

**ANALYSIS OF GENETIC DIVERSITY OF SELECTED
BANGLADESHI LOCAL CATTLE USING RAPD MARKERS**

A THESIS

BY



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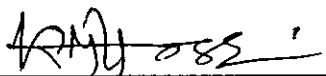
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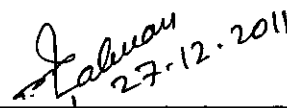
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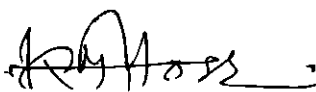
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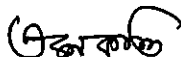
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**BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE,
LIFE SCIENCE SCHOOL, KHULNA UNIVERSITY, KHULNA-9208
BANGLADESH**

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DECLARATION

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 The findings of the research has not been/will not be published anywhere without the concurrence of the concerned supervisor/co-supervisor and the author.


29.12.11

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Dedicated to

My beloved parents

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ABSTRACT

Indigenous cattle is a potential genetic resources 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, randomly amplified polymorphic DNA (RAPD) analysis of local cattle was performed using eight primers. The major objectives of the study were to find out the genetic diversity of selected cattle population of Bangladesh and to suggest their improvement and conservation program. This experiment was conducted in the Animal Biotechnology and Cell Culture and Molecular Biology Laboratory under Biotechnology and Genetic Engineering Discipline, Khulna University (KU), Khulna, Bangladesh. DNA was extracted from twenty local cattle of the southern part of Bangladesh and the extracted DNA was observed by gel electrophoresis. Eight cattle specific primers were used in the study and two primers were capable of priming polymorphic amplification patterns. Random amplification of polymorphic DNA and Polymerase Chain Reaction (RAPD-PCR) analysis were carried out using DNA samples of twenty local cattle. Only unambiguous, reproducible and scorable polymorphic fragments were taken into consideration for analysis. Highest level of Nei's gene diversity value (1.00) was observed in Jes-2, Jes-3, Nar-4, Jhen-1 cattle and the low genetic value (1.00) was obtained in Khu-5, Nar-1, Nar-3, Nar-5 cattle among the twenty local cattle. The highest number of polymorphism observed was 77.50 percent. The number of monomorphism was 22.50 percent. The pair-wise genetic distance value ranged from 0.00 to 1.00. Dendrogram based on Nei's genetic indicated segregation of the twenty local cattle. The differences between the highest lowest genetic distances indicated presence the variability among the twenty genotype of cattle. The study showed the polymorphisms obtained using RAPD-PCR enabled to determine the genetic relationship and fingerprint of the cattle population. Adequate diversity in performance and adaptability can be exploited from the study result for actual improvement accruing to conservation and development of indigenous cattle resources. Therefore, to meet up the demand of dairy and beef industry of Bangladesh, the finding of the present study have an opportunity to improve the present cattle population of Bangladesh using selective breeding programs like artificial insemination, multiple ovulation and embryo transfer, *in vitro* fertilization etc.

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LIST OF ABBREVIATION

%	:	Percent
μl	:	Micro liter
μM	:	Micro molar
° C	:	Degree Celsius
A260	:	Spectrophotometer Absorbance Reading at 260 nm
A280	:	Spectrophotometer Absorbance Reading at 280 nm
AFLP	:	Amplified Fragment Length Polymorphism
Contd.	:	Continued
CTAB	:	N-Cetyl-N,N,N-Trimethyl Ammonium Bromide
d H ₂ O	:	Distilled water
DNA	:	Deoxyribonucleic acid
dNTPS	:	Deoxy nucleotide triphosphates
DW	:	Distilled water
e.g.	:	Exempli gratia = for example
EDTA	:	Ethylene Diaminetetra Acetic Acid
et al.	:	Et alia = and others
EtBr	:	Ethidium Bromide
etc.	:	et. cet. Era = and the others
gm	:	Gram
HCl	:	Hydrochloric acid
i.e.	:	id est = that is
M	:	Molar
mg	:	Milligram
mg/l	:	Milligram per litre
ml.	:	Milliliter
mM	:	Milli mole
N	:	Normal solution

Na ₂ EDTA	:	Sodium salt of ferric ethylene diamine tetraacetate
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
OD	:	Optical density
PCR	:	Polymerase chain Reaction
pH	:	Negative logarithm of hydrogen ion (H ⁺) concentration
PVP	:	Polyvinyl Pyrolidone
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribonucleic acid
SDS	:	Sodium Dodecyl Sulphate
SSR	:	Simple Sequence Repeats
UV	:	Ultra violet
viz.	:	Videlicet (= namely)
w/v	:	Weight per volume

Chapter One

INTRODUCTION

Domesticated animals, especially livestock and poultry, are an important source of protein in Asian countries including Bangladesh. Increasing this protein resource requires the conservation of diversity among indigenous livestock. In order to cope with an unpredictable future, genetic reserves that are capable of readily responding to directional forces imposed by a broad spectrum of environments must be maintained. Maintaining genetic diversity is an insurance against future adverse conditions (Mukherjee, 2000). In Bangladesh, diversity among environments and nutritional standards as well as challenges from multiple infectious agents requires diverse breeds and population. The breeds therefore act as storehouses of genetic variation which form the basis for selection and may be drawn upon in times of biological stress such as famine, drought or epidemics. The wide range of breeds and species that have evolved in various environments represent unique sets of genetic diversity (Liron *et al.*, 2006).

Cattle are large, even-toed hoofed, ruminant mammal of the genus *Bos* and family *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 (MacHagh *et al.*, 1997). *Bos indicus* and *Bos taurus* are the two distinct species of cattle. *B. indicus*, the Indian Zebu (humped) is available in the Indian subcontinent, characterized in general by lower growth rate, smaller size, late sexual maturity, poor production, and prone to parasitic attack but resistant to some infectious diseases (Mbulu and Hannote, 2005). On the other hand *B. taurus* (temperate cattle) is characterized by medium to heavy size, early sexual maturity, and has high production potentialities in terms of meat and milk. Cattle have multipurpose functions; it is used for traction of lands and cartage and produces milk and meat. Cow dung is used as manure and as fuel and a substrate for methane production. Cattle hides and skin are used for clothing, bags, shoes etc (Jewel, 1963).

In Bangladesh the total cattle population is about 23.4 million of which 11.91 million are males and 11.49 million are females. Included among the cattle population are about 3.53

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, Hariana, Sindhi, Australian breeds, Sahiwal-Friesian, etc (Yu *et al.*, 2004). 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 (Feliou, 1995).

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). It is generally accepted that the 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 (Cardellino, 2004).

Extinction of any local breed or population may result in the complete loss of some valuable alleles. It is estimated that 35% of mammalian breeds and 63% of avian breeds are at risk of extinction, and that approximately two breeds of livestock and poultry are lost each week (FAO, 2000). More particularly, it is estimated that 22% of known livestock breeds have become extinct in the last 100 years and another 27% are at varying degrees of risk (Rege & Tawah, 1999). The losses, as well as the risk to existing breeds, have been partially blamed on indiscriminate crossbreeding between exotic breeds and indigenous animals. Globally, this realization has led to efforts to study genetic diversity in livestock species in order to provide a foundation for conserving these potentially useful germplasms (Feliou, 1995).

In addition to loss of diversity, a large proportion of domestic animal breeds in the world is believed to be in danger of extinction (Cardellino, 2004). According to Hanotte and Jianlin (2005), though it may be too late for many livestock and poultry breeds in Europe, optimism in the developing world about slowing down the loss of both diversity and indigenous animals is high (Maule, 1990). The most significant threat to domestic animal diversity in the developing world, it is believed, is the sustained importation of high performing animals from developed countries. This leads to crossbreeding or even replacement of local breeds (Jewel, 1963).

Conservation of indigenous animal resources has been proposed as a method for slowing down the loss of diversity in livestock breeds through extinction. Apart from preventing

extinction, conservation of indigenous breeds is also important for the future health of the animal industry globally as they could be a resource for novel genes that can permit sustained genetic improvement as well as enable adaptation to changing breeding objectives and environments (Notter, 1999).

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 (Hassen *et al.*, 2007). 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 analysis of animal kinship relationships, behavioral and population ecology (Macneil *et al.*, 2006).

In order to ensure proper conservation and utilization of indigenous breeds, it is necessary to evaluate genetic variations that exist within and among breeds. A large proportion of indigenous livestock populations in the developing world have yet to be characterized or evaluated at phenotypic and genetic levels (Hanotte and Jianlin, 2005). In the case of Asian livestock, genetic classification of animal breeds is still needed. Most existing classification of livestock breeds in Asia including Bangladesh is based on historical, anthropological and morphological evidence, that are most often not enough for the purpose of conservation (Mwacharo *et al.*, 2006).

Relative genetic diversity can be determined using phenotypic characteristics and/or molecular markers. Phenotypic characteristics of livestock breeds as well as their adaptive characteristics are important in identifying breed attributes for immediate uses by farming communities (Syrstada, 1990). Despite this importance, most Asian breeds have not been phenotypically characterized and adaptive attributes of most indigenous livestock breeds have remained unknown. Traditional methods used to study individual genetic variability of animals and populations employed polymorphism in protein markers (Bradley *et al.*, 1996). However, these techniques lack the power to resolve differences between closely related breeds, since a great deal of genetic variation remains undetectable by using protein markers (Appa *et al.*, 1996).

Moreover, the genotype frequencies estimated from protein markers may be influenced by natural selection among alleles, making it difficult to interpret inter population comparisons. With recent developments in molecular genetics, it has been solidly established that a measure of relative genetic diversity in animal populations can be attained through description of nucleotide sequence differences and similarities in the deoxyribonucleic acid (DNA) of animals in such populations (Faulkner, 1962). Analysis of DNA has several significant advantages over protein markers for the study of molecular population genetics and systematics. First, the genotype rather than the phenotype is assayed. Second, the expression of DNA markers is not influenced by development or by environmental factors. Third, one or more sequences appropriate to a problem can be selected on the basis of evolutionary rate or mode of inheritance. Fourth, the methods are, for most part, general to any type of DNA and, fifth, DNA can be prepared from small amounts of tissues and is relatively stable, even in non-cryogenetically stored tissues. The last attribute means that genetic information on rare or endangered species can be obtained without destructive sampling and that it is possible to analyse DNA from extinct populations or species (Bruford *et al.*, 2003).

More recently, molecular data from DNA markers have received particular attention in the study of population variability because of their possible use in determining the chronology of evolutionary events. This is because DNA markers are much less subject to natural selection than are phenotypic traits (Gwakisa *et al.*, 1994).

The advent of molecular techniques has led to an increase in the studies that focus on the genetic characterization of domestic breeds using genetic markers (Giovambattista *et al.*, 2001). As a tool used in evaluating genetic variation, markers can provide useful information at different levels and purposes such as structure of animal populations, levels of gene flow, phylogenetic relationships, patterns of historical biogeography, and parentage (Feral, 2006). In addition, genetic assessment is also of interest for the design of genetic improvement programs including appropriate choice of breeds for cross breeding (Mwacharo *et al.*, 2006).

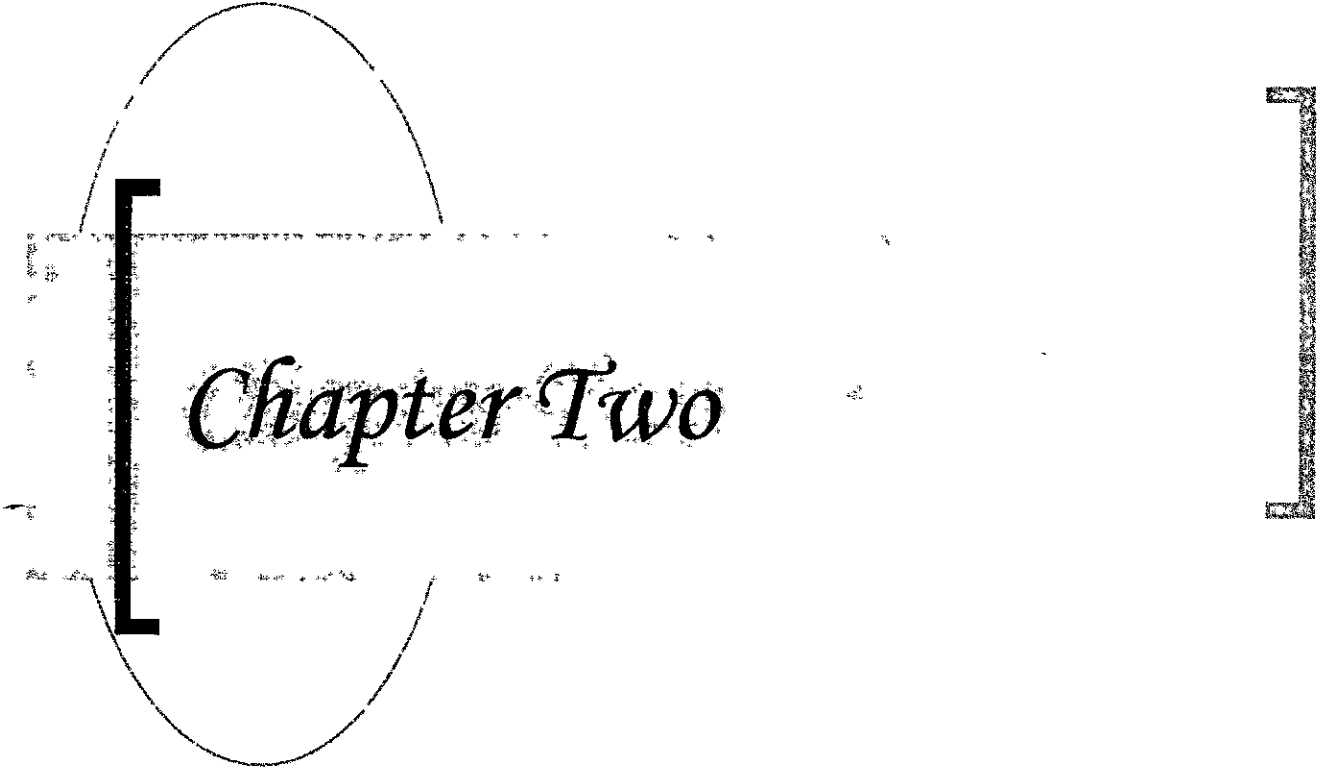
The ability to detect polymorphisms at the DNA level has led to new approaches for the genetic analysis of farm animals. Furthermore, an understanding of the pattern of genetic variability among breeds may help in the development of more rational breeding programs (Gulbaz, 2004). Genetic markers are powerful tools to investigate the phylogenic relationships and differentiation among breeds. Various molecular markers

have been identified in livestock species, such as protein polymorphism, randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), variable number tandem repeat (VNTR), single nucleotide polymorphism (SNP), short tandem repeat (STR) and microsatellite polymorphisms (Ertugrul and Alpan, 1991).

The studies on population genetic structure and genetic variation using RAPD were claimed to be quite successful (Feral, 2006). The main advantages of RAPD technique are that it is easier, less labour intensive, quicker and cheaper than the other DNA marker assays, and may also provide markers of choice for population studies, where a large number of individuals have been studied (Ozbeyaz *et al.*, 1999). The RAPD-PCR method is used to determine relationships among different breeds and individuals and to formulate breeding strategies and also used to control the pedigree records of the animals (Gulbaz, 2004).

Our understanding of genetic variation and the relatedness among cattle in most Asian countries including Bangladesh remains poor. The genetic variability of native cattle of the southern region has not been well documented so far. Therefore, the present research work has been undertaken with the following objectives:

1. To determine the genetic variability and relationship of selected native cattle.
2. To evaluate the genetic distance among experimental cattle.
3. To suggest possible improvement and conservation strategies of cattle population in Bangladesh.



Chapter Two

Review of Literature

Chapter Two

REVIEW OF LITERATURE**2.1 Description of Cattle**

Cattle are large ruminants of the family *Bovidae* and the genus *Bos*. Ruminants are mammals whose stomach has four parts such as rumen, reticulum, omasum, and abomasums. The rumen provides a pouch where fibrous plant materials are broken down by bacteria so their nutrients can be digested. Because they are ruminants, cattle can digest plant materials that serve no other human use. As herbivores, they are selective eaters, but consume a variety of types of plants (Junichi *et al.*, 2002).

From the time of their original domestication, cattle were selectively chosen to meet multiple human needs, including providing traction and transportation, meat, and milk. Cattle have provided fuel for cooking and heating, plaster for walls, manure for gardens and fields, strings for musical instruments, and clothing from hats to shoes. Originally valued for docility, cattle are increasingly bred to meet specific needs of those who raise them, process them, and eat them (Lott *et al.*, 1979).

The systematic development of cattle breeding began as a part of the industrial revolution and the renaissance of British agriculture. The enclosure movement in England in the sixteenth century not only forced rural peasant farmers from the land to work in factories, but left privatized lands in the hands of a few landed gentry who could breed the stock they desired. Breed formation started with useful local type that was then inbred until it showed uniformity (Junichi *et al.*, 2002).

In other regions of Europe, inbreeding had produced uniform and locally adapted breeds, although specialization in rearing and feeding cattle for beef occurred long after it had in Great Britain. In the United States, interest in breeding began around the turn of the twentieth century (numbers had been important up to that point). In particular, Herefords were imported because they matured early, which allowed for the slaughter of yearlings rather than the four year olds prevalent at that time. In parts of France, five to six year

olds are still preferred for their flavor, particularly if they are fattened on grass (Takeda, *et al.*, 2004).

In the course of seeking early maturing animals, many lines and traits have been lost. However, the introduction of new breeds has transformed the appearance and probably the taste, nutritional qualities, and tenderness of cattle in beef exporting nations, particularly the United States and Canada (McWhirter *et al.*, 1968).

Scientific classification

Kingdom:	Animalia
Phylum:	Chordata
Class:	Mammalia
Order:	Artiodactyla
Family:	Bovidae
Subfamily:	Bovinae
Genus	<i>Bos</i>
Species:	<i>B. primigenius</i>
Subspecies:	<i>B. p. taurus</i> , <i>B. p. indicus</i>
Binomial name	<i>Bos primigenius</i> , Linnaeus, 1758
Trinomial name	<i>Bos primigenius taurus</i> , <i>Bos primigenius indicus</i>
Synonyms	<i>Bos taurus</i> , <i>Bos indicus</i>

2.2 Species of cattle

Cattle were originally identified by Carolus Linnaeus as three separate species. These were *Bos taurus*, the European or "taurine" cattle (including similar types from Africa and Asia); *Bos indicus*, the zebu; and the extinct *Bos primigenius*, the aurochs. The aurochs is ancestral to both zebu and taurine cattle. Recently these three have increasingly been grouped as one species, with *Bos primigenius taurus*, *Bos primigenius indicus* and *Bos primigenius primigenius* as the subspecies (Takeda, *et al.*, 2004).

The aurochs originally ranged throughout Europe, North Africa, and much of Asia. In historical times its range became restricted to Europe, and the last known individual died in Masovia, Poland, in about 1627 (Krebs *et al.*, 1997). Breeders have attempted to

recreate cattle of similar appearance to aurochs by crossing traditional types of domesticated cattle, creating the Heck cattle breed.

2.3 Cattle in Asia

It is generally believed that domestication of cattle in Asia started in the Ancient East, the region with the most ancient civilization. According to Payne (1964, 1970) and Rouse (1970), the Semitic tribes of southern Arabia were the first to introduce Zebu and *Bos taurus* into Eastern, Southern, and Northern Asia. Cattle in Asia can be classified into four major groups: the humpless *Bos Taurus*; the humped *Bos indicus* (Zebu), distributed widely in Asia; *B. taurus* x *B. indicus* (Sanga), found mainly in eastern and southern Asia; and Sanga x Zebu types also known as Zenga (Reisti-Marti *et al.*, 2003). With its unique set of genetic resources, Asia is known to be one of the centers of cattle diversity and domestication (Hanotte *et al.*, 2002). However, the site of initial domestication of cattle in Asia is as yet without any consensus.

The indigenous Zebu cattle accounts for about 90 % of the total cattle population in most Asian countries (Teale *et al.*, 1993). However, indiscriminate crossbreeding of the Zebu with Taurine breeds has been a major contributor to the genetic dilution of this resource in most Asian countries. This has been accelerated by advances in new reproductive techniques, particularly artificial insemination and increased exchange of breeding stocks between regions (Moazami Goudarzi *et al.*, 1997).

Mburu and Hannotte (2005) described a study that looked at genetic diversity and population structure in 52 populations of Asian cattle using 15 microsatellite markers. The study indicated a wide range of genetic diversity among Asian cattle based on mean number of alleles, effective number of alleles, and expected heterozygosity. There was high differentiation among Asian cattle breeds. Diversity was highest in the North Asian *Bos taurus* and lowest in the western Asian cattle. The analysis supported isolation by distance in all, but the Southern Asian region, with extensive gene flow between populations. Within these, there is extensive gene flow between populations. These and other molecular studies have revealed that cattle in Asia are appropriately partitioned by origin into East, West, North, and South (Talukder *et al.*, 2003). Though both *Bos Taurus* and *Bos indicus* can be found in West Asia, Red Bororo and White Fulani are the primary cattle raised in that region. It is also the only region of Asia that still maintains a large population of apparently pure taurine breeds (Pasha K, 2000).

Several cattle breeds in Asia have been reported to be near extinction or have already been lost. Though there are no recent updates, examples of both were previously reported by Epstein (1974). Though most North Asian cattle were reported to be long or giant horned and humpless types, currently, only the N'Dama and Muturu of West Asia, the Namji of Northern Cameroon and the Kuri of Lake Chad with these characteristics can be found. In the Republic of Sudan, the humpless Nuba short-horned cattle that were previously found in the mountain region of the southern Kardofan, are now hard to find even (Alderson and Bodo, 1992). In Nigeria, the Biu cattle normally restricted to a hilly volcanic region area of the Bornu province are becoming increasingly hard to find. In Ethiopia, the Sanga cattle maintained by the Danakil and the Balla Azebo people are also very hard to find. In Uganda, the giant-horned Ankole Sanga are said to have been restricted by the advances of *Glossina morsitans* to a few isolated enclaves. Also in Tanzania, only a few herds of Ankole cattle are said to be present on the eastern side of the Tabora belt. Alderson and Bodo (1992) have also reported that in Zimbabwe, the original Amabowe have become exceedingly rare owing to the disastrous effects of the rinderpest which destroyed a large proportion of African herds.

Knowledge of the genetic variation that can be effectively measured within and between populations is important and also required for breed characterization (Hetzel and Drinkwater, 1992). The relationship among most of the Asian cattle breeds has remained poorly understood. Often, the same breed may be known by different names and equally, two breeds may be known by the same name depending on the geographical locations of such breeds (Gwakisa *et al.*, 1994). For example, in Thailand, most of the livestock species have no specific breed names but are known by the local or common names in different parts of the Country. Depending on the different geographical location and or tribe, goats are called Mbuzi, Mpongo, Imbushi; sheep are called Mbelele, Imbelele, Mpanga; and different chicken strains e.g Inkoko, Kunku. The different synonyms may represent the same or different breeds. In order to develop appropriate strategies to curb the threats of dilution and extinction it is important to quantify the genetic variation that is present among breeds.

Bangladesh, like most Asian countries, has witnessed an increased form of agriculture development and urbanization during the past few decades. To meet and sustain the increased demand for livestock products such as milk, importation of specialized cattle breeds from temperate regions has been the trend. This has placed an excessive selection

pressure against indigenous cattle. In turn this has led to the reduction in genetic variability of indigenous cattle breeds through breed substitution (Pasha K, 2000).

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 (Macneil *et al.*, 2006).

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. But again, according to Hedrick (2005), this measure is strongly influenced by the population size so that comparisons across populations with different sample sizes should be made cautiously. The effective number of alleles, n_e , which is the inverse of the expected homozygosity, is also sometimes used to measure the amount of genetic variation 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. From their results, the number of alleles, n , heterozygosity, and effective number of alleles, ranged from 4-12, 0.51-0.86 and 2.48-5.41 per locus, respectively.

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, for example, 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 Yano, 1983).

Another measure which partitions the genetic variation and provides a description of differentiation was developed by Wright (1951, 1965). This approach consists of varying types of differentiation measurements called F coefficients. One commonly reported F coefficient is F_{st} , which measures the degree of genetic differentiation based on standardized variances in allele frequencies among populations (Balloux and Goudet, 2002). Metta *et al.*, (2004), for example, genetically characterized two Indian cattle breeds, Ongole and Deoni, using coefficients F_{st} and F_{is} , the levels of differentiation and inbreeding, respectively. Phylogenetic trees often are constructed to accompany distance measurements. For example, Edwards *et al.*, (2000) constructed two Neighbor-joining trees, one in which they used the genetic distances to visualize the relationships between the endangered pustertaler-Sprinzer and three European cattle breeds and another in which they used the allele sharing statistic suggested by Bowcock *et al.*, (1994) with individual animals acting as operational taxonomical units (OTUs).

2.5 Tools to understand livestock diversity – molecular characterization

Genetic marker types that provide different estimates of genetic diversity information have been described. 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 (Hannote 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). Using published data on protein polymorphisms from approximately 1000 papers in about 216 breeds of cattle, Manwell and Baker (1980) analyzed differences among animals from different continents. Protein polymorphisms, although still used in population studies, are of limited value in the assessment of genetic variation at the level of cattle breeds. This is

largely because of the relatively low levels of polymorphism found in protein loci, resulting in a lower taxonomic limit for the resolving power of protein electrophoresis.

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.

One of the oldest molecular DNA markers used for assessing diversity is restriction fragment length polymorphisms (RFLPs). This method involves comparing the number and size of deoxyribonucleic acid (DNA) fragments produced by the digestion of DNA with various restriction enzymes. The banding pattern is generated by the presence or absence of restriction cutting sites and these in turn are produced by mutation. The fragments are blotted to membranes and probed with cloned radio-labeled DNA that binds to a single locus. This technique can be applied to nuclear DNA or to mitochondrial DNA (also to chloroplast DNA in the case of plants). It has applications in the study of genetic distance, population variation, gene flow, effective population size, patterns of historical biogeography and analyses of parentage and relatedness. For example, Theilmann *et al.* (1989) used nine RFLPs in six breeds of cattle to show that the Brahman (*Bos indicus*) differed significantly from the *Bos taurus*. Amongst the most widely used molecular markers for assessing relatedness in livestock are microsatellites. In recent years, in addition to being the markers of choice for gene mapping, they have been used in population studies to assess diversity (Wall *et al.*, 1993).

Microsatellites are highly polymorphic, densely distributed in the genome, highly variable, and relatively easy to detect using the polymerase chain reaction. As hypervariability is highly significant for detecting differences in a population and between individuals, microsatellite typing can reveal degrees of polymorphism that are easy to interpret (McWhirter *et al.*, 1968). Microsatellites have been isolated in large

numbers from most livestock species and FAO has recommended a list of microsatellite markers for genetic diversity studies that are now publicly available (<http://www.fao.org/dad>). The primary disadvantage of this technique is that a prior knowledge of the DNA sequence is required to allow the design of PCR primers. 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 (Williams *et al.*, 1990).

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 Yunnan DeHong cattle breed was closely related to the Brahman (*Bos indicus*), and the Yunnan DiQing cattle breed was closely related to the Simmental, ShortHorn, and MurryGrey (*Bos taurus*) breeds. Under the FAO recommendation, most of the livestock variation studies that have been done used microsatellite markers. The major drawback of RAPDs is that they are dominant markers and heterozygotes are typically scored as homozygotes, which decreases their information content.

2.6. Genetic Characterization of Cattle

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

enzymes and may also interfere in the quantification of nucleic acids by spectrophotometric methods (Wilkie *et al.*, 1993).

Typically, DNA, once extracted and purified, is stable when stored in TE, a Tris (hydroxymethylaminomethane) buffer at pH 8.0 containing 1 mM EDTA as a Mg²⁺-chelator (Mg²⁺ ions are required by DNases-ubiquitous enzymes which degrade DNA). Storage of DNA is usually at 4°C for short periods of time, or frozen when weeks, months, or years are involved (Clark and Christopher, 2000).

Pasha (2000) stated that after incubation at -20°C addition of cold ethanol (-17°C to -28°C) a volume of 2.5 times higher than the mixture favors DNA to precipitate. According to Puchooa (2004), once the nucleic acids are collected, they are washed in the ash butler. The purity of genomic DNA is dependent on the number of washes. A three-time wash combined with a short-run centrifugation is sufficient for DNA purification and removal of endogenous nuclease or other proteins.

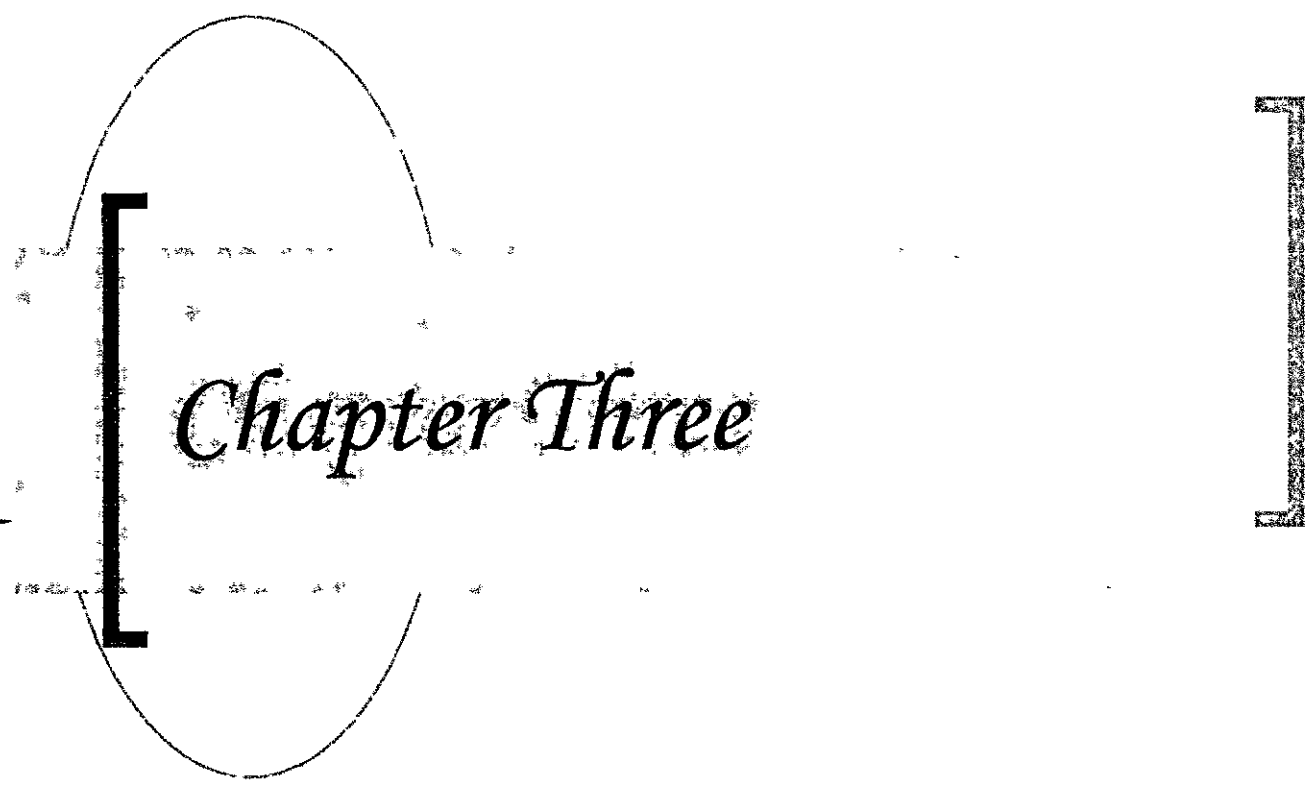
Ali, B.A. (2003) carried out a genetic analysis using RAPD markers for studying variation in four breeds of sheep (Baladi, Barki, Rahmani and Saffolk). Nineteen random primers were used to amplify DNA fragments in these breeds. RAPD patterns with a level of polymorphism were detected between breeds.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

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

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

based on allele sharing distances indicated that the inertia of the breeds is not high. Yet, the genetic differentiations between the breeds based on pairwise F_{ST} (inbreeding coefficient) values are all significant. Furthermore, the three microsatellite loci could distinguish three groups of native breeds and their crossbreeds.

Stephen *et al.* (1999) carried out an experiment on Indigenous cattle of the United Republic of Tanzania. The study was conducted to determine the genetic relationships among five Tanzanian cattle ecotypes kept by Maasai, Gogo, Sukuma and Konde communities in the country. A total of 30 random amplified polymorphic DNA (RAPD) and 198 microsatellite polymorphic alleles were used to study genetic relationships among the cattle ecotypes. The results showed closer relationships between ecotypes that are located close to one another than between ecotypes separated by greater geographical distance. Clear evidence of broad genetic diversity was observed among the studied cattle ecotypes. The diversity within the ecotypes makes genetic improvement through selection a possibility. Once they have observed that Tanzanian cattle possess different genetic attributes, farmers will have the benefit of choosing traits that will be included in future breeding programmes. The authors recommended the use of indigenous cattle genetic resources in breeding programmes rather than the usual practice of cross-breeding with exotic genotypes.



Chapter Three

Materials And Methods

Chapter Three

MATERIALS AND METHODS

This experiment was carried out at the Animal Cell Culture and Molecular Biotechnology Laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh. The sample of the experiment was collected from the different Upozilas under different Districts of the southern part in Bangladesh.

3.1 Study materials

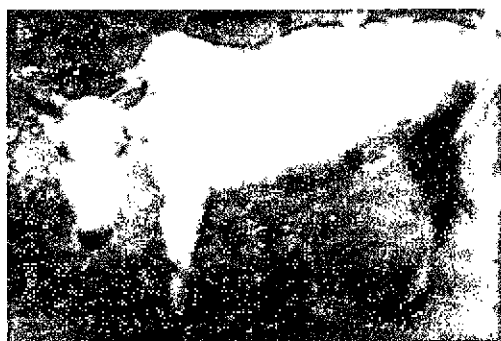
Twenty blood samples of cattle with different phenotypic and genotypic characteristics were collected from the different Upozilas under different Districts of the southern part of Bangladesh for the purpose of the research work. The different phenotypic characteristics and others information were collected by the help of cattle farmer and the respective Thana veterinary surgeons.

Table: 3.1 Cattle code no., sources and identity.

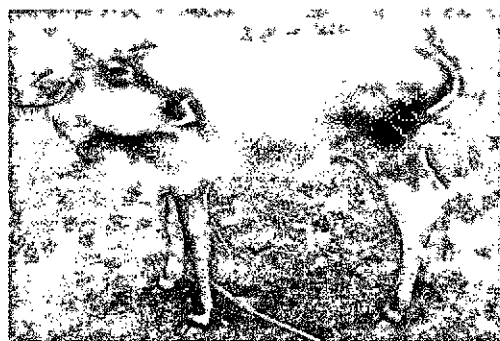
Sample No.	Cattle Code No.	Source	Cattle Identity
01	Jes-1	Jessore	Cow
02	Jes-2	Jessore	Cow
03	Jes-3	Jessore	Heifer
04	Jes-4	Jessore	Cow
05	Jes-5	Jessore	Cow
06	Khu-1	Khulna	Cow
07	Khu-2	Khulna	Cow
08	Khu-3	Khulna	Bull
09	Khu-4	Khulna	Cow
10	Khu-5	Khulna	Heifer
11	Nar-1	Narail	Bull
12	Nar-2	Narail	Cow
13	Nar-3	Narail	Bull
14	Nar-4	Narail	Cow
15	Nar-5	Narail	Heifer
16	Jhen-1	Jhenaidha	Cow
17	Jhen-2	Jhenaidha	Cow
18	Jhen-3	Jhenaidha	Bull
19	Jhen-4	Jhenaidha	Cow
20	Jhen-5	Jhenaidha	Cow

3.1.1 Selection of Animals

Native cattle were randomly selected avoiding close genetic relationships among the sampled animals from eight Upozilas under four Districts of the southern part of Bangladesh for this study. All of the cattle are indigenous breeds and containing the phenotypic and genetic variation among each other.



Code No. # Khu-2



Code No. # Nar-5



Code No. # Jhen-5



Code No. # Jes-4



Code No. # Khu-3



Code No. # Nar-3



Code No. # Jhen-2



Code No. # Jes-3

Figure 4.1: Blood sample collecting cattle.

3.1.2 Morphological Characterization

Identifying the different phenotypic characteristics of the twenty different regional breeds of cattle considering the cattle external features such as body color, cattle type, body size, cattle height, head size, ear length, horn length, horn type, eye color, tail size, and age and health condition. Morphological identification, code number and identity of selected Cattle Breeds for the research purposes are given below in the table no 3.1.

3.1.3 Blood Sample Collection

Five ml of blood was collected from each animal in 10 ml EDTA Vacutainer. The blood was gently mixed with EDTA (present in the vacutainer as an anticoagulant agent) and kept on ice to maintain low temperature in order to prevent cell lysis. Subsequently the blood samples were transported to the laboratory and stored at -20°C until the isolation of genomic DNA.

3.2. DNA Extraction

DNA was extracted from blood by following the method of Bruce A. Roe (1996), with some modifications. Quantity and quality of extracted DNA was analyzed by a T60 spectrophotometer at the wavelengths of 260nm and 280nm. The presence of DNA was confirmed by running the Extracted DNA on a 1% agarose gel.

3.2.1. Preparation of reagents for DNA isolation

Different types of reagents are used for DNA extraction. Stock solution components and concentration of Stock solution and chemicals are presented in the annex.

3.2.2: Protocol for DNA Isolation (Modified form of Roe *et al.* 1996).

1. The liquid blood samples was obtained in EDTA Vacutainer tubes and stored at -20°C freezer.
2. The frozen samples was thawed ; added 0.8ml of 1X SSC buffer with 400µl of blood in a 1.5 ml centrifuge tube and mixed well by overtaking. Centrifuge at 12000 g for 5 min.
3. Removed 1ml of the supernatant and discard into disinfectant.
4. 1ml of 1X SSC buffer was added , vortexed and centrifuged as above for 5 min and remove all of the supernatant.
5. 375µl of 0.2M sodium acetate was added to each pellet and vortexed briefly. Then added 25µl of 10% SDS and 5µl of proteinase K, vortexed briefly and incubated for 1 h at 55°C.

6. 40µl of phenol: chloroform: isoamyl alcohol was added and vortexed for 30 sec. centrifuged the sample for 10 min at 12000 g.
7. Carefully removed the aqueous layer to a new 1.5 ml microcentrifuge tube, added 1 ml of ice cold 100% ethanol, mixed and incubated for 15 min on ice.
8. Centrifuged for 10 min at 12000 g. Decant the supernatant and drain.
9. 180 µl of TE (10:1) buffer was added, vortexed and incubated at 55°C for 10 min.
10. 20 µl of 2M sodium acetate was added and mixed, added 500 of cold 100% ethanol, mixed and centrifuged for 5 min. at 12000 g in a microcentrifuge.
11. The supernatant was decanted and rinsed the pellet with 1ml of 70% ethanol. Centrifuged for 5 min. at 12000 g in a microcentrifuge.
12. The supernatant was decanted and dried the pellet in Savant Speed- Vac for 10 min (or until dry).
13. Resuspended the pellet by adding 200 of TE (10:1) buffer. Incubated overnight at 55°C, overtaking periodically to dissolve the genomic DNA. Stored the samples at -20° C.

3.2.3. Preparation of DNA samples for electrophoresis

The samples were all in the same concentration of buffer. For each sample, 9 µl of DNA sample and 2.5 µl of gel loading dye were mixed well using the micropipette. The prepared samples were then loaded slowly to allow them to sink to the bottom of the wells. A molecular weight marker DNA was also loaded on the gel. The gel was transferred carefully to the electrophoresis chamber (Whatman Biometra) keeping the gel horizontal and in a submerged condition in 1 X TAE buffer and the final level of the buffer was about 5 mm above the gel. Electrophoresis was carried out at 80V, 300mA and 300W for 1:30 minutes and the electrophoresis power supply was provided by EPS- 301 (Amersham Pharmacia Biotech). The separation process was monitored by the migration of the dye in the loading buffer. When the bromophenol blue dye had reached about three-fourths of the gel length, the electrophoresis was stopped.

3.2.4. Documentation and Detection of the DNA samples

After electrophoresis, the gel was taken out carefully from the electrophoresis chamber, washed with 2 µl ethidium bromide mixed distilled water (500ml) and placed on high performance ultraviolet light box (UV transilluminator) for checking the DNA bands. The DNA was observed as band and was photographed using a Digital Camera. Detection was carried under UV light with Digital camera for capturing photograph.

3.2.5. Quantitation of DNA Concentration and Quality by Spectrophotometer

Different DNA extraction methods showed wide variation in DNA purity and concentration. Thus, it is necessary to optimize the amount of DNA used in the RAPD analysis to achieve reproducibility and also the strong signal. Below a certain concentration of genomic DNA, Rapid Amplification of DNA is no longer reproducible (Williams *et al.*, 1993). Thus, it is essential to keep DNA above the critical concentration. It is best to do a series of RAPD reaction using a couple of primers and a set of serial dilutions of each genomic DNA to identify empirically the useful range of DNA concentration, for which reproducible RAPD patterns are obtained.

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

In each case, an exact measurement of the absorbance of DNA at 260 nm is critical. Many of the requirements of the spectrophotometer for making exact measurements at an absorbance peak are obvious, good wavelength and photometric accuracy, low stray radiant energy, noise and drift.

3.2.6 Preparation of Stock Solutions for Gel Electrophoresis

For conducting the gel electrophoresis different types of stock solutions and other reagents were prepared. Stock solution components and concentration of Stock solution and chemicals for Gel Electrophoresis are presented in the annex.

3.2.7. Preparation of the DNA sample for spectrophotometer

- The test samples were prepared by taking 2 µl of each DNA sample in the cuvette containing 2 ml sterile distilled water. The samples were mixed well by using an adjustable micropipette.

3.2.8. Recording the absorbance readings

- The reading was taken by viewing the monitor of spectrophotometer at 260 nm and 280nm respectively,
- After recording the absorbance readings, the cuvette was rinsed out with sterile distilled water, tamped out on a paper wipe, and absorbance reading for each sample was recorded in the same way. The ratios of spectrophotometric absorption readings

at 260nm and 280 nm of different samples for quality determination of DNA are shown in the table 5 in the results and discussion chapter.

3.2.9. Calculation of the Concentration of DNA

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

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

$$\begin{aligned} &= A_{260} \times \frac{\text{Volume of distilled water } (\mu\text{l})}{\text{Amount of the DNA sample } (\mu\text{l})} \times 50 \\ &= (\text{ng}/\mu\text{l}) \\ &= (\mu\text{g}/\text{ml}) \quad (\text{since } 1 \mu\text{g} = 10^3 \text{ ng}) \\ &\quad [\text{i.e. } \mu\text{g}/\text{ml} = \text{ng}/\mu\text{l}] \end{aligned}$$

A_{260} = Spectrophotometric 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 ng/ μl of DNA contained in a solution which gives the Spectrophotometer absorbance reading at 260nm equal to 1.

3.3 Primer Selection for PCR reaction:

In this study 2 RAPD primer was screened out of 8 primers (Invitrogen) due to shortage of Taq DNA polymerase which has a vital role in amplification reaction. The primers are:

Table 3.2: RAPD primers amplified in this experiment

Primer code	Sequences	(G+C) Contents %
5	5'-TCTGCCATCC-3'	70%
3	5'-ACTTCCGTCT-3'	70%

Table 3.3: RAPD primers used in this experiment

Primer No.	Sequence (5'-3')
01	AATSSGCTGG
02	TCAGCCACGT
03	ACTTCCGTCT
04	GTTGCCACCC
05	TCTGCCATCC
06	TTCCCCCAG
07	TCTGCCATCC
08	GGCCCTACAT

3.4. PCR Amplification:

The amplification conditions were based on Williams *et al.*, (1990) with some modifications. PCR reactions were performed on each DNA sample in a 20 μ reaction mix containing 4 ng genomic DNA, 2 μ l of 10x reaction buffer, 2 μ l units of Taq DNA polymerase (5 times dilution by Enzyme dilution buffer) 1 μ l (100picomol) of primer and 11 μ l of distilled water. DNA amplification was performed by thermal cycler (Eppendorf Mastercycler Gradient). The PCR samples were overlaid with one drop of mineral oil. The amplification program included an initial denaturation step of 94°C for 3 minutes followed by 40 cycles of 94°C (denaturation), 1 minute, 37°C (annealing), 1 minute and 72°C (extention) for 2 minutes. The last cycle was followed by 10 minutes incubation at 72°C and hold at 8°C temperature.

3.5 Agarose Gel Preparation For Electrophoresis:

For agarose gel electrophoresis, 50 ml of 1% agarose gel was prepared. For casting the gel the following steps were followed:

- Initially, 0.5 g agarose powder was weighed out and placed into a 250 ml conical flask.
- Then 50 ml of electrophoresis buffer (10 $\times$ TAE buffer) was added into the flask. The flask was enclosed with aluminum foil.
- The flask was then placed into a microwave oven. Using a low to medium setting, the timer was set for 2 minutes. The oven was stopped and swirled the container gently to suspend the undesired agarose.

- When the agarose solution was cooled to about 50 °C (the flask was cool enough to hold comfortably with bare hand), 4 µl from 10 mg/ml solution of ethidium bromide was added so that in the gel the concentration of ethidium bromide was maintained as 0.8 µg/ml and mixed well by gentle shaking to make DNA visible under ultraviolet light.

3.6 Gel Electrophoresis of PCR Amplified Product

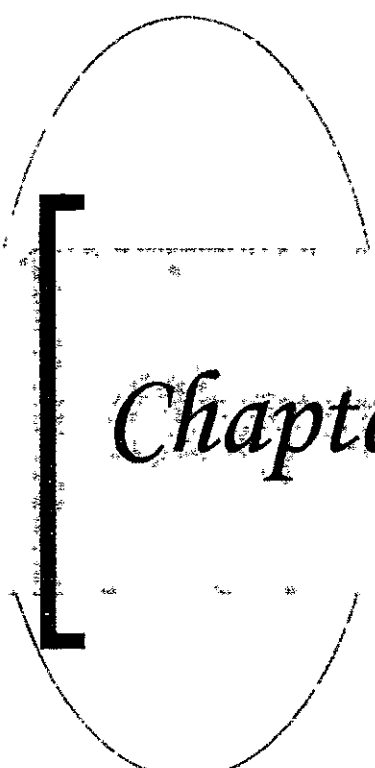
PCR products from each sample were confirmed by running 1 % agarose gel in IX TAE buffer at 40V for 80 minutes. Loading dye (5 µl) was added to the PCR products and loaded in the wells. Two molecular weight markers DNA (ladder) were also loaded on one side of the gel. After electrophoresis, the gel was taken out carefully from the electrophoresis chamber was washed with 2 µl ethidium bromide mixed distilled water 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 Gel Electrophoresis of PCR Amplified Product

Data scoring was completed by the visual analysis of RAPD photographs. Two numbers (0 & 1) are used for DNA band detection. 0 indicates the absence of DNA and 1 indicates the presence of DNA.

3.7 Data Analysis:

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



Chapter Four

Result And Discussion

Chapter Four

RESULTS AND DISCUSSION

Morphological and molecular characterizations were used to the twenty accessions and displayed different levels of variability concerning the morphological traits of local cattle. Molecular characterization was determined by using the molecular marker. RAPD markers are most useful for understanding the naturally occurring polymorphisms in living organisms.

4.1 Morphological Characterization Classification of selected cattle breeds based on morphological characters:

In this experiment the local cattle were classified based on morphological characters. Although the morphological characters are governed by environmental factors in some extent, but can be used as marker for breed identification. For morphological characterization different parameters were taken into consideration. Among these parameters body weight, body length, tail length, coat color, horn length, horn color, height, milk production, gestation period, were highly variable. Morphological characteristics of the selected cattle breeds are shown in table 4.1.

4.2. DNA extraction and quality determination

Extraction of DNA was performed using modified protocol of Bruice A. Roe (1996). The quality of DNA was confirmed by 1 percent agarose gel electrophoresis. PCR is a technique of choice for nucleic acid quantification. The DNA quality was determined by spectrophotometric absorbance at 260 nm and subjected to PCR amplification. However, successful quantification depends crucially on the quality of the sample DNA. Spectrophotometric absorbance reading at 260 nm of twenty experimental cattle shown in table 4.2.

Table: 4.1 Morphological characterizations of selected local Cattle .

Code No.	Body Color	Body Size	Cattle Height	Horn Types	Horn Length	Eye Color	Tail Size	Age (Yr)
Jes-1	White	Large	132 cm	Straight	17 cm	Blue	86 cm	05
Jes-2	Black	Medium	116 cm	Curved	12 cm	Black	81 cm	04
Jes-3	White, Black spots on head	Small	101 cm	Straight	5 cm	Blue	60 cm	04
Jes-4	White Stripes on Black	Medium	111 cm	Nil	Nil	Black	76 cm	03
Jes-5	Black	Medium	116 cm	Curved	10 cm	Black	81 cm	03
Khu-1	Red	Medium	109 cm	Curved	17 cm	Black	76 cm	04
Khu-2	White	Large	121 cm	Curved	12 cm	Black	86 cm	03
Khu-3	Black	Medium	101 cm	Straight	2 cm	Black	78 cm	02
Khu-4	Red	Small	101 cm	Straight	5 cm	Black	63 cm	03
Khu-5	White Stripes on Black	Small	96 cm	Straight	2 cm	Blue	50 cm	02
Nar-1	Red	Medium	114 cm	Curved	17 cm	Black	78 cm	03
Nar-2	Black	Medium	121 cm	Curved	15 cm	Blue	86 cm	04
Nar-3	Red	Medium	113 cm	Nil	12 cm	Black	76 cm	03
Nar-4	Black	Medium	107 cm	Straight	10 cm	Black	71 cm	04
Nar-5	Reddish, White Spots on forehead	Small	103 cm	Straight	10 cm	Black	68 cm	03
Jhen-1	Black	Small	100cm	Nil	Nil	Black	60 cm	02
Jhen-2	Black, White Spots on Whole body	Medium	116 cm	Curved	3 cm	Black	68 cm	04
Jhen-3	Red	Small	109 cm	Straight	3 cm	Black	63 cm	02
Jhen-4	Red	Large	132 cm	Curved	20 cm	Black	88 cm	05
Jhen-5	Black	Large	127 cm	Curved	21 cm	Black	86 cm	04

Table 4.2: Spectrophotometric Absorbance reading at 260 nm of twenty experimental cattle for DNA quantification.

Serial No.	Cattle Code No.	Absorbance Reading at 260 nm
1	Jes-1	0.025
2	Jes-2	0.016
3	Jes-3	0.008
4	Jes-4	0.009
5	Jes-5	0.047
6	Khu-1	0.037
7	Khu-2	0.010
8	Khu-3	0.034
9	Khu-4	0.152
10	Khu-5	0.008
11	Nar-1	0.016
12	Nar-2	0.015
13	Nar-3	0.026
14	Nar-4	0.026
15	Nar-5	0.010
16	Jhen-1	0.116
17	Jhen-2	0.013
18	Jhen-3	0.032
19	Jhen-4	0.012
20	Jhen-5	0.014

The higher DNA concentration was recorded in code no. Khu-4 and Jhen-1. The lower DNA concentration was recorded in code no. Jes-3, Khu-5. The quantified DNA sample subsequently diluted to 2ng/ μ L as per requirement for ideal PCR. The PCR did run using eight primers and among them two primers showed reproducibility. The PCR product reproducibility and banding pattern was confirmed by 1 percent agarose gel electrophoresis with 100bp ladder.

4.3. Molecular characterization

The breeds were found morphologically and genetically different as determined by the morphological and molecular characteristics. 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 amount of polymorphisms among the different genotypes of breeds. Two effective primers (Table: 3.2) were selected for determination the polymorphism of 20 genotypes.

4.4. DNA amplification by PCR and RAPD fingerprints:

Two RAPD primers were used to detect the polymorphisms of twenty cattle. Each of two primers was reproducible and capable of exhibiting polymorphic amplification patterns. Only unambiguous and scorable polymorphic fragments were taken into consideration for analysis. The amplification patterns of representative samples of cattle with two different primers have been shown in fig: 4.1 and fig: 4.2. Here the percentage of polymorphic bands was 77.50%. The frequencies of polymorphic bands varied from primer to primer. Though no accession-specific marker was identified, the high level of polymorphism revealed by the proportion of polymorphic loci (80%) indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in different accessions of local cattle. Accessions could be distinguished by a combination of fragments and differences between clusters reflected differences in frequencies rather than presence or absence of accession specific fragments.

4.4.1. Primer Selection and RAPD Analysis

Two primers (Table: 3.2) were selected that used in RAPD analysis of twenty cattle accessions amplified twenty different reproducible bands. Two different primers generated various banding patterns ranging from 1 to 5 and the average bands per primer was 4.5 and of the 9 bands scored, 7 (77.50%) were found to be polymorphic and 2 bands (22.5%) were found to be monomorphic.



Fig 4.1: RAPD pattern generated by the primer 3 with twenty genotypes of *Bos indicus* . The number corresponds to the serial number of the genotypes.



Fig 4.2: RAPD pattern generated by the primer 5 with twenty genotypes of *Bos indicus*. The number corresponds to the serial number of the genotypes.

Table 4.3: The sequence of primers used and their polymorphic bands

Primer	Sequence of the primer	Monomorphic bands	Polymorphic bands	Total bands	Polymorphism (%)
Primer 3	5'-TCTGCCATCC-3'	1	3	4	75
Primer 5	5'-TCTGCCATCC-3'	1	4	5	80
Total		2	7	9	
Average			3.5	4.5	77.50

Genetic diversity is of great significance for breeding programmes. Germplasms characterization is an important component of programmes on effective and efficient management/utilization of animal genetic resources. For successive genetic relationship morphological & molecular characterization are very effective. Morphological characterization was determined and showed genetic similarities and dissimilarities by collecting data and constructing the COMPONENT dendrogram to estimate the genetic diversity of cattle phenotypically. This some effective population size could influence the structure of the variation. Determining the degree of variability within an accession is necessary as a preliminary step for studying the genetic diversity among cattle breeds. In addition to the morphological characterization, a molecular screening is essential for determining the genetic variability. RAPD markers are the most effective method to determine the molecular characterization. A maximum of 7 polymorphic bands were obtained in PCR amplification from genomic DNA of the accessions used in present study, this was higher than the number of polymorphic bands (1.8) reported by Sureja *et al.* (2006) in ash gourd accessions. RAPD markers are randomly distributed throughout the genome, but most regions of the genome (90 %) are not expressed at the phenotypic level (Williams *et al.* 1990; Joyee *et al.*, 1999; Dahlberg 2000). As a result, markers like RAPDs may accurately assay the degree of genetic change between two genomes, but they may not necessarily reflect the divergence in terms of changes in traits of agronomic importance. In addition, the environment markedly influences the expression of most of the phenotypic traits, while detection of molecular markers is not dependent from the environmental effects.

In this experiment only 77.50% polymorphism are identified among the selected cattle breeds, which is too poor when compared with the result of those experiment conducted with cattle. This low level of polymorphism may be due to their location of origin. All of these breeds were collected Jessore, Khulna, Jhenaidha and Narail and most of them were originated in the southern part of Bangladesh. However, Dendogram show almost same relationship among the selected breeds of cattle and in both cases two different clusters were formed.

Stephen *et al.* (1999) carried out an experiment on Indigenous cattle of the United Republic of Tanzania. The study was conducted to determine the genetic relationships among five Tanzanian cattle ecotypes kept by Maasai, Gogo, Sukuma and Konde communities in the country. A total of 30 random amplified polymorphic DNA (RAPD) and 198 microsatellite

polymorphic alleles were used to study genetic relationships among the cattle ecotypes. The results showed closer relationships between ecotypes that are located close to one another than between ecotypes separated by greater geographical distance. Clear evidence of broad genetic diversity was observed among the studied cattle ecotypes. The diversity within the ecotypes makes genetic improvement through selection a possibility. Once they have observed that Tanzanian cattle possess different genetic attributes, farmers will have the benefit of choosing traits that will be included in future breeding programmes. The authors recommended the use of indigenous cattle genetic resources in breeding programmes rather than the usual practice of cross-breeding with exotic genotypes.

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

4.5. 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 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.

There was a high level of genetic variation among the studied accessions of cattle as indicated by the proportion of polymorphic loci. Estimation of higher level of genetic variation in the cattle might be consistent with the fact that it is a highly polymorphic animal. The values of pair-wise comparisons of Nei's (1979) genetic distance between 20 cattle genotypes were computed from combined data sets for the two primers ranging from 0.070 to 1 (Table4). Comparatively higher genetic distance (1) was found among Jes-2, Jes-3, Jhen-1, Nar-5. The lowest genetic distance (0) was revealed in cattle Nar-3, Jhen-3,

Table 5: Estimation of genetic variability.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

1	***																		
.3334	1	***																	
0.5	0.5	0.2	***																
.3334	0.6667	0.25	0.2	***															
.6667	0.3334	0.5	0.2	0.25	***														
0.5	0.5	0.6667	.3334	0.3334	.3334	***													
0.6	0.2	0.7143	.3334	0.4286	.1429	0.2	***												
.4286	0.4286	0.3334	0.909	0.3334	.3334	0.428	0.25	***											
0.0	1.0	0.3334	0.5	0.3334	.6667	0.5	0.6	0.6	***										
0.5	0.5	0.2	0.0	0.2	0.2	0.5	.3334	.0909	0.5	***									
.3334	0.3334	0.5	0.2	0.25	0.25	.334	.1429	0.1112	.334	0.2	***								
0.5	0.5	0.2	0.0	0.2	0.2	0.5	.333	.909	0.5	0.0	0.2	***							
0.5	0.5	0.6667	0.5	0.6667	.6667	1.0	0.6	0.4286	0.5	0.5	.3334	0.5	***						
1.0	0.0	1.0	0.5	0.6667	.6667	0.5	0.2	0.4286	1.0	0.5	.3334	0.5	0.5	***					
1.0	0.0	1.0	0.5	0.6667	.6667	0.5	0.2	0.4286	1.0	0.5	.3334	0.5	0.5	0.0	***				
.4286	0.4286	0.5556	0.2	0.25	0.25	.426	0.25	0.2	.429	.2728	.1112	.2728	.4286	.4286	.4286	***			
0.5	0.5	0.6	.3334	0.4	0.2	0.5	.3334	0.4546	0.75	.3334	0.4	.3334	0.75	0.5	0.5	.2728	***		
.5556	0.5556	0.2728	.0769	0.3334	.3334	0.6	.4546	0.2308	0.6	.1429	.334	.1429	0.6	0.6	0.6	.2308	.1429	***	
.5556	0.5556	0.4546	.2308	0.2728	.2728	0.5	0.4	0.3334	.556	.2308	.2728	.2308	.5556	.5556	.5556	.1667	.0769	.0667	**

Jhen-2, Nar-5. The range of genetic distance of twenty genotypes is (0 ± 1) and the difference between the highest and lowest genetic distance indicated the presence of variability among the 20 genotype of cattle. Genetic distance between two breeds is occurred by indiscriminate crossing, migration, and mutation or recombination breeding procedure.

4.6. Dendrogram Analysis

COMPONENT Dendrogram was constructed according to the morphological data. The Dendrogram (Fig: 4.3) shows that all the breeds were grouped into three major clusters (I), (II) & (III). Cluster I sub divided into I (A) and I (B). Cluster II subdivided into IIA (1) and IIB (2). Cattle Jes-2, Jes-5, Khu-3, Khu-5, Nar-5, Jhen-1 are closely related but cattle Jes-1, Nar-4, Jhen-2, Jhen-5 are separate. On the other hand cluster II, cattle Jes-2, Jhen-1 are closely related. Cluster I and cluster II different from Cluster III which included cattle Nar-3, Jhen-2, Jhen-3.

4.6.1. Cluster Analysis of the Genotypes Based On RAPD Analysis

Genetic similarities measured through analysis of data on the 2 RAPD markers from the twenty genotypes of *Bos indicus* cattle revealed varying degrees of genetic relatedness. Nei and Li (1979) ranged from 0 to 1. The highest dissimilarity coefficient (1) was observed between local cattle Jhen-2 and Jes-5. The lowest (0) similarity coefficient was revealed between local cattle Jes-2 and local cattle Jhen-1. Using the Nei's (1979) genetic distance of a dendrogram was constructed to obtain the clustering of the accessions.

Table 4.4: Distribution of 20 cattle accessions under different cluster.

Cluster No.		Total no. of accession in cluster	Accessions included in different clusters
I	I A	5	Jes-1, Khu-3, Khu-5, Jhen-4, Nar-4
	I B	8	Jes-3, Jes-5, Khu-2, Khu-4, Nar-1, Nar-2, Nar-5, Jhen-5
II	II A	2	Jes-4, Khu-1
	II B	2	Jes-2, Jhen-1
III		3	Jhen-3, Nar-3, Jhen-2

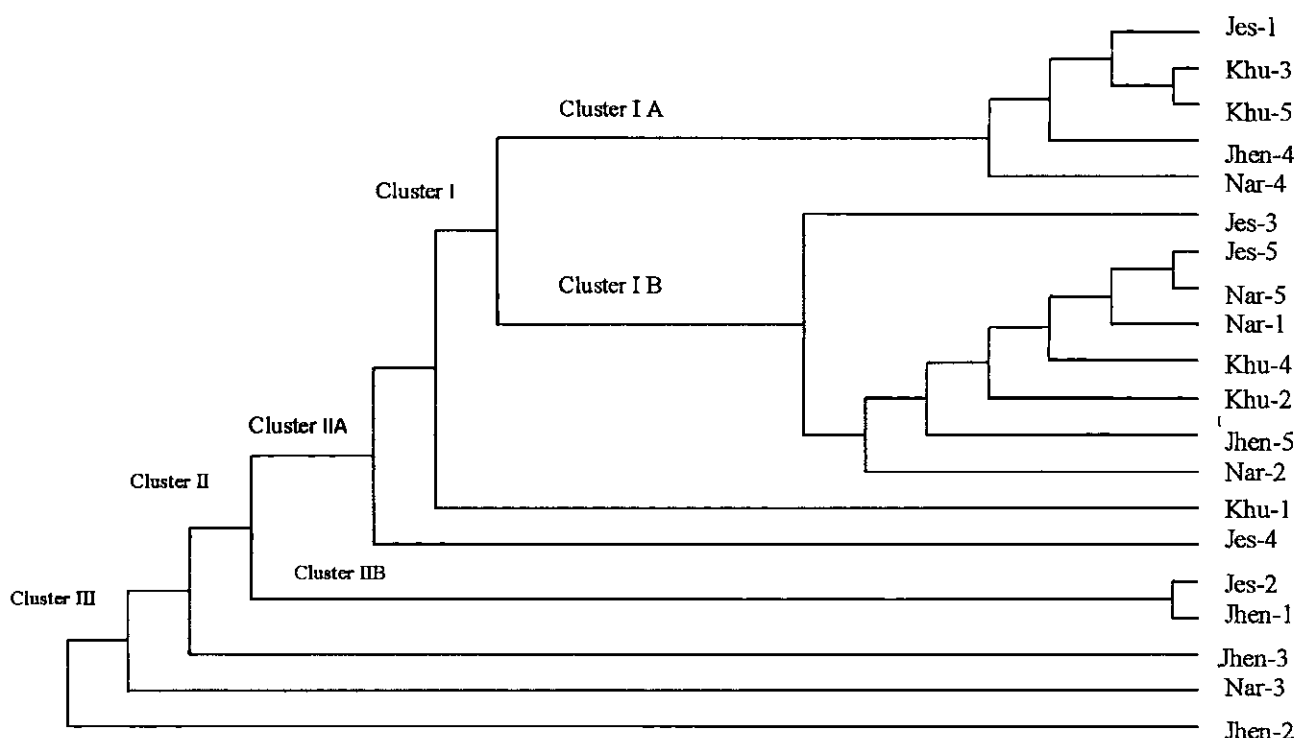


Fig 4.3: The COMPONENT Dendrogram summarizing the data on differentiation among local cattle sample.

The dendrogram shows that all the accessions were found to be grouped in three major groups designated as I, II, III. The distribution of the cluster members is shown in (Table 4.3). Cluster I is broad which includes 13 accessions. Cluster I divided into two sub-cluster (I A & I B) where sub-cluster I B includes the highest genetic distance containing value of Index-3 and sub-cluster I A includes the lowest genetic distance containing value. In Cluster II B, Jes-2, Jhen-1 sample no. showed the less genetic variation but Cluster II A showed the more genetic variation and cluster forms separately from II B. Accessions included in clusters III are Jhen-3, Nar-3, Jhen-2 which are not genetically more distant. The possible reasons for their random segregation could be the indiscriminate breeding, migration of animals, mutation in ancestors or different origin. The highest gene diversity indicates that the population is alarming to deteriorate with native or exotic blood than other population.

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 Yatano, 1983).

In another similar study, Edwards *et al.*, (2000) constructed two Neighbor-joining trees, one in which they used the genetic distances to visualize the relationships between the endangered pustertaler-Sprinzer and three European cattle breeds and another in which they used the allele sharing statistic suggested by Bowcock *et al.*, (1994) with individual animals acting as operational taxonomical units (OTUs).

CHAPTER FIVE

SUMMARY AND CONCLUSION

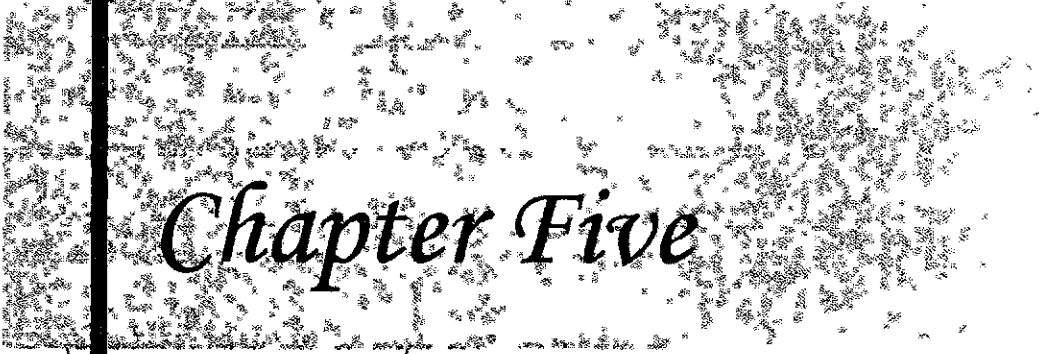
During a few decades of systematic breeding for local and exotic breeding efficiency of important characteristics such as growth and marbling have not been satisfactory. However genetic purity of basic population has been determined only by phenotypic characteristics. Expected genetic gain of performance would be based and variation of performance should be wide. When genetic uniformity of experimental materials is not established, it could be difficult to get uniform results from the feed-lot trial to produce high quality and performance of cattle. Thus, DNA marker analysis is necessary for strategies for improvement of cattle, preservation as systematic registration, identification of population for breeding, and distinguishing pure breeds from others.

This thesis research investigated the relatedness and genetic variation among twenty Bangladeshi indigenous cattle. The first objective was to compare the twenty cattle based on phenotypic characteristics and the second to determine genetic distance among these cattle using randomly amplified polymorphic DNA markers. The morphometric measurements evaluated included body length, body color, horn types, horn length, eye color, tail size and cattle height.

In the second objective of the study, the twenty Bangladeshi indigenous cattle were analyzed using RAPD markers. Two primers that were shown to be polymorphic and informative were used in the analysis. Estimation of higher level of genetic variation in the cattle might be consistent with the fact that it is a highly polymorphic animal. The values of pair-wise comparisons of Nei's genetic distance between twenty cattle genotypes were computed from combined data sets for the two primers ranging from 0.070 to 1 (Table 4). Comparatively higher genetic distance (1) was found among Jes-2, Jes-3, Jhen-1, Nar-5. The lowest genetic distance (0) was revealed in cattle Nar-3, Jhen-3, Jhen-2, Nar-5. The range of genetic distance of twenty genotypes is (0±1) and the difference between the highest and lowest genetic distance indicated the presence of variability among the twenty genotype of cattle. Genetic distance among cattle is occurred by indiscriminate crossing, migration, and mutation or recombination breeding procedure.

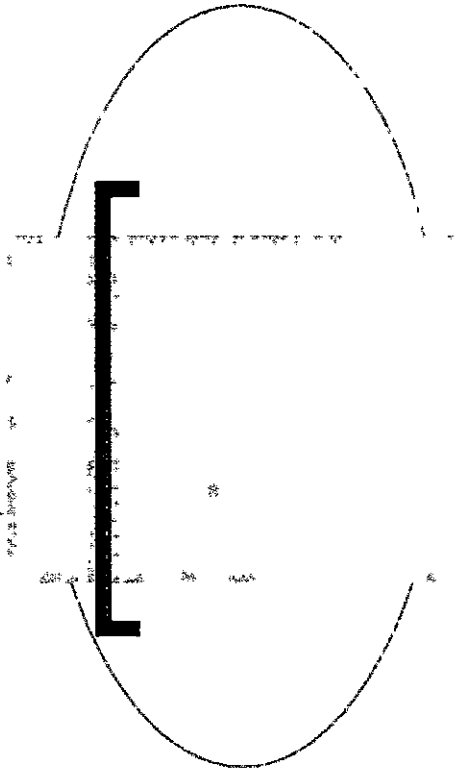
Two RAPD primers were used to detect the polymorphisms of twenty cattle. Each of two primers was reproducible and capable of exhibiting polymorphic amplification patterns ranging from 1 to 5 and the average bands per primer was 4.5 and of the 9 bands scored, 7 (77.50%) were found to be polymorphic and 2 bands (22.5%) were found to be monomorphic. Here the percentage of polymorphic bands was 77.50%. The frequencies of polymorphic bands varied from primer to primer. Though no accession-specific marker was identified, the high level of polymorphism revealed by the proportion of polymorphic loci (80%) indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in different accessions of local cattle.

Some recommendations can be made from the present study. Since the local breed largely distributed among the country, it should be given priority for conservation. For cost effectiveness, the local breed should be managed as a single management unit in a conservation program. The local cattle breeds appear, probably due to their geographic closeness, to have been extensively crossed. More research needs to be done on the Bangladeshi native cattle breeds and the choice of breeds 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 in Bangladesh for animal production.



Chapter Five

Summary And Conclusion



References

Chapter Six

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Appendix

50X TAE Buffer, pH 8.03 (For 100ml)

EDTA: 1.8609gm

Glacial acetic acid: 5.4ml

Tris-base: 24.2gm

Adjust 100 ml with distilled water.

1X TAE Buffer (For 400ml):

50× TAE buffer: 8ml

Distilled water: 392ml.

14N HCl:

Concentrated HCl: 127.51 ml in 100 ml of distilled water.

Make the final volume 250 ml with distilled water.

1M NaOH (For 100ml):

NaOH pellets: 4 gm

Distilled water: 80ml

Make the final volume 100 ml with distilled water.

0.5 M EDTA; pH 8.0 (For 250ml)

EDTA: 46.525gm

Dissolved in 100ml distilled water

Adjust pH to 8.0 with 10M NaOH

Add distilled water to 250ml.

1M Tris-HCl; pH 7.6 (For 250ml):

Tris-base: 30.275gm

Dissolved in 100ml distilled water

Adjust pH to 7.6 with concentrated HCl

Make the final volume 250 ml with distilled water.

Appendix -II

Reagents used

Name of Reagent	Name of the Company
NaCl	AnalaR ^R
Na ₂ SO ₄	Schnhardt, Germany
Tris-base	Hinweis
SDS	Hinweis
EDTA	Hinweis
Na-citrate TM Dihydrate	—
Agar	Invitrogen TM life technologies
Gel loading dye	Invitrogen TM life technologies

Appendix-III**EQUIPMENTS USED**

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