sound condition.

The author wish to express his profound sense of gratitude to my most respected teacher and supervisor Dr. Khondoker Moazzem Hossain, Professor and Head, Biotechnology and Genetic Engineering Discipline (BGE), Khulna University, Khulna, Bangladesh, for his inspiration, constant guide, valuable suggestions, sympathetic advice and unparalleled encouragement made throughout the course of study, without which this piece of work would not have taken its present shape.

The author would like to express his greatest and profound gratitude, deep appreciation and heartfelt indebtedness with best regards to his honorable co-supervisor, Dr. S.M. Mahbubur Rahman, Professor Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh, for his cordial help, suggestions, scholastic guidance and constructive criticism during this thesis work.

The author is grateful to Ministry of Science Information and Communication Technology (MOSICT) through his honorable Supervisor Professor Dr. Khondoker Moazzem Hossain, Co-supervisor, Professor Dr. S.M. Mahbubur Rahman and honourable teacher Dr. Md. Morsaline Billah, Associate Professor of BGE Discipline, Khulna University for providing financial support to conduct the research work.

The author also wishes to express his deepest sense of respect to Deputy Director, Central Cattle Breeding & Dairy Farm, Savar, Dhaka, Bangladesh for his cordial help and kind cooperation regarding collection of blood sample.

Profound gratitude and thankfulness are given to all Officer Bayer Section, Bangladesh Central Cattle Breeding & Dairy Farm, Savar, Dhaka, Bangladesh for their cordial support and kind cooperation and also Md. Shaiful Islam, Assistant Register (Lab) of BGE Discipline for his assistance during laboratory works.

The author likes to express his cordial thanks to Tarun, Maksuda, Sagor, Salim, Bulbul, Somon and other friends who sincerely helped in my laboratory work.

Finally, the author expresses his deepest sense of gratitude to his parents for their immeasurable blessing, continuous inspiration, and financial support and also for their encouragement throughout this study.

The Author

Abstract

The research work was conducted at Animal Cell Culture and Molecular Biotechnology Laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh. The major objective of the study was to find out the genetic variation and to determine the genetic distance among selected cattle population of Bangladesh. DNA was extracted from experimental cattle blood samples and the extracted DNA was observed by gel electrophoresis. Random Amplification of Polymorphic DNA analysis was carried out using DNA samples of experimental cattle. Only distinct, reproducible and scorable polymorphic fragments were taken into consideration for analysis. Twelve primers were used in RAPD analysis. Two different primers generated 106 bands of which 95 (90.48%) were polymorphic and 10 bands were common in nature. The frequencies of polymorphic bands varied from primer to primer. Though no sample-specific marker was identified, the high level of polymorphism revealed by the proportion of polymorphic loci (90.48%) indicated that RAPD markers could be considered as one of the effective tools for estimating genetic diversity. Dendrogram grouped the cattle into two major clusters: cluster (I) and (II). Again cluster (II) was sub divided into IIA and IIB, and showed genetically similar characteristics. The values of pair-wise comparisons of Nei's genetic distance among experimental cattle genotypes were computed from combined data sets for the two primers. Comparatively higher genetic distance (0.82) was found between AFSL-1360 and Sahiwal-586. The lowest genetic distance (0) was revealed between Freshian-75%-1630, AFSL-1160 and Freshian-75%-1781, Freshian-62.5%-2000. The range of genetic distance of experimental genotypes and the difference between the highest and lowest genetic distance indicated the presence of variability among the experimental genotypes of cattle. The genetic diversity of local cattle is relatively higher for a prescribed breed and therefore, has opportunities to improve those using selective crossbreeding techniques for future improvement of local cattle to yield high milk and meat products.

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LIST OF ABBREVIARTION

%	:	Per cent
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
i.e.	:	id est = that is
M	:	Molar
mg	:	Milligram
mg/l	:	Milligram Per Litre
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
PCR	:	Polymerase Chain Reaction
pH	:	Negative Logarithm of Hydrogen Ion (H^+) Concentration
PVP	:	Polyvinyl Pyrolidone
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribonucleic Acid
SDS	:	Sodium Dodecyl Sulphate
SSR	:	Simple Sequence Repeats
UV	:	Ultra Violet
viz.	:	Videlicet (= namely)
w/v	:	Weight Per Volume
Sahi	:	Sahiwal
Fre	:	Freshian

Chapter One

Introduction

INTRODUCTION

Cattle are important species of Bovidae family in Bangladesh. They are the main source of milk, meat and draught power of the country. They supply about 99% of total milk being produced in the country, 50% of the total meat sold in the market and 98% of the draught requirement of the country (BBS, 1991). The contribution of cattle to the total out-put is not static, rather varies depending upon the population size and localities (Faruque et al., 1997). According to BBS (1986), Bangladesh possesses 22.5 million cattle heads. However FAO (1992) indicates that the country now possesses 23.4 million cattle heads.

Like other developing countries, breed substitution and crossbreeding program has been practiced indiscriminately in Bangladesh for many years with exotic breed and as a result valuable genetic resources is being lost or diluted day by day. Assessment of genetic variation is a crucial element in determining breeding strategies and for effective and meaningful conservation program (Mufti et al., 2009).

Genetic diversity or similarity may be measured through genetic markers. These have been used to determine evolutionary relationship within and between species, genera or higher taxonomic categories. However, breeders tend to concentrate on specific genotypes for determination of genetic diversity, which combine traits of interest and may be used as progenitors in several breeding programs in order to introduce agro-ecological important traits. An attempt to solve the problem of maintaining pure breeds using morphological characteristics require a lot of time and effort, compare to molecular markers technique. Moreover, molecular markers are important tools in tagging desirable loci underlying the traits which have breeding importance (Kantanen et al., 1995).

Molecular markers are the molecules that could be used to trace a desired gene(s) in examined genotypes. In fact, a piece of DNA or a protein can be used as a marker. Earlier approaches that made selection of specific traits easier were based on the evaluation of morphological traits RAPDs storage proteins like hordeins. However, DNA markers seem to be the best candidates for efficient evaluation and selection of

animal population. Unlike protein markers DNA markers segregate as single genes and they are not affected by the environment as morphological markers. Presently, the PCR-based RAPD fingerprinting assays are being used for characterization of zebu cattle breeds, for detection of genetic variations in cattle and sheep (Gwakisa et al., 1994 and Allchin, 1996).

Estimation of genetic variation increasingly is being based upon information at the DNA level by various molecular markers such as, RAPD, AFLP, RFLP, SSR or Microsatellite. Among them, a RAPD marker, generated by the Polymerase Chain Reaction (PCR) is widely used since 1990's to assess infraspecific genetic variation at nuclear level. This procedure detects nucleotide sequence polymorphisms in a DNA amplification-based assay using only a single primer of arbitrary nucleotide sequence. The polymorphism between individuals result from sequence differences in one or both of the primer binding sites and are visible as the presence or absence of a particular RAPD band. Keeping in mind the above circumstances, the present research work was undertaken with the following objectives:

1. To determine the genetic variation and relatedness of experimental cattle.
2. To find out the genetic distance among experimental cattle population.

Chapter Two

Review of Literature

REVIEW OF LITERATURE

In the past decade there have been tremendous efforts globally to study genetic diversity in livestock species. This, in part, reflects a measurable indicator of an output to the Food and Agriculture Organization (FAO) global whose programme addresses the needs of both development and conservation of animal genetic resources in the different parts of the world. The efforts also reflect global concern that a surprisingly narrow range of animal species is used for agriculture (sheep, goats, cattle, pigs, buffaloes, chickens and others that have local importance to certain areas) and this pool is threatened by genetic dilution. Shrinkage of the animal genetic resource pool is, to a great extent, a result of the radical shift of livestock development from subsistence to commercial farming systems. The world is witnessing increased specialisation of animal production objectives and competitive pressures arising from the specialisation. In Bangladesh, where the role of the livestock sector in gross agricultural output is increasing, specialisation has resulted in breed replacement, where indigenous cattle are silently upgraded via artificial insemination with semen of *Bos taurus* breeds to achieve productivity gains. Although indigenous zebu cattle account for as much as 90% of the total cattle populations in Asian countries (Teale *et al.* 1993), crossbreeding with taurine breeds constitutes a significant threat to the local genetic resource. Cattle breeds of Bangladesh are of particular importance because of their adaptation to a wide range of environmental conditions, such as heat, drought and tropical diseases. It is important to know the diversity and relationship between the breeds and strains of livestock. Moreover, it is important because, quite often, the same breed may be known by different names or two breeds may be known by the same name, based on geographical locations of such populations (Gwakisa *et al.* 1994). The need to curb the threats of dilution and extinction of the African animal genetic resources, by strategic development and conservation, has never been more justifiable and is now, with the available technology, timely. Efficient strategies for conservation require sets of genetic markers, which characterise distinct populations (Kemp and Teale, 1994).

2.1. Origin and Distribution

There are many species belonging to the family are found throughout the world. The existing main groups of bovidae (domestic cattle) e.g., *Bos indicus* (India and Africa) and the other 2.Bos taurus (European), are descended from *Bos primigenius*, the original wild cattle of Europe, now no longer in existence. *Bos taurus* found in Europe and North America are all non humped cattle which were first domesticated nearly 10,000 years ago somewhere in middle East. Our indigenous zebu cattle (*Bos indicus*) or humped cattle might have been produced by selection from non-humped cattle either in South or South West Asia.The local cattle originated in Africa, Asia. The original stocks were the black, red, gray and white animals. After the New World was settled and markets began to develop for milk in America, Europe. The breed is widely distributed in Western Europe, India, China, Pakistan, Bangladesh, South America and South Africa (www.FAO.com).

2.2 Morphological characteristics

Local cattle are an important part of Bangladesh. The physical and morphological characteristics and the productive and reproductive performances of local cattle were studied. Morphometric measurements (body length, height at wither and heart girth) were taken from local cattle of different categories. In all categories of the animals, heart girth was highest followed by height at wither and body length giving the appearance of a small, compact body. Males had greater body measurements when compared to females. It is observed that the body length, body height and heart girth of adult Red Chittagon females were 114.38±1.56 cm, 107.71±0.93 cm and 139.85±1.63 cm respectively. For adult male the same measurements were 134, 125 and 168 cm respectively. The coat color of local cows was mostly black, white, gray and red brown. The tail switch was black in all animals. The horns were small to medium and curved inward with pointed tips. The ears were small and erect with a sideways orientation and had pointed tips. The face was small and narrow with a flat forehead. The body was small, compact and less fleshy. The skin was tight, the dewlap was medium and the hump was small in females and developed in males. The tail was long and reached to below the hock. Cows had small udders, teats and milk veins. The animals of this breed were usually quiet but adult males were of aggressive temperament, (Habib *et al.* 2003).

2.3 Reproductive characteristics

In Madaripur, Faridpur and Sariatpur district of Bangladesh, there are two cattle production systems: a draught-oriented system with local Deshi cattle and more milk-oriented production. Deshi animals are small. The calving interval in Deshi cows is one year long. The great majority of the bullocks and the bulls are used as draught animals. A local cow can produce 3-5 liter of milk per day (www. NCBI. Com). Indigenous cattle in Bangladesh are adapted to harsh local environmental conditions like high temperatures, drought, and tropical diseases these cattle are important resources. They also have important roles in socio-economic development. The origins of these breeds form the basis for understanding and improving them. Milk production in Bangladesh is reported to have increased from 14.9 thousand tonnes in the year 1993–94 to 16.2 thousand tonnes in the year 1997–98. This increase was due to recent government policy and to NGOs involvement (e.g. subsidies to establish small dairy farms, soft fund loans from the government as well as NGOs and improved veterinary health care) in dairy development activities, (Ahmed, 2000).

2. 4. Need for the Genetic Characterization of Livestock Population

The 'World Watch List for Domestic Animal Diversity Report documents more than 6300 breeds of breeds of livestock belonging to 30 domesticated species. These breeds were developed following domestication and natural and human selection over the past 12,000 years. The current number of breeds is likely an underestimation as a large proportion of indigenous livestock populations of the developing world, where most animal genetic resources are today found, have yet to be described at phenotypic and genetic levels. Livestock populations have evolved unique adaptation to their agricultural production system and agro-ecological environments. Their genetic diversity has provided the material for the very successful breeding improvement programs of the developed world in the 19th and 20th century, (Schearf, 2003)

However, livestock diversity is shrinking rapidly. With the exception of the wild boar - the ancestor of the domestic pig - and wild red junglefowl - the ancestor of the domestic chicken the putative wild ancestors of our major livestock species, the reservoir of genetic diversity, are now either extinct (e.g. the auroch the wild ancestor of cattle or the ancestral species of the Old World camelids) or low in numbers and threatened by extinction (e.g. wild goat populations of the Near East, vicuña from

Andean plateau, wild donkey in Africa). Among the domesticated populations, it is estimated than 1 to 2 breeds are lost every week. However, 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 at best crossbreeding. There is therefore 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. Genetic characterization through the use of molecular marker methods associated to powerful statistical approaches is providing new avenues for decision-making choices for the conservation and rational management of AnGR, (Schearf, 2003).

2.5. Molecular techniques in the study of animal genetic diversity

A variety of different molecular techniques are being used in various laboratories for the study of inter- and intra-specific genetic variation at the DNA level. The most widely used techniques are restriction fragment length polymorphisms of nuclear DNA and mitochondrial DNA, minisatellites, microsatellites, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism and sequencing of mitochondrial DNA. These techniques differ in the type of data they generate, in the way that they resolve genetic variations and in the taxonomic levels at which they may be most appropriate. As a rich selection of molecular techniques is available, choice of method to use for genetic diversity is quite often dictated by the power of the method to generate reproducible polymorphism that can either be tracked in a Mendelian fashion or can segregate a phenotypic trait in a predictable manner. However, aspects such as lack of specialised equipment, high running costs and lack of technical competence continue to impede many laboratories in research

institutions. Many researchers in Africa would benefit if laboratory technology development was paralleled by (1) simplification in technology and ultimately reduction in expense and (2) increase in analytical power per unit effort. The discovery of the polymerase chain reaction (PCR)-based RAPD technique has made a substantial contribution as a simple technique, which is affordable to a broad community working in molecular genetics with limited resources.

2. 5. 1. Principle of RAPD

The PCR-based RAPD technique is an attractive complement to conventional DNA fingerprinting. RAPD analysis is conceptually simple. Nanogram amounts of total genomic DNA are subjected to PCR using short synthetic oligonucleotides of random sequence. The amplification protocol differs from the standard PCR conditions in that only a single random oligonucleotide primer is employed and no prior knowledge of the genome subjected to analysis is required. When the primer is short (e.g. 10-mer), there is a high probability that the genome contains several priming sites close to one another that are in an inverted orientation. The technique essentially scans a genome for these small inverted repeats and amplifies intervening DNA segments of variable length. The profile of amplification products depends on the template-primer combination and is reproducible for any given combination. The amplification products are resolved on agarose gels and polymorphisms serve as dominant genetic markers, which are inherited in a Mendelian fashion (Williams *et al.* 1990). Amplification of non-nuclear RAPD markers is negligible because of the relatively small non-nuclear genome sizes. Two modifications of detection of RAPD markers have been described as DNA amplification fingerprinting (DAF) and arbitrarily primed polymerase chain reaction (AP-PCR). DAF uses short random primers of 5-8 base pairs (bp) and visualises a relatively greater number of amplification products by polyacrylamide gel electrophoresis and silver staining (Caetano-Anolles *et al.* 1991). AP-PCR uses slightly longer primers (such as universal M13) and amplification products are radioactively labelled and are also resolved by polyacrylamide gel electrophoresis (Welsh and McClelland 1990). Standard RAPD analysis is performed according to the original method (Williams *et al.* 1990) using short oligonucleotide primers of random sequence which can either be locally synthesised or are commercially available (Operon Technologies Inc., Alameda, California, USA). Only high-molecular weight, i.e. non-degraded, DNA should be subjected to RAPD

analyses. Amplified products can be resolved by gel electrophoresis on 1-2% agarose gels.

2.6. Genetic Characterization of Cattle

Gwakisa *et al.,* (1994) carried out the characterization of Zebu cattle breeds in Tanzania using random amplified polymorphic DNA markers. A total of 141 short primers, of arbitrary nucleotide sequence, were used singly in polymerase chain reactions to amplify DNA fingerprints in pools of DNA representing three Zebu cattle breeds. Two primers, which discriminated between the breed-specific DNA pools, were used further to amplify individual pool components in order to establish band frequencies of the amplified fingerprints. One of the primers (ILO 1127) amplified a RAPD fingerprint in 61% of TSZ animals but less than 6% in the other breeds, while another primer (ILO 1065) revealed a DNA sequence common to 89% of the Boran animals and less than 30% in the other two breeds. Bandsharing and mean average percentage difference calculated within and between the three breeds using RAPD fingerprint data showed a higher degree of homogeneity within than across the breeds and indicated measurable divergence between the three breeds. It is concluded that RAPD polymorphisms are useful as genetic markers for cattle breed differentiation.

Kantanen *et al.,* (1995) application for detecting genetic variation of cattle by Random amplified polymorphic DNA (RAPD).The present study investigated the use of the random amplified polymorphic DNA (RAPD) method to detect genetic variation in cattle. The animals studied consisted of samples from five Finnish cattle breeds: native Eastern (18 animals), Northern (24), Western Finncattle (24), Finnish Ayrshire (24), and Finnish Friesian (18). The cattle populations were analysed with 11 and 13 RAPD primers demonstrating the most repeatable amplification pattern. Two out of ten RAPD fragments tested by cross hybridization showed homology between the two species. The RAPD method did not prove efficient for finding new polymorphisms in either species, because we found only three polymorphic RAPD markers for cattle with different allele frequencies between the breeds. However, the interbreed and intrabreed similarity indices for cattle did not suggest any significant differentiation of the Finnish breeds, contrary to earlier results based on blood group and protein polymorphism.

Gwakisa *et al.*, (1997) studied the genetic diversity in some indigenous cattle breeds of East Africa by RAPD. The cattle breeds and their respective locations in parenthesis were Boran (Kongwa, central Tanzania), Maasai Zebu (Kongwa, central Tanzania), Iringa Red (Iringa, southern Highlands of Tanzania), Dwarf Chagga Zebu (Moshi, Kilimanjaro, northern Tanzania), Tarime (also called Sukuma, Tabora and Shinyanga; western Tanzania) and Ankole (Tabora, western Tanzania). Also included were Boran and Sahiwal breeds from Kenya and -West African N'Dama breeds sampled from the International Livestock Research Institute (ILRI) in Kenya. Twenty cattle (10 of each sex) were sampled from each breed. They have adapted to fragile environments and are influenced only by natural selection. In this study, random amplified polymorphic DNA (RAPD) marker was used to analyze Creole cattle genome polymorphism. A comparative analysis using the RAPD technique was performed in pooled DNA of three cattle breeds (Holstein Friesian, Creole and Hereford) in order to evaluate their amplification patterns. A primary screening of RAPD primers allowed to select and use those with higher percentage of GC base composition. A total of 215 loci ranging between 300 and 2500 bp were amplified. Bandsharing frequency (BSF) among breeds showed that less related fingerprints were observed between Creole and Hereford cattle (0.77), while the highest similarity frequency corresponded to Holstein Friesian compared to Hereford (0.81). Specific RAPD bands were identified in the three DNA pools and they were tested in every individual of each breed. It may be possible to isolate and sequence these bands to create breed-specific molecular markers. The identification of multiple alleles of the MSCYP 21 in Creole cattle with a heterozygosity of He = 0.846 supported the variability of this genetic resource. The use of molecular marker RAPD was proposed to establish genetic distance among American Creole cattle and possibly related ancestral Iberian breeds.

Genetic marker types that provide different estimates of genetic diversity information. The choice of the marker to use for genetic diversity is quite often dictated by the power of the method used to generate a reproducible polymorphism that can either be tracked in a Mendelian fashion or can show segregation of a phenotypic trait in a predictable manner (Hannotte and Jianlin, 2005). The choice can also be influenced by the availability of specialized equipment, operating of equipment and assays, and technical competence. The first biomarkers to be widely used in livestock

characterization were protein polymorphisms known as allozymes (Queller *et al.*, 1993). Several livestock breeds have been characterized for variations in different proteins (Di Stasio, 1997). Molecular DNA polymorphisms are now the tools of choice for the assessment of genetic diversity among livestock breeds. They have great potential for discovery of fundamental parameters or characteristics important in conservation, including past effective population size (Garrigan *et al.*, 2002), past bottlenecks (Luikart and Cornuet, 1998), inbreeding status (Ellegren, 1999; Lynch and Ritland, 1999), and sex-specific gene flow. According to Hannotte and Jianlin (2005), important assumptions that are needed for the use of genetic markers include: (i) neutrality of the polymorphisms and (ii) the use of a relatively small number of independently segregating marker loci are a good predictor of the overall genomic diversity of a population. Randomly Amplified Polymorphic DNA (RAPD) technique, like microsatellites, involves the use of PCR for genetic typing and to assess diversity (Williams *et al.*, 1990). In one study involving indigenous African cattle, Gwakisa *et al.* (1994) used RAPD markers to characterize the local Zebu (Bos indicus) cattle breeds of Tanzania. Using RAPD markers, the relatedness among the three local breeds of Tanzania was quantified. One of the primers, ILO 1127, amplified a RAPD fragment in 61% of the Tanganyika Shorthorned Zebu animals but less than 6% in the other breeds. Another primer, ILO 1065, revealed a DNA segment common to 89% of the Boran animals and less than 30% in the other two breeds evaluated. Further, the study revealed that ILO 1065 primer could be a Bos indicus-specific Y-linked polymorphism. They also showed that RAPD analysis could detect introgression. In another similar study, Yu et al. (2004) used RAPD analysis to estimate genetic diversity and relatedness of two native cattle breeds from the Yunnan province of China (DeHong cattle and DiQing cattle) and four introduced beef cattle breeds (Brahman, Simmental, MurryGrey, and ShortHorn). Using 10 primers, it was shown that the Yunnnan DeHong cattle breed was closely related to the Brahman (Bos indicus), and the Yunnan DiQing cattle breed was closely related to the Simmental, ShortHorn, and MurryGrey (Bos taurus) breeds. Under the FAO recommendation, most of the livestock variation studies that have been done used microsatellite markers. The major drawback of RAPDs is that they are dominant markers and heterozygotes are typically scored as homozygote, which decreases their information content. However, RAPD analysis could benefit most of African

laboratories in the studies of livestock due to their simplicity, affordability and relatively low technical requirements.

In one of the few studies reported, Mwacharo *et al.,* (2006) phenotypically characterized two breeds of Zebu in Kenya. They used a total of 12 morphometric measurements to show that the Masaai Zebu was different from the Kamba Zebu in Kenya. The apparent wide within-breed and between-breed variations in linear body dimensions that were observed in the study were indicative of the large genetic diversity inherent in the small East African Zebu cattle, with clearly well-differentiated breeds. The advent of molecular techniques has led to an increase in the studies that focus on the genetic characterization of domestic breeds using genetic markers (Giovambattista *et al.,* 2001). As a tool used in evaluating genetic variation, markers can provide useful information at different levels and purposes such as structure of animal populations, levels of gene flow, phylogenetic relationships, patterns of historical biogeography, and parentage (Feral, 2006). In addition, genetic assessment is also of interest for the design of genetic improvement programs including appropriate choice of breeds for crossbreeding (Mwacharo *et al.*, 2006). Though understanding genetic diversity is of importance to Africa, only a limited number of studies in livestock have been done in some African countries. For example, Gwakisa *et al.* (1994) described a study that involved characterization of Zebu cattle in Tanzania using randomly amplified polymorphic DNA (RAPD) markers. The cattle breeds included in their study were Tanganyinka Shorthorned Zebu, Boran, and the Mpwapwa. They reported that divergence between the Mpwapwa and Tanganyinka Shorthorned Zebu was on average lower than all other between comparisons. In a study of Ethiopian cattle breeds including Horro, Sheko, Arsi, Abigar and Guraghe, Hassen *et al.* (2007) reported that genetic diversity between Guraghe and Abigar were smallest followed by Guraghe and Arsi. The data developed here will provide a resource for scientists interested in germplasm conservation and will add to previous reports of genetic variation in Tanzanian and Kenyan cattle.

Jeon *et al.,* (2010) Reported that the current animal industry is both technology-intensive and globalised. Efficient molecular tools, such as DNA markers, are in demand to strengthen competitive power by maximizing the improvement of livestock

and obtaining the trust of customers by the verification of product origins. This review describes the present techniques applying DNA markers in the animal industry, with a focus on beef cattle and pigs. Preliminary data from an individual traceability assay for Hanwoo (Korean cattle) using 20 microsatellite markers is described. The industrial application of DNA techniques is limited at present, however, it is expected that DNA markers originating from trait genes, especially those of low heritability and difficult-to-measure traits, may contribute to maximizing the improvement of the major economic traits of animals in the future. 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.

Van and Nei, (2003) reported that the development of molecular biological techniques has created new possibilities for the selection and genetic improvement of livestock. The discovery of the PCR had a major impact on the research of eukaryotic genomes and contributed to the development and application of various DNA markers. DNA markers have already found wide application in parentage verification, individual identification and control of genetic disorders. The ultimate use of DNA markers would be to identify quantitative trait loci (QTL) in order to practice genotypic selection. This paper reviews DNA markers (RAPD, DFP, RFLP AFLP, minisatellites, microsatellites, SNP) and provides a brief overview of the current application of these markers in animal breeding.

Crawford, (2003) 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 characterise 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.

Brufort *et al.*, (2003) 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 conducted in the Animal Cell Culture and Molecular Biotechnology Laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh. In this chapter details of different materials used and methodologies followed for the study have been described.

3.1 Animal Selection and Blood Sample Collection

A total of 20 different cattle were selected from Savar Dairy Farm, Savar, Dhaka, Bangladesh for this study. Five ml of blood was collected from each animal in a 06 ml EDTA vaccutainer. The blood was gently mixed with EDTA (present in the vacationer as an anticoagulant agent) and kept on ice box. Subsequently the blood samples were transported to the laboratory and stored at -20°C until further use.

Fig 1. Pure Sahiwal, Pakistan

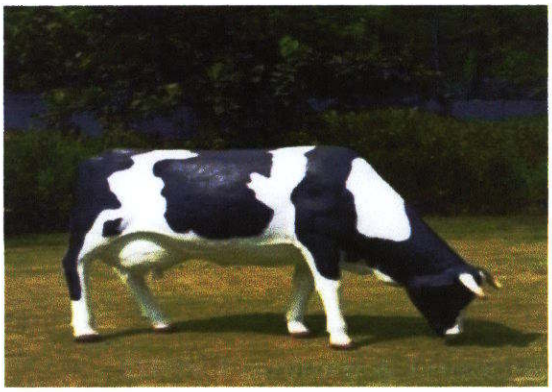

Fig 2. Pure Freshian, Australia

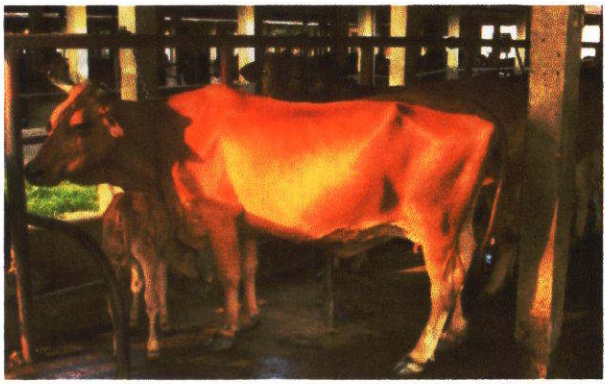

Fig 3. Local Pabna Cow, Bangladesh

Table-1: Sample identify of cattle obtained from Central Cattle Breeding & Dairy Farm, Savar, Dhaka.

SL. No.	Type of Cattle	Origin	Shade No.	Tag No.	Total No.	Sex
01	Sahiwal	Pakistan	01	645,586	02	Female
02	AFS AF(M) × S(F) Established Breed	Cross between AF×S Pakistan	03,14	757,712,754	03	Female
03	Freshian-50% AF(M) × L(F) ↓ F1 (50%)	Cross between AF×L	02	853,2627,9162	03	Female
04	Freshian-75% AF(M) × L(F) ↓ F1 (50%) × AF ↓ F2 (75%)	Cross between AF×L & F1×AF	03,12, 14	1620,1781,1630	03	Female
05	Freshian-62.5% AF(M) × L(F) ↓ F1 (50%) × AF ↓ F2 (75%) × F1 ↓ F3 (62.5%)	Cross between AF×L & F1×AF & then F2×F1 (Backcross)	03,12	2000,2303,1732	03	Female
06	AF×S×L	Cross within AF×S×L	01,12	1160,789,1360	03	Female
07	Local	Pabna	01	1315,1731, 1754	03	Female

3.2 Gel Documentation

Extracted DNA was confirmed by electrophoresis separation on 1% agarose gel staining with ethidium bromide.

3.3 Measuring of DNA Concentration and Quality

Spectrophotometer is commonly used in laboratories for the measurement of DNA concentration and purity. The concentration and purity of extracted DNA was measured by spectrophotometeric method. The DNA purity was measured by dividing the absorbance was taken at 260 nm and calculated the amount of DNA using the following formula:

DNA concentration = A260 × Dilution factor × Conversion factor

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

= (µg/ml)

= (ng/µl) [∴ µg/ml = ng/µl]

The quality of DNA was also measured by spectrophotometric method. The absorbance was taken at both 260 and 280 nm. The ratio 260/280 showed the range between of 1.8 – 2.0. Therefore, the extracted DNA was enough pure to carry out the RAPD analysis.

3.4 PCR Reaction

3.4.1 Primer Selection

In these study 2 RAPD primers was screened out of 12 primers. The primers sequences are shown in Table 3:

Table: 2. List and sequence of primers used in the experiment

Primer No.	Sequence (5'-3')	(G+C) Contents %
01	AATSSGCTGG	40%
02	TCAGCCACGT	60%
03	**TCTGCCATCC**	**80%**
04	GTTGCCACCC	60%
05	TCTGCCATCC	50%
06	**TTCCCCCCAG**	**70%**
07	TCTGCCATAC	70%
08	GGCCCTACTT	60%
09	CTTATCAGGG	70%
10	AACGGTGACC	60%
11	GGCCCTACAT	60%
12	TTGCGGCGAT	60%

3.4.2 PCR Amplification

The amplification conditions were based on Williams et al. (1990) with some modifications. PCR reactions were performed on each DNA sample in a 20 µl reaction mix containing 4ng genomic DNA, 2 µl of 10 reaction buffer, 2 U units of Taq DNA polymerase 1 µl (100picomol) of primer, 11 µl of sterile water. The PCR samples were overlaid with one drop of mineral oil. DNA amplification was performed by thermal cycler. 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 (extension) for 2 minutes. The last cycle was followed by 10 minutes incubation at 72°C and then held at 8°C.

3.4.3 Gel Electrophoresis of PCR Amplified Product

PCR products from each sample were confirmed by running 2 % agarose gel in TAE buffer at 40V for 80 minutes. Loading dye (3 µl) was added to the PCR products and loaded in the wells. Two molecular markers DNA (ladder) were also loaded on one side of the gel. After electrophoresis, the gel was taken out carefully from the electrophoresis tank and stained the gel with ethidium bromide. After staining the gel, DNA fragments were observed under UV transilluminator and photographed using gel documentation unit.

3.4.4 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, genetic distance (D) and constructing a UPGMA (Unweight Pair Group Method of Arithmetic Means) dendogram among the populations using computer program COMPONENT (Yeh et al. 1999).

Chapter Four

Results and Discussion

RESULTS AND DISCUSSION

4.1 DNA Extraction and Quantity Determination

Extraction of DNA was performed using Roe et al. 1996. The spectrophotometric absorbance readings at 260 nm of twenty experimental cattle are shown in Table 4.

Table-3: Spectrophotometric absorbance readings at 260 nm for DNA quantification experimental cattle.

Serial No.	Accession or Tag No.	Absorbance Reading at 260 nm	Quantity of DNA (ng/µl)
1	Sahi-645	0.056	277.20
2	Sahi-586	0.074	366.30
3	AFS-757	0.044	217.80
4	AFS-712	**0.031**	**153.45**
5	AFS-754	0.065	321.75
6	Fre 50%-853	0.075	371.25
7	Fre 50%-2627	0.062	306.90
8	Fre 50%-9162	0.049	242.55
9	Fre 75%-1620	0.067	331.65
10	Fre 75%-1781	0.080	396.00
11	Fre 75%-1630	0.045	222.75
12	Fre 62.5%-2000	**0.096**	**475.20**
13	Fre 62.5%-2303	0.033	163.35
14	Fre 62.5%-1732	0.067	331.65
15	AFSL-1160	0.048	237.60
16	AFSL-789	0.078	386.10
17	AFSL-1360	0.090	445.50
18	L-1315	0.046	227.70
19	L-1731	0.087	430.65
20	L-1754	0.048	237.60

N.B. - A-Australian, F- Freshian, S- Sahiwal, L-Local.

The higher DNA concentration was recorded in Freshian-62.5%-2000 cattle and the lowest DNA concentration was obtained from AFS-712 cattle. The quantified DNA sample subsequently diluted to 2ng/µl as per requirements for ideal PCR. The PCR did

run using twelve primers and among them two primers showed reproducibility. The PCR product reproducibility and banding pattern was confirmed by 2% agarose gel electrophoresis with 100 bp ladder.

4. 2 Molecular Characterization

In the present study, a polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD) marker was used to amplify cattle DNA using 12 arbitrary oligonucleotide primers. The method was able to detect the heterogeneity of amplified DNA from the cattle used in this study were polymorphic. The present study also indicates the effectiveness of RAPD analysis and the data recorded from DNA amplification were very sound for molecular characterization of twenty experimental cattle.

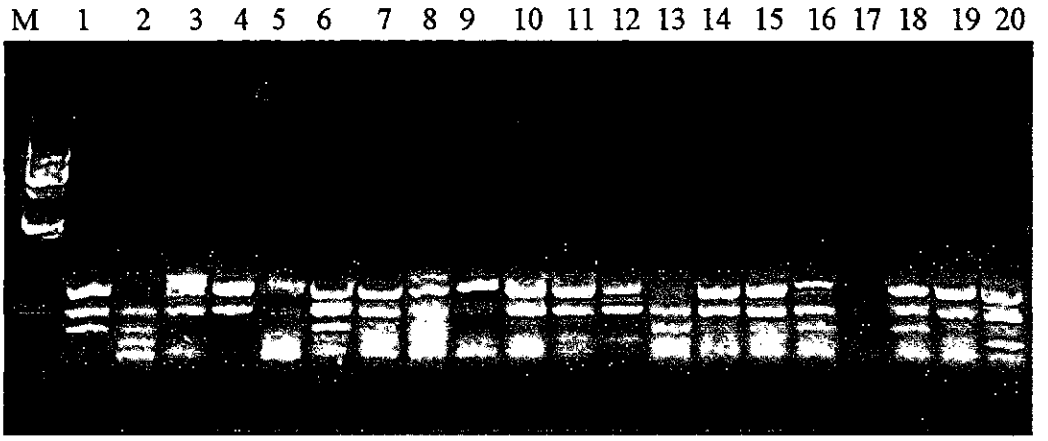

Fig. 4: RAPD profiles of twenty experimental cattle using primer 03. The number corresponds to the serial number of the genotypes Table-5.

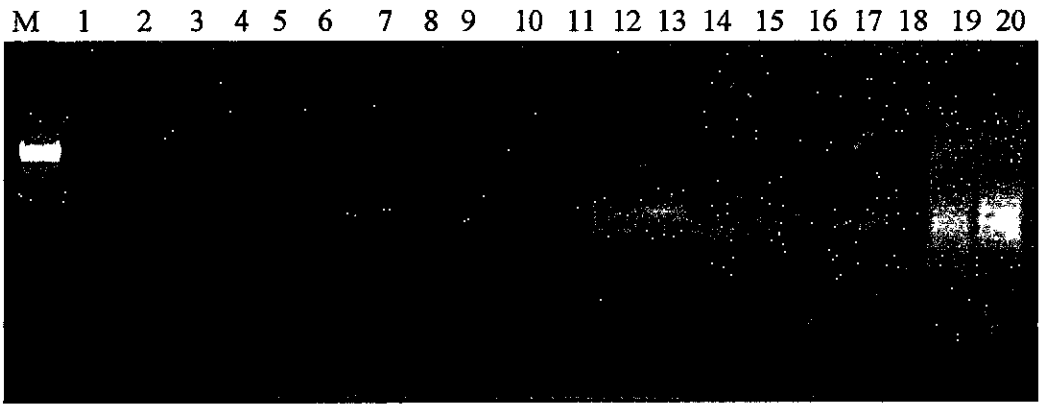

Fig. 5: RAPD profiles of twenty experimental cattle using primer 06. The number corresponds to the serial number of the genotypes Table-5.

4.2.1 DNA Fingerprinting

Twelve primers were selected that used in RAPD analysis of twenty cattle accessions amplified twenty different reproducible bands. Two different primers generated various banding patterns ranging from 1 to 8 and the average bands per primer was 2.65 and of the 106 bands scored, 95 (90.48%) were found to be polymorphic and 10 bands were found to be common in nature.

Two RAPD primers were used to detect the polymorphisms of experimental cattle. Each of two primers was reproducible and capable of exhibiting polymorphic amplification patterns. Only unambiguous and scorable polymorphic fragments were taken into consideration for analysis. The amplification patterns of representative samples of cattle with two different primers have been shown in Fig. 4 and Fig. 5. A total of 106 bands were amplified among cattle using two primers and the polymorphic bands were 95. Here the percentage of polymorphic bands was 90.48%. The frequencies of polymorphic bands varied from primer to primer. Though cattle number basis marker was identified, the high level of polymorphism revealed by the proportion of polymorphic loci (90.48%) indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in experimental cattle population. The cattle number could be distinguished by a combination of fragments and differences between clusters reflected differences in rather than presence or absence of accession specific fragments. List of primers their monomorphic and polymorphic bands are shown in Table-5.

Table-4: List of primers and their monomorphic & polymorphic bands

SL. No.	Tag No.	Primer Code	No. of Total Bands	Number of Common Bands	Number of Polymorphic Bands	Proportion of Polymorphic Loci (%)
01	02	03	04	05	06	07
1	Sahi-645	3 & 6	5	0	5	100
2	Sahi-586	3 & 6	6	0	6	100
3	AFS-757	3 & 6	7	0	7	100
4	AFS-712	3 & 6	3	0	3	100
5	AFS-754	3 & 6	5	1	4	80
6	Fre 50%-853	3 & 6	3	1	3	100
7	Fre 50%-2627	3 & 6	6	1	5	83.33
8	Fre 50%-9162	3 & 6	4	0	4	100
9	Fre 75%-1620	3 & 6	5	0	5	100
10	Fre 75%-1781	3 & 6	4	0	4	100
11	Fre 75%-1630	3 & 6	4	0	4	100
12	Fre 62.5%-2000	3 & 6	4	0	4	100
13	Fre 62.5%-2303	3 & 6	7	3	4	57.14
14	Fre 62.5%-1732	3 & 6	8	2	6	75
15	AFSL-1160	3 & 6	7	1	6	85.71
16	AFSL-789	3 & 6	5	0	5	100
17	AFSL-1360	3 & 6	5	0	5	100
18	L-1315	3 & 6	6	1	5	83.33
19	L-1731	3 & 6	5	0	5	100
20	L-1754	3 & 6	5	0	5	100
	Total	2*20=40	106	10	95	1864.51
	Average		2.65	0.25	2.38	90.48

N.B. A-Australian, F- Freshian, S- Sahiwal, L-Local.

Mufti et al. (2009) reported polymorphism and genetic relationship in different cattle populations using primers and showed a total of 92% which is higher than 83.33%. It suggests that within this period of time, the gene segregation rate has increased in these animals. In another study involving indigenous African Cattle, Gawkisa et al. (1994) used RAPD markers to characterize the local cattle (*Bos indicus*) of Tanzania (using ILO 1127 primer) and showed 72% polymorphisms which is almost similar to the present study. The degree of polymorphisms 61% compared to 29% in Taurine Cattle population (Talle et al. 2005). Estimation of higher level of genetic variation in the breeds might be consistent with the fact that they are highly polymorphic animals.

4.2.2 Genetic Distance Determination

Estimates of (Nei's & Li, 1979) genetic distance calculated all possible pairs-wise genetic distance values by the following formula:

$$d_{xy} = 1 - \{2n_{xy}/(n_x + n_y)\}$$

Where, n_x and n_y are the numbers of bands amplified in individuals x and y, respectively, and $2n_{xy}$ is the number of bands shared by those individuals.

Table-5: Estimation of pair wise genetic distance among experimental cattle using Nei's equation

Sample No.	Sahi-645	Sahi-586	AFS-757	AFS-712	AFS-754	Fre 50%-853	Fre 50%-2627	Fre 50%-9162	Fre 75%-1620	Fre 75%-1781	Fre 75%-1630	Fre 62.5%-2000	Fre 62.5%-2303	Fre 62.5%-1732	AFSL-1160	AFSL-789	AFSL-1360	L-1315	L-1731	L-1754
Sahi-645	***																			
Sahi-586	.09	***																		
AFS-757	.333	.23	***																	
AFS-712	.272	.166	.076	***																
AFS-754	.4	.454	.50	.455	***															
Fre 50%-853	.25	.333	.40	.333	.25	***														
Fre 50%-2627	.45	.333	.384	.333	.273	.33	***													
Fre 50%-9162	.333	.20	.213	.20	.333	.14	.20	***												
Fre 75%-1620	.4	.20	.166	.090	.4	.25	.27	.11	***											
Fre 75%-1781	.111	.20	.273	.20	.333	.14	.40	.25	.33	***										
Fre 75%-1630	.111	.20	.273	.20	.333	.14	.40	.25	.33	0	***									
Fre 62.5%-2000	.333	.384	.429	.20	.167	.40	.38	.45	.50	.27	.27	***								
Fre 62.5%-2303	.5	.538	.715	.538	.333	.60	.38	.64	.67	.64	.64	.29	***							
Fre 62.5%-1732	.384	.428	.333	.286	.231	.45	.42	.50	.47	.33	.33	.07	.33	***						
AFSL-1160	.333	.385	.429	.358	.16	.04	.38	.45	.50	.27	.27	0	.29	.07	***					
AFSL-789	.4	.455	.667	.456	.24	.50	.45	.56	.60	.56	.56	.33	.17	.38	.33	***				
AFSL-1360	.80	**.819**	.333	.637	.4	.75	.63	.78	.60	.78	.78	.33	.33	.23	.33	.40	***			
L-1315	.27	.667	.348	.50	.091	.33	.33	.40	.45	.40	.40	.23	.23	.29	.23	.09	.45	***		
L-1731	.2	.090	.167	.09	.4	.25	.20	0	.20	.11	.11	.33	.67	.27	.33	.60	.80	.45	***	
L-1754	.333	.728	.167	.111	.3	.50	.40	.25	.40	.33	.33	.50	.67	.54	.50	.60	.80	.64	.20	***

There was a high level of genetic variation among the studied accessions of cattle as indicated by the proportion of polymorphic loci. Estimation of higher level of genetic variation in the cattle 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 among 20 different cattle genotypes were computed from combined data sets for the 2 primers ranging from 0.0 to 0.82. Comparatively higher genetic distance (0.82) was found in AFSL-1360. The lowest genetic distance (0) was revealed between Fre 75%-1630, AFSL-1160 and Fre 75%-1781, Fre 62.5%-2000. The range of genetic distance of experimental different cattle genotypes is (0±0.82) and the difference between the highest and lowest genetic distance indicated the presence of variability among the experimental cattle.

In this study there was a high level of genetic variation among the studied samples of cattle as indicated by the proportion of polymorphic loci. Estimation of higher level of genetic variation in the experimental cattle might be consistent with the fact that it is a highly polymorphic animal (Kemp and Teale, 1994). They estimated the allele frequency characteristics of cattle using DNA markers (Buitkamp et al. 1996). More or less similar values of heterozyosity varying from 0.010 in the pantagonia Creole Cattle to 0.843 in the Argetinean Creole Cattle breed were reported by Liron et al. (2002) where they used five loci related to milk production and found similar genetic distances of average animals like the present experiment.

The selected primer can be used for analysis in other cattle germplasm. The wide range of dissimilarity values 0.00 to 1.00 suggests that the germplasm collection represents a wide genetically diverse population. RAPD markers are randomly distributed throughout the genome, but most of the genome (90%) is not expressed at the phenotypic level (Williams et al. 1990). As a result, markers like RAPDs may accurately assay the degree of genetic change between two genomes, but they may be not necessarily reflecting the divergence in term of changes in traits of agronomic importance.

For a more efficient determination of genetic diversity in cattle verities, a higher number of decamer primers showing maximum number of polymorphic band or other available marker systems could be utilized. Nevertheless, the information about the genetic diversity obtained from the present experimental animals could be used

successfully in future breeding and germplasm conservation programme for cattle production systems of Bangladesh like high yielding milk and meat production animals of Bangladesh.

4.2.3 Cluster Analysis of the Genotypes Based on RAPD Analysis

Component dendrogram was constructed according to the morphological data. The dendrogram shows that all the cattle were grouped into two major clusters (I) and clusters (II).

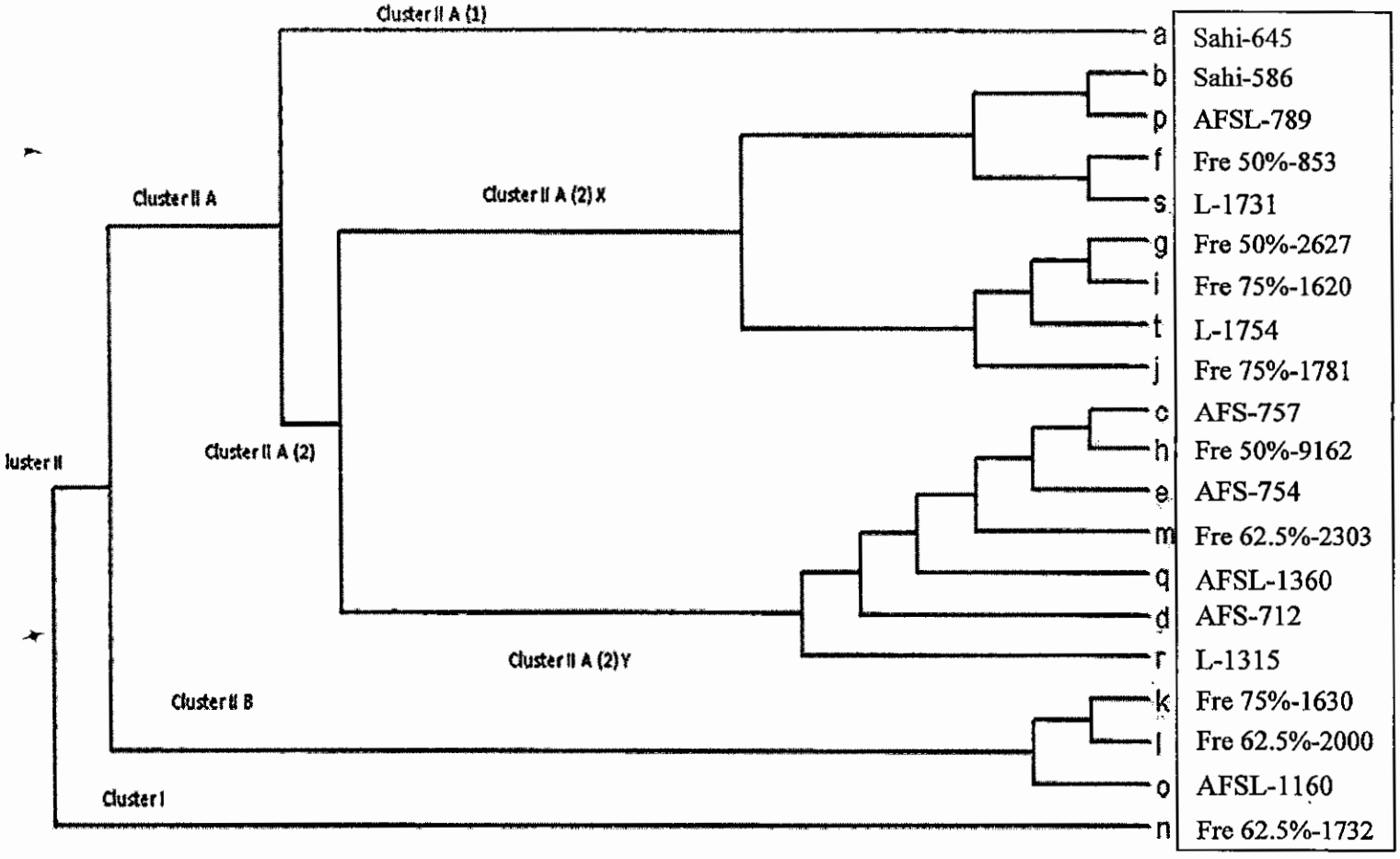

Fig. 6: The COMPONENT dendrogram summarizing the data on differentiation among twenty experimental cattle.

From the Figure-5 and Table-7, it was observed that cluster (II) sub divided into II (A) and II (B). Again, cluster II (A)-subdivided into II A (1) and II A (2). II A (2) subdivided to II A (2)X and II A (2)Y. However, due to the higher genetic distance, Fre 62.5%-1732 breeds clustered separated with cluster I cluster from II. Under cluster–II are showed

genetically similar. So they grouped into same cluster. Under cluster-I breed 14 genetically different from others breeds. Cluster II A are showed genetically different from cluster II B. II A (2) genetically same characteristics but different from II A (1). II A (2) X genetically same characteristics but different from sub-cluster II A (2) Y.

Table-6. Distribution of twenty experimental cattle samples under different clusters

Cluster No.			Total No. of Accession in Cluster	Accessions Included in Different Clusters
I			1	Fre 62.5%-1732
II	II A	II A(1)	1	Sahi-645
		II A(2)	15	Sahi-586, AFS-757, AFS-712, AFS-754, Fre 50%-853, Fre 50%-2627, Fre 50%-9162, Fre 75%-1620, Fre 75%-1781, Fre 62.5%-2303, AFSL-789, AFSL-1360, L-1315, L-1731, L-1754
	IIB	II B	3	Fre 75%-1630, Fre 62.5%-2000, AFSL-1110

The higher genetic distance due to indiscriminate breeding/crossing, immigration of animals, mutation in ancestors or due to different origin. The highest gene diversity indicates that the population is alarming to deteriorate with native or exotic blood than other population. One the other hand, the low genetic distance due to their same origin as well as lower genetic variability. In another similar study, Yu et al. (2004) used RAPD analysis to estimate genetic diversity and relationship of two native cattle breeds from the Yunnan province of China (De Hong cattle and Di Qing cattle) and four introduced beef cattle breeds (Brahman, Simmental, Murry Grey and Short Horn). Using 10 primers, it was observed that the Yunnan De Hong cattle breed was closely related to the Braham (*Bos indicus*), and the Yunnan Di Qing cattle breed was closely related to the Simmental, short horn and Murry grey (*Bos taurus*) beeds.

4. 3 Summary and Conclusion

In this experiment only 90.48% polymorphism are identified among the selected cattle breeds, which is high when compared with the result of those experiment conducted among cattle breeds. This high level of polymorphism may be due to their cross breeds among different population of different breeds. However, dendrogram show almost same relationship among the selected breeds of cattle and in both cases different clusters were formed. There is scope of cross breeding programmes for future improvement of local cattle to yield of high milk, meat products and conversation strategy.

Genetic variation is important because it provides the "raw material" for natural selection. Genetic variation is caused by variation in the order of bases in the nucleotides in genes. Genetic variation among individuals within a population can be identified at a variety of levels. It is possible to identify genetic variation from observations of phenotypic variation in either quantitative traits or discrete traits. The genetic variation in Indigenous, Cross Breed and Sahiwal Cattle is shown as Nei's gene diversity and proportion of polymorphic bands in Table-5. Table-6 shows that the Nei's gene diversity was more or less similar for all populations.

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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 dodeocyle 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	Hinweis
Agar	Invitrogen[TM] Life Technologies
Gel Loading Dye	Invitrogen[TM] Life Technologies
Primer	Bionere
dNTPs	Bionere
Taq Polymerase	Bionere

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	Froilabo
Polaroid camera	Sony
Micropipette	Eppendorf
Steri Pack Syringe	Opso Saline
Tips	Eppendorf
UV Transilluminator	UVP
Vortex Mixer; Mode:Vm-2000	STUART
Water Bath	LABSCO
PCR Machine	Bionere

Appendix -4

Protocol for DNA Isolation: Roe *et. al.*

1. Obtain the liquid blood samples in EDTA Vaccutainer tubes and store at -20°C freezer.
2. Thaw the frozen samples; add 0.8ml of 1X SSC buffer with 1 ml of blood in a 1.5 ml centrifuge tube and mix well by overtaxing. Centrifuge at 12000 g for 1 min.
3. Remove 1ml of the supernatant and discard into disinfectant.
4. Add 1ml of 1X SSC buffer, vortex, and centrifuge as above for 1 min and remove all of the supernatant.
5. Add 375µl of 0.2M sodium acetate to each pellet and vortex briefly. Then add 25µl of 10% SDS and 5µl of proteinase K, vortex briefly and incubate for 1 h at 55°C.
6. Add 120µl of phenol: chloroform: isoamyal alcohol and vortex for 30 sec. Centrifuge the sample for 2 min at 12000 g.
7. Carefully remove the aqueous layer to a new 1.5 ml microcentrifuge tube, add 1 ml of ice cold 100% ethanol, mix and incubate for 15 min at -20°C.
8. Centrifuge for 2 min at 12000 g. Decant the supernatant and drain.
9. Add 180 µl of TE (10:1) buffer, vortex and incubate at 55°C for 10 min.
10. Add 20 µl of 2M sodium acetate and mix, add 500 of cold 100% ethanol, mix and centrifuge for 1 min. at 12000 g in a microcentrifuge.
11. Decant the supernatant and rinse the pellet with 1ml of 80% ethanol. Centrifuge for 1 min. at 12000 g in a microcentrifuge.
12. Decant the supernatant, and dry the pellet in a Speedy-Vac for 10 minutes (or until dry).
13. Resuspend the pellet by adding 200 of TE (10:1) buffer. Incubate overnight at 55°C, overtaxing periodically to dissolve the genomic DNA. Store the samples at -20^0 C.