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Assessment of clonal fidelity of micro-propagated guava (*Psidium guajava*) plants by ISSR markers

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Abstract
Inter-simple sequence repeat (ISSR) was used to assess the genetic stability of micro-propagated plantlets of guava. Thirty one plantlets were chosen from a clonal collection of shoots that originally from a single mother plant. Out of 21 ISSR primers screened, 16 primers were found to produce clear, reproducible bands resulting in a total of 93 distinct bands with an average of 6.5 bands per primer. Of these 93 bands, 84 were monomorphic across all 31 of the plants tested and 9 showed polymorphisms (9.67% polymorphisms). Based on the ISSR band data, similarity indicators between the progenies and the mother ranged from 0.92 to 1.0, such a similarity indicated a very low polymorphism. These similarities indices were used to construct an UPGMA dendrogram and demonstrated that all 31 micro-propagated plants grouped together in one major cluster with a similarity level of 92%. A total of 2356 scorable bands were obtained from the full combination of primers and plantlets and only 39 (1.65%) were polymorphic across the plantlets which indicated that the micro-propagated guava is genetically stable.

Keywords: *Psidium guajava*, guava, molecular markers, reproducibility, ISSR.
Abbreviation: ISSR- Inter-Simple Sequence Repeat, AFLP- Amplified Fragment Length Polymorphism, RAPD- Random Amplified Polymorphic DNA, SSR- Simple Sequence Repeat.

Introduction

Guava (*Psidium guajava* L, *2n* = 22) belongs to the genus *Psidium* and the family Myrtaceae (Nakasone and Paull, 1998). It is a very valuable tropical and subtropical fruit representing a staple food in many countries. The largest producer countries (Mexico, Brazil, India and Thailand) grow guava for the economic importance of its fruit which is either used fresh or processed to make juice, paste, or marmalade. Fresh guavas are a good source of vitamins A and C (Singh, 2005). Guava is also used for medicinal purposes throughout the tropics for treating diarrhea and fungal infections (Garcia, 2002). As interest in use of guava increases, current methods of guava cultivation are not likely to keep up with the demand. Faster and higher plant producing strategies are needed. Clonal propagation of guava may be an efficient way to produce guava. Plant tissue culture (micropropagation) is recognized as one of the key area of biotechnology because of its potential use to regenerate elites, while conserving valuable plant genetic resources. However, the scaling up of any micro-propagation methodology carries the risk of inducing genetic variability, namely somaclonal variation amongst sub-clones of one parental line (Larkin and Scowcroft, 1981). In vitro cultivation in particular poses a problem in recovering true-to-type regenerants due to chromosomal rearrangement, gene amplification, gene mutation (Saker et al., 2000) and retrotransposon activation (Hirochika, 1993). Somaclonal variation occurs in operations which require clonal uniformity, as in the horticulture and forestry industries where tissue culture is employed for rapid propagation of elite genotypes. Clonal variation can be detected by DNA fingerprinting using different types of markers like RAPD, ISSR, or RFLP. ISSR proved to be more effective and reproducible for detecting genetic uniformity (Martins, 2004). ISSR analysis involves the use of single sequence repeat motifs in order to prime the polymerase chain reaction and thereby amplify regions between adjacent, but inversely orientated, microsatellites. The microsatellite loci have been isolated. A $(GA)_n$ and $(GT)_n$ micro-satellite-enriched library was developed to improved the type of molecular markers for genetic studies (Risterucci et al., 2005). Compared to RAPD primer, ISSR primers are longer, so they offer advantages in the detection of somaclonal variation notably with a higher degree of sensitivity, increased stringency, higher reproducibility and the dominant representation of polymorphic genetic alleles. ISSR markers have been successfully applied in the analysis of genetic fidelity in cauliflower (Leroy et al., 2000), almond (Martins et al., 2004), banana (Lakshman et al., 2007; Rout et al., 2009), *Cymbopogon martinii* var motia (Bhattacharya et al., 2010), gerbera (Bhatia et al., 2009) and *Swertia chirayita* (Joshi and Dhawan 2007). Assessment of genetic diversity of guava germplasm using a SSR marker has been reported (Rodríguez et al., 2007; Sánchez-Teyer et al., 2010). However, there is no report on assessment of genetic integrity on guava by ISSR markers. The purpose of the present study was to assess genetic fidelity of *in vitro* raised cultures by ISSR markers.

Results and Discussion

In the present study, 21 ISSR primers were used for checking the fidelity of *in vitro* generated clones among which five (7, 8, 9, 12 and 21) did not react with guava DNA. Of the primers that did react, four (2, 4, 17, and 20) displayed a positive interaction but failed to reproduce any major scor-

Table 1. List of primers, their sequence, numbers and size of the amplified fragments generated by ISSR primers in guava.

Primer name	sequence	Tm ºC	Reaction to DNA	No. of monomorphic bands	No. of polymorphic bands	Size range (bp)
Guava 1	$(AG)_8$YA	48.9	Positive, reproducible	5	1	200-1000
Guava 2	$(AG)_8$RA	48.7	Positive, not reproducible	4	1	200-1000
Guava 3	$(AG)_8$ YT	49.2	Positive, reproducible and polymorphic	7	3	200-600
Guava 4	$(AG)_8$RT	48.8	Positive, not reproducible	9	0	300-500
Guava 5	$(GA)_8$RT	47.4	Positive, reproducible	8	1	200-600
Guava 6	$(GA)_8$RG	48.4	Positive, reproducible	9	0	200-700
Guava 7	$(GA)_8$RC	48.9	Negative			
Guava 8	$(GA)_8$YG	48.8	Negative			
Guava 9	$(GT)_8$RA	51.4	Negative			
Guava 10	$(AC)_8$RG	54.3	Positive, reproducible and monomorphic	7	0	200-650
Guava 11	$(CT)_8$YA	47.1	Positive, reproducible	4	2	300-1000
Guava 12	$(CT)_8$RT	47.1	Negative			
Guava 13	$(CT)_8$RG	48.1	Positive, reproducible	9	3	300-1200
Guava 14	$(CT)_8$RC	48.6	Positive, reproducible and monomorphic	10	0	250-1300
Guava 15	$(CA)_8$RC	53.1	Positive, reproducible	7	0	200-1500
Guava 16	$(CA)_8$RA	51.7	Positive, reproducible	3	0	300-1200
Guava 17	$(GT)_8$RA	51.4	Positive, not reproducible	5	0	250-1300
Guava 18	$(GT)_8$YA	51.4	Positive, reproducible	7	0	200-1000
Guava 19	$(AG)_8$	44.7	Positive, reproducible	6	0	300-1200
Guava 20	$(GA)_8$	44.7	Positive, not reproducible	4	0	250-1300
Guava 21	$(CT)_8$	44.7	Negative			

able band, whereas 11 primers (1, 3, 5, 6, 10, 11, 13, 14, 15, 16, 18, 19) showed positive, scorable, reproducible bands (Table 1). All 11 primers generated unique sets of amplified products (a total of 93 distinct amplicons, and 3224 bands) with the size range from 200 to 1300 bp (Fig 1). These large numbers of amplicons, extended over distinct regions of the genome, are reasonably adequate for detecting somaclonal variation. This is evident by the comparable numbers of bands scored (326, 150 and 424) in various plant taxa by employing PCR-based markers assays (Martin et al., 2004; Joshi and Dhavan, 2007; Laksman et al., 2007). Examples of the monomorphic amplicons obtained for ISSR are shown (guava primer 10 and 14). Six primers produced polymorphic bands. In all, thirty nine polymorphic bands were observed. The number of bands from each of these successfully used primers varied from 3 (in 5) to 10 (in 14) per sample, where 10 displayed 7 and 13 displayed 9 bands (Fig 1) per primer. A total number of 2356 reproducible monomorphic bands were scored from the clones including their mother with an average of 6.5 bands per primer. The morphology of the *in vitro* generated clones was the same as their mother plant, which indicates that very low variation was detected among them. In the present study di-nucleotide SSRs motifs AG, GA, AC, CT, and CA were used. Three (AG, GA, CT) positive and reproducible primers (two based on AG or GA motif and two on CT) amplified distinct scorable number of bands (Table 1). Interestingly, primer guava 3 based on AG and guava 13 based on CT motif amplified clear, sharp bands with high level of polymorphism thus revealed more coverage of the genome. However, the other 4 primers produced smears that could not be scored and they were not reproducible. Five primers produced no results at all. Modification of PCR amplification conditions and staining did not improve the patterns much. We believe that the poor results with these primers were due to characteristics of the primers or to the relative abundance of priming sites in guava DNA. Primers based on $(AG)_n$ and $(CT)_n$ produced excellent fingerprint patterns, suggesting that these repeats are abundant in guava. The fingerprint quality depends on the motif abundance. Similar results were reported by other scientists. Fang et al. (1997) found in trifoliate orange, primers based on $(CA)_n$ and $(TCC)_n$ produced excellent fingerprint patterns, suggesting that these repeats were abundant. Ratnaparkhe et al. (1998) reported that in chickpea, $(AC)_n$ repeats were abundant and primers based on this repeat $(AC)_8$T were linked to the gene for resistance to fusarium wilt race 4. Blair et al. (1999) reported that in rice, $(GA)_n$ motif are more common than $(GT)_n$. Primers based on the poly (GA) motif produced significantly more bands on average (57.3±4.9) than the primers based on the poly (GT) motif (12.6±7.1). Lakshmanan et al. (2007) reported that in banana, both (GA)n and (GT)n are abundant since primers based on them produced excellent fingerprints. Joshi and Dhawan (2007) investigated that in *Swertia chirayita*, $(AG)_n$, $(GA)_n$, and $(GT)_n$ were most abundant. These primers produced 93 distinct and scorable bands in the size range of 200 bp-1300 bp with an average of 6.5 bands per primer (Table 1). Guava being a diploid and it can be assumed that limited number of bands produced by these ISSR primers would partially cover the genome. However, none of the primers showed any difference in banding pattern. Considering observed similar morphological competence of the mother plant and its clones and the displayed monomorphic banding pattern, it can be suggested that *in vitro* regenerated clones maintained their genetic integrity. Cluster analysis was performed on the basis of similarity coefficients generated from the ISSR data of the 93 scored bands. Similarity ranged from 0.92 to 1.0 among the 31 micropropagated plants (Fig 2). According to this ISSR analysis, all 31 micropropagated plants could be grouped together in a single cluster with a 92% level of similarity. Eight progenies can be grouped into the same cluster with the

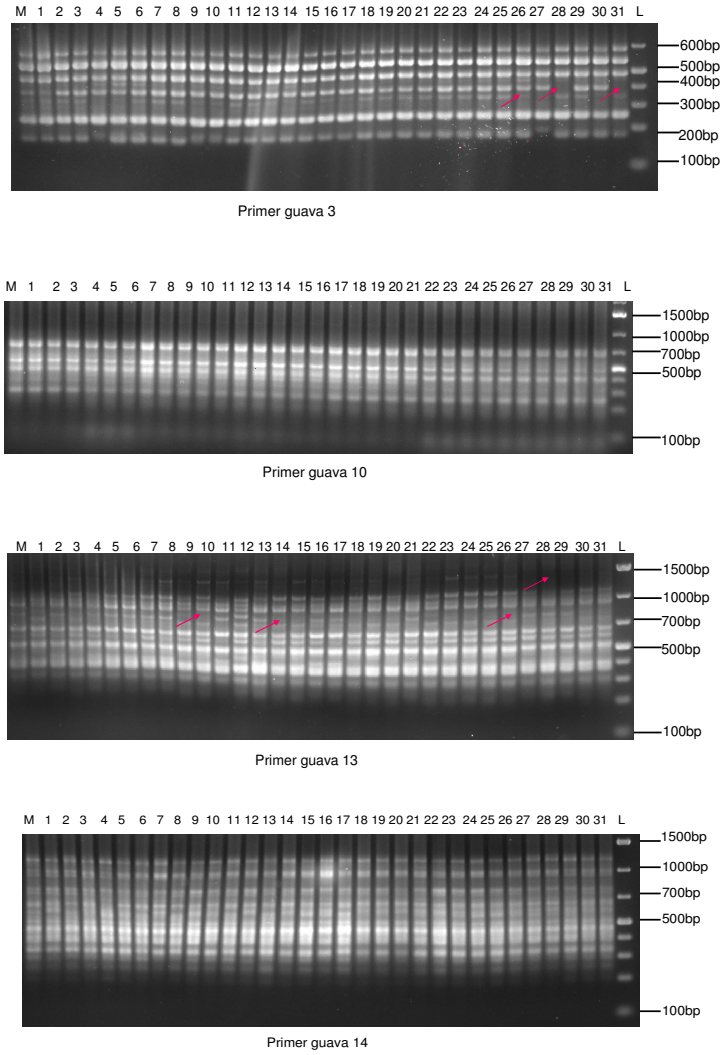

Fig1. PCR amplification products obtained with ISSR marker guava 3. Lane M, samples from the mother plant; Lane 1-31, in vitro raised clones; lane L, 1bk plus DNA ladder. There were polymorphisms bands in guava 3 and guava 13 indicated by red arrows, in some position, bands are missing for some individuals. Guava 10 and guava 14 showed monomorphic bands.

mother at a similarity level of 0.98. In view of a 2 year period of in vitro propagation, such a similarity indicated a very low polymorphism. Extensive research has been carried out on the mechanism of somaclonal variation. The level of genetic instability may be attributed to naturally occurring variation or to the accumulation of mutation during the *in vitro* culture period. In most cases, it is considered as an undesirable side-effect of *in vitro* propagation (Larkin and Scowcroft, 1981). It was recognized that the presence or absence of variations during *in vitro* propagation depends upon the source of explants and the mode of regeneration (Goto et al., 1998), including levels of growth substances, especially synthetics used (Martin et al., 2006). Axillary branch application and somatic embryogenesis are better method to generate genetic stable clones. However, organogenic differentiation is highly prone to somatic variation (Pontaroli and Camadro, 2005). As shoot tip explants directly regenerated into clones and clones cultured over a period of 24 months still maintained clonal fidelity faithfully, it is suggested that shoot tip meristem based on a micropropagation system is much more stable genetically. The protocol followed and the technique implemented (splitting and recycling of shoots in vitro) would further promote sustained long-term in vitro maintenance of shoot tip generated clones without any alteration or modification of DNA. The results revealed the significance of shoot tip explants in maintaining their regeneration frequency with morphogenetic competence. Out of twenty one ISSR primers screened, 16 primers were found to produce clear, reproducible bands. Based on the ISSR band data, similarity indicates among the progenies ranged from 0.92 to 1.0. The UPGMA dendrogram constructed according to the similarities demonstrated that all 31 micro-propagated plants can be grouped together in one major cluster with a similarity level of 0.92. Huang et al. (2009) reported that the progenies and the mother were clustered together at the similarity level of 0.91 with 2.88% polymorphic bands. A similar conclusion was reached by a study on *Phoenix dactylifera*, where 2.6 % genetic variation was detected (Saker et al., 2006). In our research, somaclonal variation rate was 1.65% across the plantlets which indicated that the micro-propagated guava is genetically stable.

Materials and methods

Plant material and in vitro culture

The initial guava cultivar used in the present study was grown in a greenhouse in a controlled environment (Liu and Yang, 2011). Shoot apices and nodal segments were used for culture initiation in vitro. After sterilization, explants were placed vertically in the G7 Magenta boxes (Magenta Corporation, Chicago, IL, USA.) containing 50 ml of Murashige and Skoog (MS) medium with 3% (v/v) sucrose, 8.88 µM BA and 0.7% Difco-Bacto agar. The multiplication medium was MS medium supplemented with 4.44 µM BA, 4.65 µM kinetin (KT) and 0.54 µM NAA, 3% (v/v) sucrose, and 0.7% Difco-Bacto agar. Cultures were incubated at 25°C under a 16-h photoperiod provided by cool-white fluorescent lamps (80–100µ mol m-2 s-1). The media was changed every three weeks to control phenolic exudation. Elongated shoots were dipped in 4.9 mM IBA for 1 min before being placed in the IBA-free rooting media (1/2MS+0.1g/L activated charcoal + 2% sucrose +0.7% Difco-Bacto agar). Rooted plantlets were successfully acclimatized in the culture room and new leaves appeared 2 to 3 weeks after acclimatization. Then, these acclimatized plantlets were transferred into the greenhouse. The leaf materials were harvested for DNA isolation after one year growth in the greenhouse. The total culture time was about 2 years.

DNA extraction and PCR amplification conditions

Total genomic DNA was extracted from 100 mg young leaf tissue from each of 31
in vitro-derived clones by using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Quality and quantity of genomic DNA was assessed by Nano drop spectrophotometer ND-1000 (Thermo Scientific, GA). ISSR primers were designed according to the guava SSR fragments in Genbank. Twenty-one ISSR primers (XXIDT Integrated DNA Technologies Int., Coralville, IA) were used for standardization of optimum annealing temperature. All the PCR components used in this study were purchased from Fishersci, Georgia. PCR amplification was performed in an Eppendorf thermal cycler

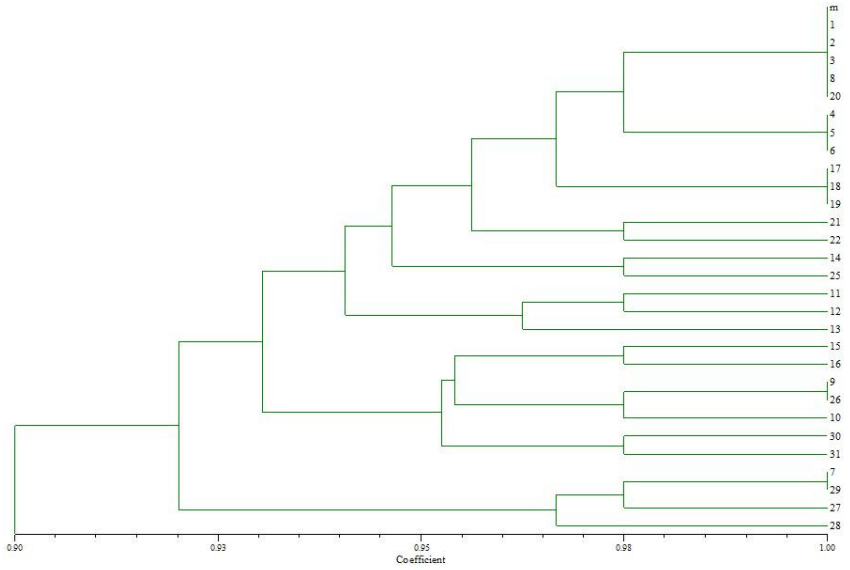

Fig 2. UPGMA based dendrogram showing the similarity among in vitro raised plants of guava and the mother plant. M is the mother plant, 1 to 31 are progenies. The similarities demonstrated that all 30 micro-propagated plants and the mother can be grouped together in one major cluster with a similarity level of 0.9.

(Eppendorf North America, Inc.). PCR was performed in a 25 µl mixture containing 25-50 ng DNA, 2.5 µl10×Taq buffer, 2.5 mM MgCl2, 0.2 mM dNTP, 1.6 µM primer, and 1U Taq DNA polymerase. The PCR consisted of an initial denaturation at 94°C for 3mins, 40 cycles comprising denaturation at 94°C for 30s, annealing at 72°C for 1 min, extension at Tm for 50sec, and a final extension step at 72°C for 10mins. Amplified products were separated by electrophoresis on 2.0% agarose gel in TAE (1×) buffer stained with ethidium bromide for 20 mins and photographs were taken using Gel documentation system (Bio-Rad Corporation, USA).

Statistical analysis

All reactions were repeated at least twice and bands that were reproducible on all runs were considered for analysis. Only distinct, reproducible and well-resolved fragments ranging from 200 to 2000 bp were selected for analysis. These bands were scored either as present (1) or absent (0) for each of the ISSR markers within the 31 micropropagated plants. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and were not scored. The size of the amplification products was estimated using a 1kb plus molecular size marker (FisherSci, Georgia). The similarity matrix was subjected to the cluster analysis of un-weighted pair group method with arithmetic average (UPGMA), and a dendrogram was generated by NTSYPC. The NTSYPC was used to perform the distance matrix and clustering analysis of the complete data set (Rohlf, 2000).

Conclusions

Twenty one ISSR primers were employed to test 30 progenies of clonal propagated guava plants. Sixteen of these 21 primers produced clear, reproducible bands with 9.67% polymorphisms. The similarity indicators between the progenies and the mother ranged from 0.92 to 1.0. Such a similarity indicated a very low polymorphism. The low somaclonal variation rate of 1.65% indicated the genetic stability of micro-propagated guava. Therefore, mircopropagation is a safe mode for multiplication of true to type plants of guava. This is also very important for commercial propagation of elite clones with desirable fruit traits.

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