MOLECULAR DIVERSITY ANALYSIS AND POLYMORPHISM STUDY IN COTTON (*Gossypium hirsutam* L.) GENOTYPES THROUGH RAPD MARKERS

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CERTIFICATE

This is to certify that thesis entitled, ***"MOLECULAR DIVERSITY ANALYSIS AND POLYMORPHISM STUDY IN COTTON (Gossypium hirsutam* L.) *GENOTYPES THROUGH RAPD MARKERS"*** *to the Faculty of AGRICULTURE, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE (MS) in BIOTECHNOLOGY,*** *emboded the result of a piece of bona fide research work carried out by* ***MD.ABDUL WAHAB****, Registration No.* ***17-08189*** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated: December, 2017

Dhaka, Bangladesh

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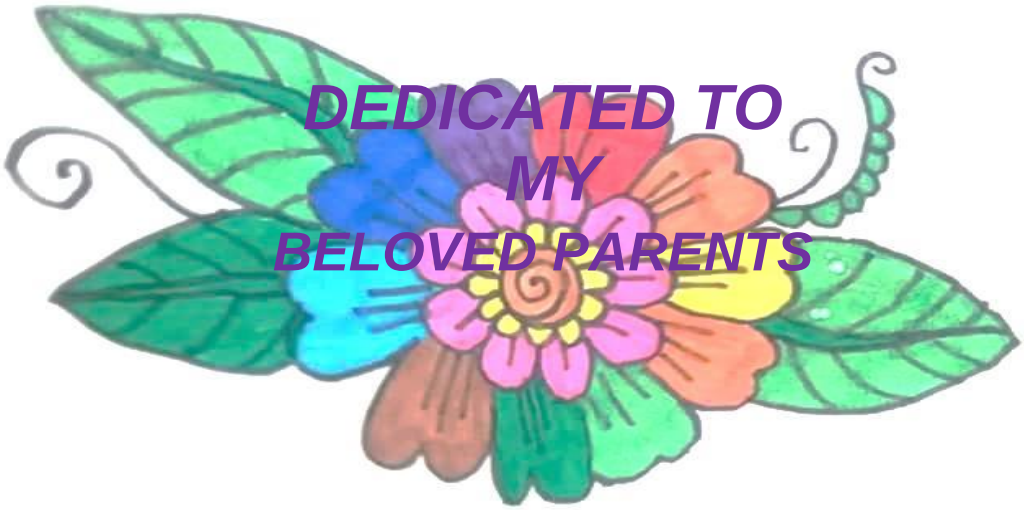
DEDICATED TO
MY
BELOVED PARENTS

ABBREVIATIONS

Full word	Abbreviation
2,4-Dichlorophenoxy acetic acid	2,4-D
Agricultural	*Argil.*
Agriculture	*Agric*.
American	*Am*
Amplified Fragment Length Polymorphism	AFLP
And others (at elli)	*et al.*
As for example	e.g.
Bangladesh Agricultural Research Institute	BARI
Bangladesh Bureau of Statistics	B.B.S
Base pair	bp
Biology	*Biol.*
Biotechnology	*Biotech.*
Botany	*Bot.*
Breeding	*Breed.*
Cotton Development Board	CDB
Continued	Cont'd
Cultivar	cv.
Culture	Cult.
Degree celsius	°C
Deoxyribonucleic acid	DNA
Distilled deionized water	ddH_20
Expansion of Cotton Cultivation Project	ECCP.
Etcetera	etc
Ethidium Bromide	Et-Br
Ethylene Diamine Tetra Acetic Acid	EDTA
Genetic Distance	GD
Genetics	*Genet.*
Gram per Liter	g/L
Hectare	ha
Horticulture	*Hort.*
International	*Int.*
Inter simple sequence repeat	ISSR
Journal	*J.*
Marker assisted breeding	MAS
Metric ton	mt
Micro liter	*µl*
Mili liter	mL
Milimetre	mm

ABBREVIATIONS

Full word	Abbreviation
Molecular	Mol.
Namely	*viz.*
Percent	%
Polymerase chain reaction	PCR
Polymorphic information content	PIC
Principle component analysis	PCA
Publication	*Pub.*
Random Amplified Polymorphic DNA	RAPD
Restriction Fragment length Polymorphism	RFLP
Research	Res.
Ribonucleic Acid	RNA
Rotation per minute	Rpm
Science	*Sci.*
Sequence Tagged Site	STS
Single nucleotide polymorphism	SNP
Simple Sequence Repeat	SSR
Sodium chloride	NaCl
Sodium Dodecyl sulphate	SDS
Species (plural)	Spp.
That is	i.e.
Tris Boric Acid EDTA	TBE
Tris-EDTA	TE
Un-weighted Pair Group Method of Arithmetic Mean	UPGMA
Ultra violet	UV
Volt	V
United States Department of Agriculture	USDA

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The Author

Desember,2017

SAU, Dhaka

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Abstract

The experiment was conducted at the Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka- 1207, Bangladesh. The period of the experiment was March 2018 to November 2018. Cotton (*Gossypium hirsutum* L.) is the leading fiber crop and the second most important oil seed crop in the world. This study aimed to analyze the molecular diversity among 9 cotton genotypes collected from Cotton Development Board, Bangladesh using the Random Amplified Polymorphic DNA primer. A total of 11 RAPD primers were used to determine polymorphism among the genotypes and finally 6 of the primers produced scorable DNA bands. Some total of with 30 DNA bands were obtained and among them 9 were polymorphic bands. The ranged of DNA amplification varied from 160 to 1500 bp. The rate of polymorphism was calculated about 31.57%. Genetic diversity ranged from 0.22 to 0.44 with an average value of 0.30. Nei's genetic distance ranging from 0.1667 to 0.6667 and most importantly PIC value ranged from 0.18 to 0.35 with an average value of 0.27. The PIC value indicates that most of the studied cotton genotypes was moderately diversified and homogenous as well as no heterozygosity found. A dendogram indicating the relative genetic similarity of the Bangladeshi cotton genotypes was constructed which followed in two major cluster (A and B) among the studied material. The results also showed that the genotypes can be separated from each other at the molecular level by taking advantage of some of the RAPD markers. The Cotton Development Board, Bangladesh committed to work on cotton improvement and so for this was the first time in CDB to investigate molecular diversity and DNA profiling in cotton using CDB collected cotton genotypes. This experiment can be used as a baseline for the future molecular research work on cotton genotypes in Bangladesh.

CHAPTER I

INTRODUCTION

Cotton is an important fiber crop of global significance. It is cultivated in tropical and sub-tropical regions of more than seventy countries the world over. The major producers of cotton are China, India, USA, Pakistan, Uzbekistan, Argentina, Australia, Greece, Brazil, Mexico, and Turkey. World harvested area in 2017-18 is estimated at 33.3 million hectares. The global yield is forecast to rise to 799.0 kg per ha. In the top three producing countries India, China and USA are project to account for 63% of the global cotton crop. India's production is forecast at, 28.5 million bales (USDA, 2017).

Cotton plays a key role in the national economy in terms of generation of direct and indirect employment in the agricultural and industrial sector. Cotton, being the king of natural fibers in preparing human apparel has played a key role in the development of civilization. Due to its importance in agriculture as well as industrial economy, it is also known as "white gold".

Cotton is the major textile fiber used by man in the world and it plays a key role in economic and social welfare (Munro, 1994). Although it is grown primarily as a fiber crop, but after the lint, the long twisted unicellular hairs are removed by ginning, the seed can be crushed to extract vegetable oil and protein rich animal food (Mathews, 1989). Cotton seed cake, an industrial byproduct of cotton, is a valuable source of protein for ruminant cattle. Cotton is the world's oldest commercial crop and one of the most important fiber crops in the global textile industry, the industry generally fails to focus on the entire value chain to ensure that those who grow their cotton also receive a living income. Up to 100 million small holder farmers in more than 100 countries worldwide depend on cotton for their income. They are at the very end of the supply chain, largely invisible and without a voice, ignored by an industry that depends on their cotton.

In Bangladesh, cotton is the most important fiber crop and it provides raw materials to domestic cotton industry containing 395 spinning mills, 1343 weaving mills, more than 3 lacs of handlooms, 2822 knitting and 5000 garment industries (BTMA, 2016).

According to Cotton Development Board in Bangladesh, present annual requirement of raw cotton for local textile industry is estimated to be 5.0 million bales (BBS, 217). Only a small portion of the total annual requirement of local spinning mills can be cover by local production. In order to increase the production and productivity of the cotton, organized cotton breeding programmes for open pollinated, hybrids, mutant variety and Bt cotton must be developed. Such a programme will require development of techniques to determine the genetic diversity of the cotton varieties and races available in Bangladesh.

Cotton industries of Bangladesh predominantly depend upon import where nearly 98% of the requirement is fulfilled by importing raw cotton from different foreign countries. In this context, it is imperative to increase cotton production in Bangladesh to feed the cotton industry, to save the hard earned foreign exchange and to attain self-sufficiency in raw cotton. Cotton production in Bangladesh may be increased either by horizontally or vertically or by both the ways. But in fact, it is almost impossible to increase cotton production horizontally because of severe competition to other crops in limited land. Yield enhancement of cotton by alternate may be possible because the productivity of cotton in Bangladesh is only 450 kg lint per hectare against world average yield of 556 kg lint per hectare. Higher yield of cotton may be achieved by selecting appropriate variety specifically suited to local ecological condition.

Gossypium includes 50 species and out of them four are cultivated (Percival and Kohel, 1990). Out of the four cultivated species, *Gossypium hirsutum* L. and Gos*sypium barbadense* L. commonly called as new world cottons are tetraploids (2n = 4x = 52), whereas, *Gossypium herbaceum* L. and *Gossypium arboretum* L. are diploids (2n = 2x = 26) and are commonly called as old world cottons

Existance of genetic diversity is an essential requirement for successful hybridization programme. In order to take the programme of development of hybrid cotton successfully, choice of suitable parent through careful and critical evaluation is of paramount importance.The application of biotechnological approaches in breeding programmes provides alternative avenues for cotton improvement through direct manipulation of genes and can produce improvements which are not possible through the classical breeding programmes.

Molecular markers have been widely used in genetic analyses, breeding studies and investigations of genetic diversity and the relationship between cultivated species and their wild parents (Sofalian *et al.,* 2009). This is because they have several advantages as compared to morphological markers, including detecting high polymorphism and independence from effects related to environmental conditions and the physiological stage of the plant.

Cotton grown in different regions shows very less genetic difference (Zhang *et al*., 2013). A narrow genetic background itself poses a threat to the survival of a plant as it makes the plant vulnerable to environmental changes and natural catastrophes. Therefore to make the plants well adapted and more resistant to diseases and other environmental stresses there is always a need to improve the quality of plant's genome i.e. to increase its genetic diversity. Researchers throughout the world are therefore trying for many years to establish varieties which display a wide genetic base. For this purpose genetic studies are being carried out to gather greater amount of information about the genome and genetic diversity of cotton and then to utilize the gathered information for the improvement of the crop's genetic base and development of improved and more adapted varieties. It is also very crucial for the conservation and maintenance of existing gene pool and the relatedness among members of that gene pool that the genome of that crop be well studied (Kim *et al*., 2001). Molecular markers are valuable tools in the characterization and evaluation of genetic diversity among different species and population. It has been reported that different markers revealed different classes of variation (Qin *et al*., 2011).

Different molecular markers are routinely used for the characterization and evaluation of genetic diversity in different plant species and population (Iqbal *et al.,* 2001). RAPD (Randomly amplified polymorphic DNAs) which is a PCR based marker has many advantages including readily being used, requiring minute amount of genomic DNA, does not need blotting and radioactive detection etc. DNA fingerprints can be generated with Random Amplified Polymorphic DNA (RAPD). RAPD markers are based on the amplification of unknown DNA sequences using single, short and random oligonucleotide sequences of arbitrary nature as primers (Chen *et al*., 2007). RAPD does not need any prior knowledge of DNA sequence, however, still reveals a high level

of polymorphism. RAPD-PCR is currently used as a tool for the assessment of genetic variability between genotypes in breeding programs. Keeping in view the role of RAPD markers in the determination of genetic diversity, the present study was carried out to determine genetic diversity among different genotypes of cotton using RAPD markers and selection of genetically diverse genotypes for future breeding programs.

OBJECTIVES

- Molecular diversity analysis of different cotton genotypes.
- DNA fingerprinting of cotton material under Bangladesh condition.
- Polymorphism study among different cotton germplasm.
- Establishment of dendogram and phylogenetic relationship among the studied genotypes.

CHAPTER II

REVIEW OF LITERATURE

Cotton is a soft, fluffy staple fiber that grows in a boll, around the seeds of cotton plants of the genus *Gossypium* and it belongs to the family of Malvaceae. The fiber is almost pure cellulose and most widely used as natural fiber cloth in textile industries predominantly. As a global commodity, cotton plays a major role in the economic and social development of emerging economies and newly industrialized countries.

Several researchers throughout the world have performed research activities on cotton genetic diversity and relationship, phylogenetic study and characterization through molecular markers like Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeat (SSR) etc. RAPD does not need any prior knowledge of DNA sequence, however, still reveals a high level of polymorphism. RAPD- PCR is currently used as a tool for the assessment of genetic variability between genotypes in breeding programs. Keeping in view the role of RAPD markers in the determination of genetic diversity, the present study was carried out to determine genetic diversity among different genotypes of cotton using RAPD markers and selection of genetically diverse genotypes for future breeding programs. Despite an important cash crop, a little information is available on genetic structure of cotton genotypes in Bangladesh. However, in these aspect a very little work has been done in Bangladesh. The most relevant literature about the present study has been reviewed here under following subheadings:

2.1 Genetic diversity studies for molecular markers

Molecular genetic markers are powerful tools to analyze genetic relationship and genetic diversity. A number of marker systems such as RAPD, AFLP, SSRs, ISSRs *etc*. are available to use in this endeavor. Restriction fragment length polymorphism (RFLPs) can be used but they are expensive and time consuming. Among the several molecular techniques, random amplified polymorphic DNA (RAPD) markers (Williams *et al.,* 1990) based on polymerase chain reaction (PCR) was shown to provide a high level of resolution equivalent to RFLPs for detecting genetic relationship (Hallden *et al.,* 1994).

2.2 **Molecular marker**

Molecular markers are reliable tools to characterize the DNA profile of plant genotypes to study the genetic diversity. According to Datta *et al.*(2011) Molecular markers are specific fragments of DNA that can be identified within the whole genome. Molecular markers are found at specific locations of the genome.

Molecular marker can identify small changes within the mapping population enabling distinction between a mapping species, allowing for segregation of traits and identity. Some studies which were conducted during the last decade of the 20th century reported numerous DNA markers that have been utilized in plant breeding programs. Apart from the application of molecular markers in the construction of linkage maps, they have numerous applications in plant breeding such as assessing the genetic variations within cultivars and germplasms (Henry, 1997).

The molecular approach for identification of plant genotypes seems to be more effective as it allows direct access to the hereditary material (Paterson *et al.,* 1991) unlike the morphological markers.

Molecular markers can be used for molecular characterization and detecting genetic variation and relationship of plants. These markers can detect the variation that arises from deletion, duplication, inversion, and/or insertion in the chromosomes. Such markers themselves do not affect the phenotype of the traits of interest because they are located only near or linked to genes controlling the traits (Mondini *et al.,* 2009).

DNA markers are accepted widely as potentially valuable tools for crop breeding such as rice (Mackill *et al.,* 1999 and McCouch *et al*., 1988), wheat and forage species (Jahufer *et al.,* 2003).

Molecular markers provide a valuable tool for genetic analysis and plant breeding (Tanksley *et al*.,1989; Rafalski and Tingey, 1993). DNA based molecular markers offer several advantages over traditional phenotypic markers and have found their own position in various fields like taxonomy, embryology, physiology, genetic engineering, etc. Molecular markers are generally classified into (i) hybridization based (non-PCR) and (ii) PCR-based markers. Hybridization based markers involve restriction digestion and hybridization of restricted DNA fragments to a labeled probe such as RFLPs, VNTRs whereas PCR based markers include amplification of isolated DNA. PCR-based markers are further divided into three

categories: (1) markers that are amplified using single primers and diversity results due to ssvariation in length and/or sequence and anchored nucleotides present at 5' or 3' termini of primers (e.g. RAPDs, SPARs, DAF, AP-PCR, ISSRs); (2) markers that are selectively amplified with two primers with 2-3 random bases at 3' end of primers (e.g. AFLP); (3) markers that are amplified using two primers that requires sequencing for construction of primers (e.g. STRs and SSRs) (Staub *et al*., 1996).

Multani and Lyon (1995) studied Australian cotton varieties by RAPD markers using silver staining and demonstrated that very closely related varieties can also be distinguished. They generated fingerprint of 12 cultivars.

Iqbal *et al*. (1997) used RAPD analysis to evaluate the genetic diversity of elite commercial cultivars of *G. hirsutum* L. Twenty-two varieties belonging to *G. hirsutum* L. and one to *G. arboreum* were analyzed with 50 random decamer primers using PCR and observed 89.1 per cent polymorphism.

Hussain *et al*. (2004) developed a simple laboratory based technique to identify resistance to CLCuV in elite commercial cotton (*Gossypium hirsutum*) cultivars and found that susceptible genotypes produced typical CLCuV symptoms upon inoculation. Virus presence in all genotypes was tested by triple antibody sandwich enzyme linked immune sorbent assay (ELISA).

Vroh *et al*. (1999) used AFLP markers to assess genetic similarity among *Gossypium* species while RFLP probes specific to chromosome segments of parental species were also used. The results indicated less percentage of genetic similarity between upland cotton and wild species while high similarity was found between upland cotton and backcross progenies

Yang *et al*. (2001) constructed a molecular map using RAPD and RFLP in the F_2 progeny from a cross between two species of *G. hirsutum* and *G. barbadense* to determine QTL location and identified three fiber length QTLs.

Shanti *et al*. (2001) used RAPD in *Gossypium hirsutum* and ten partial restorers along with three stable restorer for morphological features and RAPD banding patterns were used to

differentiate through morphology .Results proved RAPD markers to be reliable restore indicator.

Saha *et al*. (2003) described the presence of SSRs in cDNA cotton using cDNA as template. The sequences obtained were found to be conserved across *G. barbadense* and *G. hirsutum*.

Dongre and Kharbikar (2004) subjected twenty-five cotton (*Gossoypium hirsutum*) accessions from Africa, Australia, USA and India for RAPD analysis using 86 random oligonucleotide primers to study the extent of genetic variation and find out the duplicates if any. Sixty-three primers detected polymorphism.

Soregaon *et al*. (2004) used RAPD markers to analyse genetic diversity in recombinants derived from interspecific crosses of cotton (*Gossypium* species).

2.3 Random amplified polymorphic DNA (RAPD)

RAPD is the oldest PCR-based molecular marker technique it involves 10 bp random primer (Williams *et al.,* 1990). It has many advantages over RFLP technique such as non- radioactive detection, it does not require prior sequence information, it required very small amount of genomic DNA, experimental simplicity and no need for expensive equipments beyond a thermocycler and a transilluminator (Rafalski, 1997). RAPD main disadvantage is that poor reproducibility (Jones *et al.,* 1997). RAPD profile varies within and between laboratories because it is influenced by many factors like DNA concentration, reproducibility of thermocycler profiles, primer quality and concentration, choice of DNA polymerase, and pipetting accuracy (Rafalski, 1997).

Vierling and Nguyen (1992) pointed out that, the polymorphism detected between amplification products of different individuals using the short, random, single primers made RAPD marker studies good for genetic diversity, genetic relationships, genetic mapping, plant breeding, DNA fingerprinting and population genetics.

There are several advantages of RAPDs compared to other DNA based techniques. RAPD. Non-radioactive detection, multiple loci detection in single reaction, requirement of small quantity of DNA, no requirement of prior sequence information, quick and technically simple (Karp *et al*., 1997).

Hadrys *et al.* (1992) stated that RAPD-PCR technique used for examining variation in the total genome. RAPD analysis is advantageous over isozyme electrophoresis because it generates much greater numbers of loci required for genetic analysis (Kimbeling *et al.*, 1996). RAPD markers can be used as supposedly unbiased; and neutral markers for genetic mapping applications (Michelmore *et al.*, 1991), in population genetics (Haig *et al.*, 1994), taxonomy (Chapco *et al.*, 1992) as well as for genetic diagnostics.

In spite of having many usefulness of RAPD marker it have some limitation. Because of random nature of genome sampling, the RAPD assay is not an appropriate technique when the difference between the two genomes is being compared is limited to an extremely small genomic fraction. The most unavoidable problem is dominance of RAPD marker because the presence of given RAPD band does not distinguish whether its respect locus is homozygous or heterozygous or co-dominance which is possible when SSR marker is used (Rahman *et al.*, 2006).

Though having such weakness, the relative ease and speed the high degree of polymorphisms and virtually inexhaustible pool of possible genetic marker makes the RAPD technique advantageous over other molecular technique (Clark and Lanigan, 1993; Fristsch and Rieseberg, 1996). RAPD markers, in particular, have been successfully employed for determination of intra-species diversity in several plants, whereas fewer reports are available on determination of inter-species diversity (Goswami and Ranade, 1999).

2.4 Genetic diversity studies in cotton genotypes by RAPD markers

Morphological features are indicative of the genotype but are represented by only a few loci because they are not large enough. Moreover, they can also be affected by environmental factors and cultural practices. To have an accurate and reliable estimate of genetic relationships and genetic diversity assessment, there is a need of polymorphic molecular markers. Therefore, RAPD technique (Williams *et al.*, 1990) provides unlimited number of marker loci that can be used for genetic diversity and genetic

recombination analysis. It was successfully applied to characterize germplasm lines developed through interspecific hybridization. RAPD markers have been used for the estimation of genetic similarities and cultivar analysis for introgressed genes through amplified genomic regions.

Iqbal *et al.* (1997) used RAPD analysis to evaluate the genetic diversity of elite commercial cotton varieties. Twenty-two varieties belonging to *G. hirsutum* and one belonging to *G. arboreum* were analysed with fifty random decamer primers using the polymerase chain reaction (PCR). Fourty nine primers detected polymorphism in all twenty three cotton varieties while, one produced monomorphic amplification profile. They reported that, a total of 349.0 bands were amplified, of which 89.1 per cent were polymorphic.

Genetic diversity analysis of six Australian species i.e., *G. sturtianum, G. nandewarense, G. robinsonii, G. bickii, G. australe and G. nelsonii,* was made using RAPD. Among these, *G. sturtianum, G. nandewarense, G. australe* and *G. robinsonii* showed the closest affinity among the six species. Cluster analysis indicated that *G. bickii* and *G. robinsonii* have a relatively distant affinity, whereas a closed affinity exists between *G. robinsonii* and *G. australe*. A chromosome classification was proposed for the six species based on the RAPD results, in which *G. sturtianum* and *G. nandewarense* have 'C' genome, *G. bickii* has '*G' genome* and *G. australe, G. nelsonii* and *G. robinsonii* are have a new genome (Song *et al*., 1999).

Abdel *et al.* (2003) investigated the molecular nature of RAPD fragments in four *G. barbadense* cultivars. Five RAPD fragments, generated by improved RAPD-PCR technique representing polymorphic and non-polymorphic bands were analyzed at the molecular level using DNA sequence analysis. Non-polymorphic RAPD fragments showed homologies to previously characterized plant structural genes. Genetic diversity in a set of 30 elite cotton germplasm lines was studied by Pawan Kumar *et al*. (2003) which included 20 genotypes of *G. hirsutum,* seven genotypes of *G. arboreum,* one genotype each of *G. herbaceum, G. thurberi* and *G. klostzschianum*. For RAPD analysis they used 32 random primers in which only 25 showed amplification while rest failed to amplify. A total of 108 bands were amplified in 30 genotypes. They reported one primer, OLIGO 656 which amplified an 1100 bp *G. arboretum* specific band.

Gomes *et al*. (2004) used RAPD markers to assess the genetic relatedness in seven *Gossypium* species which included *G. hirsutum*, CMS lines and cultivars, *G arboreum* CMS lines, cultivars and wild species, *G. raimondii, G. bickii*, *G. thurberi, G. captis-virdis* and *G. anomolum.* Out of fourty five RAPD primers tested, twenty four primers yielded monomorphic amplified products and remaining twenty one primers amplified a total of one sixty eight fragments with an average of 9.8 fragments per primer. Out of eleven genotypes studied, *G. arboreum (*G 27) showed maximum number of DNA amplified fragments.

Hanchinal *et al*. (2004) used RAPD markers to distinguish and identify the widely adapted DCH-32 and recently released DHB-105, inter specific cotton hybrids and their parents. They reported that three polymorphic bands distinguished the DHB-105 hybrid and its parents and results help for precise identification and hybrid purity testing of the above inter specific hybrids.

Jiang Yong *et al.* (2004) conducted diversity analysis of 51 brown and green colour cottons using RAPD technique, using six random primers and reported differences in genetic relationship and similarity among the brown cottons, green cottons and brown-green cottons are not remarkable.

Rana *et al*. (2005) conducted RAPD analysis to study genetic relationship and genetic diversity of seven diploid (*G. arboretum*) 25 tetraploid (*G. hirsutum*) elite cultivars of cotton. They reported that more diversity was observed in diploid than tetraploid cotton cultivars and they identified two main clusters, one containing all the *G. arboreum* (cluster-II) and all the *G. hirsutum* cultivars (cluster-I).

Zhang *et al.* (2005) reported genetic distance among Acala 1517 genotypes ranged from 0.06 to 0.38 with an average of 0.18 on the basis of 189 SSR marker alleles, indicating a substantial genetic diversity among Acala 1517 cotton germplasm.

CHAPTER III

MATERIALS AND METHODS

This chapter constitute on the materials and methods of the experiment. The details of different materials and methodologies followed for the study have been expressed in this chapter.

3.1 Experimental location and duration

The experiment was carried out at the Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka- 1207, Bangladesh. The period of the experiment was March 2017 to November 2017.

3.2 Source of study materials

Nine cotton genotypes were used in the study. The sources of cotton seeds of the collected cotton cultivars and genotypes are presented in Table 1.

Table 1: List of cotton genotypes and their sources

Sl. No.	Genotypes name	Source of collection area
1.	CB-12	Cotton Research Center, Mahiganj, Rangpur.
2.	CB-13	Cotton Research Center, Mahiganj, Rangpur.
3.	CB-14	Cotton Research Center, Mahiganj, Rangpur.
4.	CB-15	Cotton Research Center, Mahiganj, Rangpur.
5.	Ra-16	Cotton Research Center, Mahiganj Rangpur.
6	SR-16	Cotton Research, Training & Seed multiplication farm Sreepur, Gazipur
7.	SR-17	Cotton Research, Training & Seed multiplication farm Sreepur, Gazipur
8.	DM-3	CDB, Khamarbari, Dhaka
9.	Rupali-1	CDB, Khamarbari, Dhaka

3.3 Seed germination and collection of leaf sample

Good quality, disease free, healthy cotton seeds were collected from Cotton Development Board & its research center. Soil was collected from nearby nursery and seeds were sown in nine different pot of the research farm of Sher-e-Bangla Agricultural University on May 15, 2018. All management practices were done for raising good quality seedlings from those materials. After raising seedling, in order to carry out RAPD based PCR amplification of cotton genome, fresh and young leaf samples were collected at 3 to 4 leaf stage about 30 days of germination and used as the source of genomic DNA.

3.4 Extraction of genomic DNA

Genomic DNA was extracted from the leaf sample using appropriate protocol of Phenol-Chloroform-Isoamyl alcohol method described by Islam *et al.* (2013) with some modifications. The following reagents and methods were used for the isolation of total genomic DNA.

3.4.1 Reagents used

1. Extraction buffer, pH= 8.0

Composition of extraction buffer are as follows:

- 1M Tris-HCl (pH= 8.0)
-0.5 M EDTA (Ethylene diamine tetra-acetic Acid) (pH= 8.0)
-5 M NaCl
-D.H_2O
-SDS (Sodium Dodecyl Sulphate)
-Marcapto -ethanol
-pvp (polyvinylpyrrolidone)

2. Phenol: Chloroform: Isoamyl Alcohol ratio = 25: 24: 1
3. TE (Tris-EDTA) buffer, pH=8.0

Composition of extraction TE buffer are as follows:

-1 M Tris-HCl

-0.5 M EDTA

-D.H_2O

4. Isopropanol's
5. 0.3 M Sodium Acetate
6. Absolute (100%) ethanol
7. Ethanol (70%)
8. RNAase
9. Ethidium Bromide solution

3.4.2 Equipment's required

1. Mortar and pestle
2. Water bath
3. Centrifuge
4. Vortex mixture
5. Ice maker
6. Micropipette, PCR machine, Electrophoresis system, Gel documentation system etc.

3.4.3 Reagents preparation

Stock solution for 1000 ml Extraction buffer

- 100 ml 1 M Tris HCl (p^H 8.0) was taken in a measuring cylinder.
- Then 40 ml of 0.5 M EDTA was added.
- 100 ml 5 M NaCl was mixed with the mixture.
- Finally sterilized dd.H_2O was added to make the volume upto 1000 ml.
- Then the mixture was mixed well and autoclaved.

Stock solution for 250 ml

1M Tris-HCl pH 8.0ss

- At first 30.28 g Tris was taken in a volumetric flask (500 ml)

- 100 ml dd.H_2O was added.
- pH was adjusted to 8.0 by adding HCl.
- Then sterilized dd.H_2O was added to make the volume up to 250 ml.
- The solution was autoclaved.

Stock solution for 250 ml

0.5 M EDTA. pH 8.0

- At first 46.53 g EDTA.$2H_2O$ was added in a volumetric flask (500 ml)
- 100 ml dd.H_2O was added.
- Then 4 g NaOH was added.
- pH was adjusted to 8.0 with NaOH
- Then sterilized dd.H_2O was added to make the volume up to 250 ml.
- The solution was autoclaved.
- The solution was autoclaved.

Stock solution for 250 ml

5 M NaCl

- Firstly 73.05 g of NaCl was added in 250 ml dd.H_2O.
- It was then mixed well and autoclaved.
- The solution was autoclaved.

2% SDS Stock solution for 100 ml

- 10 g of SDS was added in 100 ml of extraction buffer solution in a 250 ml beaker.
- As SDS is hazardous, so the mixture was mixed by a hot top magnetic stirrer well but not autoclaved.
- 20 ml of 10% that solution added in 80 ml dd.H_2O in a 250 ml beaker.

Stock solution for 100 ml TE Buffer

- 1 ml Tris HCl (pH 8.0) was taken in a volumetric flask (250 ml).
- Then 0.2 ml EDTA (pH 8.0) was added.
- Sterilized dd.H_2O was added to make the volume up to 100 ml.

Composition of 5X TBE buffer (1 litter)

- 54 g Tris-HCl
- 27.5 g of Boric acid
- 4.65 g of EDTA
- pH= 8.3
- Added 1000 ml of dd.H_2O and pH was adjusted at 8.3.

Phenol: Chloroform: Isoamyl Alcohol ratio= 25: 24: 1 (100 ml)

- At first 50 ml Phenol was taken in a volumetric flask (250 ml).
- Then 48 ml Chloroform was added.
- 2 ml Isoamyl Alcohol was also added and mixed well.
- The solution was stored at 4°C.

RNase A/H

- 10 mg of RNase was added to 1 ml of dd.H_2O.
- Then it was dissolved completely with the help of necessary heat (at 65°C in water bath for 30 minutes)

70% Ethanol (1000 ml)

- 700 ml absolute ethanol was mixed with 300 ml dd.H_2O

0.3 M Sodium Acetate

- 2.05 gm of Na acetate dissolved in 50 ml sterilized d.H_2O then we get 0.3 M Na acetate.

3.5 Sequential steps for DNA extraction

1. For Isolation of genomic DNA, vigorous, young, actively growing fresh leaf tissues were collected from nine different cotton germplasm.
2. Initially, healthy youngest leaves were washed thoroughly by tap water followed by de-ionized water. Then sterilized by ethanol to remove wastes and any source of foreign DNA and leaves are then dried on tissue paper

3. Approximately 100 mg of young leaves were cut into small pieces and then taken in mortar. 500 µl of extraction buffer and 100 µl of marcapto-ethanol with near about 30 mg of pvp chemical was added to it. The grind samples were taken into the 1.5 ml Eppendorf tube and then it was vortexed for 20 seconds in a vortex mixture and incubated at 65 ^{0}C for 20 minutes in hot water bath.
4. Equal volume (500 µl) of Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) was added to the tube. Then it was vortexed for 20 seconds.
5. The solution was then centrifuged for 10 minutes at 13000 rpm. The supernatant was recovered using a micro pipette tip without disturbing the lower portion and transferred into a new Eppendorf tube. Approximately 400-450 µl was taken and then equal volume of Chloroform: Isoamyl Alcohol (24: 1) was added to it. The solution was vortexed for 10 seconds.
6. Again the solution was centrifuged at 13000 rpm for 10 minutes.
7. The supernatant was taken in a separate Eppendorf tube and the lower layer was discarded.
8. The amount of the solution was multiplied with 0.6 and then same volume of Isopropanol (0.6 volume of the liquid) was added.
9. It was then tapped by finger for 20-30 seconds (the genomic DNA was visible as cotton like structure).
10. After tapping the sample was again centrifuged at 13000 rpm for 15 minutes. The liquid was discarded completely and re-precipitation of the DNA solution was done by adding 500 µl of absolute (100%) cold ethanol plus 20 µl 0.3 M Sodium acetate.
11. It was shaken gently. Tapping was done to separate pellet. The sample was centrifuged at 13000 rpm for 15 minutes. The liquid was removed completely by pouring and blotting the open tube end on fresh tissue paper.
12. The DNA pellet was then air dried for 2-3 hours. It was then dissolved in an appropriate volume (30 to 40 µl) of TE buffer and treated with RNAse at 37^{o}C in hot water bath for 15-20 minutes. Then it was spinned for 4-5 seconds.
13. Finally, the DNA samples were stored in freezer at -20^{o}C.

3.6 Confirmation of DNA preparation

The isolated genomic DNA contain large amount of RNA and pigments as contaminant and was hence purified by treatment with RNase and further precipitated. To confirm DNA preparation, 1% agarose gel was used for assessing the quality of the genomic DNA and the amount of RNA present.

3.6.1 Preparation of 1 % agarose gel

Reagents:

1. Agarose powder
2. 5X TBE Buffer (pH 8.0)
3. Ethidium Bromide

Procedure

600 mg of agarose powder was taken in a 500 ml Erlenmeyer flask containing 60 ml electrophoresis buffer (1x TBE buffer) prepared by adding 20 mL of 5X TBE buffer in 80 mL of sterile deionized water. The flask was enclosed with aluminum foil paper to prevent excessive evaporation. It was melted for about 2 to 3 minutes into a microwave oven with occasional swirling until complete disappearance of agarose particles to generate homogeneous and crystal clear suspension. Then the agarose solution was cooled to about 45-50°C (flask was cool enough to hold comfortably with bare hand) and 0.75 µl (10 mg/ml) ethidium bromide (DNA stain) was added and mixed well by gentle shaking to make the DNA visible under ultraviolet light box (Trans-illuminator). The molten gel was poured immediately on to a clean gel bed (10 x 7 x 2 cm^3; in size), that was placed on a level bench and appropriate comb was inserted parallel to the plate's edge with the bottom of the teeth about 2 mm above the plate. Air bubbles were removed by pushing away to the side using a disposable tip. After 30-45 minutes gel became completely cooled at room temperature and solidified and the comb was removed gently. The gel was then ready for loading the DNA samples.

3.6.2 Preparation of DNA samples for electrophoresis

The samples were all in the same concentration in buffer. For each sample, 3 µl dd.H_2O and 2 µl loading dye (0.25% xylene ethanol, 0.25% bromophenol blue, 30% glycerol and 1mM EDTA) and 3.0 µl of sample DNA was taken in an Eppendorf tube using 0.5-10 µl adjustable micropipette. Loading dye was used for monitoring loading and the progress of the electrophoresis and to increase the density of the sample so that it stayed in the well. Finally, 8 µl extracted DNA was added to it and mixed well. The sample was then loaded into the well of the gel and allowed them to sink to the bottom of the wells. The gel was placed in the electrophoresis chamber (Continental Lab product. Inc**.)** Keeping the gel horizontal and submerged in 1X TBE buffer (running buffer). The final level of buffer was about 5 mm above the gel. The gel tank was covered and the electrophoresis power supply was connected and turned on to move DNA from negative to positive electrode (Black to Red) through the gel. Electrophoresis was carried out at 80 V for about 55 minutes.

Table 2. Amount of DNA confirmation reagents

Components	**Amount (µl)**
Working DNA sample	3.0
D. H_2O	3.0
2X loading dye	2.0
Total volume	8.0

3.7 Working solution of DNA samples preparation

DNA concentration were adjusted to 20-25 ng/µl for doing PCR using the following formula: $V1 \times S1 = V2 \times S2$

Where,

V1 = Initial volume of DNA solution (µl)

S1 = Initial DNA concentration (ng/µl)

V2 = Final volume of DNA solution (µl)

S2 = Final DNA concentration (ng/µl)

Documentation of the DNA sample

The gel was taken from the gel chamber and was placed on an ultraviolet light box (UV transilluminator) to examine and photographed by a Gel Cam Polaroid camera. Better quality band showing DNA samples were taken for working solution preparation. Quality bands showing DNA samples were taken for quantification and working solution preparation.

3.8 Amplification of RAPD markers by PCR

3.8.1 Principle of RAPD primer amplification

For amplification of RAPD, a single oligonucleotide of arbitrary DNA sequence is mixed with genomic DNA in the presence of a thermo-stable DNA polymerase and a suitable buffer and then it is subjected to temperature cycling conditions typical to the Polymerase Chain Reaction (PCR). The products of the reaction depend on the sequence and length of the oligonucleotide, as well as the reaction conditions. At an appropriate annealing temperature the single primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance of each other (e.g., within a few thousand nucleotides) and a discrete DNA segment is produced. The presence or absence of this specific product, although amplified with an arbitrary primer, will be diagnostic for the oligonucleotide binding sites on the genomic DNA. In practice, the DNA amplification reaction is repeated on a set of DNA samples with several different primers, under conditions that result in several amplified bands from each primer. Often a single primer can be used to identify several polymorphisms, each of which matches to a different locus

3.8.2 Selection of primers

Elevan decamer RAPD primers were tested, they resulting in faint or irreproducible DNA fragments. From them seven primers were selected for this study. Seven decamer RAPD primers were **OPG 05, OPG 03, OPB 17, OPBB 06, OPBA 03, OPD 20, OPX 01** (Operon Technologies, Inc., Alameda, California, USA) and it was screened for PCR reaction in 9 genotypes of cotton. The detail of RAPD primers are given in Table 3.

Table 3. Name of RAPD primers with GC content and sequence information

Sl. No.	Primer name	Sequence (5′ to 3′)	(G+C) %
1.	**OPBA 03**	GTGCGAGAAC	60
2.	**OPBB 06**	CTGAAGCTGG	60
3.	**OPG 05**	CTGAGACGGA	60
4.	**OPX 01**	CTGGGCACGA	70
5.	**OPG 03**	GAGCCCTCCA	70
6.	**OPB 17**	AGGGAACGAG	60
7.	**OPD 20**	ACCCGGTCAC	70

3.8.3 PCR amplification

PCR reactions were performed on each DNA sample. 2X Taq ready Master Mix was used. DNA amplification was per formed in oil-free thermal cycler (Esco Technologies swift^{TM} Mini Thermal Cycler and Q-cycler). To prepare a 10.0 µl reaction mixture containing ready mix Taq DNA polymerase and other compositions were given in Table 4.

Table 4. PCR mixture composition for each cotton genotype

Reagents	Amount(µl)
2X Taq Master Mix	5.00
RAPD primer	1.50
De-ionized water	1.50
Sample DNA	2.00
Total Reaction volume	10.00

From frozen stocks of the PCR reagents i.e., 2X Taq Master Mix, primer and DNA working samples were melt, mixed by vortexing and kept on ice for maintain good quality. DNA samples were pipetted first into PCR tubes compatible with the thermo-cycler used (0.5 ml). A pre-mixture was then prepared in the course of the following order**:** reaction mixture, DNA sample and de-ionized water. Then the mixture was mixed up well and aliquoted into the tubes containing primer. The tubes were then sealed and placed in a thermal cycler and the cycling was started immediately.

3.8.4 Thermal profile for PCR

DNA amplification was performed in an oil-free thermal cycler (Esco Technologies Q-cycler). The PCR tubes were kept in the thermal cycler and the following programs were run:

Step- 1: 95 ˚C for 4 minutes – Initial denaturation

Step- 2: 95˚ C for 45 seconds – Denaturation

Step- 3: 35˚ C for 40 seconds – Primer annealing } 31 cycles

Step- 4: 72˚ C for 1 minutes – Extension

Step- 5: 72˚ C for 5 minutes – Final extension

Step- 6: 4˚ C for 10 minutes – Hold

3.8.5 Electrophoresis of the amplified products

After completion of thermal cycler reaction, each sample of PCR products were confirmed by running 2.0% agarose gel containing 1.0 µl ethidium bromide in 1x TBE buffer at 90 V for 75 minutes. Loading dye (3.0 µl) was added to the PCR products and loaded in the wells. Two Molecular weight markers 100 bp (Bio-Basic, Cat. No. M-1070-1, Canada and 1 kb DNA marker Bioneer, Korea) DNA ladder were also loaded on left side of the gel respectively. Under Ultra Violet (UV) light on a trans-illuminator RAPD bands were observed and documented by taking photograph using a Gel Cam Polaroid camera.

3.9 Data analysis

Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single allelic locus (Williams *et al.,* 1990). One molecular weight marker, 100 bp (Bio-Basic, Cat. No. M-1070, Canada and 1 kb DNA marker, Bioneer, Korea) DNA ladder was used to estimate the size of the amplification products by comparing the distance traveled by each fragment with known sized fragments of molecular weight markers. All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to their on gel

and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The band-size for each of the markers was scored using the Alpha Ease FC 4.0 software. The scores obtained using all primers in the RAPD analysis were then pooled to create a single data matrix. The individual fragments were assigned as alleles of the appropriate loci. This was used to estimate polymorphic loci. Using Power Marker version 3.25 software (Liu & K. J., 2005). The summary statistics that were determined included the following: the number of alleles, the major allele size and its frequency, gene diversity, and the polymorphism information content (PIC) value**.** The allele frequency data from POWERMARKER was used to export the data in binary format (presence of allele as "1" and absence of allele as "0"). Binary data form of allele frequency used for dendogram construction by NTSYS-pc software (Rholf & F. 2002). The unweighted pair grouping method, using arithmetic average (UPGMA), was used to determine similarity matrix following Dice coefficient with SAHN subprogram.

Polymorphic Information Content (PIC) value of a primer is calculated as:

$$PIC = 1-\sum pi^2$$

Where *pi* is the frequency of the i^{th} allele. Polymorphic Information Content was used to confirm the suitability of the primers selected for DNA fingerprinting.

Genetic similarity values defined as the fraction of shared bands between the RAPD profiles of any two individuals on same gel were calculated manually by RAPD markers of the molecular weight on the data matrix according to the following formula:

$$\text{Similarity index (SI)} = \frac{2Nxy}{Nx+Ny}$$

Where,

Nxy = Number of RAPD bands shared by individuals x and y respectively,

Nx and Ny = Number of bands in individuals x and y respectively (Chapco *et al*., 1992; Wilde *et al*., 1992 and Lynch, 1990).

The SI value ranges from 0 to 1. When SI=1.0, the two DNA profiles are identical and when SI is 0.0, there are no common bands between the two profiles.

3.10 Precautions

- To maintain a strategic distance from all types of contaminations and keep DNA pure, all dishes, micropipette tips, Eppendorf tubes, glass pipettes, de-ionized water and buffer solutions were legitimately autoclaved. Metal supplies i.e., scissors, forceps were cleaned with absolute ethanol.

- Since Ethidium Bromide (Et-Br) is an intense mutagen and carcinogenic in nature, hand gloves were utilized when taking care of anything that has been presented to Et-Br.

- Always power pack was kept turn off and the leads were unplugged before opening the electrophoresis unit to avoid electrical hazard.

- Eye protector was used while working with trans-illuminator as it produces UV radiation of 254 nm range which can cause eye damage.

- The common safety measures were kept up when performing PCR responses. All the disposables such as PCR tubes, tips, Eppendorf tubes and reagents used during preparation of PCR reactions were autoclaved. Freezing condition was maintained when necessary. Hand gloves were worn amid treatment of PCR segments. Contamination of PCR segments was maintained a strategic distance from.

CHAPTER V

SUMMARY AND CONCLUSION

Cotton (*Gossypium hirsutam)* is one of the most important fiber and cash crop grown today in Bangladesh as well as in the world. Cotton is a major source of foreign exchange for many countries around the world. Very few research work has done at molecular level in cotton in Bangladesh. For this reason, it is crying need to study more experiment on cotton for the evaluation of genetic diversity to crop improvement, breeding program and conservation purposes. This research activities performed mainly the molecular characterization of nine cotton genotypes of Bangladesh.

High-throughput DNA markers (RAPD) will be helpful in recognizing the cotton genotypes carrying desired characters and was successfully used not only to study the genetic diversity but to develop linkage maps and mapping molecular trait, which are necessary for acceleration of varietal development. In general, the choice of a molecular marker technique is based on reliability, statistical power, and level of polymorphisms.

Some total of nine cotton germplasms were used and RAPD-PCR based analysis was done by using seven primers. A total of 30 amplified products were observed in which one was monomorphic and nine were polymorphic. The number of amplified DNA bands varied between 2 to 6 with an average of 4.29 and polymorphic bands were noticed. Maximum number (2) of polymorphic bands were observed in the primer OPBA 03, OPB 17, OPBB 06 and minimum number (1) of polymorphic bands were observed in the primer OPG 05, OPG 03, OPX 01. The polymorphism percentage ranged between 25 to 50 per cent with an average of 31.57 per cent for RAPD primers while no polymorphic bands generated by primer OPD 20. The overall genetic diversity was ranged from 0.44 to 0.22 in this study. Polymorphic information content (PIC) value ranged between 0.18 to 0.35 with an average of 0.27.In principle component analysis (PCA) on 9 cotton genotypes by six RAPD primers showed into two dimensional scatter plot with four groups. Among them group- ii (CB-12) and group iii (CB-14) represents highest distance that indicates selection of materials for future cotton improvement program. Nei's genetic distance ranging from 0.1667 to 0.6667. In this experiment, inter varietal means of the pair wise similarity indices (Sij) ranged from 20 to 337.22. The highest similarity indices of 337.22 was observed in CB-12 and CB-14 varietal

pair and rest of varietal pairs showed low indices. So, this study indicates moderately genetic variation among the different cotton genotypes.

The dendogram (UPMGA) constructed from the fingerprint data obtained from nine cotton genotypes showed into two major cluster (cluster A and Cluster B).The first major cluster 'A' had divided only two minor groups. These findings demonstrate the utility and importance of molecular markers for genetic diversity analysis. Such studies can be extremely helpful for plant breeders to improve the cotton varieties for breeding program. Above all, considering the results this research work might be used as a guideline for future study.

CHAPTER VI
RECOMMENDATION

The present study indicated the preliminary assessment of genetic variation of cotton genotypes and it had some limitations in terms of limited number of individuals and cotton genotypes as well as large number of primers used and following points might be considered for sustaining genetic constituents of cotton in Bangladesh.

1. Large number of genotypes and higher number of primers should be studied with adequate replications to increase accuracy.
2. Details survey work should be conduct using more molecular markers for obtaining diagnostic loci for cotton genotypes.
3. Other molecular markers such as SSR, SNP, AFLP, ISSR, micro-satellite, etc. should be developed for cotton genotypes of Bangladesh.

CHAPTER VII

REFERENCES

Abdel, A., Abdel, G. and Zaki, E. A. (2003). DNA sequences of RAPD fragments in the Egyptian cotton *Gossypium barbadense*. *African J. Biotechnol*. **2:** 129-132.

BBS. (2017), Statistical Year Book of Bangladesh, Bureau of Statistics Ministry of Planning, Government of the People's Republic of Bangladesh, Dhaka.

Botstein, D., White, R. L., Skolnick, M. and Davis, R. W. (1980). Construction of a genetic linkage map using restriction fragment length polymorphism. *Am. J. Hum. Genet.* **32**: 314-331.

BTMA (Bangladesh Textile Mills Association), (2016). Special Bulletin on cotton import**. 2:** 15-18.

Chapco, W., Ashton, N. W., Martel, R. K .B. and Antonishyn, N. (1992). A feasibility study of the use of random amplified polymorphic DNA in the population genetics and systematics of grasshoppers. *Genome.* **35**: 569-574.

Chen, Z. J., Scheffler, B. E. and Dennis, E. (2007). "Toward sequencing cotton (*Gossypium*) genomes," *Plant Physiology*. **145:** 1303–1310.

Chen, Z. X., She, J. M., Wu, J. Y., Li, S. J. And Yue, J. X., (1992). Plant regeneration through somatic embryogenesis from suspension cultures derived protoplast of 6 cotton cultuvars (*G. hirsutum).* Curr. *Plant Sci. Biotechnol. Agric.* **15**: 115- 119.

Clark, A.G. and Lanigan, C. M. S. (1993). Prospects for estimating nucleotide divergence with RAPDs. *Mol. Biol. Evol.* **10**: 1069-1111.

Datta, D., Gupta, S., Chaturvedi, S.K. and Nadarajan, N. (2011). Molecular markers in crop improvement. *Indian Institute of Pulses Res.* 229-232

Dongre, A. B. and Kharbikar, L. L. (2004). RAPD-based assessment of genetic diversity in cotton (*Gossypium hirsutum* L.) race stock accessions. *Indian J. Genet.* **64**(2): 94-96.

Fristsch, P. and L. H. Rieseberg. (1996). The use of random amplified polymorphic DNA (RAPD) in conservation genetics. *In: Smithers, T.B. and R.K. Wanye, (eds.*), *Mol.* genetic approaches in Conservation, Oxford University Press, Oxford. 54-73.

Gomes, M., Kulothungan, G., Mehetre, S. S. and Eapan, S. (2004), Analysis of genetic relatedness in *Gossypium* species using RAPD. *Indian J. Biotechnol.* **3** : 41- 46.

Goswami, M. and Ranade, S.A. (1999). Analysis of variations in RAPD profiles among accessions of Prosopsis. *J. Genet*. **78**: 141-147.

Hadrys, H., Balicki, M. and Schierwater, B. (1992). Application of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol*. **1:** 55-63.

Haig, S. M., Rhymer, J. M. and Heckel, D. G. (1994). Population differentiation in Random Amplified Polymorphic DNA of red-cockaded woodpeckers Picoides borealis. *Mol. Ecol*. **3:** 581-595.

Hallden, C., Nilsson, N. O., Rading, I. M. and Sale, T. (1994). Evaluation of RFLP and RAPD markers in comparison of Brassica napus breeding lines. *Theoret. Appl. Genet.* **88 :** 123-128.

Hanchinal, R. R., Nadaf, H. L., Nishani, S. S., Motagi, B. N., Madhusudan, K. and Vijaykumar, A. G., (2004). Varietal identification and hybrid purity testing through RAPD markers in cotton (*Gossypium* sp.). International Symposium on Strategies for Sustainable Cotton Production – A Global Vision, 23-25 November, 2004, held at *Uni. Agric. Sci*., Dharwad, pp. 406-410.

Harisaranraj, K., Suresh and Saravanababu, S. (2009). DNA Finger Printing Analysis among Eight Varieties of Zingiber officinale Using RAPD Markers. Global Journal of Molecular Sciences. **4 (2):** 103-107.

Henry, R. J. and Nelson Thornes. (1997). Practical applications of plant molecular biology.

Hussain, S., Shah, Saif, K., Naqui, S. M. S. and Tahira Yasmin. (2004). A simple method for screening cotton germplasm against cotton leaf curl begamovirus. *Sarhad J. Agri.* **20**(3): 453-458.

Iqbal, M. J., Aziz, N., Saeed, N. A., Zafar, Y. and Malik, K. A. (1997). Genetic diversity evaluation of some elite cotton varieties by RAPD analysis. *Theoret. Appl. Genet.*, **94(1):** 139-144.

Iqbal, M. J., Reddy, O. U. K. El-Zik, K. M. and Pepper, A. E. (2001). "A genetic bottleneck in the 'evolution under domestication' of upland cotton *Gossypiumb hirsutum* L. examined using DNA fingerprinting," *Theoretical and Applied Genetics*. **103:** 547–554.

Islam, M. N., Molla, M. R. and Rahman, L. (2013). Genetic characterization of sesame varieties through RAPD fingerprinting technique in Bangladesh. *J. Sher-e-Bangla Agric. Univ.* **7(1):** 1-5.

Jahufer, M., Barret, B., Griffiths, A. and Woodfield, D. (2003). DNA fingerprinting and genetic relationships among white clover cultivars. Proceedings of the New Zealand Grassland Association **65**: 163–169

JiangYong, G. YiQing, W. MingGang, W., Liming, Z., FengJiang, L., WenBin, L., Haifeng, L., TianPeng, Z and YongRn, S. (2004). Genetic diversity analysis of brown cotton and green cotton. Herditas, Beijing. **26(1):** 63-68.

Jones, N., Ougham, H. and Thomas, H. (1997). Genome Mapping, molecular markers and Marker assisted selection in crop plants. *New phytol*. **137:**165-177.

Karp, A., Kresovich, S., Bhat, K., Ayad, W. and Hodgkin, T. (1997). Molecular tools in plant genetic resources conservation: a guide to the technologies. *IPGRI*. Rome, Italy.

Khan, A. I., Awan, F. S., Bushra S., Rana, R. M.and Khan, (2013) I. amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**:6531-6535.

Khan, A.I., Khan, I. A., Awan, F. S., Sadaqat, H. A. and Bahadur, S. (2011). Estimation of genetic distance based on RAPD between 11 cotton accessions varying in heat tolerance. *Genet. and Mol. Res*.**10:** 96-101.

Khan, S. A., Thessain, D., Askari, E., Stewari, J. Mc. D., Malik, K. A. and Zafar, Y. (2000). Molecular phylogeny of *Gossypium* species DNA fingerprinting. *Theor. Appl Genet.* **101:** 931-938.

Kim, H. J. and Triplett, B. A. (2001) "Cotton fiber growth in plant and in vitro. Models for plant cell elongation and cell wall biogenesis," Plant Physiology. **127:**1361–1366.

Kimberling, D. N., Ferreira, A. R., Shuster, S. M. and Keim, P. (1996). RAPD marker estimation of genetic structure among isolated northern leopard frog populations in the southwestern USA. *Mol. Ecol*. **5(4):** 521-529.

Kumar, N.S. and Gurusubramanian, G. (2011). Random amplified polymorphic DNA (RAPD) markers and its applications. *Sci. Vis*. **11:**116-124.

Kumar, P., Singh, K., Vikal, Y., Randhawa, L. S. and Chahal, G. S. (2003). Genetic diversity studies of elite cotton germplasm lines using RAPD markers and morphological characteristics. *Indian J. Genet. Plant Breed.* **63:** 5–10

Liu, B. and Jonathan, F.W. (2001). Inter simple sequence repeat (ISSR) polymorphisms as a genetic marker system in cotton. *Mol. Ecol. Notes.* **1**: 205-208.

Liu, B. H. (1997). Statistical genomics: linkage, mapping and QTL analysis: *CRC press*.

Liu, K. J. and Muse, S. V. (2005). Power Marker: An integrated analysis environment for genetic marker analysis. Bioinformatics. **21:** 2128–2129.

Lu H. J. and Myers, G. O. (2002). Genetic relationships and discrimination of ten influential upland cotton varieties using RAPD markers. *Theoretical and Applied Genetics* **105**: 325-331.

Lynch, M. (1990). The similarity index and DNA fingerprinting. *Mol. Biol. Evol.* **7(5):** 478484.

Mackill, D., Nguyen, H. and Zhang, J. (1997). Use of molecular markers in plant improvement programs for rainfed lowland rice. *Field crops research*. **64(1):** 177-85.

Mathews, G. A. (1989). Cotton insect pest and their management, Longman scientific and Technical Longman group UK limited, Longman House, Burnt Mill, Harlow, Assex, England. 153-157.

McCouch, S., Kochert, G. and Yu, Z. (1988). Molecular mapping of rice chromosomes. *Theoretical and Applied Genetics*. **76(6):** 815-29.

Mia, M. S., Patwary, A. K., Hassan, L., Hasan, M. M., Alam, M. A., Latif, M. A., Alam, M. M. and Puteh, A. B. (2014). Genetic Diversity Analysis of Ginger (*Zingiber officinale*) Genotypes Using RAPD Markers. *Life Sci. J.* **11(8):**90-94.

Michelmore, R.W., Paran, I. and Kesseli, R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA.* **88(21):** 9828-9832.

Michelmore, R.W., Paran, I. and Kesseli, R.V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl .Acad. Sci. USA*. **88(21**): 828-832.

Mondini, L., Noorani, A. and Pagnotta, M.A. (2009). Assessing plant genetic diversity by molecular tools. Diversity. **1(1**): 19-35.

Multani, D. S. and Lyon, B. R. (1995). Genetic fingerprinting of Australian cotton cultivars with RAPD markers. *Genome*. **38 :** 1005-1008.

Munro, J. M. (1994). Cotton and its production, insect pests of cotton, *CAB International*. **9(1):** 47-52.

Nei, M., Tajima F. A., Tateno Y.(1983). Accuracy of estimated phylogenetic trees from molecular data. *J. Mol. Evol*. **19:** 153–170.

Paterson, A. H., Tanksley, S. D. and Sorreis, M. E. (1991). DNA markers in plant improvement. *Adv Agro*. **46:** 39–90.

Percival, E. and Kohel, R. J. (1990). Distribution collection and evaluation of *Gossypium. Advances in Agronomy. **44 :** 225-228.*

Qin, Y. M. and Zhu, Y. X. (2011). "How cotton fibers elongate: a tale of linear cell-growth mode," *Current Opinion in Plant Biology*. **14:** 106–111.

Rafalski, J. A. and Tingey, S. V. (1993). Genetic diagnosis in plant breeding: RAPDs, microsatellites and machines. *Tr. Genet*. **9**: 275-279.

Rahman, M., Ahmed, N., Asif, M. and Zafar, Y. (2006). Identification of DNA markers linked with cotton leaf curl disease (*CLCD*). International Cotton Genome Initiative (*ICGI*) Work- shop, Brasilia, Brazil. 77–78.

Rahman, M., Hussain, D. and Zafar, Y. (2002). Estimation of divergence among elite cotton cultivars-genotypes by DNA fingerprinting technology. *Crop Sci*. **42:** 2137- 2144

Rahman, M., Hussain, D. and Zafar, Y. (2002). Estimation of genetic divergence among elite cotton cultivars – Genotypes by DNA fingerprinting technology *Crop Science.* **42**: 2137-2144.

Rana, M. K., Tabar, M. V. and Bhat, K.V. (2005). Genetic diversity in Indian cotton (*Gossypium Spp*.) Cultivars as reveled by RAPD analysis. *Indian J. Biotechnol***. 4 (4):** 522-527.

Rholf, F.J. (2002). NTSYS-Pc numerical taxonomy and multivariate analysis, version 2.2. *Applied Biostatics Inc*., New York, USA.

Rieseberg L. H. and Noyes, R. D. (1996). Genetic map-based studies of reticulate evolution in plants. *Trends in Plant Science.* **3:** 254–259

Roldan-Ruiz, I., Van- Eeuwijk, F. A., Gilliland, T. J., Dubreuil, P., Dillmann, C., Lallemand, J., De-Loose, M. and Baril, C. P. (2001). A comparative study of molecular and morphological methods of describing relationships between perennial ryegrass (*Lolium perenne* L.) varieties. *Theor. Appl. Genet.* **103:** 1138-1150

Saha, S., Karaca, M., Jenkins, J. N., Zipf, A. E., Reddy, O. U. K. and Kantety, R. V. (2003). "Simple sequence repeats as useful resources to study transcribed genes of cotton," Euphytica. 130: 355–364.

Saha, S., Karaca, M., Jenkins, J. N., Zikf, A. E. Reddy, O. U. K. and Kantey, Sambamurthy, J. S. V., Chamundeswari, N. and Udagasree, P. (2004). Studies on genetic divergence in introgressed lines of upland cotton (*Gossypium hirsutum* L.). International Symposium on Strategies for Sustainable Cotton Production – A Global Vision 1. Crop Improvement, 23-25 November, 2004, *Uni. Agric. Sci*., Dharwad (India).

Shanti Patil, Raveendran, T. S., Kumar, P. R. and Patil, S. (2001). Identification of fertility restorers in cotton germplasm using morphological and moelcular markers. *J. Soils Crops* **11**: 66-73.

Sofalian,O., Chaparzadeh, N. and Dolati, M. (2009) Genetic diversity in spring wheat landraces from Northwest of Iran Assessed by ISSR marker. *Not. Bot. Hort. Agrobor. Cluj*. **37(2**): 252-256

Song, G. L., Cui, R. X., Wang, K. J., Li, S. H., Zan, G. J. and Guo, J. H. (1999). Analysis of genetic diversity of Australian speices of *gossypium* using RAPD. *Acta Gossypii Simika,* **11 (2)**: 65-69.

Soregaon, C. D., Katageri, I. S., Khadi, B. M., Vamadevaiah, H.M. and Bhandari, A. (2004). Genetic diversity analysis in recombinants derived from interspecific crosses of cotton (*Gossypium* spp.*)* using RAPD markers. International Symposium on "Strategies for Sustainable Cotton Production – A Global Vision". *Crop Improv.* **23**: 23-25.

Staub, J. E., Serquen, F. C. and Gupta, M. (1996). Genetic markers, map construction and their application in plant breeding. *Hort. Sci.* **31:** 729-740.

Tanksley, S. D.,Tong, N. D., Paterson, A. H. and Boinerbale, M. W. (1989). RFLP mapping in plant breeding ; New tools for an old science. *Biotech.* **7:** 205-364.

Thormann, C. E., Ferreira, M. E., Camargo, L. E. A., Tivang, J. G. and Osborn, T. C. (1994). Comparison of RFLP and RAPD markers for estimating genetic relationships within and among cruciferous species. *Theor. Appl. Genet.* **88:** 973- 980.

USDA, Cotton and Wool Year book, Dataset, 2017.

Vierling, R. and Nguyen, H.T. (1992). Use of RAPD markers to determine the genetic relationships of diploid wheat genotypes. *Theor. Appl. Genet.* **84**: 835-838.

Vroh, B., Maquet, A., Baudoin, J. P., Du Jardin, P., Jacquemin, J. M. and Mergeai, G. (1999). Breeding for "low-gossypol seed and high gossyipol plants" in upland cotton : Analysis of trispecies hybrid and backcross progenies using AFLPs and mapped RFLPs. *Theor. Appl. Genet.* **99**: 1233-1244.

Wilde, J., Waugh, R. and Powell, W. (1992). Genetic fingerprinting of Theobroma clones using randomly amplified polymorphic DNA markers. T*heor. Appl. Genet*. **83(6-7):** 871-877.

Williams, J. G. K., Hanafey, M. K., Rafalski, J. A. And Tingey, S. V. (1990). Genetic analysis using random amplified polymorphic DNA markers. Methods Enzymology. **1218:** 704-740.

Williams, J. G. K., Kubelik, A. E., Levak, K. J., Rafalski, J. A. and Tingey, S. C. (1990): DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **28:** 6531-6535.

Yang, Y. L., Zhang, T. Z., Guo, W. Z., Shen, X. L., Yu, J. and Kohel, R. SJ. (2001). Moelcular tagging and mapping of QTLs for super quality fibre properties in upland cotton. *Yi Chuan Xue Bao.* **28**: 1151-1161.

Zhang, J. F., Lu, Y., Adragna, H. and Hughs, E. (2005). Genetic improvement of New Mexico Acala cotton germplasm and their genetic diversity. *Crop Sci*. **45(6):** 2363- 2373.

Zhang, T. Z., Yuan, Y. L., Yu, J., Guo, W. Z. and Kohel, R. J., (2003), Molecular tagging of a major QTL for fibre strength in upland cotton and its marker-assisted selection. *Theoretical Appl. Genet*. **106(2):** 262-268.

Zhang, T., Qian, N. and Zhu X. (2013). "Variations and transmission of QTL alleles for yield and fiber qualities in upland cotton cultivars developed in China," *PLoS ONE*. Article ID e57220. **8:** 2.