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Enrichment of genetic linkage maps and mapping QTLs specific to seed strength - hardness / softness - in guava (*Psidium guajava* L.)

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ABSTRACT

The present research focuses mainly on molecular mining and morphological evaluation of guava genome within a full-sib population and, thereby, mapping of quantitative trait loci related to fruit quality traits, viz., seed strength (hardness/softness) and average fruit weight. Linkage maps were enriched for both parental lines, 'Kamsari' and 'Purple Local' using a set of 60 RAPD markers following the pseudo-testcross strategy on a panel of 94 progeny. A total of 480 scorable markers were identified, of which 131 were specific to 'kamsari' and 28 to 'Purple Local', segregating as test cross markers, and, 321 showing intercross pattern common to both. 'Kamsari' spanned a total length of 1959.1cM with average marker interval distance of 3.93cM, while 'Purple Local' spanned a length of 1537.9cM with average marker interval distance of 3.29cM, by forming 11 linkage groups. Estimated genome length observed was 93.02% and 92.77% in 'Kamsari' and 'Purple Local', respectively. Composite Interval Mapping (CIM) was computed at significance of 0.05 and LOD threshold greater than 3.0, which led to detection of one major QTL for the trait of average fruit weight, and, four QTLs for the trait of seed strength (hardness/softness). Of these, two were major and two minor QTLs. Our study provides molecular mapping information on marker-assisted selection for improvement of guava in a breeding program.

Key words: Composite interval mapping, guava, linkage map, pseudo-testcross, quantitative trait loci (QTL)

INTRODUCTION

Guava (*Psidium guajava* L.), native to tropical America, is a perennial tree crop with heterozygous and heterogeneous genome comprising approximately 460 Mbp (Sara *et al*, 2012). It is a diploid with 2n=22 and belongs to the family Myrtaceae (Nakasone and Paull, 1998). Familiarly known as the Apple of the tropics / Poor man's apple, guava is one of the important and major fruit crops in India. It is a repository of nutrients, vitamins and antioxidants, and, has incredible medicinal and pharmaceutical properties (Shruthi *et al*, 2013). Guava acts as a dual-purpose fruit used as fresh fruit as well as after processing. Development of medium-sized fruits with high TSS, pink pulp and soft seeds is a major breeding objective in guava which requires basic understanding of the role of complex genomic regions controlling these traits, i.e., quantitative trait loci (QTL).

Genetic linkage maps provide ready means for localization and map-based cloning of genes, and provide the necessary infrastructure for marker assisted breeding. Besides, developing a linkage map with consistent molecular markers forms the basis for analysis of agronomically important traits. Construction of linkage maps in heterozygous species is most efficiently achieved using double pseudo-testcross mapping strategy (Grattapaglia and Sederoff, 1994). Guava genome exhibits a high degree of heterogeneity and heterozygosity (Chandra and Mishra, 2007), and, perennial nature of the crop complicates basic understanding of the genomic sites contributing to various economically important phenotypes.

Guava is still considered an orphan crop with reference to its exploration at the genomic and/or genetic level. Only limited number of reports are available on molecular profiling of the guava genome within a mapping

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population (Valdés-Infante *et al,* 2003; Rodriguez *et al,* 2007; Lepitre *et al,* 2010; Padmakar *et al,* 2015a, 2015b) or on its quantitative genetics (Valdes-Infante *et al,* 2003; Rodriguez *et al,* 2007; Ritter *et al,* 2010). Various markers have been used for molecular characterization in guava (Nimisha *et al,* 2013), of which, random amplified polymorphic DNA (RAPD) markers have been used for assessing molecular diversity (Prakash *et al,* 2002), studying genetic relatedness/diversity (Dahiya *et al,* 2002; Sharma *et al,* 2007; Ahmed *et al,* 2011; Pessanha *et al,* 2011), or determining phylogenetic relationships (Chen *et al,* 2007). Hence, in the present study, we report enrichment of the intra-specific linkage maps developed in guava using RAPD markers in a pseudo-testcross mapping configuration, and identification of fruit quality related QTLs. To our knowledge, this is the first report of a linkage map developed with SSR, SRAP and RAPD markers identifying major QTLs for the trait of seed strength (hardness/softness) in guava.

MATERIAL AND METHODS

Plant material and DNA isolation

The mapping population comprised 94 F_1 progeny obtained from a cross between two cultivars, 'Kamsari' (2n = 2x = 24) and 'Purple Local' (2n = 2x = 24), maintained in the field germplasm bank at ICAR-Indian Institute of Horticultural Research, Bengaluru, India. Total genomic DNA was extracted from young leaves of the parent plants and F_1 progeny, using modified CTAB-method (Kanupriya *et al,* 2011).

Morphological and molecular characterization

Three traits, namely, seed strength (SS) - hardness/softness, average fruit weight (FrWt) and total soluble solids (TSS) related to fruit quality, were assessed from a set of five fruits randomly selected per F_1 progeny plant, as per Dinesh and Vasugi (2010). Descriptive statistics and Pearson correlation coefficient were computed using SPSS software.

A set of 200 RAPD markers were used for screening parental lines, of which polymorphic informative markers were used for genotyping mapping population. PCR amplification was carried out in 25µl reaction mixture containing 50mM KCl, 1mM Tris-HCl (pH 8.8), 0.01% gelatin, 1.5mM $MgCl_2$, 0.2 mM of each dNTP, 0.3µM primer, 100ng genomic DNA, and 0.5 units of Taq DNA polymerase (Bengaluru Genei, India). PCR was carried out on a Master Cycler Gradient (Eppendorf AG, Hamburg, Germany) thermal cycler, as per Padmakar *et al* (2015b). Amplification products were screened on 1.5% agarose gel for confirmation of the amplification. PCR was repeated thrice for checking reproducibility of the polymorphic markers identified.

The amplicons generated were scored in a binary format by assigning '1' for presence of a band and '0' for absence of the band. Each amplicon was named after the primer name used for amplification, along with a suffix indicating the respective allele size that was amplified. In the case of fragments heterozygous with only one of the parents considered as testcross markers, segregation ratio across the mapping population was tested against a 1:1 ratio, using chi-square (χ^2) test at a significance of $p<0.05$; while, those heterozygous in both the parents were considered as intercross markers and were tested against a 3:1 ratio.

Linkage map enrichment and QTL analysis

The data generated was used for enriching the parent-specific maps developed by our group following the protocol of Padmakar *et al* (2015a). A run test (Sokal and Rohlf, 1981) was performed using the Tseries package in R (Trapletti and Hornik, 2013) to determine randomness in distribution of the markers. Genome coverage was calculated by taking the average value of linkage map length estimated, using the method of Fishman *et al* (2001), and Method 4 of Chakravarti *et al* (1994). In the methodology of Fishman *et al* (2001), average spacing of the markers is doubled, and added to the length of each linkage group; whereas, Method 4 of Chakravarti *et al* (1994) expands each linkage group by (m+1)/(m-1), where m is the number of loci mapped.

Quantitative trait loci (QTL) detection was achieved using Windows QTL Cartographer software (Wang *et al,* 2010) employing composite interval mapping (CIM) method (Zeng 1994). The walking speed chosen for all QTL was 1.0cM. Additive effects of each QTL were estimated by the Bayesian test. A QTL was declared as significant at LOD value of 3.0.

Table 1. Characteristics of trait variation in the guava mapping population

	FrWt (g)	SS (kg/cm^2)	TSS (°B)
Mean ± SE	272.9 ± 5.60	11.69 ± 0.22	9.41 ± 0.10
Min.	158.50	7.20	6.50
Max.	400.00	12.20	14.50
CV	19.90	19.00	10.50

FrWt – Average fruit weight; SS – Seed Strength (hardness/softness); TSS – Total Soluble Solids; CV – Coefficient of Variation

RESULTS AND DISCUSSION

Morphological and molecular characterization

Adequate variation was available within the fruit quality trait evaluated (Table 1). The value for fruit weight (FrWt) ranged from 158.5g to 400g, with a mean of 272.9 ± 5.60g. Similarly, this ranged from 6.5kg/cm^2 to 14.5kg/cm^2, with a mean of 11.69 ± 0.22kg/cm^2 and 7.2°B to 12.2°B, with a mean of 9.4 ± 0.10°B, for the traits of seed strength (SS) and TSS, respectively. Coefficient of variation (CV) depended strongly on a particular trait under evaluation. CV values observed were 19.9, 19.0 and 10.5 for FrWt, SS and TSS, respectively. Positive correlation was observed between the traits of SS and FrWt, significant at α=0.01, with Pearson coefficient value of r=0.40; but, a negative correlation was observed between the traits of SS and TSS, as well as FrWt and TSS at α=0.01, with a value of r=0.06 and r=0.21, respectively.

Initial screening of 200 RAPD primers in the parental lines revealed 30% polymorphism. The 60 decamers (Table 2) were used for further genotyping the mapping population.

Table 2. List of polymorphic RAPD markers used in the study

S. No.	RAPD primer	S. No.	RAPD primer
1	OPAG20	31	OPK10
2	OPAO4	32	OPK11
3	OPAO19	33	OPK17
4	OPAU2	34	OPK20
5	OPAZ11	35	OPM4
6	OPAZ14	36	OPN9
7	OPAZ15	37	OPN11
8	OPAZ16	38	OPN12
9	OPAZ18	39	OPN13
10	OPB7	40	OPN20
11	OPB19	41	OPO2
12	OPBA2	42	OPO9
13	OPBA6	43	OPO11
14	OPBA12	44	OPO12
15	OPBA13	45	OPO13
16	OPBA14	46	OPO14
17	OPBA16	47	OPO16
18	OPC2	48	OPO18
19	OPC3	49	OPP2
20	OPC8	50	OPP10
21	OPC13	51	OPP17
22	OPD8	52	OPP19
23	OPH15	53	OPQ1
24	OPK1	54	OPQ2
25	OPK2	55	OPQ3
26	OPK3	56	OPQ6
27	OPK4	57	OPQ18
28	OPK6	58	OPY1
29	OPK7	59	OPY3
30	OPK8	60	OPY9

A total of 480 scorable bands was produced, with an average of 8.00 bands per primer. Size of the amplified products ranged from 150bp to 3kb. Of the 480 bands scored, 159 (33.12%) were polymorphic and segregated as testcross markers, of which 131 markers were specific to 'Kamsari' and 28 to 'Purple Local'. The remaining 321 common fragments segregating in 3:1 ratio were treated as intercross markers. Finally, a set of 57 markers (11.87%) showing segregation distortion was identified and excluded from further mapping studies.

Linkage Map Enrichment

'Kamsari' parental map (Fig. 1) was enriched from 351 markers, leaving 53 unlinked, and grouped into 11 linkage groups (LG) spanning a length of 1951.9cM, with a mean of about 45.2 markers per LG. The LGs (Table 3) varied in genetic length from 69.9cM to 414.2cM, with a mean of 178.1cM. Average marker interval distance observed was 3.93cM ranging from 0.00cM to 50.5cM. Estimated genome length was 2,166.8cM, attributable to 90.41% of genome coverage and 2,048.3cM attributable to 95.64% of genome coverage, as per Fishman *et al* (2001) and Chakravarthi *et al* (1991), respectively. Thus, an average of the two methods resulted in genome coverage of 93.02%. In 'Purple Local', out of the 336 markers tested, 318 markers assembled into 11 LGs (Fig. 2) covering a total distance of 1537.9cM, with a mean of 42.4 markers per LG. The LGs (Table 3) varied in genetic length from 52.9cM to 256.0cM, with a mean of 139.8cM. Inter-marker separation ranged

Table 3. Characteristics of parent linkage maps

	Parent 1: Kamsari			Parent 2: Purple Local		
LG[a]	K-LG	TM[b]	cM[c]	PL-LG	TM[b]	cM[c]
1	K1	101	69.9	PL1	10	178.8
2	K2	101	102.8	PL2	101	146.3
3	K3	101	170.9	PL3	20	256
4	K4	75	194	PL4	101	92.7
5	K5	18	298	PL5	101	52.9
6	K6	6	106.2	PL6	19	187.5
7	K7	9	115.1	PL7	16	123
8	K8	31	179.9	PL8	26	128.7
9	K9	14	152.8	PL9	43	114.6
10	K10	16	155.3	PL10	20	127.3
11	K11	26	414.2	PL11	10	130.1
Total		498	1959.1		467	1537.9
Min.		6	69.9		10	52.9
Mean		45.2	178.1		42.4	139.8
Max.		101	414.2		101	256
GC[d]		93.02%			92.77%	

[a]Linkage Group
[b]Total number of markers
[c]LG length in centiMorgans (cM)
[d]Genome Coverage (estimation of)

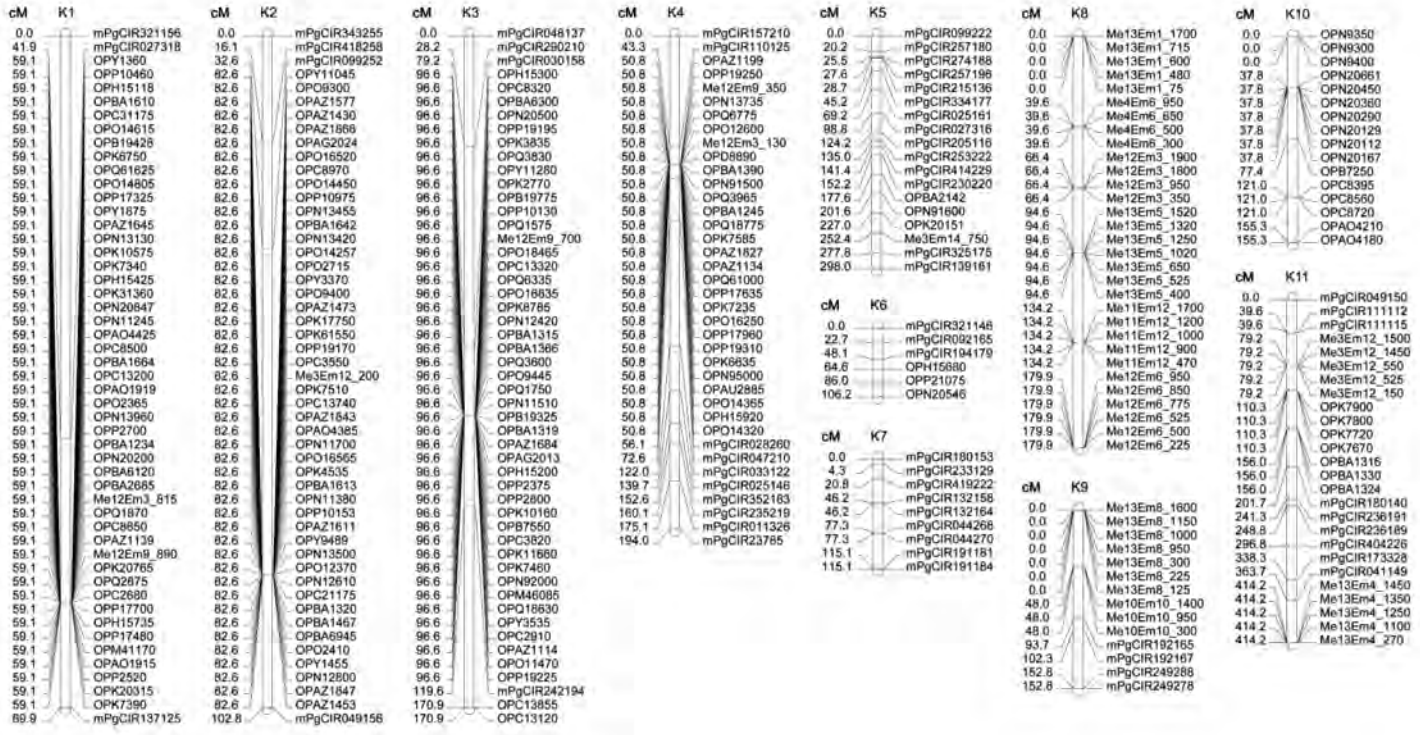

Fig 1. Genetic linkage map of 'Kamsari': Map distances in centiMorgans (cM) are indicated to the left, and loci to the right, of each linkage group

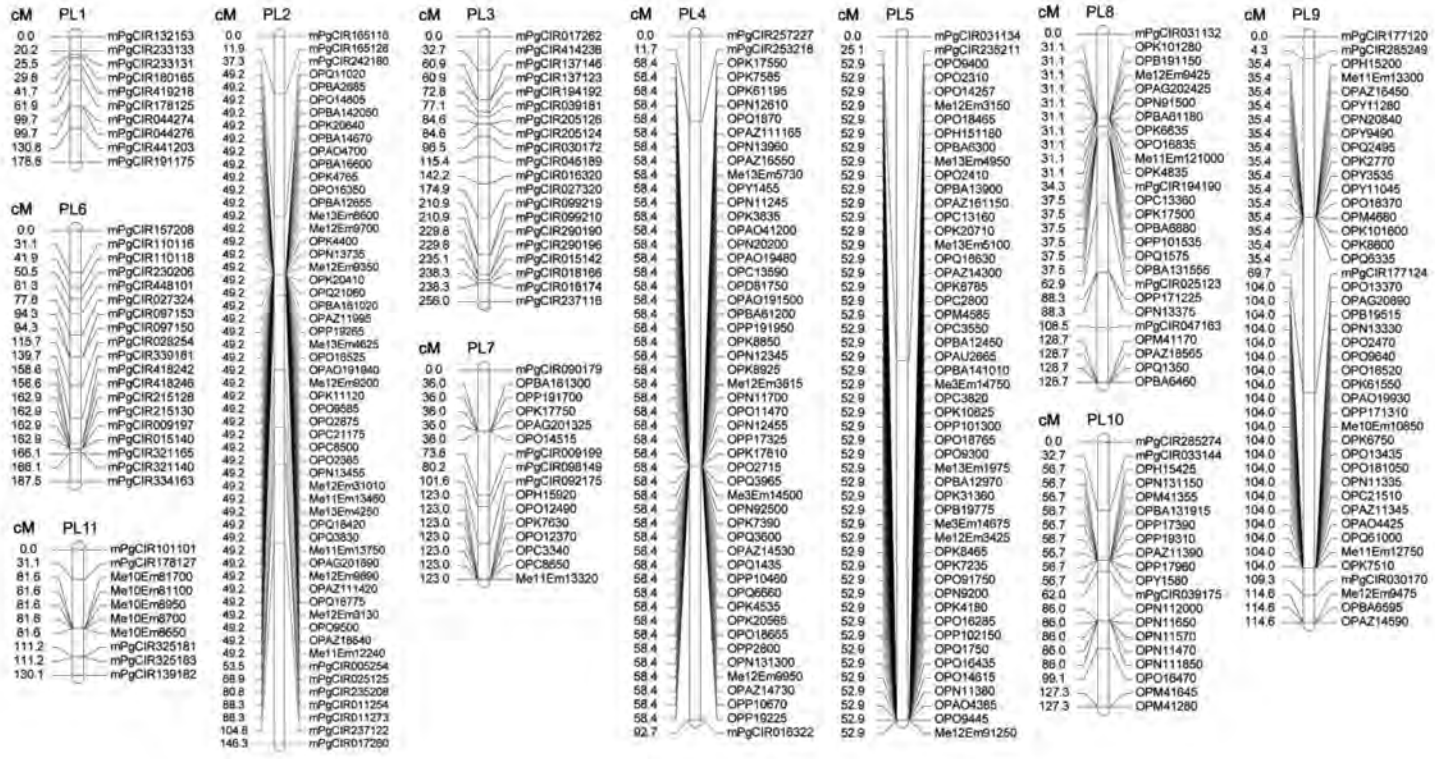

Fig 2. Genetic linkage map of 'Purple Local': Map distances in centiMorgans (cM) are indicated to the left, and loci to the right, of each linkage group

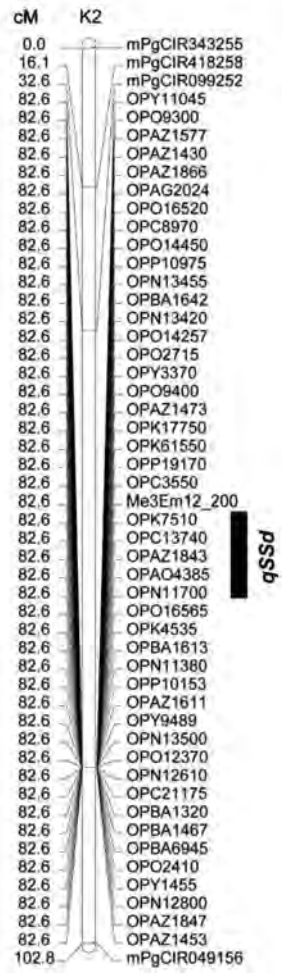

Fig 3. QTLs mapped on linkage map of 'Kamsari'

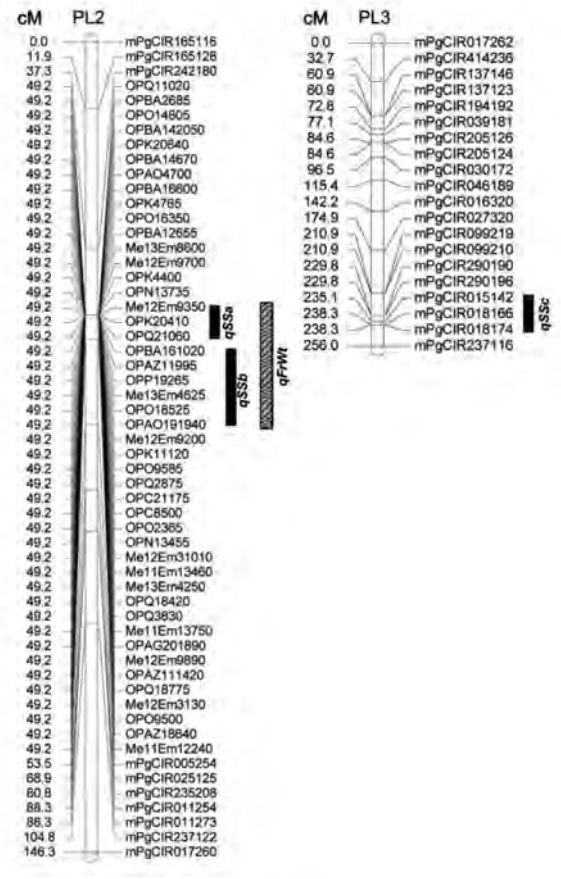

Fig 4. QTLs mapped on linkage map of 'Purple Local'

Table 4. Summary of results of QTL analyses

Trait	QTL	Linkage Group	Marker Interval	QTL position (cM)	LR	Additive effect	R^2
Average fruit weight	qFrWt	PL2	OPQ1_1020 - mPgCIR025_125	49.21	29.47	63.07	26.3
							Total R^2 26.3
Seed strength (hardness/ softness)	qSSa	PL2	OPQ1_1020 - mPgCIR005_254	51.21	174.38	4.1	43.6
	qSSb	PL2	mPgCIR005_254 - mPgCIR025_125	64.51	200.15	4.1	43.6
	qSSc	PL3	mPgCIR290_190 - mPgCIR018_166	234.81	30.36	0.83	2.3
	qSSd	K2	mPgCIR099_252 - OPY1_1045	61.61	46.16	1.11	3.6
							Total R^2 93.2

from 0.0cM to 50.5cM, with a mean marker interval distance of 3.29cM. The genome length computed was 1,705.7cM, attributable to 90.16% of genome coverage and 1,612.1cM attributable to 95.39% of genome coverage, as per Fishman *et al* (2001) and Chakravarthi *et al* (1991), respectively. The average of these two methods resulted in a genome coverage of 92.77%.

Mapping QTLs

A total of five putative QTLs was detected, with each one explaining between 2% and 43% of the phenotypic variance (Table 4). Four seed-strength QTLs (Fig. 3, 4), namely, qSSa, qSSb, qSSc and qSSd, were identified and mapped to the LGs K2, PL2-1, PL2-2 and PL3. All showed a positive additive effect and accounted for, respectively,

3.6%, 43.6%, 43.6% and 2.3% of phenotypic variance. Similarly, one QTL for average fruit weight (Fig. 3), namely qFrWt, was identified in 'Purple Local' and was mapped to the LG PL2, and contributed to 26.3% of phenotypic variance and exhibiting a positive effect.

Morphological and molecular characterization

As reported earlier by our group (Dinesh and Vasugi, 2010), a hybridization program in guava was initiated at ICAR-Indian Institute of Horticultural Research, Bengaluru, with a primary goal of developing hybrids suitable for both table purpose and processing, having fruits of uniform shape, size, good color, firm and thick pulp, good aroma, soft seeds, high TSS, high pectin and a long shelf-life. Varieties selected as parents were 'Kamsari' of medium-sized fruits, pink pulp, TSS of 9.8°B, less seed-bearing portion, strong flavour with hard seeds, and, 'Purple Local' of dark purple skin, dull-pink pulp, with soft seeds. Fruit characteristics evaluated (FrWt, SS and TSS) showed significant amount of variation within the mapping population comprising 94 progeny. Molecular exploration of guava is still in its infancy owing to a lack of availability of sufficient genomic resources. Only a few reports are available on development and application of molecular markers for characterizing guava genome. We have reported genotyping and mapping of guava genome using SSR and SRAP markers in a previous study (Padmakar *et al,* 2015a), and have majorly focused on enriching the maps developed with RAPD markers in the present work. Since SSR markers available from guava (Risterucci *et al,* 2005, 2010) and SRAP primer combinations (Li and Quiros, 2001) have been already used, RAPD markers were used here for further characterization.

Linkage mapping and QTL mapping

Construction of linkage maps in highly heterozygous species and perennial crops like guava is complicated because each parent is heterozygous, and linkage phase of the marker alleles is usually unknown (Maliepaard *et al,* 1997). However, in out-crossing species, linkage maps have been developed by a strategy known as double or two-way pseudo-testcross-mapping (Grattapaglia and Sederoff, 1994), where the F_1 population is considered as the mapping population, and this has proved efficient in mapping several heterozygous species (Xie *et al,* 2011; Lu *et al,* 2012; Sudarshini *et al,* 2014; Padmakar *et al,* 2015a, 2015b). In our present study a similar technique was employed for parent-specific linkage map enrichment of both the parents. Significant amount of difference was observed on the total distance spanned, average marker interval distance, as also the genome coverage estimated in both parental lines. In 'Kamsari', the total length of linkage map decreased from 2,553.7cM to 1959.1cM along with a reduction in average marker interval distance from 17.5cM to 3.93cM. In addition, the estimated genome coverage increased from 87.32% to 93.02%. Similarly, with 'Purple Local' the reduction observed was from 2,115.9cM to 1537.9cM, and 15.9cM to 3.29cM for the total length of linkage map and average marker interval distance, respectively, with increase in estimated genome coverage from 83.74% to 92.77%.

Marker loci showed some tendency to cluster, especially the SRAP markers. Some LGs consisted of more loci than the others. This could be due to a lack of marker polymorphism between mapping parents on some chromosomes, and/or, these might be sites on the genome representing suppressed recombination. Similar clustering was reported earlier too (Zhang *et al,* 2011; Zhang *et al,* 2013). Decamers used in the present study played the key role of missing links in the mapped SSR and SRAP markers. Thus, enriched maps were further exploited for mapping the complex QTLs governing fruit quality traits such as seed strength (SS), average fruit weight (FrWt) and total soluble solids (TSS).

Studies on understanding quantitative genetics in guava are scanty due to the complexity involved in generating mapping populations, long juvenile period of the crop, lack of adequate genomic resources, and the highly heterozygous nature of guava. Till date, only three studies are reported, that too from the same group (Valdes-Infante *et al,* 2003; Rodriguez *et al,* 2007; Ritter *et al,* 2010) on mapping of QTLs in guava. In our present study, two separate QTL analyses were performed with 'Kamsari' map (K1–K11, Fig. 1) and 'Purple Local' map (PL1– PL11, Fig. 2). We mapped five QTLs, acting on two fruit quality traits and distributed over 11 LGs (Table 4; Figs. 3, 4). Of the four seed-strength QTLs, two were responsible for a major proportion of phenotypic variance. Similarly, QTL identified for FrWt contributed a significant proportion of variance in trait. Besides, qFrWt and qSSa have been mapped very closely on LG PL2, but it is unclear whether this reflects existence of two independent loci, or that, a single locus is acting pleiotropically on these two traits. No significant QTLs were identified for the trait of TSS. This could be due to sampling bias in a mapping population based on correlation studies on the traits of SS and FrWt, as reported

earlier (Padmakar *et al*, 2015a). Detection (of major fruit quality QTLs, being spanned by the markers OPQ1_1020 - mPgCIR005_254; mPgCIR005_254 - mPgCIR025_125; mPgCIR290_190 - mPgCIR018_166 and mPgCIR099_252 - OPY1_1045) is encouraging for the prospect of applying marker-assisted breeding in improving guava to develop elite varieties with medium-sized fruits with high TSS, pink pulp and soft seeds, considered to be major breeding objectives in this crop.

To the best of our knowledge, this is the first report on SSR, SRAP and RAPD-based linkage mapping and fruit quality related QTL identification in guava. Application potential of this map in the future for guava improvement is highlighted here. Due to a lack of anchor markers between the two maps at present, additional markers (especially, more co-dominant ones) can be used to integrate the two frameworks into a single, saturated map which may be exploited for further studies on gene tagging, MAS breeding and comparative genomics.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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