A STUDY ON GENETIC DIVERSITY OF SELECTIVE LOCAL CATTLE USING RAPD MARKERS

A Thesis submitted

by

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Examination Roll No: (MS) 080719
Session: 2007-2008

Submitted to

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

Biotechnology and Genetic Engineering Discipline
Life Science School, Khulna University
Khulna-9208, Bangladesh
August, 2011

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DECLARATION

This thesis report is the original research work of the author, except as otherwise started. 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 The findings of the research has not been/will not be published anywhere without the concurrence of the concerned supervisor.

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ACKNOWLEDGEMENT

All praises go to Almighty Allah, the preserver and protector of all, whose mercy enables me to complete the thesis for the fulfilment of the degree of Master of Science in Biotechnology and Genetic Engineering.

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

It is her pleasure to express her greatest and profound gratitude, deep appreciation and heartfelt indebtedness with best regards to her honorable co-supervisor, Dr. S.M. Mahbubur Rahman, Professor of Biotechnology and Genetic Engineering Discipline, Khulna University, for his active guidance, keen interest, continuous inspiration and constructive criticism for which this work comes to light.

The author is grateful to the Ministry of Science Information and Communication Technology (MOSICT) through her honourable supervisor professor Dr. Khondoker Moazzem Hossain, honourable co-supervisor Dr. S. M.Mahbubur Rahman and honourable teacher Dr. Md. Morsaline Billah (Associate professor) for providing financial support to conduct the research work.

Special words of admiration given to Dr. Shaiad Hossain, Veterinary Surgeon, amimal hospital, Kachua, Bagerhat. The author is thankful to dresser Babu and milk recorder Habib for their active help during blood collection.

The author would like to express her cordial thanks to Arif, Sagor, Tarun, who sincerely co-operated in her Laboratory work. Special heartfelt thanks are extended to all friends for their co-operation and occasional help. Finally, the author would like to express her gratitude to her parents, husband, brother and sisters for their moral support, inspiration and encouragement throughout the work.

The Author

ABSTRACT

Randomly amplified polymorphic DNA (RAPD) analysis was performed to find out the genetic diversity and to identify the polymorphisms and to determine genetic relationship of twenty indegenious cattle of Bagerhat, Pirojpur and Khulna areas of Bangladesh. The experiment was conducted in Animal Cell Culture and Molecular Biotechnology Laboratory of Khulna University, Bangladesh. Blood samples of twenty local cattle were collected from Bagerhat, Khulna and Pirojpur districts. Among ten RAPD primers, only three primers were capable to amplify different fragments and found polymorphic 86.66%. These three primers that used in RAPD analysis of twenty experimental cattle amplified 14 different reproducible bands. Three different primers generated various banding patterns and of the 14 bands scored, 12 bands (86.66%) were found to be polymorphic and 2 bands (40%) were found to be monomorphic in nature. Genetic relations among local cattle were determined using RAPD markers that obtained total 86.66% polymorphic loci. Statistical analysis of the data, estimating the genetic distances among local cattle and sketching the cluster trees were performed using COMPONENT version 1.31. Comparatively higher genetic distance (0.90) was found among Pir-10 and Khu-11, Pir-01 and Khu-11, Khu-09 and Khu-11 cattle. The lowest genetic distance (0.05) was observed between Bag-102 and Bag-69, Bag-99 and Bag-69 cattle. The range of genetic distance of twenty cattle was 0.05-0.90. The differences between the highest and lowest genetic distance indicated the presence of variability among the twenty genotype local cattle. The genetic diversity of Pirojpur and Khulna cattle was relatively higher. The study showed that the polymorphisms obtained using RAPD-PCR enabled to determine the genetic relationships and fingerprints of local cattle population. Therefore, adequate diversity in performance and adaptability can be exploited for actual improvement accruing to conservation and development of indigenous cattle resources.

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LIST OF ABBREVIARTION

%	:	Percent
µl	:	Micro liter
µM	:	Micro molar
^{0}C	:	Degree Celsius
A260	:	Spectrophotometer Absorbance Reading at 260 nm
A280	:	Spectrophotometer Absorbance Reading at 280 nm
AFLP	:	Amplified Fragment Length Polymorphism
UPGMA	:	Unweighted Pair Group Method of Arithmetic Means
PHF	:	Pure Holstein-Friesian
d H_2O	:	Distilled water
DNA	:	Deoxyribonucleic acid
LF 50%	:	Local X Friesian 50%
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
PCR	:	Polymerase chain Reaction
pH	:	Negative logarithm of hydrogen ion (H^+) concentration
LF 75 %	:	Local X Friesian 75%
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

Chapter 1

Introduction

CHAPTER ONE

Introduction

The family of animals that includes all types of domestic cattle is known as Bovidae. Domestic cattle mostly descended from the wild aurochs (European Ox, now extinct). Domestication of cattle and their propagation at the world level started in the Neolithic ages in about 2000 BC. In Bangladesh, the total cattle population is about 24.5 million of which 11.91 million are males and 11.49 million are female including 3.53 million milking cows, 2.61 million dry cows (cows without milk), 2.13 million draught cattle, and 4.20 million improved cattle. Since the 1960s, the people of Bangladesh have been rearing three categories of cattle viz. pure breed, cross-bred, and local. The local variety is less prone to diseases and is heat tolerant. In Bangladesh the best local cattle are available in some selected areas viz. Pabna, Sirajganj, Chittagong, and Munshiganj areas (www. banglapedia. search. com).

Bangladesh is an agricultural country. Livestock, being one of the major components of agricultural output (crops, livestock, fisheries and forestry) plays a vital role in national economy, contributing about 6.5% of Gross Domestic Product (GDP) and 13% of total foreign exchange earning (DLS, 2008). The total ruminant livestock population of Bangladeshis composed of 24.0 million cattle, 34.4 million goats, 0.83 million buffalos and 1.14 million sheep (Omore *et al.,* 2002). The country has one of the highest cattle densities of 145 large ruminants/square kilo meter (sq.km) compared with 90 for India, 30 for Ethiopia, and 20 for Brazil (Karim, 1997). The numbers of dairy farms are estimated at about 1.4 million with an average herd size of 1-3 cows (Garcia, 2006). Also dairying is part of the mixed farming systems in Bangladesh (Saadullah, 2001) and a predominant source of income, nutrition and jobs (Miyan, 1996; Haque, 2009). Dairying is also considered a strong tool to develop a village micro economy of Bangladesh (Shamsuddin *et al.,* 2006) in order to improve rural livelihoods and to alleviate rural poverty. Domesticated animals, especially livestock and poultry, are an important source of protein in Bangladesh. Increasing this protein resource requires the conservation of diversity among indigenous livestock. Maintaining genetic diversity is an insurance against future adverse conditions populations. The wide range of breeds and species that have evolved in various environments represent unique sets of genetic diversity. It has been estimated that

since domestication, over 6,379 documented breed populations from 30 species of livestock have been developed globally in the last 12 thousand years (FAO, 2000). Recently, loss of genetic diversity within indigenous livestock breeds has been a major concern.

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. Now this molecular approach became a powerful and reproducible tool in genetic analysis, breeding and identification of species and/or populations. In the case of cattle, lots of researchers have been made to detect DNA polymorphisms between cattle breeds by RAPD analysis during last decade. Especially genetic markers for native cattle's in each country were strongly investigated for the improvement of their native cattle, including Japanese Black cattle (Wagyu), Germany native cattle, Zebu cattle, *Bos indicus*, and *Bos Taurus.* Over the last decade, polymerase chain reaction (PCR) technology has become a widespread research technique and has led to development of several novel genetic assays based on selective amplification of the DNA. This popularity of PCR is primarily due to its apparent simplicity and high probability of success. 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 characterization of the genome of the species. Considering the above statement, the present research work was undertaken with the following objectives:

1. To study the molecular characteristics of selective local cattle using random amplified polymorphic DNA (RAPD) markers.

2. To study the genetic diversity and relationships among experimental local cattle.

Chapter 2

Review of literature

CHAPTER TWO

Review of Literature

Isolation of genomic pure DNA and RAPD analysis has been used for 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 physiochemical analysis in general and has been reviewed in this chapter.

2. Local cattle

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., 1. *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 (www.FAO.com). The local cattle originated in Africa and 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 and 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 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 (Ahmed *et al.*, 2006).

2.3 Reproductive characteristics

In Bagerhat, Khulna and Pirojpur districts 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 important resources because they 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 cattle 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 and 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.

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 predicator 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 pairwise 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 *et al.,* 1983).

2.5 Molecular characterization of Cattle

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), population origin (Cornuet *et al.,* 1999), 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, Short horn, and Murry grey (*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 heterozygote is typically scored as homozygote, which decreases their information content.

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. 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. 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.

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 heterozygosis. 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 Zambia for example, tremendous efforts are now being made to genetically characterize the major breeds of livestock, including cattle, for conservation. 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.* (1995) 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 maximising 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.

Van Marle-Koster and Nel (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 3

Materials and Methods

CHAPTER THREE
Materials and Methods

The experiment was carried out at the Animal cell culture and Molecular Laboratory in 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

In order to minimize sampling errors two conditions were maintained in selecting local cattle:

1. Related animals were not sampled.
2. Sampling of animals which represented different eco-regional populations.

Bagerhat, Khulna and Pirojpur districts did served the best for these conditions because animals from different regions were frequently introduced and maintained in semi-intensive farming system. A total of twenty native cattle were selected for this study. Five ml blood was collected from each animal in a 10 ml EDTA vaccutainer tube. The blood was gently mixed with EDTA (present in the vacationer as an anticoagulant agent) and kept on ice box during collection and then transported to the laboratory and stored at -20°C until the isolation of genomic DNA. The cattle's identity no., source and sex are given in table 1.

3.2 Extraction of genomic DNA from blood sample

DNA was extracted from blood by following the modified method of Bruce (1996). The presence of DNA in the extracted samples was confirmed by running the sample in a 1 % agarose gel at 50V for 1.20 hours. Samples producing clear bands and visualized by UV Transilluminator. The presence of DNA was confirmed by running the extracted DNA on a 0.8– 1% agarose gel.

3.3 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.

Table 1. ***Cattle's identity no., source and sex***

SL. No.	Cattle No.	Source	Identity	Sex
1	Bag-27	Kachua, Bagerhat.	Local	male
2	Bag-99	Kachua, Bagerhat	Local	male
3	Bag-102	Kachua, Bagerhat	Local	female
4	Bag-50	Kachua, Bagerhat	Local	female
5	Bag-69	Kachua, Bagerhat	Local	female
6	Bag-03	Kachua, Bagerhat	Local	male
7	Bag-35	Kachua, Bagerhat	Local	male
8	Bag-05	Kachua, Bagerhat	Local	female
9	Pir-85	Nazirpur, Pirojpur	Local	female
10	Pir-05	Nazirpur, Pirojpur	Local	female
11	Pir-07	Nazirpur, Pirojpur	Local	male
12	Pir-10	Nazirpur, Pirojpur	Local	female
13	Pir-01	Nazirpur, Pirojpur	Local	female
14	Pir-13	Nazirpur, Pirojpur	Local	female
15	Khu-09	Pabla, Khulna.	Local	male
16	Khu-02	Pabla, Khulna.	Local	female
17	Khu-03	Pabla, Khulna.	Local	male
18	Khu-11	Pabla, Khulna.	Local	male
19	Khu-12	Pabla, Khulna.	Local	female
20	Khu-17	Pabla, Khulna.	Local	female

3.4 Protocol Used for Genomic DNA Isolation from Blood Sample

This method was adopted from Bruce (1996) with little modification from step 7 to step 11. Protocol of this method is described below:

3.4.1. Protocol

1. The blood samples were obtained in EDTA Vaccutainer 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 in a 2 ml centrifuge tube and mixed well by vortaxing. 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, vortaxed, 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 vortaxed briefly. Then 35 µl of 10% SDS and 15 µl of proteinase K was added, vortaxed briefly and was incubated for 1.5 h at 55°C.

6. Five hundred µl of phenol: chloroform: isoamyal alcohol (25:24:1) was added and vortaxed for 30 sec. The sample was centrifuged for 20 min at 12000 rmp.

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 rmp. The supernatant was decanted.

9. One ml of 70% ethanol was added and centrifuged for 10 min. at 12000 rmp in a microcentrifuge.

10. The supernatant was decanted, and pellet was dryed at room temperature for 10 minutes.

11. The pellet was dissolved by adding 50 µl of TE buffer. Then it was incubated for 30 minutes at 55°C, vortaxing periodically to dissolve the pellet. The samples were store at -20^{0}C for further use.

3.5 Quantization of Isolated DNA

In a clean microcentrifuge tube 20 µl DNA solution was mixed with 1980 µl of sterile distilled water (total 2 ml). Standard was made by adding 20 µl TE buffer in 1980 µl 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:

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

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

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

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

Here,

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

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

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

3.5 PCR reaction

3.5.1 Primer selection

In this study 3 RAPD primers were screened out of 10 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	TTCCCCCCAG	70%
07	GGTGACGCAG	70%
08	GGCCCTACAT	60%
09	GTTGCCACCC	70%
10	AACGGTGACC	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 µl reaction mix containing 2 µl of template (4ng DNA), 2 µl of 10x reaction buffer with $MgCl_2$, 2 µl of dNTPs (250 mM each), 2 units of Taq DNA polymerase, 1 µl (100picomol) of primer, 11 µl of sterile distilled water. DNA amplification was performed by thermal cycler (Eppendrof Master Cycler Gradient). 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 50^0C, 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 were confirmed by running 2 % agarose gel in 1x TAE buffer at 40V for 80 minutes. Loading dye (5 µl) was added to the PCR products and loaded in the wells. Two molecular weight markers DNA (ladder) were also loaded on one side of the gel. After electrophoresis, the gel was taken out carefully from the electrophoresis chamber, 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 4

Results and Discussion

CHAPTER FOUR

Results and Discussion

Data regarding molecular characteristics were used to evaluate different levels of variability among experimental local cattle. Molecular characterization was determined using the molecular marker. RAPD markers are most useful for understanding the naturally occurring polymorphisms in living organisms.

4.1 DNA extraction and Quantity determination

Extraction of DNA was performed using modified protocol of Bruce A. Roe (1996). The quantity of DNA was confirmed using 2% agarose gel electrophoresis. 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 on crucially on the quality of the sample DNA. The spectrophotometer absorbance readings at 260 nm of twenty experimental local cattle shown in Table 3.

Table 3: Spectrophotometric absorbance reading at 260 nm of twenty experimental local cattle for DNA quantification

Serial No.	Accession or tag no.	Absorbance reading at 260 nm	Quantity of DNA(ng/ul)
1	Bag-27	0.049	245
2	Bag-99	0.043	215
3	Bag-102	0.025	125
4	Bag-50	0.038	190
5	Bag-69	0.044	220
6	Bag-03	0.039	195
7	Bag-35	0.030	150
8	Bag-05	**0.063**	**315**
9	Pir-85	**0.023**	**115**
10	Pir-05	0.040	200
11	Pir-07	0.032	160
12	Pir-10	0.053	265
13	Pir-01	0.056	280
14	Pir-13	0.028	140
15	Khu-09	0.037	185
16	Khu-02	0.042	210
17	Khu-03	0.035	175
18	Khu-11	0.028	140
19	Khu-12	0.039	195
20	Khu-17	0.041	205

The higher DNA concentration was recorded in Bag-05 (0.063). The lowest DNA concentration was recorded from Pir- 85 (0.023). The quantified DNA sample subsequently diluted to 2ng/ul as per requirements for ideal PCR. The PCR did run using ten primers and among them three primers showed reproducibility. The PCR product reproducibility 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 local cattle DNA using arbitrary oligonucleotide primers. The method was able to detect the heterogeneity of amplified DNA from the breeds of cattle. The present study indicates the effectiveness of RAPD analysis in detecting the level of polymorphisms among the experimental local cattle. Three effective primers (Table 4) were selected for determination the polymorphism of twenty genotypes.

4.2.1. Primer Selection and RAPD Analysis

In RAPD analysis all the ten primers were 10-12 bps long with 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 73^0 C (Weising *et al.*, 1995). Three primers were selected that used in RAPD analysis of twenty experimental cattle amplified twenty different reproducible bands.

Like all limitations, 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 strictly 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). Different RAPD patterns generated by the primers are represented below.

From the figure, present study have found primer OPA-03 and OPA-05 are more reproducible and produced more distinct RAPD profile than primer OPA-02. These three primers that used in RAPD analysis of twenty experimental cattle amplified 14 different reproducible bands. Three different primers generated various banding patterns and of the 14 bands scored, 12 bands (86.66%) were found to be polymorphic and 2 bands (40%) were found to be monomorphic in nature. The frequencies of polymorphic bands varied from primer to primer. Though no experimental local cattle-specific marker was identified, the high level of polymorphism revealed by the proportion of polymorphic loci indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in different experimental cattle. Experimental cattle could be distinguished by a combination of fragments and differences between clusters reflected differences in frequencies rather than presence or absence of experimental local cattle specific fragments.

Table 4: List of primers showed polymorphism

Primer code	Sequences	(G+C) Contents %
5	5′-TCTGCCATCC-3′	60%
3	5′-TTCCCCCCAG-3′	60%
2	5′-TCTGCCACGT-3′	60%

4.2.2. DNA amplification by PCR and RAPD fingerprints

Three RAPD primers were used to detect the polymorphisms of twenty experimental local cattle. Each of three 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 three different primers have been shown in Fig 1, Fig 2 and Fig 3. A total of 14 bands were amplified among cattle breeds using three primers and the polymorphic bands were 12. The frequencies of polymorphic bands varied from primer to primer. Though no experimental local cattle-specific marker was identified, the high level of polymorphism revealed by the proportion of polymorphic loci (86.66%) indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in different experimental local cattle.

Experimental local cattle could be distinguished by a combination of fragments and differences between clusters reflected differences in frequencies rather than presence or absence of experimental local cattle specific fragments.

Different RAPD patterns generated using the primers in the present experiment have shown in Figures 1 to 3.

Fig 1: RAPD pattern generated using primer-5 of twenty experimental local cattle. The number corresponds to the serial number of the genotypes (Table 1)

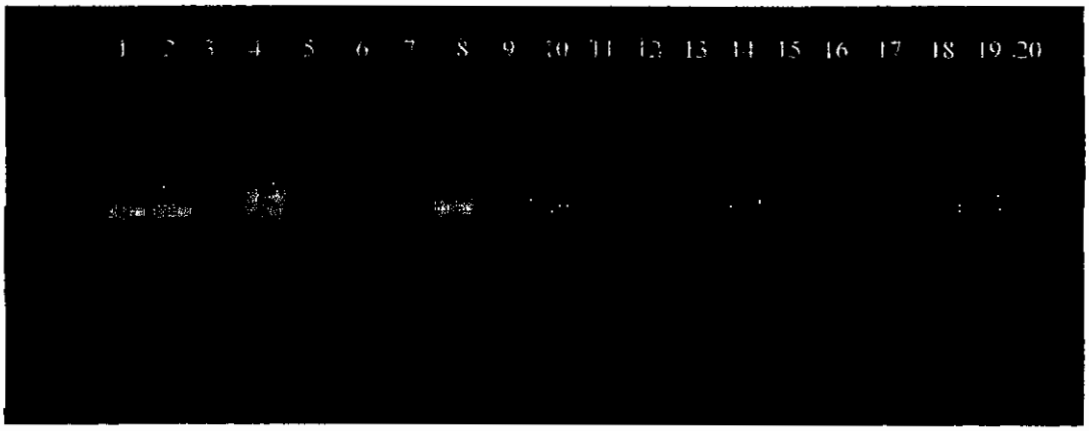

Fig 2: RAPD pattern generated using primer-3 of twenty experimental local cattle. The number corresponds to the serial number of the genotypes (Table 1)

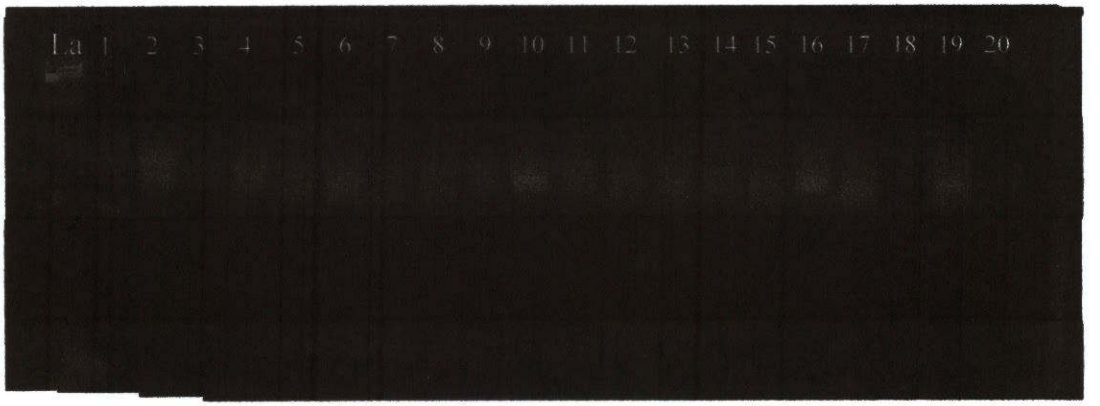

Fig3: RAPD pattern generated using primer-2 of twenty experimental local cattle. The number corresponds to the serial number of the genotypes (Table 1)

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

Serial No.	Primer code	No. of Total bands	No. of Monomorphic bands	No. of Polymorphic bands	Polymorphic Loci (%)
1	OPA-5	4	0	4	100
2	OPA-3	5	2	3	60
3	OPA-2	5	0	5	100
	Total = 3	14	2	12	Av. =86.66

4.3. Genetic Distance

Estimates of (Nei's and 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 he 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, comparatively higher genetic distances (0.90) were found among pair of Pir-10 and Khu-11, Pir-01 and Khu-11, Khu-09 and Khu-11 cattle. The lowest genetic distance (0.05) was observed between Bag-102 and Bag-69, Bag-99 and Bag-69 cattle. The range of genetic distance of twenty genotypes was 0.05-0.90. The difference between the highest and lowest genetic distance indicated the presence of variability among twenty genotype local cattle. The genetic diversity of Pirojpur and Khulna was relatively higher for prescribed local cattle. The results revealed that there was a high level (86.66%) of genetic variation among the experimental cattle as indicated by the proportion of polymorphic loci.

Estimation of higher level of genetic variation in the local cattle might be consistent with the fact that it is a highly polymorphic animal. The values of pair-wise genetic distance among twenty experimental cattle genotypes were computed from combined data sets for three primers ranging from 0.05 to 0.90 (Table 6). Total 14 bands were identified from PCR products which illustrated 86.66% polymorphism. The higher degree of polymorphism was 86.66% compared to 28 % in taurine cattle (Talle *et al.*, 2005) and 18 % in zebu cattle (Marle-Koster *et al.*, 2003).

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 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).

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

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	****																			
2	.33	***																		
3	.23	.05	***																	
4	.14	.50	.263	***																
5	. 23	.05	.05	.23	***															
6	.454	.38	.33	.45	.33	***														
7	.40	.20	.27	.80	.27	.33	***													
8	.33	.50	.07	.33	.07	.45	.40	***												
9	.25	.40	.27	.80	.27	.55	.50	.40	***											
10	.23	.20	.14	.23	.14	.16	.27	.384	.454	***										
11	.50	.33	.23	.33	.23	.09	.17	.33	.60	.230	****									
12	.80	.40	.27	.20	.27	.33	.50	.40	.90	.636	.40	***								
13	.45	.45	.50	.45	.50	.20	.33	.636	.77	.50	.454	.333	***							
14	.23	.23	.57	.07	.14	.33	.45	.230	.272	.285	.538	.636	.50	***						
15	.50	.33	.23	.50	.23	.27	.40	.333	.60	.538	.33	.20	.272	.384	***					
16	.23	.23	.28	.23	.28	.33	.27	.384	.454	.285	.23	.454	.166	.285	.384	***				
17	.55	.33	.40	.33	.40	.75	.42	.333	.428	.60	.55	.714	.75	.40	.77	.40	***			
18	.66	.71	.75	.28	.75	.55	.60	.714	.60	.75	.714	.90	.90	.75	.90	.75	.50	***		
19	.40	.20	.72	.20	.27	.44	.75	.60	.25	.454	.40	.75	.555	.272	.60	.272	.142	.60	***	
20	.75	.50	.50	.66	.55	.42	.33	.75	.66	.777	.75	.66	.428	.555	.50	.555	.60	.90	.333	***

4.4. Cluster Analysis of the Genotypes Based On RAPD Analysis

Genetic similarities measured through analysis of data using three RAPD markers from the twenty genotypes of local cattle revealed varying degrees of genetic relatedness. According to Nei and Li (1979) the genetic distance from 0 to 1. The highest dissimilarity coefficient (0.90) was observed among genotypes of local cattle. Using the Nei's (1979) genetic distance, a dendrogram was constructed to obtain the clustering of local cattle. The dendrogram shows that all the cattle were found to be grouped in two major groups designated as I, II, IIA, IIB, IIA(1), IIA(2), IIB(1) and IIB(2) . The distribution of the cluster members is shown in (Table 4). Cluster II is broad which includes seventeen local cattle. Cluster I includes the highest genetic distance. Cluster II divided into two sub- cluster (IIA & II B) & II a showed the less variation and cluster forms separately but II B again divided into sub-cluster and that cluster shows the highest genetic similarity because of their lowest (0.05) genetic distance value.

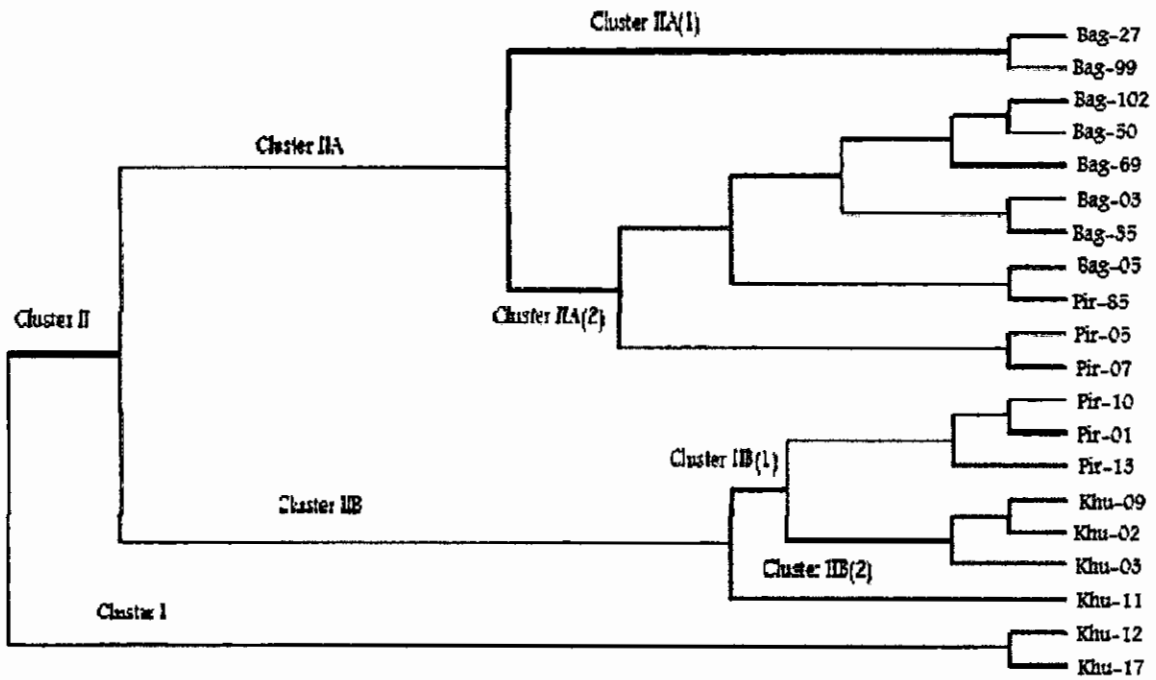

Fig 4: The unweighted pair group method with arithmetic mean (UPGMA) dendogram based on summarized data regarding differentiation among experimental local cattle

Table 7: Distribution of twenty experimental cattle under different cluster

Cluster no			Total no. of cattle in cluster	cattle included in different clusters
I			3	Khu-11, Khu-12, Kku-17
II	II A	II A(1)	9	Bag-27, Bag-99, Bag-102, Bag-50, Bag-69, Bag-03, Bag-35,Bag,-05, Pir-85
		II A(2)	1	Pir-07
	IIB	II B(1)	5	Pir-10,Pir-01, Pir-13, Khu-09, Khu-02
		II B(2)	1	Khu-03

4.5. Construction of phylogenic tree

The UPGMA dendogram based on the Nei's genetic distance differentiated the varieties into two main clusters-I and II. Cluster II is further divided into two sub-cluster, IIA and IIB which were further subdivided into II A(1), II A(2) and II B(1), II B(2) respectively.

4.6. Discussions

Genetic diversity is of great significance for breeding programmes. Germplasms characterization is an important component of programmes on effective and efficient management/utilization of animal genetic resources. All of these cattle were collected from Bagerhat, Khulna and Pirojpur and most of them were originated in the southern part of Bangladesh. RAPD markers are the most effective method to determine the molecular characterization. A maximum of 6 polymorphic bands were obtained in PCR amplification from genomic DNA of the cattle used in the present study. RAPD markers are randomly distributed throughout the genome, but most regions of the genome (86.66 %) are not expressed at the phenotypic level. 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 livestock importance. However, dendogram show almost same relationship among the selected local cattle and in both cases two different clusters were formed. Successive genetic relationship, genetic similarities and dissimilarities were determined by collecting data and constructing the UPGMA dendrogram to estimate the genetic

diversity of cattle. This effective population size could influence the structure of the variation. Determining the degree of variability within cattle is necessary as a preliminary step for studying the genetic diversity among cattle. In addition, the environment markedly influences the expression of most of the phenotypic traits, while detection of molecular markers is not dependent from the environmental effects. In this experiment only 86.66% polymorphism are identified among the selected local cattle, which is too poor when compared with the result of those experiment conducted with cattle. Mufti *et al.,* 2009 studied polymorphism and genetic relatedness in the five different local cattle population where he used twelve primers but only three were matched. This result is similar to present study. Mufti *et al.*, 2009 detected a total of 12 (92.31%) polymorphic loci which is higher than present study. That means within the time, the gene segregation rate was increased. This high level (86.66%) of polymorphism found in the present study may be due to their location of origin. The genetic difference among experimental cattle might be because of disease, environmental factors, lack of adequate feed, management etc. The higher genetic variability was observed in the cattle of Pirojpur, Khulna region compared to Bagerhat.

Considering the above parameters, diseases might be a major constraint for cattle production in Bangladesh; the extent of losses due to disease is very high. The country's climate along with the poor nutritional status of cattle contributes to a high incidence of cattle diseases, especially in the calves. The major diseases are anthrax, haemorrhagic septicaemia (HS), foot-and-mouth disease (FMD), black quarter (BQ), diseases caused by infestation with liver flukes and calf diarrhea (Ahmed, 2000). Khan *et al.* (1999) reported that most cattle suffered very badly from parasitic infestations compared with the local cattle; moreover, they reported that the incidence of parasitic diseases was very high in calves.

Dairy farms face problems with the availability of feeds and fodder; there are problems with both quality and quantity and a lack of economical technology for optimum utilisation of local feed resources. Rice straw is by far the most important crop residue, contributing >90% of feed energy available to ruminants (Tareque and Saadullah, 1988). However, animals fed on this diet fail to get adequate nutrients for maintenance and production. Efforts are being made to examine the possibilities and

economic feasibility of utilising non-conventional feeds, to improve feeding value of various agricultural and industrial by-products, and to prohibit the export of by-products such as bran, oilseed cake and molasses from Bangladesh. Furthermore, it has been established that fodder legumes can be integrated into rice production without having a negative impact on the yield of rice (Akbar *et al.* 2000).

Within production factors, labour cost is the single largest cost items all of the farming system. The higher productivity is achieved due to the use of skilled and experienced hired labour in the farm for high yielding animals which improves the management of the farms leading to increased milk production. The other reasons could be that intensive systems are operated with higher management skills and have high yielding cows (Uddin *et al.*, 2009). The livestock production executed through application of these appropriate technologies has not diverted or reduced the working hours of the beneficiaries.

Chapter 5

Summary and Conclusion

CHAPTER FIVE
Summary and Conclusion

Bangladesh is rich in cattle genetic resources, and diversity both at phenotypic and genetic level is enormous. Efforts to manage and utilize these resources efficiently are however, lacking both due to lack of awareness as well as due to weakness of Government institutions. There is a need to realize that it is our collective responsibility to leave the available genetic resources in a better form than what we inherited, to the coming generations .This thesis research investigated the relatedness and genetic variation among indigenous cattle. The first objective was to compare the cattle based on molecular characteristics and the second to determine relatedness among these using randomly amplified polymorphic DNA markers. In the second objective of the study, the indigenous cattle were analyzed using RAPD markers. Three primers that were shown to be polymorphic and informative were used in the analysis.

A genetic analysis using RAPD (Random Amplified Polymorphic DNA) markers was performed to determine the cattle-specific primers and designate the RAPD fingerprints and genetic diversities of cattle breeds an important province for cattle. The DNA samples were isolated from total of twenty animals from local cattle and three random primers were screened. Genetic relations among cattle were determined by RAPD polymorphisms. Statistical analysis of the data, estimating the genetic distances among local cattle and sketching the cluster trees were performed using COMPONENT version 1.31. The study showed that the polymorphisms generated by RAPD-PCR enable the determination of genetic relationships and fingerprints of the local cattle.

In this study, molecular characteristics were used to determine genetic relationship among these selected local cattle. The results obtained from amplified PCR products are more reliable than that of morphological character. In this experiment the highest value of Nei's genetic distance was found among Bag-03, Pir-85, Pir-10, Pir-01, Khu-09, and Khu-11and Khu-17 cattle. The lowest genetic distance was revealed between Bag-102 and Bag-69 cattle. Total 14 bands were identified from PCR products which illustrated 86.66% polymorphism. These data provides a scientific basis for future

studies on local cattle. In these study, few primers were used which were not enough to classify cattle. The present research work will provide guideline to further analysis of genetic study of local cattle. It can be concluded that overall genetic variation in each population from gene diversity and proportion of polymorphic loci points of views is relatively lower for a prescribed cattle.

The differences of genetic variations in the twenty experimental cattle were high and therefore, there is a scope for future improvement of local cattle. More research needs to be done on the native cattle and the choice of cattle for conservation must take into account any available information on productivity traits of economic value, specific adaptive features (tolerance to heat, low quality feeds, disease etc.), presence of unique genes or phenotypes, local or regional importance of a breed in production systems, and the availability of resources and infrastructure for animal production.

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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 ^{0}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 65^0 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 shaked 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 μm 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 ^{0}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 H_2O, 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.