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Random Amplified Polymorphic DNA Analysis of Olive *(Olea europaea* L.) Cultivars

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Abstract. **Seventeen olive *(Olea europaea* L.) cultivars, including oil and table olive cultivars originating from throughout the Mediterranean area, were screened using random amplified polymorphic DNA (RAPD) markers. The results indicate that a high degree of polymorphism is evident in the olive germplasm reexamined. Forty random decamer primers were screened; seventeen of these produced 47 reproducible amplification fragments useful as polymorphic markers. Each of the 17 cultivars can be discriminated with a few primers. Results were analyzed for similarity among the cultivars and a cluster analysis was performed. These analyses revealed two main groups: one comprising primarily small-fruited cultivars grown mainly for oil production, and the other characterized by having large fruit. There was no apparent clustering of olive cultivars according to their geographic origins.**

Olive *(Olea europaea)* is one of the most ancient cultivated fruit tree species in the Mediterranean basin. It is the only Mediterranean representative of the genus *Olea,* which includes 35-40 species distributed over tropical and southern Africa, south Asia, eastern Australia, New Caledonia, and New Zealand (Zohary and Hopf 1993). Traditionally, morphological and phonological traits are used to identify olive cultivars. More recently, workers have found isozymes to be useful for cultivar identification and determining patterns of relatedness among cultivars. Pontikis et al. (1980) identified 27 olive cultivars, mostly of Greek origin, using 16 enzyme systems in pollen. Trujillo et al. (1990) found that by using five pollen enzyme systems they could distinguish 134 of 155 cultivars. Ouazzani et al. (1993) distinguished 33 of 44 cultivars using 9 enzyme systems in leaf tissue. Although isozyme analysis has proved useful in olive, mainly due to the high level of isozyme polymorphism present in the species, direct analysis of DNA could increase considerably the number of markers produced. Furthermore, because isozymes are products of gene expression, differential expression by environment, tissue-specific it y, and other factors is common and may make interpretation of results difficult. Random amplified polymorphic DNA (RAPD) analysis, first described by Williams et al. (1990) and Welsh and McClelland (1990), has proven to be a useful tool for genetic typing and mapping. Bogani et al. (1994) described preliminary results of RAPD analyses of olive cultivars. They used 5 decamer nucleotide primers to generate RAPD polymorphisms among 11 olive cultivars, but found no consistent relationships. In this paper, we report the use of RAPD markers to distinguish among 17 olive cultivars, and we discuss the use of RAPDs to study relationships among cultivars of this species.

Materials and Methods

Plant materials. Seventeen *Olea europaea* cultivars were used in this study (Table 1). These were obtained from a collection maintained in experimental orchards at the Univ. of California, Davis.

DNA extraction and RAPD analysis. Young olive leaves were collected in Spring 1993 and stored at –70C before DNA extraction. Total DNA was extracted from leaf tissue following the CTAB method of Doyle and Doyle (1987) 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 pH 8, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% β– mercaptoethanol, 0.1 % $NaHSO_4$). Samples were incubated at 65C for 1 h, mixed with an equal volume of chloroform–isoamyl alcohol (24:1), and centrifuged at 2000× g for 15 min. The aqueous phase was recovered and mixed with two-thirds 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 pH 8, 0.1 mM EDTA). Extracted DNA was diluted to 10 ng·µl^{-1} and used for PCR amplification.

Forty decamer oligonucleotides (Operon Technologies, Alameda, Calif.) were used for PCR amplifications following the procedure of Williams et al. (1990), 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 $MgCl_2$, 0.00 1% gelatin, 100 µM each of dATP, dGTP, dCTP, and dTTP (Promega, Madison, Wis.), 0.4 µM primer, 0.75 units of AmpliTaq DNA polymerase (Perkin-Elmer-Cetus, Norwolk, Conn.) and 50 ng of genomic DNA. Each reaction mixture was overlaid with 25 µl of mineral oil to prevent evaporation. To destroy putative carryover products, reaction mixtures and mineral oil were placed on a UV (300 nm) transilluminator before addition of template DNA and DNA polymerase (Sarkar and Sommer, 1993). DNA amplification reactions were performed in a thermal cycler (PTC-100; MJ Research, Watertown, Mass.) programmed for 1 cycle of 2 min at 94C followed by 40 cycles of 45 sec at 92C, 1 min at 36C, 2 min at 72C, for denaturing, annealing and primer extension, respectively. The last cycle was followed by incubation for 5 min at 72C; PCR products were stored at 4C before analysis. Amplification products were analyzed by gel electrophoresis in 2910 agarose (SeaKem; FMC, Rockland, Maine) in 1 x TBE buffer, stained with ethidium bromide, and photographed under UV light using Type 57 Polaroid film. Molecular sizes of amplification products were estimated using a 123 bp DNA ladder (Sigma Chemical Company, St. Louis). All reactions were repeated at least three times using different lots of Taq polymerase and only reproducible bands were used in further analyses. Each amplification fragment

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Table 1. Olive cultivars included in this study and their country of origin.

Cultivar	Country
1 Adrouppa	Cyprus
2 Aghizi Shami	Egypt
3 Arbequina	Spain
4 Ascolana	Italy
5 Chemlali	Tunisia
6 Frantoio	Italy
7 Koroneiki	Greece
8 Manzanillo	Spain
9 Mastoides	Greece
10 Memeli	Turkey
11 Mission	United States
12 Nabali	Israel
13 Ogliarola	Italy
14 Picual	Spain
15 Sevillano	Spain
16 Sigoise	Algeria
17 Swan Hill	Australia

useful for discrimination between genotypes was named by the primer used and its approximate size in base pairs.

Statistical analysis. Data generated from RAPD analyses were analyzed using the Nei similarity index (Nei and Li 1979), which excludes common negative data, according to the following equation: Similarity = $2\ N_{ab}/(N_a + N_b)$, where N_{ab} = number of scored amplification fragments with the same molecular weight shared between genotypes *a* and *b;* N_a = number of scored amplification fragments in genotype *a,* and N_b = number of scored amplification fragments in genotype *b.*

A dendrogram was constructed based on the similarity matrix data by applying unweighed pair group method with arithmetic averages (UPGMA) cluster analysis using the NTSYS program (Exeter Software, Setauket, N.Y.).

Results and Discussion

Each of the 40 primers successfully amplified different DNA fragments as illustrated by the example of primer AH11 shown in Fig. 1. Amplification products of seventeen primers were selected for further analysis because these primers generated polymorphic amplification fragments that were conspicuous and highly reproducible (Table 2). Overall, these primers revealed 47 reproducible polymorphic amplification fragments which we selected as useful markers (Table 3). The number of reproducible polymorphic fragments per primer ranged between 1 and 5, and fragment size ranged between 315 (AI 11) and 1705 (AI05) base pairs. The relatively large number of polymorphic markers obtained with these few primers is consistent with earlier findings indicating that olive is a highly polymorphic species (Zohary and Spiegel-Roy, 1975) and confirms results obtained with isozymes (Ouazzani et al., 1993; Pontikis et al., 1980; Trujillo et al., 1990). Bogani et al. (1994) reported polymorphisms in RAPD banding patterns for three of the five primers they examined in their analysis of eleven olive cultivars, but the number of polymorphic markers obtained from these three primers is unclear from their data.

Table 2. List of the 17 primers and their sequences which produced polymorphic markers among the cultivars studied.

Primer	Sequence	Polymorphic fragments (no.)
AH01	TCGGCAACCA	4
AH02	CACTTCCGCT	3
AH09	AGAACCGAGG	2
AH11	TCCGCTGAGA	3
AH12	TCCAACGGCT	1
AH16	CAAGGTGGGT	4
AH17	CAGTGGGGAG	2
AH18	GGGCTAGTCA	1
AH19	GGCAGTTCTC	1
AI05	GTCGTAGCGG	4
AI08	AAGCCCCCCA	2
AI11	ACGGCGATGA	4
AI12	GACGCGAACC	2
AI13	ACGCTGCGAC	1
AI14	TGGTGCACTC	5
AI15	GACACAGCCC	5
AI16	AAGGCACGAG	3
Total		47

Figure 1, which shows the amplification pattern obtained with primer AH11, illustrates the criteria we used to consider if a band is a useful marker. In this case, only three bands (AH11-590, AH11-620, and AH11-740) were considered to be useful markers for subsequent analysis. We did not consider two other bands produced by amplification with AH11 (AH111-670 and AH11-800), although, under some circumstances, they may have potential as polymorphic markers. The fragment AH 11-670 produces bands of different intensities among the cultivars and this pattern was reproducible in all replications. Yang and Quiros (1993) have suggested that band intensity may reflect differences in the copy number of the amplified sequence among different genotypes, and Demeke et al. (1992) considered such bands to be useful polymorphic markers. The amplification pattern of AH11-800 was also highly reproducible, but its presence/absence was sometimes not clear in some cultivars. The high degree of polymorphism present among olive cultivars resulted in the generation of many reproducible polymorphic markers with relatively few primers, thus allowing application of more stringent criteria when considering a marker for further analysis.

All cultivars examined can be clearly discriminated using a few well chosen primers. Several unique markers were detected (Table

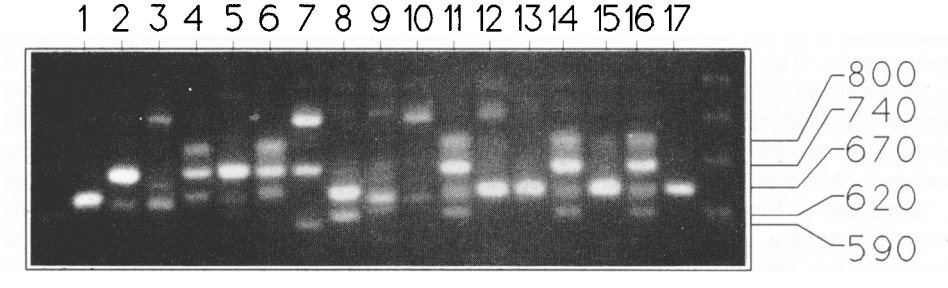

Fig. 1. RAPD pattern obtained from the 17 olive cultivars with the primer AH11. Cultivar numbers correspond to those in Table 1.

Table 3. Survey of 47 RAPD markers in 17 olive cultivars, Cultivar numbers correspond to those in Table 1. (+) Indicates presence and (-) indicates absence of the marker.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
AH01-520	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
AH01-680	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
AH01-790	–	–	–	+	–	–	–	–	–	–	+	–	–	–	+	+	+
AH01-1220	+	+	+	–	–	+	+	–	+	+	–	+	+	–	+	–	–
AH02-940	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
AH02-1000	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–
AH02-1270	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+
AH09-470	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	+
AH09-510	–	+	+	–	+	+	+	+	–	+	–	–	+	–	–	–	–
AH11-590	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
AH11-620	–	–	–	–	–	–	–	+	–	–	+	–	–	+	–	+	–
AH11-740	–	+	–	+	+	+	+	–	–	–	+	–	–	+	–	+	–
AH12-1140	–	–	–	–	–	–	–	+	–	–	+	–	–	+	–	+	–
AH16-430	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
AH16-1405	–	+	+	–	+	+	+	–	+	–	–	–	–	+	–	–	–
AH16-1525	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
AH16-1660	–	–	+	+	–	–	–	+	–	–	+	+	+	+	+	+	+
AH17-390	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	+
AH17-455	+	–	+	+	–	+	–	–	+	+	+	–	+	–	+	+	–
AH18-1160	–	–	+	–	+	+	+	–	+	–	–	–	–	–	–	–	–
AH19-955	+	–	–	–	–	+	–	–	–	+	–	+	–	+	–	–	+
AI05-730	–	+	+	–	+	+	+	–	+	–	–	–	–	–	–	–	–
AI05-1370	+	+	–	–	–	–	–	–	+	+	–	+	+	–	–	–	+
AI05-1555	–	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–
AI05-1705	+	+	+	+	+	+	+	+	–	+	+	–	–	+	+	+	+
AI08-505	–	+	+	+	–	+	–	–	–	+	–	–	–	–	–	–	+
AI08-625	–	–	–	+	+	+	–	+	+	–	+	–	+	–	+	+	+
AI11-315	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
AI11-360	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–
AI11-445	–	–	+	–	+	+	–	+	+	–	+	–	–	+	–	+	–
AI11-1040	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	+
AI12-755	–	–	+	–	–	+	+	–	+	–	+	–	–	–	–	+	–
AI12-825	+	+	–	+	–	–	–	+	–	–	+	+	+	+	+	–	+
AI13-835	+	+	+	–	+	+	+	–	+	–	–	+	–	+	+	+	+
AI14-760	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	–
AI14-800	+	+	–	+	–	+	+	–	+	+	–	+	–	–	–	–	+
AI14-975	+	–	–	–	–	–	–	–	–	–	+	–	+	–	+	+	–
AI14-1230	–	–	–	+	+	–	+	+	+	+	+	–	–	+	–	+	–
AI14-1300	–	–	–	–	+	+	+	+	–	–	+	–	–	–	–	+	–
AI15-375	+	+	+	–	+	–	+	+	+	–	+	–	–	+	–	+	+
AI15-400	+	+	–	+	–	+	–	–	–	+	–	+	+	–	–	–	–
AI15-540	+	+	+	+	+	+	–	+	–	–	+	+	+	+	+	+	+
AI15-940	–	+	–	–	+	+	–	+	–	–	+	–	–	–	–	+	–
AI15-1075	+	–	+	–	–	–	+	+	+	+	+	+	–	+	–	+	+
AI16-385	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–
AI16-955	+	–	–	–	–	+	–	+	–	–	–	+	+	–	+	–	+
AI16-985	–	+	–	+	+	–	+	–	+	–	+	–	+	+	+	+	–

3). AHO1-520 and AH16-1525 are specific to 'Chemlali', AHOl-680 and AH11-590 are specific to 'Koroneiki', AH16-430 is specific to 'Picual', and AH02-1270 is absent only in 'Mastoides'.

The Nei "estimate of similarity (Nei and Li, 1979) was used to construct a similarity matrix. Similarity values (Table 4) ranged from 0.222 for 'Nabali' and 'Chemkdi' to 0.952for 'Mission' and 'Sigoise'. The dendrogram resulting from the UPGMA cluster analysis is shown in Fig. 2. The value of the cophenetic correlation coefficient (Sokal and Rohlf, 1962), a measure of the goodness of fit between the dendrogram and the original similarity matrix, is 0.79.

As seen in the dendrogram (Fig. 2), UPGMA separates the cultivars into two main groups. The first group includes six cultivars ('Aghizi', 'Arbequina', 'Chemlali', 'Frantoio', 'Koroneiki', and 'Mastoids'). With the exception of 'Aghizi' these are small-fruited cultivars grown primarily for oil production. 'Adrouppa', 'Ascolana', 'Manzanillo', 'Mission', 'Nabali', 'Ogliarola', 'Picual', 'Sevillano', 'Sigoise', and 'Swan Hill', which comprise the second cluster, are, again with one exception, medium- to large-fruited cultivars. The exception in this group is 'Swan Hill', an ornamental, sterile cultivar that was discovered by Hartmann (1967) in an Australian table-olive orchard and, although it does not produce fruit, it maybe expected to have close affinities to the large-fruited table-olive types present in that orchard. 'Memeli', which falls outside the two groups, is a cultivar of secondary importance in the Aegean coastal area of Turkey where it originates; it produces medium-size fruit used as table and oil olives. Loukas and Krimbas (1983), in their isozyme study, obtained a comparable clustering of cultivars based on fruit size. Interestingly, of the 25 cultivars included in the Loukas and Krimbas (1983) study, only four ('Koroneiki', 'Mastoides', 'Sevillano', and 'Manzanillo') are also included here. That these similar results emerge from analysis of different olive cultivars using different approaches would seem to indicate that fruit size is a powerful separator within olive germplasm.

The data obtained in this study show no apparent relationship between the olive cultivars and their geographic origin. A similar conclusion resulted from the isozyme analyses of Ouazzani et al. (1993). In a recent study of 15 pistachio cultivars from the Middle East and the Mediterranean basin, Hormaza et al. (1994) found that clusters revealed by analyses of RAPD data were positively

Table 4. Similarity matrix generated using the Nei's estimate of similarity. Cultivar numbers correspond to those in Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	1.000																
2	0.615	1.000															
3	0.526	0.605	1.000														
4	0.471	0.513	0.368	1.000													
5	0.300	0.622	0.636	0.400	1.000												
6	0.545	0.735	0.667	0.454	0.640	1.000											
7	0.421	0.558	0.619	0.368	0.636	0.583	1.000										
8	0.457	0.400	0.462	0.514	0.537	0.444	0.410	1.000									
9	0.400	0.400	0.513	0.400	0.439	0.489	0.615	0.333	1.000								
10	0.621	0.471	0.424	0.483	0.229	0.461	0.424	0.333	0.400	1.000							
11	0.474	0.419	0.476	0.632	0.545	0.458	0.476	0.769	0.410	0.303	1.000						
12	0.800	0.514	0.412	0.467	0.222	0.450	0.353	0.452	0.323	0.560	0.353	1.000					
13	0.645	0.500	0.400	0.581	0.324	0.439	0.286	0.500	0.312	0.461	0.514	0.667	1.000				
14	0.514	0.500	0.513	0.514	0.537	0.400	0.513	0.667	0.389	0.333	0.718	0.516	0.375	1.000			
15	0.645	0.444	0.457	0.645	0.378	0.439	0.343	0.500	0.312	0.308	0.629	0.593	0.786	0.500	1.000		
16	0.474	0.419	0.524	0.579	0.591	0.500	0.524	0.718	0.461	0.303	0.952	0.353	0.457	0.718	0.629	1.000	
17	0.686	0.500	0.513	0.571	0.341	0.444	0.359	0.500	0.389	0.467	0.461	0.645	0.437	0.500	0.562	0.461	1.000

correlated with geographical origin of the cultivars studied. The difference between pistachio and olive in this regard may reflect differences in germplasm movement of these two species in the ancient world. For pistachio, it appears likely that there was relatively little widespread movement of germplasm before very recent times (Hormaza et al., 1994). The lack of any apparent correlation between DNA polymorphisms and site of cultivar origin in olive is consistent with the hypothesis that early in its domestication, olive cultivars of horticultural value were moved widely from region to region, much as happens today. In contrast to our results, however, Trujillo et al. (1990) found that isozyme polymorphisms were useful in grouping olive cultivars by their countries of origin. Furthermore, isozyme analyses indicated relationships between Spanish olive cultivars and their geographical origin (I. Trujillo, personal communication). One explanation for the apparent incongruence between the results of Trujillo's group and those reported here could be the possibility of local breeding, selection, and clonal propagation of seedlings obtained from germplasm that had previously been moved from country to country (Zohary and Spiegel-Roy 1975). This would produce local populations of related cultivars which would be evident as clusters grouping cultivars within regions. Trujillo examined material that included a number of representatives from local regions which clustered together. We did not conduct analyses at this level although we anticipate doing additional research using RAPDs to differentiate regional olive selections. The results obtained in this research show that RAPDs can be usefully applied to distinguishing olive cultivars since all the cultivars could be uniquely characterized with a relatively low number of primers. Furthermore, this approach can be an informative tool in analyses of diversity in this species.

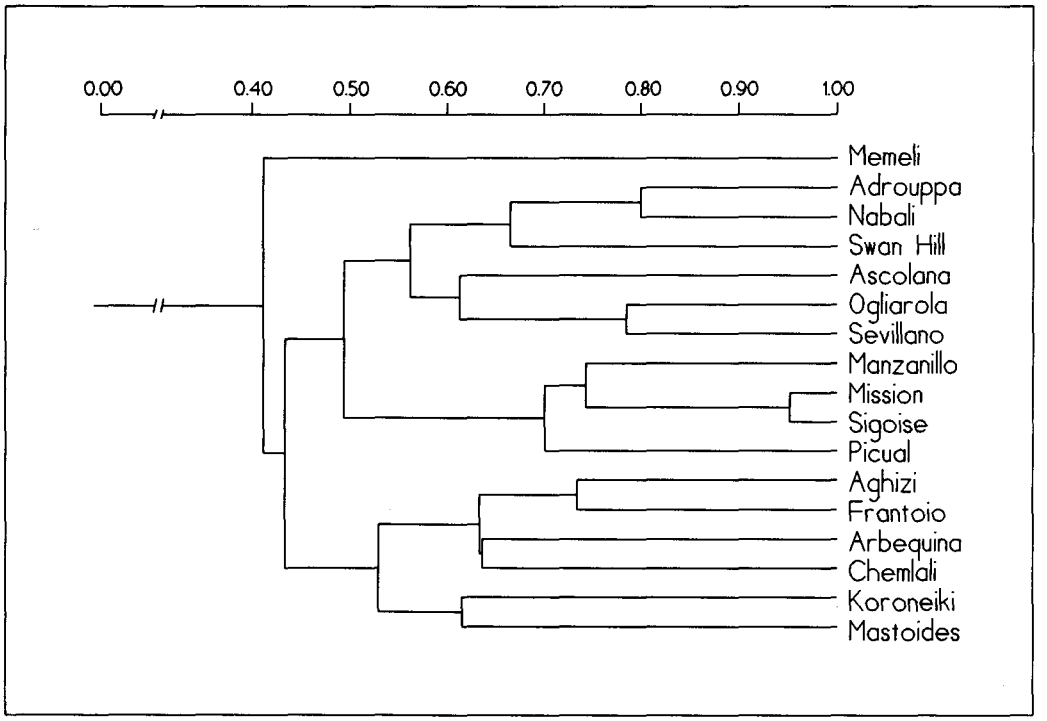

Fig. 2. Dendrogram of 17 olive cultivars generated by UPGMA cluster analysis of the similarity values given in Table 4.

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