Ampelographic description, berry oenological traits, and molecular characterisation of grapevine varieties grown in northern Greece

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Local grapevine varieties that have been traditionally grown and cultivated in the northern region of Greece, have been collected, transported by cleft grafting onto the same rootstock, and are currently grown in a vineyard at the Experimental Farm of the Aristotle University of Thessaloniki constituting our ampelographic collection (AUTh Ampelographic Collection). Forty five varieties, including Cabernet Sauvignon and Merlot as references, have been evaluated using a holistic approach: a combination of OIV ampelographic descriptors, berry oenological traits evaluation, and molecular characterization by using 10 Single Sequence Repeats (SSRs) markers. By the amplification of the 10 SSR loci a total of 87 alleles were detected with an average allele number of 9.4 per locus, while the Expected heterozygosity (gene diversity) value was averaged at 0.811. The SSR profile of the 45 varieties indicates that each variety is unique (each variety can be distinguished from all others); their genetic relationship is reflected in a dendrogram.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most important cultivated species in the world growing on approximately 8 million hectares. In Greece, an area of 67,300 ha yields wine grapes, while 21,700 and 28,000 ha yield table grapes and raisin grapes, respectively.

The widespread genetic erosion that occurred after the 2nd World War as the dramatic result of the deep transformation in viticulture policy, favored the cultivation of a handful of varieties threating vine biodiversity. Local varieties, representing valuable genetic resources, were saved from extinction in Ampelographic Collections around the world. Within such collections, the varieties are described in ampelographic terms, a practice that involves comparison of the aerial part of the vine to the OIV descriptors (OIV 1983), while the fruit morphology and analytical traits are also evaluated. The recent development of molecular biology introduced new methods to genetically clarify the identity and parentage of different grapevine varieties (Bourquin et al. 1992, Moreno et al. 1998, Dhanorkar et al. 2005) as a supplementary or alternative method to ampelography. The Aristotle University of Thessaloniki (AUTh) Ampelographic Collection was established in 50's in

the AUTh's experimental farm by cleft grafting onto the same rootstock. During the following years the collection was continuously enriched so as today it consists of 350 local grapevine varieties (collected from various regions of Greece) and 40 rootstocks and American species.

In the present study, 45 varieties of the AUTh Ampelographic Collection that had been collected from different regions of northern Greece were evaluated for their oenological traits, while their identification was performed by ampelographic description (comparison to the OIV descriptors) and by the application of molecular analysis using the SSR molecular markers.

MATERIALS & METHODS

Forty-five varieties of the AUTh Ampelographic Collection, including Cabernet Sauvignon, Merlot and Pinot noir that were used as reference varieties, were evaluated. Each variety has been assigned to a unique accession number. All 45 varieties have been described by 48 descriptors provided by OIV codes published in June 2007. The consecutive phenology stages were identified by the BBCH system, which was developed as a uniform model code for European Union and adapted for grapevine by Lorenz et al. (1994). When the BBCH code was applied to the grapevine the following 7 macro-stages were considered: "0" for bud development, "1" for leaf development, "5" for inflorescence development, "6" for flowering development, "7" for fruit development, "8" for fruit ripening, and "9" for senescence (Coombe, 1995).

At the end of veraison stage, 9 representative bunches have been selected and labeled among the ones fully developed in comparison to the expected varietal identity. Ripening time has been defined following weekly the Sugar Content by refractometric measures. Six to nine representative bunches were collected at ripening, divided into 3 samples of 2-3 bunches each and were weighted. Total Berry Weight, Berry Length and Width, Total Skin Weight, Seed Number and Weight have been measured. Juice Sugar Content was measured by a refractometer, while the Total Acidity was measured by titrating with NaOH 0.1N. Skin Total Anthocynin and Phenolic Compounds, and Seed Total Phenolics were also measured in red varieties.

For the SSR analysis, Polymerase Chain Reactions (PCRs) were performed in a volume of 20 µL including 30 ng genomic DNA, 200 mM dNTPs, 40 pmol primers, 2 µL 10X KAPATaq DNA Polymerase buffer, and 1 U KAPATaq DNA Polymerase (KapaBiosystems, Cape Town, South Africa). The following 10 pairs of primers were used in the SSR analysis: VVS2, VrZAG62, VrZAG67, VrSZAG79, VVMD5, VVMD7, VVMD27, VVMD28, VVMD32, and VVMD25**.** Forward primers were 5′ labeled with IR700/IR800 dyes. PCR amplifications were performed in a MasterCycler (Eppendorf, Hamburg, Germany) as follows: an initial step of 5 min at 94°C, followed by 35 cycles, each one including 30 s at 94°C for denaturation, 90 s at 52° to 56°C (depending on the primer) for annealing, and 90 s at 72°C for elongation. A 5 min step at 72°C was programmed as a final extension. PCR amplification products were separated by

electrophoresis in 25-cm long denaturing 6% polyacrylamide (SequaGel XR, Polymed) gels in 1x TBE buffer, and visualized using a LI-COR 4300 DNA analyzer. Results were analyzed using the LI-COR SAGAGT software.
A dendrogram was constructed using the PowerMarker program, whereas the CERVUS software (Marshall et al. 1998) was used to calculate the Polymorphic Information Content (*PIC*), the number of alleles per locus, the observed (*Ho*) and the expected (*He*) heterozygosity. The number of effective alleles (*Ne*) was calculated as *Ne*= 1 / (1-*He*).

RESULTS & DISCUSSION

1. Phenological growth stages.
The date of Bud Break (green shoot clearly visible, stage 08) fluctuated between 15 March and 15 April. Full Flowering fluctuated between 13 and 28 May, Veraison between 19 July and 12 August and Maturity between 16 August and 21 September. There were not significant differences for the Bud Break time in most of the assessed varieties although some early bud bursting varieties (Karlachanas, Moschato Samou, Mavroliatis) and some late ones (Robola, Roditis) were also found to occur.

2. Phenotyping berry oenological traits
Increased Juice Sugar Concentration was detected in most of the assessed varieties (Table 1), while the Titratable Acidity was extremely low, particularly in the white varieties (Table 2). These results could be due to particular genetic makeup and also due to environmental factors (increased temperatures over the maturation period-data not shown). Regarding Anthocyanin and Phenolic Substances remarkable differences were detected between the varieties, as was the case of the Skin Anthocyanin concentration that was very low in Mavro Mesenikola, Krasato, Mavro Kalavritino (similarly to the reference variety Pinot noir) opposing to the very high concentrations that were measured in Mavroudi Pentalofou megalorogo, Mavrodafni and Vertzami. Noticably, Mavro kalavritino, Vapsa and Mavroudi Arachovas were found with very high concentrations in Skin and Seed Phenolic substances similarly to Pinot noir.

3. Vine discrimination and identification.
The 45 varieties that were the focus of our current study were identified according to the OIV descriptors and also by the application of modern molecular methods of genetic analysis, the molecular markers SSRs. The limitations of both methods when used solely were apparent underlying the importance of using a combination of them. Enrichment of the combined approach by the use of biochemical analysis further improved the variety discrimination supporting the idea of using all the available methods rather than relying on a single one. For example, initial ampelographic observation showed that the varieties within the “Mavroudi” group were almost phenotypically identical, while incorporation of the biochemical analysis brought up significant differences. Mavroudi Pentalofou, Mavroudi Iasmou and Agiorgitiko are practically indistinguishable on morphological terms, with Mavroudi Pentalofou, however, possessing a shoot tip with more Anthocyanic Coloration and

lower density of Prostrate Hairs, compared to Mavroudi Iasmou and Agiorgitiko. Application of the molecular analysis (Table 3) confirmed the differentiation between these varieties: although all three of them are on the same sub-clade of the dendrogram (Figure 1), they are distinctly different. The seemingly superiority of the molecular approach, however, was nearly halted in the case of the two accessions of Mavroudi Iasmou (Mavroudi Iasmou 1 and Mavroudi Iasmou 2) and Mavroudi Pentalofou (Mavroudi Pentalofou 1 and Mavroudi Pentalofou 2). In both cases, accessions "1" refer to a variety possessing large berries, while accessions "2" refer to a variety with small berries. Application of the SSR analysis distinguished the two Mavroudi Pentalofou accessions but not the two Mavroudi Iasmou ones. In this case, the use of more SSRs becomes an absolute necessity; the use of 25 SSRs is under way.

Another interesting case is that of Xinomavro, Zalovitiko and Xinogaltso: although they are highly similar in their morphology, the application of the SSR analysis revealed genetic closeness only between Xinomavro and Zalovitiko clustering Xinogaltso in a distant clade (Figure 1).

4. The parameters of the genetics studies.

Observed Heterozygosity (*Ho*) is of major interest in genetic variation studies in natural populations. High values for *Ho* indicate higher genetic variability, while low values indicate little genetic variability. Comparison between the *Ho* and to what is expected under the Hardy-Weinberg Equilibrium (HWE; or Expected Heterozygosity-*He*) is very informative for the formulation of evolution hypothesis for the population under examination. When the *Ho* values are lower than the *He* values, forces such as inbreeding are likely to have occurred, while *Ho* values higher than the *He* values, an isolate-breaking effect (the mixing of two previously isolated populations) is likely to have occurred. From the data obtained in the examined Vitis population the *Ho* is always lower than the *He* for all the SSR loci indicating probable inbreeding among the varieties, in accordance with other studies of Vitis populations.

Number of alleles (*Na*) is the number of observed alleles in a specific locus in a population, while Effective number of alleles (*Ne*) is a measure of the number of alleles that would be expected in a locus in each population. It is calculated by inverting the measure of homozygosity in a locus and it can be used with co-dominant marker data such as those from SSRs, AFLPs and ISSRs. Its calculation may be affected by sample size. This measure of diversity may be informative for establishing collecting strategies. For example, we estimate it in a given sample. We then verify it in a different sample or the entire collection. If the figure obtained the second time is less than the first estimated number, this could mean that our collecting strategy needs revising.

The Polymorphism Information Content (*PIC)* value is commonly used in genetics as a measure of polymorphism for a marker locus used in linkage analysis. PIC refers to the value of a marker for detecting polymorphism within a population, depending on the number of detectable alleles and the distribution of their frequency; thus, it

provides an estimate of the discriminating power of the marker. In our case, with the exception of three markers (VVMD25, VVS2 and VVMD 32), which are moderately informative, all the remaining seven markers are highly informative.

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Table 1. Juice Sugar Content (^{0}Brix), Titratable Acidity (TA), Skin Anthocyanin (SkAnth mg.g^{-1} f.w.), *Skin Phenolic (SkPhen mg.g^{-1}f.w.), and Seed Phenolic (Seed Phen.mg.g^{-1}) content of 34 red varieties growing at AUTh Ampelographic Collection .
*: in equivalent of (+) catechine

Varieties	^{0}Brix	TA	SkAnt mg/g	SkPhen mg.g^{-1}f.w.	Seed phen mg.g^{-1}f.w.
Kokkinorobola	18,5±0,56	2,27±0,67	5,32±1,96	8,79±2,85	6,89±2,08
Mavroudi Iasmou megalorogo	21±0,5	4,9±0,26	5,63±1,55	6,43±1,67	9,08±2,74
Mavroudi Pentalofou megalorogo	23,67±0,76	3,97±0,25	18,23±3,09	11,24±1,35	12,01±3,49
Kontokladi mavro	18,5±0,5	3,37±0,32	2,68±0,25	7,99±2,31	5,66±1,07
Mavro Mesenikola	18±1,02	1,2±0,2	1,78±0,82	9,96±3,84	4,25±0,58
Vertzami	19,34±1,04	5,23±0,31	10,99±3,14	13,14±2,47	32,74±6,75
Zalovitiko	16,5±1,33	6,97±0,9	3,72±0,66	9,6±1,82	17,32±0,88
Agiorgitiko	17,34±0,28	5,37±0,21	4,47±0,47	6,52±0,97	23,17±4,21
Xinomavro	20±0,5	7,33±0,29	1,67±0,15	6,83±0,35	25,27±3,91
Vapsa	16,67±0,57	3,73±0,25	2,28±0,61	20,59±6,08	6,88±2,16
Stavroto	16,3±0,98	3,4±0,52	1,14±0,02	4,42±0,59	20,49±2,92
Mavro Vafiko	19,13±1,7	3,7±0,17	0,1±0,05	4,47±2,36	8,22±2,4
Kotsifali	17,33±1,25	3,83±0,61	1,25±0,24	14,63±1,78	15,65±1,1
Aidani Mavro	20,53±1,36	2,37±0,21	1,26±0,27	8,86±2,65	7,9±0,22
Mavro Kalavriritino	19±1,32	3,87±0,38	0,89±0,06	19,16±1,3	11,36±1,22
Mavroudi Araxovas	19,5±0,5	4,23±0,29	1,99±0,7	17,99±1,78	34,33±34,12
Karlachanas	19,5±0,5	3,5±0,1	3,85±0,88	17,5±3,33	4,43±0,62
Mavrodafni	19,6±1,27	3,33±0,58	17,59±4,54	13,45±3,48	2,83±1,19
Liatiko	17,83±0,28	3,5±0,78	1,62±0,31	12,07±4,63	14,19±1,14
Mavroliatis	21±1,32	3,47±0,25	1,82±0,57	16,46±3,1	6,85±3,33
Moschato Amvourgou	19,11±1,21	2,83±0,21	1,92±0,36	9,11±2,52	24,69±12,31
Fokiano	22,5±1,32	2,27±0,21	3,41±0,61	15,51±1,24	22,56±2,26
Agianiotiko	17,67±0,76	2,2±0,66	1,2±0,21	13,23±5,67	25,18±13,79
Kotsifaloliatiko	23,67±0,57	4,1±0,36	1,05±0,12	11,34±2,28	18,13±4,71
Cadernet Sauvignon	21,67±0,29	7,17±0,76	3,75±0,94	6,48±1,56	10,23±3,13
Bekari	16,5±0,52	2,1±0,1	3,86±0,75	10,93±2,01	12,18±4,57
Limnio	16,2±0,53	2±0,2	3,99±0,5	10,32±1,72	20,78±4,19
Xinogaltso	19,03±0,5	5,97±0,57	2,27±0,7	10,67±2,51	15,53±1,56
Krasato	20±2,64	0,89±1,42	6,05±2,76	15,64±2,27	25,55±18,88
Mavroudi Iasmou mikrorogo	22,5±1,21	4,5±0,1	4,56±0,56	6,77±1,57	15,97±1,45
Mavroudi Pentalofou mikrorogo	21,4±0,62	3,9±0,56	4,67±1,34	7,54±2,65	16,78±1,68
Merlot	22,5±1.67	6,8±0,57	4,01±1,12	7,45±1,56	11,34±2,54
Pinot noir	21,7±0,68	4,9±0,28	1,34±0,33	30,66±5,11	8,18±1,97

Table 2. Sugar Content (Brix) and Titratable Acidity (TA) of 11 white varieties growing in the AUTh Ampelographic Collection.

Varieties	^{0}Brix	TA
Lefko Naousas	17,20±1,53	4,57±0,38
Robola	17,16±0,76	3,47±0,25
Moschadrina	20,23±1,83	2,93±0,58
Mosxato Spinas	26,33±0,88	2,83±0,21
Mosxato Samou	24,33±1,95	2,27±0,21
Mosxopoula	22,5±0,95	2,2±0,66
Zoumiatiko	17,86±1,45	3,05±0,2
Savatiano	17,96±1,58	3,1±0,36
Roditis	17,83±0,67	4,07±0,12
Xinomavro lefko	18,56±0,45	5,17±0,76
Robola 1	18,21±1,67	4,1±0,36

Table 3. Genetic diversity parameters of 10 SSRs in the 43 Greek varieties and the two reference ones (Cabernet sauvignon and Merlot) growing in the AUTh Ampelographic Collection.

Locus	Na	Ne	Ho	He	P.I.C.
VVMD28	16.000	11.509	0.6222	0.872	0.8525
VVMD32	9.000	4.596	0.2889	0.612	0.5795
VrZAG67	9.000	4.860	0.4889	0.7745	0.7384
VVMD5	10.000	7.226	0.7111	0.8881	0.8661
VrZAG62	9.000	5.708	0.6889	0.8604	0.8338
VVMD25	6.000	3.796	0.1778	0.5054	0.4751
VVMD27	6.000	4.238	0.5556	0.786	0.747
VVMD7	11.000	4.942	0.4444	0.7673	0.7344
VVS2	7.000	5.796	0.2667	0.5423	0.5189
VrZAG79	11.000	5.319	0.4222	0.7016	0.6694
Mean	**9.4**	**5.793**	**0.466**	**0.731**	**0.701**

Na: *Number of Alleles*
Ne: *Effective Number of Alleles*
Ho: *Observed Heterozygosity*
He: *Expected Heterozygosity*
PIC: *Polymorphic Information Content*

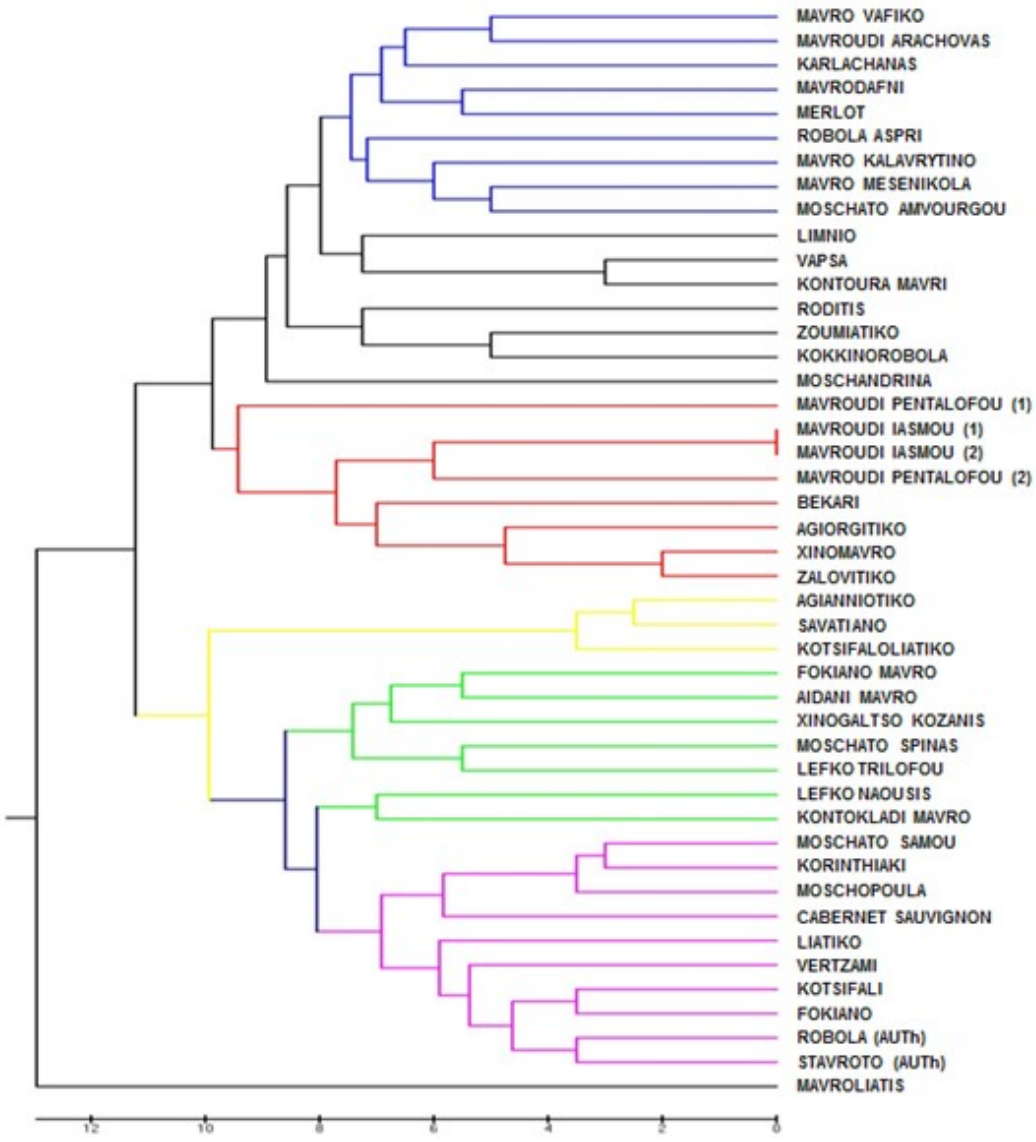

Figure 1. Dendrogram of the 43 Greek varieties and the two reference ones (Cabernet sauvignon and Merlot) based on 10 SSR loci. Mavroudi Pentalofou 1: megalorago (large berries); Mavroudi Pentalofou 2: mikrorago (small berries); Mavroudi Iasmou 1: megalorago (large berries); Mavroudi Iasmou 2: mikrorago (small berries).