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Molecular exploration of guava (*Psidium guajava* L.) genome using SSR and RAPD markers: a step towards establishing linkage map

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ABSTRACT

In the present study, molecular evaluation of two guava mapping populations (MP), MPI comprising 94 F_1 progenies and MPII comprising 46 F_1 progenies, was carried out using simple sequence repeat (SSR) and random amplified polymorphic DNA (RAPD) markers. A pseudo-test cross strategy was implemented where 'Kamsari' X 'Purple Local' and 'Purple Local' X 'Allahabad Safeda' were crossed, and, these showed variation in fruit quality traits such as seed-strength (hardness/softness), fruit weight, TSS and pulp color. A set of 30 RAPD markers was used for genotyping MPI while a set of 55 SSR markers was used for genotyping MPII. In case of MPI, 30 RAPD markers generated 214 scorable markers, of which 80 markers were specific to 'Kamsari', 14 markers to 'Purple Local' and the remaining 120 were intercross markers. As for MPII, 55 polymorphic SSR markers resulted in generation of 207 alleles (with a maximum of 4 alleles and a minimum of 3 alleles per locus), of which 108 alleles were specific to 'Purple Local' while 99 were specific to 'Allahabad Safeda'. Genotypic data thus generated can be further exploited for constructing genetic linkage maps and mapping complex QTLs governing fruit quality traits in guava.

Key words: Guava, genotyping, M13-tailed PCR, SSR, RAPD

INTRODUCTION

Guava (*Psidium guajava* L.), belonging to the family Myrtaceae, is a diploid with 2n=22 and comprises approximately 430 Mbp genome. It is popularly known as the apple of the tropics or the poor man's apple. Guava is believed to be native to Mexico (Rios *et al*, 1977) and is present throughout South America, Europe, Africa and Asia. Its edaphoclimatic adaptability, prolific bearing, hardiness to biotic and abiotic stresses and medicinal properties impart a great value to this fruit crop. Besides, it is a rich source of nutrients, vitamins and minerals; it also has a pharmaceutical potential by way of having antioxidant, antimicrobial and antidiabetic properties (Shruthi *et al*, 2013). A major traditional use of the fruit is that of an anti-diarrheal. Other uses reported include treatment of gastroenteritis, dysentery, stomach ailments, antibacterial colic pathogenic germs of the intestine (Gutierrez *et al*, 2008). Guava, a dual-purpose fruit, is used as a fresh fruit and as also after processing into a variety of forms like puree, paste, jam, jelly, nectar, syrup, ice cream or juice.

SSR or microsatellite is a PCR (Polymerase Chain Reaction)- based molecular marker technique with advantages such as abundance, high polymorphism, co-dominance and primer transferability. SSR markers are widely used for constructing genetic maps (Oliveira *et al*, 2008; Ogundiwin *et al*, 2009; Wang *et al*, 2010; Das *et al*, 2012; Pauly *et al*, 2012; Liu *et al*, 2013; Serba *et al*, 2013; Zhang *et al*, 2013). SSR markers have also been utilized for molecular characterization and genetic diversity assessment of guava germplasm resources (Nimisha *et al*, 2013). SSR markers in guava were developed by Risterucci *et al* (2005, 2010) and were applied in germplasm characterization and for assessing existing genetic variability (Risterucci *et al*, 2005; Valdés-Infante *et al*, 2007; Viji *et al*, 2010; Aranguren *et al*, 2010; Santos *et al*, 2011; Coser *et al*, 2012; Noia *et al*, 2012; José *et al*, 2012; Angélica *et al*. 2012). SSR markers have been earlier used for cultivar identification (Kanupriya *et al*, 2011), discrimination of wild guava species (Nogueira *et al*, 2012) and for assessing genetic homogeneity of guava plants derived from somatic embryogenesis (Rai *et al*, 2012). Guava SSR markers so developed have also

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been used across different species of Myrtaceae (Briceno *et al*, 2010; Rai *et al*, 2013). Additionally RAPD markers have been considered as the markers of choice for crops like guava that lack sufficient genomic resources. Here, RAPD markers were used with an objective of linkage-map enrichment by reducing inter-marker distance and substituting missing linkage between the mapped SSR and SRAP markers in guava linkage-maps (Padmakar *et al*, 2015).

A strategy frequently employed for mapping F^1 populations in tree species and in perennials is the two-way pseudo-test cross mapping strategy (Grattapaglia and Sederoff, 1994) because it has been found to be efficient for mapping several heterozygous species. Guava, being a perennial, exhibits a high degree of heterogeneity and heterozygosity (Chandra and Mishra, 2007) which makes the underlying molecular mechanisms for phenotypic expression of various economically important traits difficult to understand. Therefore, linkage maps of progenies segregating for important economic traits such as fruit quality and yield, are required to be developed. In guava, only a few reports (Valdés-Infante *et al*, 2003; Rodriguez *et al*, 2007; Lepitre *et al*, 2010) are available on construction of molecular linkage-maps. Here we report development of intra-specific linkage maps in guava using SSR markers, along with genotyping of the mapping population by RAPD markers. This could be used for further enrichment of the available linkage-maps in guava.

MATERIAL AND METHODS

Plant material

Three cultivars of *Psidium guajava* maintained in the field germplasm bank at Indian Institute of Horticultural Research, Bangalore, India, namely 'Kamsari', 'Purple Local' and 'Allahabad Safeda' were used as the parental lines for developing mapping populations. A hybridization programme was undertaken by crossing the varieties 'Kamsari' and 'Purple Local' (Dinesh and Vasugi, 2010) for developing mapping population I (MPI), and 'Purple Local' X 'Allahabad Safeda' for mapping population II (MPII) so as to develop hybrids suitable for table purpose as well as for processing. The mapping population developed was evaluated morphologically for traits like seed- strength (hardness/softness), fruit weight, TSS and pulp color.

DNA extraction

In the case of MPI, a set of 94 F^1 progenies was shortlisted (based on morphological data on seed-strength and fruit weight, as these two traits showed positive correlation) whereas, in the case of MPII, a set of 46 F^1 progenies was shortlisted (based on pulp color) for molecular characterization. Genomic DNA was extracted from mature, healthy leaf material using the modified CTAB method of Kanupriya *et al* (2011). Quality of the DNA extracted was determined on 0.8% agarose gel electrophoresis and quantified using GeneQuant UV-spectrophotometer (GE Healthcare Biosciences Ltd. U.K.)

Molecular characterization

In the case of MPII, SSR-based genotyping of Padmakar *et al* (2015) was employed using 160 SSR primers (Risterucci *et al*, 2010) to characterize parental lines along with their mapping population.

Genotyping of MPI-specific parental lines was done using a set of 200 RAPD markers (Table 1). PCR amplification was performed in 25ìl reaction mixture containing 50mM KCl, 1mM Tris-HCl (pH 8.8), 0.01% gelatin, 1.5mM $MgCl_2$, 0.2mM each of dNTP, 0.3ìM primer, 100ng genomic DNA and 0.5 units of Taq DNA polymerase (Bangalore Genei, India). PCR was performed on a Master Cycler Gradient (Eppendorf AG, Hamburg, Germany) thermal cycler, with the following temperature profile: initial denaturation at 94°C for 3 min, 40 cycles of 2s at 94°C, 2s at 35°C, and 1 min at 72°C, and a final extension at 72°C for 10 min. Amplification products were screened on 1.5% agarose gel for confirmation of amplification. PCR was performed thrice for checking the reproducibility of the polymorphic markers identified.

Data analysis

In the case of SSR markers, raw data generated from the Genetic Analyzer was analyzed and compiled using Peak Scanner V1.0 software (Applied Biosystems, USA) for detection of alleles. Allelic profile of the SSR markers within a mapping population and their segregation pattern was identified by comparison with parental profiles. Allelic data so generated was then converted into the binary format, with 1 and 0 indicating presence and absence of the corresponding allele, respectively, within that population. As for RAPD markers, polymorphic markers were scored in

Table 1. List of RAPD decamers used in the present study

S. No.	RAPD Primer
1	OPA1 – OPA20
2	OPB1 – OPB20
3	OPC1 – OPC20
4	OPH1 – OPH20
5	OPN1 – OPN20

the binary format by assigning '1' for presence of the band and '0' for absence of the band. Genetic linkage maps were constructed for the parental lines 'Purple Local' and 'Allahabad Safeda' of MPII using segregation data generated for 46 mapping-population progeny, as per Padmakar *et al* (2015).

RESULTS AND DISCUSSION

Guava is even now considered an orphan crop with respect to availability of genomic resources, as, the present day genomic technologies have still to be applied to this crop. Till date, only few reports are available on molecular characterization of germplasm and mapping populations in guava. Generating saturated genetic maps and functional markers in guava has become a major challenge which, in turn, plays a vital role in improvement of guava inbreeding programs. Hence, the present study focussed on application of biotechnological tools alongside genetics tools such as genotyping and linkage mapping. High-quality genomic DNA was extracted from a total of 94 progenies of MPI and 94 progenies of MPII along with their respective parental lines

Genotyping by SSR markers is a proven technique used in several areas of plant genetics research and development. In the case of MPII, only 120 primers (out of 160 SSR primer pairs screened) successfully amplified both the parental lines. The parental polymorphism rate observed between 'Purple Local' and 'Allahabad Safeda' was 45.83%, using a set of 55 polymorphic SSR primer pairs. Initial confirmation was done on 3% agarose gel (Fig. 1); and then, 4 primers were multiplexed into one tube and processed for gene scan analysis. High-throughput genotyping of the mapping population resulted in identification of 207 alleles, with a minimum of three alleles and a maximum of four alleles per primer. Of these, 108 alleles (52.17%) were found to be specific to 'Purple Local' and 99 alleles (47.83%) specific to 'Allahabad Safeda'. Markers showing segregation distortion were not considered for linkage analysis because these affect the detection power of QTL when QTL and SDMs, or loci, are closely linked.

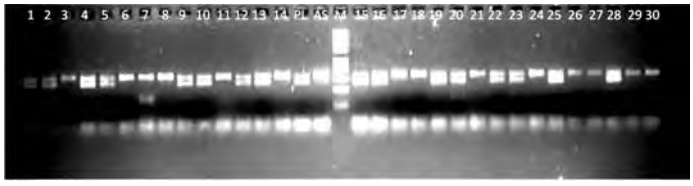

Fig. 1. Agarose gel (3%) showing amplification pattern of SSR marker among mapping population II and parental lines 'Purple Local' (PL) and 'Allahabad Safeda' (AS)

Similar omission of segregation-distorted markers in map construction was reported earlier (Grattapaglia and Sederoff, 1994; Hackett *et al*, 2000; Jones *et al*, 2003; Grando *et al*, 2003; Sudarshini *et al*, 2014) especially in the case of perennial tree crops where pseudo-test cross strategy was employed.

Independent linkage maps were constructed for each parent using double pseudo-test cross mapping strategy (Grattapaglia and Sederoff, 1994) for 'Purple Local' and 'Allahabad Safeda' of MPII only. A set of six and five linkage groups (Table 2) were observed in 'Purple Local' and 'Allahabad Safeda' respectively. Maternal 'Purple Local' map had a total of 49 marker loci distributed over six linkage groups (Fig. 2), with 13 markers remaining unlinked. This maternal framework map covered 746.5cM of the total map distance. Number of markers per linkage group ranged from three to 15. Linkage distance spanned by individual linkage groups ranged from 47.5cM (PL6) to 199.6cM (PL1). The linkage groups had an average length of 124.4cM and average marker interval length of 15.2cM. Paternal 'Allahabad Safeda' map had 44 markers distributed over five linkage groups (Fig. 3), with nine markers remaining unassigned. This paternal framework map covered 580.9cM of the total map distance, with a minimum of three and a maximum of 15 markers ordered into five linkage groups. Linkage distance spanned by individual linkage groups ranged from 23.3cM (AS5) to 245.4cM (AS4). Average length of the linkage groups was 116.2cM and average spacing was 13.2cM. As the number of markers mapped was low, the number of linkage groups observed was not equivalent to the haploid number of chromosomes. Similar study on development of partial linkage maps using just SSR markers has been reported in jute (Das *et al*, 2012). These maps can be further enriched with more markers and, thereby, used for mapping putative QTLs.

Table 2. Characteristics of parental linkage maps

	P1: Purple Local			P2: Allahabad Safeda		
LG[a]	PL-LG	TM[b]	cM[c]	AS-LG	TM[b]	cM[c]
1	PL1	15	199.6	AS1	7	53.1
2	PL2	8	118.1	AS2	11	130.0
3	PL3	10	128.7	AS3	8	129.1
4	PL4	8	113.0	AS4	15	245.4
5	PL5	5	139.6	AS5	3	23.3
6	PL6	3	47.5			
Total		49	746.5		44	580.9
Min.		3	47.5		3	23.3
Mean		8.1	124.4		8.8	116.2
Max.		15	199.6		15	245.4

[a]Linkage Group, [b]Total markers, [c]LG length (in centimorgan)

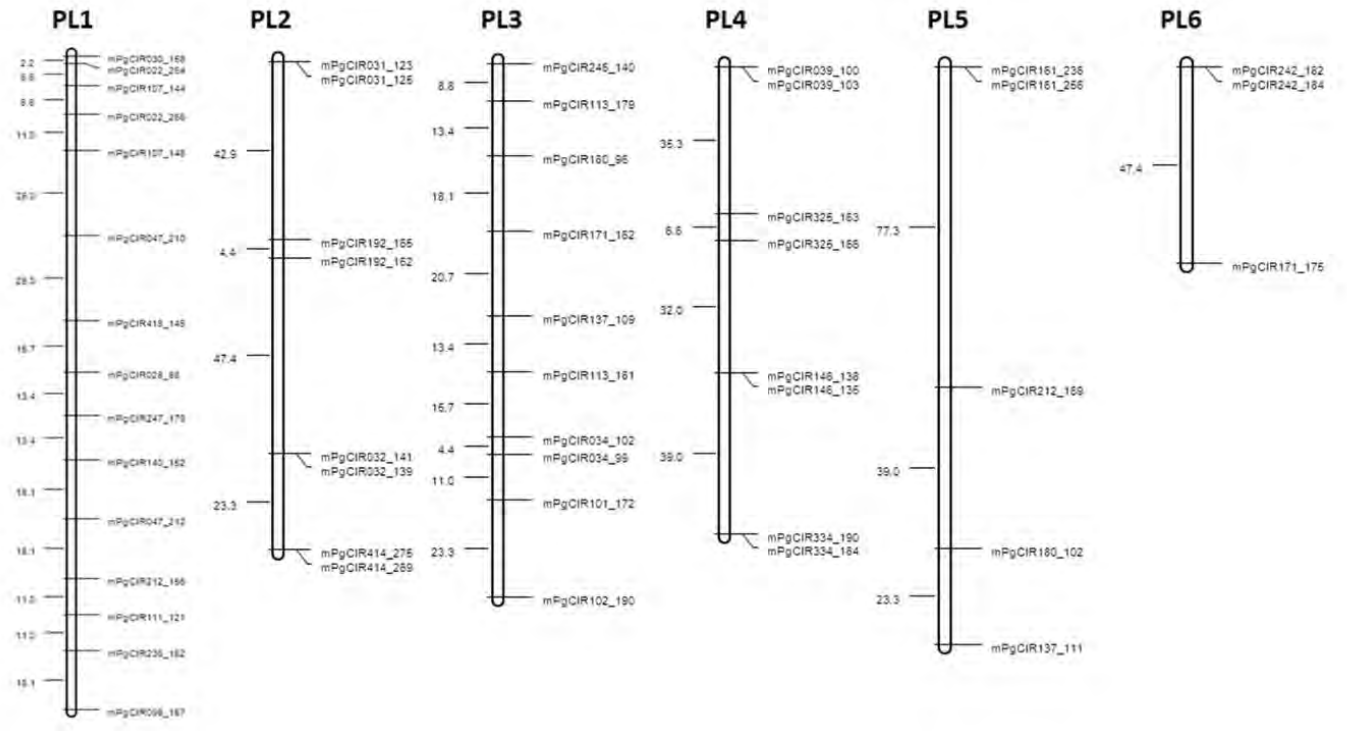

Fig. 2. Genetic linkage map of the parent 'Purple Local'; Map distances in centimorgans (cM) are indicated to the left, and loci to the right of each linkage group

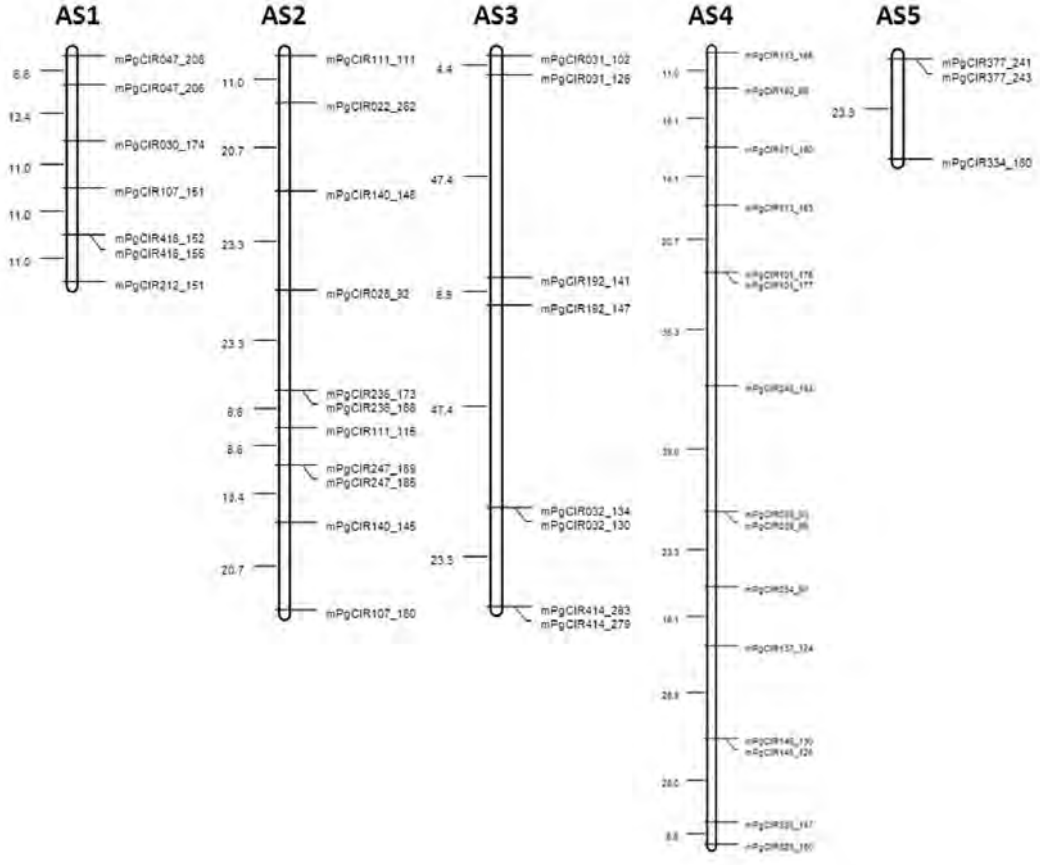

Fig. 3. Genetic linkage map of the parent 'Allahabad Safeda'; Map distances in centimorgans (cM) are indicated to the left, and loci to the right of each linkage group

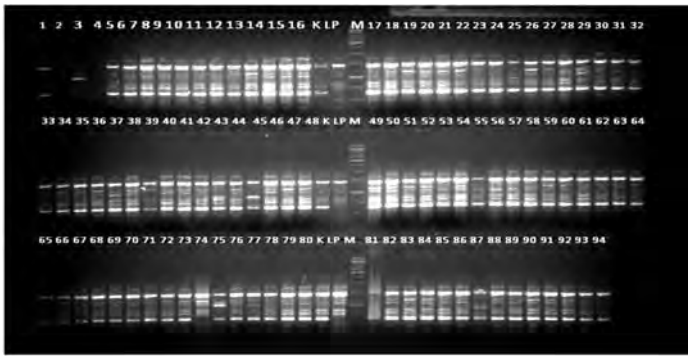

Fig. 4. Agarose gel (1.5%) showing amplification pattern of RAPD markers among mapping population I and parental lines 'Kamsari' (K) and 'Purple Local' (PL)

In the case of MPI, 100 RAPD primers were initially screened in parental lines that revealed 30% polymorphism. These 30 decamers were used further for genotyping the mapping population. A total of 214 scorable bands were produced, with an average of nine bands per primer. Size of the amplified products ranged from 250bp to 2kb (Fig. 4) Of the 214 bands scored, 94 (43.92%) were polymorphic and segregated as test cross markers, of which 80 markers were specific to 'Kamsari' and 14 markers were specific to 'Purple Local'. The remaining 120 common fragments that segregated in 3:1 ratio were treated as intercross markers. RAPD data thus generated can be further used for enriching the available genetic maps of 'Kamsari' and 'Purple Local' reported by our group (Padmakar *et al*, 2015).

Linkage maps thus constructed will be further used for enrichment and, thereby, utilized as reference maps for identifying complex Quantitative Trait loci (QTLs) associated with fruit quality traits in guava. This would also help in identifying trait specific marker(s) for executing marker assisted selection (MAS)-based guava breeding programs.

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