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Genomic Analysis of Open Pollinated Progenies of Mallika Mango (*Mangifera indica* L.) using RAPD Markers

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ABSTRACT

DNA-based RAPD (Random Amplification of Polymorphic DNA) markers have been used extensively to study genetic relationships in a number of fruit crops. A wide genetic diversity exists in mango fruit in India. In this study, seven commercial varieties of mango and with their corresponding open pollinated progenies of 'Mallika' (OPPM) were screened using RAPD markers with decamer primers of arbitrary sequence. Out of the 210 primers screened, 10 were selected which gave 86 clear and bright fragments. A dendrogram based on co-efficient of similarity implied a low degree of genetic diversity among the varieties and open pollinated progenies used for experimentation. The open pollinated progenies had deviated from their parents except for 'OPPM-5' and 'OPPM-13' grouping with their paternal parents. The hybrids 'Amrapali', 'Sindhu' and 'Mallika' were clustered with both their maternal and paternal parents. The dissimilarity matrix showed a maximum genetic difference of 17% between var. 'Ratna' and 'OPPM-13' genotype a progeny of 'Amrapali' and a low genetic difference of 2% between the variety 'Sindhu' and 'OPPM-5' genotypes, where 'OPPM-5' is an open pollinated progeny of 'Sindhu'. RAPD analysis proved to be a quick, simple and significant testing method to assess genetic diversity among mango populations studied.

Keywords: Cluster analysis, dendrogram, dissimilarity matrix, genetic variability, STATISTICA

INTRODUCTION

Mango (*Mangifera indica* L.) is one of the choice fruit crops primarily originating in the Indo-Burma region (Yonemori *et al.* 2002) and spread to tropical and subtropical regions of the world. India is a primary centre of origin for mango where it was domesticated and cultivated for 4000 years and from which cultivars spread to South East Asia and later to other tropical and subtropical regions. Presently, India cultivates the biggest germplasm in the world (Pandit *et al.* 2007). Australia, Brazil, China, USA, Sri Lanka, Thailand and South American countries are also rich in mango germplasm.

Mango belongs to the family Anacardiaceae and is popularly known as 'King of Fruits' and 'National fruit of India' with 1595 varieties worldwide. India is the world's largest mango producer contributing 52% of the global production (Sharma and Malhi 2001). Since mango is highly cross pollinated, seed propagation would lead to a high level of genetic variation. Hence, the aim of this study is to detect the genetic variability existing among the open pollinated progenies of 'Mallika' (OPPM) where 'Mallika', a hybrid progeny, was exposed to open pollination to induce variability from its origin by seven varieties ('Ratna', 'Sindhu', 'Alphonso', 'Amrapali', 'Mallika', 'Dashehari' and 'Neelum') and their progenies were tested for variability through RAPD (random amplified polymorphic DNA) markers.

Mango cultivars and related species are often identified by their morphological and agronomic traits. Precise information on the genetic relationships within the germplasm is necessary for carrying out efficient breeding programmes (Pandit *et al.* 2007). Previous research in mango using molecular markers includes isozymes (Degani *et al.* 1990), total protein electrophoretic assay (Zaied *et al.* 2007), amplified fragment length polymorphism (Eiadthong *et al.* 2000), RAPD (Deng *et al.* 1999; Karihaloo *et al.* 2003), variable number tandem repeats (Adato *et al.* 1995) and inter simple sequence repeats (Gonzalez *et al.* 2002; He *et al.* 2005).

In the various mango-growing regions, breeding attempts are always in progress for creating better cultivars (Pandit *et al*. 2007). The long juvenility phase of mango would make DNA based markers an extremely useful tool. They are used for identification of cultivars in the species (Kumar *et al.* 2001), management of germplasm collections (Karihaloo *et al.* 2003), identical cultivars often have different names (Schnell *et al.* 1995), grouping of varieties based on their similarities and divergence (Souza and Lima 2004), differentiate embryo types of mango (Lopez-Valenzuela *et al.* 1997), characterize fungal toxin tolerant mutations (Jayasankar *et al*. 1998), parentage analysis of some commercial mango hybrids (Srivastava *et al*. 2004). The present investigation attempted to genetically analyse the existing variability among the open pollinated progenies of 'Mallika' under the influence of different varieties.

MATERIALS AND METHODS

Plant materials

The plant materials used for the study comprised of seven commercial varieties of mango and 10 open pollinated progenies of 'Mallika' collected from orchards of Bangalore, India. Seeds were collected from 'Mallika' plants grown in a orchard amidst other varieties 'Dashehari' (OPPM-25), 'Neelum' (OPPM-16), 'Amrapali' (OPPM-1 and OPPM-13), 'Ratna' (OPPM-18), 'Sindhu' (OPPM-5 and OPPM-15), 'Mallika' (OPPM-10 and OPPM-26) and 'Alphonso' (OPPM-27) and maintained for 10 years. Recently matured leaves from the seven commercial varieties and 10-15 days-old leaves from the sown seeds (OPPM) were harvested and used for DNA extraction. Approximately 50 g of leaves from each

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variety and progenies were collected, washed using distilled water, wiped with 70% (v/v) ethanol, then dried in oven at 40°C for 20 h and powdered by using a 'Remi' mixer for 45 to 60 s, prior to storage at room temperature in sealed plastic bags.

DNA extraction

All the reagents and chemicals were obtained from Bangalore Genei, Bangalore, India of molecular biology grade. DNA was extracted from the dried leaf powder of mango by the cetyl trimethyl ammonium bromide (CTAB) method according to a modified protocol of Tai and Tanksley (1990). Specifically, 2 g of dried leaf powder was mixed with 10 ml extraction buffer, preheated to 65°C, containing 100 mM Tris-base, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 3% (w/v) CTAB, 2% polyvinyl pyrrolidone and 1% β-mercaptoethanol, then incubated at 65°C for 30 min with gentle shaking. The mixture was cooled to room temperature, to which 10 ml cold 24:1 (v/v) chloroform: isoamylalcohol was added and the contents were mixed well. After centrifugation at 6,000 × *g* for 15 min at 4°C, the supernatant was transferred to a fresh tube and the chloroform: isoamyl alcohol step was repeated until a clear supernatant was obtained. To the supernatant, 5 M NaCl was added (0.5 v/v) and mixed gently followed by addition of 0.8 volume of cold isopropanol to precipitate the DNA. The mixture was incubated overnight at 4°C and then centrifuged at 8,000 × *g* for 20 min. The resulting pellet was washed with 70% (v/v) ethanol, air-dried and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Two µg RNase (Bovine pancreatic ribonuclease) was added to each sample which was incubated for 3 h at 37°C, mixed with an equal volume of phenol and centrifuged at 6,000 × *g* for 20 min. at room temperature. This step was followed by a washing with an equal volume of 1: 1 (v/v) phenol: chloroform and then with chloroform alone. DNA was precipitated overnight at 4°C with 0.5 volume of 5 M NaCl and 1 volume of cold isopropanol and the resulting pellet obtained after centrifugation was dissolved in TE buffer, analysed on an agarose gel and quantified using a spectrophotometer (ND-8000, NanoDrop Technologies, Wilmington, USA).

PCR amplification

The PCR amplification protocol followed was according to Williams *et al*. (1990) with minor modifications. Of the 200 primers (10-mer) screened using bulk DNA, 10 showing prominent bands were selected for RAPD-PCR analysis (**Table 1**). Reproducibility of the primers was tested by repeating the PCR amplification three times under similar conditions. PCR reactions were carried out in a volume of 25 µl containing 25 ηg template DNA, 150 µM each dNTP, 1.5 mM $MgCl_2$, 1.5 unit *Taq* DNA polymerase (Sigma-Aldrich Chemicals, Bangalore, India), 5 pmol primer (OPA, OPB, OPC, OPD, OPE, OPF, OPG, OPH, OPI, OPJ and OPK series, Operon Technologies, Alameda, CA, US) in PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.05% (v/v) NP40 and 0.05% (v/v) Triton X-100). Screening of primer was done according to Prakash *et al*. (2002) to save time, cost and to reject primers that are not informative for the analysis. Amplifications were performed in a MJ Research PTC-100 Thermocycler (Bio-Rad Laboratories, Bangalore, India), programmed for an initial denaturation at 94°C for 4 min., followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, primer extension at 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were resolved in a 1.2% (w/v) agarose gel, visualised and documented using an Alpha Digidoc system (Alpha Innotech, San Leandro, CA, US).

RAPD profile analysis

Amplified fragments from each primer was manually scored for their presence (1) or absence (0) and a matrix of the different RAPD phenotypes of all eleven primers was assembled for statistical analysis by STATISTICA computer package (STATISTICA for Windows, Stat Soft Inc, Tulsa. OK, USA, 1996). The sizes of the fragments were estimated using 500 bp standard DNA markers (Bangalore Genei, Bangalore, India), co-electrophoresed with the PCR products. A genetic dissimilarity matrix was developed using Squared Euclidean Distances, which estimates all pair-wise differences in the amplification products (Sokal and Sneath 1973) and a cluster analysis was based on Wards method using a minimum variance algorithm (Ward 1963).

RESULTS AND DISCUSSION

This study showed the molecular markers generated by PCR reaction using 10 decamer oligonucleotides can be used to differentiate the varieties and OPPM genotypes in *Mangifera indica* species. The use of molecular markers to study the relationship among the different cultivars within *M. indica* has been previously reported by Karihaloo *et al.* (2003), Schnell *et al*. (1995) and our group (Kumar *et al*. 2001). However, studies on molecular differences with respect to cross pollination have not been reported. In this study, the accessions consisted of seven commercial varieties and 10 OPPM genotypes. Open pollination was expected between 'Mallika' and other varieties.

Despite mango trees being monoecious, numerous reports concerning insect fauna attending mango flowers and fruitset have been reported. Pollination in mango is mediated by insects rather than wind (Davenport and Núñez-Elisea 1997). A large number of insect species have been shown to pollinate mango, including wasps, bees, large ants and large flies (Anderson *et al*. 1982). Chadha and Singh (1964) reported zero fruitset on bagged panicles and 1.6% fruit set on unbagged panicles. Similarly, Bhatia *et al*. (1995) recorded zero fruit set on bagged panicles as compared with 4.3% set on unbagged panicles that allowed insect-free access. Similarly, Galán-Saúco *et al*. (1987) reported the production of mangos under greenhouse conditions showed no fruit set and when bees and other insects had free access, there was a significant increase in fruit set. Thus proves the effect of open pollination in mango and their progeny. The variety 'Mallika' is used as the female parent since; the probability of being the male parent is low due to mango auto-incompatibility (Cordeiro *et al*. 2006).

DNA was extracted from 2 g of dried leaf powder obtained from preferably recently matured leaves, as mature leaves were highly fibrous and rich in polyphenols and

Table 1 RAPD-PCR primers. The sequence and level of polymorphism of selected polymorphic primers in *Mangifera indica*.

S.No	Primer	Sequence (5′-3′)	№ of monomorphic bands	№ of polymorphic shared bands	№ of polymorphic unique bands	Total № of bands
1	OPA 20	GTTGCGATCC	4	7	1	12
2	OPB 01	GTTTCGCTCC	2	2	0	4
3	OPB 18	CCACAGCAGT	2	3	1	6
4	OPC 11	AAAGCTGCGG	3	7	1	11
5	OPC 12	TGTCATCCCC	2	13	0	15
6	OPC 20	ACTTCGCCAC	3	3	0	6
7	OPD 01	ACCGCGAAGG	4	4	2	10
8	OPD 06	ACCTGAACGG	5	4	0	9
9	OPD 07	TTGGCACGGG	3	2	0	5
10	OPJ 06	TCGTTCCGCA	3	4	1	8
Total			31	49	6	86

Fig. 1 Gel profile of mango using RAPD-PCR primer OPC-12. Lanes 1-7: amplification profile obtained using the varieties 'Ratna', 'Sindhu', 'Alphonso', 'Amrapali', 'Mallika', 'Dasheheri', and 'Neelum'. Lanes 8-17: amplification profile obtained using the OPPM genotypes 'OPPM-1', 'OPPM-5', 'OPPM-10', 'OPPM-13', 'OPPM-15', 'OPPM-16', 'OPPM-18', 'OPPM-25', 'OPPM-26', and 'OPPM-27'. Lane M, standard (500 bp) DNA markers.

Fig. 2 Dendrogram showing RAPD marker-based genetic relationships among 7 varieties and 10 OPPM genotypes of mango.

polysaccharides that hinder the extraction of PCR-quality DNA, as in jack-fruit (Simon *et al.* 2007). A primary screening of 210 random primers resulted in selection of 10 primers that produced clear and reproducible fragment patterns (**Table 1**). The amplification profiles of total genomic DNA from seven commercial varieties and 10 OPPM genotypes with 10 random primers produced a total of 86 fragments ranging in size from 230 bp to 2.75 kbp with an average of 8.6 bands per primer. Identical observations were observed using 20 RAPD primers by Ahmad *et al.* (2008) with an average of 7.5 bands per primer but Schnell and Knight (2003) reported an average of 11 bands per primer. Of the 86 bands, 49 (56.97%) were polymorphic and shared between at least two individuals, 31 (36.05%) were monomorphic and common to all the individuals. Six (6.98%) were polymorphic and unique. The number of fragments produced by a primer ranged from 4 (OPB 01) to 15 (OPC 12). The pattern of RAPD fragments produced by the random primer OPC-12 is shown in **Fig. 1**.

The dissimilarity matrix obtained using Euclidian Distance (Sokal and Sneath 1973) is shown in **Table 2**. The highest genetic dissimilarity among the genotypes (16%) was observed between 'Ratna' and 'Amrapali', while the least genetic dissimilarity (4%) was noticed between 'Amrapali' and 'Sindhu'. Among the OPPM genotypes, the highest genetic dissimilarity (13%) was observed between 'OPPM-5' and 'OPPM-15', 'OPPM-15' and 'OPPM-25', while the least genetic dissimilarity (2%) was noticed between 'OPPM-13' and 'OPPM-15' which were genetically closer to 'Amrapali' and 'Sindhu', 'OPPM-13' and 'OPPM-26' progenies were mostly related to 'Amrapali' and 'Mallika'. The progenies 'OPPM-16' and 'OPPM-26' were more related to 'Neelum' and 'Mallika', respectively. A notable genetic difference of 17% was observed between the hybrid 'Ratna' and 'OPPM-13' and a low genetic difference of 2% was observed between the hybrid 'Sindhu' and 'OPPM-5', where 'OPPM-5' is the open pollinated progeny of 'Sindhu'. The accessions 'OPPM-5' and 'OPPM-15' were morphologically identical to the maternal parent 'Mallika' despite as an open pollinated progeny of 'Sindhu' (paternal parent). The dendrogram clusters 'OPPM-5' and 'Sindhu' together with a genetic difference of 2%, in contrast to 'OPPM-15' in the same cluster with 11% genetic difference. This variation shows the allelic contribution of both the parents and also demonstrates the efficiency of DNA-based RAPD marker as a powerful tool to identify minute genetic differences among morphologically identical progenies and not altered by growth and environmental conditions.

The dendrogram clustered the genotypes into two major clusters ('A' and 'B') with 12 and 5 genotypes, respectively at a 4.8 linkage distance. The major cluster 'A' was divided into two minor clusters 'A_1' and 'A_2' at 3.7 linkage distance. The minor cluster 'A_1' was divided into two groups (I and II) at 3.1 linkage distance with eleven and one genotypes, respectively. Among the genotypes of group I, vars. 'Ratna' and 'Alphonso' were grouped together at one linkage distance. Genetically Alphonso is one of the parents of Ratna hybrid. Similarly, the hybrid 'Sindhu' and 'OPPM-5' the open pollinated progeny of 'Sindhu' were clustered together at one linkage distance and also clustered with 'OPPM-26' progeny of 'Mallika' and 'OPPM-16' to form a cluster at 1.3 linkage distance. The open pollinated progenies 'OPPM-1' and 'OPPM-10' were clustered together at one linkage distance and grouped with 'OPPM-25' and 'OPPM-15' at 1.3 linkage distance. It is clear from the dendrogram that all the OPPM progenies were placed closely with one another except in the case of 'OPPM-13' (**Fig. 2**).

The group II in cluster 'A' consisted of 'OPPM-27' probably a progeny of 'Alphonso' with its paternal parent in the same major cluster. The minor cluster 'A_2' consisted of

Table 2 Genetic dissimilarity matrix of seven commercial varieties and ten OPPM genotypes of mango based on polymorphism of RAPD markers.

Ratna	0																
Dashehari	10	0															
Alphonso	9	9	0														
Amrapali	16	10	9	0													
Mallika	14	10	11	6	0												
Sindhu	14	10	7	4	6	0											
Neelum	13	13	10	5	7	5	0										
OPPM-1	12	10	9	4	10	4	7	0									
OPPM-5	14	10	7	4	6	2	3	6	0								
OPPM-10	12	10	7	4	8	6	5	6	4	0							
OPPM-13	17	11	10	3	7	5	4	7	3	5	0						
OPPM-15	13	13	10	9	11	11	14	9	13	11	12	0					
OPPM-16	15	9	8	3	9	7	6	7	5	5	2	12	0				
OPPM-18	8	4	7	10	10	10	11	8	10	8	11	11	9	0			
OPPM-25	10	4	7	10	10	10	13	10	10	10	9	13	7	6	0		
OPPM-26	15	9	8	3	7	5	6	7	3	5	2	12	2	11	7	0	
OPPM-27	11	11	6	7	11	7	6	7	7	5	6	12	4	9	9	6	0

'OPPM-16'. The Major cluster 'B' consisted of five genotypes and was segregated into two minor clusters 'B_1' and 'B_2' with four and one genotypes, respectively. The genotypes 'Amrapali' and 'OPPM-13', probably a progeny of 'Amrapali' clustered at 1.0 linkage distance, subsequently with the varieties 'Neelum' and 'Dashehari'. The present investigation spaces varieties 'Neelum' and 'Alphonso' in different clusters. Similarly, 'Neelum' and 'Alphonso' were placed in separate clusters when analysed by He *et al.* (2007) using ISSR marker for chloroplast DNA but contrarily, Eiadthong *et al.* (1999) reported that 'Neelum' and 'Alphonso' were grouped together using SSR primers. The minor cluster 'B_2' with one variety 'Mallika' clustered with other genotypes of the group at 3.0 linkage distance.

This study is identical with our previous report (Kumar *et al.* 2001) in clustering the mango varieties/hybrids. The varieties 'Amrapali', 'Dashehari', 'Neelum' and 'Mallika' were clustered together and the varieties 'Alphonso' and 'Sindhu' were grouped in separate cluster in both our studies. It is also noted that predominantly, most of the open pollinated progenies are clustered together. Rahman *et al.* (2007) reported that varieties 'Mallika' and 'Amrapali' paired close to each other with the highest intervarietal similarity index of 87.30%. Previous reports on clustering pattern of Indian varieties of mango differentiated the North and South Indian cultivars into different clusters as reported by Karihaloo *et al.* (2003) and Ravishankar *et al.* (2000) using RAPD markers. Such groupings were observed to be changed by the increase of sample size and by change in selected cultivar set (Pandit *et al.* 2007). Duval *et al.* (2006) showed that the clustering pattern of the accessions were in accordance with their geographical origin and their known history. In contrast, our present study completely merges the varieties irrespectively of their origin. This was evident from the low bootstrap values in the dendrograms and also the probability of shared bands among any two cultivars. This could be because of the cultivar spreading in the recent past and higher rate of new cultivar generation in India (Pandit *et al.* 2007). It is also noted that the hybrids are clustered together in cluster 'A' 'Ratna' ('Alphonso' x 'Neelum') was grouped with 'Alphonso' and 'Sindhu' ('Ratna' x 'Alphonso') was clustered with both its parent. In cluster 'B' 'Amrapali' ('Dashehari' x 'Neelum') and 'Mallika' ('Neelum' x 'Dashehari') were grouped with both their parents. This observation elaborates the efficiency of RAPD as a dominant marker, amplifying identical alleles within the morphologically distinct varieties. The open pollinated progenies were not clustered with their maternal parent ('Mallika'), except for 'OPPM-13'. Contrastingly, Cordeiro *et al.* (2006) reported that the plantlets of the pollinated progeny showed little polymorphism and had the highest similarity with the female parent.

Understanding the spatial organization of genetic diversity within plant populations is of critical importance for the development of strategies designed to preserve genetic variation (Brown and Briggs 1991; Hamrick *et al.* 1991). It has been shown that species with limited gene flow, i.e. with restricted seed and/pollen movement, have considerably more among-population variation for total amount of genetic diversity (Schoen and Brown 1991). Since, mango is highly cross pollinated, seed propagation would lead to high level of genetic variations and the future of this fruit depends on the selection of high quality cultivars (Tous and Ferguson 1996). In this study open pollinated progenies deviated from their parents except for 'OPPM-5' and 'OPPM-13', which grouped with their paternal parents. This study effectively revealed the use of RAPD markers in estimating genetic diversity among varieties and their open pollinated progenies of mango, which could be the first step towards crop improvement programme in mango where land, time, effort and money could be saved.

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