International Journal of Natural Sciences (2011), 1(3):62-67

www.ijns.net

pISSN : 2221-1012
eISSN : 2221-1020

Molecular characterization of guava (*Psidium guajava* L.) germplasm by RAPD analysis

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[Received: August 17, Accepted: September 26, 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 people use it to treat acute diarrhea, cough and intestinal spasmodic diseases. In the present study, morphological and molecular characterizations were used to display different levels of variability. 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 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 yield 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 V 22). Based on the cluster analysis, the *P. guajava* samples have morphological difference were grouped independently. The results suggested that RAPD is useful for the discrimination of uncultivated, cultivars *P. guajava* for high economy.

Key words: Guava, Germplasm, RAPD, Marker, Dendrogram.

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. Guava 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 [1]. There are many varieties of Cultivated and uncultivated guavas in Bangladesh. 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 [2].

Guavas are often included among superfruits, being rich in dietary fiber, vitamins A and C and folic acid[3]. Essential oils from guava leaf show anti-cancer activity in vitro [4]. Its leaves and bark are used as an effective remedy to treat and prevent diseases such as headache, cough [5, 6], spasm, inflammatory, pyrexia, acute diarrhea [7], colic, flatulence, and gastric pain [8]. It is also use in industry as chew-sticks, tannins and astringents [9, 10].

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. Among the different types of molecular markers, Randomly Amplified Polymorphic DNAs (RAPD) are useful for the assessment of genetic diversity [11] owing to their simplicity, speed and relatively low-cost [12]; analyzing genetic relationships, tagging traits for use in marker-assisted selection, and for the rapid construction of a genetic linkage map [13] compared to other types of molecular markers. Molecular markers were considered as powerful tools in the assessment of genetic diversity within and between plant populations [14].

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.

MATERIALS AND METHODS

Plant Materials Collection

The experiment was conducted at molecular horticulture laboratory of Agrotechnology Discipline of Khulna University, Bangladesh, from December 2010 to April 2011. The younger leaves of guava tree

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Table 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	””	-

were collected from three selected locations Khulna, Shatkhira and Jessore of Bangladesh (Table 1).

Table 2: PCR master mixer

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

Guava leaves were collected from December to January, and then the leaves were preserved at -80^0 C in Lab for further investigation. 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; medium size was between 5.5cm to 7 cm diameter and small size less then 5.5cm diameter.

DNA Extraction

Table 3: 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

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 DNAzol protocol is fast and permits efficient isolation of genomic DNA from a variety of plant tissues [15]. 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. 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 sample was centrifuged at 12,000 rpm for 10 minutes and the supernatant was discarded. 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. 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. The DNA quantification was carried out using a spectrophotometer. The concentrations of absorbance were calculated by using the following formula [16].

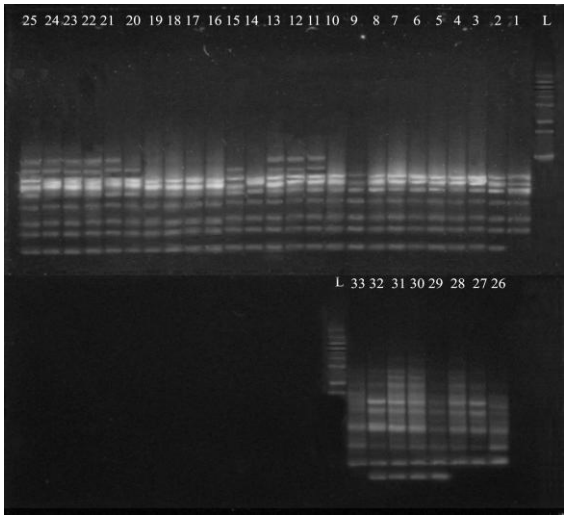

Fig 1: RAPD pattern generated by the primer A02 genotypes of Psidium guajava. The numbers show the serial number of the genotypes and "L" indicate Ladder.

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

PCR Reaction

Primers were selected based on mainly GC content. Primers that have GC content more than 60% are suitable for RAPD analysis [17]. Final subsets of four primers OPA 02, OPA 03, OPS 07 and OPS 08 were exhibited good quality banding patterns and sufficient variability was selected for further analysis. PCR amplification was carried out according to the modified protocol of Williams *et al.* [11] with minor modifications (Table 2).

The first amplification cycle consisted of the following steps: 1) Initial denaturation at 94^{o}C for 2 min; 2) Denaturation at 94^{o}C for 1 min; 3) Annealing at 35^{o}C for 1 min; 4) Elongation or extension at 72^{o}C for 1 min; 5) Cycle to step 2 for 32 more times and 6) Final extension at 72^{o}C for 5 min.

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.

RAPD Analysis

Numerical value was considered to construct the dendogram and presence of band (1) and absence of band (0) value consider for these RAPD analysis. The unweighted pair group method with arithmetic averages (UPGMA)-based dendrogram was constructed using the component 2.0 (CPW3) and Genetic distance was calculated all possible pairs-wise genetic distance values by the Nei's and Li's [18] formula using GelQuest software.

RESULTS AND DISCUSSION

Molecular characterization

In the present study, a polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD) 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 2) were selected for determination the polymorphism.

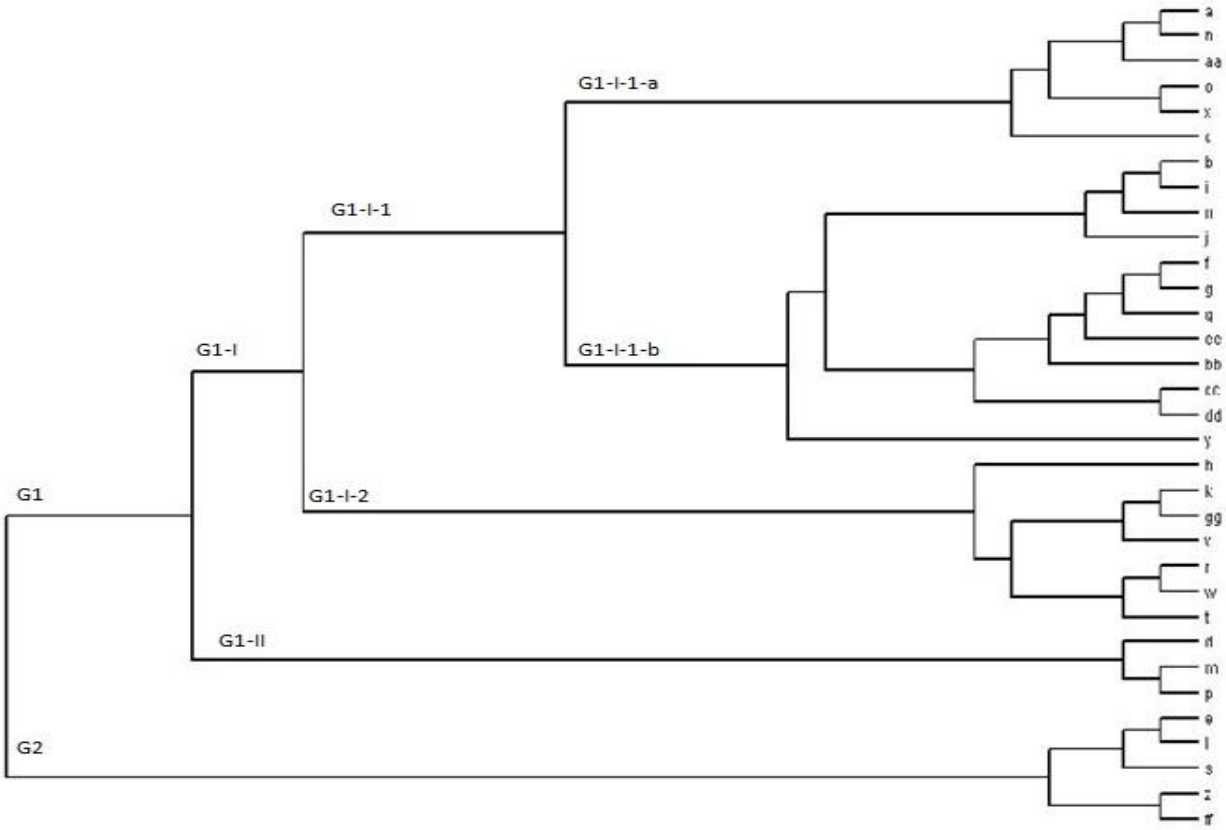

Fig 2: Unweighted Pair Group Method of Arithmetic Mean (UPGMA) dendrogram based on Nei's and Li's [18] genetic distance, according to RAPD analysis of 33 variety of guava

Primer Selection and RAPD Analysis

To enhance the polymorphism of genotypes of guava, two 10-mer (OPS07 & OPS08) and two 12-mer (OPA02 & OPA03) primers were used for DNA amplification. Only two primers (OPA02 & OPA03) were selected for fingerprinting. Two primers generated various banding patterns ranging from 5 to 9 of which 33.19% were polymorphic. The highest percent of polymorphic loci (37.5%) was observed for primer A 03 and the lowest (28.57%) was from primer S 08 (Table 3).

Since the amplification directed by these two primers produced significant polymorphic RAPD patterns as shown in Figure 1. Each of Two primers was reproducible and capable of exhibiting polymorphic amplification patterns and hence, they were used in the subsequent analysis with more number of DNA samples of guava germplasm. The amplification patterns of representative samples of guava variety with two different primers have been shown in Figure 1.

Genetic Distance

Estimates of genetic distance calculated all possible pairs-wise genetic distance values by the Nei's and Li's [18] 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. The values of pair-wise comparisons of genetic distance between 33 guava genotypes were computed from 0.5253 to 0.6631. 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 is 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.

Dendogram

Thirty three genotypes on the dendrogram were distinguished and divided into two major groups (Fig 2) based on Nei's and Li's [18] genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA). UPGMA dendrogram was constructed according to the morphological data. The dendrogram (Fig 2) 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.

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: 2) it was found that most of the cultivated guavas collected from Khulna University gerplasm were grouped under G1-I-1 cluster. We did not find any similarity between 11 cultivated variety 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 Indian in cluster G1-I-1-b found no similarity with others. The commercial culture variety Polly is also an exceptional variety and was found similarity with variety V14.

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.

ACKNOWLEDGEMENTS

I am so much grateful to the members of Molecular Horticulture laboratory, Khulna University and the donor organization of the project "Evaluation of fruits Germplasm available in the southwestern region of Bangladesh on the basis of their physicochemical characteristics and RAPD analysis" for financial support and conduction of the research work.

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