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Molecular Genetic Diversity Analysis of Commercial Mango (*Mangifera indica* L.) Cultivars Employed as Parents in Hybrid Development in India

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Mango hybridization programmes use commercial cultivars as parents. Twenty three such popular cultivars of mango belonging to different regions of India were analyzed by employing multi-locus marker techniques to measure the genetic diversity existing among them. Twelve unanchored ISSR primers (114 markers) and 15 AFLP primer-pair combinations (1,073 markers) revealed average gene diversity over loci to be 0.231 and 0.257 respectively. Mango cultivars from southern India were found to be significantly different ($p<0.001$). In order to decipher how these markers are inherited, three popular hybrids 'Amrapali', 'Mallika' and 'Ratna' were compared with their parents for band sharing information. Number of ISSR markers shared between pairs of parents and their hybrid was nearly 20% more than that of AFLP markers, endorsing the conserved nature of ISSR profiles. Our observations on (i) genetic relatedness among popular cultivars and (ii) band sharing pattern among parents and hybrids have implications on mango germplasm collection and breeding activities.

Key Words: AFLP, Band sharing, Diversity analysis, ISSR, Mango hybrids

Introduction

Mango (*Mangifera indica* L.) is the most important fruit of tropics and nearly all the world's monoembryonic cultivars have Indian pedigree (Mukherjee, 1953). Most of the present day popular mango cultivars in India have originated as chance seedlings that were maintained through vegetative propagation (Mukherjee, 1972). In addition to thousands of cultivars developed as chance seedlings, India has produced many commercially successful hybrid mango cultivars. However, despite the wide range of available genetic variation and ease with which the selected hybrid can be vegetatively propagated, very few parents have generally been employed in mango hybridization efforts in India. For instance, Indian Agricultural Research Institute (IARI), New Delhi and Central Institute of Sub-tropical Horticulture (CISH), Lucknow often use 'Dashehari' or 'Amrapali'; Indian Institute of Horticultural Research (IIHR), Bangalore and Fruit Research Station, Vengurla choose 'Alphonso'; and IARI, New Delhi and Fruit Research Station, Sangareddy employ 'Neelum' as parents.

Because few genotypes have been employed as parents (based, mostly on fruit traits and adaptation), extensive knowledge of genetic base of such cultivars turns out to be a prerequisite for developing sustainable breeding and systematic conservation strategies. Consequently, it becomes indispensable to plan, explore, assemble, establish and characterize large number of individual genotypes bearing the name of these cultivars, in order to conserve and exploit maximum allelic variability available within elite cultivars. Nevertheless, cultivation of different cultivars under the same name together with existence of many synonyms for popular cultivars e.g. 'Langra' (12), 'Dashehari' (5), 'Bangalora' (10), 'Alphonso' (8) can render germplasm utilization complicated (Pandey, 1984). Under such circumstances, unambiguous identification and meticulous diversity analysis can save a lot of investment in terms of time, effort and cost.

Traditionally, genetic characterization in mango has been carried out by means of morphological characters (IPGRI, 2006; PPVFRA, 2008). However, influence of environmental and developmental factors, existence of limited variation and subjective nature of evaluation of these traits have often posed problems (Adato *et al.*, 1995). Since 1990's various types of DNA markers have been employed to complement the characterization procedure including RAPD (Schnell *et al.*, 1995), ISSR (Eiadthong *et al.*, 1999), AFLP (Kashkush *et al.*, 2001), cpSSR (Xin-Hua *et al.*, 2007), SSR (Ravishankar *et al.*,

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2011), SCOT (Luo *et al.*, 2010), CAPS (Shudo *et al.*, 2013) and SNP (Kuhn, 2014). The markers have been used extensively to characterize germplasm accessions of mango in the USA (Schnell *et al.*, 1995), India (Karihaloo *et al.*, 2003, Ravishankar *et al.*, 2011), Iran (Shamili *et al.*, 2012), Australia (Dillon *et al.*, 2013), China (Gao *et al.*, 2013), *etc.*, with various objectives and varying degrees of success and utility. An overview of use of different marker techniques in mango genetic analysis showed that all the markers were good enough, when used suitably, for diversity analysis in an outcrossing tree species like mango.

From the available information in mango, the genetics of inheritance of various horticultural characters appears to be unclear. However, such information along with scorable markers is extremely useful in understanding the genetic relatedness between the putative parents to decide which parental types ought to be used in hybridization programmes. For instance, leaf flavor for fruit flavour, higher phloem: xylem ratio, higher phenolics in the apical bud and low stomatal density for dwarfness etc. have been identified as valuable morphological markers. Use of molecular marker profiles as a complementary approach was shown to enhance the effectiveness of pre-selection (Krishna and Singh, 2007). Comparison of morphological and physiological traits between hybrids and parents would only present part of a picture. Molecular marker analysis is expected to provide a genome wide comparison both in expressed and unexpressed regions. Srivastava *et al*. (2007) and Hemanthkumar and Vasanthaiah (2009) reported clustering of cultivars on the basis of common parentage whereas Srivastava *et al*. (2004) actually attempted to identify genetic relatedness between hybrids and parents on the basis of various multi-locus markers. However, these attempts reduced the band sharing into similarity coefficients and comparisons were generally based on the clustering pattern alone and stopped short of actually analyzing band sharing patterns. Band sharing studies can provide vital cues to the breeders about parents, hybrids and selection process. For instance, in an experimental cross it was demonstrated that 85% of the AFLP markers exhibited Mendelian segregation and 15% showed distorted segregation (Kashkush *et al.*, 2001). Therefore, it would be interesting to know band-sharing pattern between the marker profiles of commercially successful Indian mango hybrids and their parents.

Among various DNA markers used in mango genetic studies, SSRs have been established as superior because of their co-dominance and reproducibility. SSRs are particularly employed in DNA fingerprinting, marker associated selection studies involving homozygous parents, and population genetics. However, SSRs with a maximum of two alleles per diploid genome are less suitable for band sharing studies in tree species where parents and offspring are highly heterozygous. Conversely, in multi-locus marker techniques a single assay can hit multiple locations on the genome to provide a possibility of greater genome coverage. PCR-based markers inter simple sequence repeats (ISSR) and amplified fragment length polymorphism (AFLP) do not need any prior information about the target genome sequences, generate large number of amplicons and are adequately reproducible.

We selected a set of popular mango cultivars employed as parents in hybridization programmes and representing different geographical regions of India, for the present study. The objectives were: (1) to assess genetic diversity among the elite cultivars based on ISSR and AFLP profiles and (3) to study band sharing pattern in mango hybrid cultivars and their parents.

Materials and Methods

Plant Material and DNA Extraction

The study was carried out in 23 selected commercial cultivars of mango (Table 1). These included 16 monoembryonic cultivars, three polyembryonic cultivars and four hybrid cultivars. Flushing tender leaves from single trees of each cultivar were used for DNA isolation by cTAB method with minor modifications established in our laboratory (Archak *et al.,* 2003). DNA was treated with RNase and purified by repeated ethanol precipitation. Quantification was carried out by fluorometry.

Marker Analysis

Twelve primers pre-selected for clarity, scorability and reproducibility of banding pattern were employed for ISSR analysis (Table 2). Fifteen fluorescently-labelled primer combinations were employed for the generation of AFLP markers (Table 3). Amplification and documentation of ISSR and AFLP markers were carried out as per established procedures (Archak *et al.,* 2003). Amplicons were scored as discrete variables, using 1 to indicate presence and 0 for absence. Variant status of the amplicon in even one of the cultivars was counted as polymorphic. A binary matrix was created by scoring bands in case of ISSR and peaks in case of AFLP. Resolving power of a primer/ primer combination

Table 1. Mango cultivars profiled in the study

Cultivars	Region of origin/ pedigree	Source[#]
'Alphonso'	Maharastra	FHT
'Amrapali'	Dashehari × Neelum	FHT
'Bangalora'	Karnataka, Tamil Nadu	FHT
'Bhadauran'	Uttar Pradesh	FHT
'Bombay Green'	Uttar Pradesh	FHT
'Chandrakaran*'	Kerala	RARS
'Chausa'	Uttar Pradesh	FHT
'Dashehari'	Uttar Pradesh	FHT
'Fazli'	Bihar	FHT
'Husnara'	Bihar	FHT
'Jamadar'	Gujarat	FHT
'Kurukkan*'	Kerala	FHT
'Lal Sundari'	Andhra Pradesh	FHT
'Langra'	Uttar Pradesh	FHT
'Mallika'	Neelum × Dashehari	FHT
MCDH-1	North East India	Goa
'Neelum'	Tamil Nadu	FHT
'Olour*'	Tamil Nadu	RARS
'Rajapuri'	Gujarat	FHT
'Rataul'	Uttar Pradesh	FHT
'Ratna'	Neelum × Alphonso	FHT
'Vanaraj'	Gujarat	FHT
'Zardalu'	West Bengal	FHT

* Polyembryonic cultivar
FHT= Division of Fruit and Horticultural Technology, Indian Agricultural Research Institute, New Delhi; RARS= Regional Agricultural Research Station, Kerala Agriculture University, Ambalavayal, Kerala; Goa= ICAR Research complex, Goa.

was calculated as the sum of band informativeness of all the markers generated by that primer. Genetic diversity was estimated by computing average gene diversity over loci (Nei, 1973). Partitioning of variance between different mango growing regions was carried out by AMOVA version 1.55 (Excoffier, 1992; Huff *et al*., 1993). Band sharing pattern of AFLP and resultant average taxonomic distance based on two-dimensional cluster were determined using JMP version 10 (SAS Institute, 2012).

Results

Marker Profiles

Twelve oligonucleotide primers produced a total of 140 ISSR markers amplifying on an average 94.9 bands per cultivar (Table 2). 81.4% of the amplicons were polymorphic. Primers $(GAAGTGGG)_2$, $(AT)_5$ $(GT)_5$, $(TA)_5$ $(GT)_5$ and $(AGG)_6$ generated only polymorphic markers, whereas more than half the markers produced by $(ACTG)_4$ were monomorphic. Fifteen primer-pair combinations generated 1228 AFLP peaks with an average of 651.7 amplicons per cultivar (Table 3). 87.4% AFLP markers were polymorphic. All the three *Mse*I-CAG combinations (J, K and L) produced only polymorphic markers.

Both ISSR and AFLP markers could clearly distinguish all the cultivars. Oligonucleotide primers displayed an average resolving power of 4.87 against 28.84 of AFLP primers (Table 2 and Table 3). This was reflected in the inability of individual ISSR primers to distinguish each cultivar whereas each AFLP primer could do so independently. Four cultivar specific ISSR markers, two for 'Lal Sundari' [$(GATA)_4$-650, $(GACAC)_4$-250] and one each for 'Rataul' [$(GACAC)_4$-1500] and 'Chandrakaran' [$(GATA)_4$-600] were obtained. Cultivar specific AFLP markers could be identified for each cultivar except for 'Dashehari'. Remarkably, 14 cultivar specific markers were identified for 'Amrapali'. AFLP primer pair *Mse*I CAC+*Eco* RI ACA (code M) generated as many as 14 cultivar specific markers.

Diversity Estimates and AMOVA

Genetic diversity of 23 mango cultivars was estimated as average gene diversity over loci. The values of overall diversity were 0.257 and 0.231 for AFLP and ISSR respectively. Distribution of the diversity was estimated by grouping cultivars of chance origin (i.e. excluding hybrids) based on geographic differentiation. The regions were – *northern* ('Bhadauran', 'Bombay Green', 'Chausa', 'Dashehari', 'Langra' and 'Rataul'), *southern* ('Bangalora', 'Chandrakaran', 'Kurukkan', 'Lal Sundari', 'Neelum' and 'Olour'), *eastern* ('Fazli', 'Husnara', MCDH-1 and 'Zardalu') and *western* ('Alphonso', 'Jamadar', 'Rajapuri' and 'Vanaraj'). Cultivars were compared, region wise, for polymorphism of markers and average gene diversity over loci (Table 4). The estimates revealed maximum diversity in the southern cultivars. AMOVA revealed that, 96.3% of the total ISSR marker diversity was attributed to within region and 3.7% to between regions, whereas AFLP markers displayed a higher between regions distribution at 7.8% (Table 5). Southern Indian cultivars were significantly different from northern and eastern Indian cultivars ($p<0.001$) as deduced by both ISSR and AFLP markers.

Table 2. Primers used for ISSR analysis

Sequence	Annealing temperature (°C)	Total bands	Polymorphism (%)	Resolving power
$(GATA)_4$	43	9	77.78	2.43
$(ACTG)_4$	45	14	42.86	1.65
$(GACA)_4$	45	17	94.12	7.74
$(GAAGTGGG)_2$	50	14	100	7.43
$(GGAT)_4$	45	7	57.14	2.58
$(AT)_5$ $(GT)_5$	52	8	100	4.52
$(TA)_5$ $(GT)_5$	52	11	100	6.76
$(GACAC)_5$	61	9	77.78	3.27
$(GTG)_6$	57	15	80	7.09
$(AGG)_6$	57	10	100	4.16
$(GTG)_4$	36	12	66.67	4.43
$(TCC)_5$	52	14	78.57	6.33
		140*	81.43**	4.87**

* Total across primers
** Mean values across primers

Table 3. Primer combinations used for AFLP analysis

Primer combination	Code	Total Bands	Polymorphism (%)	Resolving power
*Mse*I CAT+*Eco* RI ACA	A	119	87.39	42.84
*Mse*I CAT+*Eco* RI AAG	B	112	86.61	45.00
*Mse*I CAT+*Eco* RI AGC	C	60	95.00	25.30
*Mse*I CTT+*Eco* RI ACA	D	103	88.35	40.92
*Mse*I CTT+*Eco* RI AGG	E	81	77.78	29.23
*Mse*I CTT+*Eco* RI AAC	F	58	87.93	23.64
*Mse*I CTG+*Eco* RI ACA	G	113	83.19	38.57
*Mse*I CTG+*Eco* RI AGG	H	104	90.38	38.48
*Mse*I CTG+*Eco* RI AAC	I	57	73.68	16.73
*Mse*I CAG+*Eco* RI ACA	J	98	100.00	44.72
*Mse*I CAG+*Eco* RI AAG	K	43	100.00	21.59
*Mse*I CAG+*Eco* RI AGC	L	61	100.00	23.79
*Mse*I CAC+*Eco* RI ACA	M	69	84.06	18.87
*Mse*I CAC+*Eco* RI AAG	N	90	78.89	29.86
*Mse*I CAC+*Eco* RI AGC	O	60	81.67	21.93
		1228*	87.38**	28.84**

* Total across primers
** Mean values across primers

In addition, AFLP could discern between other regions as well. However, differences between east and north Indian cultivars were found to be non-significant.

Band Sharing

The six cultivars (three hybrids and their parents) analyzed, exhibited a total of 125 ISSR bands

Table 4. Comparison between different groups of mango cultivars

Group	AFLP				ISSR			
	North	South	East	West	North	South	East	West
Total number of markers	953	977	941	998	127	129	128	117
Per cent polymorphism	62.75	72.36	57.70	64.93	48.03	70.54	41.41	53.85
Average gene diversity over loci	0.235	0.271	0.238	0.266	0.187	0.268	0.175	0.226

Table 5. Analysis of molecular variance (AMOVA)

Source of variation	df	Percentage of variance	p value (1000 permutations)
AFLP			
Among regions	4	7.80	<0.001
Among cultivars within regions	18	92.20	<0.001
ISSR			
Among regions	4	3.71	<0.001
Among cultivars within regions	18	96.29	<0.001

(44% polymorphic) and 1022 AFLP bands (72.1% polymorphic). The average number of bands per genotype was more than 190 (69.5% polymorphic), which is by far the maximum compared to any of the previous reports on mango genetic analyses. Markers observed in the profiles of hybrid cultivars were shared either with only male parent (*male parent specific*) or female parent (*female parent specific*) or were common among parents and the hybrid (*shared*). Markers which could not be attributed to either of the parents were termed as *novel*. Mean number of ISSR markers shared by hybrids with both the parents were largely equal (~10%) whereas on an average hybrids shared greater number (17.9%) of AFLP markers with female parents (Table 6). ISSR profiles of 'Amrapali' and 'Mallika', the products of reciprocal crosses, revealed that 'Dashehari' contributed significantly higher number of markers to both the hybrids irrespective of whether it was male or female parent. 'Ratna' shared higher percentage (~24%) of AFLP markers with 'Neelum' (female parent) but most remarkably, 'Amrapali' exhibited more than 20% AFLP bands that could not be assigned to either of the parents (Table 6).

Overall pairwise similarity among parents and hybrids was higher using ISSR markers (0.898), compared to AFLP (0.770). Based on AFLP, 'Amrapali' was equidistant from parents (0.275). Furthermore, assembly of banding pattern in the form of a 2D cluster (Fig. 1) illustrated that 'Amrapali' carries distinctive AFLP pattern in relation to all other genotypes particularly, its parents.

Discussion

Breeding in mango has been bringing together desirable characters from two established cultivars. Estimating the diversity existing among 23 leading cultivars therefore assumes practical significance. To ensure our estimates based on dominant markers reflect the actual state, more than thousand markers were employed of which recessive markers ("0" score) were of greater proportion.

Genetic Diversity and Distribution

Genetic diversity in 23 mango cultivars expressed as average gene diversity over loci was moderate; the

Table 6. Marker assignment* in mango hybrid cultivars

	Total markers	Female parent specific	Male parent specific	Shared	Novel
AFLP					
'Amrapali'	742	11.7	13.7	54.0	20.5
'Mallika'	612	17.8	12.6	67.3	2.3
'Ratna'	683	24.3	13.5	55.2	7.0
Mean	679	17.9	13.3	58.8	9.9
ISSR					
'Amrapali'	99	11.1	5.1	80.8	3.0
'Mallika'	109	8.3	12.8	76.1	2.8
'Ratna'	104	12.5	12.5	73.1	1.9
Mean	104	10.6	10.1	76.7	2.6

*Expressed as per cent of total markers

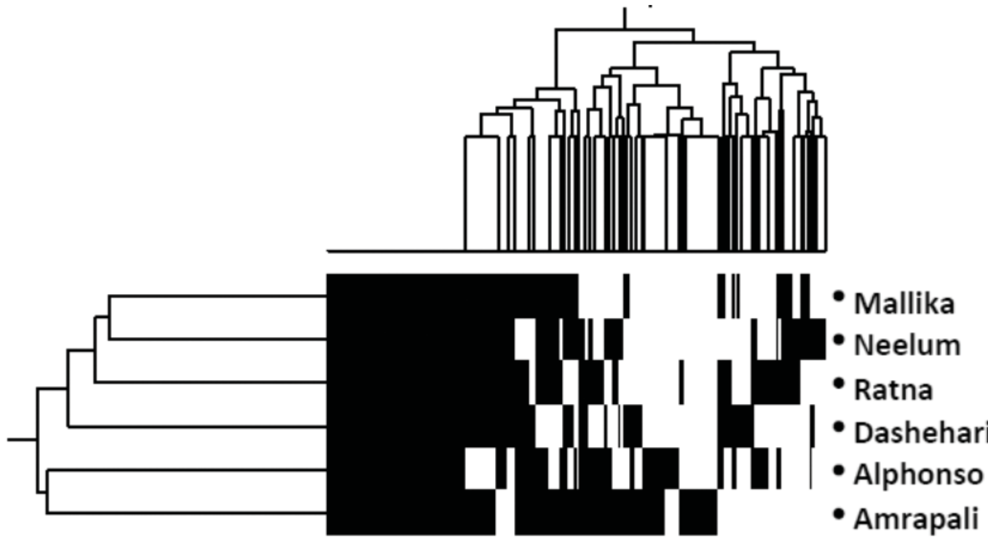

Fig. 1. Two-dimensional dendrogram of mango parents ('Dashehari', 'Neelum' and 'Alphonso') and hybrids ('Amrapali', 'Mallika' and 'Ratna') derived from 1022 AFLP markers. The OTUs are clustered horizontally and markers are grouped vertically. The binary data are represented as band profiles. Black band represents "1" and white represents "0". Note the remarkably distinct pattern of 'Amrapali'.

values were 0.257 and 0.231 based on AFLP and ISSR respectively. These values are indicative of the extent of diversity represented in the elite commercial cultivars and do not represent the overall diversity present in Indian mango germplasm. It is pertinent here for mango breeders to note that combinatorial breeding may be bringing preferred traits together but at the cost of overall genetic base (Iyer and Degani, 1997).

Mango cultivars have been selected and cultivated in different regions of India based on taste and adaptation. Cultivars could be early and late bearers, mono and polyembryonic, regular and alternate bearers, dessert type and pickling type etc. Among different mango growing regions, southern cultivars were significantly different from cultivars from northern and eastern regions as deduced by both ISSR and AFLP markers (Table 4). Moreover, majority of the molecular variation from both AFLP and ISSR data was found within rather than between regions (Table 5), which is consistent with the general trend in other out crossing species (Huff *et al.*, 1993; Nesbitt *et al.*, 1995; Hwang *et al.*, 2001). Cultivars belonging to diverse regions differ largely in their adaptability as some of them are proven ecotypes. When cultivars from one region are grown at other region, they vary in bearing characteristics, fruit quality and time of flowering. Superior chance seedlings selected as cultivars led to fixation of very high degree of heterozygosity, leading to high within-region variation.

There was no apparent clustering of 23 cultivars based on either geographical location or embryo type. Lopez-Valenzula *et al.* (1997) have reported such grouping by analyzing 15 cultivars from 4 different countries. In the present experiment, plant material belonged to different areas within India. Therefore, absence of groupings in relation to geographical location may be indicative of the *continuum of variability* existing among Indian cultivars (Mukherjee, 1972; Majumdar and Sharma, 1985). Our analysis did not cluster polyembryonic cultivars together as reported by Iyer and Degani (1997) but in contrast to observations of Ravishankar *et al.* (2004).

Band Sharing and Selection of Parents

The study was carried out in three hybrid cultivars of mango and their parents. 'Mallika' ('Neelum' × 'Dashehari') and 'Amrapali' ('Dashehari' × 'Neelum') are the two most successful regular-bearing hybrids developed by IARI, New Delhi in 1972 (Singh *et al.*, 1972). 'Mallika' has high total soluble solids content, a higher percentage of pulp, fibreless flesh and a fruit size of about 300g. 'Amrapali' is precocious, distinctly dwarf, fruit with excellent quality and very rich in vitamin A. 'Ratna' ('Neelum' × 'Alphonso') was developed in 1981 at Regional Fruit Research Station, Vengurla, Maharashtra (Salvi and Gunjate, 1989). 'Ratna' has a larger fruit size, fruit quality similar to 'Alphonso' and is free of spongy tissue.

The band sharing results of the present experiment need to be considered in the light of following facts: (i) In mango hybridization, unlike field crops, neither the parents are homozygous nor is the progeny heterozygous for all loci, and (ii) Both ISSR and AFLP are multi-locus markers, hence, apparent band sharing may be due to identity by descent or to identity by state. Therefore, the observations presented and the inferences drawn here are best taken as additional information for mango breeders and not as inheritance studies *per se*. Detailed inheritance studies require (i) pre-screening of the primers to minimize background variation, highlighting only the true differences in the inheritance pattern and (ii) both useful and discarded individuals of the hybrid progeny.

Genetic recombination events occur during the gamete formation in the parents. This may lead to changes in the primer binding sites as well as restriction sites resulting in the loss of bands or occurrence of *de novo* amplicons. Nevertheless, such events are not very common and therefore novel bands occur in negligible number. However, since AFLP and ISSR techniques generate dominant and multi-locus markers, we end up scoring variant alleles as novel bands. Based on our own studies in mango and cashew (unpublished), novel AFLP and ISSR bands may range up to 3-5% of the total number of bands. Conserved nature of ISSR markers was evident because: (i) average number of ISSR markers shared between parents and their hybrid (~77%) was nearly 20% more than those observed in case of AFLP (~59%); (ii) compared to AFLP (~10%), very few novel bands were produced by ISSR (average number of novel bands 2.6%).

Higher number of 'Neelum' specific AFLP markers observed in 'Ratna' supports the observation of fewer 'Alphonso'-like fruit and other morphological characters in 'Ratna', which necessitated backcrossing of 'Ratna' with 'Alphonso' (Salvi and Gunjate, 1989). Occurrence of 20.5% of novel AFLP bands in 'Amrapali' could not be explained on the basis of recombination events alone in the origin of the cultivar. 'Mallika' and 'Amrapali' were developed and released by Indian Agricultural Research Institute, New Delhi (Singh *et al.*, 1972). Between 1961 and 1980, 1200 hybrids were raised at IARI of which 710 had 'Neelum' as a parent. Dwarfness, regularity of bearing, and better fruit quality were the traits for which selection was carried out. There were only two successful lines with excellent fruit quality and regularity of bearing, which were subsequently released as 'Mallika' and 'Amrapali'. 'Mallika' showed vigorous habit but 'Amrapali' was the only hybrid line that had distinct dwarf stature (Sharma and Majumdar, 1989). Our observation of higher novel AFLP bands in 'Amrapali' may not be just circumstantial, but might provide clues to the marked morphological differences exhibited by the hybrid. Since 'Amrapali' has been used as a parent (mostly as a female parent) in many of the crosses dwarfness, regularity of bearing and fruit quality (Pinto and Byrne, 1993), detailed molecular analysis may pave way for marker assisted selection of the progeny. Advanced genomic tools can help scan large genome regions to provide find out if there is any correlation between observation of higher novel AFLP bands and typical morphology of 'Amrapali'.

Mango breeding remains to be a slow process without novel strategies incorporated in the programmes. As a result, conservation programmes of mango genetic resources have not translated anything into germplasm utilization. The PGR efforts in mango need to grow beyond clonal orchards and seek to characterize and conserve products of crossing programmes. Without DNA markers, screening large number of trees at younger stage is impossible. An accurate view of inheritance of DNA markers in mango can be obtained with mapped markers distributed across linkage groups. Our investigation is a preliminary attempt in demonstrating the usefulness of DNA marker inheritance study in mango.

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