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Ascertaining hybridity of progenies in mango (*Mangifera indica* L.) using microsatellite (SSR) markers

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Abstract

In the present study (2012-13& 2013-14) seven mango genotypes *viz*., Amrapali, Dushehari, Janardan Pasand, Neelam, Pusa Arunima, Sensation and Tommy Atkins were used for obtaining hybrid plantlets. Artificial hybridization was attempted using Amrapali as female and Dushehari, Janardan Pasand, Neelam, Pusa Arunima, Sensation and Tommy Atkins as male donor parent and the recovery of the hybrids was very low. Fourty-two microsatellite markers (SSRs) among seven mango parental genotypes were validated against mango plantlets obtained from the crosses Amrapali x Dashehari, Amrapali x Janardan Pasand, Amrapali x Neelam, Amrapali x Pusa Arunima, Amrapali x Sensation and Amrapali x Tommy Atkins. Fourty- two primer pairs were screened, 13 primer pairs (LMMA 2, 3, 4, 7, 8, 9, 11, 12, 16 and ESTD 1, 2, 9 and 10) were found polymorphic and utilized for ascertaining hybridity of progenies obtained from seven mango parental genotypes. Out of 13 polymorphic primers, only 3 primer pairs (LMMA 11, ESTD 9 and ESTD 10) could confirm the hybridity of progenies in different cross combinations.

Key words: Ascertaining hybridity, mango, microsatellite markers

Introduction

The highly heterozygous and allopolyploid nature of the mango with complex genetic nature and lack of knowledge on inheritance pattern of several agronomic traits and

pre-selection criteria makes the mango improvement work cumbersome (Litz 1997). Moreover, long juvenile phase, heavy fruit drop and the large progeny population and acreage required for a meaningful assessment of hybrids further complicate the process. Despite the recognized high quality of few well known mango cultivars, considerable cultivar improvement is needed in most regions of mango culture before anything approaching perfection is likely to be achieved. The excessive fruit drop at different stages of fruit development result in recovery of very few hybrid stones. It has been experienced in past that by following artificial hybridization though the recovery of hybrid fruits range between 3.5-4.5 per cent. Open pollination between parental genotypes and ascertaining the hybridity of progenies using molecular markers can significantly improve the hybrid identification in mango. Furthermore, probability does occur that in specific circumstance pistil is pollinated by undesirable pollen, and the resultant hybrid is not a true hybrid of the desired parents. It is therefore imperative to ascertain the hybridity of progenies using precise system.

In the present study, a set of 42 SSR markers (Table 1&2) have been used for detecting polymorphism among seven parental mango genotypes and informative ones were validated on hybrid progenies obtained by crossing parental mango genotypes. The simple sequence repeats (SSRs) are molecular markers characterized by their highly polymorphic nature, abundance in the genome, reproducibility and simple to use. These are ideal genetic markers for detecting differences between and within species of eukaryotes (Farooq & Azam 2002). It consist of tandemly repeated 2-7 base pair units arranged in repeats of mono-, di-, tri-, tetra- and penta-nucleotides (A,T, AT, GA, AGG, AAAG etc.) with different lengths of repeat motifs (Jonah et al. 2011).

Materials and methods

Plant material and DNA extraction

The present study was carried out on seven important mango genotypes namely, Amrapali, Dushehari, Janardan Pasand, Neelam, Pusa Arunima, Sensation and Tommy Atkins at the Experimental Orchard of the Division of Fruits and Horticultural Technology, IARI, New Delhi. The selection of mango cultivars was made on the basis of their importance in mango breeding programmes. Hand pollination was attempted using the technique described by Mukherjee et al. (1961). Artificial hybridization was attempted using Amrapali as female and Dushehari, Janardan Pasand, Neelam, Pusa Arunima, Sensation and Tommy Atkins as male donor parent (Table 3). The recovery of the hybrids was very low. The hybrids obtained from these crosses were sown in pots having soil and sand as substrate. Five hybrid plantlets from each cross combinations (there were six cross combinations) along with their parents were taken for validation purpose.

Table 1. A list of SSR primers (forward and reverse) and their base sequence

S. No.	Primer name	Sequence (5′-3′)	Tm (^{0}C)	Ta (^{0}C)
1	LMMA 1	F:ATGGAGACTAGAATGTACAGAG	56.5	53
		R:ATTAAATCTCGTCCACAAGT	51.2	
2	LMMA 2	F:AAATAAGATGAAGCAACTAAAG	50.9	47
		R:TTAGTGATTTTGTATGTTCTTG	50.9	
3	LMMA 3	F:AAAAACCTTACATAAGTGAATC	50.9	47
		R:CAGTTAACCTGTTACCTTTTT	52.0	
4	LMMA 4	F:AGATTTAAAGCTCAAGAAAAA	48.1	47
		R:AAAGACTAATGTGTTTCCTTC	52.0	
5	LMMA 5	F:AGAATAAGCTGATACTCACAC	54.0	53
		R:TAACAAATATCTAATTGACAGG	50.9	
6	LMMA 6	F:ATATCTCAGGCTTCGAATGA	53.2	50
		R:TATTAATTTTCACAGACTATGTTCA	53.1	
7	LMMA 7	F:ATTTAACTCTTCAACTTTCAAC	50.9	47
		R:AGATTTAGTTTTGATTATGGAG	50.9	
8	LMMA 8	F:CATGGAGTTGTGATACCTAC	53.3	53
		R:CAGAGTTAGCCATATAGAGTG	55.9	
9	LMMA 9	F:TTGCAACTGATAACAAATATAG	50.9	47
		R:TTCACATGACAGATATACACTT	52.8	
10	LMMA 10	F:TTCTTTAGACTAAGAGCACATT	52.8	50
		R:AGTTACAGATCTTCTCCAATT	52.0	
11	LMMA11	F:ATTATTTACCCTACAGAGTGC	54.0	53
		R:GTATTATCGGTAATGTCTTCAT	52.8	
12	LMMA 12	F:AAAGATAGCATTTAATTAAGGA	49.1	47
		R:GTAAGTATCGCTGTTTGTTATT	52.8	
13	LMMA 13	F:CACAGCTCAATAAACTCTATG	54.0	50
		R:CATTATCCCTAATCTAATCATC	52.8	
14	LMMA 14	F:ATTATCCCTATAATGCCCTAT	52.0	50
		R:CTCGGTTAACCTTTGACTAC	53.3	
15	LMMA 15	F:AACTACTGTGGCTGACATAT	53.2	50
		R:CTGATTAACATAATGACCATCT	52.8	
16	LMMA 16	F:ATAGATTCATATCTTCTTGCAT	50.9	47
		R:TATAAATTATCATCTTCACTGC	50.9	
17	MiSHRS 1	F: TAACAGCTTTGCTTGCCTCC	57.3	54
		R: TCCGCCGATAAACATCAGAC	57.3	
18	MiSHRS 4	F: CCACGAATATCAACTGCTGCC	59.8	56

		R: TCTGACACTGCTCTTCCACC	59.4	
19	MiSHRS 18	F: AAACGAGGAAACAGAGCAC	56.0	54
		R:CAAGTACCTGCTGCAACTAG	57.3	
20	MiSHRS 23	F: AGGTCTTTTATCTTCGGCCC	57.3	53
		R: AAACGAAAAAGCAGCCCA	52.0	
21	MiSHRS 26	F: TGTAGTCTCTGTTTGCTTC	54.0	50
		R: TTCTGTGTCGTCAAACTC	52.0	
22	MiSHRS 29	F: CAACTTGGCAACATAGAC	52.0	50
		R: ATACAGGAATCCAGCTTC	52.0	
23	MiSHRS 30	F: AGAATAAAGGGGACACCAGAC	57.9	53
		R: CCATCATCGCCCACTCAG	58.0	
24	MiSHRS 32	F: TTGATGCAACTTTCTGCC	52.0	50
		R: ATGTGATTGTTAGAATGAACTT	50.9	
25	MiSHRS 33	F: CGAGGAAGAGGAAGATTATGAC	58.4	53
		R: CGAATACCATCCAGCAAAATAC	56.5	
26	MiSHRS 34	F: TGTGAAATGGAAGGTTGAG	54.0	50
		R: ACAGCAATCGTTGCATTC	52.0	
27	MiSHRS 36	F: GTTTTCATTCTCAAAATGTGTG	52.8	50
		R: CTTTCATGTTCATAGATGCAA	52.0	
28	MiSHRS 37	F: CTCGCATTTCTCGCAGTC	56.0	53
		R: TCCCTCCATTTAACCCTCC	58.0	
29	MiSHRS 39	F: GAACGAGAAATCGGGAAC	54.0	53
		R: GCAGCCATTGAATACAGAG	56.0	
30	MiSHRS 44	F: AACCCATCTAGCCAACCC	56.0	53
		R: TTGACAGTTACCAAACCAGAC	55.9	
31	MiSHRS 48	F: TTTACCAAGCTAGGGTCA	52.0	50
		R: CACTCTTAAACTATTCAACCA	52.0	

Table 2. List of EST derived SSR primers (forward and reverse) and their base sequences.

S. No.	Primer name	Sequence (5′-3′)	Tm (^{0}C)	Ta (^{0}C)
1	ESTD 1	F: TGCTAATTTAGGCACTACCG	55.3	53
		R: ATCATTATCCACCTCCTCCT	55.3	
2	ESTD 2	F: TACCACTCGTAGCCTCAACT	57.3	53
		R: CCATTGTCGTTGTTGTTATG	53.2	
3	ESTD 3	F: GGGAAAGGAATTTAAAGCAT	51.2	50
		R: AAGGCATAGCTAGCACAGTC	57.3	
4	ESTD 4	F: AGAGAAGACATTTGGTGGAG	50.0	53
		R: CGCTGTTTGTTATTGTGAAA	51.2	

5	ESTD 5	F: TTGATATTGTTGTTCCCGTT R: TTAAATCTCGTCCACAAGTTC	51.2 54.0	53
6	ESTD 6	F: CTGCAAATATCTCAGGCTTC R: CAGTGCGTTAGTTGTTGATG	53.3 53.3	53
7	ESTD 7	F: ATGCATCATGTCTACCATCA R: TACTGAAAGAGCTTGGTGCT	53.2 55.3	50
8	ESTD 8	F: ATCTGTGAAATGGAAGGTTG R: AGCTGCAACATCACCAGATT	53.2 55.3	50
9	ESTD 9	F: GCTTTATCCACATCAATATCC R: TCGAACTAAAGAATTGGCAT	54.0 51.2	50
10	ESTD 10	F: GATCTGACCCAACAAAGAAC R: ACGTAGATCTGCTTAACCCA	55.3 55.3	53
11	ESTD 11	F: TTGTCTTGAAGCTATTCATT R: GGCAAGTTCTATGTTGTAAG	49.1 52.2	47

Table 3. Artificial hybridization in mango

Cross	No. of panicles crossed	No. of flowers crossed	Recovery of fruits	No. of stones germinated
Amrapali x Dushehari	29	203	14	9
Amrapali x Janardan Pasand	32	256	11	7
Amrapali x Neelam	30	180	17	11
Amrapali x Pusa Arunima	41	205	10	5
Amrapali x Sensation	28	196	18	12
Amrapali x Tommy Atkins	30	174	13	8

Five gram of young, tender and fully expanded leaves from seven mango genotypes and hybrid plantlets were collected, labeled and wrapped in aluminum foil and put in a liquid nitrogen box for inactivation of enzymes. Standard protocol of DNA isolation was carried out using CTAB (Cetyl Trimethyl Ammonium Bromide) method given by Murray & Thompson (1980) with minor modifications (added 1% PVP w/v for removal of phenols). The composition of extraction buffer was 4% w/v CTAB, 1% PVP w/v, 20 mM EDTA, 100 mM Tris-HCl (pH-8), 1.4 M NaCl, and 0.2% β-mercaptoethanol. Leaf samples were ground to fine powder in pre-chilled pestle and mortar in liquid nitrogen. Powdered material was quickly transferred to centrifuge tubes containing CTAB extraction buffer (6 ml pre-heated) and vortexed it. Then the tubes were incubated at 65°C for 1 h with intermittent shaking (after each 15 min.). After incubation, tubes were cooled to room temperature and 6 ml chloroform: isoamyl alcohol (24:1) was added. The contents were mixed by inversion for

about 5-10 minutes. Then samples were centrifuged at 12,000 rpm for 10 min. at 25^0C. Then the supernatant was transferred into new centrifuge tubes. Then nucleic acids were precipitated by adding NaCl (0.5 vol.) and chilled isopropanol (one vol.) and left it for over-night at 4^oC. Then Centrifuge tubes were spun at 10,000 rpm for 5 min. at 10^0 C. Supernatant was discarded and the DNA pellet was washed twice with 70% ethanol. The DNA pellet was dried for complete removal of ethanol. Then pellet was dissolved in 2ml TE buffer.

DNA purification and quantification

Two µl RNase A was added per 200 µl DNA solution and incubated for 1 hour at 37°C. The DNA was treated with an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) and mixed the content gently by swirling for 5 minute. Then tubes were centrifuged at 10,000 rpm for 5 min. and supernatant was taken in a fresh tube. Followed by 2 extractions were given with Chloroform: Isoamyl alcohol (24:1). RNA free purified DNA was precipitated by adding Sodium acetate (0.1 vol.) and chilled Isopropanol (2.5 vol.) to the aqueous phase and then collected by spinning at 12,000 rpm for 10 minute. Then precipitate was washed twice with 70% ethanol. The pellet was air dried and dissolved in TE buffer. DNA was run in 0.8% agarose gel to check quality and quantity. The quantity of DNA was measured in the sample by comparing with the control ƛ phage DNA indicated height and weight of DNA. Each DNA sample was diluted with sterilized double distilled water to yield a working concentration of 25-30 ng/µl.

PCR amplification

PCR amplification was carried out with 50 ng of genomic DNA, 2 µl $MgCl_2$, 1U *Taq* DNA polymerase, 1x PCR buffer without $MgCl_2$, 0.5 µM of each of primers and 200 µM of dNTPs. The volume was made up to 20 µl with sterile double-distilled water. PCR tubes containing the above components were capped and centrifuged at 10,000 rpm for 2min. to allow proper settling of reaction mixture. Thermocycling was carried out in a Perkin Elmer 9,600 thermocycler.

Gel electrophoresis

PCR amplified products were run in 3.5% high resolution agarose (Metaphor) gels. Agarose and 1X TAE buffer were mixed thoroughly and boiled for about 4 min. to dissolve the contents. Then mixture was cooled to 50°C and 2 µl ethidium bromide (1 mg/ml solution) was added per 100 ml gel. Thereafter, the gel was casted in a gel tray with properly placed combs. Polymerization of the gel was allowed for half an hour. Then combs were taken out carefully. The gel was transferred to an electrophoresis tank having an appropriate quantity of 1X TAE buffer. Two µl loading dye was added to each PCR tube containing amplified products and loaded in the wells of the metaphor gel. Three µl Gene Ruler.^{TM} (100 bp gene

ruler, Fermentas) was loaded in the first well of each gel to determine sizes of identified bands. Electrophoresis was carried out at 5 V/cm for 3 hour.

Screening of SSR markers for polymorphism and their validation on hybrid progenies

Fourty- two primer pairs were screened, 13 primer pairs (LMMA 2, 3, 4, 7, 8, 9, 11, 12, 16 and ESTD 1, 2, 9 and 10) were found polymorphic, 19 primers (LMMA 1, 4, 5, 6, 10, 13, 14, 15 and ESTD 3, 4, 5, 6, 7, 8 and 11, MiSHRS 23, 26, 29, 30 and 32) were monomorphic. Ten primer pairs (MiSHRS 1, 4, 18, 33, 34, 36, 37, 39, 44 and 48) did not amplify the DNA of any genotype. The polymorphic primer pairs were further utilized for ascertaining the hybridity of F_1 progenies obtained from different cross combinations employing seven mango genotypes. Comparison of banding pattern among parental mango genotypes and hybrid plantlets obtained from crosses Amrapali x Dushehari, Amrapali x Janardan Pasand, Amrapali x Neelam, Amrapali x Pusa Arunima, Amrapali x Sensation and Amrapali x Tommy Atkins was done to ascertain the hybrid origin of progenies.

Results and discussion

In the present study, a set of 42 SSR markers have been used for detecting polymorphism among seven parental mango genotypes and informative ones were validated on hybrid progenies obtained by crossing parental mango genotypes. Out of 42 primers, 13 primer pairs (LMMA 2, 3, 4, 7, 8, 9, 11, 12, 16 and ESTD 1, 2, 9 and 10) were polymorphic and 19 primers (LMMA 1, 4, 5, 6, 10, 13, 14, 15 and ESTD 3, 4, 5, 6, 7, 8 and 11, MiSHRS 23, 26, 29, 30 and 32) were monomorphic. Ten primer pairs (MiSHRS 1, 4, 18, 33, 34, 36, 37, 39, 44 and 48) did not amplify the DNA of any genotype. Out of 13 polymorphic primers, only three primer pairs (LMMA 11, ESTD 9 and ESTD 10) could confirm the hybridity in different cross combinations. Primer LMMA 11 confirmed the hybridity of progenies obtained from crosses Amrapali x Janardan Pasand, Amrapali x Neelam, Amrapali x Pusa Arunima, Amrapali x Sensation, Amrapali x Tommy Atkins (Fig.1&2). ESTD 9 ascertained the hybridity of progenies developed by crosses Amrapali x Sensation and Amrapali x Tommy Atkins (Fig. 3). Hybridity of progenies obtained from crosses Amrapali x Neelam and Amrapali x Tommy Atkins was ascertained by the primer pair ESDT 10 (Fig. 4&5).

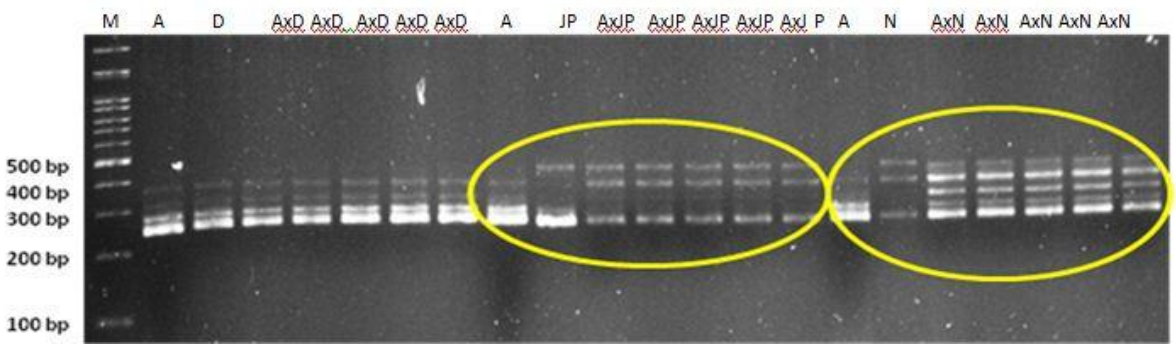

Fig. 1. Banding pattern of parental mango genotypes and their hybrids as depicted by LMMA 11 primer

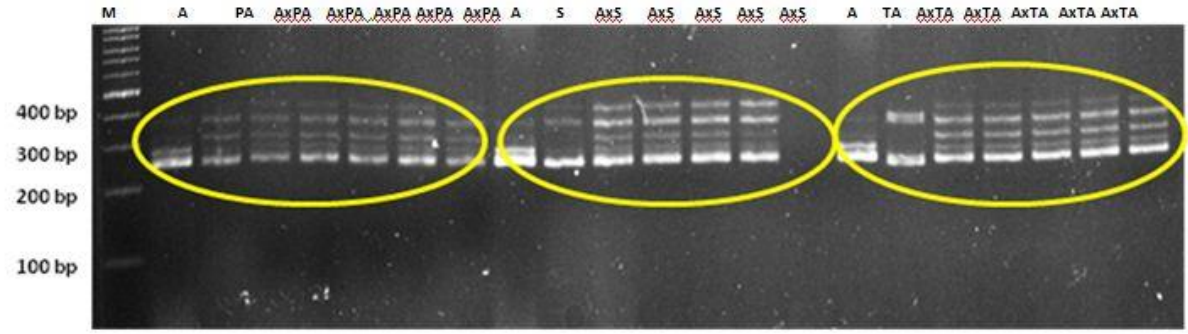

Fig. 2 Banding pattern of parental mango genotypes and their hybrids as depicted by LMMA 11 primer

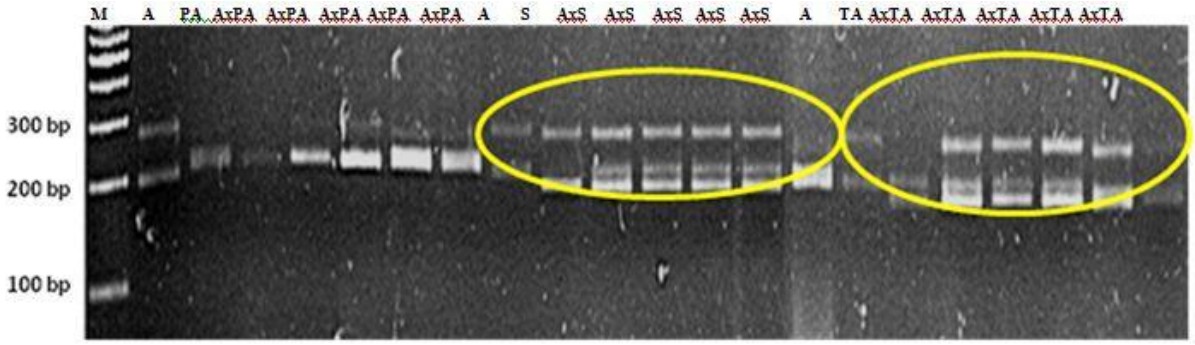

Fig. 3 Banding pattern of parental mango genotypes and their hybrids as depicted by primer ESTD 9

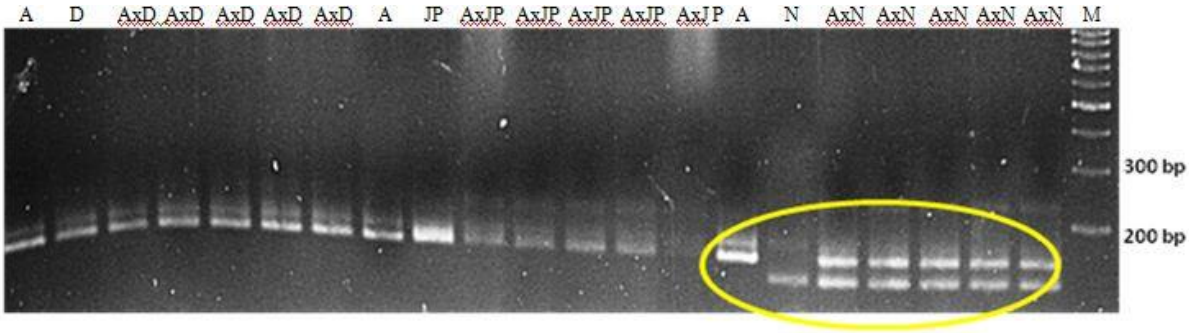

Fig. 4 Banding pattern of parental mango genotypes and their hybrids as depicted by primer ESTD 10

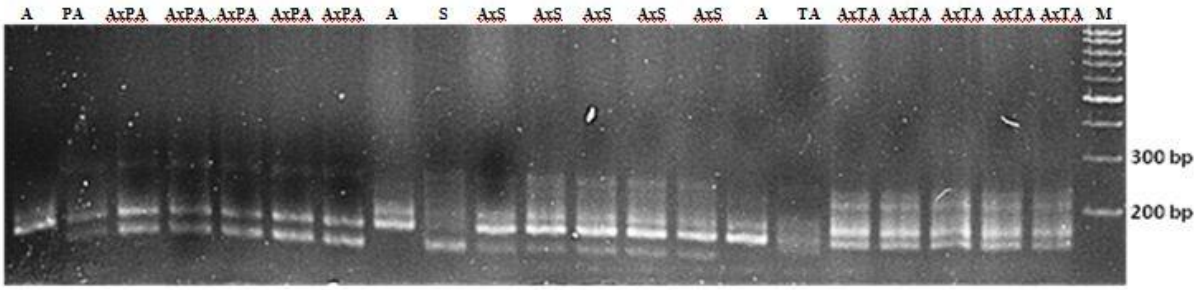

Fig. 5 Banding pattern of parental mango genotypes and their hybrids as depicted by primer ESTD 10
M- 100 bp marker, A- Amrapali, D- Dashehari,JP- Janardan Pasand, N- Neelam, PA- Pusa Arunima, S- Sensation, TA- Tommy Atkins

SSR markers are ideally suitable for DNA fingerprinting of cultivars, molecular characterization and validation of hybrids. Because of simplicity of gel pattern, it is easy to know the alleles of a locus with sequence tagged microsatellites. Therefore, they are the popular marker of identification of cultivars. The high reproducibility, information content and easy manipulation has made these markers popular in fingerprinting, diversity analysis, genotyping and hybridity testing (Powell et al. 1996). Microsatellite markers (Kashi et al. 1997; Queller et al. 1993) have been proved valuable in plant breeding due to codominant inheritance, multi-allelic, widely distributed throughout genome, easily scored analysis can be easily automated.

Hybridity analysis based on microsatellites can reduce the cost related to breeding process. In mango, SSRs can greatly accelerate the breeding process. This allowed the selection of cultivars with desirable traits at an early stage, thus permitting use of lager seedling population initially saving time, resources and space. The work of Singh et al. (2012) who studied genetic diversity in closely related mango hybrids using SSR markers and concluded that hybrids had stronger affinity towards maternal parent Amrapali. Similarly, Subashini et al. (2014) who assessed purity of *Eucalyptus* hybrid (*Eucalyptus camaldulensis* x *E. tereticornis*) using SSR markers and confirmed parentage of the hybrids with 85-100 hybrid purity index which are also in the agreement with the result of present investigation. Similarly, Lian et al. (2012) performed hybridization in castor bean (CSR24 × CSR181) and verified hybrids and genetic diversity of F_1 seeds by SSR markers and Tang et al. (2006) studied 79F_1 seedlings from *M. xiaojinensis* × *M. baccata.* The present study suggested that it is possible to identify true hybrids with accuracy in mango using microsatellite (SSR) markers and thus an approach to withdraw the inaccurate and cumbersome morphological observations. Therefore, plant breeders can discard the unwanted open pollinated seedlings at an early stage in breeding programmes of mango. The present study suggested that it is possible to identify true hybrids with accuracy in mango using microsatellite (SSR) markers and withdraw the inaccurate and cumbersome morphological observations. Therefore it is suggested that plant breeders can discard the unwanted open- pollinated seedlings at an early stage in breeding programmes in mango to achieve precision in breeding thereby evaluating useful recombinants.

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