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MICROSATELLITE ANALYSIS OF INTRA CULTIVAR DIVERSITY IN 'CHINNARASAM' MANGO FROM ANDHRA PRADESH, INDIA

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ABSTRACT

'Chinnarasam', a juicy cultivar of mango (*Mangifera indica* L.), is among the most important mangoes of an Indian State of Andhra Pradesh, whose production and quality is variable across the State. Twenty accessions of 'Chinnarasam' (CR Acc-l to CR Acc-20) were selected from 9 locations spread over 6 districts, representing all the three eco-geographical regions (Coastal Andhra, Rayalaseema and Telangana) of Andhra Pradesh. Their leaves were sampled to study intracultivar diversity following microsatellite analysis. The dendrogram generated based on unweighted pair group mean with arithmetic average, showed three major groups of accessions, which followed geographical separation. Twenty out of 109 mango-specific microsatellites validated, were polymorphic (18.34%). Microsatellites produced a total of 62 alleles, of which 31 were polymorphic (50%). The Jaccard's similarity coefficient ranged from 0.54 to 1.00. Existence of intracultivar genetic diversity (up to 46%) indicates that 'Chinnarasam' whatsoever cultivated throughout the State is not a pure clone, which allows the genetic breeding of this cultivar by means of mass selection. Microsatellite markers have proven useful in assessing intracultivar genetic diversity and identifying accessions of 'Chinnarasam' cultivar.

Key Words: Eco-geographic survey, genetic diversity, genetic relationship, SSR markers

RÉSUMÉ

'Chinnarasam', un cultivar juteux du manguier (*Mangifera indica* L.), est parmi les manguiers d'un Etat indien d'Andhra Pradesh, dont la production et la qualité sont variables à travers la région. Vingt nouvelles accessions de 'Chinnarasam' (CR Acc-l à CR Acc-20) étaient sélectionnées parmi 9 lieux de propagation sur 6 districts représentant toutes les trois régions eco-géographiques d'Andhra Pradesh (la partie cotière d'Andhra, Rayalaseema et Telangana). Leurs feuilles étaient échantillonnées pour étudier la diversité intracultivar suivant l'analyse microsatellite. Le dendrogramme généré sur base de la moyenne du groupe paire non pondérée avec la moyenne arithmétique a montré trois groupes importants d' accessions en rapport avec la séparation géographique. Vingt sur 109 microsatellites spécifiques de manguiers validés étaient polymorphes (18.34 %). Les microsatellites ont produit un total de 62 allèles parmi lesquelles 31 étaient polymorphes (50 %). Le coefficient de similarité de Jaccard variait de 0.54 à 1.00. L'existence de diversité génétique intracultivar (jusqu'à 46 %) indique que le 'Chinnarasam' cultivé à travers l' Etat n'est pas un clone pur, ce qui permet l'amélioration génétique de ce cultivar par le moyen de la sélection massive. Les marqueurs microsatellites se sont avérés utiles dans l'évaluation de la diversité génétique intracultivar et l'identification des accessions du cultivar 'Chinnarasam'.

Mots Clés: Enquête éco-géographique, diversité génétique, rapport génétique, marqueurs SSR

INTRODUCTION

Mango (*Mangifera indica* L.) is one of the members of the family Anacardiaceae in order Sapindales. It originated in the South East Asian or Indo-Burma Region (Mukherjee, 1951). It gradually spread and has become one of the choicest fruit crops of tropical and sub-tropical regions of the world, especially in Asia. Mango has been cultivated for thousands of years in India (Mukherjee, 1953). In mango, crop diversity is well represented as developed cultivars, landraces or as folk varieties in different eco-geographical regions of India. The mango has more than a thousand varieties in India (Mukherjee, 1948). Most of the cultivars of mango are the result of open pollination leading to chance seedling and then further maintained asexually (Iyer and Degani, 1997).

Juicy cultivars (sucking mangoes) of differing skin colour, stone size, sweetness and composition are available on the Indian market. Notable local juicy varieties include 'Peddarasam', 'Chinnarasam', 'Cherukurasam', and 'Panchadarakalasa' from the Krishna-Godavari zone of Andhra Pradesh State, India; these are popularly known as '*rasaalu*'. 'Chinnarasam' is a major commercial juicy cultivar in Andhra Pradesh. 'Chinnarasam' is a highly priced and major commercial juicy cultivar in the State. Most of its fresh fruits are locally consumed in hotels and super markets, only very small scale is exported to north India. In spite of its commercial importance, 'Chinnarasam' cultivar is troublesome for gardeners because of obvious dissimilarities in appearance of fruit and quality from different regions of the State.

Characterisation of intracultivar variability is important to provide scientists, producers and all interested people, with information about certain properties of some cultivars. There are different methods to characterise intracultivar variability, such as morphological, biochemical and molecular markers. Several procedures for the identification and characterisation of mango genotypes have been developed based on morphological, biochemical, agronomical or genetic traits (Krishna and Singh, 2007; Galvez-Lopez *et al*., 2009). Conventionally, morphological characteristics such as characteristics of leaves, fruits and stone were used to assess intracultivar variability in mango. Intracultivar variability of mango cultivars from India and other countries has been studied based on fruit traits by several researchers (Naik, 1971; Gan *et al*., 1981; Singh *et al*., 2009). The morphological mode of identification of intracultivar variability is slow because of long juvenile periods, and it is unstable because of environmental effects on the characteristics. In addition, the number of these traits is limited; they are unstable and they do not always enable to distinguish between closely related accessions or cultivars (Konarev, 2000).

In the last decade, various molecular techniques have been successfully applied in determining the intracultivar diversity of mango in India and abroad (Gan *et al*., 1981; Bally *et al*., 1996; de Souza and Lima, 2004; Diaz-Matallana *et al*., 2009; Rocha *et al*., 2012). Gan *et al*. (1981) employed isozymes, Bally *et al*. (1996) and de Souza and Lima (2004) employed randomly amplified polymorphic DNA (RAPDs), and Singh *et al*. (2009) and Rocha *et al*. (2012) employed inter simple sequence repeats (ISSRs). Overall, these studies showed the efficiency of different markers for the study of intracultivar genetic diversity in mango.

However, DNA-fingerprinting based on simple sequence repeats (SSRs) or microsatellites, in addition, their usefulness in mapping and breeding (Mc Couch *et al*., 1997), has become the marker of choice, because of their widespread occurrence throughout the genomes of all eukaryotic species, their co-dominant inheritance and the high level of polymorphism observed due to variations in repeat lengths. SSR analysis shows great potential for mango improvement and can be performed for variety identification, validation of parentages, and estimation of genetic variation in existing populations and characterisation of intracultivar diversity.

The aim of this study was to analyse the intracultivar diversity among 'Chinnarasam' accessions of mango in Andhra Pradesh State using SSR markers, to contribute to the maintenance and multiplication of this important collection and the improvement of 'Chinnarasam' culture in the State.

MATERIALS AND METHODS

Eco-geographic survey. Exploration surveys were undertaken during May-June, 2009 in six districts, representing all the three eco-geographical regions (*Coastal Andhra, Rayalaseema and Telangana*) of Andhra Pradesh, India. Twenty phenotypically divergent 'Chinnarasam' trees were selected (accession IDs starting with CR; CR Acc-1 to CR Acc-20), across the entire growing area of Andhra Pradesh on more than 9 different locations (Table1).

Leaf sampling. Young healthy leaves of each accession of 'Chinnarasam' mango tree were selected for molecular characterisation. The leaves were collected and maintained in styrofoam boxes with ice to be transported from the collection sites to the Biotechnology Unit (presently Institute of Biotechnology), Faculty of Agriculture, Acharya N. G. Ranga Agricultural University, Hyderabad, where they were frozen in liquid nitrogen. Then, they were stored in a freezer at -80°C, until the time of the extraction of the genomic DNA.

DNA extraction. The genomic DNA from leaf samples was extracted by a modified cetryl trimethyl ammonium bromide (CTAB) method (Porebski *et al*., 1997).

Amplification reactions. In order to select the most polymorphic primers for SSR analysis, DNA samples from the accessions of 'Chinnarasam' were amplified using 109 mango-specific SSR primers (Table 1). Elevated levels of polymorphism were detected with 20 SSRs and all of these 20 primers were employed in the subsequent SSR reactions. PCR amplification was performed in a Perkin Elmer Thermocycler (PCR-Gene Amp PCR System 9700) as per the standard protocol (Williams *et al*., 1990). Amplified products were separated by electrophoresis in a 3% metaphor-agarose gel using Tris-acetate EDTA (TAE) buffer

TABLE 1. Collection sites of 'Chinnarasam' accessions of mango

Accession	Collection site				
	Latitude	Longitude	Village	District	Region
CR Acc-1	17°37' N	78°05' E	Sangareddy	Medak	Telangana
CR Acc-2	17°37' N	78°05' E	Sangareddy	Medak	Telangana
CR Acc-3	17°37' N	78°05' E	Sangareddy	Medak	Telangana
CR Acc-4	17°37' N	78°05' E	Sangareddy	Medak	Telangana
CR Acc-5	17°37' N	78°05' E	Sangareddy	Medak	Telangana
CR Acc-6	17°37' N	78°05' E	Sangareddy	Medak	Telangana
CR Acc-7	17°37' N	78°05' E	Sangareddy	Medak	Telangana
CR Acc-8	17°37' N	78°05' E	Sangareddy	Medak	Telangana
CR Acc-9	17°07' N	82°15' E	Pithapuram	East Godavari	Coastal Andhra
CR Acc-10	17°07' N	82°15' E	Pithapuram	East Godavari	Coastal Andhra
CR Acc-11	18°34' N	83°22' E	Bobbili	Vizainagaram	Coastal Andhra
CR Acc-12	18°34' N	83°22' E	Bobbili	Vizainagaram	Coastal Andhra
CR Acc-13	13°13' N	79°06' E	Chittor	Chittor	Rayalaseema
CR Acc-14	16°46' N	80°18' E	Raghavapuram	Krishna	Coastal Andhra
CR Acc-15	16°46' N	80°18' E	Rangapuram	Krishna	Coastal Andhra
CR Acc-16	16°47' N	80°42' E	Siddarthnagar	Krishna	Coastal Andhra
CR Acc-17	16°47' N	80°42' E	Siddarthnagar	Krishna	Coastal Andhra
CR Acc-18	16°42' N	80°38' E	Pondugala	Prakasam	Coastal Andhra
CR Acc-19	16°42' N	80°38' E	Pondugala	Prakasam	Coastal Andhra
CR Acc-20	15°15' N	79°57' E	Sanampudi	Prakasam	Coastal Andhra

CR Acc = Chinnarasam accession

at pH 8.0. The amplified fragments were observed and photographed under UV light in Gel Doc System (Syngene).

Data analysis. Of the 109 SSRs validated, 20 microsatellites were highly polymorphic and were chosen for their clear pattern and high allele numbers. The number of polymorphic bands generated by each SSR primer was determined by an initial visual examination of the gel photographs, taking into account the resolution and degree of amplification (Table 2). Only distinct and clear bands of medium to strong intensity were included in the subsequent investigation. For SSR analysis, the presence of the band was scored as 1, whereas the absence of the band was scored as 0. Microsatellite data were transformed into binary scores in order to calculate similarity values between all pairs of samples according to the methods of Jaccard (1908). Based on these matrices, the genetic similarities between 'Chinnarasam' mango accessions were determined using Jaccard coefficients, and a dendrogram was constructed by applying unweighted pair group method with arithmetic average (UPGMA) feature of the NTSYS PC Software (Version 2.1) statistical analysis package (Rohlf, 2000). The polymorphic information content (PIC) of microsatellite markers was calculated according to the formula given by Powell *et al*. (1996).

RESULTS AND DISCUSSION

Polymorphism detected using SSR markers. Of the 109 mango-specific SSRs initially validated, only 20 primers were polymorphic (18.34%), which were selected for further analysis of twenty 'Chinnarasam' accessions (Table 2). Of the 62 scorable amplification fragments detected with 20 SSRs, only 31 amplification fragments were polymorphic, yielding a polymorphism rate of 50% indicating that a fairly high level of genetic variation exists among the accessions.

The large number of polymorphic bands detected clearly indicates that SSRs are highly polymorphic. PCR product size (bp) ranged from 100 (SSR-52) to 310 (SSR-20) in 20 accessions indicating that there was a wide variation in the PCR product size produced by the primers (Table 2). The level of polymorphism present in the microsatellites was variable, ranging from 2 to 4 alleles with an average of 3.10 alleles per SSR indicating that there was a wide variation in the range of polymorphic bands produced by the primers. Similar levels of polymorphism associated with SSR markers have been reported in earlier studies involving mango (Singh and Bhat, 2009; Begum *et al*., 2012).

The PIC value provides an estimate of the discriminatory power of a marker by taking into account not only the number of alleles at a locus but also the relative frequencies of these alleles. The polymorphic information content (Table 2) ranged from 0.05 (SSR-16) to 0.84 (SSR-39). Average PIC value of 0.46 for all loci indicated the moderate discriminatory power of the 20 polymorphic SSRs. Markers with high PIC values such as SSR-39, 84, 52 and 65 could be effectively used in intracultivar genetic diversity studies of 'Chinnarasam' mango. A representative of the PCR amplification product of 20 accessions of 'Chinnarasam' mango with SSR-91 is depicted in Figure 1, which yielded sufficient polymorphism to distinguish different accessions of 'Chinnarasam' mango under study. Single nucleotide changes, deletions, insertions and variable numbers of simple sequence repeats are several different mechanisms responsible for genomic polymorphism between accessions.

Dissimilarity values and cluster analysis. The largest genetic distance calculated by the Jaccard's similarity coefficient was 0.46 as evident from UPGMA cluster analysis (Fig. 2). Genetic relationships between different pairs of 20 accessions varied from 0.54 to 1.00. Genetic similarity between accessions was in the range of 54-100%. The pair-wise genetic dissimilarities ranged from 0.00 to 0.46 with a mean value of 0.23; thus showing a moderate degree of intercultivar genetic diversity at the DNA level. Bally *et al*. (1996) found a genetic dissimilarity of 0.05% among 27 accessions of 'Kensington Pride', a polyembryonic cultivar of mango using RAPD markers. Similarly, a similarity coefficient of 0.4 to 1.0 among 18 samples of the 'Rosa' variety of mango from Brazil analysed with 6 RAPD primers (A01, A09, G03, G10, N05, and M16) indicating high magnitude of intracultivar

TABLE 2. Characteristics of polymorphic microsatellite markers used in molecular analysis of intravarietal variability in 'Chinnarasam' mango cultivar

Primer	Sequence (5'-3')	Tm (°C)	Allele size range (bp)	Number of alleles†	PIC values†
SSR-8F	TTGATGCAACTTTCTGCC	53	200-224	3	0.45
SSR-8R	ATGTGATTGTTAGAATGAACTT				
SSR-15F	TTTACCAAGCTAGGGTCA	52	201-226	3	0.67
SSR-15R	CACTCTTAAACTATTCAACCA				
SSR-16F	GCTTTATCCACATCAATATCC	54	160-170	2	0.05
SSR-16R	TCCTACAATAACTTGCC				
SSR-20F	CGCTCTGTGAGAATCAAATGGT	58	295-310	4	0.32
SSR-20R	GGACTCTTATTAGCCAATGGGATG				
SSR-36F	CCTCAATCTCACTCAACA	55	215-245	2	0.40
SSR-36R	ACCCCACAATCAAACTAC				
SSR-39F	TGTCTACCATCAAGTTCG	53	150-190	3	0.84
SSR-39R	GCTGTTGTTGCTTTACTG				
SSR-46F	TCATTGCTGTCCCTTTTC	54	154–210	4	0.25
SSR-46R	ATCGCTCAAACAATCC				
SSR-52F	AAAAACCTTACATAAGTGAATC	52	207	3	0.72
SSR-52R	CAGTTAACCTGTTACCTTTTT				
SSR-59F	TTCTTTAGACTAAGAGCACATT	56	191	3	0.64
SSR-59R	AGTTACAGATCTTCTCCAATT				
SSR-61F	AAAGATAGCATTTAATTAAGGA	52	206	2	0.10
SSR-61R	GTAAGTATCGCTGTTTGTTATT				
SSR-65F	ATAGATTCATATCTTCTTGCAT	53	233	3	0.69
SSR-65R	TATAAATTATCATCTTCACTGC				
SSR-82F	TCTGACCCAACAAAGAACCA	57	108-155	3	0.25
SSR-82R	TCCTCCTCGTCCTCATCATC				
SSR-83F	AGCTATCGCCACAGCAAATC	57	190-213	3	0.67
SSR-83R	GTCTTCTTCTGGCTGCCAAC				
SSR-84F	TCTATAAGTGCCCCCTCACG	58	210-250	4	0.79
SSR-84R	ACTGCCACCGTGGAAAGTAG				
SSR-85F	GCTTGCTTCCAACTGAGACC	58	229-269	4	0.46
SSR-85R	GCAAAATGCTCGGAGAAGAC				
SSR-88F	CTGAGTTTGGCAAGGGAGAG	55	222-244	3	0.37
SSR-88R	TTGATCCTTCACCACCATCA				
MngSSR-14F	TCATTAAGCTGTGGCAACCA	59	160-192	3	0.51
MngSSR-14R	CATTGCATAGATGTGGTCATT				
MngSSR-24F	CGATGGACTTCATAAGAAGAG	58	150	3	0.18
MngSSR-24R	GCTAGCAGAATCACCTTGGTC				
MngSSR-26F	ACCTTGGTCAGGACAAAATCC	60	135-150	4	0.25
MngSSR-26R	GACTTCATAAGAAGAGGCGTC				
MngSSR-27F	CGAAACCGACTGCCTATTTT	57	158-172	3	0.62
MngSSR-27R	CCATTAATAAAGTTGTGGCCA				

Tm = Annealing temperature; PIC = Polymorphic information content

†Based on the complete set of 20 accessions of 'Chinnarasam' mango as described in Materials and Methods

Source: eurofinsmwg/operon (www.Eurofinsdna.com)

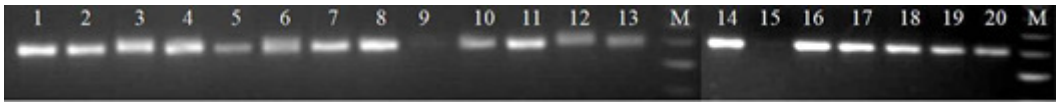

Figure 1. Amplification pattern of 20'Chinnarasam' accessions generated by SSR-91 primer.

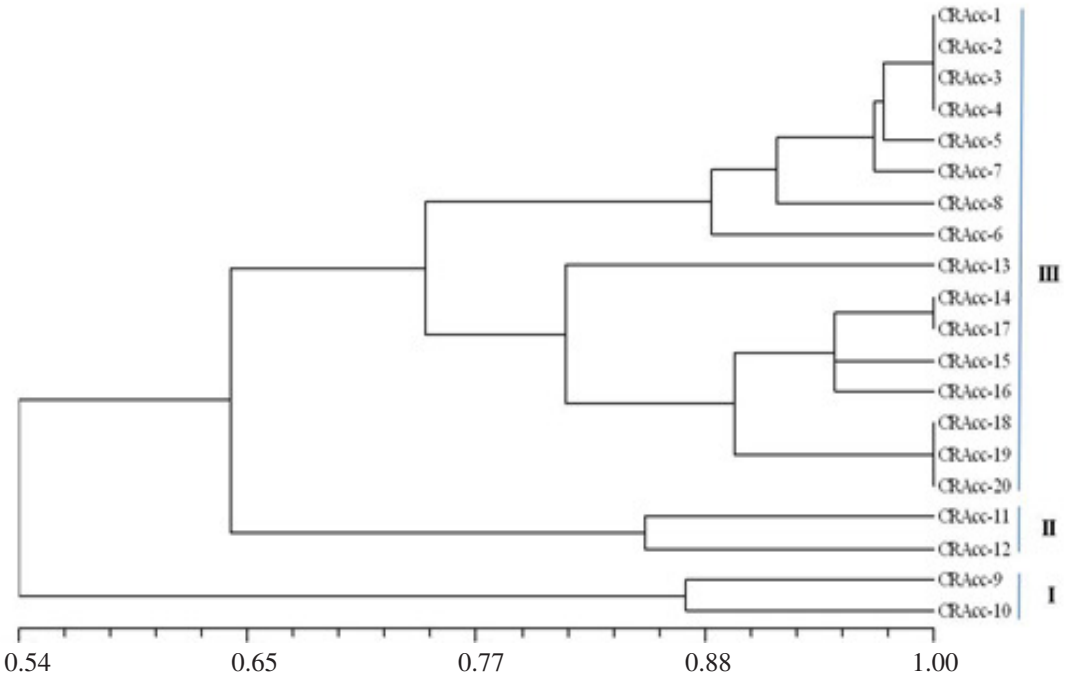

Figure 2. UPGMA dendrogram of 20 'Chinnarasam' accessions based on SSR markers.

variability in 'Rosa' mango (Souza *et al*., 2011). The level of intracultivar genetic diversity is dependent on the total sample size and type and number of molecular markers used. In the present study, the genetic dissimilarity of 0.46 among accessions although moderate, the genetic differences among the sampled materials may affect some phenotypic character that is useful for the culture. The traditional practices are likely to be responsible for the intravarietal polymorphism in 'Chinnarasam'. In the State, in general, the mango farmers procure the planting material from various public and private nurseries. The surveyed University nurseries could not meet the high demand of farmers for true-to-type grafts as evident from the fact that genetic diversity was observed among 'Chinnarasam' accessions obtained from research and repository plantings, which might be assumed to have a high level of cultivar fidelity. The separation of 4 duplicates (CR Acc-1, 2, 3, 4) from the remaining 4 accessions (CR Acc-4, 6, 7, 8) collected from research and repository plantings of the Fruit Research Station, Sangareddy, with a maximum dissimilarity coefficient of 0.886 denotes heterogeneity among the 8 accessions, which might have arisen due to the genetically variable scions used in the formation of grafted trees and their cultivation in the same location for several years. On the whole, the intracultivar diversity in 'Chinnarasam' mango could be due to poor dissemination of uniform quality planting material to mango orchardists in the State.

UPGMA tree constructed on SSR data on the basis of Jaccard's similarity coefficient (Fig. 2) clustered 20 accessions into three major groups (cluster-I, II and III) denoting distinct diversity and relationships among them. Of these three clusters, cluster-III was the largest (16 accessions), followed by cluster-II and I (2

accessions each). The first group (cluster-I) included all of the two accessions (CR Acc-9, 10) collected from Pithapuram. The second group (cluster-II) included all of the two accessions (CR Acc-11, 12) collected from Bobbili.

The third group (cluster-III) included the remaining 16 accessions (CR Acc-1 to CR Acc-8 and CR Acc-13 to CR Acc-20), of which 8 accessions from Sangareddy (CR Acc-1 to CR Acc-8), 2 accessions each from Siddarthnagar (CR Acc-16,17) and Pondugala (CR Acc-18,19) and 1 accession each from Chittor (CR Acc-13), Raghavapuram (CR Acc-14), Rangapuram (CR Acc-15), and Sanampudi (CR Acc-20). From the perusal of the geographical locality of each accession (Table 1) and their clustering pattern (Fig. 2), it could be inferred that the grouping of the accessions is not associated with their geographical location.

This study helped in the investigation of the extent of diversity among accessions in relation to their location and in the identification of a set of accessions with narrow genetic base developed from particular location. Four out of eight accessions (CR Acc-1, 2, 3, and 4) from a single collection site of Sangareddy with a similarity coefficient of 1.00 had 100% genetic similarity with each other. Similarly, the accessions from Raghavapuram (CR Acc-14) and Siddarthnagar (CR Acc-17) of the same Krishna district with a similarity coefficient of 1.00 had 100% genetic similarity with each other. Two accessions from Pondugala (CR Acc-18, 19) and one accession from Sanampudi (CR Acc-20) of the same Prakasam district with a similarity coefficient of 1.00 had 100% genetic similarity with each other.

The largest third group (cluster-III) could be further divided into several well defined sub-clusters showing a close association among local basis distinct or same accessions. The accessions CR Acc-1, 2, 3, 4 of one sub-cluster, CR Acc-14, 17 of other sub-cluster, and CR Acc-18, 19, 20 of another cluster showed high tendency to be close among themselves (100%) with the Jaccard's similarity coefficient of 1.0 as evident from SSR based dendrogram. As expected, close relationship was observed among certain accessions within the cultivar. Therefore, accessions with 100% of similarity indicate the presence of duplicates.

The high level of genetic similarity (100%) among certain accessions could be due to the fact that they were produced by vegetative propagation from a single mother plant. Most of the accessions from a single collection site were grouped together; however, some exceptions were also observed. The accessions representing one region grouped with the accessions of other region following cluster analysis indicating that the intracultivar heterogeneity in 'Chinnarasam' was so extreme. In mangoes, level of genetic diversity has been proportionate to the phenomena of allopolyploidy, out crossing and the different agro-climatic conditions in the mango growing areas.

Accession-specific markers. The genetic dissimilarity among accessions of 'Chinnarasam' cultivar precluded identifying SSR alleles unique to this cultivar. From the SSR banding pattern, it is evident that some fragments were uniquely amplified or absent in some of the accessions of 'Chinnarasam' (Table 3). SSR-8 generated a unique allele of 235 bp for CR Acc-6. MngSSR-24 generated a unique allele of 140 bp for CR Acc-6. SSR-16 generated a unique allele of 150 bp for CR Acc-9. MngSSR-27 generated a unique allele of 195 bp for CR Acc-9. MngSSR-14 generated a unique allele of 175 bp for CR Acc-10. These

TABLE 3. List of SSR primers producing unique alleles for specific accessions of 'Chinnarasam'

Accession	SSR producing specific band(s)	Size of the specific bands (bp)
CR Acc-6	SSR-8	235
CR Acc-6	MngSSR-24	140
CR Acc-9	SSR-16	150
CR Acc-9	MngSSR-27	195
CR Acc-10	MngSSR-14	175

fragments are of great interest in optical management and genetic identification of 'Chinnarasam' accessions in the germplasm collection. From these results, we conclude that the application of the SSR technique allows to discriminate all accessions of 'Chinnarasam' cultivar and to distinguish the accessions of each cultivar sampled from different geographic cultivation areas. Thus, using SSR profiling, most of the accessions of 'Chinnarasam' can be easily distinguished and identified.

CONCLUSION

On the whole, molecular analysis demonstrate that the intracultivar genetic diversity is present among the 20 accessions of 'Chinnarasam', but larger patterns of diversity based upon region are generally poor or lacking. The intracultivar variations indicate that the population within 'Chinnarasam' cultivar is genetically heterogeneous, a feature that might be exploited by breeders interested in within cultivar improvement of 'Chinnarasam' mango. Using SSR profiling, most of the accessions of 'Chinnarasam' can be easily distinguished and identified. The selection of mother plants must be performed considering the genetic similarity based on the information about SSR markers.

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