Pak. J. Agri. Sci., Vol. 50(4), 591-597; 2013
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
http://www.pakjas.com.pk

EVALUATION OF GENETIC DIVERSITY IN OPEN POLLINATED GUAVA BY iPBS PRIMERS

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DNA markers are important tools for assessing genetic diversity and relationships among species, cultivars and breeding materials. Many horticultural species are lacking genomic information. DNA markers that do not require prior knowledge of DNA sequences are therefore appealing for horticultural research. A retrotransposon-based DNA marker system, iPBS (inter primer binding sites) developed from conserved primer binding sites within retrotransposons, was used to study the genetic variation and relationships in ornamental guava. PCR from 6 iPBS primers (dominant markers) produced a total of 113 bands (52.38-100% polymorphic) ranging from 150 bp to 3000 bp, and the mean PIC value for each primer ranging from 0.1245 to 0.3698. Molecular information generated from both iPBS was separately scored in a matrix for phylogenetic dendrogram construction. The phylogenetic dendrogram based on iPBS markers reflected morphologic classifications of the accessions that were studied. The iPBS PCR-based genome fingerprinting technology in this study is low-cost and provides another effective alternative in differentiation of accessions in guava (*Psidium guajava* Linn.) and related species or genera.
Keywords: iPBS, PCR, genetic relationship, genetic diversity, guava

INTRODUCTION

Cultivated guava (*Psidium guajava* Linn., 2n = 2x = 22) belongs to the genus *Psidium* (*Myrtaceae*), and it is one of the most important fruit crops grown commercially across the tropics and sub-tropics (Hayes, 1970; Pathak and Ojha, 1993; Rodriguez *et al*., 2010). Guava fruit is generally known as the apple of tropics and sub-tropics because of the high vitamins content (A and B complex) and especially rich in vitamin C (Prakash *et al*., 2002). Originated mainly from South and Central America, wild relatives of guava include Brazilian guava (*Psidium guineense*), Mountain guava (*Psidium montanum*), Strawberry or Cherry guava (*Psidium cattleianum*), Pineapple guava (*Acca sellowiana*) and Chilean guava (*Ugni myricoides*). Approximately 130 genera and 3,000 species are categorized within *Myrtaceae* family with evergreen trees and shrubs distributed in tropics and sub-tropics of the world, and genus *Psidium* has more than 150 species comprising many edible species (Watson and Dallwitz, 2007; Jaiswal and Jaiswal, 2005; Padilla-Ramirez and Gonzalez-Gaona, 2010).

According to the amount of fruit production, India has the most mass production of guava fruit, followed by Pakistan, Mexico, Brazil, Egypt, Thailand, Columbia and Indonesia; and guava fruit production has increased 10 folds in the last five years in these countries (Pommer and Murkami, 2009). Guava plant growth is related to nutrient acquisition (Swain and Padhi, 2012) and guava branch architecture influences foliage fauna (Ghaffar *et al*., 2011). After citrus and mango guava fruit occupies 3rd position in production in Pakistan and it is extensively grown in Punjab and Sindh (Anonymous, 2012). High percentage of cross pollination in guava orchards leads toward clonal degradation as the conventional propagation is made through seeds and is one of the main reasons for low productivity in Pakistan. Therefore, it is necessary to evaluate both morphological and molecular characteristics of all the potential domestic guava germplasm resources and some major international cultivars for breeding superior varieties in Pakistan.

Similarly in Australia a range of foreign cultivars (including Hawaiian Pink, Mexican Cream, Allabad Safeeda) were imported for commercial productions as well as breeding projects in 1970s, and a series of cultivars were selected for fruit production (Menzel, 1985). However, sexual propagation (propagation from seed) is still being used by nurserymen worldwide because of economic reasons but results in lot of variations in seedlings because of cross pollination (25-40%). Such guava variants are named by the local growers according to a few morphological characters; hence the existence of synonym and homonym trees (cultivars commonly misnamed) is a major problem in guava orchards. However, rooting of cuttings is effective vegetative propagation method (Kareem *et al.,* 2013) to produce true to type and quality plants. Accurate characterization of guava cultivars and rootstocks is essential for commercial orchards and nurseries and can guarantee uniformity in the establishment of new orchards (Anonymous, 2010). The inclusion of DNA-based markers for germplasm characterization provides more basic information, and because these markers are not affected by the environment,

conclusions and interpretations could be more reliable (Sanchez-Teyer *et al*., 2010).

Genetic diversity and discrimination among individual accessions or groups of individuals or populations can be analyzed by a specific method or a combination of methods (Lepitre *et al*., 2010; Valdes-Infante *et al*., 2010; Coser *et al*., 2012; Ritter 2012). Different molecular markers such as AFLP (Valdes-Infante *et al*., 2003; Hernandez-Delgado *et al*., 2007; Sanchez-Teyer *et al*., 2010), ISTR (Rodriguez *et al*., 2004), RAPD (Chen *et al*., 2007; Feria-Romero *et al*., 2009; Ahmed *et al*., 2011; Coser *et al*., 2012) and SSR (Risterucci *et al*., 2005; Rodriguez *et al*., 2007; Aranguren *et al*., 2010;) have been used for guava germplasm analysis. Among these different types of molecular markers, microsatellites or SSRs (defined as short tandem repeats) have been widely used as an efficient tool for germplasm characterization and for management and diversity studies on *Psidium* germplasm in different countries (Briceno *et al*., 2010; Costa *et al*., 2012).

Recently iPBS markers have been developed as an alternative method to explore genetic diversity and relationships in plants (Kalendar *et al*., 2010; Kalendar *et al*., 2011; Smykal *et al*., 2011). There was a need to study the genetic diversity and structure of collected guava germplasm using SSR system and this new technique. Hence, this study was conducted to assess the strength of iPBS markers to analyze the genetic variability among open pollinated guava crop.

MATERIALS AND METHODS

***Plant Material*:** Research was conducted in PBI (Plant Breeding Institute) University of Sydney, Australia. The seeds were collected from an open pollinated fresh fruits of variety Allabad Safeeda. Seeds were washed and left to air dry for 5 days at room temperature before treatment. Seeds were treated with 1% bleach for 15 minutes. Every 20 seeds were put on moistened filter paper in a petri dish. After 2 months germinated seedlings have six leaves and young leaves were used for DNA extraction. Population of 19 seedlings (G_1, G_2, G_3, G_4, G_5, G_6, G_7, G_8, G_9, G_{10}, G_{11}, G_{12}, G_{13}, G_{14}, G_{15}, G_{16}, G_{17}, G_{18} and G_{19}) was randomly selected for DNA extraction from a collection of 100 populations of Allabad Safeeda. Six iPBS primers were applied to test their genetic diversity within the population

***DNA extraction*:** Leaves collected from Allabad Safeeda used for DNA isolation. DNA was extracted from 200 mg fresh leaves using plant DNA isolation Mini Kit Bioline) in accordance with the manufacturer's protocol. DNA concentration was estimated by a 2.0% agarose gel electrophoresis comparing with known λ DNA concentration. All isolated DNA samples were diluted to 2ng/µl and used as templates for iPBS method.

***iPBS PCR amplification*:** iPBS primers as listed in Kalender *et al.* (2010) were synthesized by Sigma Aldrich (Castle Hill, NSW, Australia) and DNA amplification was carried out by using a modified protocol of Kalendar *et al.* (2010). The PCR was performed in a 20µl reaction mixture containing 2 ng DNA, 1 time GoTaq buffer (Promega), 0.5 µM of primer (single primer), 0.2 mM dNTPs, 0.5 unit Taq DNA polymerase (GoTaq, Promega), 2.0 mM $MgCl_2$. The PCR program had an initial hot start at 95°C for 3 minutes, 40 cycles of denaturation at 94°C for 30 seconds, annealing at 45-50°C for 30 seconds and extension at 72°C for 2 minute. Following this was a final extension at 72°C for 5 minutes and then the program was terminated by holding at 10°C. The reaction was performed in Master Cycler (Eppendorf) in 0.2 ml tubes or 96-well plates. 5µl of each PCR products were electrophoreses at 70 V for 3 hours in a 1.5% (w/v) thin agarose gel with 1× TAE buffer (0.04 M Tris-acetate, 0.001M EDTA). Thin agarose gel was made on a glass plate by surface tension. One kb ladder (Fermentas, AUS) were used to estimate fragment lengths. Gels were post stained with GelRed (Biotium) for 15-30 min, and photographed using the Gel Doc-It Imaging System (UVP) at exposure rate of 1-2s.

***Data scoring and analysis*:** For each primer used, PCR was repeated twice to confirm band pattern consistency. DNA bands were sized and scored by LabWorks software (v4.5, UVP) and carefully checked manually; only the clear bands were scored and the faint bands were ignored. The same-size band for a data set was assumed to represent a single locus. For presence or absence of an iPBS band at a particular locus, the data were recorded as 1 for presence and 0 for absence to build binary matrices. Polymorphic Information Content (PIC) for dominant markers was calculated as: PIC = $1-[f^2+ (1-f)^2]$, where "f" is the frequency of the marker in the data set. The maximum PIC for a dominant marker is 0.5 for f = 0.5 (Riek *et al*., 2001). For each primer, the PIC value was the mean of calculated PIC of all loci. Polymorphic Information Content (PIC). Dendrograms were built based on Dice genetic similarity coefficient (Nei and Li, 1979) using the unweighted pair-group method with arithmetic averages (UPGMA). The iPBS binary matrices were imported into the Tree Drawing using PHYLIP (Felsenstein, 2005). The NTSYS pc2.2 software package (Rohlf, 2000) was also used for principal component analysis (PCA).

RESULTS

***Analysis of 19 open pollinated guava population DNA by 6 iPBS primers*:** A total of 19 *Psidium guajava* accessions from an open pollinated population were analyzed with 6 iPBS primers (2079, 2238, 2241, 2251, 2228 and 2376) for phylogenetic dendrogram (Fig. 2), 2D PCA analysis (Fig. 3) and 3D PCA analysis (Fig. 4). Fingerprinting of 19 open pollinated guava (*Psidium gujava* L.) DNA band patterns

from primer 2241 are represented in Fig. 1. The number of bands, number of polymorphic bands, percentage of polymorphism and mean PIC value from these 6 iPBS primers are presented in Table 1. The size of reproducible and scorable bands ranged from 150 to 3000 bp. These 6 primers amplified a total of 113 scorable bands. Primer 2238 produced 22 (the highest) and primer 2228 produced 15 (the lowest) bands. Similarly other primers as 2079, 2241, 2376, 2251, had 21, 21, 18 and 116 bands, respectively. Percentage of polymorphism for primer 2251, 228 and 2376 is as high as 100%. Primer 2238, 2079 and 2241 had 95.45%, 52.38%, 66.66% polymorphism, respectively. Primer 2251 had the highest PIC value (0.3501) while primer 2079 had the lowest (0.1245) PIC value (Table 1). These results indicated that these 6 iPBS markers used in this study revealed a wide range of genomic DNA diversity in this open pollinated guava (*Psidium guajava* L.) population.

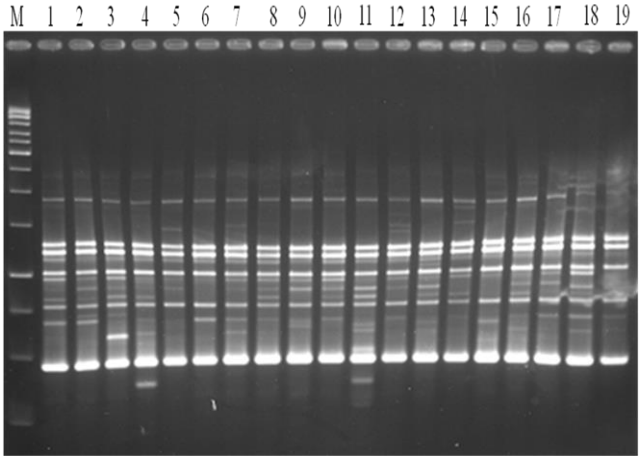

Figure 1. Nineteen open pollinated guava DNA accessions iPBS PCR band pattern from primer 2241

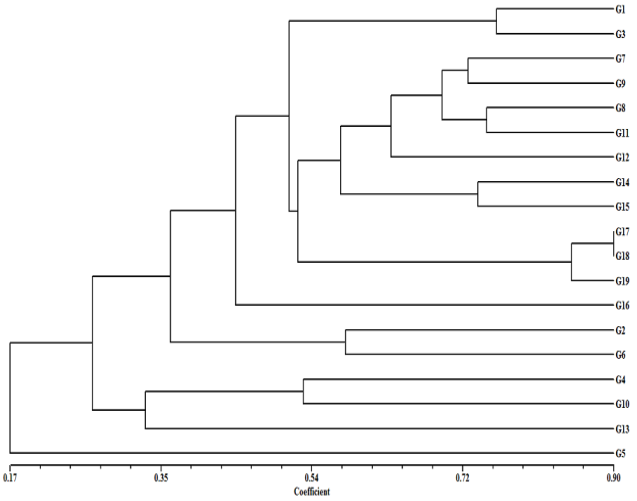

Figure 2. Dendrogram for 19 *Psidium guajava* accessions from an open pollinated population

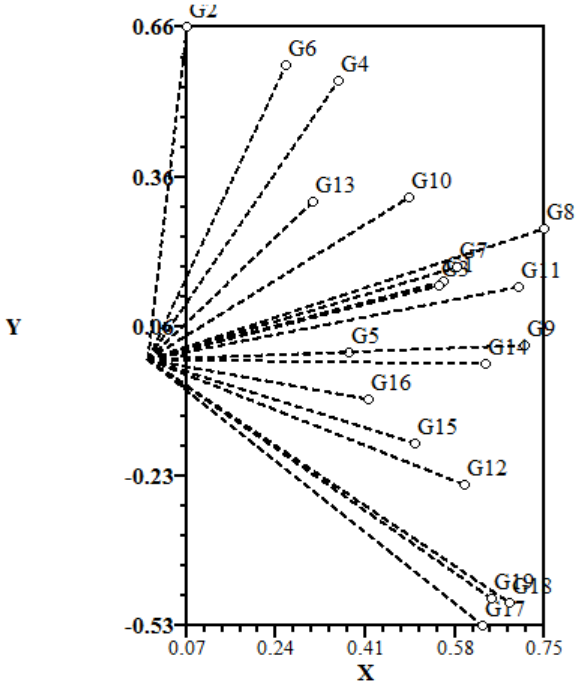

Figure 3. 2D PCA plot for of 19 guava accessions based on iPBS primers

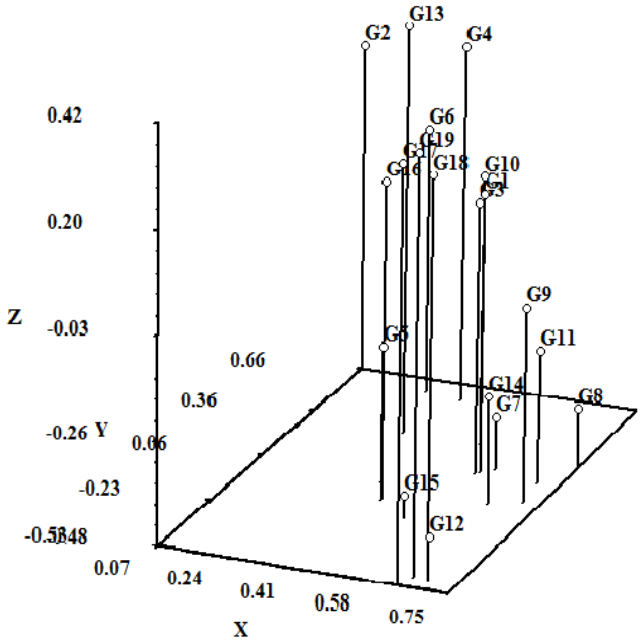

Figure 4. 3D PCA plot for of 19 guava accessions based on iPBS primers

***Analysis of molecular data and construction of phylogenetic trees*:** Binary matrices from the DNA

Table 1. iPBS primers used in the detection of polymorphism 19 accessions of *Psidium* population value is calculated as PIC = 1-[f^2+ (1-f)^2], where "f" is the frequency of the marker in the data set.

iPBS primer	Sequence (5'-3') & Ta		Species tested	Number of bands*	Number of polymorphic bands	Percentage of polymorphism (%)	Mean PIC value
2079	AGGTGGGCGCCA	55ºC	*Psidium guajava*	21	11	52.38	0.1245
2241	ACCTAGCTCATCATGCCA	59ºC	*Psidium guajava*	21	14	66.66	0.1920
2238	ACCTAGCTCATGATGCCA	55ºC	*Psidium guajava*	22	21	95.45	0.1995
2251	GAACAGGCGATGATACCA	55ºC	*Psidium gujava*	16	16	100.00	0.3501
2228	CATTGGCTCTTGATACCA	56ºC	*Psidium guajava*	15	15	100.00	0.3698
2376	TAGATGGCACCA	40ºC	*Psidium guajava*	18	18	100.00	0.2339

*Number of bands means the total accountable bands which are constantly appeared in two or three repeated experiments

fingerprints produced by the 6 primers used for guava were imported into the NTSYS-pc v2.2 software package for analysis of genetic similarity. The UPGMA dendrograms (Fig. 2) and two, three dimensional graphs from principle component analysis on guava are presented in Fig.s 3 and 4, respectively.

Figure 2 indicates that 19 accessions distributed themselves into 5 groups. Group 1 contained G_5 accession, group 2 had three accessions (G_{13}, G_{10} and, G_4), group 3 had two accessions (G_2 and G_3), group 4 had G_{16} and group 5 had twelve accessions (G_{19}, G_{18}, G_{17}, G_{15}, G_{14}, G_{12}, G_{11}, G_8, G_9, G_7, G_3 and G_1). 2D and 3D PCA analysis was also supported this result (Fig. 3, 4).

The results showed high divergence among open pollinated guava crop. Propagation through seed is big hindrance to produce true to type plant and quality fruit. These results also revealed that iPBS markers maintained desirable distinguishing power in determining genetic diversity and relationships.

DISCUSSION

Molecular markers are valuable for assessing genetic diversity and relationships in plant breeding. In this study, retrotransposon derived iPBS markers tested on guava (*Psidium guajava* L.) yielded useful information on genetic relationships among the accessions. Retrotransposons are abundant and ubiquitous components of eukaryotic genomes, especially plants (Flavell *et al.,* 1992; Waugh *et al.,* 1997). LTR retrotransposon, whose replication is through an RNA intermediate, has a LTR at the both ends. Based on their unique structure and wide dispersion in plant genomes, a number of retrotransposon-based marker systems have been developed and shown to be useful in studies of population structure, genome evolution and gene mapping. Retrotransposon-based markers were successfully applied in many plant species and genera including barley, pea, tomato, bread wheat, flax, *Oryza*, *Cucumis*, *Vitis* and *Musa* (Waugh *et al.,* 1997; Pearce *et al.,* 2000; Tam *et al.,* 2005; Gribbon *et al.,* 1999; Smykal *et al.,* 2011; Branco *et al.,* 2007; Lou and Chen, 2007; Moisy *et al.,* 2008; Teo *et al.,* 2005). The development of most retrotransposon-based marker systems, such as SSAP (sequence-specific amplified polymorphism), IRAP (inter-retrotransposon amplified polymorphism) and REMAP (retrotransposon-microsatellite amplified polymorphism), requires knowledge of the sequence of a particular part of a retrotransposon. It usually involves cloning and sequencing for each species or closely related species group. There is a lack of genomic information for many ornamental species which are not financially supported for development of efficient marker systems. The iPBS method was developed on the assumption of a universal presence of tRNA complemented primer binding site (PBS) in LTR retrotransposons (Kalendar *et al.,* 2010). Application of iPBS in guava in our study has shown it is a powerful DNA fingerprinting technique in the absence of prior sequence information. On the practical side, iPBS marker is somewhat like the RAPD marker system, but iPBS amplification conditions are more stringent than those for RAPD, especially in the case of 18 base primers. The reproducibility of iPBS is superior to RAPD; the number of bands and number of polymorphic bands generated by iPBS are usually higher than those produced by RAPD (unpublished data). Phylogenetic analysis is based on the scoring of fingerprinting bands and assumes that bands with the same size correspond to the same locus. However, similarity in band size does not necessarily indicate identity in sequence content, especially when interspecific data are compared. A study in sequence variation in SSR amplicons (Barthe *et al.,* 2012) demonstrated the complexity of amplicon sequences, which not only include the number of repeats, but also variation in sequences, including insertions, deletions and SNPs. The conclusion of Barthe *et al.* (2012) was that phylogenetic interpretation of SSR data must be with caution and therefore a combination of sequence and SSR variations in phylogenetic analysis provides greater resolution. The same situation can be applied for this study, or generally for all DNA fingerprinting data analysis. A larger dataset (larger number of markers) may reduce this limitation and minimize the problem in the interpretation of genetic relationships

when using DNA fingerprinting. In some cases when the dataset is not large or the comparison is between species, it may be necessary to sequence some bands to determine the identity. In this study, iPBS molecular differences were observed between intra-specific relationships among open pollinated guava accessions. In the interpretation of the iPBS PCR amplification profile with one specific primer, the band patterns between species were generally more polymorphic than that for plants within populations. Different primer(s) can be used to differentiate plants in the population. Results from this research proved that iPBS marker system can properly detect genetic differences not only at interspecific level but also at intra-specific level. A more detailed analysis using a large number of accessions in genus *Guava* would be necessary to clarify these findings and to make a concrete proposal regarding species classifications since some doubts exist in current botanical classification in this genus (Simoes *et al.*, 2007a,b). The iPBS molecular information revealed among *Psidium* accessions in this study demonstrated that it is possible to take advantage of the heterosis expressed in some F1 hybrids because commercial cultivars are produced between different inbred lines. Heterosis in hybrids depends upon the two parents being genetically unrelated (Dieckmann and Link, 2010; Muthoni *et al.*, 2012).

Guavas are among the most heterozygous fruit crops, and this is confirmed by the results of iPBS analysis on an open pollinated population in this study. Investigation of the genetic diversity and relationships in *Psidium* germplasm is of great importance for breeding, conservation, management and utilization of plant materials (Pommer and Murkami, 2009; Pommer, 2012). Molecular markers like iPBS add great value in assessment of genetic diversity and relationship in plant phylogenetic analysis as well as plant breeding (Kalendar *et al*., 2011).

***Conclusion*:** It is concluded that iPBS is a useful DNA fingerprinting tool for evaluation of genetic diversity and relationships of species, cultivars and breeding lines, especially for species with underdeveloped marker systems. It is a fast, low-cost and efficient molecular method applicable to plant breeding.

***Acknowledgement*:** The authors appreciate the efforts of The University of Agriculture, Faisalabad, Pakistan, Higher Education Commission, Pakistan and Punjab Agricultural Research Board for providing financial support and scientific facilities. We are also grateful to Dr. Nabil Ahmad and Dr. Shuming Luo of Plant Breeding Institute (Cobbitty), The University of Sydney for statistical data analysis.

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