

MOLECULAR CHARACTERIZATION OF SELECTED LOCAL AND EXOTIC CATTLE USING RAPD MARKERS

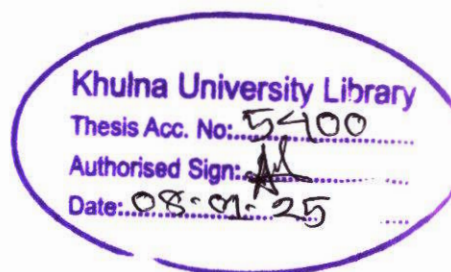
A Thesis

by

MISS. MAHFUZA KHATUN

Examination Roll No: MS- 090705

Session: 2008-2009



Submitted to

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**BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE
LIFE SCIENCE SCHOOL
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KHULNA-9208
BANGLADESH
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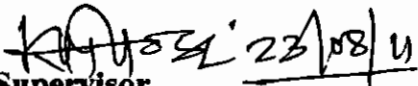
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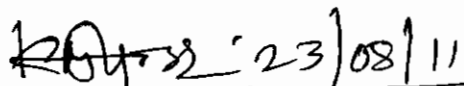
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DEDICATED TO

MY BELOVED PARENTS

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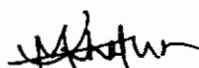
Finally, the author likes to deserve her deepest appreciation to her parents, sisters and brothers for their help, moral encouragement, admiration, understanding and all kind of logistic support.

The Author

June, 2011

DECLARATION

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 The findings of the research has not been/will not be published anywhere without the concurrence of the concerned supervisor.



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ABSTRACT

Cattle is a potential genetic resource in Bangladesh having dairy potentialities, high resistance to disease and survive harsh environmental condition while require low input supply. In order to develop the specific genetic marker for Bangladeshi native cattle and exotic breeds, randomly amplified polymorphic DNA (RAPD) analysis of local Pabna, Red Chittagong and Sahiwal cattle was performed using 12 primers. The major objective of the study was to find out the genetic diversity of selected cattle population of Bangladesh and to suggest their improvement and conservation program. The experiment was conducted in Animal Cell Culture and Molecular Biotechnology Laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh. Different blood samples were collected from Bangladesh Livestock Research Institute, cattle farm Savar, Dhaka. DNA was extracted from 20 local and exotic cattle blood samples and the extracted DNA was observed by gel electrophoresis. Randomly 12 primers were tested among them three primers were matched and found polymorphic. Random Amplification of Polymorphic DNA and Polymerase Chain Reaction analysis were carried out using DNA samples of the cattle. Genetic relations between breeds were determined by RAPD polymorphisms from a total of 56.73% loci. Statistical analysis of the data, estimating the genetic distances among cattle and sketching the cluster trees were performed using COMPONENT method. Comparatively the highest genetic distance 0.8387 was found between SL-623 and RCC-82. The lowest genetic distance 0.0303 was observed between M-5730 and M-1222. The difference between the highest and lowest genetic distance indicated the presence of variability among the 20 genotype of cattle. The study showed that the polymorphisms obtained using RAPD-PCR enabled to determine of the genetic relationships and fingerprints of the cattle population. Adequate diversity in performance and adaptability can be exploited from the study results for actual improvement accruing to conservation and development of indigenous cattle resources. The genetic diversity of Red Chittagong and Sahiwal cattle was relatively higher for a prescribed breed. Therefore, to meet up the demand of dairy and beef industry of Bangladesh, the findings of the present study have an opportunity to improve the present cattle population of Bangladesh using selective breeding programmes like artificial insemination, multiple ovulation and embryo transfer, *in vitro* fertilization etc.

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CHAPTER ONE

INTRODUCTION

highest amount of genetic diversity in these populations of livestock is found in the developing world, where record keeping is poor but the risk of extinction is high and is increasing. Recently, loss of genetic diversity within indigenous livestock breeds has been a major concern. *Bos primigenius* is a potential genetic resource in Bangladesh which is now disappearing due to indiscriminate crossing with other local and exotic cattle. It is speculated that the Red Chittagong was evolved by interbreeding among them on the basis of natural selection. Sahiwal are a breed of zebu cattle, primarily used in dairy production, from the Sahiwal district of Pakistan, a dry region of Punjab which lies along the Indian-Pakistani border (Mason, 1996). The Bangladeshi indigenous cattle breed (Pabna) and Red Chittagong breed, are of particular importance because of its favorable meat, milk, leather and draft quality although its productivity is relatively low in general. 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. Thus, it is important to identify genetic characteristics of this breed for establishing breeding parameters and conservation.

Breed characterization requires knowledge of genetic variation that can be effectively measured within and between populations (Hetzl and Drinkwater, 1992), for example, in terms of mitochondrial and nuclear DNA polymorphisms (Rassmann *et al.*, 1991; Suzuki *et al.*, 2000; Tautz, 1992). A recent technique capable of detecting DNA polymorphism is based on random amplification by the polymerase chain reaction (PCR) of DNA segments with short oligonucleotide primers, and annealing PCR (RAPD-PCR), has been successfully applied in genetic studies of various species (Michelmore *et al.*, 1991; Welsh and McClelland, 1991), as well as for characterization of bovine populations (Bardin *et al.*, 1992; Kemp and Teale, 1994). Different primers produce different RAPD polymorphisms, the variations being due to differences in spacing between primer binding. However, the ease with which many RAPD polymorphic loci can be identified without primer sequence information, and the direct reading of results from agarose gels, make the RAPD techniques useful in large outbreed populations. Here, we report results of applying the RAPD-PCR technique to the differentiation of local cattle (*Bos indicus*: Pabna, Red Chittagong) as well as exotic breed (*Bos taurus*: Sahiwal) which represent valuable animal genetic resources in Bangladesh. The primary aim of studying genetic diversity is to understand the extent of differentiation of populations within species. Population-specific genetic markers (alleles) can be generated using a range of methods available for detection of polymorphic loci. Polymorphic genetic markers are extremely useful for a number of applications, such as measurement of the amount of genetic diversity in species, discrimination between individuals, strains or species, identification of markers linked to

economically useful traits as well as analyses of animal kinship relationships, behavioural and population ecology. In the past decade there have been tremendous efforts globally to study genetic diversity in livestock species. The PCR-based RAPD technique is an attractive complement to conventional DNA fingerprinting. 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; Rothuizen and Van Wolferen, 1994).

Analysis of genetic polymorphism in higher organisms has been widely employed in estimating the genetic relatedness and diversity within and among species. Especially genetic markers for native cattle's in each country were strongly investigated for the improvement of their native cattle breeds including Bangladeshi Red Chittagong Cattle (RCC), Local breed (Pabna), Japanese Black cattle (Wagyu), Germany native cattle, Indian Zebu cattle (Sahiwal), *Bos indicus*, and *Bos taurus*. The simplicity and applicability of the RAPD technique have captivated many scientists' interests. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterisation of the genome of the species. Assessment of genetic variation is a crucial element in determining breeding strategies and for effective and meaningful conservation program (Bjornstad *et al.*, 2001). 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 (Cornuet *et al.*, 1999). 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. In an attempt to solve the problem of maintaining pure breeds using the observed morphological characteristics that require a lot of time and effort, the use of molecular markers in maintaining cattle breeds is more suitable and less time consuming. Moreover, molecular markers are important tools in tagging desirable loci underlying the traits which have breeding importance.

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 (FAO, 2004) for detection of genetic variations in cattle and sheep (Feral, 2002). 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, RAPD markers, generated by the Polymerase Chain Reaction (PCR) is widely used since 1990's to assess intraspecific genetic variation at nuclear level (Garrigan *et al.*, 2002). 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. Therefore, in order to determine the genetic variability of selective native/local, crossbred and exotic cattle population of Bangladesh, the research was undertaken with the following objectives:

1. To determine the genetic variability and relationship of selected local and exotic cattle.
2. To evaluate genetic distance among experimental cattle populations.

CHAPTER TWO

REVIEW OF LITERATURE

CHAPTER TWO

REVIEW OF LITERATURE

A conceptual framework for the study based on the ideas and concepts gathered from review work of existing literature of both theoretical and empirical nature will facilitate planning the study in a comprehensive manner. It also helps to know the previous research work carried out in the area and acts as a torch for new research. Isolation of genomic pure DNA and RAPD analysis have been used species identification, establishing genetic relationships, estimating genetic diversity on various livestock species. Since their domestication around 7000 to 9000 BC, sheep, goat, cattle have significantly influenced the economic, social and cultural fabric of human society. However, molecular characterization and its utilization are limited in Bangladesh. Hence, an attempt is made here to put together some of the closely related findings about the genetic diversity and physicochemical analysis in general and has been reviewed in this chapter.

2.1 Origin and distribution

The 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.

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. Habib *et al.* (2003) observed that the body length, body height and heart girth of adult Red Chittagong 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.

2.3 Reproductive characteristics

In 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. 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 (Ahmed, 2000). This increase was due to recent Government policy (Government subsidies to establish small dairy farms, soft fund loans from the Government as well as to improve veterinary health care) in dairy development activities. The mean estimates for milk production traits were 1368kg, 282 days and 4.9kg for lactational milk yield, lactation length and test-day milk yield, respectively. For reproductive traits of Sahiwal breed, mean estimates were 468 days, 2.2 and 1345 days for calving interval, number of services per conception and age at first calving, respectively (Ilatsia *et al.*, 2007). There was a decline in lactation milk yield and lactation length, and an increase in calving interval and age at first calving over the years. Satisfactory management and appropriate genetic improvement strategies would result in improved performance. However, to improve the production potentialities of the local cattle, efforts were made to cross breed with different exotic breeds several times in the past. The introduced breeds are Holstein-Friesian, Jersey, Sahiwal, Harians, Sindhi, Australian, Sahiwal-Friesian etc. A number of exotic pure breeds, their crossbreeds, and up-graded cattle are found in the government dairy farms, commercial dairy farms, milk pocket area, and in urban and semi-urban areas of Bangladesh. (Mufti *et al.*, 2009; Metta *et al.*, 2004).

2.4 Measures of genetic variation and differentiation in cattle

It is generally accepted that genetic variation is the raw material of evolution, without which populations cannot evolve in response to changing environments. The knowledge of how genetic variation is partitioned among populations may have important implications not only in evolutionary biology and ecology, but also in conservation biology. In order to understand the amount of genetic variation in a population, methods are required to quantify this information.

Although several measures have been used to describe genetic variation at a single locus or a number of loci, heterozygosity has remained the most widely used (Hedrick, 2005). Heterozygosity refers to the state of being heterozygous and it is believed to be a good predictor of chances for long-term survival of a population. This is because it reflects the number of genetic options available within a population. Another measure that has sometimes been used to measure genetic variation is the number of alleles observed at a given locus in a population. In a recent study in cattle, Zhou *et al.* (2005) used all these three measurements to analyze genetic diversity in breeds native to China. Based on polymorphisms of 10 microsatellite loci, they examined the genetic diversity among five native Chinese cattle breeds and also estimated the genetic differentiation and relationship within and between breeds. Distance based methods have been widely used to measure genetic differentiation among populations. These methods involve calculating pair wise distance matrices, whose entries give an estimate of the distance between pairs of individuals. Amongst distance methods, Nei's genetic distance (Nei, 1972) has been the preferred approach. Recently, MacNeil *et al.* (2006) used Nei's genetic distance metric to calculate the relationships among cattle in Chirik Island, off the coast of Alaska, and commercial breeds in North America. However, for closely related breeds within which drift is important, particularly in the developing world the modified Cavalli-sforza distance is recommended (Nei and Tatenno, 1983).

2.5 Molecular characterization of Cattle

Genetic marker types 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 (Jianlin *et al.*, 2004). 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), population origin (Cornuet *et al.*, 1999), inbreeding status (Ellegren, 1999; Lynch and Ritland, 1999), and sex-specific gene flow. According to Hanotte *et al.* (2002), 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.* (1997) 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 (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 shown that the Yunnan DeHong cattle breed was closely related to the Brahman (*Bos indicus*), and the Yunnan DiQing cattle breed was closely related to the Simmental, short horn, and Murry grey (*Bos taurus*) breeds. Under the FAO (2000) 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 homozygotes, which decreases their information content. However, RAPD analysis could benefit most of 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, 2002). 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.* (1997) 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 provide a resource for scientists interested in germplasm conservation and add to previous reports of genetic variation in Tanzanian and Kenyan cattle.

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 and 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.

RAPD, a random primer-based molecular technique, has also been used to assess diversity in plants and animals (Williams *et al.*, 1990). Hassen *et al.* (2007) used RAPDs to assess genetic variability within and among five indigenous Ethiopian cattle breeds. The analysis revealed that within breed genetic variation was much higher than that between breeds. Mbulu and Hannotte (2005) described a study that looked at genetic diversity and population structure in 52 populations of African cattle using 15 microsatellite markers. The study indicated a wide range of genetic diversity among African cattle based on mean number of alleles, effective number of alleles, and expected heterozygosity. Globally, there has been an increase in awareness about the

importance of indigenous animal breeds and the need to properly utilize and manage these resources. Awareness of the value of genetic resources in livestock has stimulated the study of the genetic diversity of native breeds. Most of such studies have been done on European cattle breeds and very little information is available concerning the genetic diversity of cattle breeds' native to Africa. However, recently there has been an increase in the number of programs and studies on livestock genetic diversity in several African countries. In most African countries including Zambia, much of this effort is attributable to the Food and Agriculture Organization (FAO, 1999) of the United Nations global program that addresses the needs of both development and conservation of animal genetic resources in different parts of Africa.

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 maximising the improvement of the major economic traits of animals in the future.

Kosgey *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.

Giovambattista *et al.* (2001) reported that early attempts at finding markers for quantitative trait loci (QTL) in the 1950s and 1960s involved searching for associations between production traits and polymorphisms at loci encoding blood groups, milk proteins and blood proteins. By the mid-1990s, linkage maps comprising mainly microsatellites covered most regions of most

chromosomes of the cow, and it was then possible to hunt for QTL using a mapping approach first proposed in *Drosophila* back in 1961. Fine mapping of QTL has eventually resulted in the identification of markers in or near particular genes. Some of these markers have been commercialized. In most cases, the actual causative quantitative trait nucleotide (QTN) remains elusive.

Marle-Koster *et al.* (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 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.

Bruford *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

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MATERIALS AND METHODS

The experiment was carried out at the Animal Cell Culture and Molecular Biology Laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh. The details of materials and methods used are described with headings and sub-headings as follows:

3.1 Animal selection and blood sample collection

A total of 20 cattle were selected for this study in which five native (Pabna), five Sahiwal (exotic) and ten Red Chittagong (Local cross-bred) cattle. Three ml of blood was collected from each animal in a 5 ml EDTA vacutainer. The blood was gently mixed with EDTA (present in the vacutainer) and kept on ice box to maintain low temperature. Subsequently the blood samples were transported to the laboratory and stored at -20°C for further use. The cattle's accession or number, sampling location and name of cattle are presented in Table 1.

Table 1: Cattle's accession or tag no., source and identity

Serial No.	Accession or tag no.	Source	Identity
1.	RCC -173	BLRI, Savar, Dhaka	Red Chittagong
2.	RCC -178	BLRI, Savar, Dhaka	Red Chittagong
3.	RCC -691	BLRI, Savar, Dhaka	Red Chittagong
4.	RCC -175	BLRI, Savar, Dhaka	Red Chittagong
5.	RCC -609	BLRI, Savar, Dhaka	Red Chittagong
6.	RCC -77	BLRI, Savar, Dhaka	Red Chittagong
7.	RCC -78	BLRI, Savar, Dhaka	Red Chittagong
8.	RCC -80	BLRI, Savar, Dhaka	Red Chittagong
9.	RCC -81	BLRI, Savar, Dhaka	Red Chittagong
10.	RCC -82	BLRI, Savar, Dhaka	Red Chittagong
11.	SL-571	BLRI, Savar, Dhaka	Sahiwal (Exotic)
12.	SL-614	BLRI, Savar, Dhaka	Sahiwal (Exotic)
13.	SL-623	BLRI, Savar, Dhaka	Sahiwal (Exotic)
14.	SL-637	BLRI, Savar, Dhaka	Sahiwal (Exotic)
15.	SL-638	BLRI, Savar, Dhaka	Sahiwal (Exotic)
16.	M-1222	BLRI, Savar, Dhaka	Native (Pabna)
17.	LF-1414	BLRI, Savar, Dhaka	Native (Pabna)
18.	M-1893	BLRI, Savar, Dhaka	Native (Pabna)
19.	M-1980	BLRI, Savar, Dhaka	Native (Pabna)
20.	M-5730	BLRI, Savar, Dhaka	Native (Pabna)



Figure1. Red Chittagong cattle

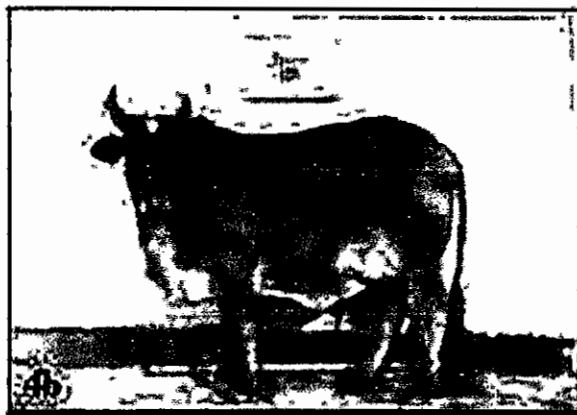


Figure 2. Local (Pabna) cattle



Figure 3. Sahiwal breed



Figure 4. Blood collection of cattle from BLRI farm

3.2 Preparation of Stock Solutions and Working Solutions

For conducting the isolation procedure, the list of stock solutions and their concentrations are presented in Appendix 2.

3.3 Protocol Used for Genomic DNA Isolation from Blood Sample

This method was adopted from Bruce A. Roe (1996) with little modification from step 7 to step

13. Protocol of this method is described below:

3.3.1. Protocol

1. The liquid blood samples were obtained in EDTA Vacutainer 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. Samples were centrifuged at 12000 rpm for 20 min.
3. One ml of the supernatant was discarded.
4. One ml of 1x SSC buffer was added, vortexed, and centrifuged as above for 10 min and all of the supernatant was removed.
5. Four hundred 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 1.5 h at 55°C.
6. Five hundred µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and vortexed for 30 sec. The sample was centrifuged for 20 min at 12000 rpm.
7. The aqueous phase was carefully removed to a new 1.5 ml microcentrifuge tube; 1 ml of ice cold propanol was added, mixed and incubated for 10 min on ice.
8. Then the samples were centrifuged for 12 min at 12000 rpm. The supernatant was decanted.
11. One ml of 70% ethanol was added and centrifuged for 10 min. at 12000 rpm in a microcentrifuge.
12. The supernatant was decanted, and pellet was dried at room temperature for 10 minutes.
13. The pellet was dissolved by adding 50 µl of TE buffer. Then it was incubated for 30 minutes at 55°C, vortexing periodically to dissolve the pellet. The samples were store at -20° C for further use.

3.4. Quantitation of Isolated DNA

In a clean microcentrifuge tube 20 μ l DNA solution was mixed with 1980 μ l of sterile distilled water (total 2 ml). Standard was made by adding 20 μ l TE buffer in 1980 μ l sterile distilled water. The quantity of DNA was measured by spectrophotometer at 260 nm wavelength. Using the absorbance readings at 260 nm, the original sample concentrations were determined according to the following formula:

$$\text{DNA concentration} = A_{260} \times \text{Dilution factor} \times \text{Conversion factor}$$

$$\begin{aligned} &= A_{260} \times \frac{\text{Volume of distilled water } (\mu\text{l})}{\text{Amount of the DNA sample } (\mu\text{l})} \times 50 \\ &= (\mu\text{g/ml}) \\ &= (\text{ng}/\mu\text{l}) \quad [\because \mu\text{g/ml} = \text{ng}/\mu\text{l}] \end{aligned}$$

Here,

A_{260} = Spectrophotometric absorbance reading at 260 nm of DNA sample.

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

Conversion factor 50 = The 50 $\mu\text{g/ml}$ of DNA contained in a solution which gives the Spectrophotometric absorbance reading at 260 nm equal to 1.

3.5 PCR reaction

3.5.1 Primer selection

In this study 3 RAPD primers were screened out of 12 primers (Bioneer). The primers have shown in Table 2.

Table 2. List and sequences of primers used in the experiment

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

3.5. 2. PCR amplification

The amplification conditions were based on Williams *et al.* (1990). PCR reactions were performed on each DNA sample in a 20 μ l reaction mix containing 2 μ l of template (4ng DNA), 2 μ l of 10x reaction buffer, 2 μ l of dNTPs (250 mM each), 2 units of Taq DNA polymerase, 1 μ l (100picomol) of primer, 11 μ l of sterile distilled water. DNA amplification was performed by thermal cycler (My Genie 96). The amplification program included an initial denaturation step of 94°C for 5 minutes (one cycle) followed by 40 cycles of 94°C (denaturation), 1 minute; 37°C (annealing), 1 minute and 72°C (extention) for 2 minutes. The final extension was performed at 72°C for 15 min (one cycle) and held at 8°C.

3.6. Preparation of 2% Agarose Gel

For agarose gel electrophoresis, 100 ml 2% agarose gel was prepared. For casting the gel the following steps were maintained:

- Initially, 2 g agarose powder was weighed out and placed into a 250 ml conical flask, containing 100 ml sterile water.
- Then 700 µl of electrophoresis buffer (50 × TAE buffer) was added into the flask. The agarose was melted by boiling using a microwave oven.
- When the agarose solution was cooled to about 50°C, 2 µl (10 mg/ml) solution of ethidium bromide was added in the gel to adjust the final concentration of ethidium bromide (0.5 µg/ml).
- Comb was set carefully attached to the tray before pouring the gel into it. The gel was polymerized within 30 minute at room temperature.

3.6.1. Gel electrophoresis of PCR amplified Product

PCR products from each sample was 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, washed with 7 µl ethidium bromide, mixed with distilled water (500 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.

3.6.2 Data analysis

Although a large number of fragments were generated from each primer, only clearly distinguishable and reproducible bands were considered and data was entered in a computer file as a binary matrix "0" coded for absence and "1" for presence of a band. 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 (1972) gene diversity, gene frequency, genetic distance (COMPONENT, Version 1.31) (Yeh *et al.*, 1999). Dendrogram was constructed using Unweighted Pair Group Method using Arithmetic Averages (UPGMA).

CHAPTER FOUR

RESULTS AND DISCUSSION

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Data regarding molecular characteristics were used to evaluate different levels of variability among the experimental cattle populations.

4.1. DNA extraction and Quantity determination

Extraction of DNA was performed using modified protocol of Bruce A. Roe (1996). The quality of DNA was confirmed by 2 percent Agarose gel electrophoresis (Figure5).

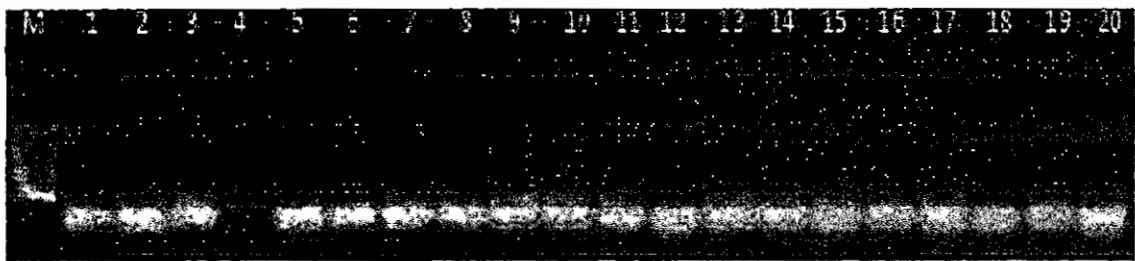


Figure 5. The gel profile of DNA extraction of experimental cattle. The number corresponds to the serial number of the genotypes shown in Table 1; M is a HindIII DNA size marker.

PCR is the technique of choice for nucleic acid quantification. The DNA quantity was determined by spectrophotometer absorbance at 260 nm and subjected to PCR amplification. However, successful quantification depends crucially on the quality of the sample DNA. The Spectrophotometer absorbance readings at 260 nm of twenty experimental cattle shown in Table 3. Using the absorbance readings at 260 nm, the original sample concentrations were determined according to the following formula:

DNA concentration = $A_{260} \times \text{Dilution factor} \times \text{Conversion factor}$

$$\begin{aligned}
 &= A_{260} \times \frac{\text{Volume of distilled water } (\mu\text{l})}{\text{Amount of the DNA sample } (\mu\text{l})} \times 50 \\
 &= (\mu\text{g/ml}) \\
 &= (\text{ng}/\mu\text{l}) \quad [\therefore \mu\text{g/ml} = \text{ng}/\mu\text{l}]
 \end{aligned}$$

The higher DNA concentration was recorded in SL-623 (495 ng/μl). The lowest DNA concentration was obtained from SL-571 cattle (165 ng/μl). Red Chittagong (RCC -178, RCC-691) and local M-1980 cattle produced 475 ng/μl DNA. The quantified DNA sample subsequently diluted to 2ng/μl as per requirements for ideal PCR.

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

Serial No.	Accession or tag no.	Absorbance reading at 260 nm	Quantity of DNA (absorbance x dilution factor x 50 ng/μl)
1.	RCC -173	0.080	400
2.	RCC -178	0.095	475
3.	RCC -691	0.095	475
4.	RCC -175	0.093	465
5.	RCC -609	0.058	290
6.	RCC -77	0.093	465
7.	RCC -78	0.067	335
8.	RCC -80	0.036	180
9.	RCC -81	0.097	480
10.	RCC -82	0.039	195
11.	SL-571	0.033	165
12.	SL-614	0.048	240
13.	SL-623	0.099	495
14.	SL-637	0.081	405
15.	SL-638	0.047	235
16.	M-1222	0.057	285
17.	LF-1414	0.038	190
18.	M-1893	0.045	225
19.	M-1980	0.095	475
20.	M-5730	0.058	290

The PCR did run using twelve primers along with the diluted DNA sample. The reproducibility of PCR product as well as banding pattern was confirmed by 2% Agarose gel electrophoresis with 100bp ladder.

4. 2. Molecular characterization

In the present study, a polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD) method was used to amplify cattle DNA using arbitrary oligonucleotide primers. The method was able to detect the heterogeneity of amplified DNA from the cattle population. The experimental cattle showed variability in genetical characteristics and the results indicate the effectiveness of RAPD analysis in detecting the level of polymorphism where three primers (Table 4) were screened out for determination the polymorphism among the experimental cattle.

4.2.1. Primer Selection and RAPD Analysis

Primer selection: In RAPD analysis, all the 12 primers were 10-12 bp long with a GC content of 60-80%. Primers with ten nucleotides and a (G+C) content of at least 50% are generally employed, because primers having high (A+T) content may cause DNA-primer hybrid melted during polymerization at 72°C (Weising *et al.*, 1995). Increased number of bands was obtained with increased GC content of the primer (Fukuoka *et al.*, 1992; Raghunathachari *et al.*, 2000). The correlation between GC content of the primer and the number of bands could be explained as the greater hydrogen bonding; because of G is pairing with C by three hydrogen bonds shows the high stability of base complementation than that of complementation of A with T by two hydrogen bonds (Fukuoka *et al.*, 1992). Sequences for the primers (three) that amplified apparent pool-specific products are shown below in (Table 4).

Table 4. List of primers showed polymorphism

Primer no.	Sequence (5'-3')	(G+C) Contents %
OP A03	TGCCTCGCACCA	80%
OP S7	GGTGACGCAG	70%
OP A09	TCTGCCATCC	60%

The cattle number could be distinguished by a combination of fragments and differences between clusters reflected in frequencies rather than presence or absence of specific fragments. From the figure, it was observed that primer A03 and S07 were more reproducible and produced more distinct RAPD profile than primer A09. Three different primers generated various banding patterns and out of the 324 bands scored, 184 bands (56.79%) were found to be polymorphic and 140 bands (43.20%) were found to be monomorphic in nature. Different RAPD patterns generated using the primers in the present experiment have shown in Figure 6 to 8.

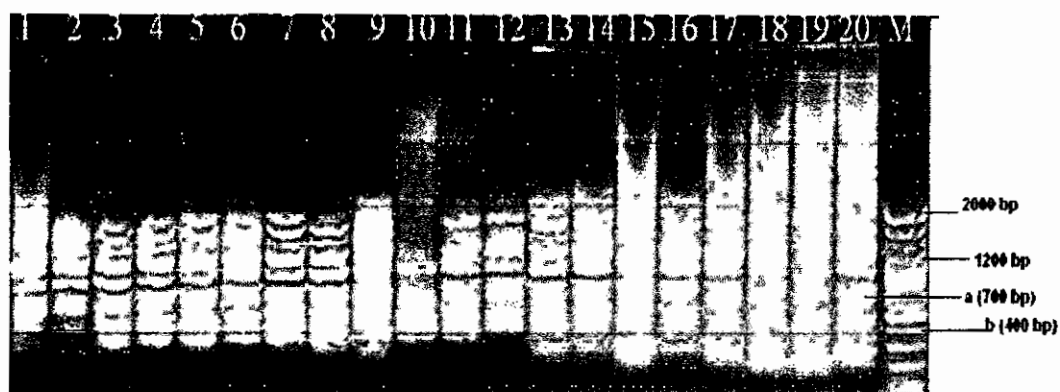


Figure 6. RAPD profiles using primer A09. The number corresponds to the serial of the genotypes shown in Table 1; M is a Hind III DNA size marker. On the gel a and b bands were monomorphic for all the experimental animals.

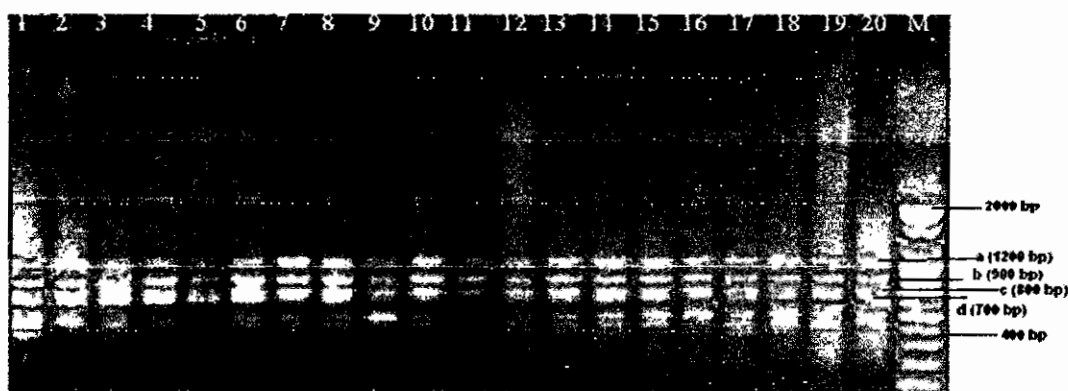


Figure 7. RAPD profiles using primer S07. The number corresponds to the serial of the genotypes shown in Table 1; M is a Hind III DNA size marker. On the gel a, b, c and d bands were monomorphic for all experimental animals.

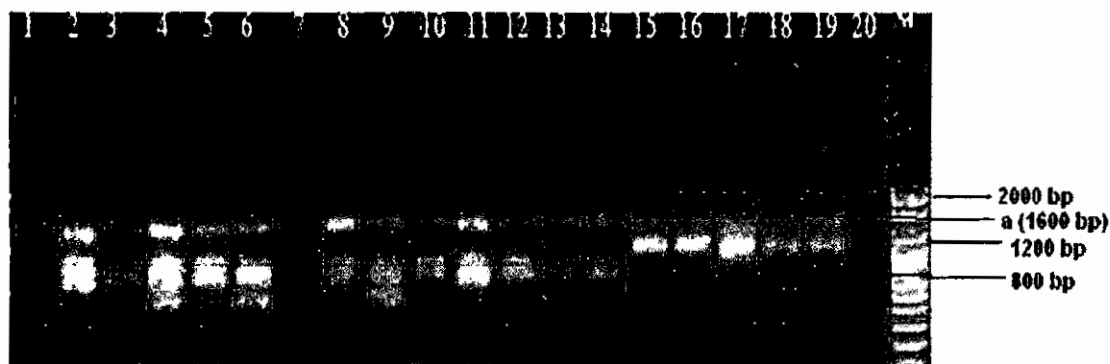


Figure 8. RAPD profiles using primer A09. The number corresponds to the serial of the genotypes shown in Table 1; M is a Hind III DNA size marker. On the gel 'a' is monomorphic for all experimental animals.

The frequencies of polymorphic bands varied from primer to primer. Primer A03, S07 and A09 produced 72.97%, 38.93% and 55.55% polymorphic loci respectively. Moreover, Primer A03

produced 148 bands in where 40 bands in the scale of 400 bp - 700 bp were monomorphic in all samples. The polymorphic fragments for primer A03 ranged from 900 bp -2000 bp. Furthermore, primer S07 and A09 produced 131 and 45 bands respectively. The range of monomorphic bands for primer S07 and A09 were 700 bp-1000 bp, 1600 bp respectively whereas, the range of polymorphic bands were 400 bp-600 bp and 800 bp-1200 bp respectively. As the number of bands common in all animals produced by primer S07 was (80) greater than the other two primers (40 for A 03 and 20 for A09), it can be concluded that S07 may be specific for species or for greater zoological classes (gender, family etc.). This point needs to be clarified in the future by a comparison with other species. Sharma *et al.* (2009) reported, the size of polymorphic bands ranged between 200 and 2500 bp using 17 operon series random primers to characterize the Malvi and Sahiwal breeds of cattle in Madhya Pradesh, India.

Table 5. List of primers and their monomorphic and polymorphic bands

Serial No.	Primer code	No. of bands	No. of monomorphic bands	Size of monomorphic bands (bp)	No. of polymorphic bands	Size of polymorphic bands (bp)	Polymorphic loci (%)
1	A03	148	40	700-400	108	2000-900	72.97
2	S07	131	80	1000-700	51	600-400	38.93
3	A09	45	20	1600	25	1200-800	55.55
Total	3	324	140		184		56.79%

Mufi *et al.* (2009) reported polymorphism and genetic relationship in five different Red Chittagong populations using 12 primers and showed a total of 12 (92.31%) polymorphic loci which is higher than 56.79%. It suggests that within this period of time, the gene segregation rate has increased in these animals.

In another study involving indigenous African cattle, Gwakisa *et al.* (1994) used RAPD markers to characterize the local Zebu (*Bos indicus*) cattle breeds of Tanzania (using ILO 1127 primer) and showed 61% polymorphism which is almost similar to the present study. The degree of polymorphism 56.79% is higher compared to 28 % in taurine cattle (Talle *et al.*, 2005) and 18 % in zebu cattle (Marle-Koster *et al.*, 2003). Estimation of higher level of genetic variation in the breeds might be consistent with the fact that they are highly polymorphic animals.

Polymorphisms at the level of individual, breed, species or gender can be produced by RAPD-PCR but it is difficult to identify the bands. No breed- or sex-specific RAPD bands were detected in this study. Nevertheless, by mean of their monomorphic band profiles, some of the investigated primers can give the information that animals not having these monomorphic bands

may not be related to these cattle. More studies should be performed on this matter in comparative genome studies.

4. 2. Genetic Distance

All possible pair-wise genetic distances were calculated according to Nei's and Li (1979) using 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. In this analysis, smaller numbers are associated with more genetically similar individuals, whereas larger numbers suggest genetically dissimilarity.

In the present experiment, the highest value of Nei's genetic distance (0.8387) was found between SL-623 and RCC-82. Lower estimate of genetic similarities indicated high degree of genetic diversity between these two cattle. This may be due to diverse origin and different purpose of raising these cattle. On the other hand, the lowest distance (0.0303) existed between M-5730 and M-1222. It indicates, the genetic relationship between local cattle population was closer than others. Sharma *et al.* (2009) calculated between breed band sharing (BS), the value for these 2 breeds obtained was lower (0.43-0.66) than within breed BS value, which was 0.70-0.93 in Malvi breed and 0.68-0.88 in Sahiwal breed, found similar genetic distances of average animals like the present experiment.

The results revealed that there was a high level of genetic variation among the studied number of cattle as indicated by the proportion of polymorphic loci. The values of pair-wise genetic distance among 20 cattle genotypes were computed from combined data sets for the 3 primers ranging from 0.8387 to 0.0303 (Table 6). Kemp and Teale (1994) identified population-specific DNA polymorphisms to detect *Bos indicus* and *Bos taurus* in West Africa. They estimated the allele frequency characteristics of cattle breeds using DNA markers (Buitcamp *et al.*, 1991; Mannen and Tsuji, 1993; Glowatzki- Mullis *et al.*, 1995; Choy *et al.*, 2001).

The selected primers used in the experiment can be used for analysis of other cattle germplasm. The wide range of dissimilarity values (0.8387 to 0.0303) suggests that the germplasm collection represents a genetically diverse population. 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; Kantanen and Maki-Tanila 1995). 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.

Heterozygosity estimates obtained for the Burlina breed were moderate to high and consistent to the value reported by Del Bo *et al.* (2001) that found an estimate of 0.68. However, such variability represents an important reservoir of genetic diversity that should be conserved. Genetic diversity is in fact important to allow genetic improvement in selected breeds as well as to facilitate rapid adaptation.

RAPD, a random primer-based molecular technique, has also been used to assess diversity in plants and animals (Williams *et al.*, 1990). Hassen *et al.* (2007) used RAPDs to assess genetic variability within and among five indigenous Ethiopian cattle breeds. The analysis revealed that within breed genetic variation was much higher than that between breeds. Mbulu and Hannotte (2005) described a study that looked at genetic diversity and population structure in 52 populations of African cattle using 15 microsatellite markers. The study indicated a wide range of genetic diversity among African cattle based on mean number of alleles, effective number of alleles, and expected heterozygosity. Globally, there has been an increase in awareness about the importance of indigenous animal breeds and the need to properly utilize and manage these resources.

Though cattle number basis marker was identified, the high level of polymorphism obtained by the proportion of polymorphic loci indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in experimental cattle population. For a more efficient determination of genetic diversity in cattle varieties, a higher number of decamer primers showing maximum number of polymorphic band or other available marker systems could be utilized. Nevertheless, if the information obtained from the present experimental animals as well as about the marker can be linked with quantitative trait loci for economically important traits, could be used successfully in future breeding and germplasm conservation programme for cattle production systems of Bangladesh.

Table 6. Estimation of pair wise genetic distance between experimental cattle populations using Nei's equation

Sample No.	RCC -173	RCC -178	RCC -691	RCC -175	RCC -609	RCC -77	RCC -78	RCC -80	RCC -81	RCC -82	SL -571	SL -614	SL -623	SL -637	SL -638	M -1222	LF -1414	M -1893	M -1980	M -5730
RCC-173	***																			
RCC-178	0.164	***																		
RCC-691	0.1111	0.2592	***																	
RCC-175	0.1851	0.1851	0.1428	***																
RCC-609	0.2592	0.1851	0.2142	0.0714	***															
RCC-77	0.1111	0.1111	0.1428	0.0714	0.1428	***														
RCC-78	0.1428	0.1857	0.1034	0.1724	0.2413	0.1724	***													
RCC-80	0.1851	0.1851	0.1428	0.2142	0.2142	0.1428	0.1034	***												
RCC-81	0.1428	0.1428	0.2413	0.1724	0.1034	0.1034	0.20	0.5724	***											
RCC-82	0.040	0.1818	0.393	0.3043	0.2173	0.3043	0.3333	0.3043	0.3333	***										
SL-571	0.1851	0.1851	0.1428	0.1428	0.1428	0.1428	0.1724	0.1428	0.1034	0.2173	***									
SL-614	0.1111	0.1111	0.2142	0.2142	0.2142	0.2857	0.1724	0.714	0.1424	0.640	0.1428	***								
SL-623	0.1034	0.2413	0.0666	0.1333	0.20	0.1333	0.0322	0.1333	0.1612	0.8387	0.1333	0.1333	***							
SL-637	0.1333	0.20	0.0967	0.1612	0.1612	0.2173	0.0625	0.6967	0.1250	0.3076	0.0967	0.0967	0.0303	***						
SL-638	0.20	0.602	0.2307	0.3846	0.3846	0.3076	0.1851	0.1538	0.2592	0.3333	0.2307	0.1538	0.1428	0.1724	***					
M-1222	0.1724	0.2413	0.0666	0.200	0.20	0.20	0.0322	0.666	0.1612	0.3600	0.1333	0.1333	0.0625	0.0431	0.1428	***				
LF-1414	0.1428	0.2142	0.1034	0.160	0.2413	0.1724	0.0666	0.344	0.1333	0.3333	0.1034	0.1034	0.0967	0.0625	0.1111	0.0322	***			
M-1893	0.20	0.20	0.2307	0.3846	0.1612	0.3076	0.1851	0.538	0.2592	0.3333	0.2307	0.1538	0.2142	0.1724	0.0603	0.1428	0.1111	***		
M-1980	0.20	0.20	0.3076	0.3846	0.1612	0.3076	0.2592	0.2307	0.2592	0.2380	0.2307	0.2307	0.2142	0.1724	0.0833	0.2142	0.8148	0.0833	***	
M-5730	0.1724	0.2413	0.0666	0.20	0.20	0.20	0.0322	0.0666	0.1612	0.3600	0.1333	0.1333	0.0625	0.0304	0.1428	0.0303	0.0322	0.1428	0.2142	**

4.2.3. Dendrogram Analysis

Estimation of genetic similarity within and between cattle and genetic distance among different cattle is an important application of the DNA based genetic markers. Dendrogram stability is an important aspect to be considered in genetic variability studies. An unweighted pair-group method of analysis (UPGMA) phylogenetic tree based on genetic distance using the COMPONENT software (version 1.31) is shown in Fig. 9. Component dendrogram depicted the relationship among twenty population of RCC, Sahiwal and local cattle. The dendrogram (Figure 9) segregated the twenty cattle into two major clusters: (I) and (II). Cluster I sub divided into I A and I B. Furthermore, cluster I A subclustered into I A (1) and I A (2). On the other hand, cluster I B subdivided into I B (1) and I B (2).

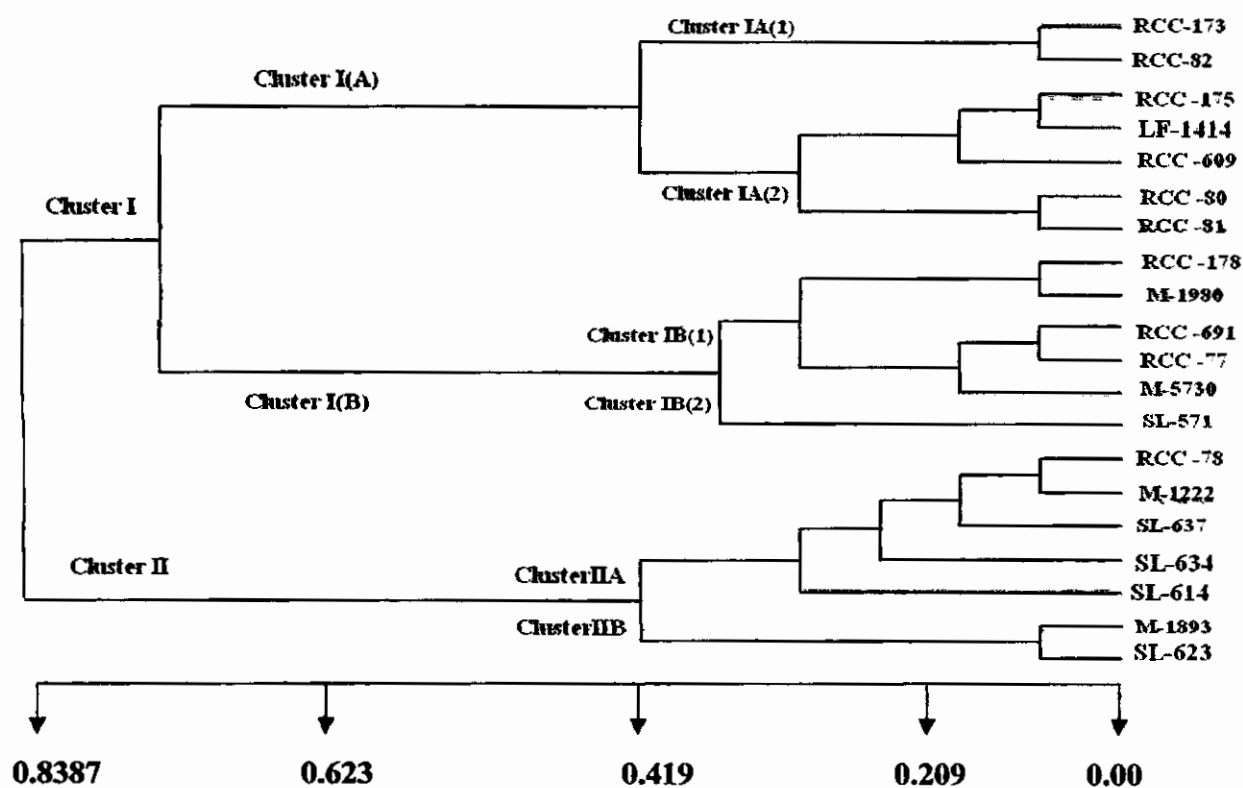


Figure 9. The unweighted pair group method of analysis (UPGMA) dendrogram based on summarized data regarding differentiation among experimental cattle

Table 7. Distribution of twenty cattle under different clusters

Cluster No.		Total no. of cattle in cluster	Cattle included in different clusters
I	IA	7	RCC-173, RCC-82, RCC-175, LF-1414, RCC-609, RCC-80 and RCC-81
	IB	6	RCC-178, M-1980, RCC -691, RCC -77, M-5730 and SL-571
II		7	RCC -78, M-1222, SL-637, SL-634, SL-614, M-1893 and SL-623

Subcluster I A comprised of RCC-173, RCC-82, RCC-175, LF-1414, RCC-609, RCC-80, RCC-81 cattle population and subcluster I B consisted of RCC-178, M-1980, RCC -691, RCC -77, M-5730 and SL-571 cattle population. On the other hand, cluster II belongs cattle RCC -78, M-1222, SL-637, SL-634, SL-614, M-1893 and SL-623. From the dendrogram, closely related indigenous population e.g., RCC and Local Pabna cattle co-existed in cluster I might be due to their same origin as well as lower genetic variability. Whereas, Sahiwal breeds randomly segregated between subcluster IB and Cluster II. The possible reasons for their random segregation could be the indiscriminate breeding/crossing, migration of animals, mutation in ancestors or due to different origin. Moreover, due to long genetic variation between SL- 623 and local RCC-82, they can be crossed for parent development. The highest gene diversity indicates that the population is alarming to deteriorate with native or exotic blood than other populations. 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 DeHong cattle breed was closely related to the Brahman (*Bos indicus*), and the Yunnan DiQing cattle breed was closely related to the Simmental, short horn, and Murry grey (*Bos taurus*) breeds. It is reported that uncontrolled hybridizations are being increased and phylogenetic relationship of the RCC and Local Pabna cattle is being closer because of regional animal cooperative systems which using RCC bulls for breeding programme since 1995 (Ahmed, 2000).

CHAPTER FIVE

SUMMARY AND CONCLUSION

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This study demonstrated that the short random oligonucleotides of arbitrary sequences can be used to successfully differentiate cattle by variation of RAPD fingerprints in breed-specific DNA pools and subsequent follow-up of prominent fingerprints in individual animals.

The higher DNA concentration was recorded in SL-623 (495 ng/ μ l). The lowest DNA concentration was obtained from SL-571 cattle (165 ng/ μ l). Red Chittagong (RCC -178, RCC-691) and local M-1980 cattle produced 475 ng/ μ l DNA.

In PCR based RAPD reaction, primer A03 and S07 showed more reproducible and distinct RAPD profile than primer A09. Out of the 324 bands scored, 184 bands (56.79%) were found to be polymorphic and 140 bands (43.20%) were found to be monomorphic in nature. The frequencies of polymorphic bands varied from primer to primer. Primer A03, S07 and A09 produced 72.97%, 38.93% and 55.55% polymorphic loci respectively. Moreover, Primer A03 produced 148 bands in where 40 bands in the scale of 400 bp - 700 bp were monomorphic in all samples. The polymorphic fragments for primer A03 ranged from 900 bp -2000 bp. Furthermore, primer S07 and A09 produced 131 and 45 bands respectively. The range of monomorphic bands for primer S07 and A09 were 700 bp-1000 bp, 1600 bp respectively whereas, the range of polymorphic bands were 400 bp-600 bp and 800 bp-1200 bp respectively. The number of bands common in all animals produced by primer S07 was (80) greater than the other two primers (40 for A 03 and 20 for A09).

From the experimental results, the highest value of Nei's genetic distance (0.8387) was found between SL-623 and RCC-82 cattle. The lowest distance (0.0303) was found between M-5730 and M-1222 cattle. The dendrogram constructed using Nei's genetic heterozygosity divided into two distinct Cluster: Cluster I and I I. Subcluster I A comprised of RCC-173, RCC-82, RCC-175, LF-1414, RCC-609, RCC-80, RCC-81 cattle population and subcluster I B consisted of RCC-178, M-1980, RCC -691, RCC -77, M-5730 and SL-571 cattle population. On the other hand, cluster II belongs cattle RCC -78, M-1222, SL-637, SL-634, SL-614, M-1893 and SL-623.

The genetic variations in the experimental cattle populations were high and therefore, there is scope of selective breeding (like ONBS) for future improvement of Red Chittagong, Sahiwal and local Cattle. RAPD markers have found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period. Therefore, RAPD technique can be performed in a moderate laboratory for most of its applications. During RAPD profiling, some problems may arise, amongst which is the relative complexity of resultant fingerprint patterns and the fact that heterozygotes cannot be distinguished from homozygotes. Thus, they require skilful scoring. Second, the method is very sensitive to reaction conditions, DNA quality and PCR temperature profiles. It is absolutely critical to maintain constant PCR reaction conditions in order to achieve reproducible results. Third, in the absence of pedigree material, the identity of individual bands in the multi-band profiles is not known and there can be uncertainty in assigning markers to specific loci. This makes RAPDs difficult to use in interpopulation or interspecific comparisons. Fourth, single bands on the gel can sometimes be comprised of several co-migrating amplification products. This makes it difficult to distinguish many of the polymorphisms apparent from PCR artifacts. Moreover, RAPD amplification occurs only at low, non-specific annealing temperatures, it is therefore likely that, depending on the surrounding DNA structures, a certain site is amplified in DNA from one animal but not from another (Rothuizen and van Wolferen, 1994).

Genetic erosion and extinction threatening an increasing number of animal species; the major consequence being a loss of global genetic diversity requires sets of genetic markers that characterize distinct populations. The local Bangladeshi Pabna cattle and Red Chittagong cross-bred are of particular importance because of its favourable meat, milk, leather and draft quality although its productivity is relatively low in general. 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. In addition, genetic assessment is also of interest for the design of genetic improvement programs including appropriate choice of breeds for crossbreeding. Thus, it is important to identify molecular characteristics of these breed/populations for undertaking proper breeding programmes and conservation strategy.

CHAPTER SIX

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APPENDICES

APPENDICES

APPENDIX-1

Chemicals and composition of DNA extraction solutions

1. 1X Standard Saline Citrate (SSC, pH 7.03)

NaCl 10gm and Na-citrate 0.441gm was added to 60 ml of distilled water and then adjusted pH 7.03. The final volume was adjusted to 100 ml with distilled water. The solution was autoclaved and stored at room temperature.

2. 0.2M Na-acetate

Na-acetate (anhydrous) 1.6406gm was added to 80 ml of distilled water. The final volume was adjusted to 100 ml with distilled water. The solution was stored at room temperature.

3. 2M Na-acetate

Na-acetate (anhydrous) 16.406gm was added to 80 ml of distilled water. The final volume was adjusted to 100 ml with distilled water. The solution was stored at room temperature.

4. 10% Stock Solution of SDS

10 g of SDS (MW=288.4) was dissolved in 80 ml of distilled water. Then the solution was heated to 65 °C to dissolve completely. SDS acts as detergent and they produce foam during dissolving. Thus, it is recommended to shake gently during dissolving to reduce foaminess. The total volume was adjusted to 100 ml with sterile distilled water. No need of autoclaving. While weighing of SDS it is recommended to wear a mask and after weighing wipe down the weighing area & balance because the fine crystals of SDS disperse easily. The solution was stored at room temperature.

5. Proteinase K

Proteinase K 20mg was added to 1 ml of distilled water. The solution was stored at 4°C.

6. Preparation of Saturated Phenol

- At first the phenol was melted in a water bath at 65° c for 15 minutes.
- Then 60 ml of melted phenol was taken in a beaker (250ml) and equal volume of 0.5M TE buffer was added.

- With a magnetic stirrer it was mixed for at least 10 minutes and then for 5 minutes it was kept without agitation for separation of two phases.
- With the help of a dropper the upper phase was removed as much as possible. The same procedure was repeated with 0.5MTE until the pH is raised up to 7.6-7.8.

7. Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) Solution

For 51 ml of this solution, 25 ml of TE saturated Phenol; 24 ml of Chloroform and 1 ml of Isoamyl Alcohol were added and mixed properly by vortexing. Mixing was done under fume hood for safety. The solution was then stored at room temperature. The solution was vortexed or shaken well before further use.

8. Stock Solution of TE (10:1) buffer (p^H 8.0)

Five ml of 1 M Tris HCl was added with 10 ml of 0.5 M EDTA. The final volume was adjusted to 500 ml with distilled water. Then sterilized through disposable filter sterilizer of 0.22 μ m pore size and stored at room temperature.

9. 70% Ethanol

Seventy ml ethanol was added to 30 ml of distilled water. The solution was stored at 4 °C.

10. 1M NaOH

NaOH pellet 4gm was added to 80 ml of distilled water. The final volume was adjusted to 100 ml with distilled water. The solution was stored at room temperature.

11. 0.5 M Stock Solution of EDTA (p^H 8.0)

46.53g EDTA (EDTA.2 H₂O, MW=372.2) was added to 100 ml of distilled water and stirred vigorously with a magnetic stirrer. The EDTA salt was not dissolved in solution until the p^H of the solution was adjusted to 8.0 by the addition of NaOH. The final volume of the solution was adjusted to 250 ml by adding distilled water. The solution was sterilized by autoclaving and stored at room temperature.

12. 1 M Stock Solution of Tris HCl (p^H 8.0)

30.28g Trizma base (MW=121.1) was dissolved in 100 ml of distilled water. The p^H of this solution was adjusted to 7.6 with concentrated HCl (10 N). About 10.5 ml of HCl (10

N) was added slowly by using dropper until the pH was adjusted to 7.6 and was kept at room temperature for several minutes. The volume of the solution was adjusted to a total volume of 250 ml with distilled water. Then it was autoclaved and stored at room temperature.

13. 1X TAE Buffer (For 1000 ml)

Tris-base: 4.845gm

EDTA: 0.745gm

Na acetate: 1.64gm

Add E-pure water to make 1000ml.

14. 14N HCl

Concentrated HCl 127.51 ml in 100 ml of E- water.

Make the final volume 250 ml with E-pure water.

15. 10M NaOH

NaOH pellets: 100 gm in 100 ml of E-pure water.

Make the final volume 250 ml with E-pure water.

16. 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. They were mixed well. The p^H of the solution was adjusted at 8.3 by mixing concentrated HCl (10 N). The final volume of the solution was adjusted to 100 ml.