Geographic Differentiation and Embryo Type Identification in *Mangifera indica* L. Cultivars Using RAPD Markers

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Abstract. Fifteen mango cultivars were examined using randomly amplified polymorphic DNA (RAPD) markers with decamer primers of arbitrary sequence. Thirteen of the 40 primers screened were informative and 109 amplified DNA bands were selected as RAPD markers. Specific RAPD markers for some mango cultivars were identified. Cluster analysis based on the 109 RAPD markers produced a dendrogram of the genetic relatedness of the 15 mango cultivars. 'Manila' and 'Carabao' were the most similar, which is in good agreement with their putative pedigrees. The four major bifurcations in the dendrogram separated correctly the genotypes into four groups according to their geographic origin. Bulk segregant analysis of polyembryonic and monoembryonic cultivars detected a specific RAPD marker for polyembryony. These markers may facilitate the management of mango germplasm for breeding purposes.

Mango is considered an allopolyploid ($2n = 40$), most probably amphidiploid, outbreeding species (Mukherjee, 1950). This fruit crop probably originated in the Indo-Burma region (Mukherjee, 1951, 1972) and has been cultivated in India and Southeast Asia for more than 4,000 years (Malo, 1970). Beginning in the 16th century, mangoes were gradually distributed to the tropical and subtropical regions around the world. It is likely that the Portuguese transported mangoes to east and west Africa, adjacent island possessions and subsequently to Brazil. It was introduced into Mexico from the Philippines by the Spaniards. The earliest introduction in the United States appears to have been in Florida from Mexico in 1833 (Mukherjee, 1951, 1972; Sauer, 1993). After the geographic spread of mango, uncontrolled pollinations produced extremely heterogeneous populations from which new selections were made that were particularly well suited to each area (Malo, 1970). The huge diversity in mango genotypes may also have

resulted from a long period of cultivation, thereby giving ample opportunity for hybridization and forces of selection to operate (Mukherjee, 1951). Little is known about the genetic control of diversity or pattern variation.

Mango genotypes are commonly divided into two general classes: 1) the polyembryonic, generally referred to as the Indochinese type, and 2) the monoembryonic as the Indian type (Rhodes et al., 1970). The occurrence of nucellar embryony (apomixis) in mango is of great horticultural importance because the nucellar seedlings are genetically identical to the mother plant, thus allowing propagation of superior clones (Sauer, 1993). Nucellar seedlings are used for rootstock propagation, but recombinant zygotic seedlings are of interest in breeding programs. The effectiveness of isozymes for distinguishing zygotic from nucellar seedlings has enabled the early identification of the progeny from crosses involving polyembryonic parents (Degani et al., 1993; Schnell and Knight, 1992). However, no reports have been published about the identification of specific markers for this trait in mango.

Historically, mango genotypes have been characterized by using morphological traits (Rhodes et al., 1970). However, phenotypically indistinguishable trees can differ genotypically (Schnell and Knight, 1992). Isozyme analysis has been used to determine parentage for some mango cultivars (Degani et al., 1990), but the isozyme polymorphism is limited.

Recently, DNA markers, such as variable number tandem repeats (VNTRs) (Adato et al., 1995) and randomly amplified polymorphic DNA (RAPD) (Schnell et al., 1995) have been used for cultivar identification and evaluation of genetic relationships in mango. These molecular markers have been proposed as tools to assist breeders in selecting desirable genotypes.

RAPD analysis involves the amplification of DNA segments, commonly using arbitrary 10-base primers to find polymorphic regions within the genome (Welsh and McClelland, 1990; Williams et al., 1990). The advantages of using RAPD, rather than other genetic markers such as restriction fragment length polymorphisms (RFLP) include low requirement for DNA and time and no need for radioisotopes. Modifications of the original method, such as analysis of bulk segregants (Michelmore et al., 1991) have further extended the importance of this technique, especially in the identification of markers linked to disease resistance genes (Koller et al., 1994; Poulsen et al., 1995).

Because of the potential benefits of RAPD analysis for the management of germplasm and breeding in mango, we estimated the genetic diversity among mango cultivars using RAPD markers. Our objectives were to determine genetic relationships and to identify specific markers for polyembryonic/monoembryonic genotypes.

Materials and Methods

Plant material. Young leaves from 15 mango cultivars (Table 1) were collected from the field germplasm collection at Campo Experimental Valle de Culiacan, Culiacan, Sinaloa, Mexico. All leaf samples were frozen for transportation to the laboratory and subsequently stored at -70°C until processed.

DNA extraction. Total DNA was extracted as described by Doyle and Doyle (1990) with modifications. Frozen leaf tissue (2 g) was ground to a fine powder in liquid N using carborundum as an abrasive. The fine powder was added to 10 mL of preheated (60°C) extraction buffer [3% (w/v) CTAB; 1.4 M NaCl; 0.1% (v/v) β -mercaptoethanol; 20 mM EDTA; 100 mM Tris-HCl (pH 8.0)] and incubated at 60°C for 30 min. The homogenate

Table. 1. Mango cultivars used in RAPD analysis.

Cultivar	Geographic origin	Embryo type
Ataulfo	Mexico	Polyembryonic
Diplomatico	Mexico	Polyembryonic
Esmeralda	Mexico	Polyembryonic
Manililla	Mexico	Polyembryonic
Oro	Mexico	Polyembryonic
Piña	Mexico	Polyembryonic
Thomas A	Mexico	Polyembryonic
Carabao	Philippines	Polyembryonic
Manila	Philippines	Polyembryonic
Haden	Florida	Monoembryonic
Kent	Florida	Monoembryonic
Keitt	Florida	Monoembryonic
Sensation	Florida	Monoembryonic
Tommy Atkins	Florida	Monoembryonic
Julie	Reunion Is.	Monoembryonic

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was extracted once with 24 chloroform : 1 isoamyl alcohol (v/v), after which the nucleic acids were precipitated overnight in cold isopropanol (-20°C). Nucleic acids were treated with RNase A and extracted once with 25 phenol : 24 chloroform : 1 isoamyl alcohol (by volume), after which the DNA was precipitated in cold ethanol (-20°C). DNA concentration was determined spectrophotometrically.

RAPD analysis. DNA amplification was based on the method described by Williams et al. (1990) using decamer primers of arbitrary sequence (Operon Technologies, Alameda, Calif.). PCR reactions were performed in a volume of 25 μL containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM MgCl_2 ; 0.1 mM each dATP, dCTP, dGTP, dTTP; 0.3 μM primer; 25 ng of genomic DNA and 3 units of *Taq* DNA polymerase. Because of the high cost of *Taq* DNA polymerase, it was produced using a recombinant *Taq* DNA polymerase purified from *Escherichia coli* (Engelke et al., 1990; Pluthero, 1993). Each reaction was overlaid with 25 μL of mineral oil. Amplification was performed in a DNA Thermal Cycler 480 (Perkin Elmer Cetus, Norwalk, Conn.) programmed for 45 cycles of 1 min at 92°C , 1 min at 35°C and 2 min at 72°C , with a final stage of 10 min at 72°C . After amplification, samples of 20 μL were loaded onto 1.4% agarose gels and electrophoresis was carried out at 5 V cm^{-1} for 3.5 h in 1 $\times$ TAE buffer [40 mM Tris-acetate (pH 8.0); 1 mM EDTA] (Sambrook et al., 1989). After staining in ethidium bromide at 2 $\mu\text{g}\cdot\text{mL}^{-1}$ for 10 min, the gels were photographed under UV with Polaroid 667 film. All reactions were repeated at least twice and genomic DNA was omitted in control reactions for each primer. Each amplified band was named by the primer used and its size in base pairs (bp).

Genetic diversity estimation. Initially 40 primers, 20 from each of kits M and X (Operon Technologies, Alameda, Calif.) were screened using the DNA from 'Julie', 'Manila', 'Piña', and 'Tommy Atkins'. Primers that generated polymorphisms among these four cultivars were tested with the remaining cultivars identified (Table 1).

The band patterns obtained by each selected primer were scored by visual inspection of the gel photographs from at least two independent experiments, and only the reproducible bands were considered. The bands were recorded as present (1) or absent (0). Genetic distance values for each pairwise comparison between genotypes were calculated as reported by Skroch et al. (1992) and a genetic distance matrix was constructed. This matrix was used to generate a dendrogram using the unweighted pair-group method with arithmetic averages (UPGMA) (Sokal and Michener, 1958) provided by the cluster procedure of the computer program SAS (SAS Institute, 1989).

Identification of markers for polyembryony/monoembryony. Polymorphic RAPD fragments were initially identified using 40 primers on bulked DNA samples of the nine polyembryonic and the six monoembryonic cultivars, on the basis of bulk segregant analysis (Michelmore et al., 1991). All 15 cultivars

were then screened individually for the presence or absence of the polymorphic markers amplified with the primers selected from the analysis of bulked standards.

Results and Discussion

Genetic diversity estimation. Of the 40 primers tested, 13 were ultimately selected for further evaluation which amplified 109 reproducible bands, 81 (74%) of which were polymorphic among the *M. indica* cultivars (Table 2). Examples of polymorphic bands generated by OPM09 primer are shown in Fig. 1. No single primer produced unique banding patterns for all 15 cultivars, which agrees with the results reported by Schnell et al. (1995). Based on combined banding patterns, all 15 mango cultivars could be identified by using 28 combinations of two selected primers.

Four mango cultivars could be distinguished from the rest by specific markers: 'Diplomatico' by OPX15-480, 'Julie' by OPM09-600 (Fig. 1), 'Manililla' by OPX18-550 and 'Piña' by OPM06-600. Markers OPM15-380, OPX01-320, and OPX20-400 were Philippines cultivars specific, while OPM20-350 and OPX20-520 were Florida cultivars specific.

Cluster analysis (UPGMA) based on the 109 RAPD markers, produced a dendrogram of the genetic relatedness of the 15 mango cultivars (Fig. 2). 'Manila' and 'Carabao' clustered together and were the most similar. 'Manila' was introduced into Mexico from the Philippines by the Spaniards (Sauer, 1993)

and is believed to be the same cultivar known as Carabao (Mathews and Litz, 1992). In the present study, seven of the 13 selected primers detected small differences (one or two bands) between these cultivars. 'Manila' has been propagated by seed and probably this genetic variation resulted from the occasional zygotic seedlings.

The four major bifurcations in the dendrogram (Fig. 2) separated correctly the genotypes into four main clusters according to their geographic origin. The UPGMA analysis of the genetic distance values obtained, using the Nei and Li (1979) model, produced a dendrogram with the same topology (not shown). The cultivars Julie, Manila, Piña, and Tommy Atkins were used to select primers because of their differing geographic origins and backgrounds and were expected to display a high polymorphism. This selection may have contributed to the separation of the genotypes according to their geographic origin in the dendrogram (Fig. 2). Certainly, this study has shown the usefulness of RAPD by identifying which primers can be used to discriminate among mango genotypes from different regions.

Within the Florida cluster, cultivars Keitt and Sensation were the most similar. 'Tommy Atkins' was a seedling of 'Haden' (Schnell et al., 1995) and clustered appropriately. However, 'Haden' clustered together with 'Kent', a seedling of 'Brooks'. Adato et al. (1995) reported that 'Kent' was genetically very close to some cultivars originated from the 'Haden' and 'Brooks' cross. These results suggest that

Table 2. Sequence of the selected primers and size of the corresponding RAPD markers.

Primer	Sequence (5' - 3')	Marker size (base pairs)
OPM05	GGGAACGTGT	950, 920, 800 ^a , 650, 620, 580, 530, 450 ^a
OPM06	CTGGGCAACT	1100, 950 ^a , 750, 700, 600, 550, 480
OPM09	GTCTTGCGGA	1600 ^a , 1150, 1100, 1000, 900, 850, 800, 750 ^a , 600, 500, 450
OPM12	GGGACGTTGG	750 ^a , 550, 500 ^a , 370 ^a
OPM15	GACCTACCAC	1050, 950 ^a , 850, 750, 650, 600, 500, 380, 320
OPM20	AGGTCTTGGG	900 ^a , 800, 750, 550, 500 ^a , 450, 350
OPX01	CTGGGCACGA	950, 850 ^a , 700 ^a , 650, 550, 450 ^a , 320, 280
OPX02	TTCCGCCACC	1100, 1050, 850, 750 ^a , 650, 500 ^a , 280 ^a
OPX08	CAGGGGTGGA	850, 800, 750, 650 ^a , 550 ^a , 450
OPX15	CAGACAAGCC	1200, 1050 ^a , 950, 850 ^a , 750, 720, 700, 600, 480
OPX17	GACACAAGCC	1350 ^a , 1000 ^a , 970, 800, 720 ^a , 670, 650, 600, 500, 450, 400, 320
OPX18	GACTAGGTGG	1100, 1000 ^a , 850, 800, 750, 650, 600, 550, 500, 450
OPX20	CCCAGCTAGA	1300, 1100 ^a , 650, 580 ^a , 550, 520, 500, 450 ^a , 400, 380, 350

^aMonomorphic marker (i.e., marker identified in all the 15 mango cultivars).

Fig. 1. DNA banding pattern produced by OPM09 primer in 15 mango cultivars: 1) 'Tommy Atkins'; 2) 'Julie'; 3) 'Sensation'; 4) 'Kent'; 5) 'Keitt'; 6) 'Haden'; 7) 'Ataulfo'; 8) 'Manila'; 9) 'Esmeralda'; 10) 'Thomas A'; 11) 'Carabao'; 12) 'Piña'; 13) 'Diplomatico'; 14) 'Manililla'; and 15) 'Oro'. Arrow indicates the 'Julie' specific marker. M = molecular size markers (1 kb ladder).

'Kent' may have resulted from a 'Haden' and 'Brooks' cross.

In Mexico, most cultivars have originated as chance seedlings with unknown parents. Because of the commercial importance of 'Manila' and some Florida cultivars, probably many accessions have originated from these cultivars. According to the dendrogram (Fig. 2), Mexico and Florida clusters were the most similar. Additional studies are necessary to decipher parentage of these mango cultivars.

The dendrogram can also be used to select the most appropriate parental material to improve horticultural traits. For example, 'Diplomatico', 'Esmeralda' and 'Thomas A' are dwarfing mango cultivars and they are genetically very close. If further improvement of the dwarfing trait is desired, it is more likely to generate sufficient genetic variability by crossing one of these cultivars with 'Julie', which is classified as dwarfing too.

Identification of markers for polyembryony/monoembryony. The polyembryonic and monoembryonic bulks differed for 29 RAPD markers. Only one of the RAPDs that was present in the polyembryonic bulk (Fig. 3A) was present appropriately in all of the polyembryonic cultivars and none of the monoembryonic cultivars (Fig. 3B). The approximate fragment size was 550 bp and was produced by OPM12 primer.

Analyses of the progenies of monoembryonic cultivars hybridized with polyembryonic ones indicate that the polyembryony is a recessive trait and is probably controlled by a single pair of genes (Sturrock, 1968). Because of the dominant nature of RAPD markers, our results suggest that polyembryony may be a dominant trait. However, segregation studies are required to know if the identified RAPD marker is linked to polyembryony in mango and to establish the inheritance mode of this trait.

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Fig. 2. Dendrogram of 15 mango cultivars constructed by UPGMA (unweighted pair-group method with arithmetic averages) cluster analysis based on 109 RAPD markers.

Fig. 3. Specific RAPD marker identification for polyembryonic mango cultivars (A) Polymorphism (arrow) detected between polyembryonic (p) and monoembryonic (m) DNA bulks by OPM12 primer. (B) DNA banding pattern produced by OPM12 primer in the 15 mango cultivars: 1) 'Tommy Atkins'; 2) 'Julie'; 3) 'Sensation'; 4) 'Kent'; 5) 'Keitt'; 6) 'Haden'; 7) 'Ataulfo'; 8) 'Manila'; 9) 'Esmeralda'; 10) 'Thomas A'; 11) 'Carabao'; 12) 'Piña'; 13) 'Diplomatico'; 14) 'Manililla'; and 15) 'Oro'. Arrow indicates the specific marker for polyembryonic cultivars. M = molecular size markers (1 kb ladder).

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