Genetic study of greek grapevine cultivars (*Vitis vinifera* L.) of the Aegean region using molecular methods and ampelographic decription

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Abstract: This study presents the results regarding the identification and discrimination of 12 greek grapevine varieties (*Vitis vinifera* L.) of the Aegean region using the ampelographic description and the molecular method AFLP (Amplified Fragment Length Polymorphism). For the ampelographic description, 24 ampelographic descriptors developed by the International Organisation of Vine and Wine were used whereas for the molecular analysis, a total of 7 primer combination with three selective nucleotides were used. These primer combinations proved to be extremely polymorphic and produced a total of more than 450 amplified fragments. The analysis of the samples was carried out using the Genetic Analyzer 310 (Applied Biosystems). For the statistical analysis of both methods, the method UPGMA and the similarity coefficients DISTand DICE were used.
From the comparative study of the degree of genetic similarity which was calculated and the relative dendrograms which were constructed in the AFLP analysis, among the grapevine cultivars studied, it was found that there is high degree of genetic similarity between the cultivars Fokiano, Arikaras, Giouroukiko, Armeletoussa, something that allows the speculation that these cultivars derive from one parent cultivar. The same can be said for the pairs of cultivars Stavrochiotiko and Serifiotiko. A separate group is consisted by the cultivars Eftakoilo, Xerichi kokkino, Xerichi mavro and Xeromachairouda with an equal high degree of genetic similarity. The above results are in accordance with those of the ampelographic description. Although further study is required, these results enhance the notion that there is a secondary creation center of Greek grapevine varieties in the greater Greek area.

Keywords: *Vitis vinifera* L., grapevine, ampelography, genetic similarity, AFLP

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 classic ampelographic (Krimbas 1943, Davidis 1967, Vlachos 1986) and biochemical (Stavrakakis 1982) methods have been used for their discrimination and classification. Later, the molecular techniques using PCR-derived DNA markers (RAPD, SSR, AFLP etc.) have been used excessively to discriminate cultivars of several plants including grapevine (Bourquin et al. 1995, Stavrakakis at al. 1997; Stavrakakis and Biniari 1998, Thomas et al. 1994, Botta et al. 1995, Bowers et al. 1996, Stavrakaki and Biniari 2008, Meneghetti et al. 2011).
The aim of this study was to identify and to discriminate 12 Greek grapevine cultivars using the ampelographic description and to determine their genetic similarities based on the AFLP analysis. For the ampelographic description, 24 ampelographic characters were measured on each grapevine cultivar during the years 2011 and 2012, following a list of descriptors developed by the International

Organization of Vine and Wine (OIV, 2009), including the preliminary minimal traits relative to shoot, mature leave, bunch and berry (Tab. 2). Ampelographic data were used to create a similarity matrix, in order to generate a dendrogram (Fig. 2), based on the DIST index, to present the morphological relationships between the cultivars, as implemented in the NTSYS-pc package 2.1 developed by Rohlf (Exeter Software, New York, USA, 1993).
For the molecular analysis, among the various polymerase chain reaction (PCR)-based DNA marker techniques used, the Amplified Fragment Length Polymorphism (AFLP) was used, which is ideal when the goal is the definition of identity among different clones of the same variety or among genetically close related cultivars, with positive results in differentiating grapevine cultivars and clones (Vignani et al. 2002, Blaich et al. 2007, Stenkamp et al. 2009, Alba et al. 2011). In this work, a total of 7 primer combination with three selective nucleotides were used to amplify genomic DNA through the polymerase chain reaction in order to identify and discriminate grapevine cultivars grown in different areas in Greece (Tab. 3).

Material and Methods

Grapevine material

Twelve grapevine cultivars grown in Greece and found in the greater Aegean region were chosen for identification (Tab. 1). Leaf material of these cultivars was obtained from the grape germplasm collection of NAGREF in Athens and from the collection of Agriculture University of Athens.

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

AFLP analysis was conducted as reported by Vos et al. (1995), following the AFLP Plant Mapping Protocol by Applied Biosystems (2007), with modifications.
For the Restriction – Ligation stage, genomic DNA (500ng) was incubated for 14-16 hours (overnight) at 20^{o}C with the presence of 3U (units) of enzyme EcoRI (5'..GAATTC..3') and 1U of enzyme MseI (5'..TTAA..3'). In each sample there were 4U T4 DNA Ligase, 1µl of EcoRI adaptor and 1 µl MseI adaptor, T4 DNA Ligase buffer, NaCl and BSA, in a final volume of 11 µl. After the incubation, 189 µl of TE buffer were added in each sample, and the products were stored at -20^{o}C. All pre-amplification and amplification reactions were performed in a Perkin Elmer DNA Thermal Cycler 9600.

For the Preselective Amplification (Preselective PCR), 4 μl of the diluted products of the restriction-ligation stage were used. For the reaction, 15 μl of AFLP Core Mix (Applied Biosystems) and 1,0 μl of AFLP preselective primer pairs (Applied Biosystems) were added in a final volume of 20 μl. The preselective PCR thermal conditions were: 2 min at 72ºC, 20 cycles of 22 sec at 94 ºC, 33 sec at 56 ºC and 2 min at 72ºC, with a final step of 30 min at 60ºC. The samples were then stored at 4ºC. After the end of the amplification, 10 μl of the pre-amplified products were checked on 1,5% (w/v) agarose gels, in TAE buffer (40 mM Tris-acetate and 1mM EDTA, pH 8), and stained in ethidium bromide ($1\mu g.ml^{-1}$). The gels were photographed on a Gel Doc 1000 (Biorad). The remaining 10 μl of the pre-amplified products were diluted with 190 μl of TE buffer and stored at 4ºC.
For the Selective Amplification (Selective PCR), 1,5 μl of the diluted preselective products were used. For the reaction, 7,5 μl of AFLP Core Mix (Applied Biosystems) and 0,5 μl of each selective primer were added in a final volume of 10 μl (the primers were EcoRI[Primer-Axx-Dye] and MseI[Primer-Cxx]). The selective PCR thermal conditions were: 2 min at 94ºC, 10 cycles of 20 sec at 94ºC, 30 sec at 66ºC (the annealing temperature was reduced in every cycle by 1ºC) and 2 min at 72ºC, 20 additional cycles of 20 sec at 94ºC, 30 sec at 56ºC and 2 min at 72ºC, with a final step of 30 min at 60ºC. The samples were then stored at 4ºC.
For the loading buffer, 13 μl of deionized formamide with 0,5 μl of GeneScan-500 (LIZ) size standard (Applied Biosystems) were added to 1,0 μl of each amplification sample. The samples were first denaturated at 94ºC for 5 min and then separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). AFLP electrophoregrams were acquired and analysed using the GeneMapper v4.0 software (Applied Biosystems), using the Local Southern Method (Fig.1).
The degree of genetic similarity (I) detected between each pair of cultivar studied was calculated using the DICE coefficient (Nei and Li, 1979) and using the NTSYS-pc package 2.1 developed by Rohlf (Exeter Software, New York, USA, 1993), generating the appropriate dendrogram (Fig.3).

Table 1: Studied cultivars and sampling areas.

Cultivar code	Cultivar (Code name)	Sampling area
1.	Eftakoilo	Institute of Vine, NAGREF, Athens
2.	Xerichi kokkino	Institute of Vine, NAGREF, Athens
3.	Xerichi mavro	Institute of Vine, NAGREF, Athens
4.	Xeromachairouda	Institute of Vine, NAGREF, Athens
5.	Serifiotiko	Institute of Vine, NAGREF, Athens
6.	Stavrochiotiko	Institute of Vine, NAGREF, Athens
7.	Arikaras	Agricultural University of Athens
8.	Armeletoussa	Institute of Vine, NAGREF, Athens
9.	Giouroukiko	Institute of Vine, NAGREF, Athens
10.	Fokiano	Agricultural University of Athens
11.	Syriki	Agricultural University of Athens

12.	Provatina	Agricultural University of Athens

Table 2: Ampelographic characterization, based on 24 OIV descriptors.

OIV Ampelographic Descriptor Code	Xerichi_kok	Xeromachairouda	Eftakoilo	Syriki	Provatina	Serifiotiko	Stavrochiotiko	Fokiano	Giouroukiko	Armeletousa	Arikaras
001	7	7	7	7	7	7	7	7	7	7	7
003	1	2	1	2	2	2	3	3	3	3	1
004	2	1	3	3	3	3	3	1	1	1	1
011	1	1	1	1	1	1	1	1	1	1	1
016	1	1	1	1	1	1	1	1	1	1	1
065	5	5	6	5	5	5	5	6	5	5	5
068	3	4	3	3	4	3	3	2	2	2	3
076	3	2	3	2	2	3	2	3	3	3	3
079	3	3	6	3	4	3	3	3	3	4	5
080	2	1	2	1	1	1	2	2	2	2	2
084	1	1	1	1	1	1	1	1	1	1	1
085	1	1	1	1	1	1	1	1	1	1	1
086	1	1	1	1	1	1	1	1	1	1	1
087	1	3	1	1	2	1	1	1	1	1	1
151	3	3	3	3	3	3	3	3	3	3	3
202	9	9	7	9	7	5	5	5	5	5	5
206	7	7	5	7	7	5	5	5	5	3	5
220	5	5	5	4	5	5	5	5	5	5	4
223	6	3	3	6	3	3	3	3	3	3	3
225	2	7	6	3	3	3	2	5	5	5	7
230	1	1	1	1	1	1	1	1	1	1	1
236	1	1	1	1	1	1	1	1	1	1	1
241	3	3	3	3	3	3	3	3	3	3	3
244	1	1	1	1	1	1	1	1	1	1	1

Table 3: Primers used for AFLP analysis (Applied Biosystems).

Primer Code EcoRI – Axx – Dye – MseI - Cxx	Number of Fragments Amplified
EcoRI – ACG JOE – MseI – CTC	78
EcoRI – ACT FAM – MseI – CAG	27
EcoRI – ACG JOE – MseI – CTA	98
EcoRI – ACA FAM – MseI – CTA	62
EcoRI – ACA FAM – MseI – CTT	59
EcoRI – AGG JOE – MseI – CAC	77
EcoRI – ACG JOE – MseI – CAC	70
Total	**471**

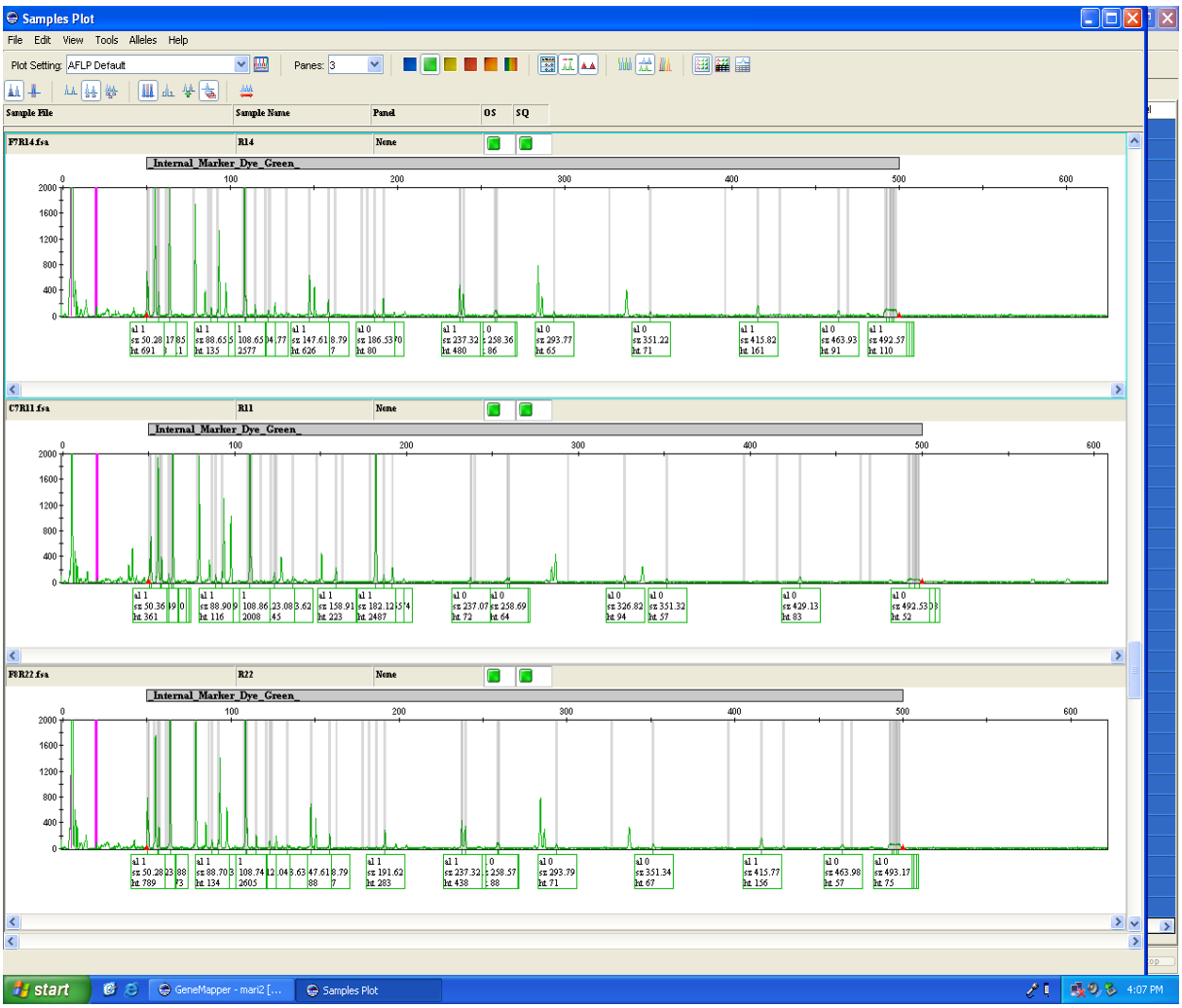

Figure 1: AFLP electrophoregrams acquired and analyzed using GeneMapper v4.0 (Applied Biosystems).

Figure 2: Dendrogram based on ampleographic descriptors showing the relationship among cultivars studied (coefficient DIST)

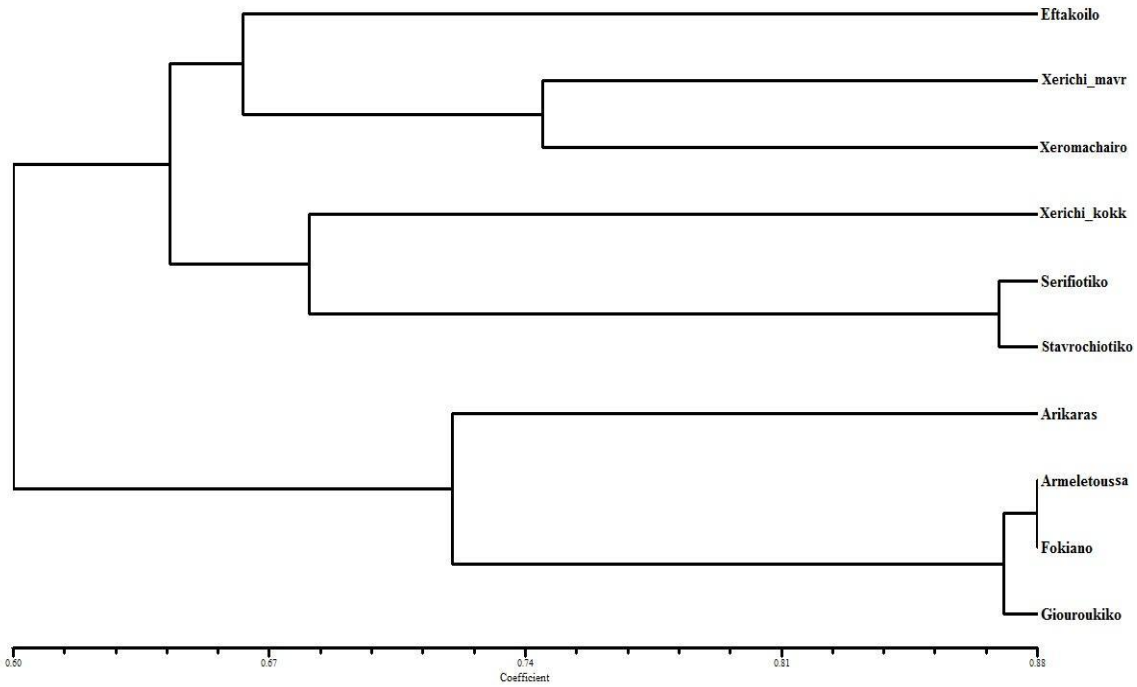

Figure 3: Dendrogram based on AFLP amplification products showing the relationship among cultivars studied (coefficient DICE)

Results and Discussion

Seven primer combinations were used to amplify genomic DNA from 12 greek grapevine cultivars. They proved to be extremely polymorphic and produced a total of more than 450 amplified fragments, discriminating all of the cultivars studied. The ampelographic data were used to obtain a dendrogram of similarity, a dendrogram which showed the formation of two main clusters, as with the molecular analysis, discriminating all of the cultivars studied. Since each similarity coefficient has a different scale, there may be difference in the degree of similarity.

The degree of genetic similarity as determined with the use of AFLP is higher than the one based on ampelogrpahic description. This can be attributed to the use of relatively low number of ampelographic characters as well as to the phenotypic fluctuation of the basic ampelographic descriptors, mainly due to the environment and cultivation technique.

These results, both with the ampelographic description as well as with the use of AFLP, confirm previous studies with the use of classic ampelography (Krimbas, 1943), that the cultivars Giouroukiko, Arikaras and Armeletoussa are closely related to Fokiano.

Specifically, the high degree of genetic similarity between the cultivars Fokiano, Arikaras, Armeletoussa and Giouroukiko, as determined with the use of AFLP and Dice coefficient (0,80), allows the notion that not only are we dealing with closely related cultivars but also that Fokiano may actually constitute the parent cultivar from which the others derived. For the first time, it is ascertained that two cultivars with different origin and names, Serifiotiko and Stavrochiotiko are closely related (0,87) and that most likely, the one originated from the other.

The above results, for the cultivars studied, indicate that these cultivars are of Eastern origin and are characterized, ampelographically, by the absence of hair (on leaves, tip, shoot) and originate from a common cultivation center.
The relatively high degree of genetic similarity observed among the cultivars studied allows the notion that we are dealing with closely related cultivars and that the Aegean region could be considered as a secondary creation center of Greek grapevine varieties. Further research is of course required.
The combination of molecular methods and ampelographic description is extremely effective for the study of the between and within genetic diversity of grapevine cultivars.

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