

Can Molecular Ampelography Identify a Grapevine Variety in the Absence of Any Ampelographic Inputs?

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Abstract

Is it possible to identify a grapevine variety without looking at the phenotype? Could someone reach a correct varietal identification solely based on molecular inputs? How trustful could the empirical names the grapevine growers use for the autochthonous grapevine varieties they cultivated for many decades? The current manuscript explores these questions and provides evidence supporting the concept that molecular ampelography—in terms of SSR data—could lead to an accurate varietal identification, while also sets the prerequisites for this to occur: i) a large number of samples collected from diverse cultivation areas should be analyzed, ii) multiple samples of the same variety should be included in the analysis, and iii) only local grapevine material should be considered and used in the analysis. Inclusion of reference samples (that have been properly described in ampelographic terms and are maintained in reference collections) increases the confidence of the outcome.

Keywords: *Vitis vinifera*, grapevine, microsatellites, SSR markers, synonyms, homonyms

1. Introduction

For centuries, optical observation was the main and only means to distinguish the varieties of a plant species, including grapevine. Traditionally, ampelography, a term that originates from the combination of the Greek words “ampelos” (grapevine) and “graphe” (imprint on paper, therefore: description) constitutes a scientific field that describes and discriminates the plethora of the grapevine varieties focusing on the phenotypic differences observed on the various organs. The degree of variability found in the shape, size, color, morphology and structure, occurrence or absence of trichomes, are used for the discrimination and the identification of grapevine varieties.

Although ampelography is generally accepted as a scientific field, heretic opinions have been expressed either considering traditional ampelography an “old technique” and “preliminary method” for describing and identifying vine varieties (Garcia-Muñoz, Muñoz-Organero, de Andrés, & Cabello, 2011), or, more radically, that traditional “ampelography is not an exact science” (Tassie, 2010). Regardless this argument, traditional ampelography served consistently the viticulture sector supplying insights into the plant material identity and providing the phenotypic description of each variety. The very definition of what is called a “variety” is based on “the expression of characteristics that results from a given genotype or combination of genotypes” (European Union-Lex [EUR-lex], 1994); therefore, the phenotype, and consequently, the means that describe the phenotype, play a major and crucial role.

It is commonly accepted that traditional ampelography provides the link between the past and the present, allowing the comparison of the varieties recovered today with those that had been described in the ampelographic works of the past. These early ampelographic works are important because they allow us to establish the existing relationship to today’s cultivated material, and also to define their geographical distribution, and draw safe conclusions about their origin and characteristics (Logothetis, 1974). However, there are “several reasons (e.g., soil-climatic conditions of the cultivation areas, vegetative mutations, cross-pollination, conscious

or unconscious mass selection by the vine-growers” that make impossible a complete and accurate comparison between the past and the present (Logothetis, 1974).

The use of traditional ampelography for grapevine variety discrimination is influenced by the terroir—the actual microclimate of the cultivation area—as well as by the cultivation techniques applied, and also by the plants’ state of health (all described briefly but adequately by This et al. (2004)). Moreover, traditional ampelographic description can only be applied in mature vines—at least four years of age—and mostly during the vegetation period rather than all year around. The ability to identify the various grapevine varieties represents an expertise that is acquired after many years of dedication on the field observation (This et al., 2004).

Enzymatic markers together with various molecular marker systems had been used to complement traditional ampelography in providing comprehensive information on the identification, characterization, genetic diversity and the population structure of numerous grapevine genotypes. In 2004 a consortium of ten partners from seven countries established a standard set of six microsatellite (Simple Sequence Repeats-SSRs) markers as substantially polymorphic to accurately identify grape cultivars (This et al., 2004). These markers were incorporated as molecular descriptors (#801 to #806) in the relevant OIV (Organisation Internationale de la Vigne et du Vin) descriptor list (OIV, 2009); a few years later, the core number of the suggested markers was increased to nine (Maul et al., 2012). Nowadays, SSR-based approaches represent the most widely accepted methodology for unravelling the patterns and the genetic structure of the grapevine germplasm: different laboratories from different countries produce comparable data, therefore, enabling direct comparison of the world’s grapevine germplasm. The term molecular ampelography has been recently introduced.

In the present study, the data of a previous research work (molecular analysis of 301 grapevine samples; Merkouropoulos, Batianis, & Mylona, 2016) has been re-evaluated in order to investigate the possibility of accurately reaching grapevine variety identification based solely on molecular data. Most of these samples correspond to rare and autochthonous varieties from distant sites of northern Greece and the south Balkans. The hypothesis whether the molecular analysis without any prior ampelographic description or knowledge could lead to the correct variety identification was questioned.

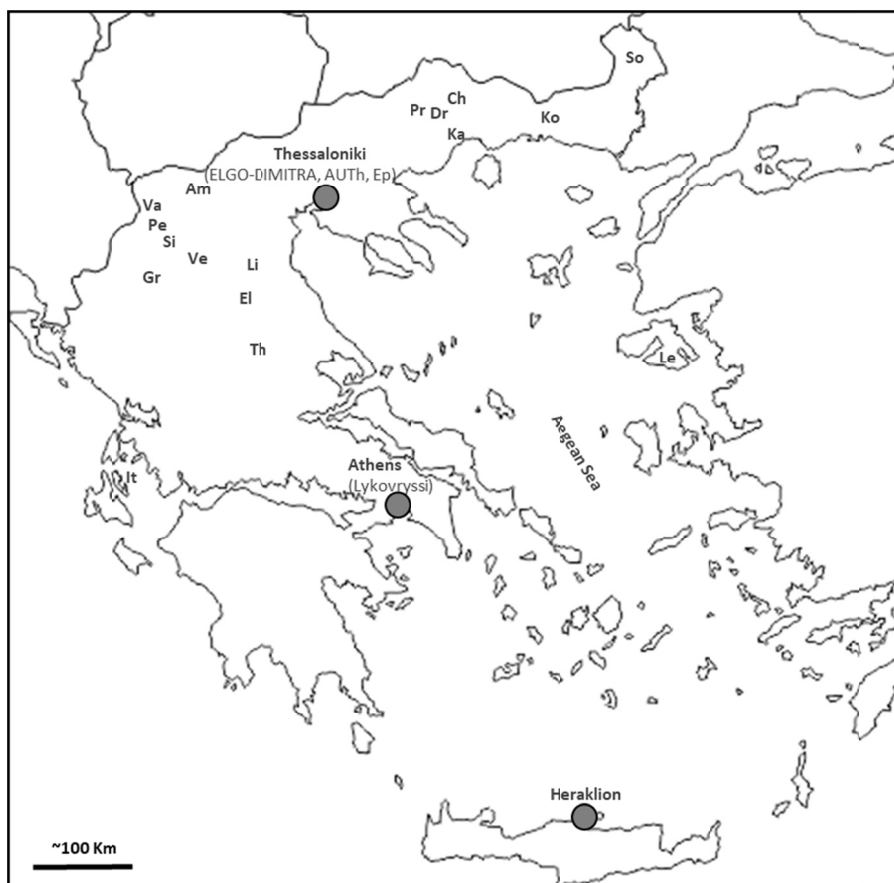


Figure 1. Collection sites of the samples analyzed in the current study. Am: Amyntaio; AUTH: Ampelographic collection of the Aristotle University (Thessaloniki); Ch: Choristi; Dr: Drama; El: Elassona; ELGO-DIMITRA: Therma, Thessaloniki; Ep: Epanomi; Gr: Grevena; It: Ithaki; Ka: Kavala; Ko: Komotini; Le: Lesvos; Li: Livadi; Pe: Pelekanos; Pr: Prosotsani; Si: Siatista; So: Soufli; Th: Thesalia; Va: Vasileiada; Ve: Velventos

2. Materials and Methods

A total of 301 samples have been analysed (Merkouropoulos, Batianis, & Mylona, 2016): 290 samples (young and healthy leaves) had been collected from various sites of northern Greece, whereas the remaining eleven samples originated from the *ex-situ* grapevine collection of the Hellenic Agricultural Organization DIMITRA (ELGO-DIMITRA) in Therma, Thessaloniki (Figure 1). During the collection of the samples, no ampelographic description was taken; in all cases, only autochthonous vine material was collected—no material that had been transferred from other places was collected. DNA from six international grapevine varieties were donated by the Julius-Kühn Institute (JKI) and were used as calibration reference (Table 1). Genomic DNA was extracted and handled as described in Merkouropoulos et al. (2015). All the collected samples were analyzed at the following ten microsatellite loci: VVS2 (Thomas & Scott 1993), VVMD5, VVMD7 (Bowers, Dangl, Vignani, & Meredith, 1996), VVMD25, VVMD28, VVMD27, VVMD32 (Bowers, Dangl, & Meredith, 1999), VrZAG62, VrZAG67, and VrZAG79 (Sefc, Regner, Turetschek, Glössl, & Steinkellner, 1999), (Table 1). The molecular data was statistically analyzed using the GenAlEx program. A dendrogram was constructed using the MEGA4 program to reveal the genetic relationships among the genotypes analyzed. In all cases, the empirical names used by the growers were taken as granted, though with a strong sense of doubt.

3. Results and Discussion

Correct variety identification is of crucial importance in viticulture, and it is even more critical when referring to the rare autochthonous material. Here, the question under investigation was whether molecular analysis based on SSR profiling could be suitable and adequate for genotype identification without any prior ampelographic inputs. The concept is that at the end of the molecular analysis, a numerical profile comprised of the allele lengths (on

the selected microsatellite loci) is created for each of the samples analysed: identical profiles are attributed to identical genotypes.

During a research expedition in the various grapevine cultivation centers of northern Greece, aiming to explore and trace rare autochthonous grapevine varieties, 290 grapevine samples had been collected. SSR analysis was performed and a dendrogram was generated showing the genetic closeness among the collected samples (Merkouropoulos, Batianis, & Mylona, 2016; Figure 2). During this activity sample collection occurred without considering the phenotype (the ampelographic characters) of the sampled vines, and taken as granted the variety names provided by the growers; it was, however, kept in mind that these suggested, empirical variety names might not be reliable. As expected, in the various clades of the SSR dendrogram, samples possessing identical or highly similar molecular profiles were grouped on the same or neighboring clades (Figure 2, Groups A to J). Reevaluating the data of that work, the attention was focused on those clades where samples possessing similar empirical names were grouped together, such as in the Group A and Group E of Figure 2. It should be noted that the majority of the sample names are not presented in Figure 2 because they are the empirical names that were given by the growers when the collections took place, therefore, they should be considered with special concern as it has not been confirmed their true identity.

Group A (Figure 2) was comprised of seven samples: four of them were empirically called ‘Sefka’ (‘Sefka-1’, -2’, -3’, -4’), a red colored grapevine variety of Bulgarian origin. These four samples had been collected from diverse and distant areas of northern Greece with no apparent plant material exchange between the grapevine growers that donated these samples. The samples have been grouped on the same clade solely on the basis of their molecular relationship. In other words: four samples that had been collected from distant areas and bearing the same empirical name, were eventually clustered together confirming their nominal relationship. Three of the ‘Sefka’ samples (‘Sefka-2’, -3’, -4’) possess an identical molecular profile, whereas the molecular profile of the fourth sample (‘Sefka-1’), although highly similar, is not identical: two alleles at the MD32 locus and one at the VrZAG79 locus differ (Table 1). Years later, in 2022, when the molecular profile of nearly all the autochthonous varieties that are maintained in the *ex-situ* national reference collection of Greece had been revealed (manuscript in preparation), the molecular profile of the three ‘Sefka’ samples (‘Sefka-2’, -3’, -4’) was compared to the ‘Sefka’ vines maintained in the reference collection (Table 1: ‘Sefka-ELGO’) to find that they were all identical. This finding supports the conclusion that similar molecular profiles are attributed to the same phenotype.

Regarding the sample “Sefka-1”, traditional ampelography could be used to define whether this sample still retains the varietal morphological characters, therefore, it is still a ‘Sefka’ (an ecotype) or a closely related variety. Novel morphological characteristics could appear due to mutations that occur naturally during plant growth (Pelsy, 2010); although these characters could be evident by simple or comprehensive phenotypic observation, it is rather likely that they would not be picked up by the application of the standard set of the nine OIV microsatellite markers. A different designation should be proposed for ‘Sefka-1’ distinguishing it from the rest of the ‘Sefka’ samples: the name of the collection area or even the name of the grower could be part of the final name of the sample/vine distinguishing it from the rest.

Table 1. Microsatellite profiles of the samples analyzed on the ten microsatellite loci

	VVS2	VVMD5	VVMD7	VVMD25	VVMD27	VVMD28	VVMD32	VrZAG62	VrZAG67	VvZAG79
Sefka-1	143 143	233 233	246 248	237 253	177 183	237 245	250 256	187 199	130 154	237 239
Sefka-2	143 143	233 233	246 248	237 253	177 183	235 245	254 270	187 199	130 154	237 241
Sefka-3	143 143	233 233	246 248	237 253	177 183	237 245	254 270	187 199	130 154	237 241
Sefka-4	143 143	233 233	246 248	237 253	177 183	237 245	254 270	187 199	130 154	237 241
Chondromavro-2	143 143	233 233	246 248	237 253	177 183	245 245	254 270	187 199	130 154	237 241
Chondromavro-ELGO	143 143	233 233	246 248	237 253	177 183	245 245	254 270	187 199	130 154	236 240
Sefka-ELGO	143 143	233 233	246 248	237 253	177 183	235 245	254 270	187 199	128 152	237 241
Sefka-ELGO/Thermi	143 143	233 233	246 248	237 253	177 183	235 245	254 270	187 199	128 152	237 241
Xinomavro-ELGO	131 131	229 231	248 248	237 239	177 179	229 245	248 250	193 203	122 136	235 247
Xinomavro-1	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	235 249
Xinomavro-2	131 131	229 231	248 248	237 239	177 179	231 245	248 254	193 203	124 138	235 249
Xinomavro-3	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	235 249
Xinomavro-Velventou	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	235 247
Palio_Xinomavro	131 131	229 231	248 248	237 239	177 179	231 231	248 250	193 203	124 138	235 251
Unknown-1	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	235 249
Unknown-2	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	235 247
Unknown-3	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	235 249
Unknown-4	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	235 249
Unknown-5	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	235 247
Unknown-6	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	235 249
Unknown-7	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	235 249
Unknown-8	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	235 249
Unknown-9	131 131	229 231	248 248	237 239	177 179	231 245	248 250	193 203	124 138	233 233
JKI-Barbera nera	131 133	223 223	248 252	237 253	183 187	237 261	250 270	191 199	130 138	241 255
JKI-Carignan noir	141 143	223 225	238 238	239 253	179 183	259 259	248 250	185 187	124 138	247 255
JKI-Merlot noir	137 151	223 233	238 246	237 247	187 189	231 235	238 238	193 193	130 138	255 255
JKI-Moschato aspro	131 131	225 233	232 248	239 247	177 191	269 269	262 270	185 195	124 138	247 251
JKI-Silvaner blau	151 153	223 229	242 246	239 247	187 191	231 237	270 270	187 203	124 158	245 247
JKI-Sultanine	143 151	231 231	238 252	237 247	179 191	221 245	248 248	187 187	124 138	243 255

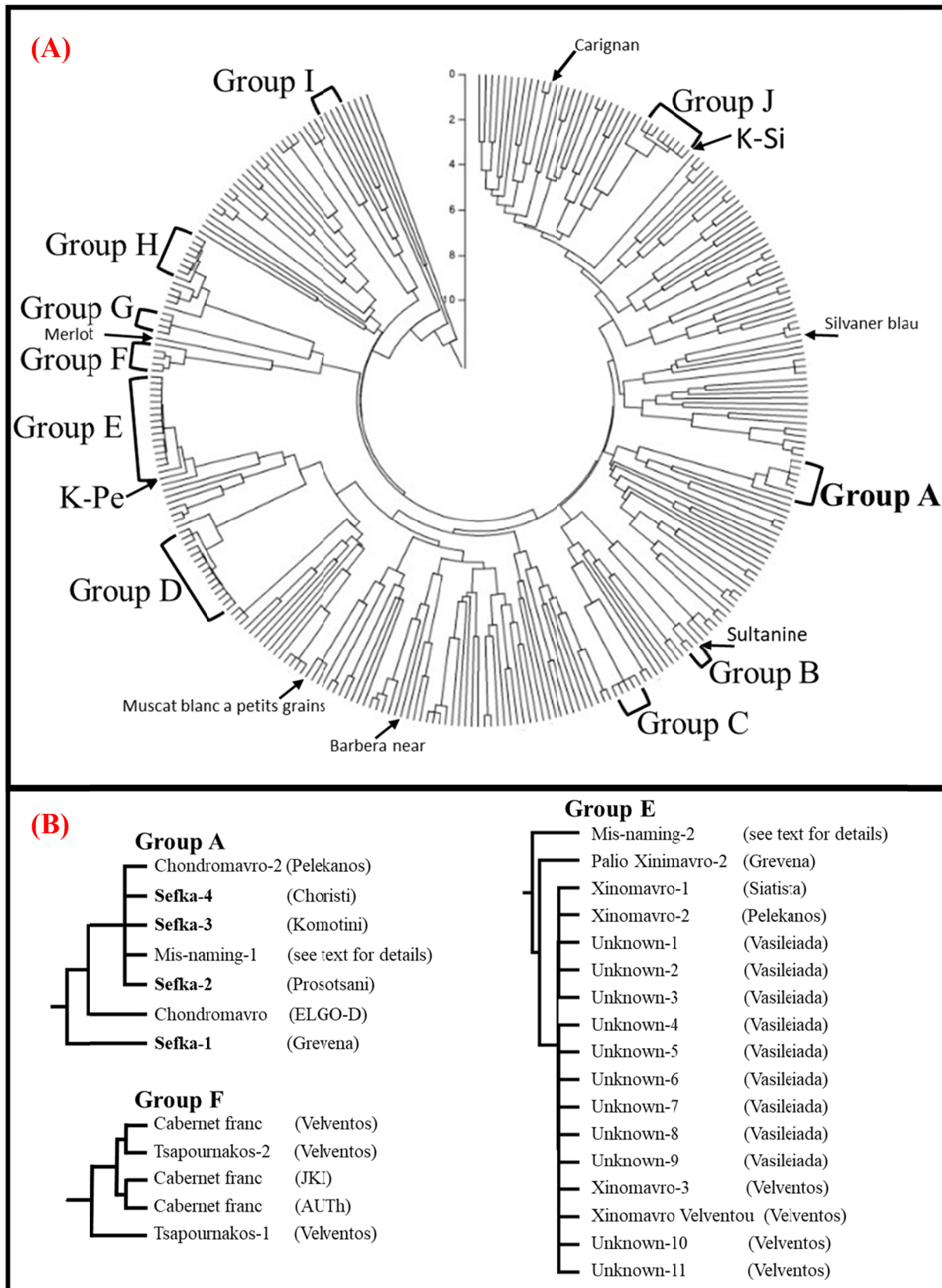


Figure 2. (A) Dendrogram derived from SSR data. K-Si: ‘Kokkinouska’ variety from Siatista; K-Pe: ‘Kokkinouska’ variety from Pelekanos; (B) Group A, Group E, Group F: magnification of the relative clades of the dendrogram

Moreover, on the same clade, two samples annotated as ‘Chondromavro’, a thick-skinned, red or dark-red wine variety that is autochthonous to the north-western part of Macedonia (Prefecture of Kozani and Prefecture of Grevena), were also grouped together with the four ‘Sefka’ samples pointing out a molecular relationship between ‘Sefka’ and ‘Chondromavro’. Three reports have been found in the Greek literature referring to this relationship: (i) an ampelographic study (Spinthiropoulou, 2000), (ii) a regional developmental study (EN.O.A.B.E., 2004), and (iii) a folklore study on the grapevine varieties of the Siatista region (Bonda-Doumanaki, 2013). In order to test this emerging relationship between ‘Sefka’ and ‘Chondromavro’, samples of these varieties were also collected from the *ex-situ* ELGO-DIMITRA reference collection, and were analysed at the same 10 genetic loci: their molecular profiles were identical (samples ‘Chondromavro-ELGO’, ‘Sefka-ELGO’, and ‘Sefka-ELGO/Thermi’ in Table 1).

A case of mis-naming was also revealed (Figure 2, sample: ‘Mis-naming-1’). The seventh sample that has been grouped in Group A, annotated as ‘Pamidi’, is a well-known variety cultivated in many countries including the north-eastern part of the Greek mainland. The corresponding reference sample (from the ELGO-DIMITRA reference collection) that had been included in the analysis, was grouped in Group J; therefore, the sample that was grouped in the ‘Sefka/Chondromavro’ group (Group A) is either a ‘Sefka’ or a ‘Chondromavro’ vine that was incorrectly named by the grapevine grower as ‘Pamidi’. Thus, a major improvement of this solely molecular approach to define unknown samples identity would be to incorporate in the analysis as many reference samples (such samples have been extensively described ampelographically) as possible. Since the summer of 2022, the molecular profile of nearly all the Greek grapevine varieties is available (Merkouropoulos, 2022; Merkouropoulos et al. in preparation). It is highly advisable to researchers that perform similar molecular or genetic studies on the autochthonous grapevine phytoresources, to request reference material from the national collection maintained by ELGO-DIMITRA (requests could be send to the authors of the current study). It is worth noting that ELGO-DIMITRA is the only official curator of the autochthonous grapevine varieties in Greece maintaining the autochthonous varieties –together with international varieties and rootstocks– in three vineyards: the oldest and larger of these vineyards is located at Lykovryssi (Athens), a copy of the Lykovryssi’s collection is located at Thermi (Thessaloniki) enriched with local varieties collected from the northern part of the country, whereas a third vineyard is located at Heraklion (Crete) comprised mostly with local varieties of Crete and the islands of the southern Aegean Sea (Figure 1).

In Group E seventeen samples were grouped together (Figure 2): fifteen of them possessed identical molecular profiles, whereas the profile of the remaining two were highly similar. Five of these samples ‘Xinomavro-1’, ‘Xinomavro-2’, ‘Xinomavro-3’, ‘Palio-Xinomavro’ and ‘Xinomavro-Velventou’; (‘palio’ means ‘old’ in Greek, whereas ‘Velventou’ is the name of a city in the Prefecture of Kozani) were annotated ‘Xinomavro’, the noblest black variety of north-western Greece. Eleven samples (‘Unknown-1’ to ‘Unknown-11’) that had been collected either from a private grapevine collection and were named ‘Vasileiada’ after the nearby village (Prefecture of Kastoria) or from the area around the city of Velventos, were identified as ‘Xinomavro’ when the analysis was completed.

A putative case of homonymy was also revealed in Group E (Figure 2; Group E, sample ‘Mis-naming-2’): a sample that was collected from Siatista (Prefecture of Kozani) with the empirical name ‘Kokkinouska’, a red variety that is cultivated in the north-western parts of the Greek mainland, was grouped in the ‘Xinomavro’ group; a second sample with the same name, however, was collected from Pelekanos (a village that is located about 20 km on the north of Siatista; Prefecture of Kozani), was grouped in the distal Group J (Figure 2; ‘K-Pe’).

As for the remaining groups:

- Group B, the Sultanine Group, is comprised of three samples: one from the JKI, and two samples from the north-western part of Greece (Figure 2, Group B). It was found that the molecular profile of the JKI sample is highly similar but not identical to the profile of the two Greek ‘Sultanine’ samples.
- Group C is the ‘Vapsa’ group. ‘Vapsa’ is a black variety that is cultivated in various sites around the country, and it is a putative subject of investigation for homonymies since it is likely that the name generally refers to material that provides color to a wine blend (data not shown).
- Group D consists almost exclusively by unknown samples collected from the private grapevine collection at Vasileiada (data not shown).
- Group F, samples of ‘Cabernet franc’ were grouped together with samples of ‘Tsapournakos’, a black variety that is cultivated in the vineyards around Velventos confirming the hypothesis that ‘Tsapournakos’ is a clone of ‘Cabernet franc’ (Figure 2, Group F).

- Group G consists of four unknown samples.
- In Group H eight samples were grouped together, two of them had been donated by the growers as ‘Cinsaut’, a third one as ‘Galliko’ (meaning ‘of French origin’) while the rest of them were called with various names (data not shown).
- In Group I four reference samples of international varieties donated by JKI were grouped together (Figure 2, Group I).
- In Group J a reference sample, ‘Pamidi’, from the ELGO-DIMITRA reference collection was incorporated in the study, and was grouped together with various varieties –their genetic relation has not yet been tested or confirmed (data not shown).

According to our findings, even when no ampelographic observation is available molecular profiling using microsatellite approaches may result in correct and trustful identification of the empirical annotations of the grapevine varieties, especially the autochthonous and rare ones, as long as:

- (i) many samples are included in the analysis,
- (ii) multiple samples of the same variety are included in the analysis, and
- (iii) the samples are collected from distant sites with no (apparent) plant material ex-change among the growers.

DNA technology provides a plethora of techniques to use for varietal identification. Many marker systems have been innovated and most of them are PCR-based (PCR: Polymerase Chain Reaction), (reviewed by Sharma & Singh, 2015). The application of various marker systems and the high-throughput next generation sequencing technologies on *Vitis* germplasm have been recently reviewed (Tympakianakis, Trantas, Avramidou, & Ververidis, 2023). Our ultimate intention in this manuscript was not to produce more molecular data regarding the molecular identification of the autochthonous grapevine vines that are in cultivation in the various cultivation centers. Our aim was to interpret the molecular data pointing out that even when no ampelographic knowledge or ampelographic experience has been acquired, correct identification is feasible as long as the above mentioned prerequisites occur. Inclusion of reference material, greatly improves the success of the analysis and shortens the time required.

The authors of the current study wish to make clear their strong belief that all scientific methods are necessary, and that the various scientific methods could and should be used complementarily, so as to result in scientific progress: traditional ampelography provides the relationships based on the phenotypic characters whereas molecular ampelography explores the relationships based on the genotype.

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Authors Contributions

Conceptualization, G.M.; methodology, G.M. and G.D.; software, G.M. and G.D.; validation, G.M. and G.D.; formal analysis, G.M. and G.D.; investigation, G.M.; resources, G.M.; data curation, G.M.; writing-original draft preparation, G.M. and G.D.; writing-review and editing, G.M. and G.D.; visualization, G.M.; supervision, G.M.; project administration, G.M.; funding acquisition, G.M. and G.D. All authors have read and agreed to the

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