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RAPD Polymorphisms Among Pistachio (*Pistacia vera* L.) Cultivars

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Abstract

The Random Amplified Polymorphic DNA (RAPD) technique was used to characterize 15 cultivars of pistachio (*Pistacia vera* L.). Thirty-three decamer oligonucleotide primers were screened and 14 of these produced 37 reproducible polymorphic bands which are useful as markers. Each cultivar exhibits a unique molecular phenotype and, as a consequence, can be uniquely identified using these selected markers. Our results suggest that RAPD analysis can provide a new alternative for pistachio cultivar identification and classification, and can be effectively incorporated into pistachio breeding programs.

The pistachio tree (*Pistacia vera* L.) is a deciduous, dioecious, and wind-pollinated member of the Anacardiaceae which also includes cashew, mango, poison ivy, poison oak, pepper tree, and sumac. There are relatively few cultivars of *P. vera* described, probably less than 100 (9). Most pistachio cultivation worldwide is based on clonally propagated scion cultivars grafted onto seedling rootstocks of other *Pistacia* species and hybrids. *P. vera* cultivars are thought to be derived from only a few primitive varieties (9). Typically, few cultivars are grown in any pistachio-producing region. The California pistachio industry, for example, relies almost exclusively on two seedling selections: 'Kerman,' a nut-producing female cultivar, and 'Peters,' a male cultivar used as a pollinizer. It is only within the last 50-60 years that breeding programs have been dedicated to *Pistacia*. As is typical for breeding tree-crop species, progress has been slow and many of the selections are still in experimental collections (1, 10, 12, 14, 17).

Few studies have been conducted in pistachio to establish intra- and interspecific genetic relationships, study patterns of inheritance, or determine breeding histories. These have traditionally been based on morphological, physiological, and biochemical data (2, 5, 7, 18). Even fewer studies (3, 8) have integrated morphological traits and isozyme markers to distinguish successfully species and varieties. A major limitation of isozyme markers, the lack of sufficient polymorphism among closely related cultivars, can be mitigated by the use of DNA-based diagnostic marker assays. Restriction Fragment Length Polymorphisms (RFLP) have been a widely used technique to produce DNA markers (11). Williams et al. (15) and Welsh and McCelland (13) developed the Random Amplified Polymorphic DNA (RAPD) assay that has proven to be a useful tool for genetic typing and mapping. This strategy uses randomly generated oligonucleotide primers to initiate Polymerase Chain Reaction (PCR) amplification of discrete but arbitrary portions of the genome. The number and sizes of the fragments generated is determined by the nucleotide sequence of the primer used and by the source of the template DNA. Because of the very large number of possible sequence permutations per primer, the number of markers detectable is potentially unlimited (16). RAPDs have

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several advantages relative to co-dominant isozyme and RFLP markers, and, as a consequence, are potent molecular markers, not only for fingerprinting and mapping purposes, but also for assessing the validity of controlled crosses, producing genetic markers linked to specific phenotypic traits, identifying clones and parental lines, and aiding in the study of population dynamics (16). We have used this technique to identify a marker linked to sex determination in pistachio using Bulked Segregant Analysis (6), and in this paper we report the use of RAPD markers for the characterization and fingerprinting of *P. vera* genotypes.

Materials and Methods

Plant material and DNA extraction

A total of fifteen cultivars of *P. vera* were used in this study (Table 1). These were obtained from a *Pistacia* collection maintained at the USDA Germplasm Repository located at the University of California Wolfskill Experimental Orchard in Winters, California. We selected female and male cultivars representing diverse pistachio-growing regions. Young leaves were collected in spring and stored at -70°C prior to DNA extraction.

Total DNA was extracted from leaf tissue following the CTAB method of Doyle and Doyle (4) with minor modifications. Young leaves (5.0 g) were ground in liquid nitrogen and mixed with 20 ml of CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% β-mercaptoethanol, 0.1% $NaHSO_3$). Samples were incubated at 65°C for 1 hour, mixed with an equal volume of chloroform-isoamyl alcohol (24:1), and centrifuged at 2,000 g for 15 min. The aqueous phase was recovered and mixed with 2/3 volume of isopropanol. The nucleic acid precipitate was recovered with a glass hook, washed with 10 ml of 10 mM ammonium acetate in 76% ethanol, dried overnight, and resuspended in 1 ml of modified TE buffer (10 mM Tris-HCl, 0.1 mM EDTA). Extracted DNA was diluted to 10ng/μl and used for PCR amplification.

RAPD analysis

Thirty-three decamer oligonucleotides (Operon Technologies, Alameda, CA, USA) were used for PCR amplifications following the procedure of Williams et al. (15), with some modifications. Amplification reactions were carried out in 25 μl volumes containing 10 mM tris-HCl, pH 8.3, 50 mM KCl, 1.9 mM $MgCl_2$, 0.001% gelatin, 100 μM each of dATP, dGTP, dCTP, and dTTP (Promega, Madison, WI), 0.4 μM primer, 0.75 units of AmpliTaq DNA polymerase (Perkin-Elmer-Cetus, Norwolk, CT) and 50 ng of genomic DNA. Each reaction mixture was overlaid with 25 μl of mineral oil to prevent evaporation. In order to destroy putative carryover products, reaction mixtures and mineral oil were placed on a UV (300 nm) transilluminator prior to addition of template DNA and DNA polymerase. DNA amplification reactions were performed in a MJ-Research Inc. (Watertown, MA) Minicycler programmed for 1 cycle of 2 min. at 94 °C followed by 40 cycles of 45 sec. 92 °C, 1 min. at 36 °C, 2 min. at 72 °C, for denaturing, annealing and primer extension, respectively. The last cycle was followed by a final incubation for 5 min. at 72°C and the PCR products were stored at 4°C prior to analysis. Amplification products were analyzed by gel electrophoresis in 2% SeaKem agarose (FMC, Rockland, ME) in 1X TBE buffer, stained with ethidium bromide and photographed under UV light using Type 57 Polaroid film. The molecular sizes of the amplification products were estimated using a 123 bp DNA Ladder (Sigma Chemical Company). All reactions were repeated at least three times using different lots of Taq polymerase and only reproducible bands were scored for further analysis. Each amplification fragment useful for discrimination between gen-

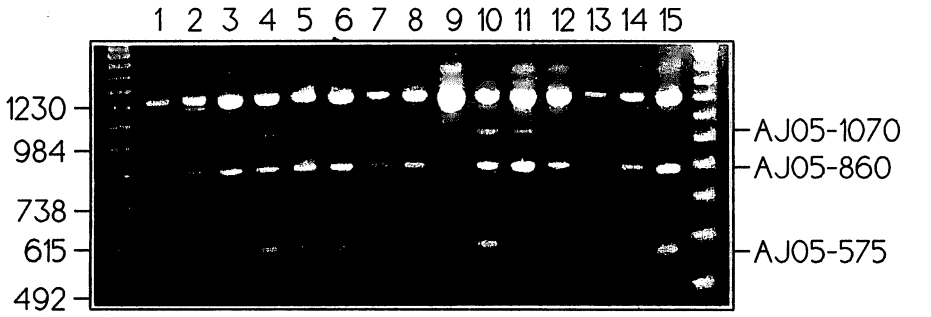


Figure 1. RAPD pattern obtained from the 15 pistachio cultivars with the primer AJ-05. Cultivar numbers correspond to those in Table 1.

otypes was named by the primer used and its approximate size in base pairs.

Results and Discussion

A total of 33 arbitrary primers were screened to compare banding patterns and to identify polymorphic fragments between 15 cultivars of *P. vera*. Almost all primers successfully amplified DNA fragments from pistachio DNA as illustrated for the primer AJ-05 in Fig. 1. Fourteen primers yielded polymorphic banding patterns among the 15 cultivars and were used for further analyses (Table 2). Overall, these 14 primers revealed 143 fragments, 37 of which were polymorphic and reproducible; these 37 were selected as useful markers. The number of amplified fragments generated varied between 6 and 14 per primer with an average of 9.9 and the number of polymorphic fragments varied from 1 to 6. The size of the amplified products ranged from 310 to 1,730 bp. All the markers were scored for presence or absence of the specific amplification product.

No cultivar-specific marker was found although two cultivars were distinguished by differences in a single marker: AJ05-860 is absent in 'Damghan' and two markers (A10-510 and AJ20-1070) are absent in 'Rashti' (Table 3). Eight markers were diagnostic for two different cultivars, six because of their presence and two because of their absence.

Even though we did not find any cultivar-specific markers, all the cultivars examined can be clearly discriminated with a few well chosen primers. For example, the primer AJ20 generated six polymorphic fragments, one of which (AJ20-1070) is absent in one cultivar ('Rashti') and two of which (AJ20-440 and AJ20-890) are useful to discriminate three cultivars ('Red Aleppo,' 'Kerman,' and 'Sfax') from all the others (Table 3). The data also indicate that genetic typing among the *P. vera* cultivars studied can be

Table 1. *Pistacia vera* cultivars included in this study. All plant materials were obtained from the USDA Germplasm Repository at the University of California, Davis.

Code #	Cultivar	Sex
1	'Peters'	Male
2	'Ask'	Male
3	'Red Aleppo'	Female
4	'Bronte'	Female
5	'Nazareth'	Male
6	'Rashti'	Female
7	'Gazvin'	Male
8	'Kerman'	Female
9	'Damghan'	Female
10	'Trabonella'	Female
11	'Sfax'	Female
12	'Iran Large'	Female
13	'Aegina'	Female
14	O2-16	Male
15	O2-18	Male

Table 2. List of 14 primers that produced polymorphic band patterns among *Pistacia vera* cultivars.

Primer	Sequence	# Polymorphic fragments
A08	GTGACGTAGG	1
A09	GGGTAACGCC	2
A10	GTGATCGCAG	3
A11	CAATCGCCGT	3
AA16	GGAACCCACA	3
AC06	CCAGAACGGA	3
AC07	GTGGCCGATG	1
AG13	GGCTTGGCGA	2
AI03	GGGTCCAAAG	1
AI12	GACGCGAACC	1
AI17	CCTCACGTCC	2
AJ05	CAGCGTTGCC	3
AJ20	ACACGTGGTC	6
AM10	CAGACCGACC	6
Total		37

assessed using 14 primers and the 37 polymorphic fragments that they generate. The fact that each cultivar has a unique molecular fingerprint will allow the use of this kind of information for breeding applications, maintenance of germplasm collections including screening for duplicate samples, and protection of proprietary and intellectual rights. In combination with phenotypic and historic information, the data generated by RAPD markers could be used to characterize new collections, and to create a scale of priorities for maintenance and regeneration of existing collections.

The results obtained in this study demonstrate that RAPD technology can be useful in the study of genetic diversity among *P. vera* cultivars. This is of potential importance in pistachio breeding as utilization of marker-aided selection depends upon the degree of polymorphism within the germplasm to be exploited. Marker-aided selection can be important in breeding tree-crop species such as pistachio where the long generation times present major impediments to timely progress in breeding. In addition, the methodology used will allow assessment of the degree of similarity among genotypes. The results obtained with this technique will help pistachio breeders to decide how to best use available germplasm in selecting parents for hybridization to obtain new genetic combinations and to introgress new genes into

Table 3. Survey of 37 RAPD markers in 15 pistachio cultivars. Cultivar numbers correspond to those in Table 1. "+" indicates presence and "–" indicates absence of the marker.

	Cultivar #														
RAPD	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A08-510	–	–	+	+	+	–	+	+	–	+	+	–	+	–	–
A09-320	+	+	+	+	+	+	+	–	–	+	–	+	+	+	+
A09-1550	–	+	–	+	+	+	–	+	–	+	+	+	+	–	+
A10-325	+	+	–	+	–	–	–	+	+	+	+	+	–	–	–
A10-510	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+
A10-1630	+	+	–	–	–	+	+	–	+	–	+	+	+	+	+
A11-900	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–
A11-950	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–
A11-1300	+	–	+	+	+	–	+	+	+	+	+	+	+	+	+
AA16-540	+	+	+	+	+	–	–	–	+	+	–	–	+	+	+
AA16-650	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–
AA16-680	+	+	+	+	+	+	+	+	–	+	+	+	–	+	+
AC06-440	–	–	–	–	–	–	–	–	+	–	–	+	–	+	+
AC06-740	+	–	–	–	–	+	+	+	+	–	–	+	–	+	+
AC06-1050	–	–	–	–	–	+	+	+	–	+	+	+	–	–	–
AC07-750	–	+	–	+	–	–	–	–	–	+	+	–	–	–	–
AG13-810	–	+	+	+	+	–	–	–	–	+	+	–	+	–	–
AG13-850	–	–	–	–	–	+	–	+	–	–	–	–	–	+	–
AI03-940	+	+	+	+	+	–	+	–	–	+	+	–	+	–	–
AI12-1370	–	+	+	+	–	+	+	+	+	+	–	–	+	+	+
AI17-510	+	+	–	–	–	–	–	–	–	–	+	–	–	–	+
AI17-980	–	–	–	–	–	–	–	–	+	–	–	+	–	–	+
AJ05-575	–	+	–	+	+	+	–	+	–	+	–	–	–	–	+
AJ05-860	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
AJ05-1070	+	+	+	+	+	+	–	+	–	+	+	–	+	–	–
AJ20-440	+	+	+	+	+	+	+	–	+	+	–	+	+	+	+
AJ20-660	–	+	–	+	–	–	–	–	–	+	+	–	–	–	–
AJ20-690	+	–	+	+	+	+	+	–	+	+	+	–	+	+	+
AJ20-860	+	–	–	–	+	–	–	+	–	–	–	+	+	–	–
AJ20-890	+	+	–	+	+	+	+	+	+	+	–	+	+	+	+
AJ20-1070	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+
AM10-360	+	+	+	–	–	+	+	+	+	–	–	+	–	+	+
AM10-520	+	–	–	+	+	+	+	+	+	+	+	+	–	+	–
AM10-810	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–
AM10-910	+	–	–	–	–	–	+	–	–	–	–	+	–	–	–
AM10-1040	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–
AM10-1230	+	–	–	–	–	+	+	+	+	–	–	–	–	+	+

locally adapted germplasm. This type of information will be particularly useful in pistachio because, as a dioecious species, traditional morphological evaluations provide no information regarding the potential contributions of male genotypes to commercially important nut characteristics. By using RAPD markers it should be possible to begin mapping linkages to these traits on the male as well as on the female genome. The method should also be useful to those concerned with the collection and maintenance of pistachio germplasm.

Further research is underway to extend our results more widely among pistachio cultivars with the aim of elucidating patterns of relatedness within *P. vera* germplasm and to provide a clearer image of the pistachio material available. In addition, we intend to use this approach to study other rootstock species closely related to *P. vera*. The long term goal will be to identify molecular markers linked to important breeding traits. An advance has been taken in that direction through the identification of a RAPD marker linked to female sex expression which will allow the screening of sex in pistachio seedlings long before they reach reproductive maturity (6).

The pistachio cultivars used in this study may be obtained through the USDA National Germplasm Repository at the University of California, Davis.

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Advancement of the Australian Disease Resistant Apple Breeding Program by Cooperation with USA Programs

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Abstract

The Australian disease-resistant apple breeding program has been conducted at Stanthorpe since 1984. High fruit quality and resistance to *Venturia inaequalis* and *Podosphaera leucotricha* are the joint aims. Visits in 1984 and 1991, by fruit breeders from the USA breeding programs, and germplasm exchanges have advanced the program. A cooperative pollination project in 1993 enabled advanced disease resistant cultivars from the USA to be incorporated into the program. Seventy-five thousand seedlings have been screened for disease resistance and are being evaluated for fruit quality.

Australian Apple Breeding

Since the 1850s nearly forty new Australian apple cultivars have been produced from organized breeding programs and by selection of chance seedlings. Growers, nurserymen, private breeders and State Departments have all played a role and several Australian cultivars have found a place in world pomology (10). Breeding for disease resistance in apples was begun in Australia by the New South Wales Department of Agriculture in the 1920s at Bathurst. The cultivar 'Redbow,' which is resistant to both apple scab (scab), *Venturia inaequalis* (Cke.) Wint., and powdery mildew, *Podosphaera leucotricha* (Ellis. & Everh.) E. S. Salmon, was released in 1961 (5). Since 1964 the Queensland Department of Primary Industries had been breeding high-quality early-season apples at the Granite Belt Horticultural Research Station (GBHRS) and three cultivars have been released to date (10). However the production of populations for selection of disease resistance was not initiated in Australia until the eminent apple breeder, the late Professor L. Fredric Hough, visited in 1984 and the subsequent importation of disease resistant seeds and scionwood from the USA. Professor Hough was the former apple breeder associated with the cooperative breeding program of Purdue University, Rutgers University and the University of Illinois, known as the PRI program (3).

In 1984 the breeding objectives were expanded to include resistance to apple scab and powdery mildew due to environmental reasons, to reduce production costs and because of the developing resistance of *V. inaequalis* to benzimidazole fungicides (1). Our breeding strategy has been to cross disease-resistant parents with different

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