# RAPD MARKER BASED GENETIC DIVERSITY ANALYSIS OF JUJUBE (*Ziziphus mauritiana* Lam.)



A THESIS
BY

**KAUSTOV BAIRAGI**
EXAMINATION ROLL NO. MS 110807

A thesis (Course No: AT- 6104) Submitted to the Agrotechnology Discipline, Khulna University, Khulna, in partial fulfilment of the requirement for the degree of

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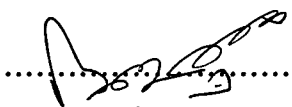

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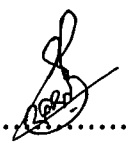

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**DEDICATED TO**

**OUR BELOVED PARENTS**

**&**

**All FARMERS**

**OF BANGLADESH**

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# ABSTRACT

The research work was carried out during June 2012 to March 2013 to determine the genetic variability of 15 selected *Ziziphus mauritiana* Lam. germplasm collected from the south-western region of Bangladesh. In the present study, molecular characterization was used to record different levels of variability concerning the morphological traits of the fruit. The 10-mer and 12-mer oligonucleotide primers were used in RAPD for amplification. Three primers, A01, A02 and S07 were able to ditect the amplification and yielded a total of 144 band patterns of which 69.74% were polymorphic. The highest percent of polymorphic loci (77.78%) was observed for primer S07 and the lowest (60.00%) for primer A01. Results were analyzed by molecular algorithm UPGMA. Fifteen genotypes on the dendrogram were identified and divided into two major groups and subgroups on the basis of genetic characteristics and GP8 is most diverged in cluster G2.1. The range of genetic distance was observed 0.090 (GP10 vs. GP07) to 0.625 (GP09 vs. GP06). Based on the cluster analysis, the *Z. mauritiana* samples that were believed to have high morphological difference were grouped independently. The results suggested that RAPD is useful to measure the genetic diversity jujube germplasm.

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# CHAPTER I

# INTRODUCTION

The genus *Ziziphus* belongs to the family Rhamnaceae and consists of 40 species in tropical and subtropical regions of the northern hemisphere. Jujube (*Ziziphus mauritiana*) is an indigenous minor fruit of Bangladesh which is available when most of the major fruits are not in the market. They are commonly named jujube, red date, Chinese date, ber, Korean date, Indian date and in some cases apple or little apple in Malaya (Reich, 1991). These species are indigenous to North Africa, Afghanistan, North India, Southern China, Malaysia, and Queensland in Australia. However, jujube is now widely distributed and has become naturalized in tropical Africa, Iran, Syria, Sri Lanka and part of the Mediterranean region (Kaarira, 1998). However, in Bangladesh the total area of land under fruit cultivation is about 141295.55 hectare and the yield per hectare is 31.03 ton. The total production of jujube in Bangladesh is about 78 thousand ton (BBS, 2011). But jujube production is not very promising because it is not cultivated carefully or commercially in Bangladesh. Though this fruit is very much popular but its production is not compatible with the country's demand. Jujube grows normally in roadside, pondside, graveyard, homestead and fallowland. From last few years improved variety of jujube gets more popularity in the country especially in Rajshahi region. Now jujube grows both in the homestead and garden. Mostly growing areas of this fruit in Bangladesh are Rajshahi, Khulna, Barisal, Comilla, Mymensingh etc. In Bangladesh the growing season of jujube is September to March within a year (Rashid *et al.*, 1987).

Jujube is a spiny, evergreen shrub or small tree up to 15 m high, with trunk 40 cm or more in diameter; spreading crown; stipular spines and many drooping branches. The tree is graceful and ornamental in appearance. The fruit can be oval, obovate, oblong or round, and that can be 2.5-6.25 cm long, depending on the variety. The flesh is white and crisp. Ber fruits are very nutritious and usually eaten fresh. Fruits are also eaten in other forms, such as dried, candied, pickled, as juice, or as ber butter (Maydell, 1986). It is quite nutritious and rich in vitamin C and second only to guava and much higher than citrus or apples. The edible portion (per 100 g) of the fruit contains moisture 83 %, sugars 5.4-10.5 g, dietary fibre 0.60 g, energy 5.92 kcal, protein 0.8g, fat 0.07g, carbohydrate 17g, calcium 25.6 mg, phosphorus 26.8 mg, iron1.1 mg , vitamin $B_1$ (0.022 mg), vitamin $B_2$ (0.029 mg) and vitamin $B_3$ (0.78 mg). The anti-oxidant content of fresh jujube is higher than strawberries, plums, apples, blueberries,

DNA testing for genetic genealogy to determine genetic distance between individuals or populations.This property enables the use of a marker, which can then be used to determine the precise inheritance pattern of the gene that has not yet been exactly localized. Recently, many molecular markers, such as restriction fragment length polymorphism (RFLP), amplified fragment-length polymorphism (AFLP), sequence characterized amplified regions (SCAR), inter simple sequence repeat (ISSR), simple sequence repeat (SSR) and random amplified polymorphic DNA (RAPD), are used in horticultural crops research. RAPD markers have been used for cultivar identification and genetic diversity analysis among 25 *Feijoa sellowiana* (Dettori and Palombi, 2000) cultivars and accessions in Italy. Molecular markers were considered as powerful tools in the assessment of genetic diversity within and between plant populations (Hossain *et al.*, 2003). Among the different types of molecular markers, randomly amplified polymorphic DNAs (RAPDs) are useful for the assessment of genetic diversity owing to their simplicity, speed and relatively low-cost (Williams *et al.*, 1990); analyzing genetic relationships, tagging traits for use in marker-assisted selection and for the rapid construction of a genetic linkage map (Stift *et al.*, 2003) compared to other types of molecular markers.

**Objective**

To estimate the genetic relationship among the fifteen selected germplasm of jujube using RAPD marker.

# CHAPTER II

# REVIEW OF LITERATURE

Although jujube is one of the most important fruit in tropical and subtropical regions of the northern hemisphere but a very little study has so far been done on molecular characterization and its utilization A review of the available information relating to the present study has been presented below.

## 2.1. Morphology of Jujube

Morton (1987) stated that the fruit of wild trees is 1.25-2.5 cm long and with sophisticated cultivation, the fruit reaches 6.25 cm in length and 4.5 cm in width. The form may be oval, obovate, round or oblong; the skin smooth or rough, glossy, thin but tough, turns from light-green to yellow, later becomes partially or wholly burnt-orange or red-brown or all-red. The plant is a vigorous grower and has a rapidly-developing tap root. It may be a bushy shrub 4 to 6 ft (1.2-1.8 m) high, or a tree 10 to 30 or even 40 ft (3-9 or 12 m) tall; erect or wide-spreading, with gracefully drooping branches and downy, zigzag branchlets, thornless or set with short, sharp straight or hooked spines. It may be evergreen, or leafless for several weeks in hot summers. The leaves are alternate, ovate- or oblong-elliptic, 2.5-6.25 cm long and 2-4 cm wide.

Rushforth (1999) opined that it is a small deciduous tree or shrub reaching a height of 5–12 metres (16–39 ft), usually with thorny branches. The leaves are shiny-green, ovate-acute, 2–7 centimetres wide and 1–3 centimetres broad, with three conspicuous veins at the base, and a finely toothed margin. The flowers are small, 5 millimetres wide, with five inconspicuous yellowish-green petals. The fruit is an edible oval drupe 1.5–3 centimetres (0.59–1.2 in) deep; when immature it is smooth-green, with the consistency and taste of an apple, maturing brown to purplish-black and eventually wrinkled, looking like a small date.

Janick *et al.* (2008) observed that it is a medium sized tree that grows vigorously and has a rapidly developing taproot, a necessary adaptation to drought conditions. The species varies widely in height, from a bushy shrub 1.5 to 2 m tall, to a tree 10 to 12 m tall with a trunk diameter of about 30 cm. The leaves are alternate, ovate or oblong elliptic with rounded apex, with 3 depressed longitudinal veins at the base. The leaves are about 2.5 to 3.2 cm long and 1.8 to 3.8 cm wide having fine tooth at margin. It is dark-green and glossy on the upper side and pubescent and pale-green to grey-green on the lower side. Depending on the climate, the

foliage of the jujube may be evergreen or deciduous. The flowers are tiny, yellow, 5-petalled and are usually in twos and threes in the leaf axils. Flowers are white or greenish white and the fruits are orange to brown, 2–3 cm long, with edible white pulp surrounding a 2-locular pyrene.

Reich (1991) stated that the fruits are a drupe, varying from round to elongate and from cherry–size to plum–size depending on cultivar.

Soliman and Hegazi (2013) opined that the fruit is a soft, juicy, drupe that is 2.5 cm diameter though with sophisticated cultivation the fruit has of size 6.25 cm long and 4.5 cm wide. The form may be oval, obovate, round or oblong; the skin smooth or rough, glossy, thin but tough. The fruit ripen at different times even on a single tree. Fruits are first green, turning yellow as they ripen. The fully mature fruit is entirely red, soft, juicy with wrinkled skin and has pleasant aroma. The ripe fruit is sweet and sour in taste.

Islam and Simmons (2006 ) opined that the plants are spinescent or thorny shrubs and trees with distinctive tri-nerved leaves, cymose inflorescence, and small perigynous flowers with the ovary surrounded by a thick nectary disc.

These variations of morphology increase its genetic diversity. This diversity is our main concern to improve the fruits

**2.2. Genetic Diversity**

Genetic diversity, the level of biodiversity, refers to the total number of genetic characteristics in the genetic make up of a species. It is distinguished from genetic variability, which describes the tendency of genetic characteristics to vary. Genetic diversity serves as a way for populations to acclimatize to changing environments. With more variation, it is more likely that some individuals in a population will possess variations of alleles which are suited for the environment. Those individuals are more likely to survive to produce offspring bearing that allele. The population will continue for more generations because of the success of these individuals (Anonymous, 2011).

When humans initially started farming, they used selective breeding which leads to monocultures: entire farms of nearly genetically identical plants. Little to no genetic diversity makes crops extremely susceptible to widespread disease. Bacteria morph changes constantly. When a disease causing bacterium changes to attack a specific genetic variation, it can easily wipe out vast quantities of the species. If the genetic variation that the bacterium is

best at attacking happens to be that which humans have selectively bred to use for harvest, the entire crop will be wiped out (Anonymous, 2008).

A very similar occurrence is the cause of the infamous Potato Famine in Ireland. Since new potato plants do not come as a result of reproduction but rather from pieces of the parent plant, no genetic diversity is developed, and the entire crop is essentially a clone of one potato, it is especially susceptible to an epidemic. In the 1840s, much of Ireland's population depended on potatoes for food. They planted namely the "lumper" variety of potato, which was susceptible to a rot-causing plasmodiophorid called *Phytophthora infestans* (Anonymous, 2008). This plasmodiophorid destroyed the vast majority of the potato crop, and left one million people to starve to death.

Genetic diversity commonly is measured by genetic distance (GD) or genetic similarity (GS = 1 - GD), both of which imply that there are either differences or similarities at the genetic level. Published applications of molecular marker-based GD in plant breeding have been limited primarily to jujube but results should apply to other species. Molecular marker-based GD also has potential for assessing changes in genetic diversity over time (Duvick, 1984) protection of intellectual property rights (Smith, 1999), registration of germplasm in countries having ratified the rules of the UPOV convention, and evaluation of new sources of germplasm for their potential to increase genetic diversity (Smith and Smith, 1992).

Obeed *et al.* (2006) carried out fruit properties and genetic diversity of 5 ber (*Ziziphus mauritiana* Lamk) cultivars and verified the occurrence of genetic diversity within the collection. The molecular characterization and fingerprint identification of the ber cultivars was conducted using the RAPD technique. The RAPD technique was able to uniquely characterize and differentiate between the five ber genotypes. Moreover, the genetic similarity tree showed that the cultivar Um-slaem is genetically distant from the other four cultivars, and the two cultivars Pakstany and Komethry were genetically identical. In the RAPD marker study of Soliman and Hegazi (2013) the genetic diversity among the three studied jujube cultivars was evaluated using RAPD and a total of 14 bands (37.84%) were monomorphic, and 23 bands (62.16%) were polymorphic. Similar results were obtained by (Peng *et al.*, 2000) who analyzed the genetic relationships of 64 Chinese date (*Z. jujuba*) cultivars using RAPD markers, and found 59.55% polymorphism. Also, 79.17% polymorphism was detected by Li *et al.* (2010) when they aimed to evaluate the genetic structure of *Z. jujuba* 'Huizao'.

Singh *et al.* (2007) stated that the genetic diversity among 47 ber accessions belonging to cultivated species (*Ziziphus mauritiana* Lam) and one wild accession of *Ziziphus nummularia* (Burm F) was investigated using Inter-Simple Sequence Repeat (ISSR) markers. A total of 167 amplification products were detected with 18 ISSR primers of which 152 (89.96%) were polymorphic. Most of the primers that produced distinct bands (14 primers out of 18) contained dinucleotide repeats. Primers based on (AC)n and (AG)n repeats produced more polymorphic bands. Genetic similarity ranging from 43.07% to 90.30% suggested that the 48 Ziziphus genotypes used in the study were divergent.

Knowledge regarding the genetic variability of any species is an essential prerequisite for its preservation and for success in breeding programs. Using RAPD markers in jujube germplasm collections, some authors have identified genotypes coming from diverse foreign regions. In the case of ber, a high genetic variability among cultivars has been confirmed by a number of studies involving morphological (Soares *et al.*, 2006; Mendonca *et al.*, 2008), isoenzyme (Gois *et al.*, 2009) and molecular (Silva, 2009) markers. This reflects the selection of jujube lines form open pollination rather than from controlled crosses. Micro-satellites also detected a high number of alleles shared for the majority of jujube genotypes in this germplasm. This suggests that most of the plant material analyzed shares a common genetic ancestry: this comes from the fact that relatively few accessions were used for breeding programmes and many hybrids derived from them were conserved in the germplasm bank. On the other hand, some individual and combined rare alleles were detected in such accessions. This information provides ground for parental selection in jujube breeding programmes and conservation strategies.



### 2.3. DNA Extraction

A fast, simple, cost-effective and reliable method is a prerequisite for any DNA extraction and subsequent downstream application. Many protocols have been used in plant DNA isolation, but because of the chemical heterogeneity of the species many of them could be applied to a limited number of species or even closely related species in some cases fail to respond to the same protocol (Weishing *et al.*,1995). Plants, especially medicinal plants contain an array of secondary metabolites. The compounds which make them interesting for molecular biology studies and hence, for DNA isolation, themselves interfere with the DNA isolation procedure. Another problem that could arise during plant DNA isolation is the necessity of liquid nitrogen for crushing the plant material as reported in most of the protocols (Ouenzar *et al.*, 1998) and lengthy procedure involved. In many laboratories the

availability of liquid nitrogen and RNAase is a limiting factor in DNA isolation. In the present study most of the concerns have been addressed. The protocol needs about an hour to prepare DNA for any molecular biology application.

A simple, efficient, reliable and cost-effective method for isolation of total genomic DNA from both young and old leaves is described. The DNA obtained was free of any contaminating proteins, polysaccharides and coloured pigments. The isolated genomic DNA was found suitable for restriction digestion and PCR amplification; hence, it can be employed for preparation of Southern blots, AFLP and cloning. The protocol overcomes the need for liquid nitrogen, RNAase and phenol-chloroform treatment, usually employed for plant DNA isolation. In terms of quantity and quality the present method has advantages over many other plant DNA isolation protocols. The protocol covers many and diverse angiospermic species belonging to different families within both dicotyledonous and monocotyledonous plants.

A unique advantage of these PCR techniques is the rapid DNA analysis of many plant samples using small quantities of DNA. Thus, a simple and rapid DNA extraction method is needed for studies, such as genetic analysis, that require large populations. Several methods for minimizing the DNA extraction steps have been reported (Berthomieu and Meyer, 1991), but they require a large amount of plant tissue and grinding of plant tissues in liquid nitrogen. Growth and management of plants and storage of the plant samples in freezers is often difficult due to space constraints.

There are many methods proposed for isolation of nuclear DNA from a variety of plant species, plant organs and tissues. Now it is considered that three main factors are critical for extraction and purification of maximally undegraded and pure genomic DNA:

1. Physical forces used to homogenize the plant tissue i.e. disruption of plant cell walls.
2. The composition of the grinding buffer.
3. Elimination of enzyme-inhibiting polysaccharides (Rogers and Bendich, 1985).

DNAzol is a complete and ready to use reagent for the isolation of genomic DNA from solid and liquid samples of animal and plant origin. The DNAzol procedure is based on the use of a novel guanidine-detergent lysing solution that hydrolyzes RNA and allows the selective precipitation of DNA from a cell lysate. Developed by (Chomczynski *et al.,* 1997), DNAzol is an advanced DNA isolation method that combines both reliability and efficiency with simplicity of the isolation protocol. The DNAzol protocol is fast and permits isolation of genomic DNA from a large number of samples of small or large volumes (Chomczynski *et al.,* 1998).

During the isolation, a biological sample is lysed (or homogenized) in DNAzol and the genomic DNA is precipitated from the lysate with ethanol. Following an ethanol wash, DNA is solubilized in water or 8 mM NaOH. The procedure can be completed in 10 - 30 minutes with a genomic DNA recovery of 70-100%. The isolated DNA can be used, without additional purification, for Southern analysis, dot blot hybridization, molecular cloning, polymerase chain reaction (PCR) and other molecular biology and biotechnology applications (Patents granted to Hoffmann LaRoche Corporation). DNAzol is stable at room temperature for at least two years after the date of purchase. DNAzol contains irritants. Handle with care, avoid contact with skin, and use eye protection (shield, safety goggles). In case of contact: wash skin with a copious amount of water and seek medical attention.

**2.4. Genetic Marker**

A genetic marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species. It can be described as a variation (which may arise due to mutation or alteration in the genomic loci) that can be observed ( Fulton and Vicente, 2003) . It demonstrates polymorphism, which means that the markers in different organisms of the same species are different. A classic example of this type of marker is the area of the DNA which codes for blood type in humans: all humans have and need blood, but the blood of individual humans can be very different as a result of polymorphism in the area of the genome which codes for blood. Genetic markers are employed in genealogical DNA testing for genetic genealogy to determine genetic distance between individuals or populations. It can be used to study the relationship between an inherited disease and its genetic cause. It can be used to create genetic maps of whatever organism is being studied (Fulton and Vicente, 2004).

Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited numbers of DNA markers. The utility of DNA-based markers is generally determined by the technology that is used to reveal DNA-based polymorphism. The restriction fragment length polymorphism (RFLP) assay (Botstein *et al.,* 1980) has been the choice for many species to measure genetic diversity and construct a genetic linkage map. However, an RFLP assay which detects DNA polymorphism through restriction enzyme digestion, coupled with DNA hybridization is time consuming and laborious.

Over the last decade, polymerase chain reaction (PCR) technology has become a widespread research technique and has led total development of several novel genetic assays based on

selective amplification of DNA (Saiki, 1989). This popularity of PCR is primarily due to its apparent simplicity and high probability of success. Unfortunately, because of the need for DNA sequence information, PCR assays are limited in their application. The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes (Williams *et al.,* 1990). The simplicity and applicability of the RAPD technique have captivated many scientists' interests.

The role of genetic markers in genetic enhancement is considered in the evaluation of genetic diversity. Traits that serve as genetic markers are by definition polymorphic; the more polymorphic the trait, the greater its potential value to germplasm management. The issue of homology may seem trivial for morphological markers, but the increasing use of molecular marker has heightened its importance (Bretting and Widrlechner, 1995).

Genetic marker screening is based on the survey of genetic diversity as revealed by variation at specific gene loci and provides information about the amount and distribution of genetic diversity within and among populations. The emphasis of this report will be on DNA based molecular techniques and how they can apply in assessing the genetic diversity of genetic resources. Some commonly used types of genetic markers are RFLP (Restriction fragment length polymorphism), AFLP (Amplified fragment length polymorphism), RAPD (Random amplification of polymorphic DNA) ,VNTR ( Variable number tandem repeat), Microsatellite polymorphism, SSR ( Simple sequence repeat), SNP (Single nucleotide polymorphism), STR (Short tandem repeat), SFP (Single feature polymorphism), DArT ( Diversity Arrays Technology) and RAD markers (Restriction site associated DNA markers).

They can be further categorized as dominant or co-dominant. Dominant markers allow for analyzing many loci at one time, e.g. RAPD. A primer amplifying a dominant marker could amplify at many loci in one sample of DNA with one PCR reaction. Co-dominant markers analyze one locus at a time. A primer amplifying a co-dominant marker would yield one targeted product. All genetic markers have the specific genomic position in particular in particular chromosome like gene and that are called loci.

Another three major types of genetic markers were stated ( Jones *et al.,* 1997).

- Morphological markers, representing phenotypic trait.
- Biochemical markers , which include allelic variants of enzyme called isozymes and
- DNA or molecular markers, which reveal sites of variation in DNA.

Morphological markers are visualized phenotypic characters such as seed color, seed shape, pigmentation flower color etc. Isozyme markers are differences in enzymes that are detected by electrophoresis and specific staining. The major disadvantages of these two types of markers are, limited in number and are influenced by environmental factors or developmental stages of the plants (Winter and Khal, 1995). However, despite these limitation, morphological and biochemical markers were often been used by Plant Breeders. The most widely use of DNA markers is due to its abundance and polymorphic nature.

**2.5. Polymerase Chain Reaction (PCR)**

PCR is a technique to synthesize DNA strand from templates by using oligonucleotide initial sequence (Primer), after 25 cycles of DNA synthesis the products of the PCR will include, 105 copies of DNA easily visualized as a discrete band of a specific size when submitted to agarose gel electrophoresis. The PCR method requires little biological material and provides a rapid method for screening large sample sizes.

Two type of primer are used in PCR analysis (i) arbitrary primers and (ii) specifically designed primers from known DNA information. Multiple arbitrary amplicon profile (MAAP) was developed for the use of single or multiple arbitrary primers (Anolles *et al.*, 1997), for RAPD analysis. It is a simple method to analyse phylogenetic relation but using arbitrary primers, low reproducibility that causes variable PCR results depending on PCR condition has been recognized as a disadvantage factor. Specifically designed primers are also use for PAPD analysis to see the specific DNA band patterns e.g. 18S rDNA. (Saiki, 1989) published the first experimental data on PCR technique has tremendously influenced research in diverse areas of biological sciences leading to an unprecedented understanding of microorganisms. Willams *et al.* (1990) used 5-10ng genomic DNA, 200µl each dNTP, 2 unit of *Taq* polymer (Perkin Elmer, USA), 10X PCR buffer and 0.22µM 10-mer random primer. Two hundred and forty primers were screened against the parent's DNA and the ones that detected polymorphism were selected for screening of the segregating population. The amplification program included and initial denaturation step of 94° C for 3 min followed by 39 cycles of 94° C 1 min: 35°C 1 min and 72°C 2 min, the last cycle was followed by 5 min incubation at 72°C and a hold at 15°C. PCR conditions can be influenced by many factors, vary on species to species for RAPD analysis, e.g. thermostable polymerase, deoxynucleotide triphosphates, Mg2+ ions, primer and DNA template concentration, and other reaction factors such as primer annealing, primer extension, denaturation time and temperature.

Some studies have shown that different brands of Taq DNA polymerase can produce dissimilar results in PCRs (Evangelopoulos *et al.*, 2001; Verweij *et al.*, 2004; Al-Soud *et al.*, 2005), and in several cases enhancers such as bovine serum albumin, casein, formamide, and polyvinyl polypyrrolidone are required to facilitate amplification. Moreover, some types of Taq DNA polymerase are more susceptible to inhibition than others, and this may cause differences in the band patterns obtained (Wilson, 1997).

**2.6. Random Amplification of Polymorphic DNA (RAPD)**

RAPD stands for Random Amplification of Polymorphic DNA. The RAPD technique was first applied by Williams *et al.* (1990). This technique uses an in vitro enzymatic reaction to specifically amplify a multiplicity of target sites in one or more nucleic acid molecules (Micheli *et al.*, 1994) to examine human DNA samples.

The scientist performing RAPD creates several arbitrary, short primers and can be applied to any species with out requiring any information about the nucleotide sequence. Selecting the right sequence for the primer is very important because different sequences will produce different band patterns and possibly allow for a more specific recognition of individual strains. PCR is used to amplify genomic DNA. The amplification products from this analysis exhibit polymorphism and thus can be used as genetic markers. The presence of band it can be calculated the genetic variability of different variety or species. Low expense, efficiency in developing a large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable.

The standard RAPD technology uses synthetic oligonucleotides (8-12 bases long) of random sequences as primers to amplify of total genomic DNA under low annealing temperatures by PCR. Amplification products are generally separated on agarose gels and stained with ethidium bromide. Welsh and McClelland (Welsh and McClelland, 1990) independently developed a similar methodology using primers about 15 nucleotides long and different amplification and electrophoretic conditions from RAPD and called it the arbitrarily primed polymerase chain reaction (AP-PCR) technique.

Although these approaches are different with respect to the length of the random primers, amplification conditions and visualization methods, they all differ from the standard PCR condition (Saiki, 1989) in that only a single oligonucleotide of random sequence is employed and no prior knowledge of the genome subjected to analysis is required. At an appropriate annealing temperature during the thermal cycle, oligonucleotide primers of random sequence bind several priming sites on the complementary sequences in the template genomic DNA

and produce discrete DNA products if these priming sites are within an amplifiable distance of each other. The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites. Recently, sequence characterized amplified regions (SCARs) analysis of RAPD polymorphisms (Paran and Michelmore, 1993) showed that one cause of RAPD polymorphisms is chromosomal rearrangements such as insertions/deletions. Therefore, amplification products from the same alleles in a heterozygote differ in length and will be detected as presence and absence of bands in the RAPD profile. The profile of RAPD bands is similar to that of low stringency minisatellite DNA fingerprinting patterns and is therefore also termed RAPD fingerprinting. On average, each primer directs amplification of several discrete loci in the genome so that allelism is not distinguishable in RAPD patterns. In other words, it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous. RAPD markers are therefore dominant.

Nandini and Chikkadevaiah (2005) established the phylogenetic relationships among sunflower hybrids genotypes and characterized them based on both morphometric traits and PCR based RAPD markers. At the molecular level, 25 scoreable bands were produced with the 5 primers with the number of bands ranging from 3 for OPH-15 to 9 for OPG-10. Of the 25 bands, 14 were polymorphic.

According to some researchers (Oliveira *et al.*, 2007), one of the main limitations of the RAPD technique is the low level of repeatability of the band pattern observed when amplification reactions have not been optimized. In the present study, repeatability and reproducibility tests revealed that the amplification reactions were carried out under adequate and optimized conditions that generated consistent RAPD bands. High levels of repeatability in RAPD loci have also been reported for the frog *Physalaemus cuvieri* (85-99%), Telles, (2005) and for a Cerrado plant *Tibouchina papyrus* (85%), Ramos *et al.*, (2008).

# CHAPTER III

# MATERIALS AND METHOD

The experiment was conducted at Molecular Horticulture Laboratory of Agrotechnology Discipline of Khulna University, from June 2012 to March 2013.

### 3.1. Plant Materials

The young leaves of jujube tree were collected from two selected location of Khulna and Jessore of Bangladesh (Table 3.1). Approximately 5 g of recently matured and tender leaves (10-15 days old) was collected, washed using distilled water, wiped with 70% (v/v) ethanol and then air dried, of which 1 g was weighed out and stored in sealed plastic bags at -80°C for further use. The genotypes were named as germplasm number sequentially from germplasm 1 to germplasm 15. The fruits of 15 genotypes exhibit morphological variation in some characters, such as fruit shape, size and taste.

Table 3.1. Plant materials used in RAPD marker analysis

| Germplasm No. | Designation | Place of Collection | Characteristics (Taste, size and shape of fruits) |
|---|---|---|---|
| 1 | Narikel kul | Khulna | Sweet, medium, oblong |
| 2 | Misty kul -1 | Khulna | Very sweet, medium, round |
| 3 | Dhaka-90 | Khulna | Slightly sweet, large, round |
| 4 | Tok kul-1 | Khulna | Sour, small, obovate |
| 5 | Taiwani | Khulna | Slightly sweet, medium, oblong |
| 6 | Dab kul | Khulna | Large, round |
| 7 | Apple kul | Khulna | Sweet, small, oval |
| 8 | Tok kul-2 | Khulna | Sour, medium, oblong |
| 9 | Hazari kul | Jessore | Slight sour, small, oval |
| 10 | Nabi kul | Jessore | Slightly sweet, small, oblong |
| 11 | Chapi kul | Jessore | Slightly sweet, medium , oblong |
| 12 | Kachhar kul | Jessore | Sour, medium, oval |
| 13 | Misty kul-2 | Jessore | Sweet, large, oblong |
| 14 | Bele kul | Jessore | Sweet, small, oval |
| 15 | Shabji kul | Jessore | Slightly sour, small, round |

### 3.2. DNA Extraction Process

Genomic DNA was extracted from the leaves by DNAzol reagent formulated for the isolation of genomic DNA from plants. The procedure is based on the use of a novel guanidine detergent lysing solution that allows the selective precipitation of DNA from the lysate (Chomczynski *et al.*, 1997). The DNAzol protocol is fast and permits efficient isolation of genomic DNA from a variety of plant tissues (Chomczynski *et al.*, 1998).

#### 3.2.1. DNA Extraction

One gram of stored (at -80°C for one day) younger leaf was taken from each sample and ground into powder with mortar and pestle. 1ml of DNAzol (Invitrogen, USA) was added and then homogenized properly. The mixture was centrifuged at 10,000 rpm for 12 minutes and transferred the supernatant to a clean sterile 1.5 microcentrifuge tube.

#### 3.2.2. DNA Precipitation

DNA was pricipitated by the addition of 0.5 ml of 100% ethanol per 1 ml of DNAzol. The samples were mixed by inverting tubes 5-8 times and were stored at room temperature for 1-3 minutes to make sure that DNAzol and ethanol mixed well to form a homogenous solution. DNA fiber became visible as quickly as a cloudy precipitate. The sample was centrifuged at 10,000 rpm for 12 minutes and the supernatant was discarded.

#### 3.2.3. DNA Purification

The DNA precipitates were washed by 75% of ethanol. After addition of ethanol, the samples were mixed by inverting the tubes 8-10 times and were stored the tubes vertically for 0.5 - 1 minutes to allow the DNA to settle to the bottom of the tubes and were removed ethanol by decanting and pippetting.

#### 3.2.4. DNA Solubilization

Remaining alcohol was removed from the bottom of a tube using a micropipette and it was dried for 30 second opening the cork of the tube at room temperature. The DNA samples were dissolved in 300µl of 8 mM NaOH by slowly passing the pellet through a pipette.

#### 3.2.5. DNA Quantification

Extraction and purification of DNA from fresh leaf tissues was done by using DNAzol protocol (Chomczynski, *et al.*, 1998). The DNA was checked in 1 percent agarose gel.

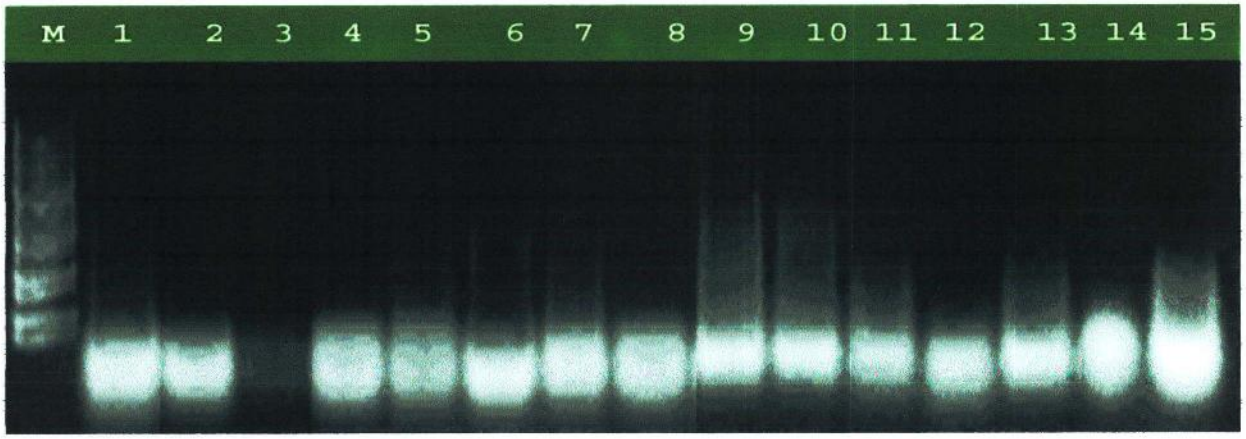


Fig. 3.1. The gel profile of *Ziziphus mauritiana* DNA extraction

DNA was quantified using UV spectrophotometer (T 60 UV-Visible, PG instrument Ltd). 2µl of isolated DNA sample from each germplasm of jujube was taken and made up to 2000µl with distilled water. The absorbance at 260 nm was measured.

The concentrations of absorbance were calculated by using the following formula.

DNA concentration (µg/ml) = ($OD_{260}$) x (dilution factor) x (50 µg DNA/ml / 1 $OD_{260}$ unit)

The DNA solution was expressed in ng/mL (Hoisington *et al.*, 1994). The final concentrations of jujube DNA stock solutions were adjusted to 100 ng/mL for PCR.

Table 3.2. Concentration of DNA



| GP No | DNA Concentration (µg/ml) | GP No | DNA Concentration (µg/ml) |
|---|---|---|---|
| 01 | 305 | 11 | 195 |
| 02 | 255 | 12 | 235 |
| 03 | 250 | 13 | 225 |
| 04 | 195 | 14 | 235 |
| 05 | 260 | 15 | 270 |
| 06 | 300 | | |
| 07 | 260 | | |
| 08 | 265 | | |
| 09 | 325 | | |
| 10 | 240 | | |

### 3.3. PCR Reaction

#### 3.3.1. Primer Selection

Primers were selected based on intensity of bands, presence of smearing, consistency within individuals and potential for population discrimination. In primer selection importance was provided on mainly GC content. Primers that have GC content more than 60% are suitable for RAPD analysis (Kubelik and Szabo, 1995). Final subsets of three primers (Table 3.2) were exhibited good quality banding patterns and sufficient variability was selected for the further analysis.

Table 3.3. Random primer used in the present study for screening

| Primer code | Sequence (5′ – 3′) | GC content (%) | *Tm; Ta |
|---|---|---|---|
| OP A01 | AGC AGC GCC TCA | 66.67% | 40 $^{0}$C; 37.4 $^{0}$C |
| OP A02 | GCC AGC TGT ACG | 66.7% | 40 $^{0}$C; 37.4 $^{0}$C |
| OP S07 | GGT GAC GCA G | 70% | 34 $^{0}$C; 35.6 $^{0}$C |

**Tm: Melting Temperature; Ta: Annealing Temperature*

**Calculation of Annealing Temperature:**

Annealing temperature was calculated by the following formula-

0.3× Tm (Primer) + 0.7×Tm (Product) -25

#### 3.3.2. PCR Amplification

PCR amplification was carried out according to the modified protocol of Williams *et al.*, (1990) with minor modifications. The following components were used to prepare PCR mixture. The total volume of PCR mixture was 17.0 µl per sample. 3µl genomic DNA was added with 17.0 µl PCR mixture and finally, total volume was made 20 µl (Table 3.3).

**Table 3.4. PCR master mixture (20µl)**

| Sl. No. | Reagents | Amount (Per sample) |
|---|---|---|
| 1 | Genomic DNA | 3µl |
| 2 | Primer (10mM) | 3µl |
| 3 | dNTPs (10mM) | 2µl |
| 4 | *Taq* DNA polymerase (2U) | 2µl |
| 5 | Reaction buffer (10x) | 2µl |
| 6 | $dH_2O$ | 8µl |
| Total | | 20µl |

### 3.3.3. DNA amplification time and Thermal profile

The first amplification cycle consisted of the following steps:

1) Initial denaturation at 94°C for 4 min
2) Denaturation at 94°C for 1 min
3) Annealing at 35°C for 1 min
4) Elongation or extension at 72°C for 2 min
5) Cycle 2 to 4 step for 40 more time.
6) Final extension 72°C for 5 min.

### 3.4. Electrophoresis of amplified products and documentation

Amplified PCR products were electrophoresed on an agarose gel (2%) in TE buffer and visualized by staining with ethidium bromide solution. Polymorphic band was observed by UV transilluminator and picture was taken by the GEL DOC.

### 3.5. RAPD Analysis

Each accession was scored manually for presence or absence of a particular amplification product. Data was analyzed by the software GelQuest and component 2.0 (CPW3) for phylogenic analysis. Dendrogram was constructed based on The Unweighted Pair Group Method using Arithmetic Averages (UPGMA). Numerical value was considered 1 for phenogram of band and 0 for absence of band to construct the dendrogram.

# CHAPTER IV

# RESULTS AND DISCUSSION

Random amplified polymorphic DNA (RAPD) is a good molecular technique to study genetic diversity as well as morphological characterization. RAPD is rapidly gained popularity to detect polymorphisms among different germplasm of fruits. Morphological and molecular characterizations were used for characterization of 15 germplasm and displayed different levels of variability concerning the morphological traits of jujube. Molecular characterization was determined by using the molecular marker. Random amplification of polymorphic DNA (RAPD) markers is most useful for understanding the naturally occurring polymorphism in living organisms.

## 4.1. Primer Selection and RAPD Analysis

It is well established that RAPD technique had been successfully used in variety of taxonomic and genetic diversity studies (Rodriguez *et al.*, 1999; Alam *et al.*, 2009) and it was found suitable for use with jujube genotypes because of its ability to generate reproducible polymorphic bands. Monomorphic bands are those which are present in all individuals, polymorphic are present in one or more but not all individuals and unique ones are present in at least one individual not in any other (Mehetre *et al.*, 2004).

However, to enhance the polymorphism of genotypes of jujube, one 10-mer (OPS07) and two 12-mer (OPA01 & OPA02) primers were used for DNA amplification (Table 3.2) and for fingerprinting. The primers generated various banding patterns ranging from 3 to 10 and yielded a total of 144 band patterns of which 69.74% were polymorphic. The highest percent of polymorphic loci (77.78%) was observed for primer S07 and the lowest (60.00%) was from primer A01. The reason of this difference could be due to the GC content of the primers. It can be mentioned in this study that the GC content of the primers was 60% to 70%. Increased number of bands was also obtained with increased GC content of the primer (Kubelik and Szabo, 1995). The correlation between GC content of the primer and the number of bands could be explained as the greater hydrogen bonding: with C by three hydrogen bonds show the high stability of base complementation than that of complementation of A with T by two hydrogen bonds (Fukuoka *et al.*, 1992).

Table 4.1. RAPD primers with corresponding bands scored and their size range together with polymorphic bands in 15 jujube germplasm

| Serial No | Primer code | No. of total band | No. of common band | Number of polymorphic band | Proportion of polymorphic loci (%) | Number of unique band |
|---|---|---|---|---|---|---|
| 1 | S07 | 9 | 1 | 7 | 77.78 | 1 |
| 2 | A01 | 5 | 1 | 3 | 60.00 | 1 |
| 3 | A02 | 7 | 1 | 5 | 71.43 | 1 |
| Total | 3 primers | 21 | 3 | 15 | 209.21 | 3 |
| Average | | 7 | 1 | 5 | 69.74 | 1 |

Since the amplification detected by these primers produced significant polymorphism and yielded a total of 144 polymorphic RAPD patterns as shown in Figure 4.2, 4.3 and 4.4. Each of three primers was reproducible and capable of exhibiting polymorphic amplification patterns and hence, they were used in the subsequent analysis with more number of DNA samples of jujube germplasm. The amplification patterns of representative samples of jujube variety with three different primers have been shown in Figure 4.1, 4.2 and 4.3.

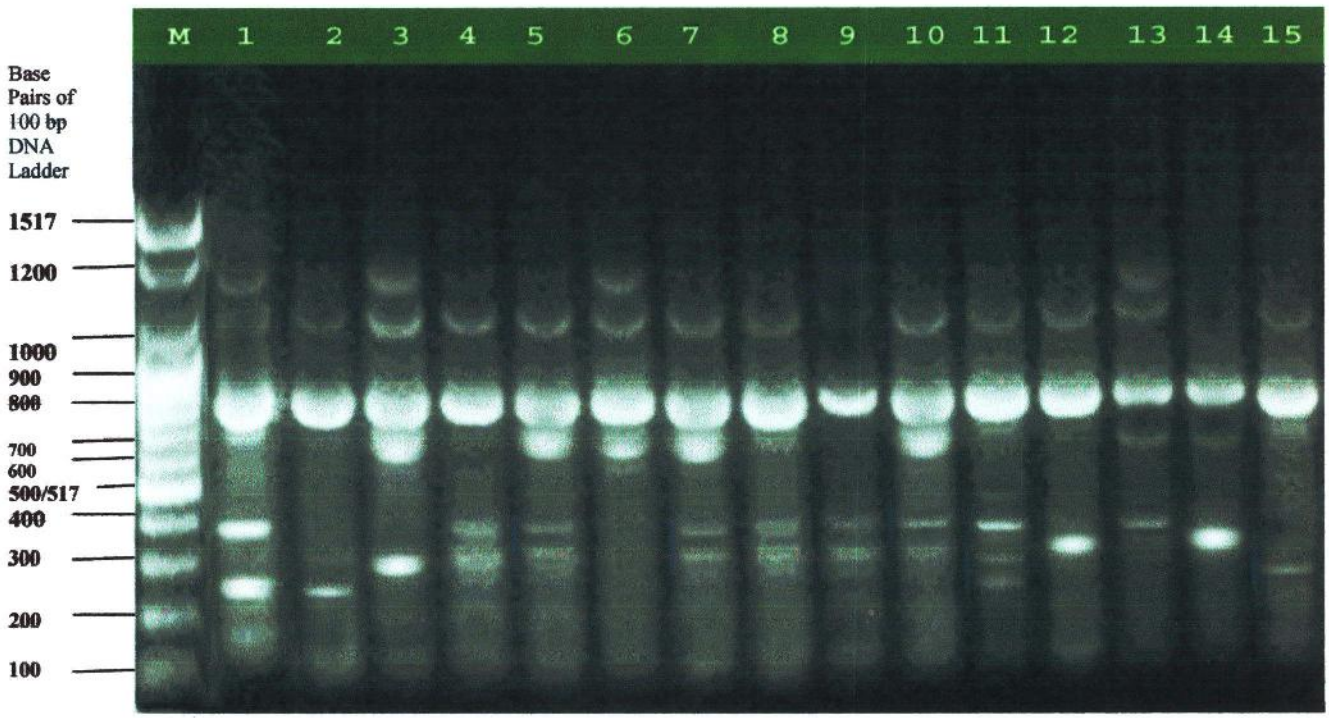


Fig.4.1. RAPD pattern generated by the primer S07 genotypes of *Ziziphus mauritiana* Numbers indicate the serial number of the genotypes and "M" indicates Ladder.

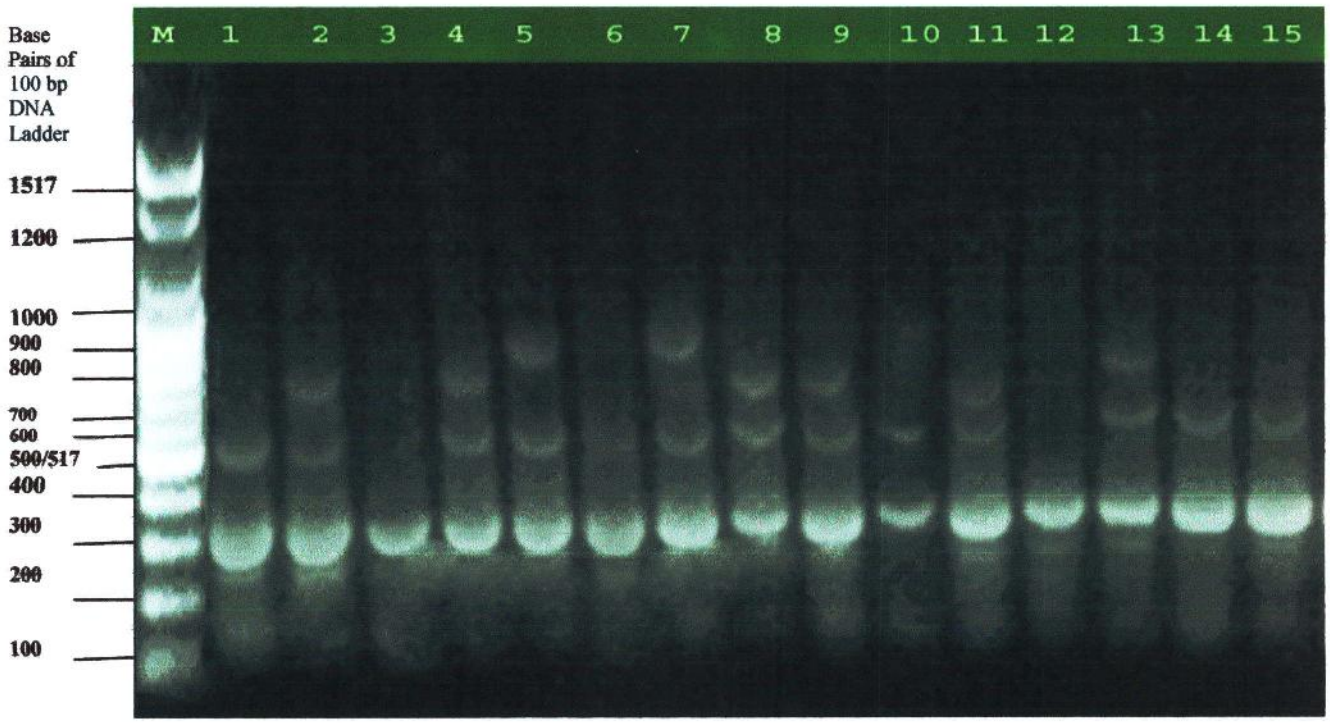


Fig. 4.2. RAPD pattern generated by the primer A01 genotypes of *Ziziphus mauritiana* Numbers indicate the serial number of the genotypes and "M" indicates Ladder.

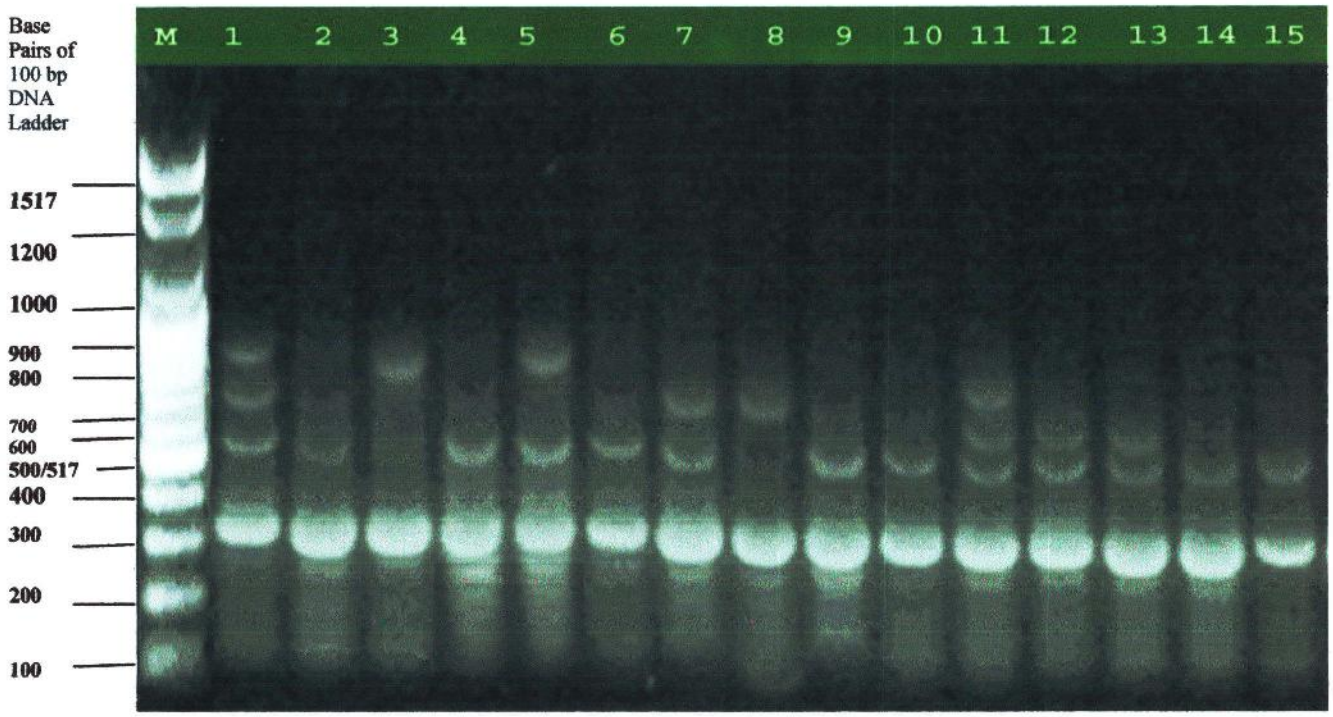


Fig. 4.3. RAPD pattern generated by the primer A02 genotypes of *Ziziphus mauritiana* Numbers indicate the serial number of the genotypes and "M" indicates Ladder.

### 4.2. Genetic Distance

Evaluation of fragments patterns was carried out by diversity index. Genetic similarity matrix (Table 4.3) was generated using GelQuest based on the marked scores of the polymorphic RAPD patterns where score 1 or 0 was assigned to the present or absent of band.

Estimates of genetic distance calculated all possible pairs-wise genetic distance values by the Nei's and Li's 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 $n_{xy}$ is he number of bands shared by those individuals.

In this analysis, smaller numbers are associated with more genetically similar individuals, whereas larger numbers suggest genetically dissimilarity. The values of pair-wise comparisons of Nei's and Li's (1979) genetic distance between 15 jujube genotypes were computed from 0.090 to 0.625 (Table 4.3). Comparatively higher genetic distance (0.625) was found between GP9 and GP6. The lowest genetic distance (0.090) was found between GP10 and GP07. There was a genetic variation among the studied jujube as indicated by the proportion of polymorphic loci. Estimated genetic variation in the jujube might be consistent with the fact that it is a polymorphic plant. The range of genetic distance of 15 genotypes is 0.090-0.62 and the difference (0.535) between the highest and lowest genetic distance indicate the presence of variability among the 15 genotype of jujube.

Table 4.2. Pair-wise genetic distance from RAPD data of different jujube germplasm

| Sample | GP1 | GP2 | GP3 | GP4 | GP5 | GP6 | GP7 | GP8 | GP9 | GP10 | GP11 | GP12 | GP13 | GP14 | GP15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GP1 | | | | | | | | | | | | | | | |
| GP2 | 0.333 | | | | | | | | | | | | | | |
| GP3 | 0.400 | 0.529 | | | | | | | | | | | | | |
| GP4 | 0.428 | 0.222 | 0.412 | | | | | | | | | | | | |
| GP5 | 0.364 | 0.368 | 0.333 | 0.263 | | | | | | | | | | | |
| GP6 | 0.368 | 0.375 | 0.200 | 0.375 | 0.412 | | | | | | | | | | |
| GP7 | 0.333 | 0.428 | 0.400 | 0.333 | 0.273 | 0.474 | | | | | | | | | |
| GP8 | 0.428 | 0.444 | 0.412 | 0.222 | 0.263 | 0.500 | 0.238 | | | | | | | | |
| GP9 | 0.428 | 0.444 | 0.529 | 0.333 | 0.368 | 0.625 | 0.238 | 0.222 | | | | | | | |
| GP10 | 0.364 | 0.368 | 0.333 | 0.263 | 0.300 | 0.412 | 0.090 | 0.263 | 0.158 | | | | | | |
| GP11 | 0.360 | 0.273 | 0.524 | 0.273 | 0.304 | 0.500 | 0.280 | 0.364 | 0.273 | 0.217 | | | | | |
| GP12 | 0.619 | 0.333 | 0.412 | 0.222 | 0.368 | 0.375 | 0.428 | 0.444 | 0.444 | 0.368 | 0.273 | | | | |
| GP13 | 0.333 | 0.428 | 0.400 | 0.333 | 0.364 | 0.263 | 0.333 | 0.333 | 0.333 | 0.273 | 0.280 | 0.333 | | | |
| GP14 | 0.474 | 0.500 | 0.333 | 0.375 | 0.412 | 0.428 | 0.263 | 0.375 | 0.250 | 0.176 | 0.400 | 0.375 | 0.368 | | |
| GP15 | 0.400 | 0.294 | 0.375 | 0.412 | 0.444 | 0.333 | 0.300 | 0.412 | 0.412 | 0.222 | 0.333 | 0.294 | 0.300 | 0.200 | |

### 4.3. Dendrogram

Morphological characterization was determined and showed genetic similarities and dissimilarities by collecting data and constructing the UPGMA dendrogram to estimate genetic diversity of jujube phenotypically. Fifteen genotypes on the dendrogram were distinguished and divided into two major groups as shown in Figure 4.5 based on Nei and Li (1979) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA). The dendrogram (Fig. 4.5) shows that all the germplasm were grouped into two major clusters G1 and G2. Cluster **G2** is subdivided into **G2.I** and **G2.2**, cluster **G2.2** is further subdivided into **G2.2.1** and **G2.2.2**, of which cluster **G2.2.2** is again subdivided into **G2.2.2.a** and **G2.2.2.b**.

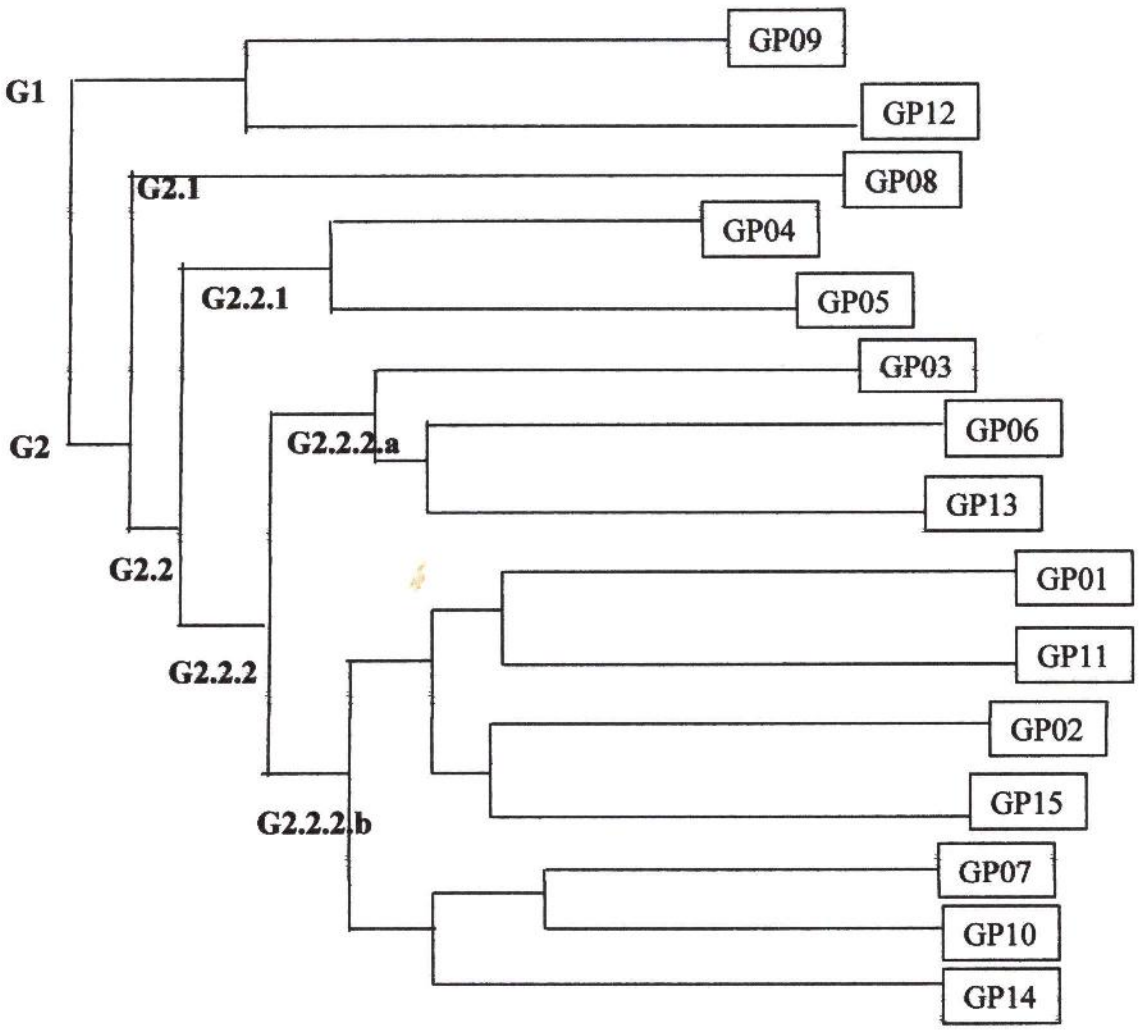


Fig.4.4. Unweighted Pair Group Method of Arithmetic Mean (UPGMA) dendrogram based on Nei's and Li's (1979) genetic distance

From the dendrogram (Fig: 4.5) it was found that the jujube germplasms collected from Khulna and Jessore were distributed under different clusters. GP8 is most diverged in cluster. G2.1. Presence of so many subclusters in G2.2 indicates sound variation among the germplasms. GP7 and GP10 under the subgroup **G2.2.2.b** showed the closest relationship between themselves

The genetic structure of plant populations reflects the interactions of different processes such as the long-term evolutionary history of the species (e.g., shifts in distribution, habitat fragmentation, and/or population isolation), mutation, genetic drift, mating system, gene flow, and selection (Slatkin, 1987; Schaal *et al.*, 1998). All of these factors can lead to complex genetic structuring within populations. Genetic diversity is of great importance to the sustainability of plant populations. The results obtained confirm the usefulness and applicability of the RAPD method in determining genetic similarity.

Based on the above results there are collections with high similarity index, even though they may be belonging to geographically different locations. High similarity indices suggest that the individuals in the population have close genetic relation among them. This situation can rise in natural populations when there is a possibility of free/random pollen flow and fertilization. The genetic similarity of the samples slightly correlated with their close geographic locations. Sources of polymorphism in RAPD assay may be due to deletion, addition or substitution of base within the priming site sequence (Williams *et al.*, 1990). High diversity is the reflection of adaptation to environment, which is beneficial to its propagation, resources conservation, the domestication of wild species and the screen of specified locus. Geographically isolated individuals tend to accumulate genetic variations during the course of environmental adaptations (Sarwat *et al.*, 2008).

Effective conservation of a species depends largely on the knowledge of patters of genetic variation. For example, the spatial structure of genetic variation can provide information for sampling strategies for *ex situ* or *in situ* conservation (Torre *et al.*, 2008). This study was an attempt to establish the genetic diversity background in *Z. mauritiana* with RAPD markers. High levels of polymorphism found in the present work showed that RAPD markers as a suitable tool for genetic diversity studies. This study could open the way for detailed research to understand all the aspects of this divergence of jujube.

# CHAPTER V

# CONCLUSION

Variability studies of 15 jujube germplasm belonging to south-western coastal zone of Bangladesh revealed a high coefficient of variation. Molecular markers have been used as a tool to investigate the plant germplasm diversity. The evidence from RAPD analysis indicates the existence of a moderate degree of genetic diversity in jujube cultivars from different authors. Banding patterns can be converted into informative data for pedigree analyses. The shortcoming of RAPD method is the reproducibility in amplification. In this study, the PCR reactions were performed in optimal conditions and informative RAPD fragments were obtained with high reproducibility. In addition, RAPD makers have proved to be effective for fingerprinting and characterizing the genetic basis of each jujube sample for assessing genetic diversity and relatedness between accessions. The geographical locations, growth altitude, and climates may contribute the polymorphic RAPD of jujube trees in Bangladesh. Even though this study showed close relationships among a few genotypes studied, there were still no duplicates. The range of genetic distance was observed 0.090 (GP10 vs. GP07) to 0.625 (GP09 vs. GP06). GP8 is most diverged in cluster G2.1. Presence of so many subclusters in G2.2 indicates sound variation among the germplasms. GP7 and GP10 under the subgroup G2.2.2.b showed the closest relationship between themselves. Although we found a good correlation between genetic and geographical data, future studies involving a large number of morphological and biochemical traits with molecular markers should have important implications for germplasm management. The result of this study is of critical importance for jujube breeding programs as well as informed and efficient management of germplasm collections.

## CHAPTER VI

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