Genetic study of grapevine varieties using molecular markers

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Abstract: RAPD and microsatellites were used in the present study as molecular markers for identification and discrimination of grapevine material and for comparison between the two methods. Thirty random decamer primers of arbitrary nucleotide were used to amplify genomic DNA through the polymerase chain reaction (RAPD-PCR) in order to identify and discriminate 28 greek and foreign grapevine cultivars (*Vitis vinifera* L.) grown in Greece and to determine the genetic similarities among these cultivars. More than 400 reproducible polymorphic fragments were generated with this method. Herefrom, the degree of genetic similarity was calculated and the dendrogram of the 28 studied cultivars was constructed. Thereafter, eight random microsatellite primers were used to amplify genomic DNA through the polymerase chain reaction (SSR analysis) for the same 28 cultivars, but the reproducible fragments of the primers used showed low discriminating ability. Evidently, for the SSR analysis is required further research with the use of a larger number of primers.

Abstract: RAPD et microsatellites ont été utilises comme des marqueurs moléculaires pour l'identification et la discrimination des cépages de vigne et pour la comparaison entre les deux méthodes. Trente decamer amorces aléatoires de l'arbitraire de nucléotides ont été utilisées pour amplifier l'ADN génomique par la réaction en chaîne par polymérase (RAPD-PCR) afin d'identifier et de distinguer les 28 grecs et étrangers cépages (*Vitis vinifera* L.) cultivés en Grèce et de déterminer les similitudes génétiques parmi eux. Plus de 400 fragments reproducibles ont été générés avec cette méthode. Donc, le degré de similitude génétique a été calculé et le dendrogramme des 28 cépages étudiés a été construit. Par la suite, huit microsatellites amorces aléatoires ont été utilisées pour amplifier l'ADN génomique par la réaction en chaîne par polymérase (SSR analyse) pour les mêmes 28 cépages, mais les fragments de reproductible les amorces utilisées ont montré faible capacité discriminante. Evidemment, pour l'analyse SSR, plus de recherché est indispensable avec l'utilisation d'un plus grand nombre d'amorces.

Introduction

The discrimination and identification of grape varieties are very difficult due to their large number. It has been estimated that there are more than 8000 grape cultivars, under 24.000 different names (Viala and Vermorel 1909). In Greece more than 700 grapevine cultivars (*Vitis vinifera* L.) are grown and for the discrimination and classification of Greek cultivars have been used classic ampelographic (Krimbas 1943, Davidis 1967, Vlachos 1986) and biochemical (Stavrakakis 1982) methods.

The aim of this study was to identify and to discriminate 28 Greek and foreign grapevine cultivars and to determine their genetic similarities based on RAPD-PCR and SSR analysis. The

first method, based on random amplified polymorphic DNA obtained by polymerase chain reaction analysis allows the direct comparisons of the genetic material of grape cultivars. The second method, based on simple sequence repeats, also allows the direct comparisons of the genetic material of grape cultivars. Both DNA markers have been used to discriminate cultivars of several plants (Carlson et al. 1991, Stiles et al. 1993) including grapevine (Bourquin et al. 1995, Grando et al. 1995, Moreno et al. 1995, Stavrakakis at al 1997; Stavrakakis and Biniari 1998, Thomas et al. 1994, Botta et al. 1995, Bowers et al. 1996). In this work, 30 decamer primers of arbitrary nucleotide sequence and 8 random microsatellite primers were used to amplify genomic DNA through the polymerase chain reaction (RAPD – PCR and SSR analysis) in order to identify and discriminate grape cultivars grown in different areas in Greece.

Material and Methods

Grapevine material

Twenty-eight grapevine cultivars grown in Greece were chosen for identification (Tab. 1). Leaf material of these cultivars was obtained from the grape germplasm collection of NAGREF in Athens, Agriculture University of Athens as well as different grapevine areas in Greece.

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 were frozen in liquid nitrogen and ground to a 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 polyvinyl-pyrrolidone (MW 40.000)]. A crude nuclei pellet was obtained by centrifugation at 7.000 rpm for 10 min at 4 ^{o}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 polyvinyl-pyrrolidone, 3% sarkosyl, 20% ethanol] and incubated at 37^{o}C for 45 min. An equal volume of chloroform/isoamyl alcohol (24:1) was then mixed in and the phases were separated by centrifugation at 14.000 rpm for 15 min. The aqueous layer was collected and 0.54 volume of frozen isopropanol (-20 ^{o}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^{-1} RNase A and incubated for 15 min at 37 ^{o}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 a 0.25 of cold isopropanol; ca. 120 μg DNA per g FW was obtained.

Amplification conditions

For both RAPD and SSR analysis the protocol reported by Williams et al. (1990) was followed with minor modifications. Amplification reactions were performed in volumes of 25 μl containing 60 ng of genomic DNA, 10 mM TRIS-Cl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 200 M each of dATP, dGTP, dCTP, dTTP, 50 ng primer and 1 unit of Taq DNA polymerase (Qiagen). Thirty random decamer oligonucleotides (Tab. 2) were used as primers for the amplification of RAPD sequences, while eight random microsatellite primers (Tab.3) were used for the amplification of SSR sequences.

Amplification was performed in a Perkin Elmer DNA Thermal Cycler 9600. For the RAPD analysis, after 5 min at 94^{o}C, 35 cycles of PCR were performed, (1 min at the 94^{o}C, 1 min at the 44^{o}C, 2 min at the 72^{o}C) followed by a 10 min at 72^{o}C for extension. For the SSR analysis, after 5 min at 94^{o}C, 35 cycles of PCR were performed, (1 min at the 94^{o}C, 1 min at T^{o}C where T the annealing temperature of each pair of SSR analysis primer, 2 min at the 72^{o}C) followed by a 10 min at 72^{o}C for extension

Gel electrophoresis

Aliquots of the RAPD products were analyzed in 2% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate and 1mM EDTA, pH 8), while aliquots of the SSR products were analyzed in 10% polyacrylamide gel electrophoresis in TBE buffer (Trizma base, boric acid, 0,5M EDTA) After staining in ethidium bromide ($1\mu g.ml^{-1}$) for the RAPD products and silver stain for the SSR products, the gels were photographed on a Gel Doc 1000 (Biorad).

All of the reactions were repeated at least twice with independently isolated genomic DNA as templates. The degree of genetic similarity (I) detected electrophoretically between each pair of cultivar studied was calculated by using the SM coefficient and using the NTSYS-pc package 1.8 developed by Rohlf (Exeter Software, New York, USA), generating the appropriate dendrogram (Fig.2).

Table 1: Studied cultivars and sampling areas.

Cultivar code	Cultivar (Code name)	Sampling area
1.	Skilopnixtis Lefkos (L_Skilopnixt)	Institute of Vine, NAGREF, Athens
2.	Skilopnixtis Mavros (M_Skilopnixt)	Institute of Vine, NAGREF, Athens
3.	Skilopnixtis (K_Skilopnixt)	Vineyards of Ionian's islands
4.	Skilopnixtis (B_Skilopnixt)	Agricultural University of Athens
5.	Skilopnixtis (P_Skilopnixt)	Vineyards of Peloponnese
6.	Skiloklima	Institute of Vine, NAGREF, Athens
7.	Limnio	Institute of Vine, NAGREF, Athens
8.	Limniona	Institute of Vine, NAGREF, Athens
9.	Traminer (L_Traminer)	Institute of Vine, NAGREF, Athens
10.	Traminer (B_Traminer)	Agricultural University of Athens
11.	Mourvedre	Institute of Vine, NAGREF, Athens
12.	Pinot Noir	Institute of Vine, NAGREF, Athens
13.	Pinot Meunier	Institute of Vine, NAGREF, Athens
14.	Pinot Gris	Institute of Vine, NAGREF, Athens
15.	Pinot Blanc	Institute of Vine, NAGREF, Athens
16.	Mavrodafni (L_Mavrodafni)	Vineyards of Ionian's islands
17.	Mavrodafni (P_Mavrodafni)	Vineyards of Peloponnese
18.	Mavrdafni (LY_Mavrodafni)	Institute of Vine, NAGREF, Athens
19.	Hondromavrodafni	Vineyards of Ionian's islands

20.	Mavrodafni agigarti (seedless)	Vineyards of Peloponnese
21.	Reniw	Vineyards of Peloponnese
22.	Fileri Tripoleos	Institute of Vine, NAGREF, Athens
23.	Mosxofilero	Vineyards of Peloponnese
24.	Mosxofilero 7/6	Vineyards of Peloponnese
25.	Fileri A22	Agricultural University of Athens
26.	Bossos (LY_Bossos)	Institute of Vine, NAGREF, Athens
27.	Bossos (LA_Bossos)	Vineyards of Ionian's islands
28.	Thiniatiko	Vineyards of Ionian's islands

Table 2: Synthetic nucleotides were used as primers for RAPDs analysis and number of bands.

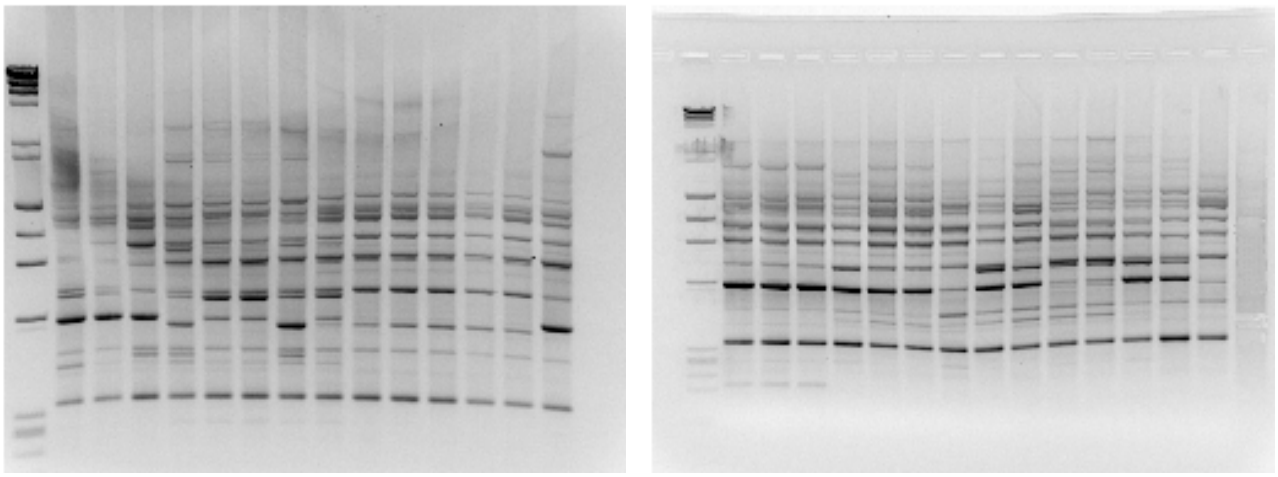
Primer code	**Nucleotide sequence 5′→ 3′**	**Total number of fragments amplified**	**Primer code**	**Nucleotide sequence 5′→ 3′**	**Total number of fragments amplified**
1224	CAGGCCCTTC	15	**OPF-14**	TGCTGCAGGT	22
1225	AGGTGACCGT	17	**OPM-02**	ACAACGCCTC	9
1226	CGCAGGATGG	18	**OPM-03**	GGGGGATGAG	15
1227	GTGTGCCCCA	8	**OPM-04**	GGCGGTTGTC	18
OPF- 01	ACGGATCCTG	9	**OPM-07**	CCGTGACTCA	15
OPF-02	GAGGATCCCT	13	**OPM-08**	TCTGTTCCCC	10
OPF-03	CCTGATCACC	11	**OPM-09**	GTCTTGCGGA	13
OPF-04	GGTGATCAGG	18	**OPM-11**	GTCCACTGTG	14
OPF-07	CCGATATCCC	11	**OPM-12**	GGGACGTTGG	13
OPF-08	GGGATATCGG	6	**OPM-13**	GGTGGTCAAG	17
OPF-09	CCAAGCTTCC	18	**OPM-14**	AGGGTCGTTC	15
OPF-10	GGAAGCTTGG	16	**OPM-15**	GACCTACCAC	20
OPF-11	TTGGTACCCC	8	**OPM-16**	GTAACCAGCC	16
OPF-12	ACGGTACCAG	10	**OPE-04**	GTGACATGCC	13
OPF-13	GGCTGCAGAA	13	**OPQ-01**	GGGACGATGG	16

Table 3: Synthetic nucleotides were used as primers for SSR analysis and their annealing temperature.

Primer code	**Nucleotide sequence 5′→ 3′**	**Annealing Temperature**
VVMD25 [F]	TTCCGTTAAAGCAAAAGAAAAAGG	56,0˚C
VVMD25 [R]	TTGGATTTGAAATTTATTGAGGGG	
ssrVrZAG79 [F]	AGATTGTGGAGGAGGGAACAAACCG	50,0˚C
ssrVrZAG79 [R]	TGCCCCCATTTTCAAACTCCCTTCC	
VVS4 [F]	CCATCAGTGATAAAACCTAATGCC	55.6˚C
VVS4 [R]	CCCACCTTGCCCTTAGATGTTA	56.7˚C

VVMD7 [F]	AGAGTTGCGGAGAACAGGAT	52,0˚C
VVMD7 [R]	CGAACCTTCACACGCTTGAT	
VVS29 [F]	CCCCAAGGCTCTGAAAACAAT	52,0˚C
VVS29 [R]	TGCAAAGCAAATAAAGCTTCC	
VVMD27 [F]	GTACCAGATCTGAATACATCCGTAAGT	56˚C
VVMD27 [R]	ACGGGTATAGAGCAAACGGTGT	
VVMD5 [F]	CTAGAGCTACGCCAATCCAA	56˚C
VVMD5 [R]	TATACCAAAAATCATATTCCTAAA	
VVMD26 [F]	GAGACGACTGGTGACATTGAGC	56˚C
VVMD26 [R]	CCATCACCACCATTTCTACTGC	

(a)



(b)

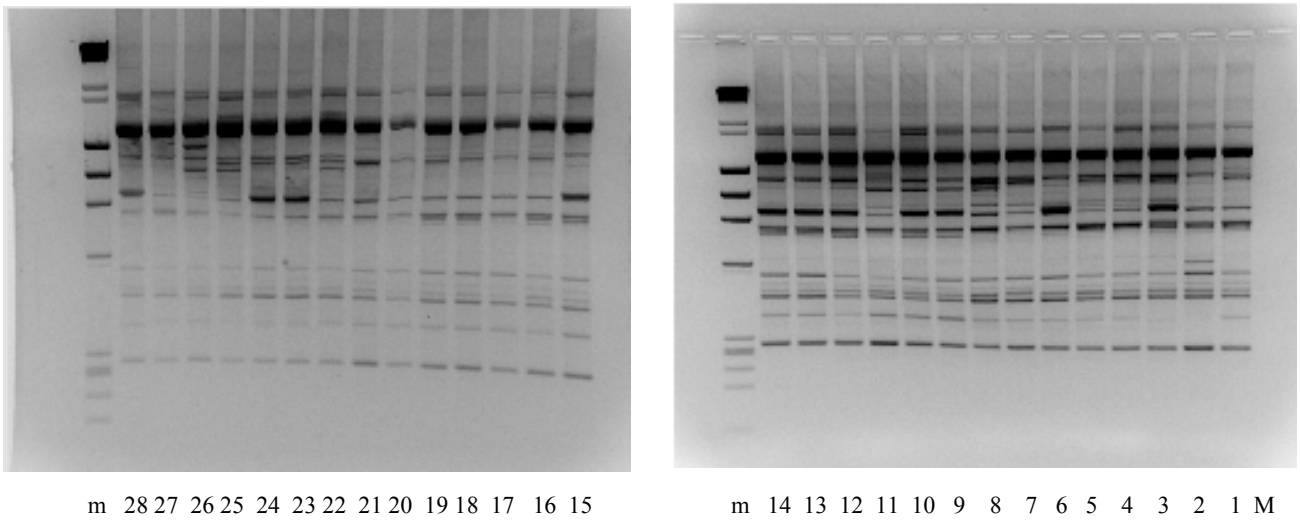


Figure 1: RAPD patterns obtained using primers 1226 (a) and OPF-09 (b) for all studied cultivars.

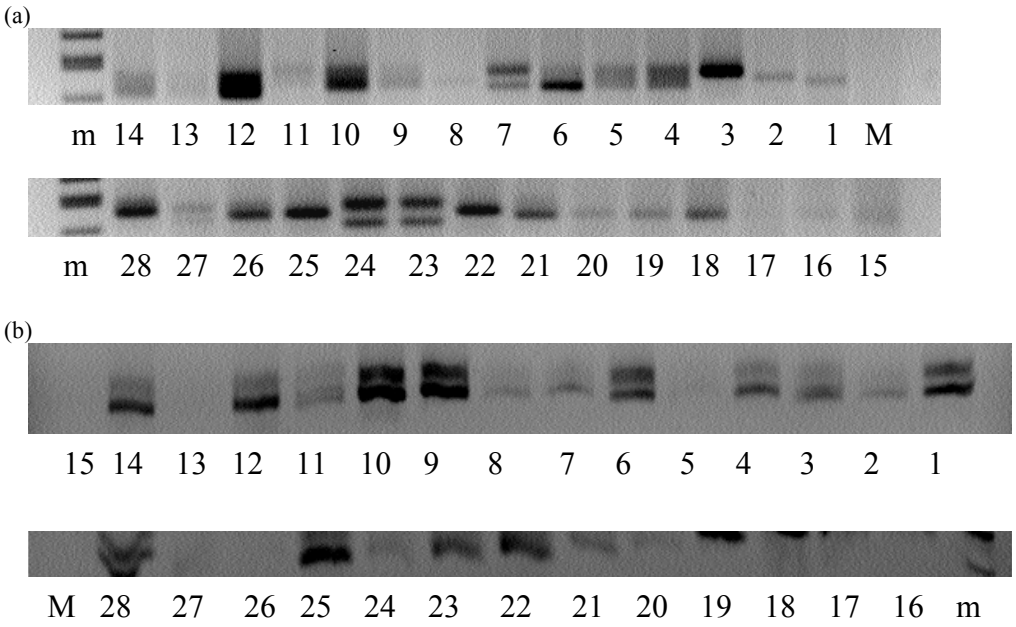


Figure 2: SSR patterns obtained using primers ssrVrZAG7 (a) and VVMD25 (b) for all studied cultivars.

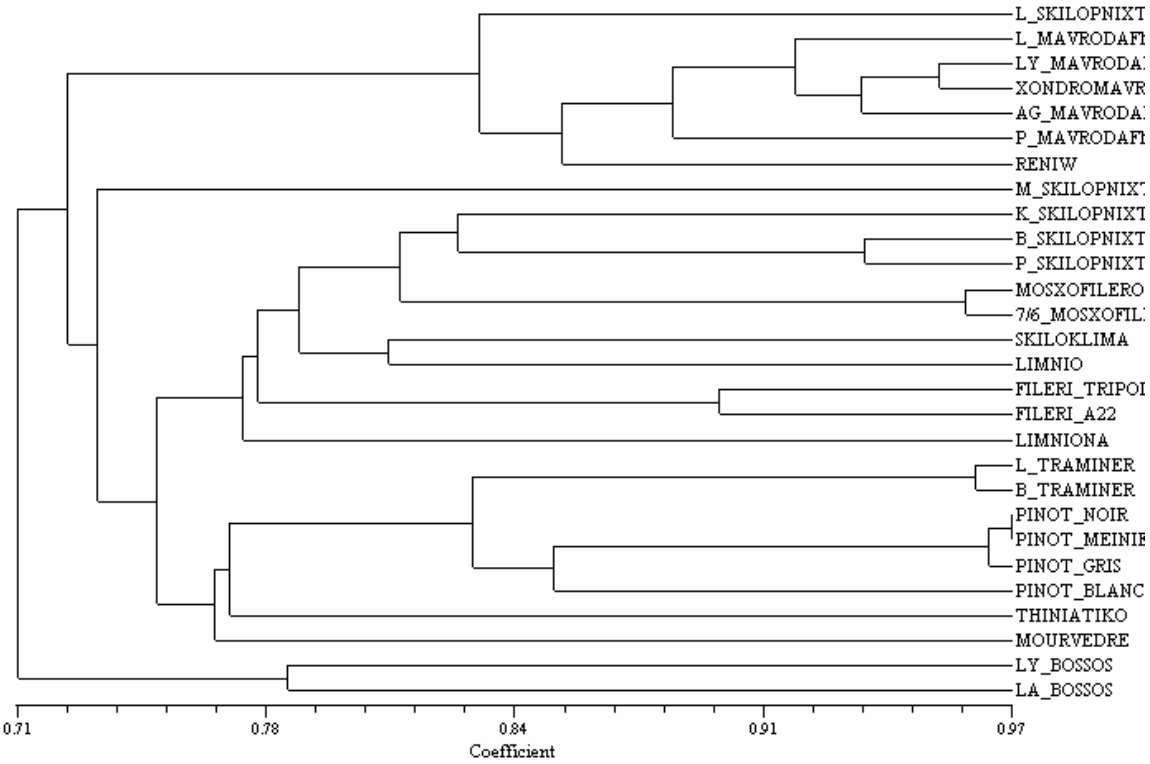


Figure 3: Dendrogram based on amplification products showing the relationship among studied cultivars

Results and Discussion

Thirty random decamer primers and eight random microsatellite primers were used to amplify genomic DNA from 28 greek grapevine cultivars. Each primer provided at least two polymorphic bands. 437 reproducible fragments (89,7% of total electrophoretic bands) were generated by these methods. The total number of amplified fragments for the RAPD analysis is shown in Tab. 2. Among the 30 RAPD primers used, the primers 1225, 1226, OPM-03, OPM-04, OPM-15, OPM-16, OPF-04 and OPF-09 have more polymorphic DNA fragments and discriminated all of the studied cultivars. For the SSR analysis, further research is required with the use of a larger number of primers. Examples of RAPD patterns amplified with primers 1226 and OPF-09 are shown in Fig. 1. Examples of SSR patterns amlplified with primers ssrVrZAG7 and VVMD25 are shown in Fig.2.

In general, there was genetic variation among grape cultivars studied with the values of the genetic similarity ranging from 0,68 to 0,97. Higher genetic similarity was found in the pairs of cultivars Pinot noir-Pinot Meunier (I= 0,97) as expected, L_Traminer-B_Traminer (I= 0,96) also expected, Limnio-Limniona (I= 0,80), Mavrodafni-Hondromavrodafni (I= 0,95) and Mosxofilero-Fileri Tripoleos (I= 0,90).

The results confirmed the ampelographic descriptions (Krimbas 1943, Vlachos 1986) that despite the relatively high degree of genetic similarity (I=0,80), the cultivars Limnio-Limniona are not the same cultivars, as believed. Also, among the cultivars Mavrodafni and the rest of its clones (Hondromavrodafni, Mavrodafni seedless etc.) there is high degree of genetic similarity, but not identity. These cultivars could have originated due to mutations of the original cultivar. The same speculation could be made for the cultivar Reniw, which was originally considered closely related to the typical Mavrodafni, but the results of this study show otherwise. The results of the present study confirm previous study (Stavrakakis et al. 1998/99) that the cultivar Mosxofilero 7/6 is considered a clone of the cultivar Mosxofilero, both of which however are different from the cultivar Fileri Tripoleos. The results show that there is genetic variation among studied greek cultivars and that RAPD-PCR and SSR are reliable and very useful methods for the identification and genetic analysis of grape cultivars.

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