Vitis **43** (1), 27–34 (2004)

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

T. HVARLEVA[1)], K. RUSANOV[1)], F. LEFORT[1, 2)], I. TSVETKOV [1)], A. ATANASSOV[1)] and I. ATANASSOV[1)]

[1)]AgroBioInstitute, Sofia, Bulgaria
[2)] Laboratory of Biotechnology and Applied Genetics, Ecole d'Ingénieurs de Lullier, Jussy, Suisse

Summary

A characterization of the Bulgarian grapevine genepool (*Vitis vinifera* L. cultivars) was initiated through microsatellite analysis. Seventy four wine and table grapevine varieties from the National List of Cultivars, were analyzed at 9 microsatellite loci: VVS2, ssrVvUCH11, ssrVvUCH 29, ssrVrZAG21, ssrVrZAG47, ssrVrZAG62, ssrVrZAG64, ssrVrZAG79 and ssrVrZAG83. The high genetic diversity (78 %) allowed accurate identification and discrimination of the cultivars. The low PI value (1.201×10^{-8}) reflects the high discriminative power of the chosen set of markers for the investigated population. Based on the microsatellite allele data, two pairs of old native varieties, Misket Cherven and Misket Vrachanski; Tamyanka and Tamyanka tvarda, were considered distinct cultivars. The synonymy of (i) Tamyanka, Italian Moscato Bianco and Greek Moschato Kerkyras and (ii) Pamid and Greek Pamidi was verified, while the putative synonymy of Mavrud and Greek Mavroudi Arachovis was rejected. Further utilization of microsatellite profiling in the management of the Bulgarian grapevine genepool is discussed.

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

Introduction

Grapevine cultivation and winemaking in Bulgaria dates back to the times of ancient Thrace. Due to its location at the crossroads between Asia and Europe, to diverse soil and climatic conditions and to social and political changes (change of frontiers, migration, large scale grapevine cultivation by cooperative farms during the second half of the last century), many different grape varieties have been cultivated in Bulgaria. Today, the National List of Cultivars of Bulgaria includes nearly 100 varieties. Many others, which are not officially registered, are cultivated on small private farms. Nowadays, the genetic pool of commercially cultivated grapevines in Bulgaria consists of old native varieties, more recently introduced widespread European cultivars and locally selected cultivars. The last group derived from crosses between old native Bulgarian varieties or outcrosses with other European cultivars. The intensive renewal of grapevine plantations, implementation of EU regulations and reshaping of national viticulture and wine industries taking place at present, require application of more efficient and reliable methods for cultivar identification and germplasm management. Considering the present social changes, urgent and well targeted efforts have to be made for better evaluation, preservation and utilization of the genetic resources of grapevine. Responding to demands for the improvement of viticulture in Bulgaria, the AgroBioInstitute in Sofia established a modern grapevine germplasm collection including the varieties from the National List of Cultivars and initiated a program for collection and genetic characterization of native and new valuable grape germplasm.

The development and use of microsatellites in grapevine was reviewed by SEFC *et al*. (2001).

During the past few years, the analysis of microsatellite alleles was proved to be a powerful method for identification of cultivars and evaluation of genetic diversity (THOMAS *et al*. 1993; THOMAS and SCOTT 1993; SEFC *et al*. 1998, 2000, 2003; MALETIC *et al.* 1999; REGNER *et al*. 2000; LEFORT and ROUBELAKIS-ANGELAKIS 2001; PELLERONE *et al*. 2001; LABRA *et al.* 2002), verification of synonyms (LOPES *et al*. 1999; CRESPAN and MILANI 2001; SCHNEIDER *et al.* 2001), parentage analyses (BOWERS and MEREDITH 1997; SEFC *et al*. 1997; BOWERS *et al*. 1999; PILIAC *et al*. 2002).

This work presents the genotyping of Bulgarian grapevine cultivars, using microsatellite markers. The Bulgarian *V. vinifera* L. cultivars listed in the National List of Cultivars and presented in the grapevine collection of AgroBioInstitute, Sofia were analyzed at 9 microsatellite loci. The determined genetic diversity and relationships between cultivars, as well as the application of the obtained data for cultivar identification are discussed.

Material and Methods

P l a n t m a t e r i a l : Seventy-four cultivars and 2 clones were sampled in the *Vitis vinifera* collection located at the AgroBioInstitute, Sofia, Bulgaria.

D N A e x t r a c t i o n : Young leaves were collected, frozen in liquid nitrogen and ground to fine powder. DNA was isolated according to LEFORT and DOUGLAS (1999).

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 : The following 9 microsatellite loci were used for the microsatellite profiling: ssrVvUCH11, ssrVvUCH 29 (LEFORT *et al*. 2002), ssrVrZAG 21, ssrVrZAG47, ssrVrZAG62, ssrVrZAG64, ssrVrZAG79, ssrVrZAG83 (SEFC *et al.* 1999) and VVS2 (THOMAS and SCOTT 1993). PCR reaction was performed in GeneAmp

Correspondence to: Dr. T. HVARLEVA, AgroBioInstitute, Dragan Tzankov No8, Sofia, Bulgaria. Fax: +359-2-963-5408, E-mail: hvarleva@abi.bg

PCR System 9700 (Applied Biosystem) in 20 µl reaction mixture containing 50 ng DNA, 1 µM of each primer,100 µM of each dNTPs, 1.5 mM $MgCl_2$ and 1U of Taq polymerase and applied PCR buffer (Amersham Biosciences). In all cases, the forward primer was labeled with Cy-5fluor label (Amersham Biosciences). The following PCR conditions were applied for all loci: 95 °C for 5 min, 10 cycles of 15 s at 50 °C (58 °C for ssrVrZAG64 and ssrVvUCH29, 65 °C for ssrVvUCH11), 15 s at 94 °C, followed by 23 cycles of 15 s at 50 °C (58 °C for ssrVrZAG64 and UCH29, 65 °C for ssrVvUCH11), 15 s at 89 °C, and, terminated immediately at 4 °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 (Amersham Biosciences). Internal size standards were produced by amplification of PUC19 fragments with sizes 100, 150, 200, 250, 300, 350, 400, 450, 500 bp. Identity 1.0 (WAGNER and SEFC 1999) was used for calculation of allele frequencies, expected and observed heterozygosity, probability of null alleles and probability of identity. The phenogram was constructed using 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

Seventy-four grapevine varieties of the Bulgarian National List of Cultivars, were genotyped at 9 nuclear microsatellite loci. The cultivar set consisted of 10 native varieties, 58 selected local cross-breds and 6 international cultivars (Tab. 1). Two clones from the native varieties Mavrud and Dimyat were analyzed as well. A few international cultivars were included in this study in order to validate microsatellite analysis through comparison with published results. This also allowed a direct study of the relationship between native Bulgarian and international cultivars.

A comparison of the detected microsatellite profiles demonstrated a high genetic diversity of the analyzed genotypes. All cultivars were found to have unique allelic profiles. The microsatellite profiles of the investigated cultivars are presented in Tab. 2 and are also available on: http// www.bulgenom.abi.bg (RUSANOV *et al.* 2003). The selected SSR markers revealed a high degree of polymorphism among the tested cultivars. The number of alleles ranged from 4 per locus ssrVrZAG83 to 10 per loci VVS2, ssrVrZAG64, ssrVrZAG79 and ssrVvUCH11 (Tab. 3). The mean number of alleles per locus was 8.1, which was higher than the value observed for the same loci by LEFORT and ROUBELAKIS-ANGELAKIS (2001). This is most probably due to the high proportion of selected cross-bred cultivars among the tested genotypes, which were derived from crosses between native Bulgarian varieties or outcrosses with international cultivars. A comparison of the utilized SSR markers with regard to their information content (number of alleles and PI value) showed that the most informative loci for investigated cultivars were ssrVrZAG79 with PI 0.06 and10 alleles and VVS2 with PI 0.09 and10 alleles respectively. The higher information content of locus ssrVrZAG79 was reported for Austrian, Italian and Greek sets of cultivars (SEFC *et al.* 1999, 2000; LEFORT and ROUBELAKIS-ANGELAKIS 2001; ZULINI *et al.* 2002). The loci ssrVrZAG83 (PI 0.25 / 4 alleles), ssrVrZAG21 (PI 0.20 / 8 alleles) and ssrVvUCH11 (PI 0.19 / 5 alleles) were found to be less informative in this study. The determined PI values were comparable with the values observed after their determination in other European grapevine populations (SEFC *et al.* 2000; LEFORT and ROUBELAKIS-ANGELAKIS 2001). The calculated cumulative probability to obtain individuals with identical profile at all 9 loci was 1.201×10^{-8}, which corresponds to a statistical potential to distinguish 13000 unrelated cultivars. This low PI value reflects the high discriminative power of the chosen set of markers for the investigated population.

The estimated values of the expected heterozygosity of the studied loci range from 0.70 at locus ssrVrZAG83 to 0.85 at locus ssrVrZAG79 with a mean value of 0.78. Correspondingly, the observed heterozygosity (the percentage of heterozygous individuals among all tested ones) varies between 0.60 at locus ssrVrZAG 83 to 0.89 at locus ssrVrZAG 64, with a mean value of 0.77 (Tab. 3). The observed heterozygosity is higher than the expected one at the 4 loci ssrVrZAG 47, ssrVrZAG 62, ssrVrZAG 64 and ssrVvUCH11, it was lower at the loci ssrVrZAG 21 and ssrVvUCH29, and significantly lower for the loci ssrVrZAG 79 and ssrVrZAG 83. The higher positive values of the estimated probability of null alleles for the last two loci (0.083 and 0.057, Tab. 3), could explain the described substantial lower values of the observed heterozygosity. High positive values of probability of null alleles at the loci ssrVrZAG 79 and ssrVrZAG 83 were reported also by LOPES *et al.* (1999); LEFORT and ROUBELAKIS-ANGELAKIS (2001) and ZULINI *et al*. (2002). The observed heterozygote deficiency could also be a result of the constrains of breeding techniques employed during the development of the cultivars, as proposed by SEFC *et al.* (1999).

In order to characterize further the structure of Bulgarian grapevine genepool, a phenogram based on the genetic similarity of the investigated varieties was constructed (Figure). The high genetic diversity allows discrimination of all analyzed cultivars using the selected set of microsatellite markers. In contrast, the two clones, Mavrud 1 and Mavrud 2, derived from the old native variety Mavrud, have identical alleles at all tested loci. Similarly the clone Dimyat 4/24 showed the same allelic profile as the native variety Dimyat. The phenogram demonstrated an evenly distribution of the native Bulgarian cultivars, as three of them, Misket cherven, Mavrud and Pamid, are plotted within a large cluster with the international cultivars Cabernet Sauvignon, Chardonnay, Merlot, Pinot noir. Although the phenogram indicates more genetic similarity rather than kinship (SEFC *et al.* 1999; PELLERONE *et al.* 2001), most of the offspring cultivars are grouped close to their parental varieties. For example, the cultivars Trakijska slava (Mavrud x Pamid), Buket (Mavrud x Pinot noir), Evmolpia (Mavrud x Merlot), Kuklenski mavrud (Mavrud x Supersaver) are placed close to the parent

Table 1

Grapevine cultivars investigated: white (B), red and black (N), table (T), wine (W). Accession numbers are according to the *V.vinifera* L. collection of ABI. ID numbers of Bulgarian cultivars presented in the European Vitis database are shown

Local native cultivars	Berry colour	Use	Accession number	ID
Bolgar	B	T	53	
Dimyat	B	W	36	14687
Gamza	N	W	5	
Mavrud cl.1	B	W	6	
Mavrud cl.2	B	W	6B	
Misket Cherven	B	W	37	25986
Misket Vrachanski	B	W	38	
Pamid	N	W	8	1313
Shiroka Melnishka	N	W	7	
Tamyanka	B	W	32	
Zarchin	N	W	9	
Local cross-bred cultivars				
Afrodita	B	T	94	
Aheloj	B	W	48	2329
ArmiraB	T	59		
Buket	N	W	11	2111
Brestovitsa	B	T	61	
Cherna Perla	N	T	79	
Chernomorski Brilyant	B	W	52	
Chernomorski Eleksir	B	W	51	
Diana	B	T	62	
Dimyat cl.4/24	B	W	36B	
Druzhba	B	T	70	1691
Dunav	N	T	82	
Evmolpiya	N	W	14	
Hebros	N	W	21	978
Hybrid 42/82	N	T	107	
Hybrid 52/41	N	T	105	
Hybrid 53/12	N	T	106	
Hybrid 53/7	N	T	104	
Hybrid VI-4	B	T	88	14209
Kamchiya	B	W	44	1235
Kondarev	B	T	102	72
Kondarev 6	B	T	91	
Kondarev 10	N	T	100	
Lyubimets	N	T	80	
Maritsa	N	T	83	
Mavrud Kuklenski	N	W	15	
Mechta	B	T	69	
Melnishki Rubin	N	W	18	
Misket Plevenski	N	T	77	
Misket Rusenski	N	T	78	
Misket Sandanski	B	W	41	1238
Misket Sungurlarski	B	W	42	
Misket Trakijski	B	T	67	2046
Misket Varnenski	B	W	39	1236
Nadezhda	B	T	58	
Naslada	B	T/W	71	107
Orfej	B	W	49	
Plovdivska Malaga	N	W	101	
Pomorijski Biser	B	W	47	

Table 1, continued

Local native cultivars	Berry colour	Use	Accession number	ID
Prista	B	T	65	
Pyrvenets	N	T	84	
Ranna Melnishka	N	W	17	
Ranno bez seme	B	T	92	
Riesling BulgarianB	W	45	1302	
Rubin	N	W	12	2180
Rusalka	B	T	89	
Rusalka cl.1	B	T	90	
Rusalka cl.3	N	T	99	
Rusensko bez seme	B	T	93	
Rusensko Edro	B	T	64	
Ryahovo	B	T	66	
Siyana	N	T	103	
Sredets	N	T	85	
Super ran Bulgar	B	T	57	1304
Ticha	N	T	86	
Trakijska Slava	N	W	19	985
Velika	N	T	81	
Veren	B	T	63	
Vita	B	T	98	
International cultivars				
Cabernet Sauvignon cl.R5	N	W	1	
Chardonnay cl.6/24	B	W	25	
King Ruby	N	T	95	
Merlot cl.ENTAV 181	N	W	2	
Michele Palieri	N	T	75	
Pinot Noir cl.ENTAV 115	N	W	3	

Mavrud. For these known parentages the microsatellite profiles of the offsprings were in agreement with those of known parents.

The obtained data allowed to identify several varieties. Due to the similarities of the ampelographic characteristics and names, two of the native Bulgarian cultivars: Misket cherven and Misket Vrachanski, were suspected to be closely related. Microsatellite analysis showed that they share only 38 % of the allele profiles and are placed in two different large clusters in the phenogram. On the other hand, in several regions of Bulgaria Misket Vrachanski is grown under the name Tamyanka Tvarda (translated: Tamyanka Hard), which rises the possibility for this cultivar to be a synonym of the old variety Tamyanka. Although both cultivars, Misket Vrachanski and Tamyanka are plotted quite closely in the phenogram they only shared 83 % of studied alleles and were considered distinct varieties.

Since similar microsatellite loci were used to characterize native cultivars grown in other countries, it allows a direct evaluation of genetic similarity with the studied Bulgarian native cultivars. Thus a comparison of the data obtained from this study with those from the Greek Vitis Microsatellite Database (LEFORT and ROUBELAKIS-ANGELAKIS 2001) demonstrates that the analyzed native Bulgarian variety Mavrud is homonym with the Greek cultivar Mavroudi Arachovis, differing at 7 out of 9 loci. On the contrary, the other two native cultivars, Bulgarian Pamid and Greek Pamidi, having identical allele profiles, were found to be synonyms. Based on ampelographic studies, it was considered that another native Bulgarian variety, Tamyanka, which was supposed to originate from Asia Minor had several synonyms in other European countries, *e.g.* Moscato Bianco in Italy, Muscat de Frontignan in France and Muscadel Menude Blanco in Spain. A comparison of microsatellite profiles of Tamyanka and Moscato bianco at 6 loci (CRESPAN and MILANI 2001), and Tamyanka and Greek Moschato Kerkyras at 9 loci (LEFORT and ROUBELAKIS-ANGELAKIS 2001) indicated that these three cultivars are identical. No clear synonymy and homonymy data were found for the other native Bulgarian cultivars involved in this study, Shiroka Melnishka, Gymza, Zarchin, Misket Cherven, Misket Vrachanski and Dimyat.

The results of this study allow a reliable identification of all analyzed cultivars of the Bulgarian National List of Cultivars. The described data demonstrate the high genetic diversity within the Bulgarian grapevine genepool. A microsatellite-based characterization, management and utilization of the Bulgarian grapevine resources were initiated with the present work. Further collection and study of genetic relationship of native grapevine germplasm are in progress.

Table 2

Genetic profiles of two clones, 6 international 68 Bulgarian *V. vinifera* L. cultivars analyzed at 9 microsatellite loci. Allele sizes are given in base pairs

No.	Cultivar	VVS2	VVS2	ZAG21	ZAG21	ZAG47	ZAG47	ZAG62	ZAG62	ZAG64	ZAG64	ZAG79	ZAG79	ZAG83	ZAG83	UCH11	UCH11	UCH29	UCH29
1	Afrodita	132	134	190	206	161	172	185	199	139	159	246	256	188	188	248	264	211	211
2	Aheloj	142	142	206	206	157	167	193	199	137	163	250	258	188	190	244	246	211	309
3	Armira	132	132	200	206	161	172	187	203	143	159	246	256	188	194	244	264	205	309
4	Bolgar	132	134	190	214	163	163	185	187	137	139	242	250	188	194	244	244	207	207
5	Brestovitsa	132	134	194	200	157	172	185	203	139	159	254	254	188	188	246	250	209	309
6	Buket	144	150	200	206	159	163	187	193	139	141	236	238	200	200	250	250	207	289
7	Cabernet Sauvignon cl. R5	138	150	200	206	153	167	187	193	139	159	246	246	200	200	246	264	211	287
8	Chardonnay cl. 6/24	136	142	200	206	159	167	187	195	159	163	242	244	188	200	250	264	289	297
9	Cherna Perla	132	150	200	200	157	163	187	203	143	159	254	254	194	200	244	250	211	211
10	Chernomorski Brilyant	132	142	200	206	157	157	195	203	141	143	236	254	188	188	246	246	207	211
11	Chernomorski Eleksir	142	142	200	206	157	157	185	203	143	159	236	254	188	194	246	246	207	211
12	Diana	134	142	200	202	157	159	185	187	159	159	254	258	188	194	244	264	211	211
13	Dimyat	140	142	200	202	157	159	187	203	143	159	236	258	188	194	246	250	207	211
14	Dimyat cl.4/24	140	142	200	202	157	159	187	203	143	159	236	258	188	194	246	250	207	211
15	Drujba	134	134	190	196	157	159	187	191	195	195	254	254	188	200	244	246	211	289
16	Dunav	134	142	200	200	157	172	185	203	159	159	254	256	188	188	250	264	211	211
17	Evmolpiya	132	142	206	206	157	161	187	187	139	141	236	258	200	200	248	250	295	297
18	Gymza	132	134	206	206	163	172	187	203	143	163	248	248	188	190	244	250	211	309
19	Hebros	140	154	200	204	159	172	195	203	159	159	246	258	188	194	244	244	289	303
20	Hybrid 42/82	134	148	200	204	157	167	185	187	139	159	254	258	188	194	244	250	207	211
21	Hybrid 52/41	134	150	200	206	172	172	185	187	141	163	256	256	188	194	246	264	211	303
22	Hybrid 53/12	132	134	200	206	172	172	185	187	137	159	256	256	188	188	246	264	211	211
23	Hybrid 53/7	134	150	200	206	157	172	187	203	141	159	254	254	188	194	246	264	211	303
24	Hybrid IV	132	148	190	200	172	172	191	199	143	159	256	256	194	194	246	246	211	309
25	Kamchiya	144	150	200	200	163	167	187	203	159	163	250	250	188	200	244	248	211	289
26	King Rubi	132	150	206	206	172	172	187	203	137	139	256	256	188	194	246	246	211	303
27	Kondarev	134	148	200	206	157	159	185	187	143	197	254	258	188	190	244	244	211	289
28	Kondarev 10	148	150	190	200	157	172	187	199	161	165	246	246	188	194	244	246	303	309
29	Kondarev 6	148	154	190	200	172	172	187	191	159	159	254	256	194	194	244	246	211	303
30	Ljubimets	132	134	200	206	163	172	185	187	137	143	254	258	190	194	246	246	211	289
31	Maritsa	132	134	200	200	157	159	185	187	143	159	250	250	194	200	246	250	209	211
32	Mavrud cl.1	132	144	206	206	157	159	187	193	137	159	236	242	194	200	248	250	207	297
33	Mavrud cl.2	132	144	206	206	157	159	187	193	137	159	236	242	194	200	248	250	207	297
34	Mavrud Kuklenski	144	150	206	206	157	163	187	193	137	139	236	238	200	200	250	250	289	295
35	Mechta	142	148	190	206	159	172	191	203	159	197	256	258	188	200	246	264	211	211
36	Melnishki Rubin	132	136	202	206	157	159	187	203	143	159	242	250	188	188	246	264	211	289

No.	Cultivar	VVS2	VVS2	ZAG21	ZAG21	ZAG47	ZAG47	ZAG62	ZAG62	ZAG64	ZAG64	ZAG79	ZAG79	ZAG83	ZAG83	UCH11	UCH11	UCH29	UCH29
38	Michele Palieri	134	136	202	206	163	172	193	193	143	197	250	256	194	194	246	264	211	211
39	Misket Cherven	134	142	202	206	161	172	187	193	137	143	250	258	194	200	250	250	211	297
40	Misket Plevenski	132	134	190	200	157	163	185	185	139	159	238	254	200	200	246	250	209	209
41	Misket Rusenski	134	134	202	206	157	163	185	185	141	159	238	254	188	200	246	250	207	211
42	Misket Sandanski	132	132	202	206	157	157	185	195	143	159	242	254	188	188	244	246	207	211
43	Misket Sungurlarski	132	142	206	206	153	161	187	187	137	139	246	258	200	200	244	250	211	297
44	Misket Trakijski	132	142	200	200	157	157	187	203	143	161	254	258	188	200	246	250	211	211
45	Misket Varnenski	142	150	200	206	159	167	187	193	139	143	250	258	188	188	246	246	211	287
46	Misket Vrachanski	132	132	200	206	157	157	185	187	159	159	250	254	188	188	244	250	211	297
47	Nadezhda	132	132	200	200	163	172	185	185	137	159	250	254	188	194	244	246	205	205
48	Naslada	132	148	190	200	163	172	191	193	139	143	254	254	190	200	244	246	211	289
49	Orfej	136	142	200	206	167	172	187	187	139	141	244	250	200	200	246	250	289	297
50	Pamid	134	142	200	206	161	167	187	187	139	143	242	250	190	200	250	264	207	297
51	Parvenets	150	154	200	200	157	172	187	195	159	163	246	246	188	194	244	244	209	303
52	Pinot Noir cl.ENTAV 115	136	150	200	206	163	167	187	193	139	163	238	244	188	200	246	250	289	289
53	Plovdivska Malaga	136	142	190	206	161	167	187	187	139	143	244	258	190	200	248	250	211	297
54	Pomorijski Biser	142	142	200	206	159	161	181	193	139	139	254	258	190	194	246	248	207	297
55	Prista	134	148	190	206	157	163	185	185	139	159	242	254	188	200	244	246	207	211
56	Ranna Melnishka	132	142	202	204	159	169	195	195	143	159	242	258	190	190	244	250	207	309
57	Ranno bez seme	134	144	190	214	157	159	185	187	143	159	246	254	188	194	244	244	207	303
58	Riesling Bulgarian	132	142	200	206	157	167	187	193	137	139	236	244	188	200	244	250	207	287
59	Rubin	132	138	202	206	157	172	193	203	137	159	250	250	194	194	244	244	207	289
60	Rusalka	134	144	190	202	157	172	187	199	139	159	254	254	188	194	244	264	209	295
61	Rusalka 1	132	154	202	206	157	163	199	203	139	159	242	254	194	200	244	246	209	209
62	Rusalka 3	144	154	202	202	157	172	187	199	139	159	254	256	194	200	244	244	211	297
63	Rusensko bez seme	132	134	200	214	163	172	187	199	137	143	250	254	194	194	244	246	207	211
64	Rusensko Edro	134	148	200	200	157	172	185	203	137	159	254	254	188	188	250	264	211	211
65	Ryahovo	132	142	190	200	157	159	187	191	139	141	254	258	188	194	244	250	211	211
66	Shiroka Melnishka	132	132	196	202	157	159	187	195	143	143	242	250	188	188	244	246	207	211
67	Siyana	134	148	200	204	163	172	187	191	143	197	242	242	194	194	244	246	211	211
68	Sredets	134	142	190	190	163	163	187	203	143	197	238	250	188	200	246	250	207	211
69	Super ran Bulgar	132	132	190	200	157	163	185	191	137	197	250	254	188	188	244	246	211	211
70	Tamyanka	132	132	206	206	157	172	185	195	141	159	250	254	188	188	246	250	211	297
71	Ticha	134	134	190	206	163	163	187	191	139	197	238	242	188	200	244	244	207	211
72	Trakijska Slava	142	144	206	206	159	161	187	193	143	159	236	250	200	200	246	264	207	207
73	Velika	134	148	190	202	163	172	187	191	139	159	242	242	194	194	244	246	209	309
74	Veren	134	148	202	206	163	172	185	203	139	159	250	250	188	194	246	250	207	289
75	Vita	132	134	202	206	157	159	187	203	139	159	246	248	188	194	244	248	211	289
76	Zarchin	132	144	206	206	163	172	187	193	137	143	248	250	188	190	244	248	211	211

Table 3

Genetic parameters of 9 microsatellite loci used for analysis of 74 grapevine cultivars: the number of alleles, expected (He) and observed (Ho) heterozygosity, probability of identity (PI) and frequency of null alleles

Loci	Number of alleles	H_e	H_o	Probability of idendity (PI)	Probability of null alleles
VVS2	10	0.8273	0.8243	0.093023	0.001649
SsrVrZAG21	8	0.7300	0.7162	0.205516	0.007970
SsrVrZAG47	8	0.8023	0.8243	0.122509	-0.012209
SsrVrZAG62	8	0.7836	0.8514	0.124770	-0.037985
SsrVrZAG64	10	0.8074	0.8919	0.111473	-0.046729
SsrVrZAG79	10	0.8575	0.7027	0.065356	0.083366
SsrVrZAG83	4	0.7047	0.6081	0.253210	0.056668
UCH11	5	0.7495	0.8108	0.191522	-0.034999
UCH29	10	0.7712	0.7361	0.116343	0.019821
Total	73			1.201170x10-8	
Mean	MNA=8.1	0.78	0.7739		

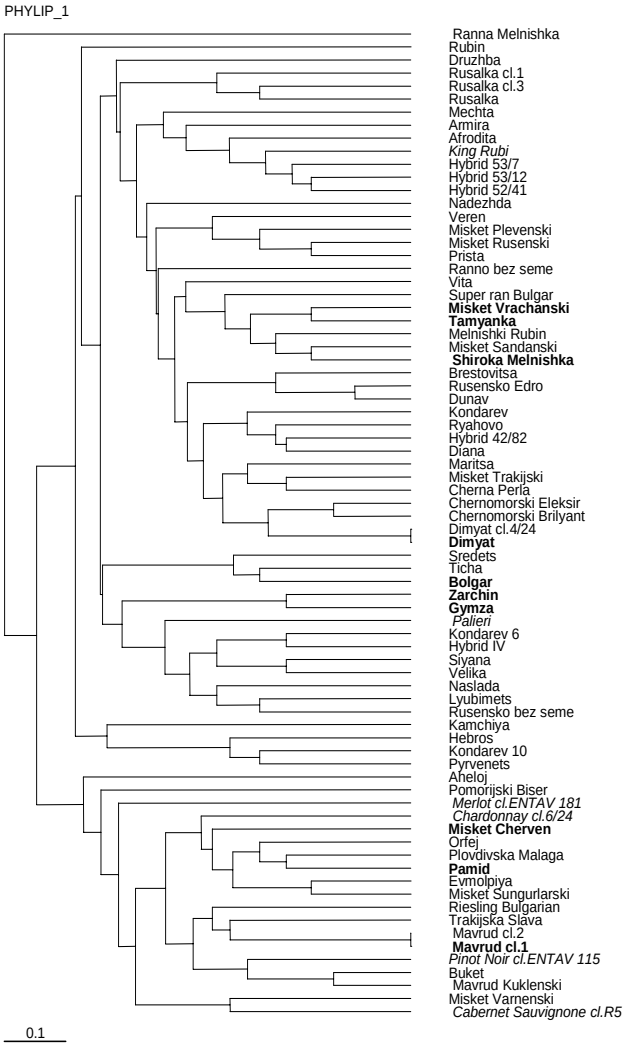

Figure: Phenogram of 74 Bulgarian *V. vinifera* L. cultivars and two clones. The native varieties are given in bold and international cultivars in italics. Since two names, Misket Vrachanski and Tamyanka Tvarda, are used for one accession, they are only represented in the phenogram under the name Misket Vrachanski.

Acknowledgements

This work was financially supported by the 5th Framework Program of the European Union, project No:ICA1CT200070001

References

Bowers, J. E.; Meredith, C. P.; 1997: The parentage of classic wine grape, Cabernet Sauvignon. Nat. Genet. **16**, 84-87.

Bowers, J.; Boursiquot, J.; This, P.; Chu, K.; Johansson, H.; Meredith, C. P.; 1999: Historical genetics: The parentage of Chardonnay, Gamay, and other wine grapes of Northeastern France. Science **285**, 1562-1565.

Crespan, M.; Milani, N.; 2001: The Muscats: A molecular analysis of synonyms, homonyms and genetic relationships within a large family of grapevine cultivars. Vitis **40**, 23-30.

Felsenstein, J.; 1989: Phylogeny inference package. Cladistics **5**, 164-166.

Labra, M.; Moriondo, G.; Schneider, A.; Grassi, F.; Failla, O.; Scienza, O.; Sala, F.; 2002: Biodiversity of grapevines (*Vitis vinifera* L) grown in Aosta Valley. Vitis **41**, 89-92.

Lefort, F.; Douglas, G. C.; 1999: An efficient micro-method of DNA isolation from mature leaves from four hardwood tree species *Acer, Fraxinus, Prunus* and *Quercus.* Ann. For. Sci. **56**, 259-263.

Lefort, F.; Kyvelos, C.; Zervou, M.; Edwards, K.; Roubelakis-Angelakis, K.; 2002: Characterization of new microsatellite loci from *Vitis vinifera* and their conservation in some *Vitis* species and hybrids. Mol. Ecol. Notes **2**, 20-21.

Lefort, F.; Roubelakis-Angelakis, K.; 2001: Genetic comparison of Greek cultivars of *Vitis vinifera* L. by nuclear microsatellite profiling. Am. J. Enol. Vitic. **52**, 101-108.

Lopes, M.; Sefc, K.; Eiras, D.; Steinkellner, H.; Laimer da Camara Machado, M.; da Camara Machado, A; 1999: The use of microsatellites for germplasm management in a Portuguese grapevine collection. Theor. Appl. Genet. **99**, 733-739.

Maletic, E.; Sefc, K.; Steinkeller, H.; Kontic, J.; Pejic, I.; 1999: Genetic characterization of Croatian grapevine cultivars and detection of synonymous cultivars in neighbouring regions. Vitis **38**, 79-83.

Minch, E.; Ruiz-Linares, A.; Goldstein, D.; Feldman, M.; Cavalli-Sforza.; 1997: Microsatv1.5d: A computer program for calculating various statistics on microsatellite allele date (http://hpgl.stanford.edu/projects/microsat/)

Page, R.; 1996: Treeview: An application to display phylogenetic trees on personal computer. Comput. Appl. Biosci. **12**, 357-358.

PELLERONE, F.; EDWARDS, K.; THOMAS, M.; 2001: Grapevine microsatellite repeats: Isolation, characterisation and use for genotyping of grape germplasm from Southern Italy. Vitis **40**, 179-186.

PILIAC, J.; MALETIC, E.; KONTIC, J.; DANGL, G.; PEJIC, I.; MIROSEVIC, N.; MEREDITH, C.; 2002: The parentage of Posip bijeli, a major white wine cultivar of Croatia. Vitis **41**, 83-87.

REGNER, F.; STADLBAUER, A.; EISENHELD, C.; KASERER, H.; 2000: Genetic relationship among Pinots and related cultivars. Am. J. Enol. Vitic. **51**, 7-14.

RUSSANOV, K.; LEFORT, F.; ATANASSOV, A.; ATANASSOV, I.; 2003: The Bulgarian plant genomics database: A web-backed molecular genetics database for plant biotechnology and management of plant genetic resources in Bulgaria. Biotechnol. Biotechnol. Eq. **17**, 3-5.

SCHNEIDER, A.; CARRA, A.; AKKAK, A.; THIS, P.; LAUCOU, V.; BOTTA, R.; 2001:Verifying synonyms between grape cultivars from France and Northwestern Italy using molecular markers. Vitis **40**, 197-203.

SEFC, K.; LOPES, M.; LEFORT, F.; BOTTA, R.; ROUBELAKIS- ANGELAKIS, K.; IBANEZ, J.; PEJIC, I.; WAGNER, H.; GLÖSS, J.; STEINKELLNER, H.; 2000: Microsatellite variability in grapevine cultivars from different European regions and evaluation of assignment testing to assess the geogrphic origin of cultivars. Theor. Appl. Genet. **100**, 498-505.

SEFC, K.; REGNER, F.; GLÖSS, J.; STEINKELLNER, H.; 1998: Genotyping of grapevine and rootstock cultivars using microsatellite markers. Vitis **37**, 15-20.

SEFC, K.; REGNER, F.; TURETSCHEK, E.; GLÖSSL, J.; STEINKELLNER, H.; 1999: Identification of microsatellite sequences in *Vitis riparia* and their applicability for genotyping of different *Vitis* species. Genome **42**, 367-373.

SEFC, K.; LEFORT, F.; GRANDO, M.; SCOTT, K.; STEINKELLNER, K.; THOMAS, M.; 2001:Microsatellite markers for grapevine: A state of the art. In: K. A. ROUBELAKIS-ANGELAKIS (Ed.): Molecular Biology and Biotechnology of Grapevine. Kluwer Acdemic Publishers, The Netherlands.

SEFC, K.; STEINKELLNER H.; LEFORT, F.; BOTTA R.; DA CAMARA MACHADO, A.; BORREGO PALANCO L.; MALETIC, E.; GLÖSSL, J.; 2003: Substantial genetic contribution of local wild vines to European grapevine cultivars. Am. J. Enol. Vitic. **54,** 15-21.

SEFC, K.; STEINKELLNER, H.; WAGNER, H.; GLÖSS, J.; REGNER, F.; 1997: Application of microsatellite markers to parentage studies in grapevine.Vitis **36**, 179-183.

THOMAS, M.; MATSUMOTO, S.; CAIN, P.; SCOTT, N.; 1993: Repetitive DNA of grapevine: Classes present and sequences suitable for cultivar identification. Theor. Appl. Genet. **86**, 173-180.

THOMAS, M.; SCOTT, N.; 1993: Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs). Theor. Appl. Genet. **86**, 985-990.

WAGNER, H.; SEFC, K.; 1999: IDENTITY 1.0 Centre for Applied Genetics, University of Agricultural Sciences, Vienna. (http://www.boku.ac.at/zag/forsch/identity.htm).

ZULINI, L.; RUSSO, M.; PETERLUNGER, E.; 2002: Genotyping wine and table grape cultivars from Apulia (Southern Italy) using microsatellite markers. Vitis **41**, 183-187.

Received August 6, 2003