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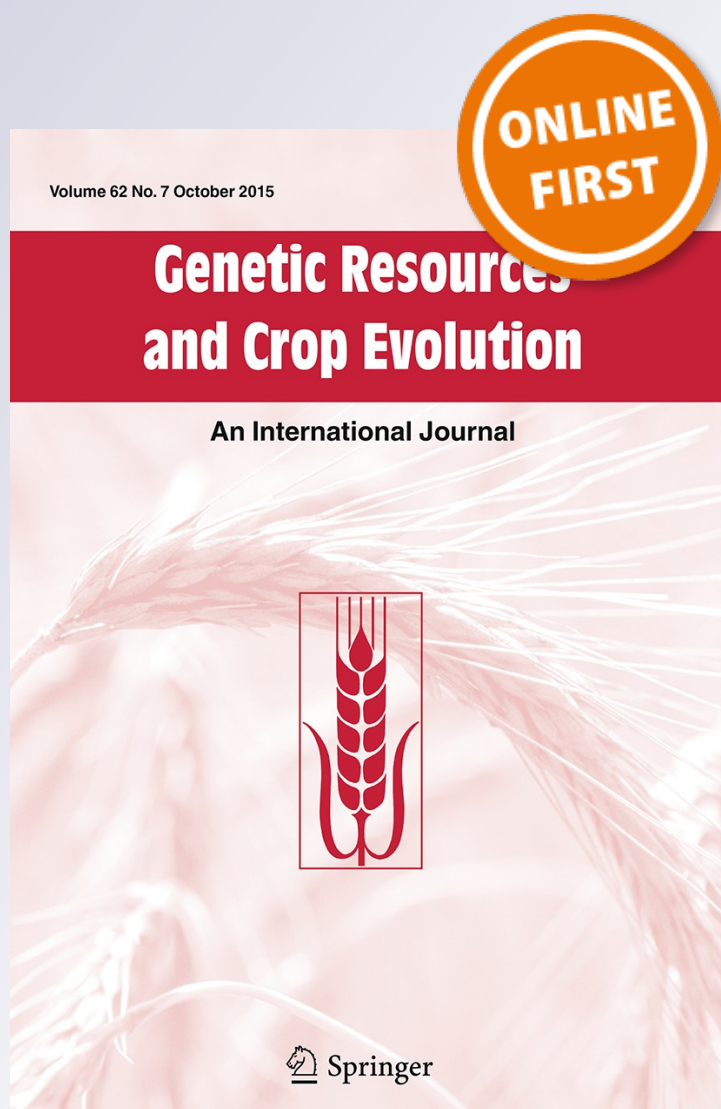
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Molecular variability and phylogenetic relationships of guava (*Psidium guajava* L.) cultivars using inter-primer binding site (iPBS) and microsatellite (SSR) markers

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Abstract The main objective of this research was to add genotyping information to previous phenotyping findings of Pakistani guava for the purposes of selective breeding. Inter-primer binding site (iPBS) and microsatellite (SSR) markers were used to assess the molecular variation and genetic structure of 51 promising Pakistani guava (*Psidium guajava* L.) genotypes which were then compared with 19 others from different geographical regions across the world. PCR of 6 iPBS primers (dominant markers) produced

a total of 97 bands (96.63 % polymorphic) ranging from 100 to 2800 bp and the mean PIC for primers ranged from 0.1687 to 0.3522. The mean unbiased expected heterozygosity (0.183), Shannon's information index (0.275) and average fixation index (F_{st} or inbreeding) (0.925) indicated a high level of inbreeding among the accessions tested. Multi-locus DNA fingerprints using 18 SSR loci unambiguously differentiated all accessions and demonstrated an absence of duplicated samples. Diversity analysis revealed a total of 172 alleles (from 124 to 553 bp) ranging from 2 to 17 with a mean value of 9.56 alleles per locus. The mean unbiased expected heterozygosity (0.091), Shannon's information index (0.130) and mean inbreeding coefficient (0.854) also indicated a high level of inbreeding among the accessions. Ordination and cluster analysis from both iPBS and SSR markers showed that the genetic relationships between all accessions could be separated into geographic origin, specifically Pakistan, Mexico, Hawaii and India. Guava accessions cultivated in Pakistan and wild guava germplasm are highly divergent and possess abundant genetic diversity. The iPBS PCR-based genome fingerprinting technology used in this study is low-cost and provides an effective alternative in differentiating accessions of guava and their related species or genera.

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Keywords Genetic diversity · Germplasm · Plant
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Introduction

Cultivated guava (*Psidium guajava* L., $2n = 2x = 22$) in the genus *Psidium* (family Myrtaceae) is one of the most important fruit crops grown commercially across the tropics and sub-tropics (Bajinath et al. 1982; Hayes 1970; Pathak and Ojha 1993; Rodriguez-Medina et al. 2010). Guava fruit is generally known in India as the 'apple of the tropics and sub-tropics' because of its high vitamin content (A and B complexes) and because it is especially rich in vitamin C (Prakash et al. 2002). Originating mainly from South and Central America, wild relatives of guava include Brazilian guava (*Psidium guineense* Sw.), Mountain guava (*Psidium montanum* Sw.), Strawberry or Cherry guava (*Psidium cattleianum* Sabine), Sartre guava [*Calyptrorpsidium sartorianum* (O. Berg) Krug et Urb.], Pineapple guava [*Acca sellowiana* (O. Berg) Burret] and Chilean guava [*Ugni myricoides* (Kunth) O. Berg]. Approximately 130 genera and 3000 species are categorized within the Myrtaceae and the genus *Psidium* has more than 150 species, many of which produce edible fruits (Jaiswal and Jaiswal 2005; Padilla-Ramirez and Gonzalez-Gaona 2010; Watson and Dallwitz 2007). However, there is limited genetic information about guava, which could underpin future selective breeding programs as well as genetic studies.

In terms of fruit production, India produces the most guava fruit, followed by Pakistan, Mexico, Brazil, Egypt, Thailand, Columbia and Indonesia. Guava fruit production has increased tenfold in the last 5 years in these countries (Pommer and Murkami 2009). In Pakistan, guava fruit is extensively produced in Punjab and Sindh provinces and ranks third after citrus and mango in terms of production. However, the prevalence of cross-pollination in established new orchards is one of the main reasons for low productivity in Pakistan (FAO 2002). The morphological characteristics of our 51 Pakistani accessions have been previously analysed by Mehmood et al. (2014). This study is designed to analyse the molecular characteristics of all the potential domestic guava germplasm resources in Pakistan as well as other accessions from different geographical regions, so that superior varieties could be bred.

In Australia, a range of foreign guava cultivars (including Hawaiian Pink, Mexican Cream, Allabad Safeeda) imported in the 1970s for commercial production and breeding projects led to a series of

cultivars being released for commercial fruit production (Menzel 1985). By comparison, however, local growers in Pakistan replenish their trees from sexual propagation (propagation from seed). No commercial cultivars are available. Since these growers name their cultivars according to a few morphological characters and locality, the naming system is confused. Accurate characterization of guava cultivars and rootstocks is essential for commercial orchards and nurseries as this can guarantee uniformity in the establishment of new orchards (Pakistan Statistical Yearbook 2010). This can be supported by inclusion of DNA-based markers (such as the iPBS and SSR marker systems) for germplasm characterization to provide more basic genetic information. As markers are not affected by the environment, conclusions and interpretations regarding genetic variation will be more reliable (Sanchez-Teyer et al. 2010).

Genetic diversity and discrimination among individual accessions or groups of individuals or populations can be analysed by a specific method or a combination of methods (Cosser et al. 2012; Lepitre et al. 2010; Mehmood et al. 2014; Ritter 2012; Valdes-Infante et al. 2010). Different molecular markers such as AFLP (Hernandez-Delgado et al. 2007; Sanchez-Teyer et al. 2010; Valdés-Infante et al. 2003), ISTR (Rodríguez et al. 2004), RAPD (Ahmed et al. 2011; Chen et al. 2007; Cosser et al. 2012; Feria-Romero et al. 2009) and SSR (Aranguren et al. 2010; Risterucci et al. 2005; Rodriguez et al. 2007) have so far 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 in different countries as efficient tools for germplasm characterization, for plant management and for diversity studies on *Psidium* germplasm (Briceno et al. 2010; da Costa et al. 2012).

iPBS markers were developed as an alternative method to explore genetic diversity and relationships in plants (Kalendar et al. 2010, 2011; Smykal et al. 2011). This marker system was recently used for fingerprinting in apricot (Baranek et al. 2012) and *P. guajava* (Mehmood et al. 2013). The present research aimed to investigate genetic diversity and structure of our assembled guava germplasm (51 cultivars from 6 Pakistani regions and 19 accessions collected in Australia) using the new iPBS technique together with SSR markers. The study was conducted as part of the guava research program at the Institute of

Horticultural Science, University of Agriculture, Faisalabad, and included research undertaken at Plant Breeding Institute, Cobbitty.

Materials and methods

Plant materials

The plant materials used in this study are listed in Table 1. Seventy accessions of *P. guajava* and several related species were used for iPBS and SSR analysis. Fifty-one accessions of *P. guajava* varieties were collected from different regions in Punjab (Faisalabad, Sheikhpura, Sahiwal, Cheshtian Mandi, Bahawalpur) and in Khyber Pukhtoonkhwa (Peshawar) provinces (Fig. 1). The accessions were selected on the basis of fruit shape, fruit size, flesh colour, seed weight and seed number for future breeding projects. Nineteen accessions were collected from the germplasm collection at the Plant Breeding Institute, Cobbitty; those 19 accessions included 1 *U. myricoides*, 2 *A. selowiana*, 3 *P. catteleianum* and 13 *P. guajava* (Hawaiian Pink, Beamount, Mexican Cream, Allabad Safeeda and nine hybrids involving them).

DNA extraction and quantification

Young fresh leaves collected from *P. guajava* and related species were used for DNA isolation. DNA was extracted from 200 mg of fresh leaves using the plant DNA isolation Mini Kit (Bioline, Australia) in accordance with the manufacturer's protocols. Quality and quantity of DNA was checked following 2.0 % agarose gel electrophoresis by comparison with known λ DNA concentrations. Portion of the isolated DNA were diluted in molecular grade water to 2 ng/ μ L concentration and used as templates for PCR.

iPBS PCR amplification

iPBS primers listed by Kalendar et al. (2010) and used in this study were from Sigma Aldrich (Castle Hill, NSW, Australia). DNA amplification was carried out by a slightly modified protocol from Kalendar et al. (2010). PCR were performed in 20 μ L reaction mixtures containing 2 ng genomic DNA, 1 time GoTaq buffer (Promega), 0.5 μ M of primer (single primer), 0.2 mM dNTPs, 0.5 U Taq DNA polymerase

(GoTaq, Promega) and 2.0 mM $MgCl_2$. The PCR program had an initial hot start at 95 °C for 3 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 38–56 °C for 30 s and with an extension at 72 °C for 2 min. There was a final extension at 72 °C for 5 min and the program was terminated by holding at 10 °C. The reaction was performed in a Bio-Rad T100™ Thermal Cycler with 0.2 mL tubes or 96-well plates. A 5 μ L sample of each PCR product was electrophoresed at 70 V for 3.5 h in a 1.5 % (w/v) thin agarose gel with 1 \times TAE buffer (0.04 M Tris–acetate, 0.001 M EDTA). A Thermo Scientific GeneRuler 1 kb ladder (Fermentas, Australia) was 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 an exposure of 1–2 s.

SSR PCR amplification

Eighteen SSR primer combinations from Risterucci et al. (2005) were used, and the allele size range, EMBL accession numbers and repeat motif for these 18 primers were listed in the website <http://rda.ebi.edu.au/default.jsp;jsessionid=5FCDF0E3B5BF388387C0ED60FA48EEFA?d-7523440-p=2&collectionId=4769&view=preview>. PCR were performed on 10 μ L volumes of reaction mixture containing 2 ng of genomic DNA, 1 time GoTaq PCR buffer (Promega), 0.5 μ M forward primer (labeled with M13) and 0.5 μ M reverse primer, 0.2 mM dNTPs, 0.05 μ M IRDye 700 or IRDye 800, 0.5 unit Taq DNA polymerase (GoTaq, Promega) and 2.0 mM $MgCl_2$. A 19 base pair M13 sequence (CACGACGTTG-TAAAACGAC) was 5'-tailed with all the forward primers. DNA accessions 1–35 used IRDye 700 while accessions 36–70 used IRDye 800. The PCR program had an initial hot start at 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 45–50 °C for 30 s and extension at 72 °C for 2 min, after which there was a final extension at 72 °C for 5 min before the program was terminated by holding at 10 °C. 3 μ L of the PCR product was separated on 2.0 % agarose gel electrophoresis to check the presence of specific bands. Due to the characteristics differences between the IRDye 700 and IRDye 800, add 15 μ L dye (0.5 % Fuchsin dye + 100 % formamide + 0.5 M EDTA) to PCR samples 1–35, and 10 μ L to accessions 36–70 for optimizing band pattern images shown on the LI-COR

Table 1 Seventy guava (*Psidium guajava* L.) accessions used in iPBS and SSR analysis

Accession	Cultivar name	Origin	Genus	Species	Characters
SW1	Surahi	Sahiwal, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape
SW2	Moti Surahi	Sahiwal, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (large size)
SW3	Gola	Sahiwal, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round large pink flesh
SW4	Gola	Sahiwal, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round large size
SW5	Gola	Sahiwal, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (pink flesh)
SW6	Surahi	Sahiwal, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape
SW7	Gola	Sahiwal, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round
SW8	Gola	Sahiwal, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (pink flesh)
SW9	Sada Bhar Gola	Sahiwal, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (cream blush)
SW10	Gola	Sahiwal, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (low seeded)
SW11	Desi Gola	Sahiwal, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (very small size)
CH12	Gola	Chishtian Mandi, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (pink flesh)
CH13	Gola	Chishtian Mandi, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (pink flesh)
CH14	Sada Bhar Surahi	Chishtian Mandi, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (cream colour)
CH15	Sada Bhar Surahi	Chishtian Mandi, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (cream colour)
CH16	Desi Surahi	Chishtian Mandi, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (small size)
CH17	Surahi	Chishtian Mandi, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (pink flesh)
FSD18	Larkana Surahi	Faisalabad, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (long neck)
FSD19	Gola	Faisalabad, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round
FSD20	Larkana Surahi	Faisalabad, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (long neck)
FSD21	Larkana Gola	Faisalabad, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (medium size)
FSD22	Larkana Gola	Faisalabad, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (medium size)
FSD23	Gola	Faisalabad, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (pink flesh)
FSD24	Kareala	Faisalabad, Pakistan	<i>P.</i>	<i>guajava</i> L.	Bitter gourd shape
FSD25	Bangladeshi variety	Faisalabad, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (very large size)
FSD26	Gola	Faisalabad, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (pink flesh)
FSD27	Gola	Faisalabad, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (pink flesh)
BWP28	Gola	Bahawalpur, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (dark pink flesh)
BWP29	Sour Surahi (pink flesh)	Bahawalpur, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (sour taste)
BWP30	Gola	Bahawalpur, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round
BWP31	Moti Surahi	Bahawalpur, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (large size)
BWP32	Sada Bhar Gola	Bahawalpur, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (cream blush)
BWP33	Gola	Bahawalpur, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round
BWP34	Surahi	Bahawalpur, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape
BWP35	Mota Gola	Bahawalpur, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (large size)
PSH36	Sindhi	Peshawar, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round
PSH37	Gulabi	Peshawar, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (pink flesh)
PSH38	Ramzani	Peshawar, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round
PSH39	Riaz	Peshawar, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round
PSH40	Thandiani	Peshawar, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape

Table 1 continued

Accession	Cultivar name	Origin	Genus	Species	Characters
PSH41	PG-001	Peshawar, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (medium size)
PSH42	PG-005	Peshawar, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape medium size)
PSH43	PG-013	Peshawar, Pakistan	<i>P.</i>	<i>guajava</i> L.	Gola (medium size)
PSH44	Gola	Peshawar, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (low seeded)
SHP45	Moti Surahi	Sheikhupura, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape (large size)
SHP46	Mota Gola	Sheikhupura, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (large size)
SHP47	Chota Gola	Sheikhupura, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round (very small size)
SHP48	Surahi	Sheikhupura, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape
SHP49	Gola	Sheikhupura, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round
SHP50	Gola	Sheikhupura, Pakistan	<i>P.</i>	<i>guajava</i> L.	Round
SHP51	Surahi	Sheikhupura, Pakistan	<i>P.</i>	<i>guajava</i> L.	Pear shape
G1	Pineapple Guava	Australia	<i>Acca</i>	<i>sellowiana</i> (O. Berg) Burret	Grey leaves
G2	Cherry Guava	Australia	<i>P.</i>	<i>cattleianum</i> Sabine	Very small fruit
G3	Pineapple Guava	Australia	<i>A.</i>	<i>sellowiana</i> (O. Berg) Burret	Grey leaves
G4	Unknown	Australia	<i>P.</i>	<i>guajava</i> L.	White flesh large fruit
G5	Unknown	Australia	<i>P.</i>	<i>guajava</i> L.	Red/white large fruit
G6	Black Chilean Guava	Australia	<i>Ugni</i>	<i>myricoides</i> (Kunth) O. Berg	Small black cherry type
G7	Unknown	Australia	<i>P.</i>	<i>guajava</i> L.	White flesh small seed
G8	Hawaiian Pink	Australia	<i>P.</i>	<i>guajava</i> L.	Pink flesh large fruit
G9	Allabad Safeeda	Australia	<i>P.</i>	<i>guajava</i> L.	White flesh large fruit
G10	Beamount	Australia	<i>P.</i>	<i>guajava</i> L.	Pink flesh large fruit
G11	Cherry Guava	Australia	<i>P.</i>	<i>cattleianum</i> Sabine	Very small fruit
G12	Unknown	Australia	<i>P.</i>	<i>guajava</i> L.	Pink flesh
G13	Unknown	Australia	<i>P.</i>	<i>guajava</i> L.	Pink flesh large fruit
G14	Unknown	Australia	<i>P.</i>	<i>guajava</i> L.	Pink flesh
G15	Unknown	Australia	<i>P.</i>	<i>guajava</i> L.	Pink flesh
G16	Yellow Cherry Guava	Australia	<i>P.</i>	<i>cattleianum</i> Sabine	Very small fruit
G17	Mexican Cream	Australia	<i>P.</i>	<i>guajava</i> L.	Large fruit
G18	Unknown	Australia	<i>P.</i>	<i>guajava</i> L.	Large fruit
G19	Unknown	Australia	<i>P.</i>	<i>guajava</i> L.	Large fruit

machine. All PCR samples were denatured at 94 °C for 5 min, and placed the plate on ice before loading.

The polyacrylamide gel solution includes 20 mL KB Plus 6.5 % Gel Matrix, 150 µL ammonium persulfate solution (APS) and 25 µL tetramethylethylenediamine (TEMED). LI-COR DNA Analyzer Model 4300 for SSR markers was adopted using SAGA^{GT} software to image the band pattern for each primer.

Data scoring and analysis

For each primer, PCR was performed three times to confirm band pattern consistency. DNA bands were sized and scored by LabWorks software (v4.5, UVP)

and carefully checked manually; only clear bands were scored and faint bands were ignored. Bands with the same size were assumed to represent a single locus. For each locus data were recorded using '1' for presence of a band and '0' for absence so as to build a binary matrix.

Summary statistics for each group of accessions relating to allelic richness, heterozygosity, genetic diversity, number of alleles and Shannon's Information Index were computed using GenAIEx 6.5 (Peakall and Smouse 2006, 2012). Shannon's Information Index was calculated following the method of Lewontin (1972). Definition of inbreeding coefficient followed Wright (1965). Duplicates in the data set were checked by multi-locus matching. Accessions with

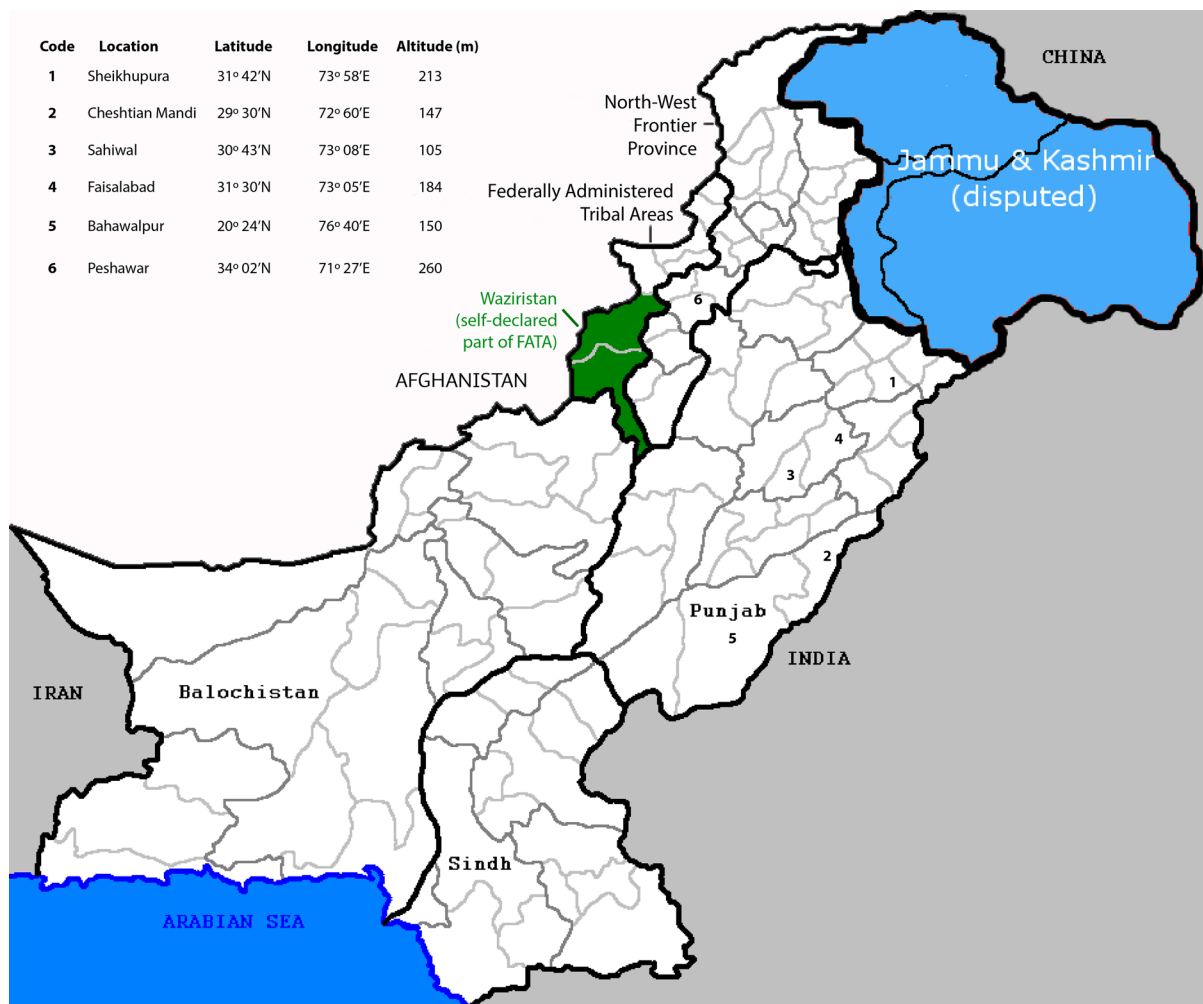


Fig. 1 Map of Pakistan showing collection sites for 51 guava accessions

different names that were completely matched at all the loci were considered as duplicates. Pair-wise genetic distance was calculated using the DISTANCE procedure implemented in GenAIEx 6.5. This program was also applied for a principal coordinate analysis (PCoA). Dendrograms were built based on a Dice genetic similarity coefficient (Nei and Li 1979) using the unweighted pair-group method with arithmetic averages (UPGMA). The matrix data were imported into Tree Drawing software from PHYLIP (Felsenstein 2005) for dendrogram construction.

Cross-species transferability of SSR primers

All 18 SSR loci were evaluated for amplification on six wild guava accessions namely *U. myricoides* (1),

A. sellowiana (2) and *P. cattleianum* (3). Procedures for sample collection, DNA isolation, PCR amplification and detection of SSR profiles were as described above for *P. guajava*.

Results

iPBS marker system

DNA polymorphism for six iPBS primers

Seventeen iPBS primers were initially screened for polymorphism across the 70 guava accessions. Six primers (2079, 2228, 2238, 2251, 2376, 2241) were selected for iPBS PCR amplifications because of the

Table 2 Six iPBS primers used in the detection of polymorphism among 70 guava (*Psidium guajava*) accessions

iPBS primer	Sequence (5'-3')	Ta (°C)	Number of bands ^a	Number of polymorphic bands	Percentage of polymorphism (%)	Mean PIC value ^b
2079	AGGTGGGCGCCA	55	22	22	100	0.2906
2251	GAACAGGCGATGATACCA	55	16	14	87.5	0.2729
2238	ACCTAGCTCATGATGCCA	55	19	19	100	0.3301
2228	CATTGGCTCTTGATACCA	56	13	12	92.3	0.3068
2376	TAGATGGCACCA	40	13	13	100	0.3522
2241	ACCTAGCTCATCATGCCA	59	14	14	100	0.1687

^a Total accountable bands consistently appearing in two or three repeated experiments

^b PIC value is calculated as $PIC = 1 - [f^2 + (1 - f)^2]$, where f is the frequency of the marker in the data set

large number of polymorphic bands they generated (Table 2). The sizes of reproducible and scorable bands ranged from 100 to 2800 bp. The iPBS fingerprinting pattern of the 70 accessions from primer 2079 are shown in Fig. 2. The number of unique banding patterns among the 70 accessions validated the use of iPBS markers for the identification of guava DNA. Furthermore, the six primers we used amplified a total of 97 scorable bands with 96.63 % polymorphism indicating a high degree of genetic variability. The information from these six primers, including band polymorphism and mean polymorphism information content (PIC) values, is included in Table 2. Primer 2079 produced the highest number of bands (22) and primers 2228 and 2376 generated the lowest (13). Primer 2376 had the highest PIC value (0.3522) whereas primer 2241 had the lowest (0.1687). These results indicate that the iPBS marker system used in this study can reveal a wide range of genomic DNA

diversity in guava (*P. guajava* L.) and its related species.

Heterozygosity and diversity of species

Summary statistics for each of the eight groups of accessions including number of different alleles, number of effective alleles, Shannon's information index, expected heterozygosity and unbiased expected heterozygosity are listed in Table 3. Expected heterozygosity values (H_e) ranged from 0.1218 (CH) to 0.296 (G), with an average of 0.183, whereas unbiased expected heterozygosity (uH_e) ranged from 0.138 (SHP) to 0.308 (G) with an average of 0.194. The Shannon's information index among the eight groups ranged from 0.193 (CH, SHP) to 0.442 (G), with an average of 0.275. The mean fixation index F_{st} or inbreeding coefficient is 0.925. Among all the six locality groups from Pakistan, accessions from

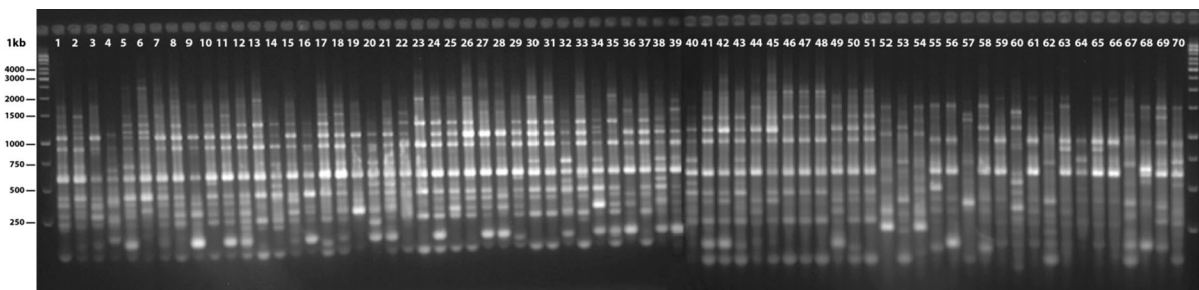


Fig. 2 PCR banding pattern for 70 DNA accessions using iPBS primer 2079. Identification for accessions codes were listed in Table 1

Table 3 Summary statistics for 70 guava (*Psidium guajava*) accessions assessed with six iPBS primers (Kalendar et al. 2010)

Groups	N	Na	Ne	I	He	uHe
SW	11.000	1.500	1.399	0.353	0.234	0.245
CH	6.000	0.944	1.218	0.193	0.128	0.140
FSD	10.000	1.189	1.298	0.260	0.173	0.182
BWP	8.000	1.133	1.238	0.217	0.143	0.153
PSH	9.000	1.156	1.230	0.218	0.141	0.149
SHP	7.000	1.078	1.217	0.194	0.128	0.138
O	6.000	1.389	1.386	0.322	0.219	0.239
G	13.000	1.744	1.515	0.442	0.296	0.308
Mean	8.750	1.267	1.313	0.275	0.183	0.194

N number of sample size, *Na* number of different alleles, *Ne* number of effective alleles, *I* Shannon's information index, *He* expected heterozygosity, *uHe* unbiased expected heterozygosity

Sahiwal (SW) had the highest expected heterozygosity (0.234), unbiased expected heterozygosity (0.245) and Shannon's information index (0.353). Groups from

Chishtian Mandi (CH) and Sheikhpura (SHP) recorded the lowest expected heterozygosity (0.122), unbiased expected heterozygosity (0.138) and Shannon's information index (0.193).

Principal component analysis and cluster for iPBS markers

Results of principal coordinate analysis presented a spatial representation of the relative genetic distances among individual accessions revealed five distinct groups (Fig. 3). The plane of the first three PCoA axes accounted for 37.86 % of the total variation (first axis = 20.14 %, second = 10.66 %, third = 7.06 %). Most of the Pakistani accessions (except SW3 and SW4) clustered in the first group and were distributed to the left of the plane. The second group comprised of five accessions, viz. G9 (Allabad Safeeda), G4, G13, SW3 and SW4. A third group clustered in the lower centre of the right plane included G5, G10, G19 and Mexican Cream (G17). Hawaiian Pink (G8) and five accessions (G7, G12, G14, G15 and G18) separated as the fourth

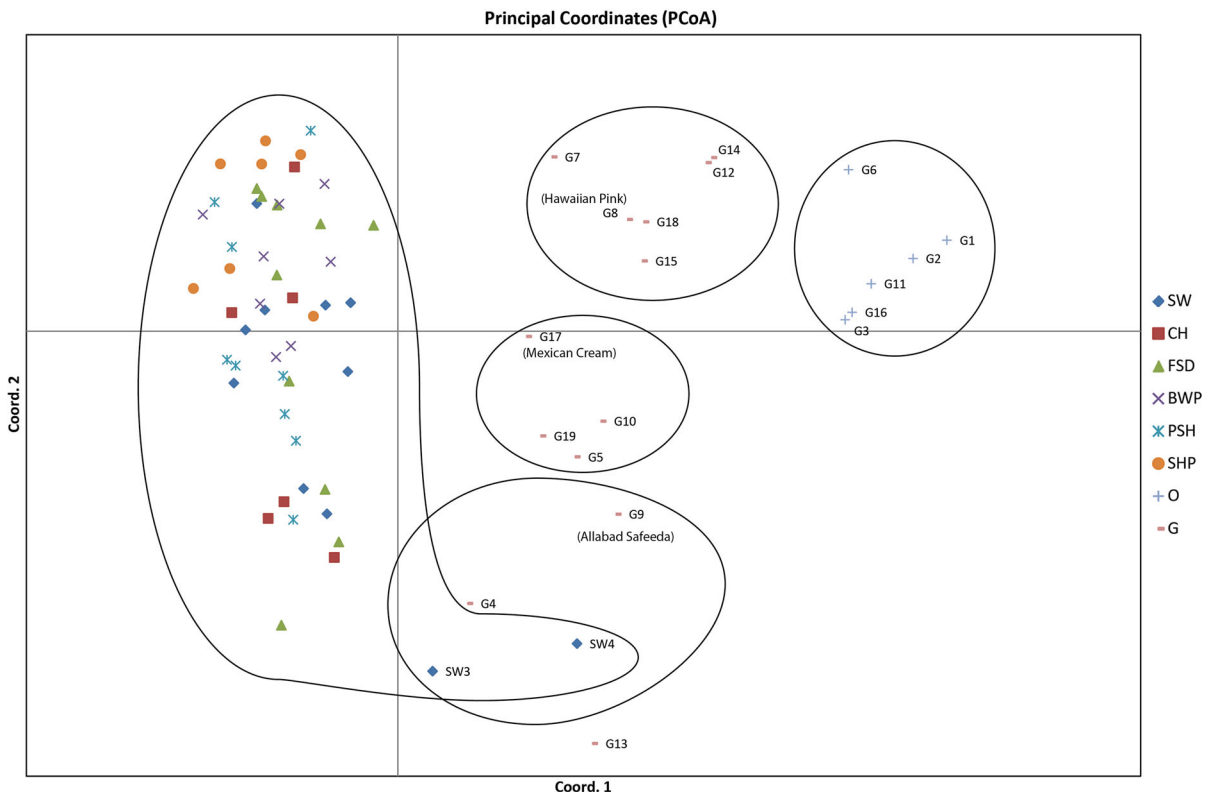


Fig. 3 Principle coordinate analysis of 70 guava accessions with six iPBS primers. Cluster are represented by *different marker types*

group on the left of the upper right plane. The fifth group on the right of the upper right plane had six wild guava accessions including Black Chilean Guava (*U. myricoides*) G6, Pineapple Guava (*A. sellowiana*) G1 and G3, and 3 Cherry Guava (*P. cattleianum*) accessions G2, G11, G16.

The un-weighted pair group method with arithmetic mean (UPGMA) dendrogram placed the 70 accessions into five clusters (Fig. 4), supporting results from the principal coordinate analysis. The first cluster comprised identical accessions to those revealed in the PCoA. This cluster had 49 Pakistani accessions (SW1, SW2 and SW5 to SHP51) and displayed a wide range of characters such as fruit shape, fruit size and flesh colour. Cultivars BWP29 (Sour Surahi, pink flesh), SHP49, SW5 and SW2 were clustered as a Pakistani sub-group. The second cluster contained Australian accessions G4 and G5 and Mexican Cream (G17). The third cluster included Hawaiian Pink (G8) Beaumont (G10) and six other Australian accessions. In this cluster, accessions Hawaiian Pink and Beaumont grouped closely and therefore accurately reflected their parent-offspring relationship. The fourth cluster

comprised four accessions, including Pakistani accessions SW3 and SW4, an Australian hybrid (G13), and Indian cultivar Allabad Safeeda (G9). Significantly, this cluster all had large round fruits. The fifth cluster included six accessions of related species as listed.

SSR marker system

DNA polymorphism generated by 18 SSR primers

Eighteen guava-specific microsatellite primer pairs were applied to screen the 70 guava accessions for genetic identification. Significant molecular variability was detected among all accessions (Table 4). The sizes of reproducible and scorable bands ranged from 124 to 553 bp. The SSR band patterns for 70 guava accessions from primer mPgCIR16 are shown in Fig. 5. The unique banding patterns from these accessions suggest that microsatellite markers have the potential to distinguish individual guava accessions. The 18 primer pairs amplified 172 alleles, with frequencies of 2–17 per locus and an average 9.56. Primer information and mean PIC values are also listed in Table 3. Primer pairs

Table 4 Eighteen SSR primers used in the detection of polymorphism among 70 guava (*Psidium guajava*) accessions

SSR primers	Forward primer	Reverse primer	Ta (°C)	Alleles*	PIC
mPgCIR02	M13AGTGAACGACTGAAGACC	TTACACATTCAGCCACTT	54	4	0.3382
mPgCIR03	M13TTGTGGCTTGATTTCC	TCGTTTAGAGGACATTTCT	55	2	0.1413
mPgCIR05	M13GCCTTTGAACCACATC	TCAATACGAGAGGCAATA	54	9	0.7516
mPgCIR07	M13ATGGAGGTAGGTTGATG	CGTAGTAATCGAAGAAATG	52	6	0.3339
mPgCIR08	M13ACTTTCGGTCTCAACAAG	AGGCTTCTACAAAAGTG	55	8	0.6567
mPgCIR09	M13GCGTGTGCTATTGTTTC	ATTTCTTCTGCCTTGTC	54	10	0.9498
mPgCIR10	M13GTTGGCTCTATTTTGGT	GCCCCATATCTAGGAAG	54	9	0.4583
mPgCIR11	M13TGAAAGACAACAAACGAG	TTACACCCACCTAAATAAGA	54	17	0.9558
mPgCIR14	M13TAAACACAACAAGGGTCA	CAGTTTTCATATCGTCCTC	54	3	0.2006
mPgCIR15	M13TCTAATCCCTGAGTTTC	CCGATCATCTCTTTCTTT	53	9	0.4478
mPgCIR16	M13AATACCAGCAACACCAA	CATCCGTCTCTAAACCTC	54	12	0.8371
mPgCIR17	M13CCTTTCGTCATATTCACTT	CATTGGATGGTTGACAT	54	11	0.7926
mPgCIR18	M13TAAGCTGCATGTGTGC	ATGGCTTTGGATGAAA	54	16	0.5586
mPgCIR19	M13AAAATCCTGAAGACGAAC	TATCAGAGGCTTGCAATA	53	14	0.5380
mPgCIR20	M13TATACCACACGCTGAAAC	TTCCCCATAAACATCTCT	54	11	0.2652
mPgCIR21	M13TGCCCTTCTAAGTATAACAG	AGCTACAAACCTTCCTAAA	53	12	0.9537
mPgCIR22	M13CATAAGGACATTTGAGGAA	AATAAGAAAGCGAGCAGA	54	9	0.9482
mPgCIR25	M13GACAATCCAATCTCACTTT	TGTGTCAAGCATACCTTC	53	10	0.9014

All forward primers were 5'-tailed with the 19 base pair M13 sequence (CACGACGTTGTAACGAC)

* Number of consistent bands in two or three repeated experiments. PIC value is calculated as $PIC = 1 - \sum p_i^2$

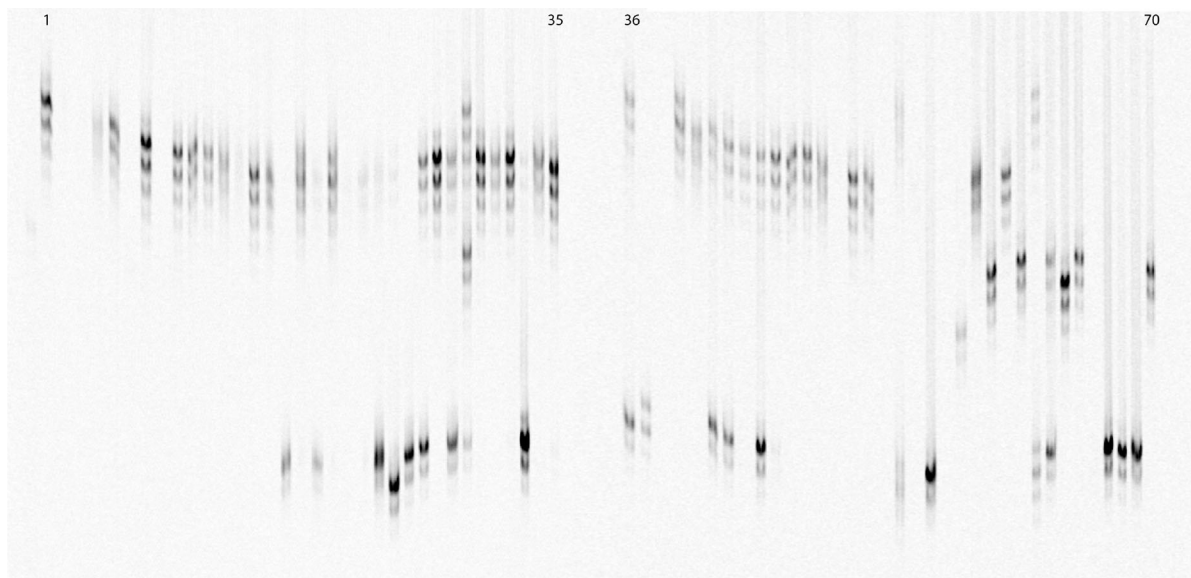


Fig. 5 SSR PCR banding pattern for 70 guava accessions generated from primer M13-CIR 16. The forward primer was 5'-tailed with the 19 base pair M13 sequence (CACGACGTTGTA AAC

GAC). Accessions 1–35 used IRDye 700 and accessions 36–70 used IRDye 800. Identification of accessions were listed in Table 1

Table 5 Summary statistics for 70 guava (*Psidium guajava*) accessions assessed with 18 SSR primers

Groups	N	Na	Ne	I	He	uHe
SW	11	0.651	1.127	0.119	0.077	0.080
CH	6	0.529	1.115	0.095	0.065	0.070
FSD	10	0.686	1.137	0.121	0.080	0.084
BWP	8	0.581	1.085	0.080	0.052	0.055
PSH	9	0.547	1.095	0.086	0.057	0.060
SHP	7	0.384	1.049	0.039	0.027	0.029
O	6	1.035	1.240	0.237	0.153	0.167
G	13	1.116	1.276	0.263	0.172	0.179
Mean	8.75	0.691	1.141	0.130	0.085	0.091

See Table 3 for abbreviations

mPgCIR11 and mPgCIR18 produced the largest number of bands (17 and 16), whereas mPgCIR3 generated the smallest (2). PICs ranged from 0.1413 to 0.9537 with an average of 0.6127 per locus. Nine primers (mPgCIR05, mPgCIR08, mPgCIR09, mPgCIR11, mPgCIR16, mPgCIR17, mPgCIR21, mPgCIR22, mPgCIR25) gave PIC values higher than 0.6. Primer pair mPgCIR21 had the highest PIC value (0.9537), indicating that this was the most informative locus. These

results indicated that SSR markers can also reveal genomic DNA diversity in guava (*P. guajava*) and its related species as can the iPBS method.

Heterozygosity and species diversity of SSR markers

Summary statistics for each of the eight groups of accessions including number of different alleles, number of effective alleles, Shannon's information index, expected heterozygosity and unbiased expected heterozygosity are listed in Table 5. Expected heterozygosity values (He) ranged from 0.027 (SHP) to 0.172 (G), with an average 0.085, whereas unbiased expected heterozygosity (uHe) ranged from 0.029 (CH) to 0.179 (G) with an average of 0.091. The Shannon's information index among the eight groups of accessions ranged from 0.039 (SHP) to 0.263 (G), with an average of 0.130. The average inbreeding coefficient was 0.854. Among the six locality groups in Pakistan, those from Faisalabad (FSD) had the highest expected heterozygosity (0.080), unbiased expected heterozygosity (0.084) and Shannon's information index (0.121). Groups from Shekhupura (SHP) had the lowest expected heterozygosities (0.027), unbiased expected heterozygosity (0.029) and Shannon's information indices (0.039).

Principal component analysis and clustering by 18 SSR primers

Individual genotype matching using pair-wise comparisons did not detect matching pairs, suggesting that each accession analysed was a distinct genotype. Results of principal coordinate analysis performed to present spatial representation of the relative genetic distance among individuals revealed five distinct groups (Fig. 6). The plane of the first three PCoA axis accounted for 59.03 % of the total variation (first axis = 27.23 %, second = 16.77 % and third = 15.13 %). Most of the Pakistani accessions (except for SW3 and SW4) clustered in the first group and were distributed on the left of the plane. The second group comprised of eight accessions including Hawaiian Pink (G8), Beamount (G10) and six accessions (G4, G5, G12, G13, G15 and G19). Cultivar Mexican Cream (G17) together with four accessions (G7, G18, SW3, SW4) were clustered in the upper centre of the right plane as the third group. The Indian cultivar Allabad Safeeda (G9) and G14 were separated as the fourth group on the right of the upper right plane. The

fifth group on the lower right plane had the six wild guava accessions.

The un-weighted pair group method with arithmetic mean (UPGMA) dendrogram placed the 70 guava accessions into six clusters similar to the above iPBS results (Fig. 7), which was supported by similar results from the principal coordinate analysis. The first cluster comprised of accessions as those revealed in the upper left plane in the PCoA had 24 Pakistani accessions (SW1, SW2 and SW5 to SW11; BWP28 to BWP34; SHP45 to SHP51), and displayed a wide range of characters such as fruit shape, fruit size and flesh colour. Cultivars from these three provinces further separated as three sub-groups according to their geographic origin. The second cluster comprised nine accessions; 2 Pakistani (SW3, SW4), four Australian hybrids (G7, G12, G13, G14), Hawaiian Pink (G8), Beamount (G10) and Indian cultivar Allabad Safeeda (G9). The Hawaiian cultivars [Hawaiian Pink (G8) and Beamount (G10)] and the Indian cultivar Allabad Safeeda (G9) were separated into two sub-groups. Accessions in this cluster all had large round fruits. The third cluster contained 15 Pakistani accessions

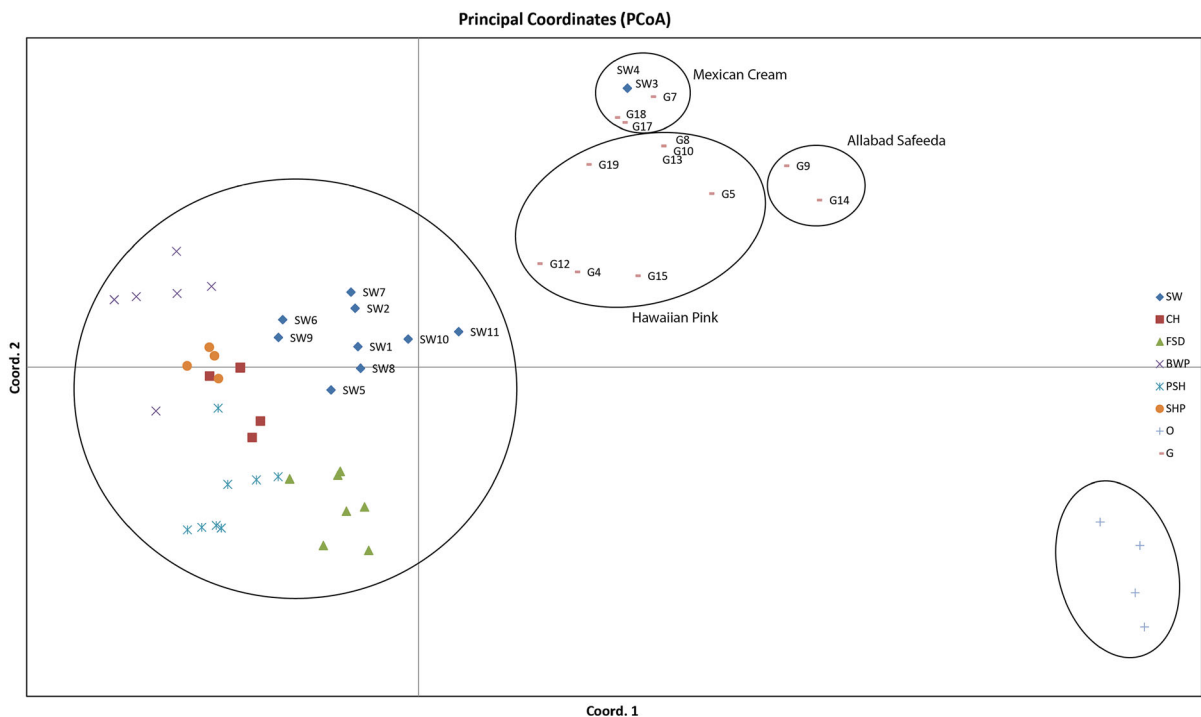


Fig. 6 Principal coordinate analysis of 70 guava accessions with 18 SSR primers. Identification of eight groups of accessions corresponded to samples listed in Table 1

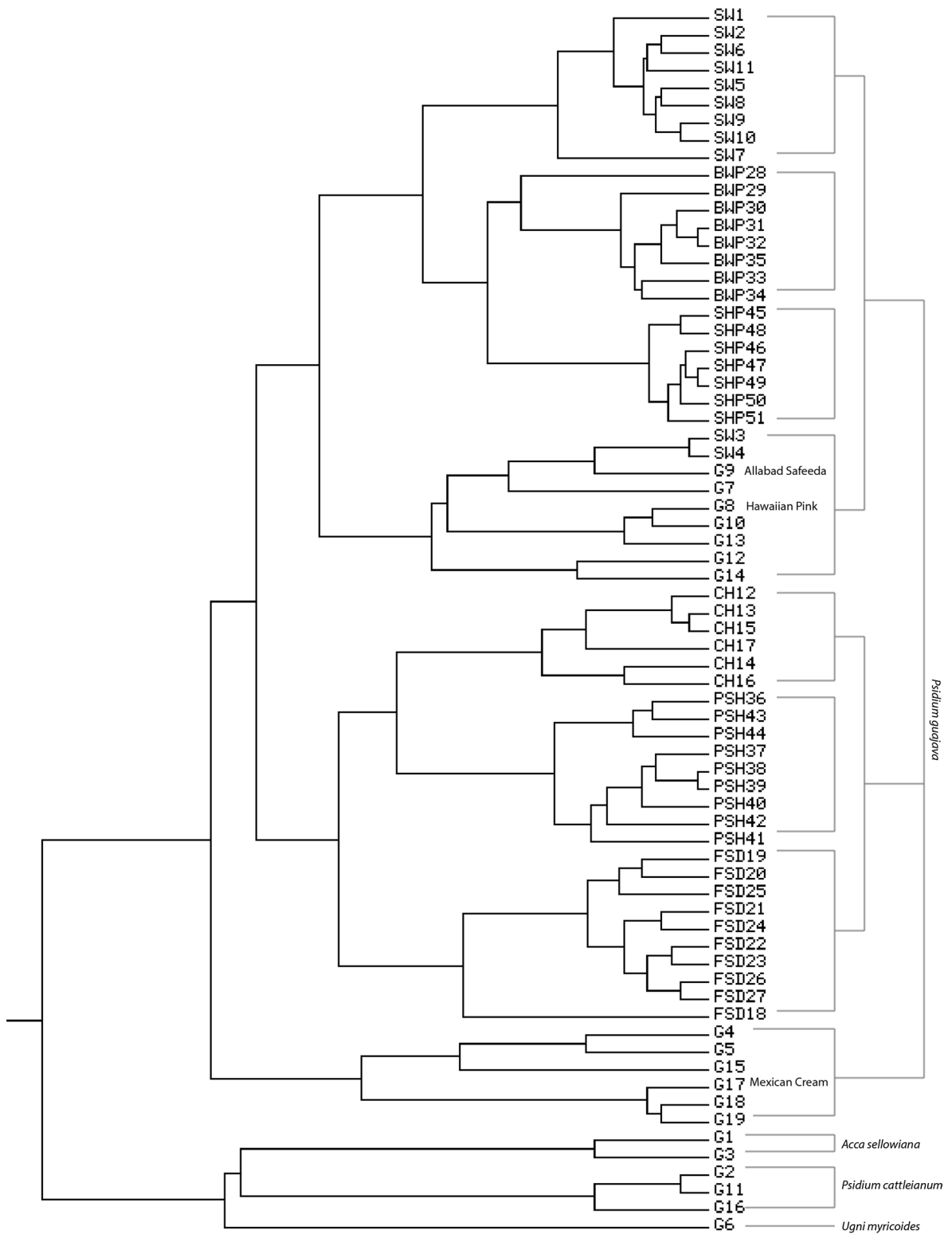


Fig. 7 Dendrogram for 70 guava accessions generated using data from 18 SSR primers. Accession codes correspond to samples listed in Table 1

(CH12 to CH17, PSH36 to PSH44). Cultivars from these two provinces further separated as two sub-groups. The fourth cluster included 10 Pakistani accessions (FSD18 to FSD27). The fifth cluster included Mexican Cream (G17) and five other Australian accessions (G4, G5, G15, G18, G19). The sixth cluster comprised the related species (Fig. 7).

Discussion

Investigation of the genetic diversity and relationships in *Psidium* germplasm is important for breeding, conservation, management and utilisation of the materials (Pommer 2012; Pommer and Murkami 2009). Accurate identification of accessions in a germplasm collection is an important challenge faced in crop improvement projects. For germplasm identification, molecular marker systems such as the iPBS and SSR can add to the assessment of genetic diversity and relationships in plant phylogenetic analysis as well as selective plant breeding because they are objective and offer reproducible means of identification, independent of environmental influences. The use of iPBS markers provided valuable information on grapes (*Vitis vinifera* L.) (Guo et al. 2014). In our study, polymorphic iPBS and SSR markers enabled identification on a range of *P. guajava* accessions including their related wild species and genera. This yielded valuable information on the genetic relationship amongst these accessions (Fig. 7).

Both the iPBS and SSR systems distinguished accessions into separate groups with similar distinguishing power at the species and genus level. Both methods could group all 70 accessions studied into clusters according to their genetic backgrounds which underpin their species and genus categorisation. For example, both marker systems distinguished the 64 cultivated *P. guajava* accessions from the six wild guava accessions at both the species (*P. cattleianum*) and genus levels (*A. sellowiana*; *U. myricoides*). The level of information generated by iPBS and SSR markers suggest that both methods can be important for diversity studies in guava and related species and genera.

Ninety-seven iPBS bands were obtained from six primers, generating substantial polymorphism (96.63 %). The average numbers of iPBS bands were far more than reported by Gailite and Rungis (2012),

Baranek et al. (2012) and Guo et al. (2014) found. The 12-mer primer 2376 produced the lowest number of 13 bands whereas another primer 2079 generated the highest number of 22. Hence the length of the individual primer was not relevant to the number of bands amplified in this study.

The levels of unbiased expected heterozygosity (0.194 for iPBS, 0.091 for SSR) in the present study is comparable to that reported by Sittler et al. (2014) in which guava germplasm in the US had an average uHe value of 0.2 when using microsatellite markers. The high values of average inbreeding coefficient (0.925 for iPBS, 0.854 for SSR) also suggest that cross-incompatibility may play a significant role in hindering the effectiveness of creating true hybrids and recombining of favourable alleles from parental clones. Controlled hybridisation between cross-compatible parents may be needed for the development of new guava cultivars. However, since we evaluated only a small set of iPBS data, there is a possibility that the low heterozygosity and low Shannon's Information Index (0.275 for iPBS, 0.130 for SSR) were partially due to the sampling bias of our iPBS and SSR markers.

The general consensus for a good molecular marker system is that it should be polymorphic and evenly distributed across the entire genome; it should distinguish between genetic differences; it should be cheap, quick, and easy to use, requiring minimal amounts of DNA; and it should require no previous knowledge of the genome (Odong et al. 2011). The results from our study confirm that the iPBS method exhibits all these characteristics.

On the other hand, the SSR method also has advantages. These include reproducibility and high frequency and dispersion throughout the genome (both in coding and non-coding regions), high polymorphism, co-dominance, and transferability to related taxa (Wuensch 2009). A disadvantage, however, is that the SSR system actually requires previous genomic DNA information for the design of primer pairs and as mentioned previously, the search strategy for repetitive sequences and the design of the primers employed were only for these related species. Consequently, the laborious and costly procedure in designing SSR primers limits the number of species that can be assayed (Dayanandan et al. 1997). However, it needs to be noted that an ultimate limitation of the SSR method is that it requires a large number of primers.

Unfortunately at this stage of current research on guava, there are only a limited number of available primers. We used the 18 pairs developed by Risterucci et al. (2005) in their original research on guava.

Within the species *P. guajava*, both the iPBS and SSR systems separated the 64 cultivated guava accessions into clusters according to locality; that is, Pakistani, Indian, Hawaiian and Mexican sub-groups. The level of information generated suggests that accessions collected from the same geographical region or breeding program tended to group together, indicating that despite the widespread distribution of guava in the tropical world and more than 100 years of cultivation, germplasm exchange among regions has been limited. Furthermore, results from this study showed that only a small number of guava cultivars have been used in breeding programs.

In Pakistan, guava breeding is highly dependent on seed propagation with subsequent selection being made according to specific characteristics like fruit quality and/or plant vigour according to local farmer preferences (Mehmood et al. 2013). Our results showed that cultivars collected from each of the six separate areas within Pakistan tended to be closely related, indicating a common genetic basis. Such knowledge has implications for future germplasm collection, management and selective breeding projects. The results also indicate that cultivated Pakistani guava (*P. guajava*) is genetically diverse within its own specific grouping whilst being significantly different from other accessions from India, Hawaii, Mexico and Australia. This would infer that intracrossing between Pakistani cultivars and accessions from other geographic areas could enhance hybrid vigour that could be captured with asexual propagation.

Both the iPBS and the SSR methods placed two Pakistani cultivars (SW3 and SW4) with Indian cultivars in one cluster. This result suggests that guava may have diverged into separate evolutionary lines in Pakistan and India following the separation in 1947. Alternatively, cultivars SW3 and SW4 could have been introduced into Pakistan from India more recently. Further research would be needed to clarify this finding.

The analysis of molecular genetic diversity and relationships of germplasm collections has mainly been based on multivariate statistical analysis, such as hierarchical cluster analysis, principal-component analysis, and multidimensional scaling, usually using

agronomic data (Odong et al. 2011). New methods have been developed using molecular markers and our results indicate that the related software of Tree Drawing using PHYLIP (Felsenstein 2005) and GenAIEx 6.5 (Peakall and Smouse 2006, 2012) are more suitable for molecular genetic data analysis.

It is a well-known phenomenon that the botanical classification within the *Myrtaceae* family suffers from a number of errors and it is a continuous task for botanists to eliminate such mistakes (Van Wyk 1980; Van Wyk and Botha 1984). The genus *Acca* did not exist until Landrum (1990) suggested that it be separated from *Psidium* based on certain morphological differences. Dettori and Palombi (2000) reaffirmed this position. The iPBS and SSR results from this study confirm the validity of separation since *Acca* emerged as a separate group from *P. guajava* and *P. cattleianum*. This indicates the potential power of these marker systems in distinguishing genetic diversity at both the species and genus levels. Future research using the iPBS system could target other genera within the Myrtaceae.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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