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Genotyping *Vitis vinifera* L. cultivars of Cyprus by microsatellite analysis

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Summary

Twelve native *Vitis vinifera* L. cultivars of Cyprus were genotyped at 11 highly polymorphic microsatellite markers. The obtained microsatellite allelic profiles allowed precise identification and discrimination of all tested cultivars. Each cultivar had a unique allelic profile. The low PI value (5.1 x 10^{-9}) demonstrated the high descriptive power of the chosen markers for the investigated set of grapevines. Three cases of synonymy of Cyprus cultivars with cultivars grown in other countries under different names were verified: (1) Cyprus Malaga and Muscat of Alexandria, (2) Cyprus Lefkas and Greek Verdzami, and (3) Cyprus Moscato, Bulgarian Tamyanka and Italian Moscato Bianco. The homonymy of Cyprus Sideritis and Greek Sideritis as well as of Cyprus Mavro and Bulgarian Mavrud was shown not to rely on genetic similarity.

K e y w o r d s : SSR, microsatellite, *Vitis vinifera*, cultivar identification, synonym.

Introduction

In Cyprus cultivation of grapevine for wine production dates back to the earliest days of colonization of the island about 3000 BC (Galet 1993). Nowadays, grapevines cultivated in Cyprus, especially those for wine production, are traditional local varieties. Several western European cultivars like Cabernet Sauvignon, Syrah, Riesling and Chardonnay, were introduced into Cyprus by the replanting scheme of the Ministry of Agriculture and Natural Resources, starting in 1970 (Roumbas 1993).

Among the local cultivars 15 are considered indigenous (Galet 1993). Evaluation and preservation of unique grapevine germplasm in Cyprus, as well as the implementation of European Union regulations concerning viticulture and winemaking, require accurate denomination of local varieties. Most of them have been characterized in the last century by Moillefert (Galet 1993). Recently, in the context of a research program of the Agricultural Research Institute (ARI) the characterization of grapevine germplasm by microsatellite markers has been initiated. Characterization of Cyprus native varieties by microsatellite analysis completes and specifies the existing ampelographic data, which were very often influenced by growing conditions and the health status of plants. Due to their high polymorphism, random distribution and co-dominant Mendelian inheritance, microsatellite markers are most reliable for cultivar identification (Sefc *et al.* 2001). They were successfully used in several countries for identification of grape cultivars (Thomas and Scott 1993; Thomas *et al.* 1993; Sefc *et al.* 1998, 2000, 2003; Lefort and Roubelakis-Angelakis 2001; Pellerone *et al.* 2001; Labra *et al.* 2002, Zulini *et al.* 2002), verification of synonyms (Lopes *et al.* 1999; Crespan and Millar 2001; Schneider *et al.* 2001; Maletic *et al.* 1999) and discrimination of clones (Riaz *et al.* 2002, Regner *et al.* 2000).

In this study 12 native cultivars of Cyprus are characterized by microsatellite analysis aiming to identify and discriminate them and to evaluate the existing genetic diversity. The relationship between native Cyprus, Bulgarian, Greek and some western European grape cultivars is claryfied.

Material and Methods

P l a n t m a t e r i a l : Leaf samples were taken from 12 *Vitis vinifera* L. cultivars, represented by 37 plants in the prebasic *V. vinifera* collection of ARI, located in the ARI Research Station at Zygi.

D N A e x t r a c t i o n : Leaf samples were ground to fine powder in liquid nitrogen and DNA extraction was performed according to Lefort and Douglas (1991).

P C R a n d m i c r o s a t e l l i t e a n a l y s i s : Analysis was performed at the following 11 microsatellite loci: VVS2 (Thomas and Scott 1993), VVMD5, VVMD7 and VVMD27 (Bowers *et al.* 1996, 1999), ssrVrZAG21, ssrVrZAG47, ssrVrZAG62, ssrVrZAG64, ssrVrZAG79 and ssrVrZAG83 (Sefc *et al.* 1999), and ssrVvUCH29 (Lefort *et al.* 2002). PCR reaction was performed in a GeneAmp PCR System 9700 (Applied Biosystem) in 20 µl reaction mix containing 50 ng DNA, 1 µM of each primer, 100 µM of each dNTPs. 1,5 mM $MgCl_2$ and 1U of Taq polymerase (Amersham Biosciences). In all cases, the forward primer was labelled with Cy-5fluor label. The two step protocol (Smith *et al.* 1995) was used for amplification of all loci: 95 °C for 5 min, 10 cycles of 15 s at 50 °C (58 °C for ssrVrZAG64 and 55 °C for ssrVvUCH29), 15 s at 94 °C, followed by 23 cycles of 15 s at 50 °C (58 °C for ssrVrZAG64 and 55 °C for ssrVvUCH29), 15 s at 89 °C. Fragment analysis of the obtained PCR products was carried out on an ALF Express II sequencer (Amersham Biosciences) and alleles were sized with the software Allele Locator 1.03. Internal standards were produced by amplification of PUC19

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fragments with sizes 100, 150, 200, 250, 300, 350, 400, 450, 500 bp. Allele frequencies expected an observed heterozygosity, probability of identity and probability of null alleles were calculated using the software Identity 1.0 (WAGNER and SEFC 1999). The phenogram was constructed with Microsat software (MINCH *et al.* 1997) for calculation of genetic distances in [-log (proportion of shared alleles)]. The distance matrix obtained from Microsat was processed with KITSCH from the PHYLIP package (FELSENSTEIN 1989) and TREEVIEW (PAGE 1996).

Results and Discussion

Twelve local wine and table grape cultivars were genotyped at 11 microsatellite loci. The plants were previously collected from different areas of Cyprus in the context of a clonal and sanitary selection program for local Cyprus varieties (IOANNOU 2000). The list of plants, their accession number in the collection, berry color and their use are shown in Tab. 1. For each cultivar, except Morocanella and Moscato, two or more accession numbers were available.

T a b l e 1

Grapevine cultivars investigated: white (B), red or black (N), table variety (T), wine variety (W). Accession numbers according to the *V. vinifera* collection of ARI

Cultivar	Accession number	Berry colour	Use
Aspro X	9, 26	B	W
Lefkas	4, 10, 13	N	W
Malaga	16, 19	B	T/W
Maratheftiko	3, 8, 15, 21, 23, 24	N	W
Mavro	1, 6, 18, 22, 23	N	W/T
Morokanella	5	B	W
Moscato	2	B	W
Oftalmo	11, 12	N	W
Sideritis	46, 47, 48, 49, 50	N	T
Spourtico	7, 20, 27	B	W
Verigo	41, 42, 43, 44, 45	B	T
Xynisteri	14, 17, 25	B	W/T

T a b l e 2

Genetic profiles of 12 Cyprus varieties analyzed at 11 microsatellite loci. Allele sizes are given in base pairs

Cultivars	VVS2		ZAG21		ZAG47		ZAG62		ZAG64		ZAG79		ZAG83		UCH29		VVMD5		VVMD7		VVMD27	
Aspro X	132	134	206	206	157	165	191	199	143	159	240	246	188	188	211	297	226	230	236	244	177	187
Lefkas	132	142	190	206	159	167	185	195	143	143	242	242	188	190	209	211	226	230	236	236	181	189
Malaga	132	148	190	206	157	172	185	203	139	141	246	254	188	188	211	297	226	230	246	248	177	193
Maratheftiko	134	134	206	206	157	157	191	203	143	143	246	246	188	188	297	309	226	230	244	244	179	179
Mavro	132	148	190	202	159	172	187	203	139	159	246	246	188	194	211	297	230	236	236	242	181	193
Morokanella	132	142	202	202	165	172	199	203	139	159	246	246	188	188	211	297	226	236	236	242	179	179
Moscato	132	132	206	206	157	172	185	195	141	159	250	254	188	188	211	297	226	234	230	246	179	193
Oftalmo	134	148	200	202	157	163	203	203	141	159	236	246	190	194	211	297	230	236	246	250	179	185
Sideritis	150	154	190	206	163	172	199	199	143	159	242	254	194	194	289	289	232	244	236	246	185	185
Spourtico	134	134	202	206	157	172	191	203	159	163	250	250	190	194	207	209	226	236	244	246	179	193
Verigo	144	150	190	202	172	172	191	203	139	143	250	250	188	194	209	295	230	238	236	248	193	193
Xynisteri	132	142	202	206	165	172	191	199	145	159	256	256	190	190	211	211	226	226	236	244	187	193

Microsatellite profiles of cultivars are presented in Tab. 2. The different accessions for a given cultivar were found to have identical profiles. The set of markers used revealed high level of polymorphism among investigated cultivars and enabled determination of a unique allelic profile for each cultivar.

The number of alleles ranged from 3 at locus ZAG83 to 7 at loci VVS2, ssrVvUCH29, ssrVrZAG79, VVMD5, VVMD7 and VVMD27 (Tab. 3). At all loci a lower number of alleles was observed in comparison with the number of alleles found for Greek and Bulgarian cultivars (LEFORT and ROUBELAKIS-ANGELAKIS 2001; HVARLEVA *et al.* 2004), especially for locus ssrVrZAG21 (4 alleles) and locus ssrVrZAG83 (3 alleles). The mean number of alleles per locus was 6.1, which is lower than that obtained in other publications (LOPES *et al.* 1999; SEFC *et al.* 2000; LEFORT and ROUBELAKIS-ANGELAKIS 2001; HVARLEVA *et al.* 2004). The lower mean number of alleles can be explained by the reduced number of tested cultivars. This value agreed well with the corresponding values of some European sets of cultivars when they were calculated for samples containing 13 cultivars (SEFC *et al.* 2000).

The expected heterozygosity (gene diversity) ranged from 0.600 at locus Z83 to 0.790 at loci VVMD27 and ssrVrZAG79, with a mean value 0.742. The observed hetero-

Table 3

Genetic parameters of 11 microsatellite loci used for the analysis of 12 varieties

Loci	Number of alleles	Observed hetero-zygosity (Ho)	Expected hetero-zygosity (He)	Probability of identity (PI)	Probability of null alleles
VVS2	7	0.833	0.784	0.1349	-0.0274
VVMD5	7	0.916	0.739	0.1878	-0.1017
VVMD7	7	0.833	0.7847	0.1367	-0.0272
VVMD27	7	0.666	0.7916	0.1290	+0.0697
ssrVrZAG21	4	0.666	0.659	0.3033	-0.0041
ssrVrZAG47	6	0.833	0.743	0.1820	-0.0517
ssrVrZAG62	6	0.833	0.777	0.1483	-0.0312
ssrVrZAG64	6	0.833	0.756	0.1744	-0.0434
ssrVrZAG79	7	0.500	0.791	0.1277	+0.1627
ssrVrZAG83	3	0.416	0.600	0.3621	+0.1149
UCH29	7	0.833	0.7465	0.1750	-0.0497
Total	67			5.17658×10^{-9}	
Mean	6.1	0.742	0.742		

zygosity varied between 0.416 at locus ssrVrZAG83 to 0.916 at locus VVMD5 and was higher than the expected one at 8 out of 11 loci. The observed heterozygote deficiency, especially at loci ssr VrZAG79 and ssrVrZAG83, indicates the probability of null alleles at these loci. The mean value of observed hterozygosity was equal to the expected one (0.742).

A comparison of microsatellite markers with regard to their information content revealed that the most informative loci for the investigated set of cultiars were ssrVrZAG79 and VVMD27, both with a probability of identity (PI-probability of obtaining identical profiles) value of 0.12 and 7 alleles, as well as VVS2 and VVMD7, both with a PI=0.13 and 7 alleles. Less informative loci are ssrVrZAG83 with PI=0.36 and 3 alleles and ssrVrZAG21 with PI=0.30 and 4 alleles, respectively.

It is known that the information content of the markers is not equal in different sets of cultivars, due to the predominance of some alleles in a particular population (Sefc *et al.* 2000). The lower information content of loci ssr VrZAG83 and ssrVrZAG21 was certainly due to the small number of alleles and the uneven distribution of allele frequencies. Locus ssrVrZAG83 was represented by3 alleles, one of them having 54 % of allele frequencies. For locus ssrVrZAG21, two alleles accounted for a total frequency of 75 %, while another two had a total frequency of 25 %.

Most of the PI values of the loci used to characterize Cyprus cultivars were comparable with the values of corresponding loci obtained for grapevine cultivars originated from different European regions (Sefc *et al.* 2000). An exception was locus VVMD5, reported to have highest information content for distinction of European grape varieties (Sefc *et al.* 2000) with a PI value varying between 0.08 and 0.13. For the varieties of Cyprus, locus VVMD5 was found to be less informative, with PI=0.18, as the sum of the frequencies of two alleles (226 and 230 bp) resulted in 67 % while the remaining 5 alleles had a total frequency of 33 %. The total value of probability to obtain identical cultivars by using this set of markers is 5.1765×10^{-9}. This low PI value agrees with corresponding values assessed for cultivars from different regions of Europe (Sefc *et al.* 2000).

In order to compare microsatellite profiles of cultivars from Cyprus and Bulgarian *V. vinifera* collections, 7 local Bulgarian varieties and 4 varieties originating from western Europe were genotyped at three additional loci VVMD5, VVMD7 and VVMD27, which previously had not been used for characterization of the Bulgarian cultivars (Hvarleva *et al.* 2004; Tab. 4).

Table 4

Genetic profiles of 7 native Bulgarian varieties and 4 widespread varieties originating from western Europe analyzed at 3 microsatellite loci. Allele sizes are given in base pairs

Cultivars	VVMD5		VVMD7		VVMD27	
Cabernet Sauvignon cl. R5	230	238	236	236	175	189
Chardonnay cl. 6/24	232	236	236	240	181	189
Dimyat	238	244	236	246	179	181
Gamza	224	224	244	252	185	185
Mavrud	230	238	236	246	179	181
Merlot cl. ENTAV181	224	234	236	244	189	191
Misket Cherven	234	244	236	246	179	179
Misket Vrachanski	230	238	246	250	183	193
Pinot Noir cl. ENTAV115	226	236	236	240	185	189
Shiroka Melnishka	232	244	236	236	179	181
Zarchin	224	224	244	244	185	193

The name of the Cyprus cultivar Moscato and its aromatic flavour suggest that it belongs to the family of Muscats. Comparison of the microsatellite profiles of Cyprus and Bulgarian cultivars revealed that the Cyprus Moscato and the Bulgarian variety Tamyanka have identical profiles at all 11 investigated loci. The synonymy of the Bulgarian Tamyanka with the Greek Moschato Kerkyras at 9 loci (LEFORT and ROUBELAKIS-ANGELAKIS 2001) and with the Italian Moscato Bianco at 6 loci (CRESPAN and MILANI 2001), has previously been shown (HVARLEVA *et al.* 2004). Thus all cultivars mentioned above are considered synonyms of the same variety.

The Cyprus variety Malaga is known to be a synonym of Muscat of Alexandria (GALET 1993), which was probably introduced into Cyprus from Egypt. Out data strongly support the ampelographic data, since the microsatellite profiles of the Cyprus Malaga determined in this study and that of Muscat of Alexandria, published by LOPES *et al.* 1999 and presented also in the Greek Vitis Database (http://www.biology.uoc.gr/gvd), were identical. Both cultivars have identical genotypes at 10 common loci and thus they were defined as synonyms.

According to the ampelographic data, the Cyprus cultivar Lefkas is a synonym of the Greek variety Vertzami, widely cultivated on the island Lefkada where it is called Vartzami, Marzavy and Mavro (GALET 1993). Comparison of our data for Lefkas with those published for Verdzami (LEFORT and ROUBELAKIS-ANGELAKIS 2001) revealed that both varieties have identical microsatellite profiles at 10 common loci, confirming the synonymy of these varieties.

It is known that the name of a variety is very often related to its phenotypic characteristics and this can lead to mistakes concerning the identity. Two pairs of suspected synonymy, based on the similarity of the names of the varieties were checked. Comparison of the microsatellite profiles of the Cyprus Mavro and the Bulgarian Mavrud led us to reject suspected synonymy for these two cultivars, since they shared only 47 % of their alleles.

Two varieties from the Cyprus and Greek collections (LEFORT and ROUBELAKIS-ANGELAKIS 2001), grown under the same name Sideritis were tested for genetic identity. They had identical profiles at 4 out of 11 loci and thus they were considered homonyms.

The relationship between native Cyprus native Bulgarian and some varieties originating from western Europe is shown in the dendrogram, which demonstrates the separation of 23 varieties in three main clusters, based on their similarity, calculated as a proportion of shared alleles (Figure). An exception is the variety Sideritis which is plotted far away from all other varieties. This variety has been grown for a long time in Cyprus but its origin is unclear.

The distribution of varieties in the dendrogram corresponds to their main area of cultivation. The first cluster contains 4 varieties originated from western Europe. The second one contains 6 out of 7 native Bulgarian varieties and the Cyprus variety Lefkas. As was shown in this study, Lefkas is a synonym of the Greek variety Vertzami (cultivated elsewhere in Greece), which would explain that this variety is closer to Bulgarian than to Cyprus local varieties. The third cluster includes most of the Cyprus varieties (9 out of 12) and Bulgarian Misket Vrachanski. It contains 4 pairs of varieties with the highest similarity between Moscato and Malaga (64 % shared alleles), followed by Marateftiko/Aspro and Morocanella/Mavro, with 60 % shared alleles for each pair.

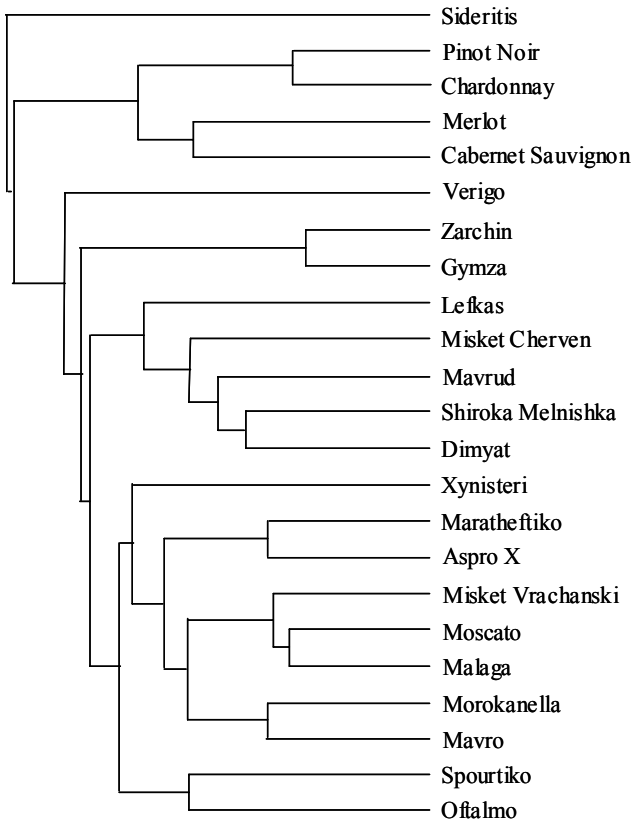

Figure: Phenogram of 12 Cyprus, 7 Bulgarian and 4 west European varieties.

It is not surprising that the Bulgarian variety Misket Vrachanski is plotted in the group of Cyprus Muscats. It has previously been shown (HVARLEVA *et al.* 2004) that this variety is very close to the Bulgarian Tamyanka and, consequently, to Cyprus Moscato (64 % shared alleles) and Malaga.

The data obtained in this study provide genetic information, which helps to identify the unique grapevine varieties of Cyprus and to reveal their relationship with varieties cultivated in other countries.

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