MOLECULAR CHARACTERIZATION OF SOME SELECTED GUAVA (*Psidium guajava L.*) GERMPLASM BY RAPD ANALYSIS

A Thesis By

Bulbul Ahmed

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DECLARATION

This thesis report is the original research work of the author, except as otherwise stared. 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 The findings of the research has not been/ will not be published anywhere without the concurrence of the concerned supervisor.

Bulbul Ahmed
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Dedicated To.....

All Punctual teachers of Khulna University

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The Author
May 2, 2011

Abstract

Psidium guajava L. is a perennial fruit tree in subtropical and tropical areas. In Bangladesh, *P. guajava* has been used as edible fruits and pharmacological plants by the people to treat acute diarrhea, cough and intestinal spasmodic diseases. The classification and functional identification of *P. guajava* remains unsolved these days. In the present study, morphological and molecular characterizations were used to displayed different levels of variability concerning the morphological traits of guava. Molecular marker random amplified polymorphic DNA (RAPD) was used for the molecular identification of 33 *P. guajava* germplasm from three selected south-western location of Bangladesh. Among them, eleven were commercially cultivated germplasm and the rest twenty two were collected from local farmers. The 10-mer and 12-mer oligonucleotide primers were used in RAPD to amplify. Four primers, A02, A03, S07 and S08, were able to direct the amplification and yielded a total of 252 band patterns of which 33.19% were polymorphic. The highest percent of polymorphic loci (37.5%) was observed from primer A03 and the lowest (28.57%) was from primer S08. Results were analyzed by molecular algorithm UPGMA and Neighbor-Joining. Thirty-three genotypes on the dendrogram were identified and divided into two major groups and subgroups on the basis of morphological characteristics and also on the uncultivated and commercial cultivars. The range of genetic distance was observed 0.5253 (Jelly and Thai) to 0.6631 (V30 and V22). Based on the cluster analysis, the *P. guajava* samples that were believed to have high morphological difference were grouped independently. The results suggested that RAPD is useful for the discrimination of uncultivated, cultivars to use *P. guajava* for high economy.

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Introduction

Psidium guajava L., is a shrub, perennial and evergreen fruit tree up to 25 feet (8 m) tall, commonly named guava, found in subtropical and tropical regions. It is common in the backyards. The branches are crooked, bringing opposite leaves. The flowers are white, incurved petals, 2 or 3 in the leaf axils; they are fragrant, with four to six petals and yellow anthers. The fruit is small, 3 to 6 cm long, pear-shaped, reddish-yellow when ripe. It is native to South American countries and was introduced to subcontinent by the Portuguese during 17th century (Menzel, 1985). There are many varieties of Cultivated and uncultivated guavas in Bangladesh. There are many cultivated guava varieties including L-49, Kazi, Chinese, Jelly, Polly, Bomby, Thai and Kashi etc. As Bangladesh is in subtropical area and its land is fertile, farmers get benefited easily by cultivating guava. Bangladesh is in eleventh position for the production of guava (Jain *at al,*. 2009).

P. guajava has high nutritional content and is especially rich in vitamin C. Guavas are often included among superfruits, 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 (*P. guajava*) fruit contains about four times the amount of vitamin C as an orange (Hassimotto *et al.* 2005). Guavas contain both carotenoids, polyphenols and galloccatechin (Jimenez-Escrig *et al.*, 2001); guaijaverin, leucocyanidin and amritoside (Kaljee *et al.,* 2004); the major classes of antioxidant pigments giving them relatively high potential antioxidant value among plant foods (Mahattanatawee *et al.*, 2006). Essential oils from guava leaf show anti-cancer activity in vitro (Mukhtar *et al.*, 2006). It's leaves and bark are used as an

effective remedy to treat and prevent diseases such as headache, cough (Khan *et al.,* 1985 and Jaiarj *et al.*, 1999), spasm, inflammatory, pyrexia, acute diarrhea (Lin *et al.,* 2002), colic, flatulence, and gastric pain (Lozoya *et al.,* 2002). It is also use in industry as chew-sticks, tannins and astringents (Lutete *et al.,* 1994 and Nadkarni *et al.*, 1999).

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 guava trees in indigenous is a more systematic way, specific genetic markers for guavas 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 *et al.*, 2000) cultivars and accessions in Italy and 41 genotypes of guava 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 (Williams *et al.*, 1990) owing to their simplicity, speed and relatively low-cost (Rafalski and Tingey, 1993); 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 33 indigenous genotypes of guava in Khulna region (Khulna, Shatkhira and Jessore) of Bangladesh. The study is aimed to see the morphological variations through phenotypic characteristics among thirty three germplasm and understand the distribution of guavas in indigenous by molecular markers analyses. The preliminary results are useful in the discrimination of guava species. It is crucial to identify guavas, which may have potential to be developed variety.

Review of Literature

Guava is the common name for any of the various tropical shrubs and small trees comprising the New world genus *Psidium* of the myrtle family (Myrtaceae), characterized by tough, dark, opposite leaves and an edible fruit.

The term "guava" appears to derive from Arawak guayabo "guava tree", via the Spanish guayaba. According to Ellshoff *et al.* (1995) *P. guajava* was first named by Linneaus in 1753 as Ruehle (1964) stated. It is believed that in 17th century the Spaniards transported the guava tree of the Pacifc to the Philippines and India. Another term for guavas is pera . It is common around the western Indian Ocean and probably derives from Spanish or Portuguese, which means "pear", or from some language of southern India, though it is so widespread in the region that its origin cannot be clearly discerned any more. Pera itself is used in Malayalam, Sinhala and Swahili. In Marathi it is peru, in Bengali pearah (wikipedia).

2.1. Morphology of Guava

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). 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). 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.

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 adapt to changing environments. With more variation, it is more likely that some individuals in a population will possess variations of alleles that are suited for the environment. Those individuals are more likely to survive to produce offspring bearing that allele. The population will continue for more generations because of the success of these individuals (U.S. Geological Survey). When humans initially started farming, they used selective breeding to pass on desirable traits of the crops while omitting the undesirable ones. Selective breeding leads to monocultures: entire farms of nearly genetically identical plants. Little to no genetic

diversity makes crops extremely susceptible to widespread disease. Bacteria morph and change constantly. When a disease causing bacterium changes to attack a specific genetic variation, it can easily wipe out vast quantities of the species. If the genetic variation that the bacterium is best at attacking happens to be that which humans have selectively bred to use for harvest, the entire crop will be wiped out (cheetah.org).

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* (evolution.berkley.edu). 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 guava 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 *et al.,* 1992).

Using RAPD markers in guava germplasm collections, some authors have identified genotypes coming from diverse foreign regions (Prakash *et al.,* 2002). Nevertheless, overall interpretation of the genetic relationships among guava accessions with AFLP (Valdes-infante *et al.,* 2003) and SSR (Rodriguez *et al.,* 2007) in Cuba indicates the absence of separate clusters representing local and foreign germplasm. This reflects the selection of guava lines form open pollination rather then from controlled crosses. Micro-satellites also detected a high number of alleles shared for the majority of guava 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 th other hand, some individual and combined rare alleles were detected in such accessions. This information provides ground for parental selection in guava breeding programmes and conservation strategies (Jain *et al.*).

2.3. DNA Extraction

A fast, simple, cost-effective and reliable method is a pre-requisite for any DNA extraction and subsequent downstream application. Many protocols have been used in plant DNA isolation, but because of the chemical heterogeneity of the species many of them could be applied to a limited number of species or even closely related species in some cases fail to respond to the same protocol (Weishing *et al.*,1995). Plants, especially

medicinal plants contain an array of secondary metabolites. The compounds which make them interesting for molecular biology studies and hence, for DNA isolation, themselves interfere with the DNA isolation procedure. Another problem that could arise during plant DNA isolation is the necessity of liquid nitrogen for crushing the plant material as reported in most of the protocols (Ouenzar *et al.*, 1998) and lengthy procedure involved. In many laboratories the availability of liquid nitrogen and RNAase is a limiting factor in DNA isolation. In the present study most of the concerns have been addressed. The protocol needs about an hour to prepare DNA for any molecular biology application.

A simple, efficient, reliable and cost-effective method for isolation of total genomic DNA from both young and old leaves is described. The DNA obtained was free of any contaminating proteins, polysaccharides and coloured pigments. The isolated genomic DNA was found suitable for restriction digestion and PCR amplification, hence, it can be employed for preparation of Southern blots, AFLP and cloning. The protocol overcomes the need for liquid nitrogen, 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. 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 *et al.*, 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 P. Chomczynski (1997), DNAzol is an advanced DNA isolation method (U.S. patent no. 5,945,515) that combines both reliability and efficiency with simplicity of the isolation protocol. The DNAzol protocol is fast and permits isolation of genomic DNA from a large number of samples of small or large volumes (Chomczynski *et al.*, 1998).

During the isolation, a biological sample is lysed (or homogenized) in DNAzol and the genomic DNA is precipitated from the lysate with ethanol. Following an ethanol wash, DNA is solubilized in water or 8 mM NaOH. The procedure can be completed in 10 - 30 minutes with a genomic DNA recovery of 70-100%. The isolated DNA can be used,

without additional purification, for Southern analysis, dot blot hybridization, molecular cloning, polymerase chain reaction (PCR) and other molecular biology and biotechnology applications (Patents granted to Hoffmann LaRoche Corporation). DNAzol is stable at room temperature for at least two years after the date of purchase. DNAzol contains irritants. Handle with care, avoid contact with skin, and use eye protection (shield, safety goggles). In case of contact: Wash skin with a copious amount of water and seek medical attention.

2.4. Genetic Marker

Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited numbers of DNA markers. The utility of DNA-based markers is generally determined by the technology that is used to reveal DNA-based polymorphism. Currently, the restriction fragment length polymorphism (RFLP) assay (Botstein *et al.*, 1980) has been the choice for many species to measure genetic diversity and construct a genetic linkage map. However, an RFLP assay which detects DNA polymorphism through restriction enzyme digestion, coupled with DNA hybridization, is, in general, time consuming and laborious. Over the last decade, polymerase chain reaction (PCR) technology has become a widespread research technique and has led total development of several novel genetic assays based on selective amplification of DNA (Saiki, 1989). This popularity of PCR is primarily due to its apparent simplicity and high probability of success. Unfortunately, because of the need for DNA sequence information, PCR assays are limited in their application. The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of

genetic markers for a variety of purposes (Williams *et al.,* 1990). The simplicity and applicability of the RAPD technique have captivated many scientists' interests.

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

Genetic marker screening is based on the survey of genetic diversity as revealed by variation at specific gene loci and provides information about the amount and distribution of genetic diversity within and among populations. The emphasis of this report will be on DNA based molecular techniques and how they can apply in assessing the genetic diversity of genetic resources.

2.4.1. Types of Genetic Markers

Some commonly used types of genetic markers are

- RFLP (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 *et al.,* 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 synthesized 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 use in PCR analysis i. arbitrary primers and ii. specifically designed primers from known DNA information. Multiple arbitrary amplicon profile (MAAP) was developed for the use of single or multiple arbitrary primers (Anolles *et al.*, 1997), for RAPD analysis. It is a simple method to analysis phylogenetic relation but using arbitrary primers, low reproducibility that causes variable PCR results depending on PCR condition has been recognized as a disadvantage factor. Specifically designed primers are also use for PAPD analysis to see the specific DNA band patterns e.g. 18S rDNA.

Saiki et al. (Saiki *et al.*, 1985) published the first experimental data on PCR technique has tremendously influenced research in diverse areas of biological sciences leading to an unprecedented understanding of microorganisms. 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.

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 exibit 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 *et al.,* 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 *et al.*, 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. Theprofile 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.

Tseng-Wei Chen (2006) shows the RAPD analysis of thirty two Taiwan variety of *Psidium guajava* L. for variety development. Chikkadevaiah and Nandini (Nandini *et al.*, 2005) established the phylogenetic relationships among sunflower hybrids genotypes and characterizes 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.

Materials and Method

3.1. Plant Materials Collection

The experiment was conducted at molecular horticulture laboratory of Agrotechnology Discipline of Khulna University from December 2010 to April 2011. The younger leaves of guava tree were collected from three selected locations Khulna, Shatkhira and Jessore of Bangladesh (Table 3.1). Guava leaves were collected from December to January, then the leaves were preserved at -80^0 C in Lab for further use. The fruits of 33 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 7cm diameter and medium size was between 5.5cm to 7 cm diameter.

Table 3.1: Plant materials used in RAPD marker analysis

Germplasm No	Variety	Place of collection	Morphological characteristics and test
1-a	Polly	Germplasm center, Khulna University,	Sweet, Small, Round
2-b	Kazi	””	Medium sweet, large
3-c	Jelly	””	Sour, medium
4-d	Chinese	””	High sweet, Small, Round
5-e	L-49	””	Sweet, Medium
6-f	Kashi	””	Sweet, Medium
7-g	Thai	””	Sweet, Small, Round
8-h	Local	Khulna	Sweet, Medium
9-i	””	””	Sweet, Large
10-j	””	””	Sweet, Medium, Perennial
11-k	””	””	Very Large
12-l	””	””	Sweet, Small
13-m	””	””	very sweet, Small, Perennial
14-n	””	””	Sweet, Round, Large

15-o	””	Shatkhira	Sweet, Small
16-p	””	””	Sweet, Medium
17-q	””	””	Sweet, Large
18-r	””	””	Medium
19-s	””	””	Sweet, Large
20-t	””	””	Sweet, Large
21-u	””	Jessore	Large
22-v	””	Shatkhira	Sweet, Large, Round
23-w	””	””	Sweet, Large, longer
24-x	””	Jessore	Sweet, Small
25-y	””	””	Sweet, Large, Perennial
26-z	””	””	Medium
27-aa	””	Shatkhira	Sweet, Large
28-bb	””	””	Sweet, Large
29-cc	””	””	Sweet, Large
30-dd	Unknown	Germplasm center, Khulna University	-
31-ee	””	””	-
32-ff	Bomby	””	-
33-gg	Unknown	””	-

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).

I. DNA Extraction

The collected plant leaves were rinsed with distilled water, 50mg of younger leaf parts were taken and stored at -80°C for one day. Leaf samples were ground into powder with

mortar and pestle and 1ml of DNAzol was added and then homogenized properly. The mixture was centrifuged at 12,000 rpm for 10 minutes and transferred the supernatant to another 1.5 ml clean tube.

II. DNA Precipitation

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

III. DNA Purification

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

IV. DNA Solubilization

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

V. DNA Quantification

The DNA quantification was carried out using a spectrophotometer. One micro liter each of the guava DNA extracts was diluted with 99μL of deionized water and the absorbance at 260

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

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

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

3.3. PCR Reaction

I. Primer Selection

Primers were selected based on intensity of bands, presence of smearing, consistency within individuals and potential for population discrimination. Primers were selected based on mainly GC content. Primers that have GC content more than 60% are suitable for RAPD analysis (Anne *et al.*, 1995). Final subsets of four 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 (%)	Annealing Temp.(Ta)
OPA 03	TGCCTCGCACCA	66%	37
OPA 02	GCCAGCTGTACG	66%	37
OPS 07	GGTGACGCAG	70%	36
OPS 08	GTCCACACGG	70%	36

Calculation of Annealing Temperature:

Annealing temperature was calculated by the following formula-

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

II. PCR Amplification

PCR amplification was carried out according to the modified protocol of Williams *et al.* (1990) with minor modifications. The following components were used to prepare PCR mixture. The total volume of PCR mixture was 15.5 µl per sample. 2µl genomic DNA was added with 15.5µl PCR mixture and finally the total volume was made 17.5 µl (Table 3.3).

Table 3.3: PCR master mixture (10µl)

Serial. No.	Reagents	Amount Per sample
1	Genomic DNA	2µl
2	Primer (10nM)	1µl
3	dNTPs(10mM)	2µl
4	*Taq* DNA polymerase(2U)	1.5µl
5	Reaction buffer(10x)	2µl
6	dH_2O	10µl
Total		18.5µl

III Temperature Profile

The first amplification cycle consisted of the following steps:

1) Initial denaturation at 94°C for 2 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 1 min
5) Cycle to step 2 for 32 more times
6) Final extension at 72°C for 5 min.

3.4. Electrophoresis of the amplified products and documentation

Amplified PCR products were electrophoresed on an agarose gel (2%) in TAE 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 to construct the dendogram and presence of band (1) and absence of band (0) value consider for these RAPD analysis.

Results and Discussion

4.1. 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 for 33 germplasm were used and displayed different levels of variability concerning the morphological traits of guava. Molecular characterization was determined by using the molecular marker. Random amplification of polymorphic DNA (RAPD) markers is most useful for understanding the naturally occurring polymorphisms in living organisms.

4.1.1. Morphological Characterization

Morphological characteristics of thirty three germplasm of guava 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 two indigenous 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 consume. Similarly, eleven commercial varieties were used to show the similarity and dissimilarity among the local germplasm.

4.1.2. Molecular characterization

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

4.1.2.1 Primer Selection and RAPD Analysis

To enhance the polymorphism of genotypes of guava, two 10-mer (S07 & S08) and two 12-mer (A02 & A03) primers were used for DNA amplification (Table 3.2). Only two primers (A02 & A03) were selected for fingerprinting. Two primers generated various banding patterns ranging from 5 to 9 and yielded a total of 252 band patterns of which 33.19% were polymorphic. The highest percent of polymorphic loci (37.5%) was observed for primer A03 and the lowest (28.57%) from primer S 08. The reason of this difference could be due to the GC content of the primers. It can be mentioned that 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 *et al.,* 1995). Fukuoka *et al.,* (1992) got bands per primer with 40% and 6.1 bands per primer with 50% GC content for rice varieties. The correlation between GC content of the primer and the number of bands could be explained as the greater hydrogen bonding: with C by three

hydrogen bonds show the high stability of base complementation than that of complementation of A with T by two hydrogen bonds (Fukuoka *et al.,* 1992).

Table 4.1. RAPD primers with corresponding bands scored and their size range together with polymorphic bands observed in 33 guavas Germplasm.

Serial No	Primer code	No. of total band	No. of common band	Number of polymorphic band	Proportion of polymorphic loci (%)
1	A02	9	6	3	33.34
2	A03	8	5	3	37.50
3	S07	6	4	2	33.34
4	S08	7	5	2	28.57
Total	4 primers	30	20	10	132.75
Average		7.5	5	2.5	33.19

Since the amplification directed by these two primers produced significant polymorphism and yielded a total of 252 polymorphic RAPD patterns as shown in Figure 4.1. Each of the two primers was reproducible and capable of exhibiting polymorphic amplification patterns and hence, they were used in the subsequent analysis with more number of DNA samples of guava germplasm. The amplification patterns of representative samples of guava variety with two different primers have been shown in Figure 4.1 and 4.2

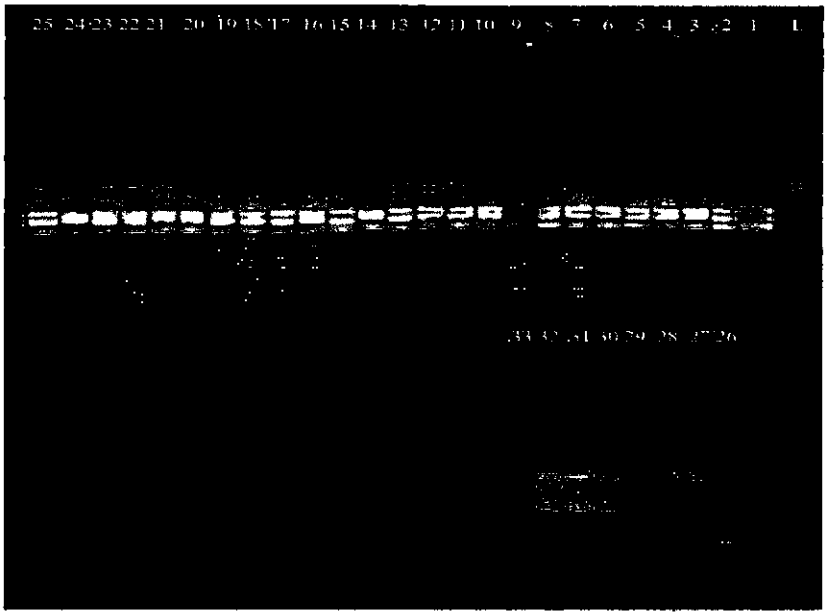

Fig 4.1. RAPD pattern generated by the primer A02 genotypes of *Psidium guajava.* The numbers show the serial number of the genotypes and "L" indicate Ladder.

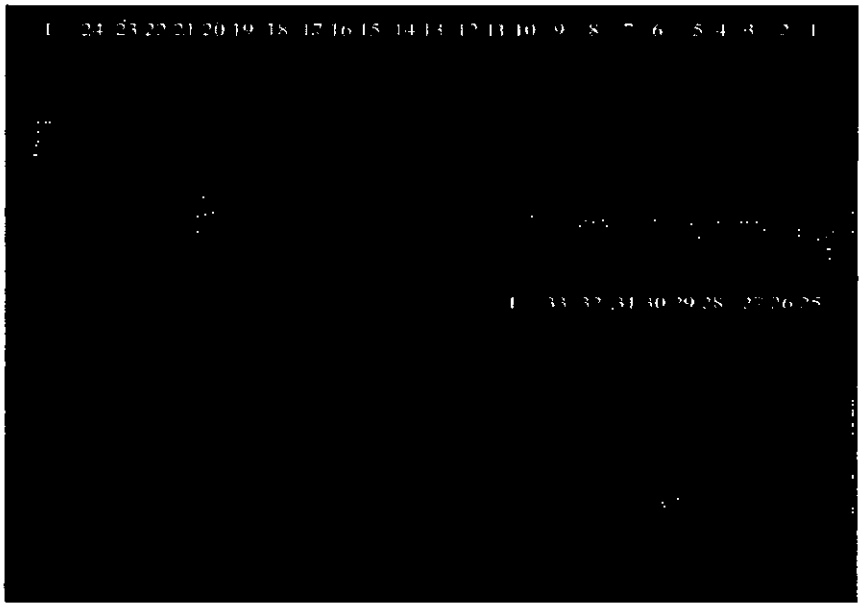

Fig 4.2. RAPD pattern generated by the primer A03 genotypes of *Psidium guajava.* The numbers show the serial number of the genotypes and "L" indicate Ladder.

4.1.2.2. Genetic Distance

DNA amplification was repeated four times. Genetic similarity matrix (Table 4.2) 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 band.

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

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

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

The values of pair-wise comparisons of Nei's (1979) genetic distance between 33 guava genotypes were computed from **0.5253** to **0.6631** (Table 4.2). Comparatively higher genetic distance (**0.6631**) was found between V30 vs. V22. The lowest genetic distance (**0..5253)** was found between V3 and V7.

There was a genetic variation among the studied of guava as indicated by the proportion of polymorphic loci. Estimated genetic variation in the guava might be consistent with the fact that it is a polymorphic plant. The range of genetic distance of 33 genotypes was **0.5253-0.6631** and the difference between the highest and lowest genetic distance indicated the presence of variability among the 33 genotype of guava.

Table 4.2: Pariwise distance values between guava genotypes included in several varietals types. Calculations were based on RAPD band data.

	V1	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V2	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29	V3	V30	V31	V32	V33	V4	V5	V6	V7	V8
V1	-																															
V10	.5597	-																														
V11	.6035	.5741	-																													
V12	.5740	.5963	.5617	-																												
V13	.6551	.5688	.5783	.6084	-																											
V14	.5928	.5625	.5808	.6024	.5671	-																										
V15	.6285	.5928	.5765	.5976	.6047	.6149	-																									
V16	.5882	.5671	.5765	.6140	.6127	.6149	.5780	-																								
V17	.6271	.6243	.6080	.5882	.6035	.5896	.6089	.5852	-																							
V18	.5515	.5988	.6150	.5610	.6343	.6127	.6000	.5673	.5747	-																						
V19	.6127	.6095	.5930	.6059	.5799	.6149	.6180	.6023	.6167	.6158	-																					
V2	.6264	.6095	.5988	.5952	.6264	.6127	.6390	.6464	.5829	.6292	.6389	-																				
V20	.5917	.6048	.6047	.6257	.6400	.5941	.6369	.6215	.6354	6193	.5977	.5872	-																			
V21	.6094	.5890	.5723	.5590	.6012	.6199	.5655	.6307	.6057	.5455	.5988	.6364	.5602	-																		
V22	.6227	.5917	.6158	.5714	.6114	.6292	.6319	.6319	.5922	.5747	.5932	.6067	.5966	.5896	-																	
V23	.5903	.5688	.5868	.6084	.6316	.5928	.6127	.6286	.6575	.5941	.5882	.6497	.5833	.5843	.5954	-																
V24	.6082	.6131	.5799	.6012	.5917	.5858	.6519	.6444	.6045	.5872	.5977	.6193	.6092	.6342	.5886	.6477	-															
V25	.6215	.6421	.5862	.6150	.6136	.6389	.6034	.5955	.6324	.6467	.6111	.6467	.6374	.6464	.6614	.5977	.5989	-														
V26	.6024	.6074	.5818	.5598	.5767	.5964	.6000	.6082	.6229	.5808	.5663	.5893	.6358	.6048	.6070	.6024	.6118	.6328	-													
V27	.5610	.5915	.5833	.6131	.6279	.5723	.5765	.6012	.6236	.5906	.6250	.5655	.5799	.5893	.5920	.5868	.5965	.6023	.5732	-												
V28	.5460	.5590	.6023	.5818	.5893	.5833	.6425	.5872	.6257	.6012	.5954	.6405	.5906	.6082	.5614	.5723	.6149	.6201	.6012	.5774	-											
V29	.6059	.5854	.6105	.5818	.5808	.5833	.5536	.5621	.6023	.6012	.6348	.6012	.6149	.6477	.6484	.6059	.6149	.6278	.5928	.5941	.6047	-										
V3	.5818	.5776	.6035	.5915	.5904	.5843	.6207	.6364	.6193	.6185	.6364	.6264	.6081	.6177	.6035	.6071	.6000	.6215	.5939	.5783	.5976	.5808	-									
V30	.5824	.6036	.5790	.5833	.5824	.5930	.5886	.6124	.6413	.6484	.6354	.6180	.5838	.6092	.6631	.6070	.5673	.5899	.5774	.6114	.6057	.5647	.6069	-								
V31	.5882	.5843	.6171	.6379	.6207	.5824	.6102	.6257	.6541	.6464	.6180	.6000	.6215	.5988	.6319	.6047	.6057	.5955	.6000	.5422	.6348	.5954	.6127	.5723	-							
V32	.5954	.5833	.5673	.6047	.5954	.5896	.5523	.5690	.6000	.6145	.5852	.6145	.6429	.6136	.6378	.5954	.6429	.6250	.6384	.5756	.5614	.5943	.5790	.5955	.6011	-						
V33	.6071	.5776	.5697	.5472	.6154	.6257	.6127	.6127	.6271	.6264	.6047	.5941	.5663	.5843	.6271	.5818	.5917	.6292	.6108	.5952	.6140	.5808	.5732	.5906	.5799	.6114	-					
V4	.6250	.6303	.5617	.5839	.6250	.5679	.5549	.6221	.6047	.6279	.6140	.6036	.6337	.6108	.6085	.6000	.6177	.5988	.5688	.5793	.6154	.5818	.6084	.6243	.5893	.6047	.5652	-				
V5	.5663	6131	.5965	.5928	.6243	.6185	.5389	.5562	.6045	.6035	.6215	.6035	.6171	.6185	.6045	.5749	.6328	.6145	.5868	.5882	.5988	.5655	.6322	.5838	.5814	.5723	.5663	.6095	-			
V6	.5732	.6121	.5783	.5563	.6154	.6095	.6207	.5965	.6035	.6264	.5799	.5602	.6243	.6012	.6035	.6474	.5833	.5814	.5767	.5432	.5636	.5723	.5988	.5740	.5714	.5273	.5818	.6084	.5576	-		
V7	.5964	.5926	.5758	.6145	.5964	.5988	.5687	.5941	.5765	.6082	.6264	.6322	.6140	.6154	.6171	.6048	.6221	.6114	.6000	.5843	.6279	.6199	.5253	.5965	.6105	.5930	.6131	.5350	.6140	.6048	-	
V8	.6480	.6221	.6444	.6421	.6171	.6500	.5909	.5909	.6284	.6124	.5988	.6201	.6257	.5954	.5899	.6328	.6180	.6154	.5366	.6057	.6000	.6158	.6328	.6167	.6145	.6284	.6092	.5602	.6023	.6012	.5906	-
V9	.5843	.5890	.5976	.5767	.6257	.6036	.6069	.6069	.6292	.6207	.6149	.6047	.6264	.6358	.5896	.6012	.6023	.6000	.5438	.5808	.5749	.5749	.5404	.5848	.6070	.6292	.6012	.5679	.5774	.5843	.5818	.5954

4.1.2.3. Dendogram

Thirty three genotypes on the dendrogram were distinguished and divided into two major groups as shown in Figure 4.3 based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA). UPGMA dendrogram was constructed according to the morphological data. The dendrogram (Fig: 4.3) shows that all the germplasm were grouped into two major clusters G1 & G2. Cluster G1 subdivided into G1-I and G1-II, Cluster G1-I subdivide into G1-I-1 and G1-I-2 and Cluster G1-I-1 subdivided into G1-I-1-a and G1-I-1-b.

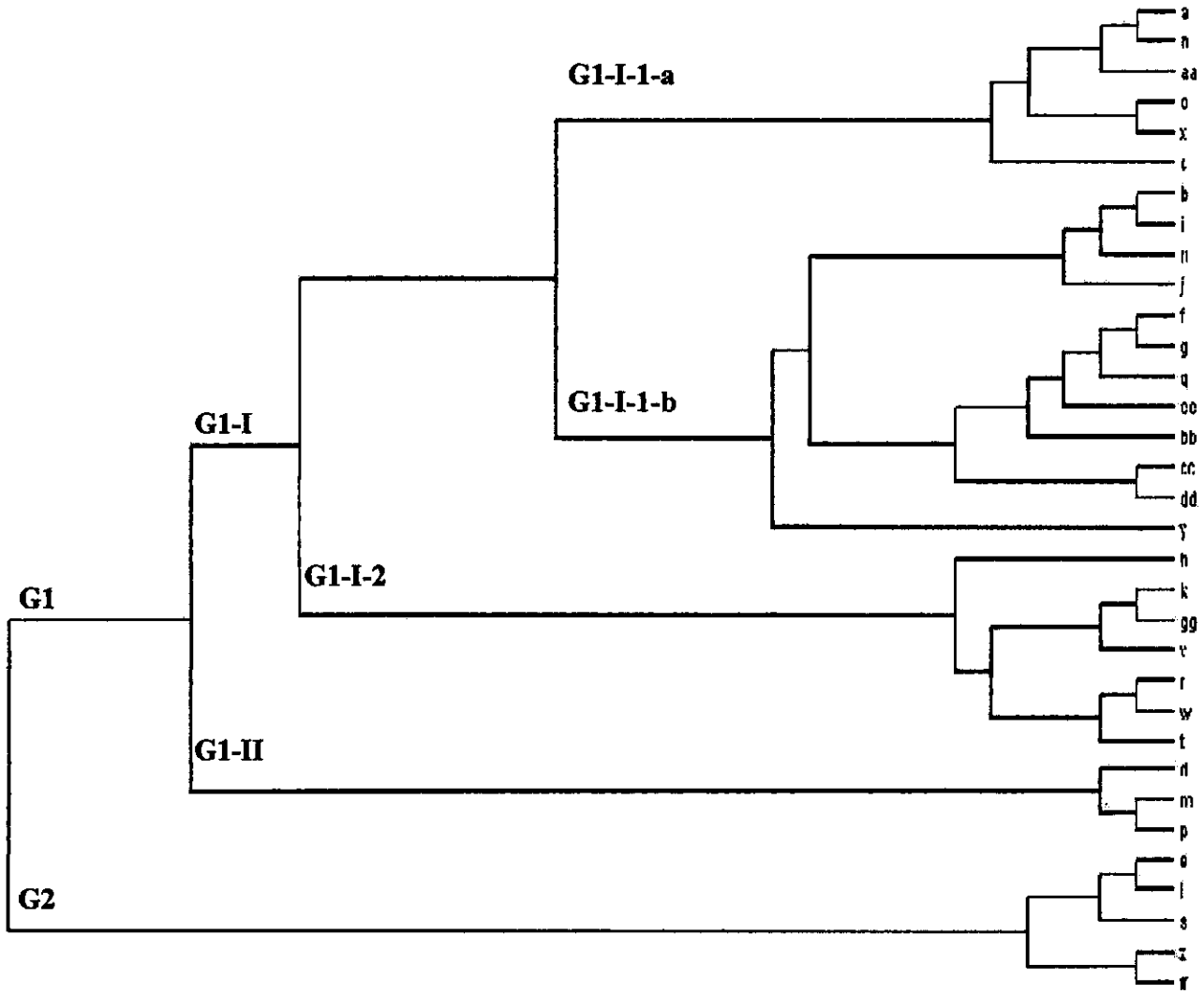

Fig 4.3. Unweighted Pair Group Method of Arithmetic Mean (UPGMA) dendrogram based on Nei's (1979) genetic distance, according to RAPD analysis of 33 germplasm of guava.

Morphological characterization was determined and showed genetic similarities and dissimilarities by collecting data and constructing the UPGMA dendrogram to estimate the genetic diversity of guava phenotypically. From the dendrogram (Fig: 4.3) it was found that most of the cultivated guavas collected from Khulna University gerplasm centre were grouped under G1-I-1 cluster. We did not find any similarity between 11

cultivated germplasm and found similarity between cultivated and uncultivated variety, such as Polly & V14, Kazi & V9, V30 &V29, V33 & V11, L-49 &V12 and V32 & V26 as their shape, size and taste of fruits was similar. V28 was mutated variety originated from Indian in cluster G1-I-1-b found no similarity with others. The commercial cultured variety Polly is also an exceptional variety and was found similarity with variety V14.

4.2. Conclusion

Recently molecular markers have been used as a tool to investigate the plant germplasm diversity. 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 commercial guava or local guavas. The RAPD analysis is useful in the fingerprinting of each guava sample. The geographical locations, growth altitude, and climates may contribute the polymorphic RAPD of guava trees in Bangladesh. This result is beneficial for further research on the guava functionality.

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