Application of Random Amplified Polymorphic DNA Markers for Identification of Marula Genotypes

Feiga Gutman, Avinoam Nerd, and Yosef Mizrahi

The Institutes for Applied Research, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

Dudy Bar-Zvi and Dina Raveh

Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

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Abstract. Twenty-four genotypes of marula (*Sclerocarya birrea* subsp. *caffra*) were characterized using randomly amplified polymorphic DNA (RAPD) analysis. A distinct band pattern was obtained for each of the trees, using as few as four arbitrary 10-mer primers. Trees propagated vegetatively by grafting showed identical fingerprints. These results suggest that RAPD markers provide a useful system for documenting the identity of marula genotypes.

Marula (Anacardiaceae) is a large, dioecious, deciduous tree native to southern Africa (Shone, 1979). The yellow, plum-sized fruit has an aromatic, sweet-sour flesh, rich in ascorbic acid, and a large stone with an edible kernel. Fruits are consumed fresh or utilized for processing into liqueurs, nectar, and jelly (Weinert et al., 1990). The nut (kernel) is an important source of oil. In southern Africa, fruits are usually collected from native stands. The range of crops that can be grown commercially in hot desert regions is very limited. To determine the commercial potential of the tree as a multipurpose crop, vegetative performance and fruiting of seed-propagated plants were investigated in introduction plots located in various parts of the Negev Desert of Israel (Nerd and Mizrahi, 1993). The plants are tolerant of high summer temperatures (47 °C) and irrigation with brackish water. Selected phenotypes were planted in additional test plots (Goldman et al., 1996). Selection was based on yield, time of fruit abscission, and fruit characteristics. Visual identification of the selected plant material is difficult because some of the trees are morphologically similar, and morphological traits may vary with environmental conditions. In principle, this disadvantage can be circumvented by direct identification of genotypes using DNA fingerprinting techniques (Caetano-Anolles and Gresshoff, 1994; Mulcahy et al., 1993; Tingey

and Tufo, 1993; Williams et al., 1990). Randomly amplified polymorphic DNA (RAPD) analysis has recently been employed to identify cultivars of many fruit crops, such as apple (*Malus ×domestica* Borkh.) (Gardiner et al., 1996), grape (*Vitis* sp.) (Qu et al., 1996), strawberry (*Fragaria ×ananassa* Duch.) (Graham et al., 1996), and cranberry (*Vaccinium macrocarpon* Ait.) (Novy and Vorsa, 1996). The objective of the present study was to examine whether RAPD markers could serve for the identification of marula genotypes.

Plant material. The study was completed in 1997 on 12 seed-propagated plants and 12 vegetatively propagated clones grafted onto marula seedlings at two sites in the Israel Negev Desert, viz., Beer Sheva and Revivim (4- and 6-year-old orchards, respectively). Batches of seeds collected from natural stands either in the center of Botswana (Gaborone region), in northeastern South Africa (Kruger Park), or from a marula plot established from seeds in southern Israel were used as sources for seed (Table 1). Selected trees from the sites of seed collection were used for vegetative propagation. The trees were irrigated and fertilized year round (Nerd and Mizrahi, 1993).

RAPD analysis. DNA was extracted from 1 g of young leaves, using the method described by Porebski et al. (1997), and its concentration was estimated by gel electrophoresis on 0.6% agarose, followed by comparison with a DNA sample of known concentration. Reactions were carried out in 25-μL volumes containing: 75 mM Tris-HCl, pH 8.8; 20 mM (NH₄)₂SO₄; 0.01% Tween 20; 2.5 mM MgCl₂; 200 μM each of dNTPs; 0.4 μM primer; 0.8–2.4 ng of genomic DNA; and 1 unit of Taq DNA polymerase (Advanced Biotechnologies, U.K.). The primers used were the twenty 10-base oligomeric primers from Operon Kit A (Operon Technologies, Alameda, Calif.). Amplification was performed using an Omnigene Thermocycler (Hybaid, Teddington, U.K.). Reaction conditions included four cycles of 1 min at 94 °C, 3 min at 36 °C, and 3 min at 72 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 36 °C, and 1 min at 72 °C. Amplified DNA products were resolved on a 1% agarose gel, visualized by ethidium bromide staining.

Table 1. Origins and characteristics of investigated marula trees.

Specimen	Propagation method	Origin ^z	Gender ^y	Abscission time ^x	Fruit size ^w
BS 1-7	Grafting	South Africa	F	ND	Large
BS 1-8	Grafting	Botswana	F	ND	Large
BS 1-9	Grafting	Botswana	F	ND	Large
BS 2-8	Grafting	Botswana	F	ND	Large
BS 3-8	Grafting	Botswana	F	ND	Large
BS 4-8	Grafting	South Africa	F	ND	Large
BS 6-1	Grafting	South Africa	F	ND	Large
BS 6-3	Grafting	South Africa	F	ND	Large
BS 6-5	Seed	Israel	M	---	---
BS 6-7	Seed	Israel	M	---	---
BS 6-9	Grafting	South Africa	H	Intermediate	Large
Q 2	Grafting	Israel	F	Intermediate	Small
Q 17	Grafting	Israel	F	Late	Medium
Q 31	Grafting	Israel	M	---	---
R 2-4	Seed	South Africa	F	Late	Small
R 3-21	Seed	Botswana	M	---	---
R 4-12	Seed	South Africa	M	---	---
R 4-17	Seed	South Africa	M	---	---
R 4-18	Seed	Botswana	F	Intermediate	Medium
R 5-4	Seed	Botswana	F	Intermediate	Large
R 5-11	Seed	Botswana	F	Early	Small
R 5-20	Seed	South Africa	F	Late	Medium
R 5-23	Seed	South Africa	M	---	---
R 5-24	Seed	Botswana	F	Early	Medium

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^zPlant material for propagation was obtained from natural stands in the Gaborone region of Botswana and in the northeastern region of South Africa, and from a marula plot of seedlings located in the south of Israel.

^yF = female; H = hermaphrodite; M = male.

^xND = not determined; early = July–September; intermediate = October–December; late = January–March.

^wSmall = 20–30 g; medium = 30–50 g; large = 50–70 g.

Table 2. Results of the amplification of marula DNA using twenty 10-bp primers.

Primer	Sequence (5' to 3')	No. of amplified fragments	No. of polymorphic fragments
A01	CAGGCCCTTC	6	3
A02	TGCCGAGCTG	2	—
A03	AGTCAGCCAC	6	1
A04	AATCGGGCTG	5	—
A05	AGGGGTCTTG	7	—
A06	GGTCCCTGAC	0	—
A07	GAAACGGGTG	5	—
A08	GTGACGTAGG	2	—
A09	GGGTAACGCC	5	—
A10	GTGATCGCAG	4	—
A11	CAATCGCCGT	4	—
A12	TCGGCGATAG	0	—
A13	CAGCACCCAC	11	7
A14	TCTGTGCTGG	7	—
A15	TTCCGAACCC	2	—
A16	AGCCAGCGAA	0	—
A17	GACCGCTTGT	0	—
A18	AGGTGACCGT	10	8
A19	CAAACGTCGG	6	—
A20	GTTGCGATCC	0	—

Fig. 1. RAPD patterns obtained from marula trees: (A) Amplification products obtained using primer A01. Lanes 1-8, DNA from trees BS6-1, BS6-3, BS6-9, BS4-8, BS3-8, BS2-8, BS1-9, and BS1-7; M - molecular size marker, bp (IDNA/Eco 471). (B) Amplification products obtained using primer A03. Lanes 1-8, DNA from trees BS6-1, BS6-3, BS6-9, BS4-8, BS3-8, BS2-8, BS1-9, and BS1-7; M - molecular size marker, bp (IDNA/Eco 471). (C) Amplification products obtained using primer A13. Lanes 1-8, DNA from trees BS1-8, BS1-9, BS6-5, BS6-7, Q31, R2-4, R3-21, and R4-12; M - molecular size marker, bp (IDNA/Eco 471). (D) Amplification products obtained using primer A18. Lanes 1-8, DNA from trees R2-4, R3-21, R4-12, R4-17, R4-18, R5-4, R5-11, and R5-20; M - molecular size marker, bp (IDNA/Eco 471).

and photographed. Polymorphic bands were scored as + (band present) or - (band absent). The reproducibility of the method was tested by comparing three different accessions of the same tree.

Results and Discussion

Marula trees used in the investigation and their origin and characteristics are presented in Table 1. For trees in Beer Sheva (designated by the letters "BS"), which had not yet begun to fruit at the time of the experiment, information on gender and fruit size was subsequently

supplied by breeders. Data cited for Revivim (trees designated with a "Q" or "R") were obtained in the orchard, where trees were in the second year of fruiting. Female trees showed a high degree of diversity with respect to time of abscission and fruit size.

Twenty 10-mer primers were used to amplify marula DNA. Fifteen out of the 20 primers tested gave from two to 11 reproducible RAPD bands from all templates (Table 2). Of these 15 primers, four (A01, A03, A13, and A18) gave bands that were unique to a subset of the trees (Fig. 1A-D), whereas 11 primers amplified DNA fragments that failed to show

polymorphism among the 24 marula trees tested.

The largest numbers of specific bands were obtained with primers A13 and A18 (7 and 8, respectively). Patterns produced using primers A01 and A03 showed a lower level of polymorphism (Table 3). Based on RAPD patterns obtained with primer A18, tree-specific patterns emerged for 14 trees. Ten of the remaining trees could be grouped into four subsets, each showing the same pattern: BS1-7, BS6-1, Q31; BS1-8, BS1-9, BS4-8; R2-4, R3-21; and R4-17, R4-18. Within each subset, trees were further distinguished using primer A13. A subset of the trees BS1-7, BS6-1, Q31 was distinguished using polymorphic bands A13-0.6 and A13-0.9. These bands also allowed us to distinguish between trees R4-17 and R4-18. In the subset of trees BS1-8, BS1-9, and BS4-8, genotype BS1-8 was identified using band A13-0.9. Distinction not revealed by primers A13 and A18 emerged between genotypes BS4-8 and BS1-9, R2-4 and R3-21 when primer A03 (A03-0.9) was used, whereas primer A01 revealed a unique band in BS6-5. The identification of marula clones using RAPD markers is summarized in Fig. 2.

Thus, four primers sufficed to uniquely characterize all 24 trees tested by number and size of fragments. Initial screening with multiple primers is essential in order to select markers yielding distinctive patterns for each given genotype (Fritsch et al., 1993). While many authors report difficulties in reproducing RAPD patterns from one experiment to another (e.g., Sweeney and Danneberger, 1997), we found that different DNA preparations from the same marula trees gave totally reproducible fingerprints. Moreover, vegetatively propagated grafts exhibited an RAPD pattern identical with that of the mother tree (Fig. 3).

All 24 trees tested presented different RAPD patterns, although some of them were morphologically identical. Furthermore, clones originating from the same geographical area revealed different DNA fingerprints. The patterns are reproducible, as evidenced by their conservation in vegetatively propagated grafts. DNA fingerprinting of marula should therefore become the method of choice for registering proprietary cultivars.

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Table 3. RAPD summary of marula genotypes obtained with different primers.

Primer:	A18								A13						A01				A03	
	Fragment size (kb)																			
Tree	>2.0	1.3	0.9	>0.9	>0.6	0.6	0.5	0.4	>1.3	1.3	<1.3	>0.9	0.9	<0.9	0.6	0.9	0.6	0.4	0.9	
BS 1-7	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	+	-	+	-	
BS 1-8	-	-	-	+	-	-	-	-	+	-	-	-	+	-	+	-	-	+	+	
BS 1-9	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	+	+	
BS 2-8	-	-	+	+	-	+	-	+	+	-	-	-	+	-	+	-	-	+	+	
BS 3-8	-	-	-	+	-	-	+	-	+	-	-	-	-	-	+	+	-	+	+	
BS 4-8	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	+	-	
BS 6-1	-	-	-	+	-	+	-	-	+	-	-	-	-	-	+	-	-	+	+	
BS 6-3	+	-	-	+	-	-	+	-	+	-	+	-	+	+	+	-	-	+	+	
BS 6-5	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	-	+	
BS 6-7	+	-	-	+	-	-	-	-	-	+	-	+	-	-	+	+	-	+	+	
BS 6-9	-	-	-	+	-	+	-	+	+	-	-	-	+	+	+	-	-	+	+	
Q 2	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	
Q 17	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	
Q 31	-	-	-	+	-	+	-	+	+	-	-	+	+	-	-	-	-	+	+	
R 2-4	-	-	+	-	+	-	-	-	+	-	-	-	+	-	+	-	-	-	+	
R3-21	-	-	+	-	+	-	-	-	+	-	-	-	+	-	+	-	-	-	-	
R 4-12	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	
R4-17	-	+	-	+	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	
R 4-18	-	+	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	
R 5-4	-	-	+	-	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	
R 5-11	-	-	+	-	+	-	+	-	-	-	-	-	+	-	+	-	-	-	-	
R 5-20	-	-	+	+	+	-	-	-	+	-	-	-	+	-	+	-	-	-	+	
R 5-23	-	-	-	+	+	-	+	-	+	-	+	+	+	-	-	-	-	-	+	
R 5-24	-	-	-	+	+	-	-	-	+	-	-	-	+	-	-	-	-	-	+

Fig. 2. Identification of marula genotypes using RAPD markers. Polymorphic bands included A18-2.0, A18-1.3, A18-0.9, A18>0.6, A18-0.6, A18-0.5, A13-0.9, A13-0.6, and A03-0.9.

Fig. 3. RAPD patterns of marula produced with primer A18 and DNA preparations from mother trees and vegetatively propagated grafts. Lane 1, Q2-mother tree; lanes 2-4, Q2-grafts; lane 5, Q17-mother tree; lanes 6-8, Q17-grafts; M - molecular size marker, bp (IDNA/Eco 471).

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