

MOLECULAR CHARACTERIZATION OF SOME SELECTED SAPOTA

(*Achras sapota*) GERMPLASM BY RAPD ANALYSIS

A Thesis

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AGROTECHNOLOGY DISCIPLINE

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By

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Examination Roll No: MS-100813

in partial fulfillment of the requirement for the degree of MS in Horticulture

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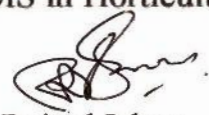
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DECLARATION

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 The findings of the research has not been/ will not be published anywhere without the concurrence of the concerned supervisor.

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Dedicated To.....

All Punctual teachers of Khulna University



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Abstract

Achras sapota is a perennial fruit tree in subtropical and tropical areas. In Bangladesh, it has been used as edible fruits. The classification and functional identification of *Achras sapota* remains unsolved these days. In the present study, genetic variation and relationship among 15 sapota germplasm were analyzed using Random Amplified Polymorphic DNA (RAPD). Three oligonucleotide primers A02, A03 and S07 were used to direct the amplification which yielded a total of 57 band patterns out of that 48.89% were polymorphic. The highest percent of polymorphic bands (66.67%) was observed for primer A03 and the lowest (20%) was from primer S07. 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 morphological characteristics. The range of genetic distance was observed 0.112 to 0.5. Based on the cluster analysis, the *Achras sapota* samples that were believed to have high morphological difference were grouped independently. The results suggested that RAPD is useful for the discrimination of uncultivated, cultivars and potential *Achras sapota* of high economy.



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Chapter-I INTRODUCTION

Sapota belongs to the family *Sapotaceae*, Scientific name: *Achras sapota*. It commonly known as the sapodilla. It is a long-lived, evergreen tree native to southern Mexico, Central America and the Caribbean. It was introduced to the Philippines during Spanish colonization. It is grown in huge quantities in Bangladesh, India, Pakistan and Mexico. Sapodilla is known as *sapodilla* in Guyana, Grenada, and Trinidad & Tobago, *zapote* in Colombia, Honduras, El Salvador, Cuba, and the Dominican Republic, *nispero* in Costa Rica, Cuba, Puerto Rico, Nicaragua, and Venezuela, *dilly* in the Bahamas, *naseberry* in Jamaica and other parts of the Caribbean, *sapoti* in Brazil and Haiti, *chico* or *tsiko* in the Philippines and *chicosapote* or *chicozapote* in Mexico, Hawaii, southern California and southern Florida. It is called sobeda in Bangladesh.

It is known as *chikoo* in India and Pakistan and *sapota* in some parts of India (Tamil Nadu, Kerala, Karnataka, Andhra Pradesh), *sapathilla* or *rata-mi* in Sri Lanka, *sobeda/sofeda* in eastern India and Bangladesh, *sabudheli* in Maldives, *sawo* in Indonesia and *saos* in West Sumatra, *hồng xiêm* (lit. Siamese persimmon), *lồng mứt* or *xa pô chê* in Vietnam, *lamoot* in Thailand, Laos and Cambodia (Campbell and Lara, 1982).

Sapodilla can grow to more than 30 m (98 ft) tall with an average trunk diameter of 1.5 m (4.9 ft). The average height of cultivated specimens, however, is usually between 9 and 15 m (30 and 49 ft) with a trunk diameter not exceeding 50 cm (20 in). It is wind-resistant and the bark is rich in a white, gummy latex called chicle. The ornamental leaves are medium green and glossy. They are alternate, elliptic to ovate, 7–15 cm long, with an entire margin. The white flowers are inconspicuous and bell-like, with a six-lobed corolla.

The fruit is a large ellipsoid berry, 4–8 cm in diameter, very much resembling a smooth-skinned potato and containing two to five seeds. Inside, its flesh ranges from a pale yellow to an earthy brown color with a grainy texture akin to that of a well-ripened pear. The seeds are black and resemble beans, with a hook at one end that can catch in the throat if swallowed. The fruit has a high latex content and does not ripen until picked, where upon the fruit softens to a firmness and appearance very similar to that of a fuzzy, brown-skinned kiwifruit (Kulwal *et al.*, 1958).

The fruit has an exceptionally sweet, malty flavor. Many believe the flavor bears a striking resemblance to caramel or a pear candied with brown sugar. The unripe fruit is hard to the touch and contains high amounts of saponin, which has astringent properties similar to tannin, drying out the mouth.

The trees can only survive in warm, typically tropical environments, dying easily if the temperature drops below freezing. From germination, the sapodilla tree will usually take anywhere from five to eight years to bear fruit. The sapodilla trees yield fruit twice a year, though flowering may continue year round (Pantin, 1991).

Sapota is propagated through seed, air layering and softwood cuttings, using IBA (2,000 ppm) treatment are also successful methods for sapota propagation. But plants which are raised through air layering or cuttings establish poorly and are vulnerable to wind damage (Malo, 1970). For air layering, 1-2 years old, 45-60 cm, long juvenile matures shoots of pencil thickness with plenty of healthy dark green leaves are selected. On the base of such shoots, a ring of bark (2.5-3cm wide) is removed on which IBA+NNA (10,000ppm) each in lanolin paste is applied, covered with rooting media like sphagnum moss, vermiculite or garden soil and wrapped with

plastic. The roots emerge in 1-3 months and then layers are slowly separated from the mother tree and established in nursery. They are planted in the next season.

Sapodilla is rich in dietary fiber (5.6 g/100g), which makes it a good bulk laxative. The fiber content helps relieve constipation episodes and helps protect the mucous membrane of the colon from cancer causing toxins by firmly binding to them. The fruit is rich in antioxidant poly-phenolic compound tannin. Tannins are a compound family of naturally occurring poly-phenols that neutralize acids by precipitating proteins. Sapota contains good amount of antioxidant vitamins like vitamin C and vitamin A. Vitamin A is essential for vision. It is also required for maintaining healthy mucus membranes and skin. Consumption of natural fruits rich in vitamin A has been known to protect from lung and oral cavity cancers. So also, consumption of foods rich in vitamin C helps body develop resistance against infectious agents and scavenge harmful free radicals.

Fresh ripen sapodilla are good source of minerals like potassium, copper, iron and vitamins like folate, niacins and pantothenic acid. These compounds are essential for optimal health as they involve in various metabolic processes in the body as cofactors for the enzymes (Whitman, 1966).

In addition with medicinal value here are some serving tips:

- Fresh fruit sections are a great addition to fruit salads.
- Sapodilla-milk shake is a favorite drink in Asia.
- It is also use in ice-creams, cakes, pies etc.

Morphological traits are traditional phenotypic markers for the identification of plants. They may change with the cultivation and growth environment so that the identification is confusing. In

order to identify sapota trees in indigenous is a more systematic way, specific genetic markers for sapotas are developed. 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 *et al.*, 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 DNA (RAPD) are useful for the assessment of genetic diversity (Williams *et al.*, 1990) owing to their simplicity, speed and relatively low-cost 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. Random amplified polymorphic DNA (RAPD) depended on polymerase chain reaction (PCR). In the present study, RAPD markers are used to identify 15 indigenous genotypes of sapota in Shatkhira and Jessore districts of Bangladesh. The study was aimed to see the morphological variations through phenotypic characteristics among 15 germplasms and understand the distribution of sapotas in indigenous by molecular marker analyses.

The objectives of the present research are:

1. Molecular characterization of selected sapota germplasm by RAPD.
2. To estimate the genetic diversity in sapota germplasm.



Chapter- II

REVIEW OF LITERATURE

Sapota (*Achras sapota*) is a species of tree native to southern Mexico. Today, the tree is cultivated not only in Mexico, but also in Central America, the Caribbean, and South Florida for its fruit, which is commonly eaten in many Latin American countries.

2.1. Morphology of Sapota

Sapota is a large and highly ornamental evergreen tree that can reach a height of 15 to 45 meters (60 to 140 feet) at maturity. Like most fruit trees, it is mainly propagated by grafting, which ensures the new plant has the same characteristics as the parent, especially its fruit. Air layering and softwood cuttings, using IBA (2,000ppm) treatment are also successful methods for sapota propagation. But plants which are raised through air layering or cuttings establish poorly and are vulnerable to wind damage. It is also considerably faster than growing trees by seed. The leaves are medium green and glossy. They are alternate, elliptic to ovate, 7–15 cm long, with an entire margin. The white flowers are inconspicuous and bell-like, with a six-lobed corolla. The fruit is about 10 to 25 cm (4 to 10 inches) long and 8 to 12 cm (3 to 5 inches) wide and has orange flesh (Smith and Smith, 1992).

The fruit is eaten raw or made into milkshakes, smoothies, ice cream and fruit bars. Some consider the fruit to be an aphrodisiac. Some beauty products use oil pressed from the seed, otherwise known as sapayul oil. The brown skin has a texture somewhat between sandpaper and the fuzz on a peach. The fruit's texture is creamy and soft. A mamey

sapote is ripe when the flesh is pink when a fleck of the skin is removed. The flesh should give slightly, as with a ripe kiwifruit (Morera, 1982).

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 adapt to changing environments. With more variation, it is more likely that some individuals in a population will possess variations of alleles that 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 (U.S. Geological Survey). When humans initially started farming, they used selective breeding to pass on desirable traits of the crops while omitting the undesirable ones. Selective breeding leads to monocultures: entire farms of nearly genetically identical plants. Little to no genetic diversity makes crops extremely susceptible to widespread disease. Bacteria morph and change 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 (Morera, 1982).

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*. This plasmodiophorid destroyed the vast majority of the potato crop, and left one million people to starve to death (Kubelik and Szabo, 1995).

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 (Weir, 1990). Published applications of molecular marker-based GD in plant breeding have been limited primarily to sapota 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).

Using RAPD markers in sapota germplasm collections, some authors have identified genotypes coming from diverse foreign regions (Prakash *et al.*, 2002). Nevertheless, overall interpretation of the genetic relationships among sapota accessions with AFLP (Valdes-infante *et al.*, 2003) and SSR in Cuba indicates the absence of separate clusters representing local and foreign germplasm. This reflects the selection of sapota lines from open pollination rather than from controlled crosses. Micro-satellites also detected a high number of alleles shared for the majority of sapota 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 sapota breeding programmes and conservation strategies (Jain and Priyadarshan, 2002).

2.3. DNA Extraction

A fast, simple, cost-effective and reliable method is a pre-requisite 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, RNA ase and phenol-chloroform treatment, usually employed for plant DNA isolation. In terms of quantity (up to 174 $\mu\text{g g}^{-1}$ FW) and quality ($A_{260}/A_{280} = 1.6$ to 1.9) 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. In addition, growth and management of plants and storage of the plant samples in freezers is often difficult due to space constraints.

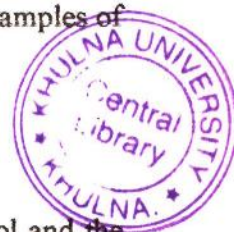
There are many methods proposed for isolation of nuclear DNA from a variety of plant species, plant organs and tissues. Now it is considered that three main factors are critical for extraction and purification of maximally undegraded and pure genomic DNA:

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 (1997), DNAzol is an advanced DNA isolation method (U.S. patent no. 5,945,515) 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 (Fukuoka *et al.*, 1992).



2.4. Genetic Marker

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. Currently, 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, in general, 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 markers has heightened its importance (Bretting *et al.*, 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.

2.4.1. Types of Genetic Markers

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)
- 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 by Jones *et al.* (1997) viz.

1. Morphological markers, representing phenotypic trait
2. Biochemical markers , Which include allelic variants of enzyme called isozymes and
3. 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 types 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 analysis 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 used for RAPD analysis to see the specific DNA band patterns e.g. 18S rDNA.

Saiki *et al.* (1985) 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. Williams *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 an 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 influence 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.

2.6. Random Amplification of Polymorphic DNA (RAPD)

RAPD (pronounced "rapid") 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 without 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.

Chikkadevaiah and Nandini (Nandini and Chikkadevaiah, 2005) established the phylogenetic relationships among sunflower hybrids genotypes and characterizes 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.

However though no statistical data are available for sapota, the fruits are considered to be of great importance in the south western region of Bangladesh, because of the growing demand for the products derived from them and the increasing interest in improving cultivation of the species.

Chapter- III

MATERIALS AND METHOD

The experiment was conducted at molecular horticulture laboratory of Agrotechnology Discipline of Khulna University, from October 2011 to April 2012.



3.1. Collection of plant materials

The younger leaves of sapota tree were collected from two selected locations Shatkhira and Jessore districts of Bangladesh (Table 3.1). Sapota leaves were collected from October to December, then the leaves were preserved -80°C in Lab for further use. The fruits of 15 genotypes exhibit morphological variation in some characters, such as fruit shape, size and taste. The fruit size was measured into three categories, e.g. large, medium and small. Large size was more than 8cm diameter and medium size was between 5.5cm to 8 cm diameter.

Table 3.1: Plant materials used in RAPD marker analysis

SI	Germplasm number	Name of the farmer	Location of collection	Morphological characteristics
01	1	Golam Rasul Biswas	Vill:Ghoshnagar; Post:Ghuni; Union: Ghoshnagar; Upazilla:Bhagarpara; Dist.:Jessore	Small, Round
02	2	Ratan Mondol	Vill:Ghoshnagar; Post:Ghuni; Union:Basuria; Upazilla:Bhagarpara; Dist.:Jessore	Large, Globose
03	3	Benoy Debnath	Vill:Ghoshnagar; Post:Ghuni; Union:Basuria; Upazilla:Bhagarpara; Dist.:Jessore	Medium, ovoid
04	4	Dheran Kumar Debnath	Vill:Ghoshnagar; Post:Ghuni; Union:Basuria; Upazilla:Bhagarpara; Dist.:Jessore	Small, Round
05	5	Dr. Sanker Kumar Debnath	Vill:Ghoshnagar; Post:Ghuni; Union:Basuria; Upazilla:Bhagarpara; Dist.:Jessore	Medium, Ovoid

06	6	Sheik Boni Amin	Vill+ Post+ Union =Moutala; Upazilla= Kaligong, Dist= Shatkhirā	Ovoid, Medium
07	7	Sheik Shahidul Islam	Vill+ Post+ Union =Moutala; Upazilla= Kaligong, Dist= Shatkhirā	Small, Round
08	8	Sheik Siddique	Vill: Maziyara; Post+ Upazilla= Tala; Union:Sadortala ; Dist.: Shatkhirā	Medium
09	9	Sheik amin Uddin	Vill: Ghona; Post: Gopalpur; Union: Islam kathi; Upazilla: Tala; Dist.: Shatkhirā	Large
10	10	Md. Anis Sheik	Vill: Ghona; Post: Gopalpur; Union: Islam kathi; Upazilla: Tala; Dist.: Shatkhirā	Medium, round
11	11	Khohinur Sheik	Vill: Ghona; Post: Gopalpur; Union: Islam kathi; Upazilla: Tala; Dist.: Shatkhirā	Very Large
12	12	Habibur Rahman	Vill+Post+Union = Sagordhari; Upazilla: Kashobpur; Dist.:Jessore	Small
13	13	Rhamot Ali	Vill+Post= Bhoga; Union: Shurighata; Upazilla: Kashobpur; Dist.:Jessore	Small, round
14	14	Md. Rouf	Vill+Post= Bhoga; Union: Shurighata; Upazilla: Kashobpur; Dist.:Jessore	Round, Large
15	15	Md. Inser Ali Molla	Vill: Barba; Post: Bhusandia; Union: Jamdia; Upazilla:Bhagarpara; Dist.:Jessore	Small

3.2. Procedure of DNA extraction process

Genomic DNA was extracted from the leaves by DNAzol (U.S. patent no. 5,945,515) 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).

I. Preparation of plant material for isolation of DNA

Plant leaves were rinsed with distilled water, 50 mg of younger leaf parts were taken and stored at -80°C for one day. Leaf samples were ground into powder with mortar and pestle and 1 ml of DNAzol was added and then homogenized properly. The mixture was centrifuged at 12,000 rpm for 10 minutes and transferred the supernatant to another 1.5 ml clean tube.

II. DNA precipitation

DNA was precipitated from the lysate/ homogenated 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 was mixed well to form a homogenous solution. DNA became visible as quickly as a cloudy precipitate. The sample was centrifuged at 12,000 rpm for 10 minutes and the supernatant was pipetted.

III. DNA purification

The DNA precipitate samples 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 pipetting.

IV. DNA solubilization

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

V. DNA Quantification

The DNA quantification was carried out using a spectrophotometer. One micro liter each sapota DNA extracts was diluted with 99 μ l of deionized water and the absorbance at 260 nm were measured. The concentrations of absorbance were calculated by using the following formula.

$$\text{DNA concentration } (\mu\text{g/ml}) = (\text{OD}_{260}) \times (\text{dilution factor}) \times (50 \mu\text{g DNA/ml}) / (1 \text{ OD}_{260} \text{ unit})$$

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

OD= Optical Density

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. Primers were selected based on mainly GC content. Primers that have GC content more than 60% are suitable for RAPD analysis (Anne *et al.*, 1995). Final subsets of three primers (Table 3.2) were exhibited good quality banding patterns and sufficient variability was selected for further analysis.

Table 3.2: Random primer used in the present study for screening

Primer code	Sequence (5' – 3')	GC content (%)	*T _m ; T _a
OP A00	ATC AGC GCA CCA	58%	38 ⁰ C; 36.8 ⁰ C
OP A02	GCC AGC TGT ACG	66.7%	40 ⁰ C; 37.4 ⁰ C
OP A03	TGC CTC GCA CCA	66.7%	40 ⁰ C ; 37.4 ⁰ C

GC – Guanine + Cytosine

T_m = Melting Temperature

T_a = Annealing Temperature

Calculation of annealing temperature:

Annealing temperature was calculated by the following formula-

$$0.3 \times T_m (\text{Primer}) + 0.7 \times T_m (\text{Product}) - 25$$

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 15.5 μl per sample. 2 μl genomic DNA was added with 15.5 μl PCR mixture and finally, total volume was made 17.5 μl (Table 3.3).

Table 3.3. PCR master mixture (10 μl)

Sl. No.	Reagents	Amount (Per sample)
1	Genomic DNA	3 μl
2	Primer (10 nM)	3 μl
3	dNTPs (10 mM)	2 μl
4	<i>Top</i> DNA polymerase (2U)	2 μl
5	Reaction buffer (10x)	2 μl
6	dH ₂ O	8 μl
Total		20 μl

3.3.3. Temperature 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 to step 2 for 40 more time.
- 6) Final extension 72°C for 5 min.

3.4. Electrophoresis of the 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 were 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. The unweighted pair group method with arithmetic averages (UPGMA)-based dendrogram was constructed using the component. Numerical value was considered to construct the dendrogram and presence of band (1) and absence of band (0) value consider for these RAPD analysis.

3.6. Genetic Distance

Estimates of genetic distance calculated all possible pair-wise genetic distance values by the following formula:

$$d_{xy} = 1 - \{2n_{xy} / (n_x + n_y)\}$$

Where, n_x and n_y are the numbers of bands amplified in individuals x and y , respectively, and $2n_{xy}$ is the number of bands shared by those individuals. In this analysis, smaller numbers are associated with more genetically similar individuals, whereas larger numbers suggest genetically dissimilarity.

3.7. Genetic similarity

Estimates of genetic similarity calculated all possible pair-wise genetic similar values by the following formula:

$$d_{xy} = \{2n_{xy}/(n_x + n_y)\} \times 100\%$$

Where, n_x and n_y are the numbers of bands amplified in individuals x and y , respectively, and $2n_{xy}$ is the number of bands shared by those individuals.

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 sapota. Molecular characterization was determined by using the molecular marker. Random amplification of polymorphic DNA (RAPD) markers is most useful for understanding the naturally occurring polymorphisms in living organisms.

4.1. Molecular characterization

In the present study, a polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD) method was used to amplify *Achras sapota* DNA using arbitrary oligonucleotide primers. The method was able to detect the heterogeneity of amplified DNA from the germplasm of *Achras sapota*. The study indicated the effectiveness of RAPD analysis in detecting the amount of polymorphisms among the different genotypes of sapota. Three effective primers (Table 3.2) were selected for determination of the polymorphism.

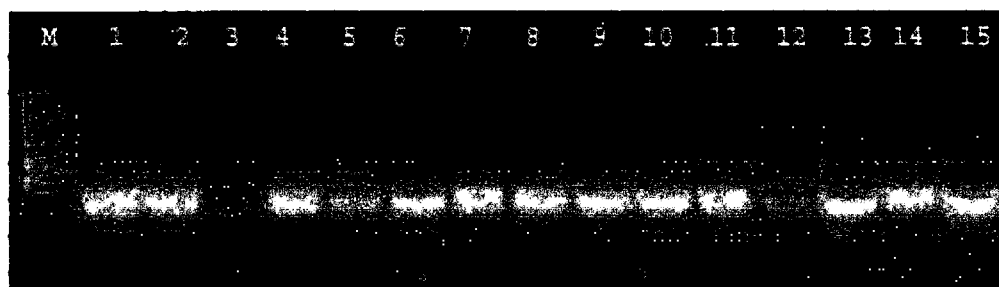


Fig 4.1. The gel profile of *Achras sapota* DNA

PCR is the technique of choice for nucleic acid quantification. The DNA quantity was determined by 260 nm and the UV spectrophotometer readings showed that the presence of DNA for the PCR stock. However, successful quantification depends crucially on the quality of the sample DNA analyzed. Whereas, Puchooa (2004) reported the yield of DNA per gram of leaf tissue extracted using a UV-VIS Spectronic Genesys spectrophotometer at 260 nm. Similarly, Katarina *et al.* (2006) confirmed the quality of DNA by 0.8 percent Agarose Gel Electrophoresis and DNA quantity was determined by 260 nm and quantified DNA was subjected to PCR amplification. Like others it was quantified the purified DNA at 260 nm and the UV spectrophotometer readings (Table-4.1) showed that the presence of DNA at high concentration and subsequently diluted the DNA to 100 ng/mL as per requirements for ideal PCR.

Table 4.1. UV spectrophotometer readings at 260nm showed the presence of DNA

GP No	OD at 260 nm
01	0.045
02	0.038
03	0.028
04	0.029
05	0.024
06	0.027
07	0.021
08	0.11
09	0.006
10	0.035
11	0.023
12	0.039
13	0.087
14	0.025
15	0.054

4.1.1 Primer selection and RAPD analysis

To enhance the polymorphism of genotypes of sapota, one 10-mer (OPS07) and two 12-mer (OPA02 & OPA03) primers were used for DNA amplification (Table 3.2). Only two primers (OPA02 & OPA03) were selected for fingerprinting. One primer generated various banding patterns ranging from 3 to 5 and yielded a total of 57 band patterns of which 48.89% were polymorphic. The highest percent of polymorphic bands (66.67%) was observed for primer A 03 and the lowest (20%) was from primer S 07. The reason of this difference could be due to the GC content of the primers. It can be mentioned that in this study. 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 *et al.*, 1995). Fukuoka *et al.*, (1992) got bands per primer with 40% and 6.1 bands per primer with 50%

GC content for rice varieties. 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 4.2. RAPD primers with corresponding bands scored and their size range together with polymorphic bands observed in 15 sapota Germplasm

Serial No	Primer code	No. of total band of germplasm	No. of common band	Number of polymorphic band	Proportion of polymorphic bands (%)
1	A02	5	2	3	60
2	A03	6	2	4	66.67
3	S07	5	4	1	20
Total		16	8	8	146.67
Average		5.33	2.67	2.67	48.89

Since the amplification directed by these two primers produced significant polymorphism and yielded a total of 57 polymorphic RAPD patterns as shown in Figure 4.2. Each of two 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 sapota germplasm. The amplification patterns of representative samples of sapota variety with three different primers have been shown in Figure 4.2, 4.3 and 4.4

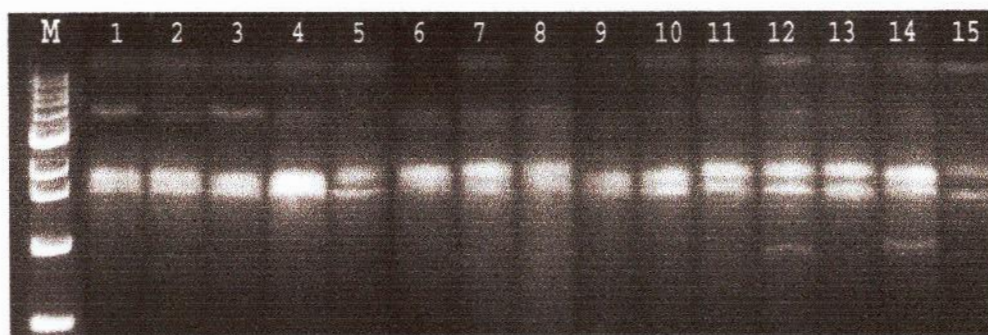


Fig 4.2. RAPD pattern generated by the primer A02 genotypes of *Achras sapota*.

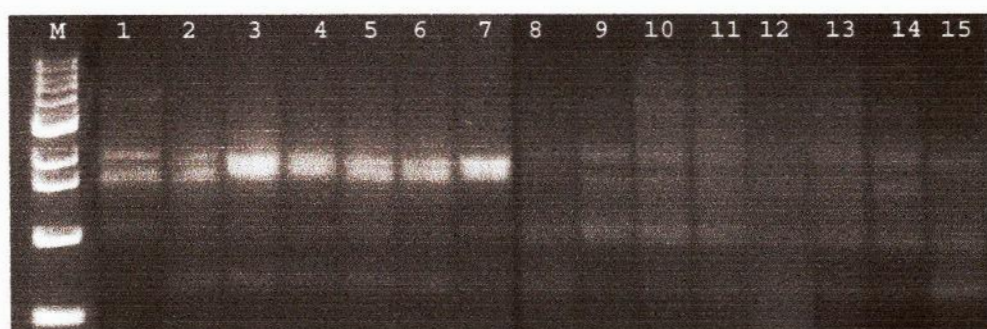


Fig 4.3. RAPD pattern generated by the primer A03 genotypes of *Achras sapota*.

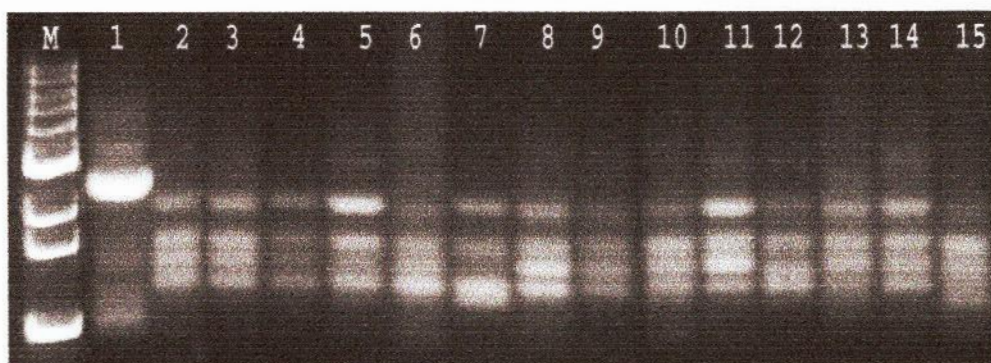


Fig 4.4. RAPD pattern generated by the primer S07 genotypes of *Achras sapota*.

The numbers show the serial number of the genotypes and "M" indicates Ladder.

4.1.2. Genetic Distance

DNA amplification was repeated three times. Genetic similarity matrix (Table 4.2) was generated using Gel Quest based on the marked scores of the polymorphic RAPD patterns where score 1 or 0 was assigned to the present or absent band.

The values of pair-wise comparisons of Nei's (1979) genetic distance between 15 sapota genotypes were computed from 0.112 to 0.5 (Table 4.2). Comparatively higher genetic distance (0.5) was found between germplasm no-1 vs. germplasm no-5, between germplasm no-3 vs. germplasm no-4, between germplasm no-1 vs. germplasm no-6, between germplasm no-1 vs. germplasm no-10, between germplasm no-1 vs. germplasm no-14. The lowest genetic distance (0.112) was found between germplasm no-4 vs. germplasm no-5, between germplasm no-3 vs. germplasm no-11, between germplasm no-4 vs. germplasm no-11, between germplasm no-10 vs. germplasm no-11, between germplasm no-2 vs. germplasm no-12, between germplasm no-3 vs. germplasm no-12, between germplasm no-4 vs. germplasm no-12, between germplasm no-5 vs. germplasm no-12, between germplasm no-6 vs. germplasm no-12, between germplasm no-10 vs.

germplasm no-12, between germplasm no-11 vs. germplasm no-12, between germplasm no-11 vs. germplasm no-14, between germplasm no-12 vs. germplasm no-14.

There was a genetic variation among the studied of sapota as indicated by the proportion of polymorphic loci. Estimated genetic variation in the sapota might be consistent with the fact that it is a polymorphic plant. The range of genetic distance of 15 genotypes is 0.112-0.5 and the difference between the highest and lowest genetic distance indicated the presence of variability among the 15 genotype of sapota.

Table-4.3. Genetic diversity index from RAPD data of different sapota germplasm

	Gp ₁	Gp ₂	Gp ₃	Gp ₄	Gp ₅	Gp ₆	Gp ₇	Gp ₈	Gp ₉	Gp ₁₀	Gp ₁₁	Gp ₁₂	Gp ₁₃	Gp ₁₄	Gp ₁₅
Gp ₁	-														
Gp ₂	25%	-													
Gp ₃	25%	25%	-												
Gp ₄	25%	25%	50%	-											
Gp ₅	50%	25%	25%	11.2%	-										
Gp ₆	50%	25%	25%	25%	25%	-									
Gp ₇	14.2%	14.2%	14.2%	14.2%	14.2%	14.2%	-								
Gp ₈	42.8%	14.2%	14.2%	14.2%	14.2%	14.2%	33.4%	-							
Gp ₉	42.8%	14.2%	14.2%	14.2%	14.2%	14.2%	33.4%	33.4%	-						
Gp ₁₀	50%	25%	25%	25%	25%	25%	14.2%	14.2%	14.2%	-					
Gp ₁₁	33.4%	33.4%	11.2%	11.2%	33.4%	33.4%	25%	25%	25%	11.2%	-				
Gp ₁₂	33.4%	11.2%	11.2%	11.2%	11.2%	11.2%	25%	25%	25%	11.2%	11.2%	-			
Gp ₁₃	42.8%	14.2%	14.2%	14.2%	14.2%	14.2%	33.4%	33.4%	33.4%	14.2%	25%	25%	-		
Gp ₁₄	50%	25%	25%	25%	25%	25%	14.2%	14.2%	14.2%	25%	11.2%	11.2%	14.2%	-	
Gp ₁₅	42.8%	14.2%	14.2%	14.2%	14.2%	14.2%	14.2%	33.4%	33.4%	14.2%	25%	25%	33.4%	14.2%	-

Gp = Germplasm Number



4.1. 3. Genetic Similarity

Inter-population genetic similarity indices for different germplasm have been presented in table 4.4. In the present study the inter-population similarity index value was the highest between germplasm no-4 vs. germplasm no-5, between germplasm no-3 vs. germplasm no-11, between germplasm no-4 vs. germplasm no-11, between germplasm no-10 vs. germplasm no-11, between germplasm no-2 vs. germplasm no-12, between germplasm no-3 vs. germplasm no-12, between germplasm no-4 vs. germplasm no-12, between germplasm no-5 vs. germplasm no-12, between germplasm no-6 vs. germplasm no-12, between germplasm no-10 vs. germplasm no-12, between germplasm no-11 vs. germplasm no-12, between germplasm no-11 vs. germplasm no-14, between germplasm no-12 vs. germplasm no-14 (88.8%). On the other hands the lowest genetic distance (50%) was found between germplasm no-1 vs. germplasm no-5, between germplasm no-3 vs. germplasm no-4, between germplasm no-1 vs. germplasm no-6, between germplasm no-1 vs. germplasm no-10, between germplasm no-1 vs. germplasm no-14. The higher

Table-4.4. Genetic similarity index from RAPD data of different sapota germplasm

	Gp1	Gp2	Gp3	Gp4	Gp5	Gp6	Gp7	Gp8	Gp9	Gp10	Gp11	Gp12	Gp13	Gp14	Gp15
Gp1	-														
Gp2	75%	-													
Gp3	75%	75%	-												
Gp4	75%	75%	50%	-											
Gp5	50%	75%	75%	88.8%	-										
Gp6	50%	75%	75%	75%	75%	-									
Gp7	85.8%	85.8%	85.8%	85.8%	85.8%	85.8%	-								
Gp8	57.2%	85.8%	85.8%	85.8%	85.8%	85.8%	66.6%	-							
Gp9	57.2%	85.8%	85.8%	85.8%	85.8%	85.8%	66.6%	66.6%	-						
Gp10	50%	75%	75%	75%	75%	75%	85.8%	85.8%	85.8%	-					
Gp11	66.6%	66.6%	88.8%	88.8%	66.6%	66.6%	75%	75%	75%	88.8%	-				
Gp12	66.6%	88.8%	88.8%	88.8%	88.8%	88.8%	75%	75%	75%	88.8%	88.8%	-			
Gp13	57.2%	85.8%	85.8%	85.8%	85.8%	85.8%	66.6%	66.6%	66.6%	85.8%	75%	75%			
Gp14	50%	75%	75%	75%	75%	75%	85.8%	85.8%	85.8%	75%	88.8%	88.8%	85.8%	-	
Gp15	57.2%	85.8%	85.8%	85.8%	85.8%	85.8%	85.8%	66.6%	66.6%	85.8%	75%	75%	66.6%	85.8%	-

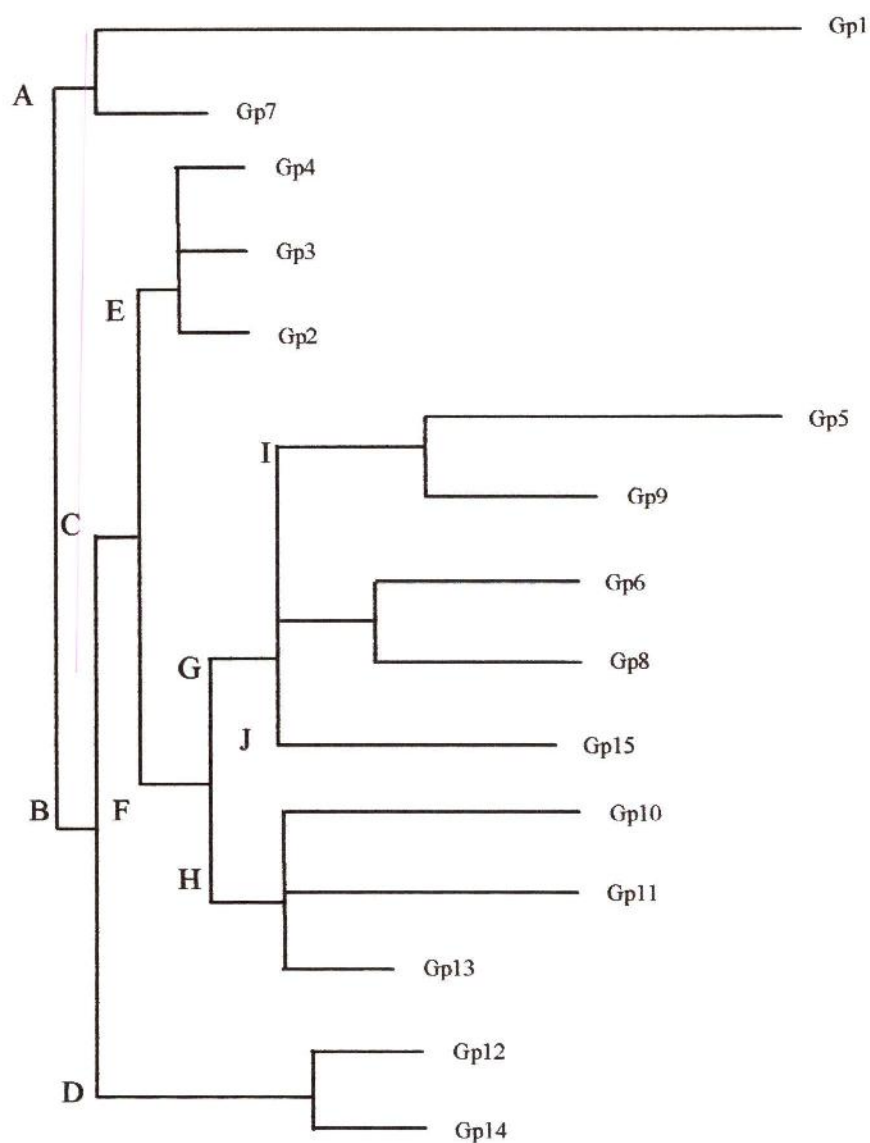
Gp = Germplasm Number

genetic similarity among these germplasm clearly indicates the genetic relatedness between the germplasm.

4.1.4. Dendrogram

The dendrogram (Fig: 4.5) shows that all the germplasm were grouped into two major clusters A & B. Cluster B subdivided into C and D, Cluster C subdivided into E and F and Cluster F subdivided into G and H, Cluster G subdivided into I and J.

Morphological characterization was determined and showed genetic similarities and dissimilarities by collecting data and constructing the UPGMA dendrogram to estimate the genetic diversity of sapota phenotypically. From the dendrogram (Fig: 4.5) it was found that most of the cultivated sapotas collected from local germplasm were grouped under C cluster. We did not find any similarity between germplasm no-1 and germplasm no-7 and found similarity among germplasm such as germplasm no-2, germplasm no-3, germplasm no-4, germplasm no-5, germplasm no-6, germplasm no-9, germplasm no-10, germplasm no-11, germplasm no-12, germplasm no-13, germplasm no-14 and germplasm no-15 as their shape, size and taste of fruits was similar. Germplasm no-1 is an exceptional variety and was found similarity with germplasm no-7.



Here, Gp = Germplasm Number

Fig 4.5. Unweighted Pair Group Method of Arithmetic Mean (UPGMA) dendrogram based on Nei's (1979) genetic distance, according to RAPD analysis of 15 variety of sapota.

Chapter- V

CONCLUSION

A study on “Molecular Characterization of Some Selected Sapota (*Achras sapota*) germplasm by RAPD Analysis” of 15 germplasm of sapota was studied in the Molecular Horticulture Laboratory, Agrotechnology Discipline of Khulna University, Khulna during October 2011 to April 2012. The objective of this study was to estimate the genetic diversity in sapota germplasm using RAPD markers. RAPD makers have proved to be effective for characterizing the genetic basis of *Achras sapota* for assessing genetic diversity and relatedness between accessions. In this study, the PCR reactions were performed in optimal conditions and informative RAPD fragments were obtained with high reproducibility. RAPD analysis is efficient and accurate for the investigation of distribution of local sapota. Even though this study showed close relationships among a few genotypes studied, there were still no duplicates. 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 sapota breeding programs. This result is beneficial for further research on the sapota functionality.

Chapter VI

REFERENCE

Anolles, G. C., Callahan, L.M. and Gresshoff, P.M. (1997). The origin of bermudagrass (*Cynodon*) offtypes inferred by DNA amplification fingerprinting. *Crop Sci*, 37: 81–87.

Berthomieu, P. and Meyer, C. (1991). Direct amplification of plant genomic DNA from leaf and root pieces using PCR. *Plant Mol Biol*; 17: 555–557.

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

Bretting, P. K. and Widrechner, M. P. (1995). Genetic Markers and Plant Genetic Resource Management. In: *Plant Breeding Reviews*, Janick, J. (Eds.). John Wiley and Sons, New York, pp: 11-86.

Campbell, C.W. and Lara, S.P. (1982). Mamey sapote cultivars in Florida. *Proc. Fla. State Hort. Soc.*, 95: 114–115.

Chomczynski, P., Mackey, K., Drews, R. and Wilfinger, W. (1997). DNAzol: A reagent for the rapid isolation of genomic DNA. *BioTechniques*, 22, 550-553.

Chomczynski, P., Wilfinger, W. and Mackey, K. (1998). Isolation of Genomic DNA from Human, Animal, and Plant Samples with DNAzol Reagents. *Biotechnology International*, 185-188.

Dettori, M. T. and Palombi, M. A. (2000). Identification of *Feijoa sellowiana* Berg accessions by RAPD markers. *Sci. Hortic.*, 86: 279-290.

Duvick, D. N. (1984). Genetic Contributions to Yield Gains of U.S. Hybrid Maize, 1930 to 1980. In W. R. Fehr (ed.) *Genetic Contributions to Yield Gains of Five Major Crop Plants*. CSSA Special Publication No. 7. Crop Science Society of America, American Society of Agronomy, Madison, WI, USA. p. 15-47.

Fukuoka, S., Hosaka, K. and Kamijima, O. (1992). Use of random amplified polymorphic DNA (RAPDs) for identification of rice accessions. *Japanese Journal of Genetics*, 67, 243–252.

Hoisington, D. Khairallah, M. and Gonzalez-de-Leon, D. (1994). Laboratory Protocols: CIMMYT Applied Biotechnology Center. Second Edition, Mexico, D.F.: CIMMYT

www.cheetah.org. "Introduction to Genetic Diversity." Cheetah Conservation Fund. (2002). 19 Mar. 2008.

Hossain, M. B., Awal, A., Rahman, M.A., Haque, S. and Khan, H. (2003). Distinction between cold-sensitive and - tolerant Jute by DNA polymorphism. *J. Biochem. Mol. Biol.*, 36:427-432.

Jain, S. M. and Priyadarshan, P. M. (2002). Breeding Plantation Tree Crops: Tropical Species. Pp-108.

Jones, N., Ougham, H. and Thomas, H. (1997). Markers and mapping: we are all geneticists now. *New Phytologist*, 137: 165-177.

Katarina C, Stebih D, Dreo T, Zel J and Gruden, K (2006). Critical points of DNA quantification by real-time PCR-effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. *BioMed Central Biotechnology*. 6(37): 1472-6750.

Kubelik, A. R. and Szabo, L. J. (1995). High-GC primers are useful in RAPD analysis of fungi. Volume 28, Number 4, 384-389, DOI: 10.1007/BF00326438

Kulwal, L.V., Tayde, G.S. and Deshmukh, P.P. (1958). Studies on softwood grafting of sapota. *PKV. Res. J. (India)*, 9(2): 33-36.

Malo, S.E. (1970). Propagation of the mamey sapote. *Proc. Trop. Reg. Am. Soc. Hortic. Sci.*, 18: 165-174.

Micheli, M.R., Bova, R., Pascale, E. and Ambrosio, E. D. (1994). Reproducible DNA fingerprint with the random amplified polymorphic DNA (RAPD) method. *Nucl. Acids Res.*, 22: 1921-1922.

Morera, J.A. (1982). *El zapote*. Genetic Resources Unit. Turrialba, Costa Rica, CATIE/GTZ., 200-210.

Nandini, R. and Chikkadevaiah (2005). DNA fingerprinting of sunflower genotypes (*Helianthus annuus* L.). *HELLA*, 28 (42): 9-18.

Ouenzar, R. B., Hartmann, C., Rode, A. and Benslimane, A. (1998). Date palm DANN mini-preparation without liquid nitrogen. *Plant Molecular Biology Reporter*, 16: 263-269.

Pantin, D. (1991). Mamey feature. Pantin's mamey conservations with Donald Pantin, mamey grower. *Trop. Fruit World*, 2(1): 2-17.

Paran, I and Micheltore, R. W. (1993). Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor. Appl. Genet.*, 85:985-993.

Prakash, D. P., Narayanasway, P. and Sonder, S. N. (2002). Analysis of molecular diversity in guava using RAPD markers. *J. Hortic. Sci. Biotech.*, 77: 287-293.

Puchooa, D. (2004). A simple, rapid and efficient method for the extraction of genomic DNA from lychee (*Litchi chinensis* Sonn.). *African Journal of Biotechnology*. 3(4): 253-255.

Quilantan Carrero, J. (1979). Propagación vegetativa del mamey zapote. *Proc. Trop. Reg. Am. Soc. Hortic. Sci.*, 23: 180-182.

Rogers, S. O. and Bendich, A. J. (1985). Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol.*, 5:69-76.

Saiki, R. K. (1989). The design and optimization of the PCR. In: *PCR Technology - Principles and applications for DNA amplification*. Editor: Erlich HA. New York: Stockton Press, Chapter, 1:7-16.

Smith, O. S. and Smith, J. S. C. (1992). Measurement of genetic diversity among maize hybrids; A comparison of isozymic, RFLP, pedigree, and heterosis data. *Maydica*, 37:53-60.

Smith, P. (1999). Are weak patent rights a barrier to US exports? *Journal 283 of International Economics*, 48: 151-177.

Stift, G, Pachner, M. and Lelley, T. (2003). Comparison of RAPD fragment separation in agarose and polyacrylamide gel by studying Cucurbita species. *Cucurbit Genet. Coop. Rep.*, 26: 62-65.

Valdés-Infante, D., Becker, D., Rodríguez, N., Velázquez, B., González, G., Sourd, D., Rodríguez, J., Ritter, E. and Rohde, W. (2003). Molecular characterization of Cuban accessions of guava (*Psidium guajava* L.), establishment of a first molecular linkage map and mapping of QTLs for vegetative characters. *J. Genet. & Breed.*, 57:349-358.



Weir, B. S. (1990). *Genetic Data Analysis: Methods for discrete population genetic data*. Sunderland, Massachusetts: Sinauer Associates, Inc. 377 pp.

Weishing, K, Nybom, H, Wolff, K, and Meyer, W. (1995). DNA isolation and purification. In: *DNA fingerprinting in plants and fungi*, CRC Press, Boca Raton, Florida. pp 44-59.

Welsh, J. and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucl. Acids Res.*, 18: 7213-7218.

Whitman, W.F. (1966). The green sapote, a new fruit for South Florida. *Proc. Fla. State Hortic. Soc.*, 78: 330-336.

Williams, J. G. K., Kubelik, A. R., Livak, K.J. and Rafalski, J. A. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, 18: 6531-6535

Winter, P. and Khal. (1995). Molecular marker technologies for plant improvement. *World Journal of Microbiology and Biotechnology*, 11: 438 - 448.