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A combined approach involving ampelographic description, berry oenological traits and molecular analysis to study native grapevine varieties of Greece

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Summary

A combined approach involving phenotypical characterization (ampelographic description and oenological traits) and molecular analysis was applied on 91 accessions of native Greek grape varieties plus 3 references, all conserved in the Ampelographic Collection of the Aristotle University of Thessaloniki. The accessions were described in accordance to 48 OIV descriptors. Their berry oenological traits were determined at maturity to detect a high juice sugar concentration in most of the assessed varieties, whereas the titratable acidity was found to be extremely low, particularly in the white accessions. Moreover, skin anthocyanin and phenolic content fluctuated from 0.09 to 39.4 mg·g^{-1} f.w. and from 2.05 to 30.65 mg·g^{-1} f.w. respectively, whereas seed phenolic content was in the range of 2.83 and 32.72 mg·g^{-1} f.w. Finally, the discriminative SSR analysis confirmed the differences and similarities among the analyzed varieties as can be evinced from the phylogenetic analysis where close genetic relationship has been detected between 'Fokiano' and 'Armeletousa', 'Moschato Spinas' and 'Moschato Samou', 'Vilana' and 'Asprouda Patron', and 'Mouchtouri' and 'Mavro Spetson'. In all these occasions, the parts of each pair possess similar morphological characteristics.

K e y w o r d s : *Vitis vinifera*; Greek grapevine varieties; microsatellites; SSRs.

Introduction

Grapevine is cultivated in Greece since antiquity (Valamoti 2011, Banilas *et al*. 2009). Nowadays, viticulture occupies an area of about 117,000 ha of which 67,300 ha yield wine grapes, 21,700 ha table grapes and 28,000 ha raisin grapes. The diverse geographical terrain of the country creates many microenvironments, with cold winters and cool summers in the mountainous regions to soft winters and hot summers in the southern areas and the islands. The combination of such geographical and climate conditions results in a range of local varieties that have been completely adapted to the local conditions favoring an environmentally friendly agricultural system of low inputs. The local varieties, however, have been neglected especially during the second half of the 20th century due to the outstanding performance of the international varieties. To prevent and avoid their loss the Aristotle University of Thessaloniki (AUTh) established its own Ampelographic Collection in the 1950's in AUTh's experimental farm. Over the following years, the Collection was enriched with varieties collected from different regions of the country, so that today it is one of the three most important grapevine conservation sites in Greece. The importance of the Collection became evident in recent years: the neglected and underappreciated local varieties, which represent a valuable genetic reservoir, could provide the material for the making of products with novel characteristics that could establish their own way into the international markets. Therefore, evaluation of the local varieties became an urgent demand and a first priority in the design of the national oenological strategy and management. The initial step, however, towards this direction is to accurately identify and extensively characterize the various available local genotypes in order to use them accordingly (Korir *et al*. 2012).

Traditionally, description and discrimination of vine varieties was performed by ampelographic means, which relies on the comparison of their morphology (This *et al*. 2004, Krimbas 1943, Logothetis 1947). This approach based on visual observation and performed by a "small and declining number" of experts, is influenced by the environment and the life history of each plant (This *et al*. 2004). Such limitations have been largely overcome by the use of biochemical methods which successfully discriminate grapevine varieties since the '70s (Stavrakakis 1982). Microsatellites (Simple Sequence Repeats- SSRs) represent a powerful tool for various genetic studies, including plant genotyping, because they are highly reproducible and polymorphic (Olivera *et al.* 2006, and references therein). As for studies on grapevine, SSRs have been widely in use, so as a set of six microsatellites have been included into the OIV (OIV: The International Organization of Vine and Wine) descriptor list (OIV 2009).

In the present work, implemented in the frame of the COST Action FA1003 "Grapenet: East-West Collaboration for Grapevine Diversity Exploration and Mobilization of Adaptive Traits for Breeding", a total of 94 accessions (91 autochthonous Greek accessions from the AUTh's Ampelographic Collection plus 3 well known international varieties as reference) have been analyzed at 10 microsatellite loci. In addition, their ampelographic description by

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48 OIV descriptors has been performed, while at the same time carpological and berry analytical traits were measured at the ripening stage according to the protocols proposed during the COST Action FA1003. The aim of the current study was to analyze comprehensively the set of 91 Greek varieties in order to define clearly their relationship in ampelographic and genetic level. The selection of the Greek accessions was made on the ground of their future dynamics as they have been judged by our working experience.

Material and Methods

The experiment was conducted over the years 2012 and 2013. Ninety-one Greek accessions from the AUTh's ampelographic collection plus 'Cabernet-Sauvignon', 'Pinot Noir' and 'Soultanina' as reference varieties, have been described by 48 OIV descriptors (OIV 2009). Relationships among the OIV descriptors (parameters) were studied by linear regression using the SPSS statistical program. Principal Component (PC) and Cluster (C) analysis were used to evaluate the most important parameters that contributed to the vine variety separation into different groups according to their morphological traits (OIV descriptors). At ripening, total berry weight, berry length and width, total skin weight, seed number and weight have been measured. Juice sugar content and total acidity were measured by refractometer and by titrating with NaOH 0.1N, respectively. Skin total anthocyanin and phenolic compounds, and seed total phenolics were also measured in red varieties (DI STEFANO *et al*. 1989).

For the SSR analysis, polymerase chain reactions (PCRs) were performed in a volume of 20 μL including 30 ng genomic DNA, 200 mM of each dNTP, 10 pmol primers, 4 μL 5X MyTaq Reaction Buffer, and 1u MyTaq DNA Polymerase (Bioline, UK). The 10 pairs of primers used, included the OIV core set: VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, VrSZAG79 corresponding to OIV801 to OIV806 descriptors (THIS *et al.* 2004, OIV 2009), along with VrZAG67 VVMD28, VVMD32, and VVMD25**.** Forward primers were 5´-end fluorescently labeled with different fluorophores. The dyes that were used for 5' primer labeling were FAM, HEX, ROX and TAMRA. Primers were custom labeled according to each dye's absorption and emission wavelength and the length of the amplified product, in order to avoid overlapping during electrophoresis. PCR amplifications were performed in a 96-well Veriti® Thermal Cycler (Applied Biosystems, USA) as follows: 1 cycle [95 °C, 2 min], 35 cycles [95 °C, 15 s; 52 to 60 °C (depending on the primer), 15 s; 72 °C, 10 s], and 1 cycle [72 °C, 20 min]. PCR fragments were separated using capillary electrophoresis in a 3730xl DNA Analyzer (Applied Biosystems, USA). Data analysis, sizing and genotyping were performed using the GeneMapper (version 4.0) software.

A dendrogram was constructed using the MEGA5 program, whereas the CERVUS software (MARSHALL *et al*. 1998) was used to calculate the observed heterozygosity (Ho), the expected heterozygosity (He), the number of alleles (Na), the number of effective alleles (Ne) and the fixation index (F).

Results and Discussion

OIV descriptor evaluation: According to the PC analysis, which transforms the original data set (OIV descriptors) into a smaller set of uncorrelated new variables (Principal Components), 16 components have been produced in a decline series of their importance, explaining 70.84 % of the total variability among the different varieties. Each component is strongly correlated with a set of the initial OIV descriptors so we can estimate their contribution to the variability. The OIV descriptors strongly correlated with the 10 first components are presented in Tab. 1. For example, the OIV 053 descriptor ("Young leaf: density of prostrate hairs between main veins on lower side of blade - 4th leaf") contributed better to variability compared to the OIV 303 descriptor ("Time of beginning of berry ripening- veraison") or the OIV 070 descriptor ("Mature leaf: area of anthocyanin coloration of main veins on upper side of blade"). Cluster analysis separated the varieties in particular groups according to their morphological characteristics (Figure not shown).

Berry oenological traits: An important variability among the varieties analyzed was the total soluble solid content. During the maturity period (August-September) grape maturity was defined; no changes

Table 1

Evaluation of the OIV descriptors and their contribution to the variability of the vine varieties

Principal Components									
1	2	3	4	5	6	7	8	9	10
% Contribution to variability									
7.17	6.61	5.09	4.49	4.46	4.42	4.3	4.16	4.12	4.05
Related OIV descriptors									
053	502	506	079	220	074	007	087	081-1	303
084	504	225	080	221	051	008	003	076	070
004	068	075	072	503					

Table 2

Indices of genetic diversity per locus. Na: no. of alleles; Ne: no. of effective alleles; Ho: observed heterozygosity; He: expected heterozygosity; F: fixation index

Locus	N	Na	Ne	Ho	He	F
VVS2	94	13,000	5,398	0,723	0,815	0,112
VVMD5	94	10,000	6,440	0,745	0,845	0,118
VVMD27	94	11,000	5,313	0,840	0,812	-0,035
VVMD7	93	14,000	5,530	0,806	0,819	0,016
VrZAG62	88	10,000	6,225	0,909	0,839	-0,083
VrZAG79	93	10,000	5,495	0,731	0,818	0,106
VVMD32	94	15,000	5,481	0,734	0,818	0,102
VVMD25	94	10,000	4,630	0,883	0,784	-0,126
VVMD28	93	15,000	7,617	0,860	0,869	0,010
VrZag67	92	17,000	7,812	0,728	0,872	0,165
Mean	92,900	12,500	5,994	0,796	0,829	0,038

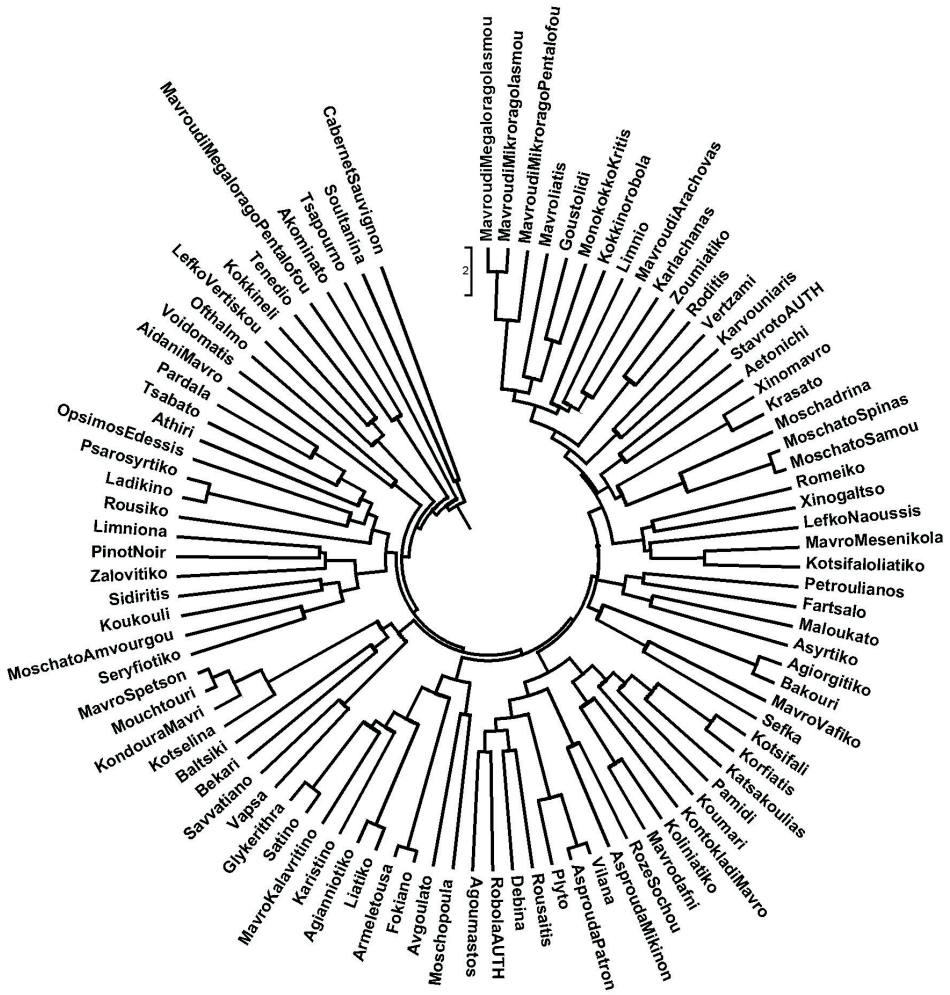

Figure: Dendrogram of the 94 accessions examined using the unweighted pair group method with the allelic data obtained from the 10 microsatellite loci analyzed.

in °Brix degree measurements (according to the COST working group instructions) were recorded in weekly collected samples. The °Brix degree ranged between the lowest value of 14.4 % for 'Voidomatis' and the highest value of 27.50 % for 'Koumari'. A significant number of varieties showed high or very high levels. The titratable acidity was generally low to very low with some exceptions, such as the 'Xinomavro' group ('Xinomavro', 'Zalovitiko', and 'Xinogaltso' with 7.33, 6.97, 5.97 $g \cdot L^{-1}$ tartaric acid, respectively), which were found to have medium acidity content.

An important variability was detected for anthocyanin and phenolic content of the red varieties. Skin anthocyanin and phenolic content fluctuated from 0.09 to 39.4 $mg \cdot g^{-1}$ f.w. and from 2.05 to 30.65 $mg \cdot g^{-1}$f.w. respectively, whereas the seed phenolic content between 2.83 and 32.72 $mg \cdot g^{-1}$ f.w..

Parameters of the genetic studies: In the current study, 91 Greek autochthonous grapevine accessions were genotyped at 10 loci. The analysis was informative since all analyzed loci were polymorphic among grapevine accessions, producing a total of 125 different alleles with a mean of 12.5 different alleles per locus, varying between 10 (VVMD5, VVMD25, VrZAG62, VrZAG79) and 17 (VrZAG67). Observed Heterozygosity (Ho) is defined as the number of individuals heterozygous per locus (AVISE 2001); the higher the Ho values the higher the genetic variability it is. The values of Observed Heterozygosity (Ho) ranged from 72.3 % to 90.9 % with a mean of 79.6 % (Tab. 2). The Expected Heterozygosity (He), or gene diversity, ranged between 78.4 % and 87.2 %, with a mean value of 82.9 %. Although, in three occasions (VVMD27, VrZAG62, and VVMD25) Ho values were higher than the He values, the mean Ho value was lower than the He value (79.6 % and 82.9 %, respectively) indicating probable inbreeding among the varieties. The Fixation Index is a measure of the difference in the allele frequency between two populations. Although it ranges from 0 (meaning complete sharing of genetic material) to 1 (meaning no sharing), it practically receives values much less than the maximum.

Genetic and morphological identification: The genetic analysis using the molecular marker approach ended up revealing that the microsatellite profile of three pairs of the analyzed accessions differ only in one allele out of a total of 20 alleles examined: 'Moschato Spinas' and 'Moschato Samou' at VVS2, 'Vilana' and 'Asprouda Patron' at VVS2, and 'Fokiano' and 'Armeletousa' at VrZAG67, while another pair of accessions, 'Mouchtouri' and 'Mavro Spetson', differ in both alleles of a single loci (VVMD28). All these molecular findings confirm the high levels of similarity observed by optical observation: in the case of the 'Fokiano' and 'Armeletousa', their ampelographic similarity was known since the early '60s (LOGOTHETIS and VLACHOS 1963) suggesting that they are putative synonyms.

'Xinomavro' is one of the noblest Greek varieties that is cultivated in the northern parts of the country. According to previous microsatellite work, performed before the proposal and establishment of the 6 microsatellites as the basic markers for grapevine genotyping (THIS *et al.* 2004, OIV 2009), 'Xinomavro' was considered almost identical to 'Krasato' due to the fact that these varieties shared 16 identical alleles out of a total of 20 (Greek Vitis Database: http://gvd.biology.uoc.gr/gvd). Our results confirm the close relationship between 'Xinomavro' and 'Krasato' since both accessions possessed identical microsatellite profiles in 8 loci, whereas the two accessions differed in both alleles of the remaining two loci (VVMD25 and VrZAG67). Therefore they should not be considered as synonyms. Ampelographic description showed that 'Xinomavro' and 'Krasato' share many ampelographic characters however they differ in some others. For instance, there are no well-formed lateral sinus on the 'Xinomavro' blade, whereas there are deep superior and inferior ones for the 'Krasato' blade.

According to the Greek National Catalogue, 'Xinogaltso' is considered as a synonym to 'Xinomavro'. According to our molecular findings, however, they are clustered in different sub-clades as they differ in 11 of the 20 alleles examined (Figure).

Another three pairs of accessions, namely 'Ladikino' and 'Psarosyrtiko', 'Satino' and 'Glykerithra', and 'Agiorgitiko' and 'Bakouri' share the same alleles at 9 loci (18 identical alleles out of a total of 20). The two remaining alleles occur in different loci (VVMD32 and VrZAG67 for the first pair, and VVMD7 and VrZAG62 for the remaining two pairs), therefore there is a strong indication of a parent/progeny relationship. Similarly, 'Liatiko' and 'Agianniotiko', and 'Mavroudi Mikrorago Iasmou' and 'Mavroudi Megalorago Iasmou' share 17 alleles, with two of the remaining three alleles at the same loci (VrZAG62 for the first pair and VrZAG67 for the second pair).

Conclusions

Although for millennia selection of *Vitis* genotypes was based on phenotypic characterization, only recently with the advent of high throughput methods and molecular tools, it was possible to accurately genotype a high number of *Vitis* varieties. Among these methods, microsatellites represent a useful tool to discriminate plant varieties including those of grapevine. The current study showed that in four pairs ('Fokiano' and 'Armeletousa', 'Moschato Spinas' and 'Moschato Samou', 'Vilana' and 'Asprouda Patron', and 'Mouchtouri' and 'Mavro Spetson') there is only one allele out of 20 examined that differs. Moreover, high degree of genetic relationship was detected between the pairs 'Ladikino' and 'Psarosyrtiko', 'Satino' and 'Glykerithra', 'Agiorgitiko' and 'Bakouri', 'Liatiko' and 'Agianniotiko', and 'Mavroudi Mikrorago Iasmou' and 'Mavroudi Megalorago Iasmou' which share the same 17 or 18 alleles of the total of 20. Despite the genotyping work performed, we believe that still additional molecular work is needed in order to safely suggest any synonym or homonym annotation.

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