MOLECULAR CHARACTERIZATION OF HOGPLUM (*Spondias dulcis* L.) GERMPLASM AVAILABLE IN THE SOUTH-WESTERN REGION OF BANGLADESH BY RAPD ANALYSIS

A Thesis

Submitted to

AGROTECHNOLOGY DISCIPLINE

KHULNA UNIVERSITY

By

Md. Shawkat Hossain

Examination Roll No. MS 100816

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

KHULNA, BANGLADESH

May, 2012

KHULNA UNIVERSITY

AGROTECHNOLOGY DISCIPLINE

FINAL ORAL EXAMINATION OF THE MS IN HORTICULTURE

THESIS

The undersigned certify that they have read, and recommended to the Agrotechnology Discipline for acceptance, a MS in Horticulture Thesis entitled:

MOLECULAR CHARACTERIZATION OF HOGPLUM (*Spondias dulcis* L.) GERMPLASM AVAILABLE IN THE SOUTH-WESTERN REGION OF BANGLADESH BY RAPD ANALYSIS

By

Md. Shawkat Hossain

Examination Roll No. MS 100816

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

.....

Prof. Dr. Md. Abdul Mannan
Professor
Agrotechonology Discipline
Khulna University, Khulna
(Supervisor)

.....

Prof. Md. Rejaul Islam
Professor
Agrotechonology Discipline
Khulna University,Khulna
(Co-supervisor)

Chairman, Examination Committee
And Head, Agrotechnology Discipline
(The signature of the Chairman does not necessarily signify that the Chairman has read the Thesis)

May, 2012

DECLARATION

This thesis is entirely based on the candidate's own investigation except for quotations and citations which have been duly acknowledged and no part of this paper has been accepted nor is it being previously or concurrently submitted for any degree.

Submitted By
Md. Shawkat Hossain
Examination Roll No. MS 100816
Session: 2009-2010

Supervisor
Dr. Md. Abdul Mannan
Professor
Agrotechnology Discipline
Khulna University
Khulna-9208
Bangladesh.

DEDICATED TO

OUR BELOVED PARENTS

&

All FARMERS

OF BANGLADESH

ACKNOWLEDGEMENT

The author would like to express with utmost humble gratitude and endless praises to Omnipotent Almighty Allah, who enabled him to complete the thesis successfully for the fulfillment of the degree of Master of Science in Horticulture.

The author has much pleasure to express his heartfelt respect, deep sense of gratitude, immense indebtedness and deep appreciation to honorable supervisor Dr. Md. Abdul Mannan, Professor, Agrotechnology Discipline, Khulna University, Khulna, for his worthy guidance, valuable suggestions, constructive criticism, encouragement and continuous supervision throughout the research work and preparation of thesis.

The author wish to express his enormous appreciation and heartiest indebtedness to Md. Rejaul Islam, Professor, Agrotechnology Discipline, Khulna University, Khulna, for his guidance, kind co-operation and constructive criticism during the period of conducting this work.

The author expresses his gratitude and thanks to Professor Md. Monirul Islam, Head, Agrotechnology Discipline, Khulna University, Khulna for his love, encouragement and constructive advice with a view to preparing the paper.

The author extends his gratefulness to all the teachers of the Agrotechnology Discipline, Khulna University, Khulna, for their valuable advice, suggestions and constructive criticism.

He also expresses his gratefulness to Rubel, Mita and Debjani, the MS students of Agrotechnology Discipline, Khulna University, Khulna, who helped the author to complete the research.

Special thanks are extended to library authority of Khulna University, Seminar library authority of Agrotechnology Discipline, Khulna University, Khulna, from where the authors found valuable information to complete the thesis.

Also thanks are extended to donor organization of the project entitled by "Evaluation of fruit (*Mangifera indica, Artocarpus heterophyllus, Psidium guajava, Zizyphus jujuba, Citrus grandis, Achras sapota and Spondias dulcis*) germplasm available in the southwestern region of Bangladesh on the basis of their physico-chemical characteristics and genetic variability by Random Amplified Polymorphic DNA (RAPD) analysis" funded by USDA under which the research work was done.

Finally the author wishes to express his boundless gratitude to parents and other members of his family whose sacrifices, inspiration, and continuous blessings opened the gate and paved the way for his higher studies.

May, 2012

The author

ABSTRACT

The research work was carried out to determine the genetic variability of 20 selected *Spondius dulsis* L. germplasm collected from the south-western region of Bangladesh during October 2011 to March 2012. Hog plum 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 hog plum remains unsolved these days. In the present study, morphological and molecular characterizations were used to record different levels of variability concerning the morphological traits of the fruit. Random Amplified Polymorphic DNA (RAPD) marker was used for the molecular identification. Among the 20 selected germplasm, fourteen were commercially cultivated germplasm. The 10-mer and 12-mer oligonucleotide primers were used in RAPD for amplification. Three primers, A01, A02, and S01, were able to ditect the amplification and yielded a total of 362 band patterns of which 63.06% were polymorphic. The highest percent of polymorphic loci (66.67%) was observed for primer A02 and the lowest (60.00%) was from primer S01. Results were analyzed by molecular algorithm UPGMA and Neighbor-Joining. Twenty genotypes on the dendrogram were identified and divided into two major groups and subgroups on the basis of genetic characteristics. The range of genetic distance was observed 0.091 (GP09 vs. GP04) to 0.389 (GP14 vs. GP13). Based on the cluster analysis, the *S. dulsis* samples that were believed to have high morphological difference were grouped independently. The results suggested that RAPD is useful for the discrimination of cultivars and selection of potential hog plum clones of high economy for crop improvement.

CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Hog plum (*Spondias dulcis* L.) is a small, aromatic and delicious fruit belongs to the family Anacardiaceae under the genus *Spondias*. The genus consists of seventeen species, seven of which are native to the neotropics and other ten are native to tropical Asia. They are commonly named hog plums, spanish plums and in some cases golden apples (Mitchell and Daly, 1998).

Native to Melanesia through Polynesia, *S. dulcis* has been introduced into tropical areas across the world. At first in 1782, this species was introduced into Jamaica and among other places such as Cuba, Haiti, the Dominican Republic, Trinidad, Venezuela etc. Although the United States Department of Agriculture (USDA) received seeds from Liberia in 1909, *S. dulcis* has yet to become popular in America (Morton, 1987). However, in Bangladesh the total area of land under fruit cultivation is about 1,81,000 hectare and the yield per hectare is 8.24 ton. But hog plum 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 nearly 15% of the country's demand. Hog plum grows both in the forest and garden. Mostly growing areas of this fruit in Bangladesh are Barisal, Khulna, Sylhet, Satkhira etc. In our country the growing season of hog plum is June to September among a year (Rashid *et al.*, 1987).

The hog plum tree produces small, inconspicuous white flowers in terminal panicles, assorted male, and female. Its oval fruits, 2.5 to 3.5 inch (6.25-9 cm) long, are long-stalked and are produced in bunches of 12 or more. The average weight of the fruit is about 70-130 g. Over several weeks, the fruit fall to the ground while still green and hard, turning golden-yellow as they ripen (Morton, 1987).

This fruit has high calorific and nutritional value. The edible portion (per 100 g) of the fruit contains moisture 83.2%; mineral matter 0.6 g; fibre 1.0 g; energy 66 kcal; protein 1.1 g; fat 0.1 g; carbohydrate 15 g; calcium 15 mg; iron 3.9 mg; carotene 800 mg; phosphorus 11.0 mg; vitamin B_1 (0.28 mg); vitamin B_2 (0.04 mg) and vitamin C (92 mg). This fruit can be used as medicine. The fruit is astringent and it is stated to be useful in bilious dyspepsia. It is eaten either green or dry as an antidote for wounds caused by poisonous arrows. The plant is reported to have anti-tubercular properties. The bark is also useful in dysentery and diarrhoea. The bark is used for tanning (Rashid *et al.*, 1987).

Hog plum is most commonly used as a food source. Its fruit may be eaten in raw form. The flesh of raw hog plum is crunchy and a little sour. If the crisp sliced flesh is stewed with a little water and sugar and then strained through a wire sieve, it makes a most acceptable product, much like traditional applesauce but with a richer flavor. With the addition of cinnamon or any other spices desired, this sauce can be slowly cooked down to a thick consistency to make a preserve very similar to apple butter. Besides this unripe fruits are eaten in curries or green salads or made into jelly, pickles or relishes, or used for soups and stews (Daulmerie, 1994). The ripe fruits are used to make jams, preserves, juices (Geurts *et al.*, 1986).

The climate of Bangladesh is suitable for hog plum production but it is not cultivated widely. By determining the physical, chemical and morphological characteristics of this fruit it can be possible to obtain its nutritive value and other characteristics. Such types of findings will be used to increase the production of it by inspiring the farmers and other concern people. As a seasonal fruit it is essential to produce several products of hog plum to minimize the losses due to the lack of storing facilities and to enhance the scope to consume its product not only for a season but also for all the year round.

But 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 hogplum trees in indigenous is a more systematic way, specific genetic markers for hogplums 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 polymorphism 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 and 41 genotypes of hogplum in India (Prakash *et al.*, 2002). 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. Random amplified polymorphic DNA (RAPD) depended on polymerase chain reaction (PCR).

In the present study, RAPD markers are used to identify 20 indigenous genotypes of hogplum in Khulna and Jessore district of Bangladesh. The research is aimed to estimate the genetic diversity of hog plum germplasm using RAPD markers.

CHAPTER II

REVIEW OF LITERATURE

Although hog plum is one of the most important tropical fruit but a very little study has so far been done on physicochemical characteristics and developed products of this fruit in the world. A review of the available information relating to the present study has been presented below.

2.1. Morphology of Hog plum

Rashid *et al.* (1987) reported that two types of hog plum cultivate in Bangladesh. Each fruit of both types to be 6 to 8 cm long, 5 to 7 cm wide and weighing 100 to 350 g or more. Bailey (1950) observed that tree of hog plum is a medium to long length tree with compound leaves 30 to 40 cm long. Ten to fifteen fruits borne in terminal clusters. The ovoid shaped fruit is a drupe and generally 4 to 5 cm long.

Persad (1997) stated that tree of golden apple is a medium-sized (15-25 m) long tree with olive green hairless leaves of 2 to 6 cm length. The fruit is an ellipsoid to ovoid drupe (4-12 cm length; 3-8 cm diameter) and can weigh up to 450 g.

Campbell and Sauls (1994) observed that fruit length is 2.5 to 5 cm (1-2 inch) in golden apple. Fruit is globular to ovoid. Fruits of some selections are irregular in shape. Fruit is a drupe with a tough skin, edible pulp of varying thickness and a single large seed. Seeds are difficult to separate from the pulp and often have strong woody fibers projecting into the pulp.

Franquin *et al.* (2005) observed that average fruit length, diameter and weight of mature green golden apple are 7.1 cm, 5.4 cm and 116.4 g, respectively.

Popenoe (1974) opined that hog plum is a widely distributed tropical tree. The fruits, leaves and wood of the tree are utilized in many ways. The oval to roundish fruit is usually 5 cm long and has a thin skin which colour could be changed at ripening stage.

McMillan (1986) reported about hog plum that its tree is rapid-growing, attaining a height of 60 ft (18 m) in its homeland; generally not more than 30 or 40 ft (9-12 m) in other areas.

Long-stalked fruits dangle in bunches of a dozen or more; oval or somewhat irregular and 2.5 to 3.5 inch (6.25-9 cm) long, with thin but tough skin.

Arif and Sheeba (2010) reported about hog plum that it is simple, succulent, fibrous and drupe type of fruit. The Epicarp becomes greenish yellow when ripe. Ovoid or oblong fruit is 7 to 8 cm long and 4-5 cm in diameter. Ayoka *et al.* (2008) stated that *Spondias mombin* grew in the rain forest and in the coastal area. The tree of this fruit can be reached to a height of 15 – 22 inch. The fruit is 1.5-3.5 inch long oval yellow plum which has a leathery skin and a thin layer of fruit pulp with a very exotic taste. It hangs in numerous clusters of more than a dozen on the tree.

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 makeup 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 to pass on desirable traits of the crops 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 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 (Weir, 1990). Published applications of molecular marker-based GD in plant breeding have been limited primarily to hog plum 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).

Mendonca *et al.* (2008) carried out a physicochemical analysis (weight of fruits, weight of stones, amount of pulp, total soluble solids, total titratable acidity, etc.) of the fruits from 18 of the *Spondias mombin* and verified the occurrence of genetic diversity within the collection.

Based on the physicochemical characteristics of the fruits, Soares *et al.* (2006) also demonstrated the presence of diversity among 14 genotypes distributed in the municipality of Teresina, and stressed the possibility of selecting superior individuals for direct use in the improvement of commercial orchards. Genetic diversity in populations of golden apple has also been observed in studies involving isoenzyme markers in which the observed heterozygosity was greater than that expected (Gois *et al.*, 2009; Silva, 2009).

In the RAPD marker study of Gois *et al.* (2008) 17 golden apple plants collected in the State of Sergipe of Brazil exhibited a genetic similarity of 46.77% (range 21-78%). Based on an estimate of the genetic diversity of 12 golden apple accessions using inter-simple sequence repeat (ISSR) markers, Silva (2009) claimed that there was no correlation between genetic similarity and the origin of the accessions.

Lima *et al.* (2011) evaluated genetic variability among 32 *Spondias mombin* accessions using RAPD technique. The 21 primers employed in the final analysis produced 145 fragments, 79% of which were polymorphic. Two of the accessions were the most unrelated and, hence, most suitable for initial crossings in order to obtain high levels of segregation. They concluded, based on the repeatability and reproducibility tests, that the RAPD technique is reliable and efficient for revealing the genetic diversity of the fruits, which will be useful for genetic improvement programs. Authors also said that the results presented reveal the presence of genetic variability in the accessions among the Germplasm Collections of Embrapa Meio-Norte of Brazil and can be exploited in future breeding programs.

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 hog plum germplasm collections, some authors have identified genotypes coming from diverse foreign regions. In the case of amra, 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 hog plum lines form open pollination rather then from controlled crosses. Micro-satellites also detected a high number of alleles shared for the majority of hog plum 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 hog plum 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 (up to 174 µg g -1 FW) and quality (A260 /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. 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 (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.

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, 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.

2.4.1. Types of Genetic Markers

Some commonly used types of genetic markers are

- RFLP (or Restriction fragment length polymorphism)
- AFLP (or Amplified fragment length polymorphism)
- RAPD (or Random amplification of polymorphic DNA)
- VNTR (or Variable number tandem repeat)
- Microsatellite polymorphism, SSR (or Simple sequence repeat)
- SNP (or Single nucleotide polymorphism)
- STR (or Short tandem repeat)
- SFP (or Single feature polymorphism)
- DArT (or Diversity Arrays Technology)
- RAD markers (or 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) vise.

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 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 .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 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.

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 (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 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.

Chen *et al.* (2006) showed the RAPD analysis of thirty two Taiwan variety of *Psidium guajava* L. for variety development. Nandini and Chikkadevaiah (2005) established the phylogenetic relationships among sunflower hybrids genotypes and characterized them based on both morphometric traits and PCR based PAPD 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).

However, though no statistical data are available for hog plum, the fruits are considered to be of great importance in the southwestern region of Bangladesh because of the growing demand for the products derived from them and the increasing interest in improving cultivation of the species. To meet this demand we should find out variety through the observation of genetic diversity. So the aim of this experiment was to study the genetic diversity of hog plum available in the southwestern region of Bangladesh.

CHAPTER III

MATERIALS AND METHOD

3.1. Plant Materials Collection

The experiment was conducted at molecular horticulture laboratory of Agrotechnology Discipline of Khulna University, from October 2011 to March 2012. The younger leaves of hogplum tree were collected from two selected location 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 20. The fruits of 20 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 5 cm diameter and medium size was between 4 cm to 6 cm diameter.

Table 3.1. Plant materials used in RAPD marker analysis

Germplasm No.	Designation	Place of collection	Morphological characteristics
1	Local	Jessore	Sweet, medium, oblong
2	Local	Jessore	Medium sweet, large, oblong
3	Local	Jessore	Sour, medium, ovoid
4	Local	Jessore	Sweet, small, globose
5	Local	Jessore	Sweet, medium, ovoid
6	Local	Jessore	Sour, medium, ovoid
7	Local	Jessore	Sweet, small, globose
8	Local	Jessore	Sweet, medium, ellipsoid
9	Local	Jessore	Medium sour, large, ovoid
10	Local	Jessore	Sweet, medium, oblong
11	Local	Jessore	Slight sour, very large, ovoid
12	Unknown	Jessore	Sweet, small, globose
13	Belati amra	Jessore	very sour, small, globose
14	Belati amra	Jessore	Slight sweet, large, oblong
15	Belati amra	Khulna	Sour, small, round
16	Unknown	Khulna	Sweet, medium, ovoid
17	Unknown	Khulna	Sweet, large, ellipsoid
18	Local	Khulna	Sour, medium, ovoid
19	Local	Khulna	Slight sweet, small, oblong
20	Local	Khulna	Sour, small, round

3.2. 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).

3.2.1. DNA Extraction

One gram of stored (at -80°C for one day) younger leaf parts were taken and samples were 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 another 1.5 ml clean tube.

3.2.2. DNA Precipitation

Precipitate DNA from the lysate/homogenate by the addition of 0.5 ml of 100% ethanol per 1 ml of DNAzol was used for the isolation. 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 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 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 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

The DNA was checked in 1 percent agarose gel electrophoresis and quantified using UV spectrophotometer (T 60 UV-Visible, PG instrument Ltd). 2µl of isolated DNA sample from each germplasm of hogplum 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)/(100 OD_{260} unit)

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

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 (Kubelik and Szabo, 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 (%)	*Tm; Ta
OP A01	AGC AGC GCC TCA	66.67%	40^0C; 37.4^0C
OP A02	GCC AGC TGT ACG	66.7%	40^0C; 37.4^0C
OP S01	GTC CAC ACG G	70%	34^0C ; 35.6^0C

**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 3.3. 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. 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 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. The unweighted pair group method with arithmetic averages (UPGMA)-based dendrogram was constructed using the component. Numerical value was considered (1 for presence of band and 0 for absence of band) to construct the dendogram.

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 20 germplasm and displayed different levels of variability concerning the morphological traits of hog plum. 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. Morphological Characterization

Morphological characteristics of 20 germplasm of hog plum have been given in Table 3.1 on the basis of their size, shape and taste. The varieties were showed morphological similarities and dissimilarities. Among the twenty, ten germplasm performed best by considering the sweetness, size and shape. Farmers would like to cultivate those Germplasm for the best performance and demand as human consumption.

4.2. Molecular characterization

Extraction and purification of DNA from fresh leaf tissues was done by using DNAzol protocol (Chomczynski, *et al.*, 1998). The quality of DNA was confirmed by 1 percent Agarose Gel Electrophoresis (Figure 4.1).

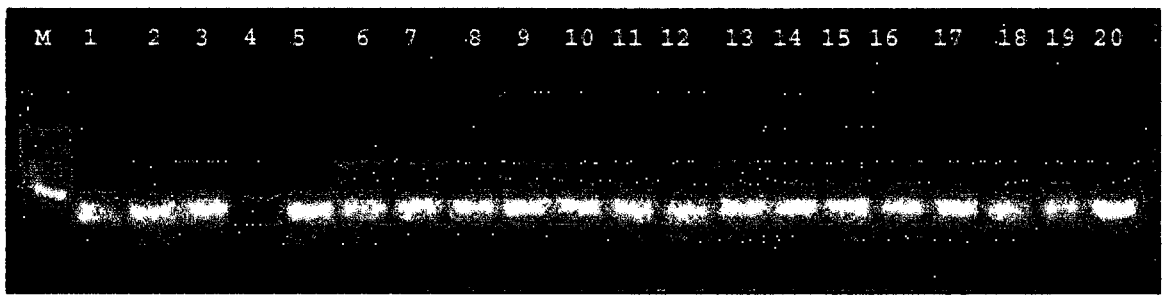

Figure 4.1. The gel profile of *Spondias dulcis* DNA extraction

PCR is the technique of choice for nucleic acid quantification. The DNA quantity was determined by 260nm 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 we 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 we 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	260 nm	GP No	260 nm
01	0.034	11	0.021
02	0.027	12	0.024
03	0.041	13	0.037
04	0.046	14	0.027
05	0.039	15	0.02
06	0.014	16	0.019
07	0.02	17	0.041
08	0.065	18	0.031
09	0.038	19	0.034
10	0.03	20	0.038

In the present study, a polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD) method was used to amplify *Spondias dulcis* DNA using arbitrary oligonucleotide primers. The PCR product reproducibility and banding pattern was confirmed by 1.5% and 2% Agarose Gel Co-Electrophoresis with 100bp ladder. In which it was found that the perfect and clear banding pattern revealed from 2% Agarose Electrophoresis. The method was able to detect the heterogeneity of amplified DNA from the germplasm of hog plum. The study indicated the effectiveness of RAPD analysis in detecting the amount of polymorphisms among the different genotypes of hog plum. Three effective primers (Table 3.2) were selected for determination of polymorphism.

4.2.1 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 hog plum 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 hogplum, one 10-mer (OPS01) 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 362 band patterns of which 63.06% were polymorphic. The highest percent of polymorphic loci (66.67%) was observed for primer A02 and the lowest (60.00%) was from primer S01. 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 4.2. RAPD primers with corresponding bands scored and their size range together with polymorphic bands observed in 20 hog plum 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	A01	8	3	5	62.5	2
2	A02	9	3	6	66.67	1
3	S01	10	4	6	60.00	1
Total	3 primers	27	10	17	189.17	4
Average		9	3.33	5.67	63.06	1.33

Since the amplification detected by these primers produced significant polymorphism and yielded a total of 362 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 hog plum germplasm. The amplification patterns of representative samples of hog plum variety with three different primers have been shown in Figure 4.2, 4.3 and 4.4.

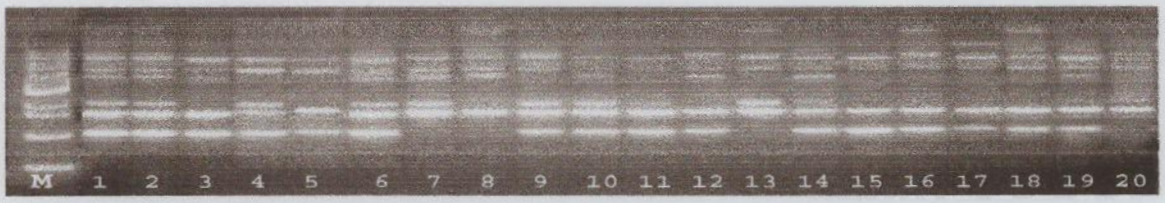

Figure 4.2. RAPD pattern generated by the primer A 01 genotypes of *Spondias dulcis*
Numbers show the serial number of the genotypes and "M" indicates Ladder.

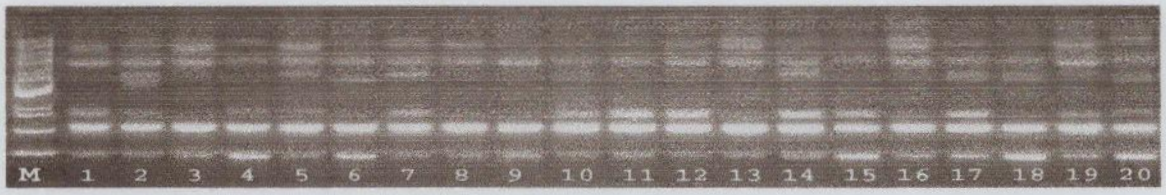

Figure 4.3. RAPD pattern generated by the primer A 02 genotypes of *Spondias dulcis*
Numbers show the serial number of the genotypes and "M" indicates Ladder.

Figure 4.4. RAPD pattern generated by the primer S 01 genotypes of *Spondias dulcis*
Numbers show the serial number of the genotypes and "M" indicates Ladder.

4.2.2. 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.

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

Sample	GP1	GP2	GP3	GP4	GP5	GP6	GP7	GP8	GP9	GP10	GP11	GP12	GP13	GP14	GP15	GP16	GP17	GP18	GP19	GP2
GP1																				
GP2	0.156																			
GP3	0.158	0.163																		
GP4	0.189	0.190	0.143																	
GP5	0.200	0.250	0.212	0.250																
GP6	0.128	0.227	0.189	0.278	0.294															
GP7	0.211	0.163	0.222	0.143	0.212	0.351														
GP8	0.189	0.286	0.200	0.353	0.313	0.222	0.314													
GP9	0.167	0.268	0.235	0.091	0.290	0.200	0.235	0.273												
GP10	0.171	0.130	0.282	0.263	0.222	0.200	0.231	0.316	0.297											
GP11	0.211	0.209	0.278	0.143	0.212	0.297	0.222	0.371	0.176	0.179										
GP12	0.128	0.182	0.135	0.222	0.118	0.211	0.189	0.222	0.200	0.250	0.243									
GP13	0.263	0.256	0.167	0.200	0.273	0.189	0.222	0.257	0.235	0.385	0.222	0.243								
GP14	0.263	0.209	0.333	0.257	0.212	0.243	0.222	0.314	0.294	0.179	0.222	0.243	0.389							
GP15	0.273	0.316	0.290	0.200	0.143	0.313	0.290	0.267	0.241	0.235	0.161	0.250	0.355	0.161						
GP16	0.263	0.256	0.167	0.257	0.273	0.297	0.333	0.143	0.294	0.333	0.333	0.297	0.222	0.333	0.226					
GP17	0.250	0.156	0.263	0.243	0.143	0.333	0.211	0.351	0.278	0.171	0.158	0.179	0.316	0.263	0.212	0.316				
GP18	0.351	0.286	0.257	0.294	0.188	0.222	0.314	0.353	0.273	0.263	0.257	0.222	0.257	0.257	0.267	0.314	0.189			
GP19	0.128	0.182	0.189	0.222	0.118	0.158	0.189	0.278	0.257	0.200	0.243	0.105	0.243	0.135	0.250	0.297	0.231	0.222		
GP20	0.278	0.220	0.235	0.091	0.290	0.257	0.176	0.333	0.188	0.297	0.235	0.257	0.176	0.294	0.241	0.294	0.278	0.273	0.257	

**Nei's diversity coefficient generated by UPGMA analysis*

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 (1979) genetic distance between 20 hog plum genotypes were computed from 0.091 to 0.389 (Table 4.3). Comparatively higher genetic distance (0.389) was found between GP14 vs. GP13. The lowest genetic distance (0.091) was found between GP09 vs. GP04.

There was a genetic variation among the studied of hog plum as indicated by the proportion of polymorphic loci. Estimated genetic variation in the hog plum might be consistent with the fact that it is a polymorphic plant. The range of genetic distance of 20 genotypes is 0.091-0.389 and the difference (0.298) between the highest and lowest genetic distance indicate the presence of variability among the 20 genotype of hog plum.

4.2.3. Genetic Similarity

Inter-population genetic similarity indices for different population pairs have been presented in table 4.4. Percence of genetic similarity among the selected germplasms was carried out by diversity index. This was calculated from the values of Nei and Li (1979) pair-wise comparisons. Here the smaller numbers indicates genetically dissimilarity, whereas larger numbers suggest genetically similar individuals. The range of genetic distance of 20 genotypes is 61.11 to 90.91 (Table 4.4). Comparatively higher genetic distance (90.91) was found between GP09 vs. GP04. The lowest genetic distance (61.11) was found between GP14 vs. GP13.

Table 4.3. Genetic similarity index from RAPD data of different hogplum germplasm

Sample	GP1	GP2	GP3	GP4	GP5	GP6	GP7	GP8	GP9	GP10	GP11	GP12	GP13	GP14	GP15	GP16	GP17	GP18	GP19	GI
GP1																				
GP2	84.44																			
GP3	84.21	83.72																		
GP4	81.08	80.95	85.71																	
GP5	80.00	75.00	78.79	75.00																
GP6	87.18	77.27	81.08	72.22	70.59															
GP7	78.95	83.72	77.78	85.71	78.79	64.86														
GP8	81.08	71.43	80.00	64.71	68.75	77.78	68.57													
GP9	83.33	73.17	76.47	90.91	70.97	80.00	76.47	72.73												
GP10	82.93	86.96	71.79	73.68	77.78	80.00	76.92	68.42	70.27											
GP11	78.95	79.07	72.22	85.71	78.79	70.27	77.78	62.86	82.35	82.05										
GP12	87.18	81.82	86.49	77.78	88.24	78.95	81.08	77.78	80.00	75.00	75.68									
GP13	73.68	74.42	83.33	80.00	72.73	81.08	77.78	74.29	76.47	61.54	77.78	75.68								
GP14	73.68	79.07	66.67	74.29	78.79	75.68	77.78	68.57	70.59	82.05	77.78	75.68	61.11							
GP15	72.73	68.42	70.97	80.00	85.71	68.75	70.97	73.33	75.86	76.47	83.87	75.00	64.52	83.87						
GP16	73.68	74.42	83.33	74.29	72.73	70.27	66.67	85.71	70.59	66.67	66.67	70.27	77.78	66.67	77.42					
GP17	75.00	84.44	73.68	75.68	85.71	66.67	78.95	64.86	72.22	82.93	84.21	82.05	68.42	73.68	78.79	68.42				
GP18	64.86	71.43	74.29	70.59	81.25	77.78	68.57	64.71	72.73	73.68	74.29	77.78	74.29	74.29	75.33	68.57	81.08			
GP19	87.18	81.82	81.08	77.78	88.24	84.21	81.08	72.22	74.29	80.00	75.68	89.47	75.68	86.49	75.00	70.27	76.92	77.78		
GP20	72.22	78.05	76.47	90.91	70.97	74.29	82.35	66.67	81.25	70.27	76.47	74.29	82.35	70.59	75.86	70.59	72.22	72.73	74.29	

4.2.4. Dendogram

Morphological characterization was determined and, showed genetic similarities and dissimilarities by collecting data and constructing the UPGMA dendrogram to estimate genetic diversity of hog plum phenotypically. Twenty 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). UPGMA dendrogram was constructed using neighbor joining method of cluster analysis on the basis of distance matrix.

The dendrogram (Fig. 4.5) shows that all the germplasm were grouped into two major clusters G1 and G2. Cluster **G1** is subdivided into **G1.I** and **G1.2**, and cluster **G1.I** is subdivided into **G1.I.1** and **G1.I.2.** Again cluster **G2** is subdivided into **G2.I** and **G2.2**, cluster **G2.I** is subdivided into **G2.I.1** and **G2.I.2**, while cluster **G2.2** is subdivided into **G2.2.1** and **G2.2.2**, and cluster **G2.2.1** is subdivided into **G2.2.1.a** and **G2.2.1.b**.

From the dendrogram (Fig: 4.5) it was found that the hogplum germplasms collected from Khulna were distributed under different clusters. We did not find any similarity between them and found similarity between two Belati amra. GP7 is most different in cluster G1. Unknown variety GP12 (cluster G2.I.2), GP16 (cluster G1.I.1) and GP17 (cluster G2.2.1.b) showed similarity with local variety. Presence of so many subclusters indicates sound variation among the germplasms.

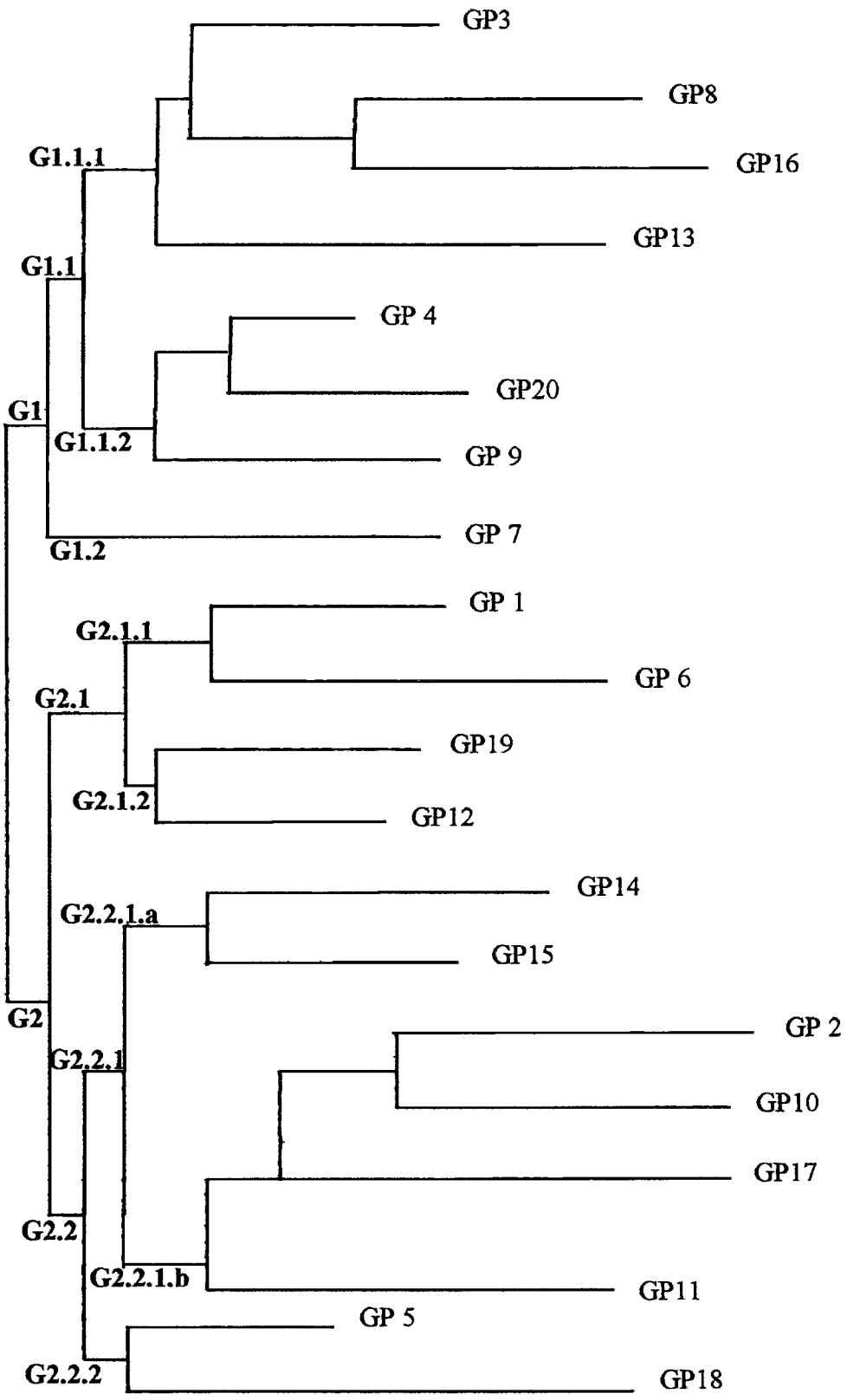
GP3
GP8
G1.1.1
GP16
GP13
G1.1
GP 4
GP20
G1
G1.1.2
GP 9
GP 7
G1.2
GP 1
G2.1.1
GP 6
G2.1
GP19
G2.1.2
GP12
GP14
G2.2.1.a
GP15
G2
GP 2
G2.2.1
GP10
GP17
G2.2
G2.2.1.b
GP11
GP 5
G2.2.2
GP18

Figure 4.5. Unweighted Pair Group Method of Arithmetic Mean (UPGMA) dendrogram based on Nei's (1979) genetic distance, according to RAPD analysis of 20 germplasm

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 *S. dulsis* 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 hog plum.

CHAPTER V

CONCLUSION

Variability studies of 20 *S. dulsis* germplasm belonging to south-western coastal zone of Bangladesh revealed a high coefficient of variation. Recently 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 hog plum 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. RAPD analysis is efficient and accurate for the investigation of distribution of local/commercial hog plums. In addition, RAPD makers have proved to be effective for fingerprinting and characterizing the genetic basis of each hog plum sample for assessing genetic diversity and relatedness between accessions. The geographical locations, growth altitude, and climates may contribute the polymorphic RAPD of hog plum trees in Bangladesh. 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 hog plum breeding programs as well as informed and efficient management of germplasm collections.

CHAPTER VI

REFERENCES

Alam, M.A., Pallavi, G., Aswini, K.G., Gyan, P.M. and Pradeep, K.N. 2009. Assessment of genetic diversity among *Podophyllum hexandrum* genotypes of northwestern Himalayan region for Podophyllotoxin production. Indian J. Biotech., 8: 391-399.

Al-Soud, W.A., Ouis, I.S., Li, D.Q., Ljungh, S. 2005. Characterization of the PCR inhibitory effect of bile to optimize real-time PCR detection of Helicobacter species. FEMS *Immunol. Med. Microbiol.*, 44: 177-182.

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.

Anonymous. 2008. Introduction to Genetic Diversity. Cheetah Conservation Fund. 2002. [www.cheetah.org].

Anonymous. 2008. Monoculture and the Irish Potato Famine. Understanding Evolution. Berkley University. <evolution.berkley.edu>

Anonymous. 2011. National Biological Information Infrastructure. *Int. Genet. Divers.* U.S. Geological Survey.

Arif, M. and Sheeba, F. 2010. Pharmacognostic Investigation and Authentication of Potentially Utilized Fruit *Spondias mangifera* (willd). *Int. J. Pharma. Clin. Res.*, 2(1): 31-35.

Ayoka, A.O., Akomolafe, R.O., Akinsomisoye, O.S. and Ukponmwan, O.E. 2008. Medicinal and Economic Value of *Spondias mombin*. *African J. Biomed. Res.*, 11: 129 – 136.

Bailey, L.H. 1950. The standard cyclopedia of horticulture. The Macmillan Publishing Company Ltd; New York. pp. 392-393.

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 Widrlechner, 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 Sauls, J.W. 1994. *Spondias* in florida. Fruit crops fact sheet. Florida cooperative extension services, University of Florida, Gainesville. Fact Sheet HS-63.

Chen, T.W., Chai, N.C., Wang, C.Y. and Shyu, Y.T. 2006. Molecular identification and analysis of *Psidium guajava* L. from indigenous tribes of Taiwan. *J. Food and Drug Anal.*, 15(1): 82-88.

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

Chomczynski, P., Wilfinger, W. and Mackey, K. 1998. Isolation of genomic DNA from human, animal, and plant samples with DNAzol reagents. *Biotech. Int.*, pp. 185-188.

Daulmerie, S. 1994. Investigations on Golden Apple production with particular reference to post-harvest technology and processing. Port of Spain, Trinidad and Tobago: Inter-American Institute for cooperation on Agriculture. pp. 1-10.

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. pp. 15-47.

Evangelopoulos, A., Legakis, N. and Vakalis, N. 2001. Microscopy, PCR and ELISA applied to the epidemiology of amoebiasis in Greece. *Parasitol. Int.*, 50: 185-189.

Franquin, S., Marcelin, O., Aurore, G., Reynes, M. and Brillouet, J.M. 2005. Physico-chemical characterisation of the mature-green Golden apple (*Spondias cythere* Sonnerat). *Fruits.*, 60(3): 203-210.

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

Geurts, F., Blaak, G. and Baradj, E.T. 1986. Genetic resources of tropical and subtropical fruits and nuts (excluding Musa). *Int. Board Plant Genet. Resources.*, pp. 18-19.

Gois, I.B., Silva-Mann, R., Ferreira, R.A. and Ferreira, R.A. 2008. Diversidade Genética de Indivíduos de *Spondias lutea* L., por Marcadores RAPD, Procedentes do Baixo São Francisco Sergipano. *In:* Livro de Resumos do XVIII Encontro de Iniciação Científica e IV Encontro de Pós-Graduação da Universidade Federal de Sergipe, Aracaju. Available at [http://www.posgrap.ufs.br/down/Livro_Resumos_18EIC_4EPG.pdf]. Accessed August 10, 2010.

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

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.

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 Biotech.*, 6(37): 1472-6750.

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

Lima, A.T.B., de Souza, V.A.B., Gomes, R.L.F. and Lima, P.S.C. 2011. Molecular characterization of cajá, *Spondias mombin* (Anacardiaceae) by RAPD markers. *Genet. Mol. Res.*, 10 (4): 2893-2904

McMillan, R.T. 1986. Serious diseases of tropical fruits in Florida. *Proc. Fla. State Hortic. Soc.*, 99: 224-227.

Mehetre, S.S., Gomes, M. and Eapen, S. 2004. RAPD analysis of hybrid nature of the offspring of *Gossypium hirsutum* x G. raimondii. *Curr. Sci.*, 84, 24-28.

Mendonca, R.U., Moura, C.F.H., Alves, R.E. and Figueredo, R.W. 2008. Caracterização Física de Frutos da Cajazeira (*Spondias mombin* L.) Oriundos da Região Meio-Norte do Brasil. *In:* Anais do XX Congresso Brasileiro de Fruticultura and LIV Annual Meeting of the Interamerican Society for Tropical Horticulture, Vitória. 4p.

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.

Mitchell, J.D. and Daly. D.C. 1998. "The "tortoise's caja" - a new species of *Spondias* (Anacardiaceae) from southwestern Amazonia" *Brittonia.*, 50(3): 447-451.

Morton, J.F. 1987. Ambarella, *In:* Fruits of Warm Climates. J.F. Morton publisher. Florida, 6(3): 240-244.

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

Nei, M. and Li, W.H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. U. S. A.*, 76: 5269-5273.

Oliveira, A.C.B., Caixeta, E.T., Zambolim, E.M. and Zambolim, L. 2007. Aplicação Técnica de Marcadores Moleculares no Melhoramento de Plantas. Documentos IAC No. 81. Instituto Agronômico de Campinas, Campinas. Accessed August 10, 2010.

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.

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

Persad, C. 1997. Golden apple. *Spondias cytherea* Sonn. Tree. *Tropic. Fruits Newsl.*, 23: 11-13.

Popenoe, W. 1974. Mannual of tropical and subtropical fruits. Hafner Press, New York. 474p.

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 J. Biotech.*, 3(4): 253-255.

Ramos, J.R., Telles, M.P., Diniz-Filho, J.A., Soares, T.N., *et al.* 2008. Optimizing reproducibility evaluation for random amplified polymorphic DNA markers. *Genet. Mol. Res.*, 7: 1384-1391.

Rashid, A., Kader. A. and Hossain, M.A. 1987. Bangladesher Fol. Published by: Krishi somprosaron odhidoptor, Bangladesh. pp. 161-163.

Rodriguez, J.M., Berke, T., Engle, L. and Nienhuis, J. 1999. Variation among and within *Capsicum* species revealed by RAPD markers. *Theor. Appl. Genet.*, 99: 147-156.

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, H.A. New York: *Stockton Press*, 1:7-16.

Sarwat, M., Das, E.S. and Srivastava, E.P.S. 2008. Analysis of genetic diversity through AFLP, SAMPL, ISSR and RAPD markers in *Tribulus terrestris*, a medicinal herb. *Plant Cell Rep.*, 27: 519–528.

Schaal, B.A., Hayworth, D.A., Olsen, K.M., Rauscher, J.T. and Smith, W.A. 1998. Phylogeographic studies in plants: problems and prospects. *Mol. Ecol.*, 7: 465-474.

Silva, C.J.D. 2009. Caracterização Genética de Cajazeiras (*Spondias mombin* L.) (Anacardiaceae) por Meio de Marcadores Moleculares. Master's thesis, Universidade Federal Rural de Pernambuco, Recife.

Slatkin, M. 1987. Gene flow and the geographic structure of populations. *Science.*, 236: 787-792.

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? *J. Int. Econ.*, 48: 151–177.

Soares, E.B., Gomes, R.L.F., Carneiro, J.G.M. and Nascimento, F.N. 2006. Característica física e química de frutos de cajazeira. *Rev. Bras. Frutic.*, 28: 518-519.

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

Telles, M.P.C. 2005. Estrutura Genética Populacional de *Physalaemus cuvieri* Fitzinger, 1986 (Anura: Leptodactylidae) e Padrões de Ocupação Humana no Estado de Goiás. Doctoral thesis, Universidade Federal de Goiás, Goiânia. Gois, I.B., Silva-Mann, R. and Ferreira, R.A. 2009. Diversidade genética de *Spondias lutea* L. em uma população do baixo São Francisco sergipano, por meio de isoenzimas. *Sci. For. Piracicaba.* 37: 55-60.

Torre, A., Lopez, S., Yglesias, E. and Cornelius, J.P. 2008. Genetic (AFLP) diversity of nine *Cedrela odorata* populations in Madre de Dios, southern Peruvian Amazon. *Forest Ecol. Manag.*, 255: 334–339.

Verweij, J.J., Blange, R.A., Templeton, K., Schinkel, J. 2004. Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR. *J. Clin. Microbiol.*, 42: 1220-1223.

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.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.*, 18: 6531-6535.