ASSESSMENT OF GENETIC VARIABILITY AMONG SELECTIVE GUAVA (*Psidium guajava* L.) VARIETIES BY RAPD ANALYSIS

A Thesis submitted

By

Farzana Alam
Examination Roll No. MS 080711
Session: 2007-2008

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Khulna-9208, Bangladesh**

May, 2011

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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 The findings of the research has not been/ will not be published anywhere without the concurrence of the concerned supervisor.

Farzana Alam

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Dedicated

To

My Beloved Late Mother

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ABSTRACT

This study was conducted to assess the genetic diversity using random amplified polymorphic DNA (RAPD) variation of twelve varieties of guava (*Psidium guajava* L.) growing in southern part of Bangladesh. Morphological characterization of guava varieties showed a wide range of variation. Highest variability was observed in Poly and Jelly varieties. DNA was extracted from twelve guava varieties by using modified CTAB method and was quantified by mini-gel method for RAPD analysis. RAPD analysis was carried out and only unambiguous, reproducible and scorable polymorphic fragments were taken into consideration for analysis. Polymerase chain reaction (PCR) with 5 arbitrary 10-mer and 5 arbitrary 12-mer oligonucleotide primers produced a total of 50 bands of which 75.23 per cent were polymorphic. The highest percentage of polymorphic loci (100%) was observed for primer A and the lowest (50%) was for A03 primer. The UPGMA dendogram revealed the segregation pattern and the difference of evolutionary changes from origin for each variety. Guava varieties were separated into two main groups, one of them was made up Chineese, Jelly, Kazi, Apple, L-49, and Local-2. The other one was made up Local-1, Local-3, Poly, Kashi, Thai and Bombay. The range of genetic distance was observed 0.2054 (Apple and Kazi) to 0.7837(Bombay and Local-3). The highest genetic distance 0.784 indicating Apple and Kazi derived from different origin and could be utilized in breeding programme for improving trait of interest. This scientific information could be used for further improvement of guava genotypes through breeding program.

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Abbreviations

μM = Micro Molar

°C = Degree Celsius

CTAB = N-Ctyle-N,N,N-Trimethyle Ammonium Bromide

DH2O = Distilled Water

e.g. = exempli gratia=for example

EDTA = Ethylenediamineteraacetic Acid

et al.= et alia = and other people

Et-Br = Ethedium Bromide

Etc = et cet era = and others

gm = Gram

HCl = Hydrochloric Acid

i.e.= id est = that is

M = Molar

Mg = Miligram

Mg/l = Miligram per litter

ml = milliliter

mM = Mili molar

NaCl = Sodium Chloride

pH = Acid Base Measurement of Solution

PVP=Polyvinyl Pyrolidonev

viz = videlicet=namely

Chapter One

Introduction

1. Introduction

One of the most gregarious of fruit, the guava, *Psidium guajava* L., of the myrtle family (Myrtaceae), is almost universally known (Morton, 1987). This is native to tropical South America, but it is cultivated in many tropical and subtropical part of the world. It is one of the 50 most well known edible tree fruits of the tropical and subtropical climate. Several species are grown commercially. Apple Guava and its cultivars are those most commonly traded internationally. It claims to be the fourth most important fruit in India (Chandra and Mishra, 2002)

Guava tree is easy to recognize because of its smooth, thin, coppered bark that flakes off, showing the greenish layer beneath. Young twigs are quadrangular and downy. The leaves are evergreen, opposite, short-petioled, oval or oblong-elliptic, somewhat irregular in outline; 7-15 cm long, 3-5 cm wide, leathery, with conspicuous parallel veins, and more or less downy on the underside. The white flowers, borne singly or in small clusters in the leaf axils, are 1 in (2.5 cm) wide, with 4 or 5 white petals which are quickly shed, and a prominent tuft of perhaps 250 white stamens tipped with pale-yellow anthers. The fruit, exuding a strong, sweet, musky odor when ripe, may be round, ovoid, or pear-shaped, 5-10 cm long, with 4 or 5 protruding floral remnants (sepals) at the apex; and thin, light-yellow skin, frequently blushed with pink. When immature and until a very short time before ripening, the fruit is green, hard, gummy within and very astringent (Morton, 1987).

The genus *Psidium* contains about 150 species. In Bangladesh, common cultivars of guava are kazi, deshi, swarupkhathi, lal peyara, kushum payara etc. Kazi peyara is the most

popular variety with commercial cultivation taking all over Bangladesh. Popular cultivars in India are Apple Colour, Behat Coconut, Lucknow 42, Lucknow 49, Banaras, Chittidar, Allahabad safeda, Baruipur, Pear shaped, Seedless etc (Chandra and Mishra, 2002).

According to Saswati, (2010), *Psidium guajava* had the chromosome count of 2n = 44 with a karyotype formula $2A^{\&\&}_{sm}+8B_m+24C_{nm}+10D_{sm}$. . The karyotype of the species was slightly asymmetric with a little variation in chromosome length. The chromosomes are small sized and mostly represented by median types of chromosomes. There are 4 pairs of median (m), 12 pairs of nearly-median (nm) and 6 pairs of sub-median (sm) chromosomes in which one pair has secondary constriction. On the basis of primary constrictions and size of chromosomal complements, the chromosomes are grouped into four morphological types: Type A Small chromosomes (1.80 ± 0.57 μm) bearing two constrictions, primary and secondary; both are sub-median in position. Type B Chromosomes are short sized measuring from. 46 ± 0.24 μm to 1.10 ± 0.16 μm and with median primary constriction. Type C Short sized chromosomes with nearly median primary constrictions and are measured from 1.80 ± 0.22 μm to 1.19 ± 0.20 μm. Type D Chromosomes with sub – median primary constriction and size range is 1.88 ± 0.46 μm to 1.25 ± 0.37 μm. Minute details of the chromosomal complements has been characterized by having karyotype formula chromosomal complements was 1.48 μm with a size range of 1.10 μm – 1.88 μm and with a TF value 40.07%. Meiotic analysis of pollen mother cells of *P. guajava* revealed 22 normal bivalents . No meiotic irregularities like secondary association were recorded in their meiotic cells. Anaphase distribution of bivalent chromosomes was normal.

For an effective breeding program, information concerning the extent and nature of genetic diversity within and between species is a prerequisite for any crop improvement. It is particularly useful for organizing germplasm, characterizing individual accessions and genotypes, identification of cultivars as well as a general guide in selection of the parents for hybridization to maximize the expression of heterosis (Singh, *et al.*, 2006). In addition, an unambiguous, reliable, fast and cost-effective assessment of genetic diversity is important for determining the uniqueness and distinctiveness of the phenotypic and genetic constitution of genotypes to protect breeder's intellectual property rights (Franco, *et al.*, 2001).

Guava is an allogamous fruit crop, which is highly heterozygous. Several guava cultivars have emerged as a result of seedling selection and seedling of these cultivars are being commercially exploited through seed propagation which has indirectly given rise to several types which are not true to the commercial type and vary in several characters from the parent population. Morphological characters may not be reliable to discriminate between closely related guava genotypes although several morphological traits such as fruit colour, leaf shape and size may be useful but they lose their usefulness for assessment of genetic diversity between closely related guava genotype. Molecular markers can be gainfully employed to discriminate between species and cultivars of guava. Recently, few reports have appeared on use of RAPD marker for genetic assessment of guava (Prakash, *et al.*, 2002).

Molecular markers are especially advantageous for improving qualitative and quantitative traits in perennial fruit crops that is otherwise time consuming and difficult to tag the genes conferring resistance to pathogens, tolerance to abiotic stresses, quality parameter and quantitative traits. By using marker-assisted selection, the breeder could select molecular markers that are tightly linked to resistant genes for identifying resistant genotypes. Unfortunately, information pertaining to molecular assisted breeding is lacking in guava and there is an urgent need to develop genetic map of guava in days to come (Chandra and Mishra, 2002).

Among the different types of molecular markers, RAPDs are useful for the assessment of genetic diversity (Williams, *et al.*, 1990) owing to their simplicity, speed and relatively low-cost (Rafalski and Tingey, 1993) compared to other molecular markers. RAPD markers have been used extensively to classify accessions, identify cultivars and hybrids (Meng, *et al.*, 1996), and analyse genetic diversity (Lee, *et al.*, 1996; Levi, *et al.*, 2001; Garcia, *et al.*, 2001; Silberstein, *et al.*, 1999; Gwanama, *et al.*, 2000; Sureja, *et al.*, 2006). The RAPD approach depends on polymerase chain reaction (PCR). It can rapidly differentiate closely related individuals. RAPD Marker is highly suitable for quick fingerprinting, 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).

DNA polymorphism can be used as molecular markers and is considered to have significant impact on applied plant breeding. The polymerase chain reaction (Sakai *et al.*, 1985; Mulis and Faloona, 1987) provides a rapid safe and efficient method for screening large

population. Unlike Southern blot analysis, PCR can directly distinguish polymorphism including insertion or deletion and point mutation events. Besides, RAPD analysis provideds DNA polymorphism even among related species rapidly and feasibly (Williams *et al.*, 1990). RAPD fingerprinting can be used to trace genetic or epigenetic changes at the genome level (Schmitt *et al.*, 2001).

After considering the above points, this research work was conducted with following purposes.

1. To assess the morphological variations through phenotypic study among selected guava varieties.
2. To investigate the genetic variabilities and cluster analysis of selected guava varieties at the genomic level.
3. To select parents for better and more production

Chapter Two

Review of Literature

2. Literature Review

2.1 Preface

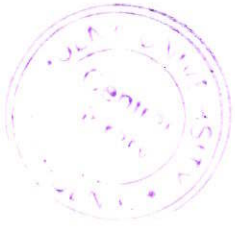
Guava (*payara*) is a berry like fruit of any of various myrtaceous trees or shrubs of the genus *Psidium*, especially *P. guajava* (family Myrtaceae). It originated in tropical America (Mexico to Peru), where it still occurs in the wild. Guava is often called the "apple of the tropics". The plant was introduced by the Portuguese to the Indian subcontinent by the early 17th century. Guava stands fifth in production among the most important fruit crops of Bangladesh and can be grown all over the country. The annual production is about 45,000 m tons in an area of about 10,000 ha. The districts of Barisal, Pirojpur, Jhalokathi, and Chittagong are the main guava producing areas. Some of the important varieties are known by the name of the places where these are grown commercially. Thus Swarupkathi is from Barisal, Mukundapuri from Brahmanbaria and Kanchannagar from Chittagong. (Banglapedia, http://www.banglapedia.org/httpdocs/HT/G_0215.HTM).

Guava is important in international trade and domestic economy of several countries in warmer climates (Menzel, 1985). Because of its easy cultivation under variable soils and climates, high nutritional value, and popular uses in such processed products like juice, preserves, and dairy or bakery items, guava is favorite of billions of people in the tropical and subtropical countries but not so much in the temperate regions including the United States. Due to their astringent properties, mature guava fruits, leaves, roots, bark, and immature fruits are used in local medicines to treat gastroenteritis, diarrhea, and dysentery (Morton 1987; Purseglove, 1968). Guava consumption has been reported to significantly reduce serum total cholesterol, triglycerides, and blood pressure with the opposite effect (an

explicit increase) in high-density lipoprotein (HDL) or good cholesterol (Singh *et al.* 1992). Furthermore, high concentrations of pectin in guava fruit may play a significant role in the reduction of cholesterol and thereby decrease the risk of cardiovascular diseases. (Yadava, 1996.)

2.1.1 Morphology

The guava plant grows symmetrically dome-shaped with broad, spreading, low-branching canopy and a shallow-rooted small tree of 3 to 10 m in height, branching close to the ground and often heavily suckering from the base of the trunk. The green to reddish-brown and smooth bark on older branches and trunk peels off in thin flakes. The four-angled young twigs of guava are easily distinguished. The simple leaves of guava are opposite, 10 to 15 cm long, oval to oblong-elliptic, smooth, and light green in color. The perfect epigynous flowers 25 to 30 mm in diameter with four incurved white petals and a large tuft of white stamens with yellowish anthers, are borne solitary or in clusters of 2-3 in leaf axils on new growth from mature wood. Self-pollination is conspicuous (60% to 75%) since even isolated trees produce good crop; however, the distribution of cross-pollination by insects, is about 35% (Purseglove, 1968; Menzel, 1985). Based on the cultivar, guava fruit could be an ovoid, spherical or pyriform berry topped by calyx lobes. Generally, guava fruits measure 4 to 10 cm in diameter and weigh from 100 to 450 g; Guava has a blend of sweet and acid flavors when fully ripe and characteristically a rather penetrating aroma (Rathore, 1976; Wilson, 1980; Thonte and Chakrawar, 1982; Menzel, 1985; Morton, 1987; Yadava, 1994). From fruit set to maturity, guava takes 150 days. Most of the famous cultivars contain numerous small, hard, yellowish-cream colored seeds which are imbedded in the soft pulp. However,



seedless cultivars are available in many countries and have great potential to become very popular worldwide in the future.

2.1.2 Cultivation

The guava is successfully cultivated in a wide range of growing conditions. It is fairly well adjusted to different rainfall while soil pH ranging from 4.5 to 8.2 is proper for desirable plant performance. While guava trees tolerate poor soils, fruit production is substantially enhanced when grown in rich soils under proper management. As a general rule, guava requires very little attention. Nevertheless, guava trees can be grown as cordons on wire fence. Trees can be planted from 2.5 to 8 m in any combination for rows and tree spacing. The most common tree spacings are 3 x 5 m and 5 x 6 m with trees established along the contours. (Yadava, 1996.) In order to enhance fruit production and minimize requirements for labor, Mohammed *et al.*, (1984) developed an intensive system for guava growing. They tested plant densities of 27,000 (60 x 60 cm), 37,000 (90 x 30 cm), and 73,000 (45 x 30 cm) plants/ha in combination with the use of growth regulators for plant size control. Highest yield of good quality fruits was obtained at density of 27,000 plants/ha.

2.1.3 Harvesting and Storage

Immature guavas do not ripen off the trees; fruits may soften, but never develop abundant color, and typical flavor associated with good eating enjoyment (Menzel, 1985; Yadava, 1994). Over-ripe fruits drop. There are no visible physical appearances or chemical indices of fruits that consistently reflect the appropriate stage of fruit maturity for harvest. Fruit harvesting should be carried out when the fruit is fully developed, matured, and began to

show signs of color change from green to yellowish. Harvested fruits should be wrapped individually in paper towels and packed in padded layers before shipping or refrigeration. Wrapping of guava fruits suppresses weight loss and preserves glossiness (Morton, 1987). Storage of wrapped guavas at cool temperatures extends post-harvest life up to 5 weeks with retention of healthy fruit quality and no significant reduction in nutrient contents; however, fruits stored unwrapped loose moisture and shininess (Yadava, 1994, 1996). Desiccation, browning of fruit skin tissue with concomitant loss of flesh firmness, and the high rate of physiological loss of weight limit the storage life of guava fruit (Singh *et al.*, 1992). Treatment of harvested guavas with 100 mg L^{-1} morphactin increases storage life of fruit by restricting fungal decay and curtailing loss of color, weight, sugars, ascorbic acid, and non-volatile organic acids (Prasad and Shukla, 1979; Morton, 1987). Furthermore, guava fruits should be packed in the natural posture (with the pedicel end of the fruit kept upward) in order to retain better quality for longer periods of time (Siddiqui *et al.*, 1991).

2.1.4 Nutritional value

According to Healthaliciousness.com (2008), guavas are often included among super fruits, being rich in dietary fiber, vitamins A and C, folic acid, and the dietary minerals, potassium, copper and manganese. Having a generally broad, low-calorie profile of essential nutrients, a single common guava fruit contains about four times the amount of vitamin C as an orange. However, nutrient content varies across guava cultivars. Although the strawberry guava (*P. littorale* var. *cattleianum*), notably containing 90 mg of vitamin C per serving, has about 25% of the amount found in more common varieties, its total vitamin C content in one serving still provides 100% of the Dietary Reference Intake for adult males.

Guavas contain both carotenoids and polyphenols like (+)-gallocatechin. (+)-gallocatechin has been identified as a bio-antimutagenic compound in guava leaves (Matsuo, *et al.*, 1994). The presence of major classes of antioxidant pigments – give them relatively high potential antioxidant value among plant foods (Jiménez-Escrig, *et al.,* 2001, Hashimotto *et al.*,2005, Mahattanatawee *et al.* 2006) The pigments are produced the fruit skin and flesh. Guavas that are red-orange have more pigment content as polyphenol, carotenoid and pro-vitamin A, retinoid sources than yellow-green ones (Wrolstad, 2001).

Table 2.1 Nutritive value of Guava

Common Guava, per 165 g of individual fruit portion	Amount
Calories	112
Moisture	133 gm
Dietary Fiber	8.9 g (36%)
Protein	4.2 g (8%)
Fat	1.6 g (2%)
Ash	2.3 g
Carbohydrates	23.6 g (8%)
Calcium	30 mg (3%)
Phosphorus	66 mg (7%)
Iron	0.4 mg (2%)
Potassium	688 mg (20%)
Copper	0.4 mg (19%)
Beta-carotene (Vitamin A)	1030 IU
Ascorbic acid (Vitamin C)	377 mg
Thiamin (Vitamin B_1)	0.1 mg (7%)
Riboflavin (Vitamin B_2)	0.1 mg (4%)
Niacin (Vitamin B_3)	1.8 mg (9%)
Folic acid	81 mcg (20%)

% Daily Value in parentheses. Nutrient data source: US Department of Agriculture National Nutrient Database from Nutritiondata.com

2.2 Genetic Diversity

Assessment of genetic diversity is important in plant breeding if improvement of fitness is deemed essential. For assessment of genetic diversity, molecular markers have been generally superior to morphological, pedigree, heterosis, and biochemical data (isozymes and chromatography) (Melchinger *et al.*, 1991; Melchinger, 1993). Genetic diversity is commonly 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 pumpkin but results should be applicable to other species. Molecular marker-based GD also has potential for assessing changes in genetic diversity over time protection of intellectual property rights (Smith *et al.*, 1990), 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).

An equally important application of molecular marker-based GD may be in the selection of parents for crossing in a breeding program. This application deserves serious attention because breeders currently rely primarily on pedigree and performance data for choosing parents in breeding programs. Using molecular markers to select parents has the potential to allow simultaneous maintenance of genetic diversity and performance. Dudley *et al.*, (1992) presented one application of molecular markers for choosing parent. Using molecular markers to choose parents likely will require establishment of a relation between GD and genetic variation, and many of the same conditions necessary for predicting hybrid performance may be required for choosing parents. Using molecular markers as a diagnostic tool to survey new or exotic germplasm for novel genetic diversity also may be possible. It

is unlikely, however, that this use will be possible with random genomic or cDNA clones because molecular marker-based genetic diversity will not guarantee genetic diversity for the traits of interest. Screening with probes of expressed genes with known function offers the greatest potential in this area.

Genetic divergence or genetic diversity is the process in which two or more populations of an ancestral species accumulate independent genetic changes (mutations) through time, often after the populations have become reproductively isolated for some period. In some cases, subpopulations living in ecologically distinct peripheral environments can exhibit genetic divergence from the remainder of a population, especially where the range of a population is very large. The genetic differences among divergent populations can involve silent mutations (that have no effect on the phenotype) or rise to significant morphology changes. Genetic divergence will always accompany reproductive isolation, either due to novel adaptations or due to genetic drift, and is the principle mechanism underlying speciation.

The divergence analysis has a definite role to play during efficient choice of divergent parents in any plant breeding program. Its importance in the improvement of a crop has been stressed in both self and cross –pollinated crops (Murty and Anand,1966; Gaur, *et al.*,1978). The quantification of genetic diversity through biometrical procedures (Anderson,1957) has made it important to know the source of genes for a particular trait within the available germplasm.

2.3 DNA Extraction

DNA extraction and separation by agarose gel electrophoresis is a simple and exciting process. PCR techniques such as RAPD (Williams, *et al.*, 1990), CAPS, STSs (Mazur, *et al.*, 1995), microsatellite (Panaud, *et al.*, 1996), and AFLP (Vos, *et al.,* 1995), have been used in plants for molecular mapping, identification of genotypes associated with genes of interest, and genetic diversity studies. 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, *et al.*, 1991; Edwards, *et al.*, 1991; Tomas, *et al.,* 1989), 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).

The use of liquid nitrogen for grinding of the plant material and homogenization buffers which contain CTAB (Watson and Thompson, 1986) or polyamines like spermine and spermidine (Hewish and Burgoyne, 1973) as well as the addition of EtBr to inhibit the endogenous nuclease activity (Luthe and Quatrano, 1980) allowed to obtain a high molecular weight plant DNA. Most of these methods for extraction and especially for the purification of plant DNA have employed caesium chloride density gradients to eliminate proteins, RNA and the severe polysaccharides contaminations. This method replaced successfully the classical method of phenol/chloroform purification of DNA (Razin, 1988) but it is not conveniant and time consuming since buoyant density centrifugation is required. Recently a rapid and simple method has been proposed for purification of DNA from human cells (Boom, *et al.,* 1990). Briefly, this method is based on both the lysing and nuclease-inactivating activities of the chaotropic agent guanidinium thiocyanate (GuSCN) together with the high binding properties of silica particles or diatoms to DNA in the presence of this agent. The use of diatoms has great significance for the preparation Celite (the fossilized cell walls of unicellular algae) for purification of crude nuclear lysate DNA or phenol-deproteinized DNA from excised cotyledons of *Cucurbita pepo L.* (zucchini).

Doyel and Doyel (1987) stated the standard protocol used in their laboratories is based on the cetylrimethylammonium bromide (CTAB) procedure of total DNA isolation.

According to Doyel and Doyel (1990), DNA can be successfully isolated from och plants by simply increasing the CTAB concentration in the isolation buffer. CTAB binds to fructans and other polysaccharides and forms complexes that are removed during subsequent chloroform extraction. When choosing a particular protocol, one should also keep in mind

those different kinds of experiments demand varying levels of DNA purity. For example, DNA fingerprinting analyses based on restriction and hybridization experiments require relatively pure high molecular weight DNA, while polymerase chain reaction (PCR) may also work on rather crude and somewhat degraded DNA sample (Berthomieu and Meyer, 1991).

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. 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 to the development of several novel genetic assays based on selective amplification of DNA (Erlich, 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; Welsh and McClelland, 1990). The simplicity and applicability of the RAPD technique have attracted many scientists from diverse avenues over the techniques.

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 and Widrlechner, 1995).

Genetic markers should be easily scored, negligible effects on plant growth, rapidly, safely, and inexpensive scored (Muray, 1988, Smith, 1989; Chunwongse, *et al,.* 1993). Polymorphic DNA is thought to provide ideal genetic markers because (i) nucleotide sequence variation is selectively neutral (Nei, 1987); (ii) certain complication reducing heritability of protein may be minimized; (iii) three distinct genomes as nuclear, chloroplast, mitochondria, may each involve according to different modes and tempos (Bretting and Widrlechner, 1995).

2.4.1 Uses of Genetic Marker

Genetic markers can be used to study the relationship between an inherited disease and its genetic cause (for example, a particular mutation of a gene that results in a defective protein). It is known that pieces of DNA that lie near each other on a chromosome tend to be inherited together. This property enables the use of a marker, which can then be used genetic markers have to be easily identifiable, associated with a specific locus, and highly polymorphic, because homozygotes do not provide any information. Detection of the marker can be direct by RNA sequencing, or indirect using allozymes. Some of the methods used to study the genome or phylogenetics are RFLP, AFLP, RAPD and SSR. They can be used to create genetic maps of whatever organism is being studied.

Genetic Markers have also been used to measure the genomic response to selection in livestock. Natural and artificial selection leads to a change in the genetic makeup of the cell. The presence of different alleles due to a distorted segregation at the genetic markers is indicative of the difference between selected and non-selected livestock (Rayaa, *et al.*, 2002). There was a debate over what the transmissible agent of CTVT (canine transmissible venereal tumor) was. Many researchers hypothesized that virus like particles were responsible for transforming the cell, while others thought that the cell itself was able to infect other canines as an allograft. With the aid of genetic markers, researchers were able to provide conclusive evidence that the cancerous tumor cell evolved into a transmissible parasite. Furthermore, molecular genetic markers were used to resolve the issue of natural transmission, the breed of origin (phylogenetics), and the age of the canine tumor (Murgia, *et al.*, 2006).

2.5 Polymerase Chain Reaction

PCR-based marker techniques have been employed extensively for confirming genotypes of organisms at the level of species and population. The PCR method requires little biological material and provides a rapid method for screening large sample sizes. PCR markers have been developed using either arbitrary primers or specifically designed primers from known DNA information such as repetitive sequences multiple arbitrary amplicon profile (MAAP) was developed for the use of single or multiple arbitrary primers (Caetano-Anolles, 1994; Caetano-Anolles and Gresshoff, 1997; Caetano-Anolles *et al.*, 1991), and includes the techniques of RAPD (Williams *et al.*, 1990), AP-PCR (Welsh and McClelland, 1990; Welsh *et al.*, 1990), and DNA amplification fingerprinting (DAF) (Caetano- Anolles *et al.*, 1992).

Because of its simplicity, the RAPD method has been used widely in studies of genetic diversity and in examining phylogenic and taxonomic relationship (Caetano-Anolles and Gresshoff, 1997). However, in PCR techniques using arbitrary primers, low reproducibility that causes variable PCR results depending on PCR condition has been recognized as a disadvantage factor.

The PCR conditions for RAPD analysis can be optimized by varying the many components, namely, type and concentration of 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. All these factors can influence a PCR reaction, and this is exacerbated by the fact that not all processes and mechanisms involved in such reactions are as yet understood fully (Wolff, *et al.*, 1993).

2.6 Amplification of Polymorphic DNA (RAPD)

RAPD is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8-12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction. Due to advances in molecular biology techniques, large numbers of highly informative DNA markers have been developed for the identification of genetic polymorphism. In the last decade, RAPD technique based on the PCR has been one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers, and thus do not require prior knowledge of a DNA sequence. 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 although reproducibility of the RAPD profile is still the centre of debate.

The standard RAPD technology (Williams, *et al.*,1990) utilizes short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR. Amplification products are generally separated on agarose gels and stained with ethidium bromide. Decamer primers are commercially available from various sources (e.g., Operon Technologies Inc., Alameda, California). 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. PCR amplification with primers shorter than 10 nucleotides [DNA amplification fingerprinting (DAF)] has also been used producing more complex DNA fingerprinting profiles (Caetano-Annoles, *et al* 1991). 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 (Erlich, *et al;* 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, *et al.*, 1993 and Bardakci, *et al., 1*999) 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.

RAPD is an inexpensive yet powerful typing method for many bacterial species. RAPD profiles, example Silver-stained polyacrylamide gel showing three distinct RAPD profiles generated by primer OPE15 for Haemophilus ducreyi isolates from Tanzania, Senegal, Thailand, Europe, and North America (Erlich, *et al.,* 1989). 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.

Chapter Three

Materials and Methods

3. Materials and Method

3.1 Plant materials

Twelve varieties of Guava were selected for this investigation. All varieties exhibit morphological variation in some characters, such as fruit shape, fruit size, fruit color, fruit taste and content of seeds etc. Accessions of guava varieties are presented in table 3.1.

Table 3.1 List of *P.guajava* species tested

Accession no	Variety	Sampling location
1.	Chineese	Germplasm conservation area, Khulna University
2.	Apple	Germplasm conservation area, Khulna University
3.	Kashi	Germplasm conservation area, Khulna University
4.	Kazi	Germplasm conservation area, Khulna University
5.	Poly	Germplasm conservation area, Khulna University
6.	Jelly	Germplasm conservation area, Khulna University
7.	Thai	Germplasm conservation area, Khulna University
8.	Bombay	Germplasm conservation area, Khulna University
9.	L-49	Germplasm conservation area, Khulna University
10.	Local-1	Khulna University Campus
11.	Local-2	Khulna University Campus
12.	Local-3	Khulna University Campus

3.2 Genomic DNA extraction

The modified CTAB method (Doyel and Doyel, 1987) was adopted to extract genomic DNA from the plant leaves at Plant Biotechnology Laboratory of Khulna University, Khulna, Bangladesh.

3.2.1 Sample collection:

Fully expanded young leaves were collected for isolation of genomic DNA. Leaf sample were collected from germplasm conservation centre of Khulna University. Initially, leaves were washed in distilled water and water was removed by soaking on sterile filter paper.

3.2.2 Extraction of DNA

DNA extraction was done by using modified CTAB method. Strict hygiene condition was maintained during the DNA extraction process.

Fully expanded cleaned young leaves (0.1g) was grinded into fine paste in presence of 10 volume of isolation buffer [10% PEG, 0.35M Sorbitol, 0.1M Tris (pH 8.0), 0.5% ß-mercaptoethanol]. The paste was collected in a microfuse tube (2ml). The homogenate was centrifuged at 10000 rpm for 15 min at 4° C. The pellet was suspended in 5 volume of lysis buffer [0.35M sorbitol, 0.1M Tris (pH 8.0), 0.5% ß-mercaptoethanol] and the mixture was incubated for 30 minutes at room temperature. An equal volume of preheated CTAB buffer [2% CTAB, 0.1M Tris, 20mM EDTA, 1.4M NaCl, 1% PVP] was added, mixed gently and incubated at 65° C for 30 minutes. Then an equal volume of chloroform:isoamylalcohol (24:1) was added and mixed gently. After centrifugation at 10000 rpm at 20° C for 15 minutes, the aqueous phase was collected in a clean microfuse tube (1.5 µl) and DNA was

precipitated by adding 0.8 volume of chilled 2-isopropanol. The precipitated DNA was recovered by centrifugation at 10000 rpm for 10 minutes at 4° C. The pellet was then washed with chilled 70% ethanol containing 3M sodium acetate with gentle agitation. After centrifugation at 10000 rpm for 10 minutes at 4° C the supernatant was discarded and DNA pellet was air dried and dissolved in 50 µl of TE buffer [10mM Tris (pH 8.0), 1mM EDTA]. Two microlitre of RNase [10 mg/ml] was added and incubated at 37° C for an hour. Finally the DNA sample was stored at -20° C until further use.

3.3 DNA confirmation using gel electrophoresis

The extracted DNA samples from guava leaves are shown in fig. 3.1. The concentration of extracted DNA ranged from 1.25 to 3.0 µg, which was enough to perform RAPD analysis. After Gel electrophoresis and staining, the gel was taken out carefully from the electrophoresis chamber and placed on high performance ultraviolet light box (UV transilluminator) for checking the DNA bands. The DNA was visualized as band and photographed using a gel documentation system (Gel DOC).

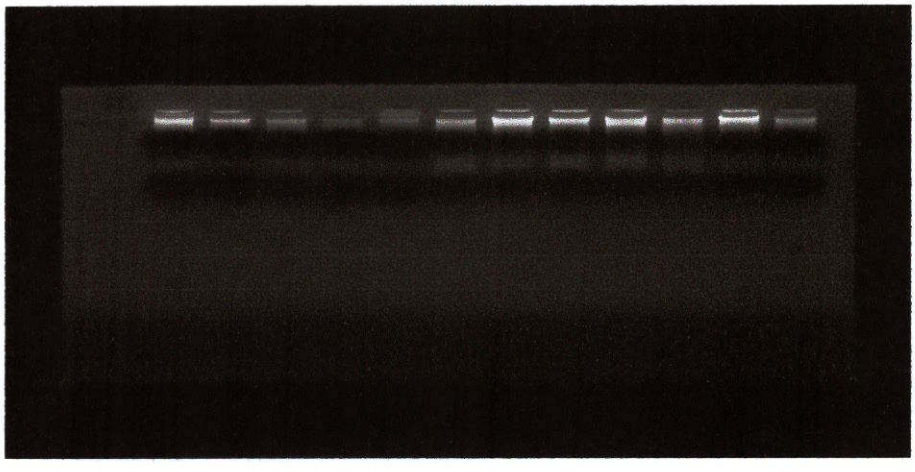


Fig: 3.1 Extracted DNA from 12 varieties of *P.guajava*

3.4 Quantification of DNA concentration

Before PCR amplification, it is important to know the concentration of genomic DNA because different DNA extraction methods produced DNA of widely different quantity. Thus, it is necessary to optimize the amount of DNA to achieve reproducibility and strong signal in PCR assay. Excessive template may result smears or lack of clearly defined bands in the gel. On the other hand, too little templete will give non-reproducible patterns (Williams *et al.*, 1993). Lambda (λ) DNA standards were used for quantitation of DNA.

3.5 PCR reaction

3.5.1 Primer selection

Primers (Bioneer Corporation, Korea and Invitrogen, USA) were selected based on mainly GC content. Primers that have higher GC content (more than 60%) are suitable for RAPD analysis.

Table 3.2: Random Primer Used in the Present Study for Screening

Primer code	Sequence (5' – 3')	GC content (%)
OPA	GAACGGACTC	60%
OPB	AATSSGCTGG	60%
OPC	AACGGTGACC	60%
OPD	TCTGCCATCC	60%
OPE	TTCCCCCCAG	70%
A00	ATCAGCGCACCA	58%
A01	AGCAGCGCCTCA	66%
A02	GCCAGCTGTACG	66%
A03	TGCCTCGCACCA	66%
A05	CCGCAGTTAGAT	50%

Calculation of Annealing Temperature:

Annealing temperature should be calculated by the following formula-

0.3× Tm (Primer) + 0.7×Tm (Product)

3.5.2 PCR amplification:

Genomic DNA polymorphism was determined by the random amplified polymorphic DNA (RAPD) method (Williams et al., 1990; Tingey and Del Tufo, 1993). Five 10 mer primers and five 12 mer primers were used for DNA amplification (Table 2). Amplification was performed in 0.2 ml Eppendorf tubes in 10 µl reaction mixture containing PCR buffer [10 mM Tris-HCl; pH 8.5; 40 mM KCl; 0.1% Tween 20; 2 mM gelatin and 1.5mM $MgCl_2$]; additional 1.5 mM $MgCl_2$ was also added; 200 µM each of dATP, dCTP, dGTP, dTTP; 0.004 µM primer; 2 units of *Taq* DNA polymerase (Bioneer Corporation, Korea); and 4 ng template DNA. Amplification was conducted in a thermocycler (Eppendorp Master Cycler) for 5 min at 94°C (preliminary denaturation) for single cycle, Than 1 min at 94°C (denaturation), 1 min at 35°C (annealing) and 2 min at 72°C (elongation). Steps 2 to 4 were repeated 30 times. Final elongation was performed for 5 min at 72°C.

3.5.3 Gel electrophoresis and scoring of polymorphisms:

After the PCR reaction, gel loading dye (6µl) [10 mM Tris-HCl (pH 7.6), 0.3% bromophenol blue, 0.3% xylene cyanol FF, 30% glycerol, 60 mM EDTA] was added to each tube. Amplified products were analyzed by electrophoresis in 2% agarose gel at 50 V for 1.5 hr in 1×TAE buffer. A Lambda/Hind III marker was run for each agarose gel, for estimation of fragment size. The gel was stained with ethidium bromide (0.5 µg/ml in 1×TAE buffer), and visualized under UV Trans illuminator. Gels were photographed using a gel documenter fitted with camera.

3.6 RAPD Analysis

Polymorphism was scored for the presence of band (1) or absence of band (0) of a particular amplification product. Data was analyzed by the software component 2.0 (CPW3) for phylogenic analysis. The unweighted pair group method with arithmetic averages (UPGMA) based dendrogram was constructed from distance matrix. The possible pair-wise genetic distance values were calculated according Nei and Li (1979)

$$d_{xy} = 1 - [2n_{xy} / (n_x = n_y)],$$

where, n_x and n_y are the numbers of bands amplified in individuals x and y, respectively, and n_{xy} is the number of bands shared by those individuals. The distances between each pair of accessions were used to construct a dendrogram using the unweighted pair group method with arithmatic averages (UPGMA).

3.7 Analysis of morphological data

Morphological data can be obtained by measuring the tree, leaves and fruits. Fruit height was measured in meter. For fruits, 3 to 4 mature fruits were collected from same or different trees. Fruit length and diameter is measured three times. Then final value was obtained by average of those values. Fruits shapes were measured by adjusting the fruit shape chart.

Chapter Four

Results and Discussion

4. Results and Discussion

Morphological and molecular characterizations were used to reveal the levels of variability among 12 varieties of guava. Molecular characterization was determined by using the molecular marker RAPD.

4.1 Morphological characterization

Morphological characteristics of twelve commonly available varieties of guava have been presented in **Table 4.1** where traits like length, width, fruit color, shape, seed production rate and taste are considered. Trees of Kazi and L-49 are small sized (3.9m and 2.3-3.4m respectively), in contrast to trees of Kashi which is taller (5.2m). All the varieties except Poly are green to light green in color, L-49 has primrose yellow and variety Poly is reddish in color. Fruits of four varieties (Chineese, Thai, Poly and Local-2) are round and another five varieties (Jelly, Bombay, L-49, Local-1andlocal-3) are rounded ovoid in shape. Apple, Kashi and Kazi are spherical, ovoid and globose in shape, respectaively. Thai variety contains less numbers of seed comparing with other varieties. Apple, kasha and bombay contain medium number of seeds. High amount of seeds is observed in L-49. Only Poly is red fleshed guava. Jelly guava has pinkish colored flesh and sour taste. Kazi variety is considered as the most popular commercial variety in Bangladesh.

Fig 6.1(a): Fruit and fruit section of Bombay

Fig 6.1(b): Fruit and fruit section of Apple payara

Fig 6.1 (c): Fruit and fruit section of Chineese

Fig 6.1 (d): Fruit and fruit sect of Jelly

Fig 6.1(e): Fruit and fruit section of Kashi

Fig 6.1 (f): Fruit and fruit section of Kazi

Fig 6.1 (g): Fruit and fruit section of L-49

Fig 6.1 (h): Fruit section of Poly

Fig 6.1 (i): Fruit and fruit section of Thai

Table 6.1 Morphological analysis of selective guava varieties:

No. of variety	Name of variety	Average plant height (m)	Fruit length (cm)	Fruit diameter (cm)	Fruit color	Fruit shape	Taste of fruit	Seed Content
V1	Chinese	3.8	3.7	4.3	Yellowish green	Round	High sweet	Low
V2	Apple	4-5.2	5.3	5.7	Light green	Spherical	Sweet	Medium
V3	Kashi	5.25	6.5	5.8	Light yellow	Ovoid	Sweet	Medium
V4	Kazi	3.9	8.0	7.0	Yellowish green	Globose	Medium sweet	Low
V5	Poly	4-4.5	5.2	4.8	Blackish red	Round	Sweet	Low
V6	Jelly	4.2-4.6	7.3	6.6	Yellowish green	Rounded ovoid	Sour	Low
V7	Thai	3.8-4.4	5.8	5.9	Light green	Round	Sweet	Very low
V8	Bombay	4.5-5	5	4.8	Yellowish green	Rounded ovoid	Sweet	Medium
V9	L 49	2.3-3.4	5.5	6	Primrose yellow	Rounded ovoid	sweet	High
V10	Local-1	4.5	6.8	6.5	Yellowish green	Rounded ovoid	Sweet	Low
V11	Local-2	5.0	5.2	5.9	Light green	Round	Medium sweet	Low
V12	Local-3	5.2	5.3	5.5	Light green	Rounded ovoid	Sweet	Medium

4.2 Molecular characterization

Polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD) method was used to amplify *Psidium guajava* DNA using arbitrary oligonucleotide primers. This method was able to detect the heterogeneity of amplified DNA from twelve varieties of *P.guajava*. The present study indicates the effectiveness of RAPD analysis in detecting the amount of polymorphisms among the different genotypes of guava. Ten effective primers (**Table 3.2**) were used for determination the polymorphism of 12 guava varieties.

4.2.1 Primer selection and RAPD analysis

Ten primers (Table 3.2) that were used in RAPD analysis of 12 guava varieties could amplify different genomic regions and produced different banding patterns, ranging from 2 to 10 and the average bands per primer was 6.25. Of the 50 bands scored, 40 (**75.23%**) were found to be polymorphic and 10 bands were found to be shared in nature. The polymorphism is low comparing to mango (85.71%) (Farzana *et al.*, 2009). The reason of this difference could be that the GC content of the primers. It is mentioned that in this study, the GC content of the primers was 60 to 70%, on average 60%. Increased number of bands was obtained with increased GC content of the primer (Fukuoka *et al.*, 1992; Raghunathachari *et al.*, 2000). Fukuoka (1992) got average 0.8 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; because of G is pairing with C by three hydrogen bonds shows the high stability of base complementation than that of complementation of A with T by two hydrogen bonds (Fukuoka *et al.*, 1992). In this investigation primers were selected according

to their performance with mango, citrus and sweet gourd (Farzana *et al.*, 2009). In this case the average level of polymorphic bands was (5.0) per primer.

Table 4.2: RAPD primers with corresponding bands scored and their size range together with polymorphic bands observed in 12 guava varieties.

Serial no	Primer code	No. of total band	Number of common band	Number of polymorphic Band	Proportion of polymorphic loci (%)
1	A	10	0	10	100
2	B	5	1	4	80
3	C	7	2	5	71.43
4	D	5	2	3	60
5	E	5	1	4	80
6	A01	9	1	8	88.89
7	A03	2	1	1	50
8	A05	7	2	5	71.43
Total	**8 primers**	**50**	**10**	**40**	**601.75**
Average		**6.25**	**1.25**	**5.0**	**75.23**

The frequencies of polymorphic bands varied from primer to primer. Though no accession-specific marker was identified, the high level of polymorphism revealed by the proportion of polymorphic loci (**75.23%**) indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in different accessions of guava. RAPD is considered to be one of the major techniques commonly used in germplasm characterization and similar studies have been carried out in horticultural crops including cherimoya (Rahman *et al.*, 1997), mango (Farzana *et al.*, 2010), banana (Das *et al.*, 2009) and guava (Tseng *et al.*,

2007). Maximum number of shared bands has been observed for primer A03 and primer A gives maximum polymorphic bands

The agarose gel showing polymorphisms observed with three primers 'A' 'A01' 'A05' and 'C' were given in fig 4.2

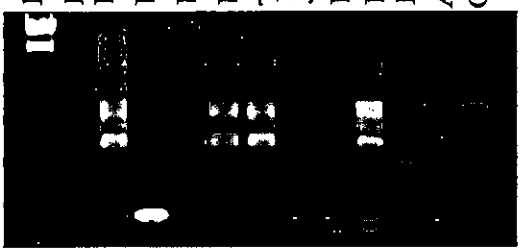


Fig 4.2(a): RAPD profile for primer A

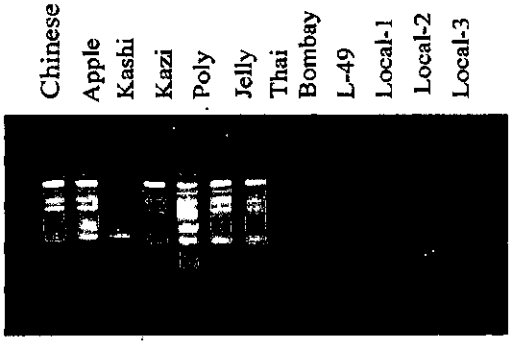


Fig 4.2(b): RAPD profile for primer A01

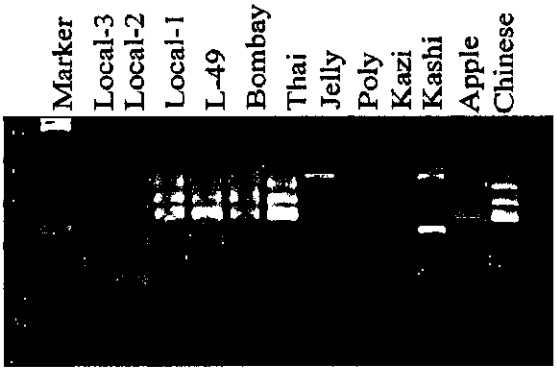


Fig 4.2(c): RAPD profile for primerA05

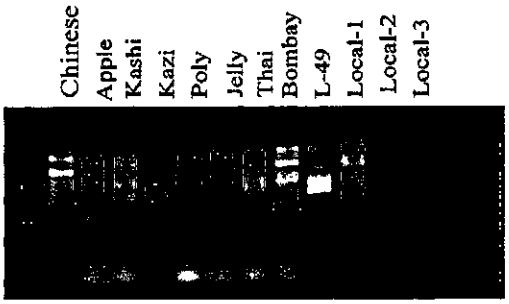


Fig 4.2(d): RAPD profile for primer C

Maximum number of shared bands was amplified with primer A, C, D, A05 but primer A and A01 amplified maximum number of polymorphic bands.

4.3. Estimation of genetic distance

Estimates of genetic distance (Nei and Li, 1979) calculated all possible pairs-wise genetic distance values by the following formula:

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

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

Genetic similarities measured through analysis of data on the 8 RAPD markers from the 12 genotypes of *Psidium guajava* revealed varying degrees of genetic relatedness. Genetic distance was ranged from 0.205 to 0.784. The highest dissimilarity coefficient (0.784) was observed between genotypes local-3 vs Bombay. The lowest (0.205) similarity coefficient was revealed between the Kazi and Apple. Using the Nei's 1979 genetic distance a dendrogram was constructed to obtain the clustering of the accessions.

Table 6. 3: Pair wise genetic distance values in of 12 guava varieties

	Chinese	Apple	Kashi	Kazi	Poly	Jelly	Thai	Bombay	L-49	Local-1	Local-2	Local-3
Chinese	-											
Apple	0.273	-										
Kashi	0.655	0.375	-									
Kazi	0.4328	**0.205**	0.323	-								
Poly	0.491	0.442	0.509	0.452	-							
Jelly	0.490	0.439	0.592	0.448	0.435	-						
Thai	0.3333	0.333	0.574	0.4 00	0.517	0.407	-					
Bombay	0.571	0.548	0.741	0.460	0.608	0.745	0.356	-				
L-49	0.547	0.661	0.5686	0.533	0.708	0.727	0.404	0.265	-			
Local-1	0.560	0.571	0.562	0.614	0.689	0.561	0.630	0.478	0.488	-		
Local-2	0.464	0.419	0.444	0.365	0.490	0.660	0.500	0.538	0.714	0.783	-	
Local-3	0.707	0.574	0.692	0.625	0.722	0.750	0.778	**0.784**	0.765	0.706	0.730	-

4.4. Cluster analysis of the genotypes based on RAPD analysis

Genetic distance was ranged from 0.2054 to 0.7837. The highest dissimilarity coefficient (0.7837) was observed between genotypes local-3 vs Bombay. The lowest (0.2054) similarity coefficient was revealed between the Kazi and Apple. Using the Nei's 1979 genetic distance a dendrogram was constructed to obtain the clustering of the accessions. The dendrogram shows that all the accessions were found to be grouped in two major groups designated as I and II. The distribution of the cluster members is shown in **Table 8**. Cluster I is broad which includes 7 accessions. Cluster II includes the highest distance.

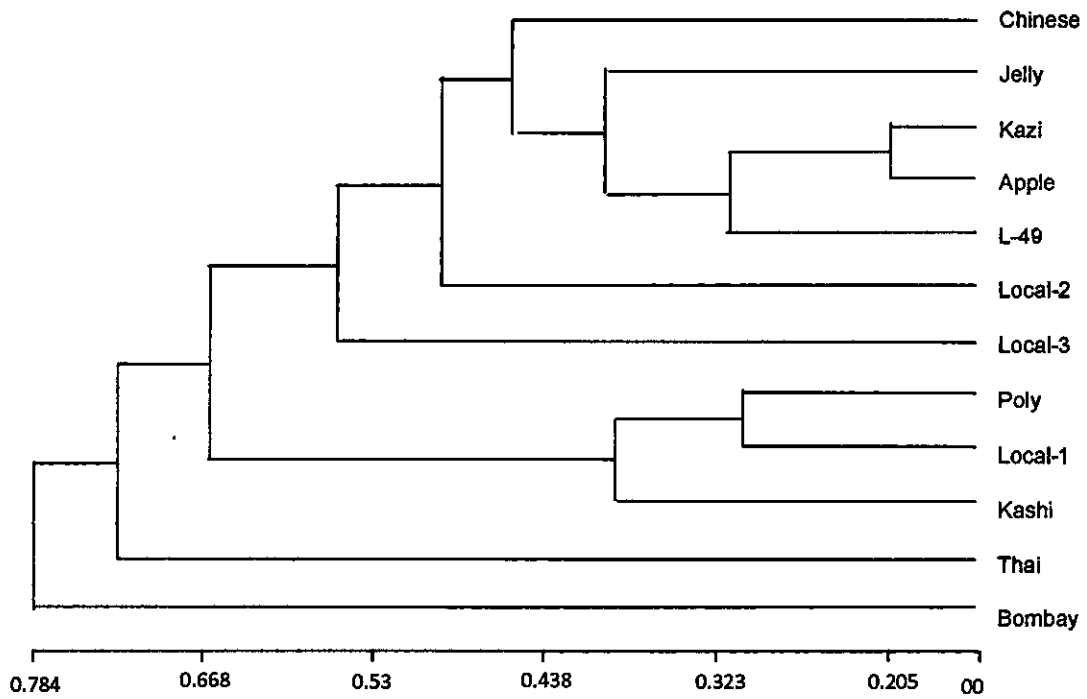


Fig.4.3. The UPGMA dendrograme based on Nei's genetic distance (1978) summarizing the data on differentiation between twelve guava samples.

Chapter Five

Summary and Conclusion

5. Summary and Conclusion

This research work was conducted in Plant Biotechnology Laboratory at Khulna University, Khulna and samples were collected from Gerplasm Conservation Centre of Khulna University as well as other location in Khulna University Campus. Morphological characters analyzed through the phenotypic traits including the leaf colour, length and width, fruit colour, shape, seed content, taste etc. Twelve selective varieties of guava were considered for morphological and molecular characterization. Kazi variety are considered as commercial variety regarding the high yielding performance compared to others.

RAPD analysis was conducted using ten random primers in order to characterize 12 different guava genotypes based on genetic variability and relatedness among them. In the RAPD technique, alleles were separated on 2% agarose gel electrophoretically, stained with ethidium bromide, and visualized on UV transilluminator.

Eight primers produced 50 scorable bands in this study, of which 40 were considered as polymorphic and the percentage of polymorphism was 75.23. The primer A amplified maximum number of polymorphic bands (10). The range of genetic distance was observed between 0.2054 (Kazi & Apple) to 0.7837 (Bombay & Local-3). Nei's genetic distance differentiated among the varieties formed clustered. Clustered was showed the genetic similarities and dissimilarities among the varieties.

The RAPD analysis discovered sufficient genetic variations among the guava varieties as this technique has frequently been used for the detection of genetic variability in plants. The advantages of this approach are its rapidity, simplicity and the absence of any need for prior genetic information of the plant. RAPD fingerprint patterns obtained are consistent irrespective of plant source and age. However, only ten polymorphic markers are not sufficient to cover guava genome particularly the regions influencing the expression of quantitative traits. Hence, sufficient and more efficient RAPD primers showing maximum number of polymorphic bands or other available markers systems could be utilized for analysis of guava. In addition, RAPD fingerprint data could be complied with gene bank data, thereby selection based on genetic linkage between DNA bands and loci of commercial important trait could be established. The result of the present study can also be used to study relationship of guava germplasm among each other for further improvement through cross breeding.

Chapter Six

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Appendix

7. Appendix

Composition of buffer and solution used

1. Composition of Isolation buffer

Components	Concentration
Polyethylene Glycol	10%
Sorbitol	0.35M
Tris-HCL(pH 8.0)	0.1M
β −mercaptoethanol	0.55

2. Composition of lysis buffer

Components	Concentration
Sorbitol	0.35M
Tris-HCL(pH 8.0)	0.1M
β −mercaptoethanol	0.5%

3. Composition of CTAB Buffer

Components	Concentration
Cetyltrimethylammonium Bromide(CTAB)	2%
Tris-HCL(pH 8.0)	0.1M
Ethylenediaminuteettraaceyic (EDTA)	20mM
NaCL	1.4M
PVP	1%
β −mercaptoethanol	0.5%

4. Composition of TE Buffer

Components	Concentration
Tris-HCL (pH 8.0)	10mM
Ethylenediaminuteettraaceyic (EDTA)	1mM

5. Composition of TAE Buffer

Components	50 x	10 x
Tris – HCl (pH 8.0)	242.2 gm	4.8gm
EDTA	0.05M	0.001M
Glacial acetic acid	57.1mM	1.14ml

6. Composition of 6xdye marker

Components	Volume ml
Bromophenol blue	25
Xylene cyanol	25
Glycerol	3
Sterile water	7

7. Flowchart of DNA extraction

0.2gm fully expanded young leaves

↓

Leaves were grinded to fine paste under isolation buffer

↓

The paste was dispended into 2×2 ml microfuge tube

↓

Centrifugation of the paste at 10, 000 rpm for 10 min

↓

Pellet was suspended by vortex in lysis buffer
and incubated at room temperature for 30 minute

↓

Equal vol. of CTAB buffer was added and incubated at 65°c for 30 min.

↓

Equal vol. of chloroform isoamylalcohol 24:1 was added and gently mixed

↓

Centrifugation at 10, 000 rpm at 25°c for 15 minute

↓

The aqueous phase was collected in a sterile clean tube

↓

Equal vol. of 2-isopropanol was added and mixed gently then kept on ice for 10 min

↓

Centrifugation at 11,000rpm for 15 min and pellet was washed with 70% ethanol

↓

The supernatant was discarded, pellet was air dried and dissolved in TE buffer

↓

5 ul of RNase was added and incubated at 37°c for 30 min

8. Flow chart of polymer gel preparation

2% agarose (0.8 gm) was mixed with 40ml distilled water

↓

400 ul 50×TAE buffer was added

↓

The solution was boiled in oven to dissolve the agarose

↓

Poured on gel casting tray and combs were set on gel solution

↓

The tray was then kept at room temperature for 30 min.