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RAPD and minisatellite markers for genetic diversity and relationship in guava varieties

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Abstract

PCR based Random Amplified Polymorphic DNA (RAPD) and Directed Amplification of Minisatellite DNA (DAMD) markers were used to study the genetic diversity and relatedness among 22 guava accessions comprising commercial cultivars, breeding lines 'and unimproved cultivars. DNA isolated by CTAB method was used for amplification of 96 markers by using 7 RAPD primers and 56 workers generated by 40 DAMD primers. Genetic distance matrix based on Jaccard's coefficient revealed maximum distance between Purple Guava and Allahabad Safeda (43%), whereas minimum distance was as low as 5.4% between two breeding lines HPSI-20 and HPSI-26. Interestingly half-sib progenies CISH-G-1 to CISH-G-6 had slightly more distance ranging from 10.8-24.0%. The clustering revealed that most of the cultivars/accessions originated from Indo-Gangetic plains are grouped together. DAMD was found to suitably cluster the cultivars from exotic origin or having exotic parentage.

Key words: RAPD, DAMD, genetic diversity, relationship, neighbour-joining, Jaccard's coefficient

Introduction

Guava (*Psidium guajava*) is exquisitely delicious, aromatic, nutritious and remunerative fruit crop. Beside its hardiness, productivity and adaptability under varied agro-climatic conditions, it responds very well to various agronomic manipulations, indicating high level of genetic homeostasis for reacting towards such practices. Even though, its origin has been evinced in tropical America (Mexico-Peru), it flourishes exceedingly well in almost all parts of India. Among cultivars, substantial variation exists in terms of growth pattern, yielding behavior, fruit characters (skin, shape etc.) and physico-chemical composition, aroma, pulp to seed ratio, seed characters, susceptibility to diseases, as documented by various workers [1]. Mitra and Bose [2] attributed fruit shape, size, colour and texture to be the main morphological characters used for varietal differentiation. The genetic improvement programme have mainly utilized the existing variability and resorted to selections and sometimes hybridization. As a result, most of the commercial varieties are selections from seedling progenies of open- pollinated seeds. Use of molecular marker techniques like RAPD, DAMD etc., are modern tools for complementing morphological characterization, removal of ambiguities (in systematics) and also aid documentations for generating DNA profiles necessary to protect our genetic resources.

The RAPD assay [3] has been utilized in diversity analysis, mapping and genotype identification in subtropical fruits [4, 5]. The polymorphism detected by RAPD is due to DNA sequence variation at primer binding site and DNA length differences between primers binding site. Similarly single primer amplification targeting minisatellite regions possessing SSR's is due to presence of variable number of VNTR's [6]. Horticultural characteristic are the basis of cultivar identification in guava as in other fruit crops. The present study was undertaken for assessing the genetic variability present in cultivated commercial varieties and breeding lines. An assessment of genetic diversity for organization of the germplasm and differentiation has also been attempted along with rapid characterization of the studied germplasm. Use of two marker systems aim at comparing RAPD with DAMD and the picture of genetic diversity revealed by them can help in reviewing the constraints regarding their use in characterizing genetic resources.

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Materials and methods

Plant materials

Leaf sample from 22 accessions consisting CISH-G-1, CISH-G-3 (Lalit), CISH-G-4 (Shweta), CISH-G-5, CISH-G-6, Allahabad Safeda, Sardar, Behat Coconut, Chittidar, Phoolpur Red, PFS1, PF1, Nasik, Pant Prabhat, HPSI 16, HPSI 20, HPSI 26, KG Guava, Florida Seedling, Philippine Guava, Hongkong, and Seedless were obtained from field gene bank. Young leaves were harvested and brought to laboratory for DNA extraction. Leaves were cleaned and total genomic DNA was isolated using CTAB method [7].

PCR using RAPD primers

The basic protocol reported by Williams *et al*. [8] for PCR was followed for RAPD with slight modification. All PCR reactions were carried out in a final volume of 25µl reaction mixture containing 25-30ng template DNA, 200uM of each dNTPs, 1.5mM - $MgCl_2$, 5pmoles primer, 1X Taq polymerase buffer, 1 unit of Taq DNA polymerase (Bangalore Genei). The amplification was performed in programmable thermal cycler (H. B. Cycler Helena Biosciences (U.K.) with following programme: initial denaturation at 94°C for 5min followed by 45 cycles of, 94°C for 1min, 35°C for 1min, 72°C for 2min and final extension at 72°C for 10 min. The amplified DNA was separated in 1.5% (w/v) agarose gel containing ethidium bromide in 1X TBE buffer at constant voltage ($5Vcm^{-1}$) for 3 hours. These were photographed under UV excitation using Alpha Digi Doc system (Alpha Innotech Corporation).

PCR using minisatellite core sequence primer

DNA amplification was carried out according to Zhou *et al*. [9]. PCR reactions were carried out in a final volume of 25µl reaction mixture containing 25-30ng template DNA, 10µM Tris HCl, 50 mM KC1, 2mM $MgCl_2$, 0.2mM primers, 200uM dNTPs, 1 unit of Taq DNA polymerase and 5pmoles primer. DNA Amplification was carried out by initial denaturation at 92°C for 2min followed by 40 cycles of 92°C for Imin, 55°C for 2min and 72°C for 2min and final extension at 72°C for 5min. The amplified product was run and visualized as described above.

Data analysis

The amplification products from all the primers were scored by presence *vs* absence (scoring as 1/0). The genetic similarity among accessions was determined by Jaccard's coefficients and dendogram was constructed based on Neighbour Joining method with the help of FreeTree software ver 0.9.1.50 [10]. To evaluate the robustness of the tree, bootstrapping was performed using winboot programme. The phenogram was reconstructed 500 times and the frequency with which groups were formed was used to indicate the strength of groups. The trees were viewed, annotated and printed using TreeView program [11].

Results and discussion

RAPD analysis

A total of 11 decamer oligonucleotides, were used to screen the 22 accessions of guava in the present study. Among these 7 primers (Table 1) displaying repeatable, discrete, distinct bands were selected for amplifying the DNA. These yielded 96 markers which ranged from 300-3000 bp, with 9-16 bands per primer at a rate of 13.7 bands per primer. The primer OP A-19 was most polymorphic (Table 3 and Fig. 1a) yielding 14 polymorphic and 2 monomorphic bands, where as OPF-13 yielded 6 polymorphic and 3 monomorphic bands. At the same time for OPF-13 cv. Behat Coconut was characterized by presence of only 3 bands, exhibiting null alleles at other loci.

Table 1. Oligonucleotide sequence details of RAPD primers

Name of RAPD primer	Sequence 5'->3'	No. of bases
OPA2	TGCCGAGCTG	10
OPA19	CAAACGTCGG	10
OPA20	GTTGCGATCC	10
OPC20	ACTTCGCCAC	10
OPF13	GGCTGCAGAA	10
OPF20	GGTCTAGAGG	10
OPG3	GAGCCCTCCA	10

Distance matrix of the RAPD data was calculated using the Jaccard's genetic distance coefficient analysis. The value obtained for each pair wise comparison of RAPD fragments are shown in Table 4. Genetic distances among the accessions comprising commercial cultivars, half-sib populations and important landraces, ranged from 0.054 (between HPSI 20 and HPSI 26) to 0.430 (between Purple Guava and Allahabad Safeda). Similarly low values for genetic distances were also obtained between HPSI 16 and HPSI 20 and among

half-sib population CISH-G1 to CISH-G6 (0.108-0.24). Based on the Jaccard's coefficient, generated dendrogram revealed that PFS1 occupied most distal position forming separate shoot (Fig. 2). The remaining genotypes could be broadly classified into 3 major clusters, which have further subdivisions. Half-sib progeny CISH-G-I, Lalit, Shweta, CISH-G-5 and CISH-G-6 are grouped together forming separate cluster, HPSI 16, 20 and 26 form another cluster and so on. Bootstrap analysis of trees robustness was also performed and the values are given on the nodes. The RAPD analysis also revealed putative cultivar specific amplicons which are given in Table 5.

DAMD Analysis

Out of the 5 minisatellite primers tested, four gave (Table 2) distinct polymorphic products. These cumulatively produced 36 polymorphic and 19 monomorphic amplicons. Primers 33.6b, M13 and HVR were informative primers for generating cultivars specific bands (Table 4 and Fig. 1b). Primer HVR yielded lowest

Table 2. Oligonucleotide sequence details of DAMD primers

Name of RAPD primer	Sequence 5'->3'	No. of bases
M13	GAGGGTGGCGGTTCT	15
HVR	CCTCCTCCCTCCT	13
HVB	GGTGTAGAGAGAGGGGT	18
33.6b	AGGGCTGGAGG	11

Table 3. Oligonucleotide and minisatellite core sequence primers yielding polymorphic and monomorphic bands for genetic diversity analysis

Name of primer	No. of polymorphic bands	No. of mono-morphic bands
OPA2	8	5
OPA19	14	2
OPA20	7	8
OPC20	9	4
OFF 13	6	3
OPF20	9	6
OPG3	6	9
M13	9	6
HVR	4	4
HVB	13	4
33.6b	10	5

Table 4. Putative cultivar specific PCR markers in guava

Marker	Size of band (approx value)	Cultivars
OPA19	1.6kb	Pant Prabhat
33.6b	0.64 kb	Purple guava
M13	2.0kb	HPSI 20

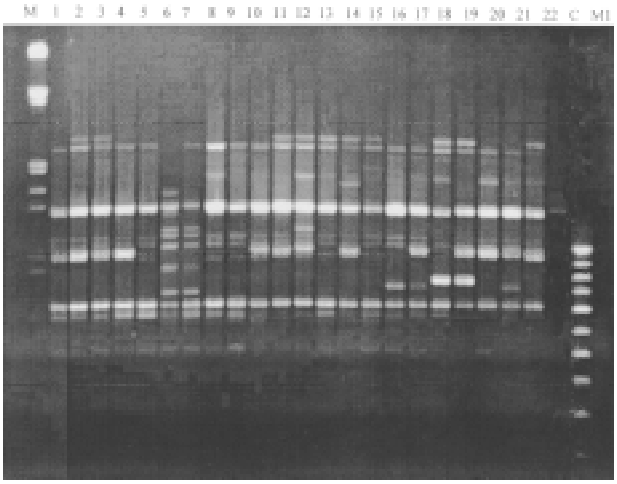

Fig. 1a. DNA fingerprinting of guava varieties obtained by RAPD analysis using OPA 19 primer

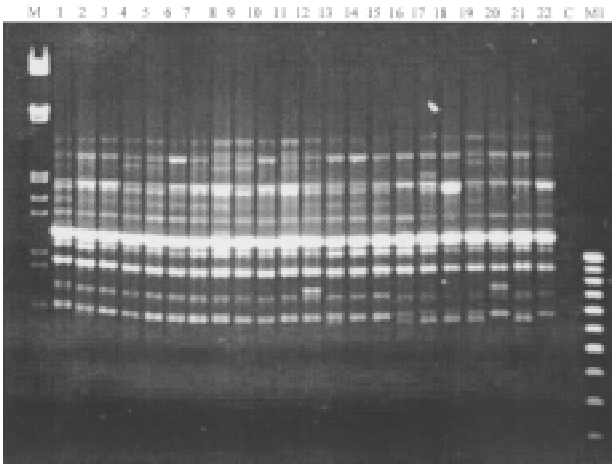

Fig. 1b. DNA fingerprinting of guava varieties obtained by DAMD analysis using M 13 primer: Lane 1: CISH-G-1, Lane 2: Lalit, Lane 3: Shweta, Lane 4: CISH-G-5, Lane 5: CISH-G6, Lane 6: Allahabad Safeda, Lane 7: Sardar, Lane 8: Behat Coconut, Lane 9: Chittidar, Lane 10: Phoolpur Red, Lane 11: PFS1, Lane 12: Nasik, Lane 13: PF1, Lane 14: Pant Prabhat, Lane15: Seedless, Lane 16: HPSI 16, Lane17: HPSI20, Lane18: Purple guava, Lane 19: Hongkong, Lane 20: Florida Seedling, Lane 21: HPSI 26, Lane 22: K.G. guava, Lane C: Negative control, Lane M: EcoRI/Hind III double digested Marker and Lane M1: 100 bp ladder

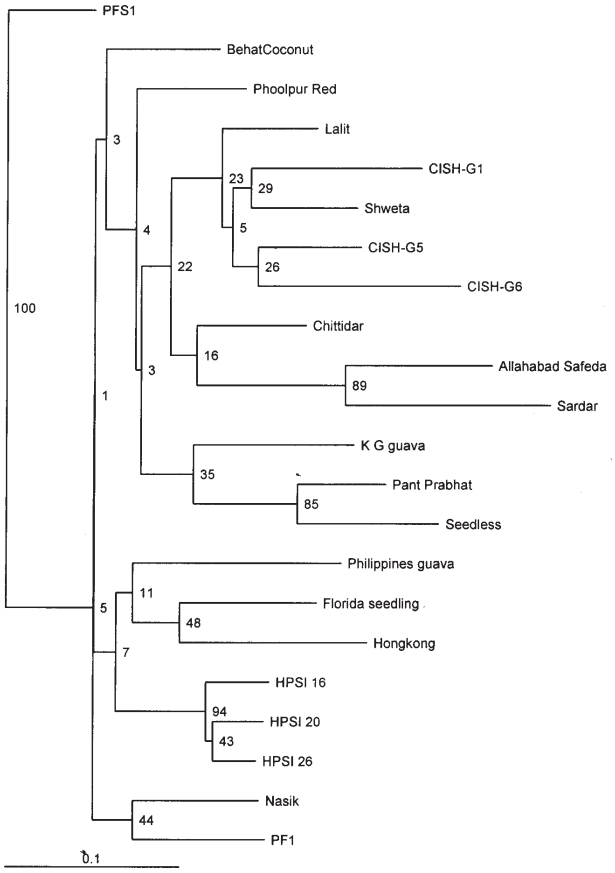

Fig. 2. NJ tree generated after 500 replicate bootstrapping from pair-wise distance data obtained by RAPD primers

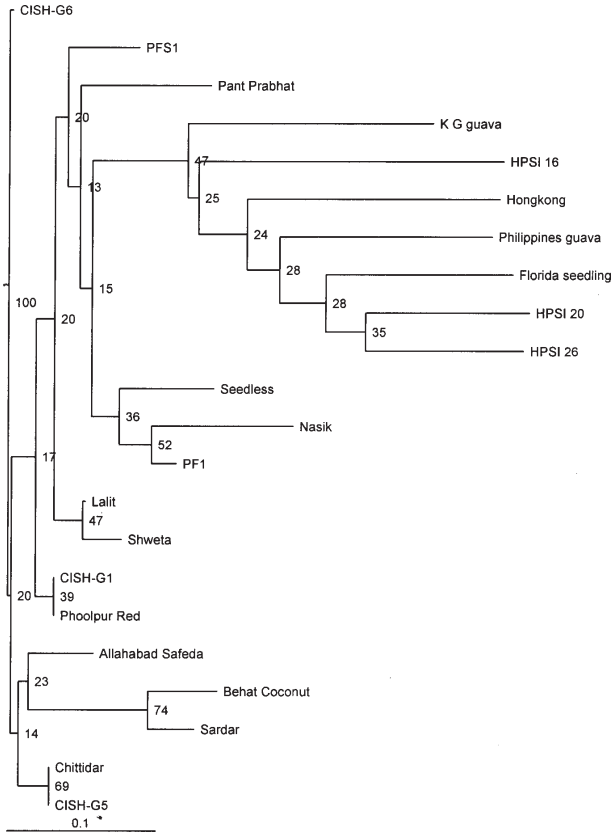

Fig. 3. NJ tree generated after 500 replicate bootstrapping from pair-wise distance data obtained by DAMD primers

number of amplification products, i.e., 8 bands 700 bp to 3 kb in size, HVB amplified 17 bands 300 bp-3 kb in size and 33.6 yielded 15 bands 300bp-3 kb in size. HVB was most informative displaying 76.4% polymorphism.

The distance values based on minisatellite analysis are presented in Table 4. The genetic distance among the sample accessions ranged from as low as 0.001 (between Phoolpur Red and CISH-G-1 and also CISH-G-5 and Chittidar) to 0.466 between HPSI 16 and Behat Coconut. NJ tree constructed on the basis of DAMD distance coefficient was comparatively more spread and showed random clustering, CISH-G-6 being most distant and forming a separate shoot (Fig. 3). CISH-G-5 and Chittidar were grouped together, and so were Sardar, Behat Coconut with Allahabad Safeda. Rest of the cultivars/accessions formed diverse groups among themselves.

The two methods RAPD and DAMD have employed single primer amplification and these enjoy wide usage, because of being less expensive, and not needing genomic nucleotide sequence data. The RAPD and DAMD target different genomic regions that necessarily do not overlap, and together provide adequate coverage of the genome to be analysed. While DAMD targets the minisatellite rich regions, RAPD generate banding profiles representing several disparate regions. Present analysis of diversity based on 96 markers is expected to saturate the genome at density of one marker per 5.5 mbp in diploids, which is sufficient to infer the diversity/relatedness among the guava individuals (presuming genomic size of guava to be similar to eucalyptus n = 11) [12]. The analysis suggested that diversity is low to moderate, genetic similarity being as high as 94.6 to 57.0% (based on Jaccard's matrix). Perusal of data indicated that CISH-G-1 to CISH-G-6, Chittidar, Allahabad Safeda and Sardar are closely related, which indicates that these accessions have common parental material. Sardar is a selection from open-pollinated Allahabad Safeda, whereas Chittidar closely relates to Allahabad Safeda with distinct spots on the fruit surface. The CISH-G-1 to

CISH-G-6 are selections from half-sib population of the parent material originated from Allahabad area, probably from Allahabad seedlings. Obviously the genetic distance between all these 8 accessions was less, Phoolpur Red also originated from seedling population of Allahabad Safeda. Surprisingly KG guava, which may not have originated from Indo-Gangetic plains with dominance of Allahabad Safeda, is also member of the cluster, comprising all these accessions. Another important finding of distance matrix is that Seedless guava, a triploid found quite nearer to Pant Prabhat, phylogenetically. They also show similarity in leaf characters thus corroborating molecular data.

Accessions other than these, Philippine Guava, Florida Seedling, Hong Kong, HPSI 16, HPSI 20 and HPSI 26 were different from Allahabad Safeda and its relatives, which is also corroborated by distant geographical origin of these accessions and dissimilarity in morphology of fruits. The study was quite adequate in revealing the correspondence between morphological traits and molecular markers to a great extent. The low distance index (43%), reflects high similarity among the studied accessions, as also earlier reported [5, 13].

The clustering pattern obtained by employing DAMD was somewhat different than that produced by RAPD. Interestingly half-sib populations of CISH selections were placed with different genotypes, but most of them were from Indo-Gangetic plains and nearer to Allahabad Safeda in phylogenetic relationship. The technique efficiently clustered the accessions which had exotic origin of parental lines. The most important cultivars of this country Sardar and Allahabad Safeda have been grouped in the same cluster which show that morphological difference among these two cultivars are not manifested at genetical level targeted by the satellite DNA.

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