

## Identification and discrimination of eight Greek grape cultivars (*Vitis vinifera* L.) by random amplified polymorphic DNA markers

by

M. N. STAVRAKAKIS<sup>1</sup>), K. BINIARI<sup>1</sup>) and P. HATZOPOULOS<sup>2</sup>)

<sup>1</sup>) Laboratory of Viticulture, Agricultural University of Athens, Greece

<sup>2</sup>) Laboratory of Molecular Biology, Agricultural University of Athens, Greece

**S u m m a r y :** Fifteen decamer primers of an arbitrary nucleotide sequence were used to amplify genomic DNA by polymerase chain reaction (PCR-RAPD) in order to identify and discriminate between 8 cultivars of *Vitis vinifera* L., grown at the Island of Crete. Over 140 reproducible polymorphic fragments were generated by this method. Each grape cultivar showed a unique banding pattern for more than 5 of the primers used. Herefrom, the degree of genetic similarity was calculated and the dendrogram of the 8 cultivars was constructed. The results show that RAPD is a reliable and very useful method for the identification and genomic analysis of grape cultivars.

**Key words :** dendrogram, grape cultivars, PCR, RAPD markers, *Vitis vinifera*, similarity index.

### Introduction

In Greece more than 300 grapevine cultivars of *Vitis vinifera* L. are grown, the majority of which are single or multiple hybrids (LOUKAS *et al.* 1983); they are maintained by clonal propagation. For the identification, discrimination and classification of Greek grape cultivars traditional ampelographic (KRIMBAS 1943; LOGOTHETIS 1947; VLACHOS 1986) and biochemical (STAVRAKAKIS 1982) methods have been used. The first method is based on a qualitative and/or quantitative description of morphological characteristics of leaves, young shoots, shoot tips and berries. These ampelographical characters vary depending on the environmental and the cultural conditions (BACHMANN and BLAICH 1988; BENIN *et al.* 1988). The most systematic and complete description of about 200 Greek grape cultivars was carried out by KRIMBAS (1943), who used a classification system based on the ratio of mean length of the berries versus mean length of their seeds. Among the biochemical markers those which are based on isozyme polymorphic patterns have proven to be useful to identify and to discriminate Greek grape cultivars and more than 70 cultivars have been identified by this method (STAVRAKAKIS 1982, 1990, 1991; STAVRAKAKIS and LOUKAS 1983, 1985; STAVRAKAKIS *et al.* 1995).

A new method based on random amplified polymorphic DNA (RAPD) by polymerase chain reaction analysis (PCR) allows direct comparison of genetic material of grapevine cultivars. DNA molecular markers seem to be an excellent tool for genetic identification and genome analysis; they have been used to differentiate cultivars of several plant species (CARLSON *et al.* 1991; HU and QUIROS 1991; KLEIN-LANKHORST *et al.* 1991; KRESOVICH *et al.* 1992; STILES *et al.* 1993) including the grapevines (BOWERS *et al.* 1993; BÜSCHER *et al.* 1993, COLLINS and SYMONS 1993; BOURQUIN *et al.* 1995; GRANDO *et al.* 1995; MORENO *et al.* 1995; BINIARI *et al.* 1996).

The aim of this paper is to identify and discriminate the main grape varieties grown in Crete, to determine the genetic similarities among these cultivars and to relate the findings to those obtained by using enzyme polymorphisms.

### Materials and methods

**Grapevine material:** Eight wine grapevine cultivars (*Vitis vinifera* L.) grown at the Island of Crete were chosen for identification: Daphni, Vidiano, Vilana and Plyto (white) Kotsifali, Ladhikino, Liatiko and Romeiko (red).

**DNA extraction:** Grapevine DNA was extracted from young and fully expanded leaves according to THOMAS *et al.* (1993) with minor modifications. 1 g of leaves from individual vines was frozen in liquid N<sub>2</sub> and ground to fine powder, thawed and resuspended in 12.5 ml of buffer A [0.25 M NaCl, 0.2 M TRIS-Cl (pH 8.0), 50 mM EDTA, 0.1 % v/v 2-mercaptoethanol, 2.5 % w/v polyvinylpyrrolidone (MW 40,000)]. A crude nuclei pellet was obtained by centrifugation at 4,000 g for 10 min at 4 °C. The pellet was resuspended in 2.5 ml of extraction buffer B [0.5 M NaCl, 0.2 M TRIS-Cl (pH 8.0), 50 mM EDTA, 1% v/v 2-mercaptoethanol, 2.5 % w/v polyvinylpyrrolidone, 3 % sarkosyl, 20 % ethanol] and incubated at 37 °C for 45 min. An equal volume of chloroform/isoamyl alcohol (24:1) was then added and mixed; the phases were separated by centrifugation at 16,000 g for 15 min. The aqueous layer was collected and 1.6 ml of isopropanol (-20 °C) was added to precipitate the DNA. The DNA was fished and resuspended in 300 µl TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) containing 15 µg·ml<sup>-1</sup> RNase A and incubated for 15 min at 37 °C. Protein was removed by the addition of a half volume of 7.5 M ammonium acetate, followed by centrifugation and the DNA in the supernatant was precipitated with 0.25 ml of cold isopropanol; ca. 120 µg DNA per g FW was obtained.

**Amplification conditions:** For RAPD analysis the protocol reported by WILLIAMS *et al.* (1990) was followed with minor modifications. Amplification reactions were performed in 25 µl containing 60 ng of genomic DNA, 10 mM TRIS-Cl pH 8.8, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1 % Triton X-100, 200 µM each of dATP, dGTP, dCTP, dTTP, 50 ng primer and 1 unit of DNA polymerase (Biometra). The surface was covered with 30 µl of mineral oil (Sigma). Fifteen random decamer oligonucleotides were used as primers for

Table 1

Synthetic deoxyribonucleotides used as primers for amplification of grape cultivar DNA and the unique pattern (+) generated by each primer

Primer code	Nucleotide sequence (5' to 3')	Total number of fragments amplified	VL	VD	KO	LI	RO	DA	LA	PL
A OPF-5	CCGAATTCCC	1	-	-	-	+	+	-	-	+
OPF-6	GGGAATTCGG	4	-	-	-	-	-	-	-	-
OPF-7	CCGATATCCC	7	-	+	+	-	-	-	-	-
OPF-8	GGGATATCGG	9	+	+	+	-	-	+	+	-
OPF-9	CCAAGCTTCC	13	-	-	-	+	+	+	+	+
OPF-13	GGCTGCAGAA	11	+	+	+	+	-	-	+	+
OPF-14	TGCTGCAGGT	9	+	-	-	-	+	+	-	-
OPF-17	AACCCGGGAA	11	-	+	+	+	+	+	+	+
OPF-18	TTCCCGGGTT	8	+	+	+	+	+	+	+	+
OPF-19	CCTCTAGACC	9	+	-	+	+	-	+	+	+
OPF-20	GGTCTAGAGG	11	+	+	+	+	+	+	+	+
B 1224	CAGGCCCTTC	9	-	+	-	-	-	+	-	-
1225	AGGTGACCGT	10	+	+	+	-	-	-	-	-
1226	CGCAGGATGG	8	-	-	-	-	+	+	+	+
1227	GTGTGCCCCA	11	-	-	-	-	+	-	+	+

VL: Vilana, VD: Vidiano, KO: Kotsifali, LI: Liatiko, RO: Romeiko, DA: Daphni, LA: Ladhikino, PL: Plyto

the amplification of RAPD sequences (Tab. 1). Amplification was performed in a Techne PHC-3 DNA Thermal Cycler. After 5 min at 94 °C, 34 cycles of PCR were performed, (1 min at 94 °C, 1 min at 44 °C, 2 min at 72 °C) followed by 10 min at 72 °C for extension.

**Gel electrophoresis:** Aliquots of the RAPD products were analyzed in 1.4 % agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate and 1mM EDTA, pH 8). After staining in ethidium bromide (1µg·ml<sup>-1</sup>) the gels were photographed on a UV transilluminator. All reactions were repeated at least twice with independently isolated genomic DNA as templates.

The degree of genetic similarity detected electrophoretically between each pair of cultivars studied (Tab. 2) was calculated using the NTSYS-pc package 1.8 developed by ROHFL (Exeter Software, New York, USA), generating the dendrogram.

## Results and Discussion

Two groups of single, arbitrary 10-mer oligonucleotide primers were used to amplify genomic DNA from 8 grape cultivars. The primers belonging to group A revealed a greater degree of genetic polymorphism than the primers of group B. Each primer regardless of the group to which it belonged provided at least two polymorphic bands. Among the 15 primers used the primers OPF-8, OPF-13, OPF-17, OPF-18 and OPF-20 have generated more polymorphic DNA fragments. Examples of RAPD patterns amplified with primers OPF-8, OPF-14, OPF-18, OPF-20 are shown in Fig. 1 a-d.

Although the electrophoretic patterns of the amplification products of genomic DNA proved to be quite complex, the grape cultivars studied showed a unique banding pattern for more than 5 primers. Thus the cultivar Vilana had unique patterns in each of 8 primers (OPF-8, 13, 14, 17, 18, 19,

Table 2

Genetic similarity values between eight grape cultivars as determined from the analysis of 15 different primers

Cultivar	Vilana	Vidiano	Kotsifali	Liatiko	Romeiko	Daphni	Ladhikino	Plyto
Vilana	—							
Vidiano	0.673	—						
Kotsifali	0.717	0.700	—					
Liatiko	0.698	0.607	0.663	—				
Romeiko	0.639	0.524	0.610	0.654	—			
Daphni	0.620	0.483	0.605	0.560	0.591	—		
Ladhikino	0.666	0.564	0.609	0.605	0.649	0.583	—	
Plyto	0.607	0.562	0.654	0.576	0.553	0.568	0.614	—

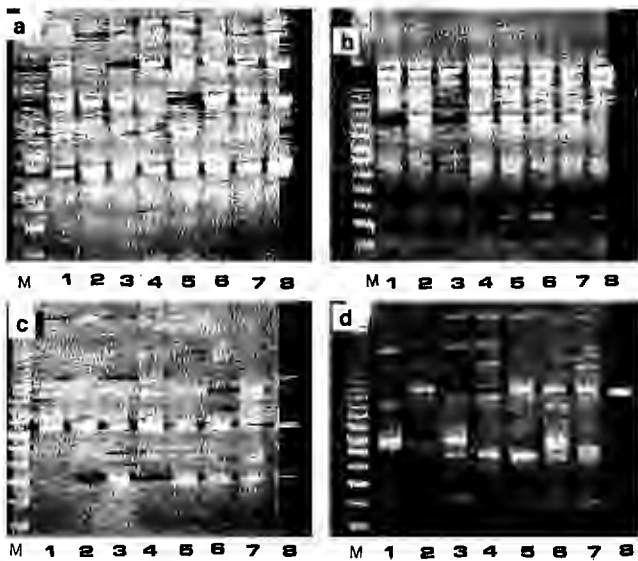


Fig. 1: Amplification patterns of polymorphic DNA from 8 grape cultivars generated by primers OPF-8 (a), OPF-14 (b), OPF-18 (c) and OPF-20 (d). 1.Plyto, 2.Ladhikino,3.Daphni 4.Romeiko, 5.Liatiko, 6.Kotsifali, 7.Vidiano, 8.Vilana. M: 100-bp molecular weight ladder (Pharmacia Biotech).

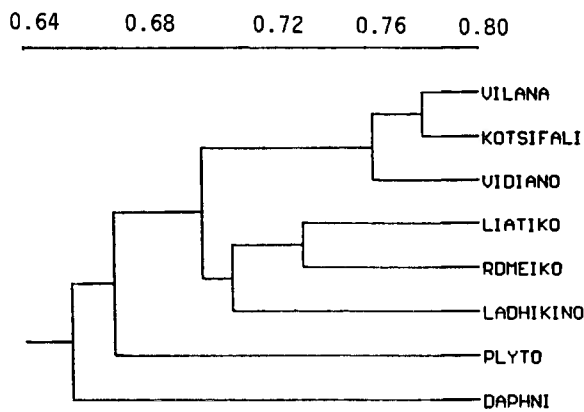


Fig. 2: Dendrogram based on 140 RAPDs probes showing the relationships among 8 grape cultivars studied.

20 and 1225), Vidiano in each of 8 (OPF-7, 8, 13, 17, 18, 20 and 1224, 1225), Kotsifali in each of 8 (OPF-7, 8, 13, 17, 18, 19, 20 and 1225), Liatiko in each of 7 (OPF-5, 9, 13, 17, 18, 19, 20), Romeiko in each of 8 (OPF-5, 9, 14, 17, 18, 20 and 1226, 1227), Daphni in each of 9 (OPF-8, 9, 14, 17, 18, 19, 20 and 1224, 1226), Ladhikino in each of 10

primers (OPF-7, 8, 9, 13, 17, 18, 19, 20, 1226, 1227) and Plyto in each of 10 primers (OPF-5, 8, 9, 13, 17, 18, 19, 20 and 1225, 1226). Thus the 8 cultivars could be identified by using one primer with a unique pattern or by combining two primer systems, e.g. OPF 17-OPF 18.

The values of genetic similarity (Tab. 2) ranged from 0.717 for the most closely related cultivars (Vidiano - Kotsifali) to 0.483 for the most distant related varieties (Vidiano-Daphni). Kotsifali, Vidiano and Vilana grouped to a single bunch of the tree, although Kotsifali is a red wine cultivar with different ampelographical and morphological characters (Fig. 2). On the other hand, unexpectedly, the RAPD data indicated that Liatiko was more closely related to Romeiko than to cv. Kotsifali. The degree of genetic similarity (I) between each pair of the 5 cultivars Vilana, Kotsifali, Liatiko, Romeiko and Ladhikino was calculated by using the formula

$$I = \frac{\sum c_i}{\sum (c_i + u_i)}$$

where  $c_i$  is the number of common (homologous) bands between two cultivars for the primer  $i$  and  $u_i$  is the number of uncommon (non-homologous) bands. The degree of genetic similarity (I) ranges from 0 (no common bands) to 1 (all bands in common). The 5 cultivars were also studied by STAVRAKAKIS (1982). Tab. 3 shows the values of I between all possible pairs of the 5 cultivars. For each pair two values are given, one calculated from data of STAVRAKAKIS (1982) (based on the enzyme systems leucine aminopeptidase, esterase, peptidase, tetrazolium oxidase, phosphogluco-mutase and -glycerophosphate dehydrogenase) and the other from the present study. The former is indicated by S and the latter by P. It is obvious that the values of I as calculated in the present study are always higher than those of the previous study, except for Liatiko-Vilana indicating a higher similarity at the genotype level.

The correlation coefficient between these pairs of values is 0.2992 (Spearman Rho) and 0.1451 (Kendall Tau b). The very small numerical values of the correlation coefficient mean that the data obtained from the study of the enzyme systems do not correlate well with those from the DNA analysis.

It can be concluded that the molecular markers generated by the polymerase chain reaction through the method

Table 3

The degree of genetic similarity (I) (x1000) between 5 grape cultivars calculated from the data given by STAVRAKAKIS (1982) (S) and from the present study (P)

Cultivar	Vilana		Kotsifali		Liatiko		Romeiko		Ladhikino		
	S	P	S	P	S	P	S	P	S	P	
Vilana	_____										
Kotsifali	523	717	_____								
Liatiko	736	698	631	663	_____						
Romeiko	280	639	250	610	291	654	_____				
Ladhikino	375	666	347	609	333	605	550	649	_____		

of random amplified polymorphic DNA are more effective than the biochemical markers based on enzymes to identify and discriminate grape cultivars.

### Acknowledgements

We would like to thank Professor P. J. KALTSIKES for critical reading of the manuscript and Dr. P. BEBELI for help to construct the dendrogram. This research was supported in part by a grant of the EPET project.

### References

- BACHMANN, O.; BLAICH, R.; 1988: Isoelectric focusing of grapevine peroxidases as a tool for ampelography. *Vitis* **27**, 147-155.
- BENIN, J.; GASQUEZ, J.; MAHFOUDI, A.; BESSIS, R.; 1988: Caractérisation biochimique des cépages de *Vitis vinifera* L. par électrophorèse d'isoenzymes foliaires: Essai de classification des variétés. *Vitis* **27**, 157-172.
- BINIARI, K.; STAVRAKAKIS, M. N.; HATZOPOULOS, P.; 1996: Identification of grape cultivars with RAPD markers. Proc. of the Hellenic Scientific Society of the Horticulture Science Conference, Thessaloniki **5**, 372-378.
- BOURQUIN, J. C.; OTTEN, L.; WALTER, B.; 1995: PCR-RLFP analysis of *Vitis*, *Ampelopsis* and *Parthenocissus* and its application to the identification of rootstocks. *Vitis* **34** (2), 103-108.
- BOWERS, J. E.; BANDMAN, E. B.; MEREDITH, C. P.; 1993: DNA fingerprint characterization of some wine grape cultivars. *Amer. J. Enol. Viticult.* **44**, 266-274.
- BÜSCHER, N.; ZYPRIAN, E.; BLAICH, R.; 1993: Identification of grapevine cultivars by DNA analysis: Pitfalls of random amplified polymorphic DNA techniques using 10mer primers. *Vitis* **32**, 187-188.
- CARLSON, J. E.; TULSIERAM, L. K.; GLAUBITZ, J. C.; LUK, V.; KAUFFELDT, C.; RUTLEDGE, R.; 1991: Segregation of random amplified DNA markers in F1 progeny of conifers. *Theor. Appl. Genet.* **83**, 194-200.
- COLLINS, G. G.; SYMONS, R. H.; 1993: Polymorphisms in grapevine DNA detected by the RAPD-PCR technique. *Plant Mol. Biol. Repr.* **11**, 105-111.
- DAVIDIS, X. O.; 1967: An Introduction to Ampelography. Privately printed, Athens, 315 pp. (in Greek).
- FREGONI, M.; 1991: Origines de la Vigne et de la Viticulture. Musumeci Ed. Quart (Valle d'Aoste).
- GRANDO, M. S.; DEMICHELLI, L.; BIASETTO, L.; SCIENZA, A.; 1995: RAPD markers in wild and cultivated *Vitis vinifera*. *Vitis* **34**(1), 37-39.
- HU, J.; QUIROS, C. F.; 1991: Identification of broccoli and cauliflower cultivars with RAPD markers. *Plant Cell Rep.* **10**, 505-51.
- JEAN-JAQUES, I.; PEFONTAINE, A.; HALLET, J. N.; 1993: Characterization of *Vitis vinifera* cultivars by Random Amplified Polymorphic DNA markers. *Vitis* **32**, 189-190.
- KLEIN-LANKHORST, R. M.; VERMUNT, A.; WEIDE, R.; LIHARSKA, T.; ZABEL, P.; 1991: Isolation of molecular markers for tomato (*Lycopersicon esculentum*) using random amplified polymorphic DNA (RAPD). *Theor. Appl. Genet.* **83**, 108-114.
- KRESOVICH, S.; WILLIAMS, J. G. K.; McFERNON, J. R.; ROUTMAN, J.; SCHAAL, B. A.; 1992: Characterization of genetic identities and relationships of *Brassica oleracea* L. via random amplified polymorphism DNA assay. *Theor. Appl. Genet.* **85**, 190-196.
- KRIMBAS, V.; 1943: Greek Ampelography. Vol. I. Ministry of Agriculture, Athens (in Greek) 266 pp. + 83 plates.
- LOGOTHETIS, V.; 1947: An Introduction to Ampelography. Athens, 183 pp. (in Greek).
- LOUKAS, M.; STAVRAKAKIS, M. N.; KRIMBAS, K.V.; 1983: Inheritance of polymorphic isoenzymes in grape cultivars. *J. Heredity* **74**, 181-183.
- MORENO, S.; GORGORGENA, V.; ORTIZ, J.; 1995: The use of RAPD markers for identification of cultivated grapevine (*Vitis vinifera* L.). *Scientia Horticult.* **62**, 237-243.
- STAVRAKAKIS, M. N.; 1982: Identification of grape cultivars (*Vitis vinifera* L.) by pollen isozyme polymorphisms. Ph.D Thesis. Agric. Univ. of Athens, Athens, 95 pp.
- -; 1990: Comparative study of grape cultivars of the "Mavroudia" group. *Agric. Res.* **14**, 19-29.
- -; 1991: Comparative study of synonymus of the wine grape cultivar Savvatiano. *Agric. Res.* **15**, 191-201.
- -; LOUKAS, M.; 1983: The between and within grape cultivars genetic variation. *Scientia Horticult.* **19**, 321-334.
- -; - -; 1985: Identification of grape cultivars by pollen isozyme polymorphisms. *Agric. Res.* **9**, 347-357.
- -; SIMINIS, X.; BINIARI, K.; 1995: Genetic variation between and within several Greek grape cultivars grown in Santorini. *Agric. Res.* (in press).
- STILES, J. I.; LEMME, C.; SONDUR, S.; MORSHIDI, M. B.; MANSCHARDT, R.; 1992: Using randomly amplified polymorphic DNA for evaluating genetic relationships: analysis of the relationship among 'Solo' and other papaya cultivars. *Theor. Appl. Genet.* **85**, 697-701.
- THOMAS, M. R.; MATSUMOTO, S.; CAIN, P.; SCOTT, N. S.; 1993: Repetitive DNA of grapevine: Classes present and sequences suitable for cultivar identification. *Theor. App. Genet.* **86**, 173-180.
- VLACHOS, M.; 1986: Ampelography. Thessaloniki, 518 pp (in Greek).
- WILLIAMS, J. G. K.; KUBELIK, A. R.; LIVAK, K. J.; RAFALSKI, J. A.; TINGEY, S. V.; 1990: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**, 6531-6535.

Received June 16, 1997