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SSR markers reveal genetic diversity in closely related mango hybrids

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ABSTRACT

Forty eight mango hybrids were analyzed using 17 simple sequence repeat (SSR) markers. Which detected 59 scorable loci, of which 45 were polymorphic. The size of the alleles detected ranged from 100 to 480 bp. SSR markers was highly polymorphic with an average of 3.47 alleles per primer. SSRs gave moderate values of Polymorphis Information Content (PIC) and heterozygosity. The genetic relationship among mango hybrids, based on Jaccard's Similarity Coefficient values ranged from 0.38 (between H-1-13 and H-6-8) to 0.97 (between H-13-4 and H-13-7). The dendrogram, based on UPGMA cluster analysis, grouped the mango hybrids into three major groups. Cluster 'A' comprised of the five most diverse hybrids, namely, H-12-2, H-9-6, Pusa Arunima, H-9-5 and H-1-13. Two unique fingerprints were identified in hybrid H-9-6. The size of unique fingerprints ranged from 90 (MiSHRS-18) to 350 bp (MiSHRS-39). The Principal Coordinate Analysis also exhibited more or less similar distribution of mango hybrids. The tendency of clustering among mango hybrids revealed that they had stronger affinity towards female parent Amrapali.

Key words: Mango hybrids, micro-satellite markers, genetic relatedness, fingerprinting.

INTRODUCTION

Mango (*Mangifera indica* L.) is widely cultivated in India and reportedly, there are over 1,000 varieties found in the country (Singh, 17). However, there is a lot of confusion in nomenclature of the mango cultivars, which is attributed to the lack of systematic approach in nomenclature. Characterization of the available germplasm is a prerequisite for their conservation as well as utilization in the future breeding programmes. Genetic characterization serves the twin purposes of the identification of genotypes and estimation of their genetic relatedness (Ravishankar *et al*., 13). Traditionally, the genetic variation in mango was estimated using morphological markers and isozymes. These techniques, however, do not provide an accurate estimation of variation and could lead to misidentification or duplication of genotypes. The incorrect labeling of accessions and ambiguous identification of individuals is a limitation that impedes progress in mango improvement programmes. Precise characterization of genetic variation at the molecular level is possible using DNA-based markers.

Different molecular markers such as randomly amplified polymorphic DNA (RAPDs; Bajpai *et al*., 1), amplified fragment length polymorphism (AFLPs; Eiadthong *et al*., 3), inter-SSRs (Pandit *et al*., 11) and simple sequence repeats (SSRs; Duval *et al*., 2; Schnell *et al*., 16; Viruel *et al*., 20) have been employed for genetic diversity assessment in mango. SSRs have gained considerable importance in genetic studies owing to their desirable attributes such as hyper-variability, multiallelic nature, codominant inheritance and reproducibility. Assessment of the genetic structure of closely related populations is also possible with SSRs. Based on informative and robustness, the use of SSRs has been preferred to determine the genetic relationships among mango genotypes. Keeping in view these advantages, we assayed the closely related newly developed mango hybrids with microsatellite markers.

MATERIALS AND METHODS

Forty eight hybrids of mango derived from different cross combinations, were included in the present experiment (Table 1). Of these hybrids, 47 have been developed by the Division of Fruits and Horticultural Technology, IARI, New Delhi. Some of these hybrids have been release for commercial cultivation. A few others are considered potential for future release. Additionally, mango hybrid Ratna, which represents western Indian mango germplasm pool, was also included in the present study.

Fresh young leaves (5.0 g) were collected from each plant for DNA extraction. Genomic DNA was extracted by the cetylhexadecyl-trimethyl ammonium bromide (CTAB) method (Saghai-Maroof *et al*., 15) with minor modifications. Leaf samples were ground to fine powder in liquid nitrogen. Polyvinyl pyrrolidone (100 mg) was added and the powdered leaf material was

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Table 1. List of mango hybrids used in the study.

Sl. No.	Hybrid	Parentage
1.	Pusa Pratibha	Amrapali × Sensation
2.	H-1-3	Amrapali × Sensation
3.	H-1-5	Amrapali × Sensation
4.	Pusa Shreshth	Amrapali × Sensation
5.	H-1-8	Amrapali × Sensation
6.	H-1-9	Amrapali × Sensation
7.	H-1-10	Amrapali × Sensation
8.	H-1-11	Amrapali × Sensation
9.	H-1-12	Amrapali × Sensation
10.	H-1-13	Amrapali × Sensation
11.	H-2-3	Amrapali × Lal Sundari
12.	H-2-4	Amrapali × Lal Sundari
13.	H-2-5	Amrapali × Lal Sundari
14.	Pusa Peetambar	Amrapali × Lal Sundari
15.	H-2-7	Amrapali × Lal Sundari
16.	H-2-10	Amrapali × Lal Sundari
17.	H-2-11	Amrapali × Lal Sundari
18.	H-2-14	Amrapali × Lal Sundari
19.	H-2-2	Amrapali × Lal Sundari
20.	H-3-7	Amrapali × Sensation
21.	H-6-1	H-8-11 × IIHR-95
22.	H-6-2	Amrapali × Sensation
23.	H-6-6	Amrapali × Alphonso
24.	H-6-7	Amrapali × Alphonso
25.	H-6-8	Amrapali × Alphonso
26.	H-6-13	Amrapali × Sensation
27.	H-7-4	Amrapali × Sensation
28.	H-8-11	Amrapali × Sensation
29.	H-11-1	Amrapali × Sensation
30.	H-11-2	Amrapali × Sensation
31.	H-11-3	Amrapali × Sensation
32.	H-9-1	Amrapali × Sensation
33.	Ratna	Neelum × Alphonso
34.	H-9-3	Amrapali × Sensation
35.	H-9-4	Amrapali × Sensation
36.	H-9-5	Amrapali × Sensation
37.	H-9-6	Amrapali × Sensation
38.	H-9-8	Amrapali × Sensation
39.	H-9-9	Amrapali × Pusa Arunima
40.	H-12-2	Amrapali × Sensation
41.	Pusa Arunima	Amrapali × Sensation
42.	H-13-2	Amrapali × Sensation
43.	H-13-3	Neelum × Dashehari
44.	H-13-4	Amrapali × Sensation
45.	H-13-7	Amrapali × Sensation
46.	H-13-8	Amrapali × Sensation
47.	H-15-2	Amrapali × Sensation
48.	H-15-4	Amrapali × Sensation

Contd...

quickly transferred to centrifuge tubes containing 20 ml pre-heated (60°C) extraction buffer [2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1% (v/v) ß-mercaptoethanol, 100 mM Tris-HCl [pH 8.0] and incubated at 60°C for 1 h, with intermittent shaking. The homogenate was cooled to room temperature and extracted with 10 ml 24:1 (v/v) chloroform-isoamyl alcohol, followed by centrifugation at 4,000 × g for 20 min. and separation of clear aqueous phase. To this were added 2.5 ml 5 M NaCl and 10 ml iso-propanol, and the mixture was stored overnight at -20°C to precipitate the DNA. This was centrifuged at 5,000 x g for 10 min. and the supernatant decanted, retaining the pellet. The pellet was washed twice with 70% (v/v) ethanol, and then air-dried. The dried pellet was re-suspended in 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Contaminating RNA was removed by digestion with 10 µg RNaseA (Banglore Genei Pvt. Ltd., Bangalore, India) for 30 min. at 37°C. The DNA was purified further by extracting it twice with an equal volume of phenol, followed by an equal volume of 1:1 (v/v) phenol-chloroform and finally with an equal volume of chloroform. The DNA was precipitated by the addition of two volumes of chilled absolute ethanol and centrifugation at 10,000 × g for 5 min. The final pellet was dissolved in 0.5 ml TE buffer. DNA concentrations were determined using a spectrophotometer, and the quality checked by electrophoresis in 0.8% (w/v) agarose gel.

The PCR reactions were performed on Perkin Elmer 9,600 thermocycler (USA). Each PCR reaction consisted of 1.5 µl of 10x reaction buffer, 0.20 µl of 10 mM dNTPs (133 µM), 1.5 µl each of forward and reverse primers (5 pmol), and 2.5 µl of genomic DNA (10 ng/µl), 0.15 µl of *Taq* DNA polymerase (0.75 u; Vivantis Technologies) in a final reaction volume of 15

µl. The PCR reaction profile was: DNA denaturation at 95°C for 5 min. followed by 35 cycles of 94°C for 1 min., primer annealing at 55°C for 1 min., 72°C for 1 min. and finally, 72°C for a final extension of 10 min.

Each band was treated as one SSR marker. Scoring of bands was done from the photographs. Homology of bands was based on their migration distance in the gel. The presence of a band was scored as '1', absence of a band as '0' and missing datum was denoted by '9'. The genetic divergence among mango hybrids was evaluated by calculating the Jaccard's similarity coefficient. The pair-wise similarity matrix between cultivars was determined by the index of Nei and Lie (10). The similarity matrix was subjected to the cluster analysis of unweighted pair group method for arithmetic mean (UPGMA) and a dendrogram was generated. These computations were performed using the program NTSYS-PC Ver. 2.11 (Rohlf, 14). The probability of identity of SSR markers was calculated by the formula suggested by Paetkau *et al*. (12).

RESULTS AND DISCUSSION

Initially 25 SSR primers were used for generating banding profile, out of which 17 primers gave consistent and discrete bands. Two typical SSR profiles are shown in Fig. 1. The details with respect to band statistics are furnished in Table 2. The 17

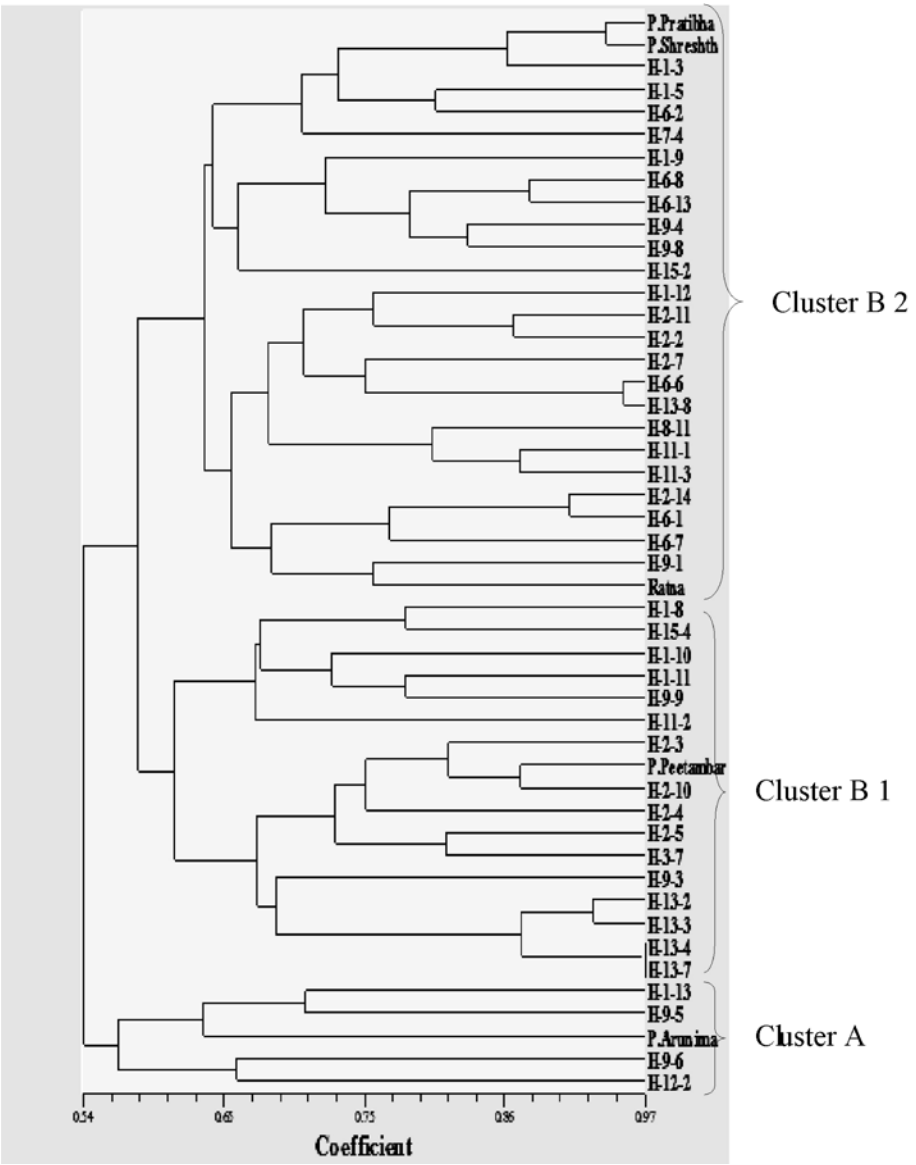

Fig. 1. Dendrogram based on UPGMA analysis of 48 mango hybrids using SSR markers (P = Pusa).

Table 2. Summary statistics of the 17 micro-satellite markers across 48 mango hybrids.

Locus	Gene Bank Acc. No.	Primer sequence (forward/reverse)	Repeat motif	N_A	Size range (bp)	H	PIC	No. of fingerprints	No. of unique fingerprint (s)	Probability of identity
MiSHRS-1	AY942817	F: TAACAGCTTTGCTTGCCTCC R: TCCGCCGATAAACATCAGAC	$(CT/AG)_{14}$	3	180-220	0.044	0.073	3	1	0.2314
MiSHRS-18	AY942819	F: AAACGAGGAAACAGAGCAC R: CAAGTACCTGCTGCAACTAG	$(AAC/GTT)_8$	3	100-120	0.256	0.144	3	1	0.5931
MiSHRS-23	AY942820	F: AGGTCTTTTATCTTCGGCCC R: AAACGAAAAAGCAGCCCA	$(TATG/CATA)_7$	6	140-250	0.413	0.295	12	0	0.7385
MiSHRS-29	AY942822	F: CAACTTGGCAACATAGAC R: ATACAGGAATCCAGCTTC	$(TG/CA)_9$	2	180-200	0.042	0.041	2	0	0.1215
MiSHRS-30	AY942823	F: GAATAAAGGGGACACCAGAC R: CCATCATCGCCCACTCAG	$(GTTGTGT/ACACAAC)_3$	3	220-300	0.238	0.171	3	1	0.5168
MiSHRS-32	AY942824	F: TTGATGCAACTTTCTGCC R: TGTGATTGTTAGAATGAACTT	$(CA/TG)_9$	2	210-230	0.340	0.251	2	0	0.5505
MiSHRS-33	AY942825	F:CGAGGAAGAGGAAGATTATGAC R: CGAATACCATCCAGCAAAATAC	$(CGG/CCT)_7$	2	250-270	0.321	0.248	2	0	0.5816
MiSHRS-36	AY942827	F: GTTTTCATTCTCAAAATGTGTG R: CTTTCATGTTCATAGATGCAA	$(CT/AG)_{15}$	3	200-250	0.067	0.042	3	0	0.2332
MiSHRS-37	AY942828	F: CTCGCATTTCTCGCAGTC R: TCCCTCCATTTAACCCTCC	$(AG/CT)_9$	3	140-250	0.489	0.249	4	0	0.7668
MiSHRS-39	AY942829	F: GAACGAGAAATCGGGAAC R: GCAGCCATTGAATACAGAG	$(GTT/AAC)_8$	4	380-480	0.117	0.023	5	1	0.7952
MiSHRS-48	AY942831	F: TTTACCAAGCTAGGGTCA R: CACTCTTAAACTATTCAACCA	$(GA/TC)_{15}$	3	200-400	0.422	0.283	4	0	0.7456
LMMA1	AY628373	F: ATGGAGACTAGAATGTACAGAG R: ATTAAATCTCGTCCACAAGT	$(GA)_{13}$	6	120-250	0.326	0.208	12	0	0.8848
LMMA2	AY628374	F: AAATAAGATGAAGCAACTAAAG R: TTAGTGATTTTGTATGTTCTTG	(GA)11	4	300-400	0.337	0.247	5	0	0.8067
LMMA3	AY628375	F: AAAAACCTTACATAAGTGAATC R: CAGTTAACCTGTTACCTTTTT	(GA)16	3	120-250	0.108	0.073	4	0	0.8658
LMMA4	AY628376	F: AAAAACCTTACATAAGTGAATC R: CAGTTAACCTGTTACCTTTTT	(GA)13	5	230-350	0.204	0.219	5	0	0.7589
LMMA8	AY628380	F: CATGGAGTTGTGATACCTAC R: CAGAGTTAGCCATATAGAGTG	(GA)12	3	270-300	0.359	0.242	3	0	0.7491
LMMA10	AY628382	F: TTCTTTAGACTAAGAGCACATT R: AGTTACAGATCTTCTCCAATT	(GA)10	4	160-225	0.264	0.201	4	1	0.7429

*N_A = Number of alleles, bp = Base pairs, H = Heterozygosity, PIC = Polymorphic Information Content

SSR primers generated 59 scorable bands in mango hybrids, of which 45 were polymorphic (84.9%). The number of alleles detected varied from 2 (MiSHRS 29, MiSHRS 32 and MiSHRS 33) to 6 (MiSHRS 23 and LMMA 1). The average number of alleles per primer pair was 3.47. The allele size ranged from 100 bp (MiSHRS 18) to 480 bp (MiSHRS 39). Earlier, Shareefa (19), and Nayak (9) reported similar values of SSR polymorphism (71 to 81.8%), number of alleles and allele size in mango. In present experiment, most of the SSR primers detected multiple loci, which can be attributed to the allopolyploid nature of mango (Mukherjee, 7).

The characteristics of PCR products, namely, the Polymorphism Information Content (PIC) and heterozygosity (H) are presented in Table 2. In present experiment, SSR markers gave low PIC values ranging from 0.023 (MiSHRS 39) and 0.295 (MiSHRS 23). The average PIC value for MiSHRS primer series was 0.165, whereas it was 0.198 in LMMA primer series. The heterozygosity of SSR markers used in this study ranged from 0.042 (MiSHRS-29) to 0.489 (MiSHRS-37). The average heterozygosity value for MiSHRS primer series was 0.249, whereas it was 0.266 in LMMA primer series. Our results with respect to PIC and heterozygosity values are consistent with the findings of Shareefa (19), and Nayak (9). PIC values of these markers were also low to moderate in Florida mango cultivars (Schnell *et al*., 16).

Genetic relationships among mango hybrids were determined based on the Jaccard's pairwise similarity coefficients. We obtained moderate degree of genetic diversity, with Jaccard's similarity coefficient values ranging from 0.38 (between H-1-13 and H-6-8) to 0.97 (between H-13-4 and H-13-7), with the mean value of 0.59. The dendrogram generated from the Unweighted Pair Group Arithmetic Average (UPGMA) cluster analysis broadly placed 48 mango hybrids into two major clusters (Fig. 2). Cluster 'A' comprised of the five most diverse hybrids, namely, H-12-2, H-9-6, Pusa Arunima, H-9-5 and H-1-13. Cluster 'B' was again bifurcated into two sub-clusters, namely, Cluster 'B' 1 and Cluster 'B' 2. Cluster 'B' 1 consisted of 17 hybrids and Cluster 'B' 2 consisted of 26 hybrids. The dendrogram revealed that H-13-4 and H-13-7 were the most similar hybrids with 97% similarity. Contrary to this, hybrids H-1-13 and H-6-8 were the most divergent with a diversity value of 62%. Three dimensional scatter diagram of PCA was generated for supplementing the findings reported in the cluster analysis. The principal co-ordinate analysis of 48 mango hybrids also generated more or less similar distribution of the hybrids.

The Jaccard's similarity values (38 to 97%) clearly depicted rich genetic variability in the hybrid population studied. Our findings are supported by the earlier studies on genetic diversity analysis in mango using different marker systems (RAPD, Bajpai *et al*., 1; IISRs, Singh, 18). The rich genetic variation found, in hybrid progeny could be attributed to the cross pollinated nature of mango crop, high degree of heterozygosity and high discriminatory power of the

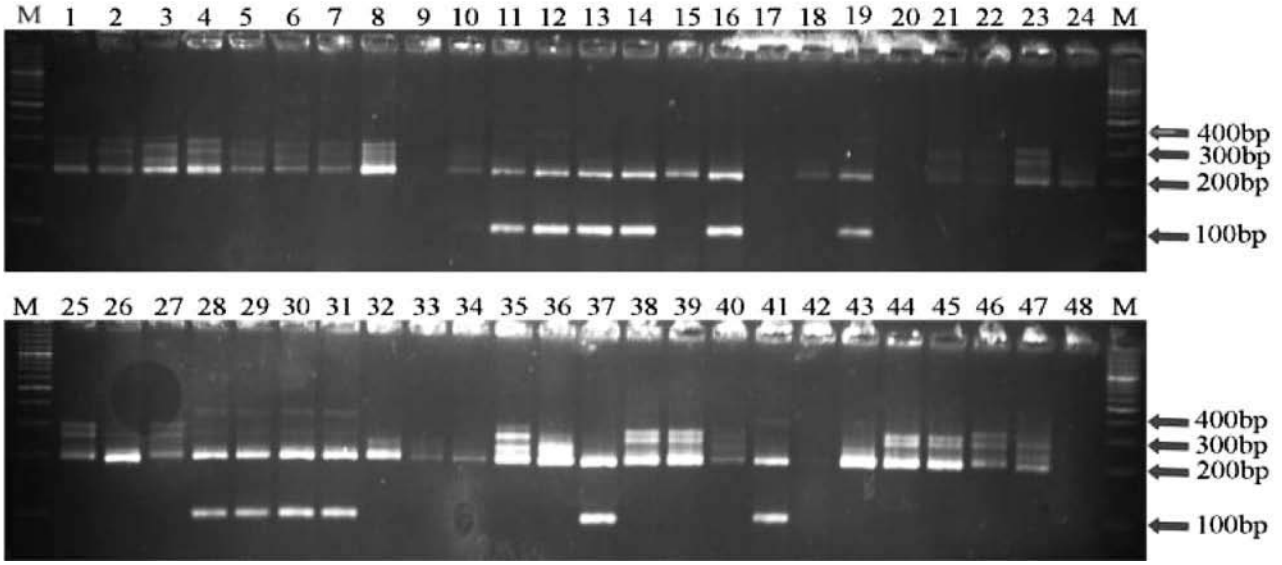

Fig. 2. Typical agarose gel electrophoresis profiles of DNA from 48 mango hybrids using SSR primers MiSHRS-23. Lanes marked 1 to 48 are the DNAs of each of the 48, respectively (hybrid names are given in Table 1). M indicates GeneRuler™ 100 bp DNA ladder.

SSR markers. Again, the diverse genetic backgrounds of parents seem to have contributed to rich genetic variation observed in hybrid population. Amrapali, the female parent of most of the hybrids, has been derived from the cross of 'Dashehari' and 'Neelum', representing the north and south Indian mango germplasm pool, respectively. The male parents of hybrids, namely, Sensation, Lal Sundari, Alphonso, Dashehari and others also represent different genetic and geographical backgrounds. Thus, this diverse parents could have resulted in high genetic variability in F_1 population.

As with other vegetatively propagated clonal crops, the differences among mango hybrids can result from epigenic modifications in response to the environment (Kaeppler *et al*., 5). Somatic and bud mutations also play a minor role in clonal polymorphism in woody plants. Naik (8) observed significant variation among the trees of same clones in mango with respect to fruit characteristics and tree performance. It could be expected that most of the somatic mutations that occur during plant growth would have no effect on phenotype, although they could be identified at the molecular level.

The grouping of the hybrids in dendrogram was more or less based on their parentage. The hybrids related to each other by descent were placed together. Owing to parental similarity, hybrids H-12-2, H-9-6, Pusa Arunima, H-9-5 and H-1-13 constituted a separate cluster. They are progenies of Amrapali × Sensation cross. A similar trend was noted with respect to clustering of H-13-4 and H-13-7 (offsprings of Amrapali × Sensation), Pusa Pratibha and Pusa Shreshth (progenies of Amrapali × Sensation) and Pusa Peetambar and H-2-10 (progenies of Amrapali × Lal Sundari). The tendency of clustering among mango hybrids revealed that they had stronger affinity towards female parent Amrapali. Shareefa (19) also reported clustering of H-1-1 (released as Pusa Pratibha) and H-1-6 (released as Pusa Shreshth) together.

The genotypes analyzed could be distinguished unambiguously using the combined molecular profiles from 17 primer pairs. Number of fingerprints generated, number of unique fingerprints and probability of identity of SSR markers are furnished in the Table 2. A total of 76 fingerprints were identified and those generated by individual SSR primers ranged from 2 to 12. The highest numbers of fingerprints (12) were generated by primer pairs MiSHRS-23 and LMMA-1 each. However, these two primers failed to identify any unique fingerprints. Five SSR loci each detected one unique fingerprint in five mango hybrids.

Interestingly, the hybrids in which unique fingerprints were detected were the progenies of Amrapali × Sensation combination. Two unique fingerprints were identified in hybrid H-9-6. The size of unique fingerprints ranged from 90 (MiSHRS-18) to 350 bp (MiSHRS-39). The probability of identity, which measures the probability of any two randomly drawn genotypes are expected to have identical allele frequencies and random assortment as calculated for each primer pairs. The probability of chance identity varied from 0.1215 for MiSHRS-29 to 0.8848 for LMMA-1. The average probability of identity was higher in LMMA series as compared to MiSHRS series. In general, most of the SSR primers generated moderate to high degree of probability of identity indicating that the DNA fingerprints of these genotypes are highly genotype specific.

DNA fingerprinting can be employed for individual identification of cultivars or rootstocks for different horticultural purposes, such as breeders' rights, identification of pollen parent(s) and determination of genetic relatedness (Lavi *et al*., 6). The potential of SSR markers in fingerprinting is well established in mango (Viruel *et al*., 20; Shareefa, 19). Unique fingerprints are genotype and marker specific alleles. Such alleles are unique in the sense that they are produced by a particular marker and are present only in one genotype and absent in all other analyzed accessions. The presence of unique fingerprints may be explained by the high mutation rates at SSR loci (Henderson and Petes, 4). Presence of unique allele indicates the diverse genetic base of a genotype. In the present study, we identified unique fingerprints in hybrid progenies of Amrapali and Sensation. These hybrids represent wide genetic base as both of the parents are of different geographical origin. Amrapali (Dashehari × Neelum) carries the genes from north and south Indian mango germplasm. Similarly, Sensation represents the mango gene pool from Florida. It is assumed that sometimes unique alleles may serve as indicators of a particular region of the genome specific to a particular trait of horticultural importance. The genotypes carrying the unique alleles may prove useful for introducing diversity in the future mango breeding programmes.

The application of DNA fingerprinting technology has the potential of significantly improve mango breeding projects in terms of cost, time and efficiency by enabling eventual use of marker-assisted selection (MAS) and reduction in the number of backcross generations needed for gene introgression. Our results indicated that SSR markers are useful not only for varietal identification and detection of duplicate entries, but also for the use in future mango breeding programmes to design crosses that maximize genetic variability with the objective of developing superior hybrids suited to emerging consumer demands.

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