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Genetic characterization and relationships of traditional grape cultivars from Serbia

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Summary

Reference genetic profiles were generated for 12 traditional grapevine cultivars of Serbia through a genotyping approach that included the "core set" of 9 SSR markers for genetic identification and further 13 common microsatellites for strengthening genetic relationship analysis. Consistent matching with SSR markers of grapevines cultivated in neighbouring countries or maintained in European germplasm collections was found for most of the genotypes, suggesting possible synonyms and revealing that 'Muskat Krokan' corresponds to 'Muscat fleur d'Oranger' and two 'Tamjanika' cultivars are identical to 'Moscato Giallo' and 'Moscato Rosa'. When compared with germplasm representing the classical eco-geographic grouping of grapevine cultivars, Serbian non-Muscat genotypes clustered within the Convar pontica subconvar balcanica taxon thus supporting their indigenous origin.

K e y w o r d s : *Vitis vinifera,* molecular markers, SSR, genetic relationships, Balkan region.

Introduction

Serbia is located in the central Balkans, at the crossroads between Asia and Europe, on the line dividing various nations and civilizations of the East and the West. Due to its favourable climate and geological characteristics, the area of the Balkans is an ancient wine-growing region and both Serbia and many neighbouring countries have a long viticulture tradition.

The first reported occurrence of *Vitis vinifera* in Balkan dates in the Neolithic period in the form of wild grape (Buric 1972). In the beginning of the 2nd millennium B.C., domesticated grapevines were found in the Southern Balkans (Logothetis 1970). Early traces of viticulture and winemaking in the territory of Serbia are vessels from the Iron Age (~ 400 BC) and the Bronze Age (~ 200 BC). Buric (1972) stated that, based on fossil remains found in the territory of Croatia, Bosnia and Herzegovina, we may even assume that the grapevine has been independently domesticated in these regions. In addition, Buric (1972) further stated that historians Dio Cassius (40-110 AD) and Strabo (63/64 BC - ca. AD 24) described the Illyrian and Celtic grapevine from the Pannonia region (located in current Serbia, Croatia, Hungary and Romania).

Turbulent history of the Balkans and the changes of different cultures affected the ups and downs in the development of viticulture. During Middle Ages, the Roman Empire promoted viticulture and spread its cultivation in the Balkans. Upon their arrival to the Balkans (600-900 AD), the Slavic peoples discovered grapevines and took to its cultivation. In medieval Serbia, viticulture progressed thanks to the feudal authorities and the monasteries on whose properties the grapes were grown. In addition, wine became a true national beverage among the common people. With establishment of the Ottoman Empire, Serbian viticulture focussed more on cultivation of table grape varieties, mainly through introduction of new varieties from the Middle East. At the end of the 19th century, Serbian viticulture shared the same fate as the European, due to the expansion of disease-causing agents from America, resulting in devastation of many vineyards. After a recovery period, new areas under grapevine were established with wine varieties introduced mainly from France and table grape varieties of various origins. Along with the introduced varieties, many smaller manufacturers as well as large state-owned companies also started growing native varieties, such as 'Prokupac', 'Smederevka', 'Plovdina', 'Tamjanika' and several other varieties of minor importance.

As stated by Dettweiler (1993), the identification of plant material by ampelography sometimes results in misinterpretation and a more objective characterization of local cultivars is required. Molecular marker profiles enable a direct comparison of the similarity of genotypes at the DNA level and serve as a valuable adjunct to morphological description. Here we present the first application of the SSR markers to the Serbian grapevine germplasm in order to provide reference descriptors for the identification and evaluation of genetic relationships of local cultivars. Moreover, this study aims to support a development of the regional germplasm collection of native grapevines in order to preserve agricultural biodiversity.

Material and Methods

Woody canes of 22 grapevines putatively corresponding to 12 varieties were sampled in the collection "Radmilovac" (YUG09) maintained by the Faculty of Agriculture at the University of Belgrade, the collection "Sremski Karlovci" (YUG016) maintained by the Faculty of Agriculture at the University of Novi Sad and in old vineyards in the Župski and Negotinski vine growing districts (Tab. 1).

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Table 1

Grapevine accessions included in this study and distinct genetic profiles obtained with 10 SSR markers

Putative variety	Accession/sample name	Berry colour	Source/location	SSR profile
1	**Ćilibarka**	1	YUG016 collection	1
2	**Ružica** / Kevidinka	2	YUG016 collection	2
3	**Kreaca**	1	YUG016 collection	3
4	**Muskat Krokan**	1	YUG016 collection	4
5	**Plovdina** 1	5	YUG016 collection	5
5	Plovdina 2	5	Vineyard – Župsko v.	5
5	Plovdina 3	5	Vineyard – Župsko v.	5
5	Slankamenka crvena	5	YUG016 collection	5
6	**Prokupac** 1	6	YUG016 collection	6
6	Prokupac 2	6	YUG09 collection	6
6	Prokupac 3	6	Vineyard – Župsko v.	6
6	Prokupac 4	6	Vineyard – Župsko v.	6
7	**Smederevka**	1	Vineyard – Župsko v.	7
8	**Sremska Zelenika**	1	YUG016 collection	8
9	**Tamjanika Crna** NG1	6	Vineyard – Negotinsko v.	9
9	Tamjanika Crna NG2	6	Vineyard – Negotinsko v.	9
10	Tamjanika Bela 1	1	YUG016 collection	10
10	**Tamjanika Bela**	1	Vineyard – Župsko v.	11
10	Tamjanika Bela 3	1	Vineyard – Župsko v	10
10	Tamjanika Bela NG	1	Vineyard – Negotinsko v.	11
11	**Tamjanika Crvena**	2	Vineyard – Župsko v.	10
12	**Začinak**	6	Vineyard – Negotinsko v.	12

Note: Accession names in bold agreed with variety name

DNA was extracted from flakes of cambium tissues frozen in liquid nitrogen and ground to a fine powder in a mortar according to the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) protocol. All accessions were first genotyped at 10 microsatellite loci combined in 4 multiplex panels as follows: VVS2, VVMD32 and VVMD28; VMC1B11, VVMD27 and VVMD7; VrZAG62 and VrZAG79; VVMD5 and VVMD25. Primer sequences and nomenclature are reported in Thomas and Scott (1993), Bowers *et al.* (1996, 1999) and Sefc *et al.* (1999). The marker VMCB11 (GenBank BV681754) was developed by the Vitis Microsatellite Consortium (Agrogene, Moissy Cramayel, France).

Next, non-redundant genotypes were analyzed at further 12 SSR loci in order to apply the complete set of markers proposed by Laucou *et al.* (2011).

Simultaneous PCR amplifications were carried out in a final volume of 12.5 µL containing 10 ng of genomic DNA, 0.25 mM of each dNTPs, 2mM $MgCl_2$, 1.5 U Taq DNA Polymerase (Gold Taq®; Applied Biosystems, Foster City, CA, USA). Depending on the locus, primer concentrations ranged from 0.2 to 0.6 µM. Reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems) using the following profile: a hot start of 95 °C for 7 min, 30 amplification cycles of 45 s at 95 °C, 1 min at 54 °C, 30 s at 72 °C, and a final extension step of 1 h at 72 °C.

PCR products (0.5 µl) generated by two or three different fluorescence dye-labeled primers were mixed with 9.3 µl of formamide and 0.2 µl of the GeneScan™ 500 ROX® Size Standard (Applied Biosystems). DNA fragments were denatured and size fractioned using capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems). GeneMapper v3.5 (Applied Biosystems) was used for the estimation of allele sizes.

In order to compare SSR genotypes between different studies, allele sizes were harmonized based on the marker profile of common grapevine cultivars 'Pinot noir' or 'Cabernet Sauvignon'.

The molecular profiles at 22 SSR loci of the Serbian varieties were subjected to cluster analysis together with homologous profiles of 31 accessions belonging to the FEM-IASMA germplasm collection (ITA362). The last were shown to represent the classical eco-geographic grouping of grape cultivars (Negrul 1938) within a population of ca. 900 unique genotypes of *V. vinifera* (Emanuelli and Grando, pers. communication). A dissimilarity matrix-based tree was calculated using an unweighted neighbor-joining method implemented in Darwin software package v5.0 (Perrier *et al.* 2006). The SSR genotype of three grape rootstock varieties were used as an outgroup.

Evaluation of OIV descriptors was carried out for 11 of the distinct varieties identified in this study and which accessions were available in the YUG016 and YUG09 collections (Tab. 2).

Results and Discussion

Twenty two grapevine accessions analyzed in this study with 10 SSR markers generated 12 distinct molecu-

Table 2

(a) Genotypes at 10 SSR loci of the grapevine accessions used in this study (identical genotypes were merged) and matched found with data reported in literature or SSR profile databases.
(b) Extended profile of SSR markers for the unique genotypes identified in this study

a)

SSR profile	VVMD5	VVMD7	VVMD25	VVMD27	VVMD28	VVMD32	VMC1B11	VVS2	VrZAG62	VrZAG79	Matching	Source of data
1	235:239	239:249	242:250	177:183	245:259	259:271	169:175	134:142	199:203	251:251	Cornichon blanc*	GrapeGen06 DEU098-1993-191
2	235:241	239:249	242:256	179:179	235:249	253:271	181:183	130:142	187:193	253:259	Kövidinka	FEM-IASMA 1061
3	241:247	239:247	242:250	177:179	235:237	253:265	171:185	130:142	187:203	251:259	Kreaca	GrapeGen06 DEU098-1980-240
4	227:237	247:249	242:250	177:183	247:267	241:271	175:189	130:130	185:203	251:255	Muscat Fleur d'Oranger	FEM-IASMA1328 GrapeGen06 FRA139-570Mtp11
5	225:247	239:239	250:256	181:187	249:259	253:257	181:183	132:140	187:187	243:251	Pamid	Dzhambazova et al. (2009)
6	225:227	249:249	242:256	179:183	247:261	271:271	183:183	140:144	193:199	243:251	Prokupac	GrapeGen06 DEU098-1980-304
7	241:247	239:249	250:256	177:179	235:247	251:265	171:185	142:142	187:203	237:259	Dimyat	Dzhambazova et al. (2009), FEM-IASMA642
8	225:241	239:247	240:250	177:193	227:257	251:271	171:185	130:130	187:203	249:255	Szerémi	Galbács et al. (2009)
9	237:241	239:249	242:256	177:193	249:267	265:271	167:185	130:132	185:187	249:255	Moscato Rosa	FEM-IASMA1309, GrapeGen06 ITA388-1934
10	227:241	239:249	242:256	177:177	239:249	259:271	183:187	130:140	185:187	249:255	Moscato Giallo	FEM-IASMA3000, GrapeGen06 ITA388-R#1094
11	227:237	239:247	250:256	177:183	259:269	265:271	183:183	130:132	193:203	251:255	No match	
12	225:247	249:255	242:256	183:193	229:251	271:271	181:183	132:144	193:203	249:251	No match	
Cabernet Sauvignon	229:237	239:239	240:250	173:187	235:237	241:241	183:183	136:150	187:193	247:247		
Cabernet Sauvignon	n+10:n+18	n+8:n+8	n+4:n+14	n:n+14	n+18:n+20	n+5:n+5	n+18:n+18	n+16:n+30	n+14:n+20	n+10:n+10		
Pinot Noir	227:237	239:243	240:250	183:187	219:237	241:271	167:173	134:150	187:193	239:245		
Pinot Noir	n+6:n+16	n+8:n+12	n+4:n+14	n+10:n+14	n+2:n+20	n+5:n+35	n:n+6	n+14:n+30	n+14:n+20	n+2:n+8		

Tab. 2 continued

b)

SSR Profile	VVMD24	VVIN16	VVIQ52	VVIH54	VVIV37	VMC4f8	VVMD21	VVIN73	VMC4f3	VVIB01	VVIP31	VVIV67
1	209:217	151:153	82:84	166:172	160:176	111:111	243:249	266:268	167:175	295:295	183:195	362:374
2	207:211	151:153	78:84	168:178	162:162	111:123	257:257	266:266	173:209	291:295	179:185	356:364
3	207:207	151:151	80:84	150:166	170:170	111:123	249:257	266:266	117:173	295:295	175:195	356:362
4	207:211	149:159	78:80	164:166	152:162	113:119	249:265	264:266	167:173	295:295	183:193	360:362
5	207:211	151:151	78:80	166:168	152:162	111:111	249:259	266:268	187:229	295:295	177:195	356:360
6	207:211	151:151	78:82	168:168	158:162	111:119	249:249	266:268	167:169	295:295	177:189	362:362
7	207:207	151:151	78:80	166:168	158:162	111:111	249:257	266:266	167:173	295:295	183:183	362:362
8	207:207	151:151	78:84	150:178	158:162	111:111	249:249	258:266	173:203	295:295	173:195	356:364
9	207:217	149:151	78:80	164:166	162:162	111:119	243:265	258:264	173:207	291:295	187:195	356:374
10	207:211	149:159	78:80	164:178	160:162	125:125	255:265	266:266	167:183	295:295	183:183	352:374
11	207:211	157:159	80:84	164:166	162:162	113:123	265:265	264:266	167:173	295:295	173:193	360:370
12	207:217	151:151	78:78	164:178	162:162	111:119	257:259	264:266	167:167	291:291	177:187	356:362
Cabernet Sauvignon	207:215	153:153	78:84	166:180	162:162	113:119	249:257	266:270	173:179	291:291	189:189	362:370
Pinot Noir	213:215	151:159	84:84	164:168	152:162	113:119	249:249	266:268	173:179	289:295	179:183	362:370

lar profiles (Tab. 1). Different samples collected from the same putative grapevine variety in the collections or in old vineyards showed identical molecular genotypes except for 'Tamjanika Bela' accessions which showed two diverse DNA profiles. One identical genotype was found between 'Tamjanika Bela 1' and 'Tamjanika Bela 3' and it was also shared with the 'Tamjanika Crvena' accession suggesting a potential misnaming. A second profile was determined for 'Tamjanika Bela' and 'Tamjanika Bela NG' accessions. Varieties with prefix 'Tamjanika' in the name are all Muscat type cultivars. Major trait variations within 'Tamjanika' are related to skin color, aroma intensity and sex of flower. Skin can be blue or black ('Tamjanika Crna'), reddish ('Tamjanika Crvena'), yellowish ('Tamjanika Žuta', not included in this study) and greenish ('Tamjanika Bela') (Tab. 3). 'Tamjanika Crna', 'Crvena' and 'Žuta' have female type of flowers, while 'Tamjanika Bela' has hermaphrodite flower. Identical SSR genotypes occurred also between 'Slankamenka Crvena' and the 'Plovdina' accessions but those are known synonyms being the first a common name for 'Plovdina' cultivars in the province of Vojvodina in the North of Serbia.

All 10 loci tested were quite polymorphic in the population (Tab. 2 a), with a number of alleles per locus ranging from 4 for VVMD7 and VVMD25 to 13 for VVMD28, for a total of 68 alleles. Allele length was in the range reported for *V. vinifera* cultivars (This *et al.* 2004, Ibáñez *et al.* 2009) and in particular fell within both high and low frequent SSR markers observed in the group of accessions from the Balkans held in the INRA Domaine de Vassal repository (Laucou *et al.* 2011).

Nine of the microsatellite markers used for the identification step belonged to the 'core set' of markers chosen by the international grape community (GrapeGen06 EU project) for the characterization of regional cultivars of Europe (Bacilieri and This 2010). This allowed the comparison of markers to the SSR profiles reported in the European Vitis Database (www.eu-vitis.de) and with SSR profiles published in previous studies or generated from the accessions of the FEM-IASMA germplasm collection.

All but two genotypes did match the SSR profiles of grapevine cultivars as reported in Tab. 2 thus revealing that some genetic resources are represented at least in one different European germplasm repository whereas others may be synonyms for minor varieties cultivated in neighbouring countries or elsewhere.

Kreaca, Ružica and Sremska Zelenika are autochthonous varieties of the Pannonian plain. (*Convar pontica, subconvarietas balcanica,* Negrul 1938). They are spread in the Northern part of Serbia (Vojvodina), Hungary and Romania. In Serbia, 'Kreaca' is also called 'Banat Riesling' because it is the most common cultivar in Serbian and Romanian Banat. In Romania, 'Kreaca' is called 'Creata' and 'Creata de Banat' (Nemeth 1967, Zirojevic 1974). The SSR profile perfectly matched that of one 'Kreaca' accession conserved in the DEU098 collection at Institut für Rebenzüchtung Geilweilerhof, Germany. The variety 'Ružica' is called 'Red Dinka' and also 'Kevidinka' in Serbia. The last is a version of the Hungarian name 'Kövidinka' which was also the name of one accession with the same markers profile identified in the FEM-IASMA collection. 'Sremska Zelenika' is considered as a rare native variety of the geographical area Srem, located in Serbia (Vojvodina) and in Croatia. The accession shared the same SSR genotype with one Hungarian Szerémi cultivar described by Galbács *et al.* (2009) and in fact Szerémi Zöld means Sremska Zelenika.

The accessions named Tamjanika (from *tamjan*, incense) are considered the oldest Serbian autochthonous cultivars. As Muscat varieties they were included in the

Table 3

Primary and secondary OIV descriptors of grapevine cultivars evaluated in this study

Cultivar	SSR Profile	OIV CODES 004	084	202	204	206	220	223	225	504	505	506
		Density of prostrate hairs on the shoot tip	Density of prostrate hairs between main veins on lower side of blade	Bunch length	Bunch density	Length of peduncle of primary bunch	Berry length	Berry shape	Berry color of skin	Yield per m^2	Sugar content of must	Total acidity of must
Ćilibarka	1	5	1	9	5	5	7	9	1	9	3	7
Ružica	2	9	9	3	3	3	3	3	2	5	5	5
Kreaca	3	7	5	3	7	1	3	3/4	1	5	5	5
Muskat Krokan	4	1	1	5	3	7	3	2	1	3	3	5
Plovdina	5	1/3	7	5	5	1	5	7	5	5	1	7
Prokupac	6	5/7	5	5	5/7	3	5	2	6	7	3	7
Smederevka	7	5/7	5	5	3/5	5/7	5	4	1	9	5	7
Sremska Zelenika	8	7/9	5/7	3	5	5	3	3/4	1	7	5	7
Tamjanika Crna	9	5	1	3	3	3/5	3	5	6	/	7	9
Tamjanika Bela	11	5	1	5	3	7	3	3/4	1	/	5	9
Tamjanika Crvena	10	5	3	5	5	5	3	3	2	5	7	5
Začinak	12	3	9	3	7/9	3	3/5	3	6	5	5	7

eco-geographical group *Convar orientalis, Convarietas caspica* by Negrul (1938). The genotype of 'Tamjanika crna NG' matched the SSR profile of the 'Moscato Rosa' accessions maintained in the FEM-IASMA collection. The variety is called 'Rosenmuskateller' in South Tyrol and 'Muskat Ruža Porečki' in Croatia (Costantini *et al.* 2001) and according to Maletic *et al.* (1999) should be native to Dalmatia (Croatia). However, 'Tamjanika' is related more to the medieval vineyards of the Nemanjić dynasty in the central and eastern part of Serbia (Stojanovic and Toskic 1948). The existence of 'Tamjanika Crna' in eastern Serbia, where grapevines were cultivated in the Roman Empire, suggests a possible route of its spreading from East to central Europe. 'Tamjanika Bela' and 'Tamjanika Crvena' are widespread in central Serbia, in the Župa vineyards and slightly less in eastern Serbia. The present study revealed that accessions 'Tamjanika Bela' 1, 'Tamjanika Bela' 3 and 'Tamjanika Crvena' have the same markers profile of the true-to-type variety 'Moscato Giallo', a yellow-skinned member of the Muscat family sometimes called 'Gold-muskateller' in Northern Italy and Germany. 'Tamjanika Bela' and 'Tamjanika Bela NG' did not match either to reference Muscats or other cultivar profiles consulted, therefore excluding the synonymy with the Bulgarian variety Tamyanka identified as 'Moscato Bianco' by Hvarleva *et al.* (2004).

The accession 'Muscat Krokan', on the other hand, is cultivated only at the location called the "Pearl Island" in the Banatsko-Potisko vine growing district and its origin was not known. This study determined the genetic identity with true-to-type 'Muscat Fleur d'Oranger', a variety apparently derived from a cross between 'Chasselas' and 'Moscato Bianco' based on evidences provided by Schneider *et al.* (2008).

'Smederevka', 'Prokupac', 'Plovdina' and 'Začinak' are considered autochthonous varieties that belong to *Convar pontica, Convarietas balcanica*. 'Smederevka' is grown in many Serbian vine growing districts and got its name since it was cultivated in the vicinity of Smederevo at the time of the Roman Empire in the 3th century B.C. (Jiricek 1923). Serbian variety 'Smederevka' and Bulgarian 'Dimyat' were suggested to be synonyms on the basis of morphological descriptors (Avramov 1991). This has been confirmed with the SSR markers, since the profile of 'Smederevka' matches that reported by Hvarleva *et al.* (2004) and Dzhambazova *et al.* (2009) for 'Dimyat' accessions of ancient cultivars conserved at AgroBioInstitute of Sofia (Bulgaria). 'Prokupac' and 'Začinak' are considered old Serbian autochthonous grapevine varieties as well. 'Prokupac' is common in all Serbian winegrowing districts, especially in southern Serbia, while 'Začinak' is mainly grown in eastern Serbia (Timok vine growing district). The 'Prokupac' sample analyzed in this study perfectly matches the SSR profile of the 'Prokupac' accession maintained in the DEU098 collection. On the other hand, no synonyms nor homonyms were found for 'Začinak'. 'Plovdina' is a variety traditionally grown along with 'Prokupac' in the same vineyards. The comparison of the SSR profile suggests that the Serbian 'Plovdina' could be synonym of the Bulgarian 'Pamid' which in turn was found to be identical to the Greek cultivar 'Pamidi' by Hvarleva *et al.* (2004).

Finally 'Ćilibarka' is a domesticated table grape cultivar which is thought to originate from the Middle East and that is mainly cultivated in gardens. The variety was much

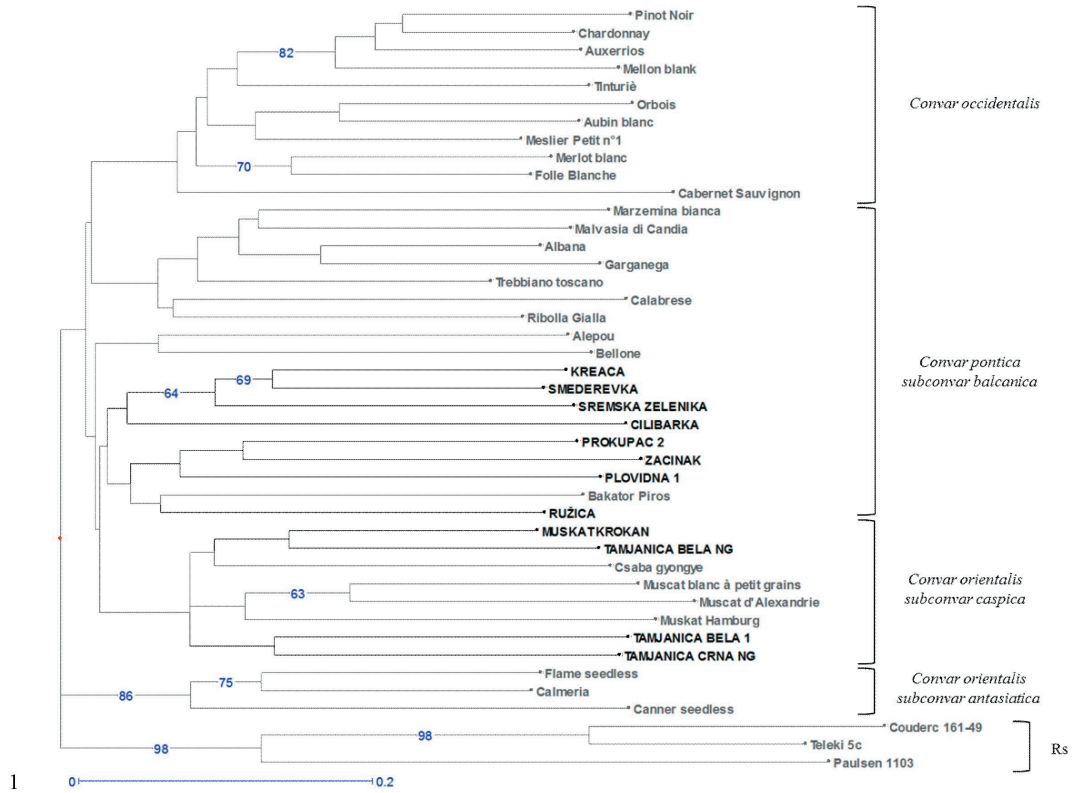

Figure: Neighbor joining unweighted tree based on a dissimilarities matrix calculated from SSR alleles at 22 loci for 40 *V. vinifera* accessions and three rootstocks (*Vitis* sp.) as an outgroup. Only bootstraps superior to 60 are presented. Serbia varieties are shown in capital letters.

more common before the phylloxera crisis (AVRAMOV 1991) and might have different names. This research found an accession with identical SSR profile within the DEU98 collection, however its name 'Cornichon Blanc' is not certain. Further research is necessary to provide evidence of the synonymy of the cultivars. The genetic relationship among the Serbia varieties were examined by building a neighbor- joining unweighted tree based on a dissimilarities matrix calculated from SSR alleles (Figure). In addition to the 12 unique marker profiles obtained in this study, the SSR profiles of 28 grape cultivars of different origin were also considered, with three rootstocks (*Vitis* sp.) as an outgroup. The dendrogram showed four major clusters representing the eco-geographical classification proposed by NEGRUL (1938). A clear-cut division between Muscats and non-Muscat populations was found with 'Muskat Krokan', 'Tamjanica Bela NG', 'Tamjanica Bela 1' and 'Tamjanica Crna NG' actually included in the cluster attributable to the *Convar orientalis subconvar caspica* taxon. All the other traditional Serbian genotypes grouped closely together moving the Hungarian variety 'Bakator Piros' from the Italian and Greek cultivars used to represent the *Convar pontica subconvar balcanica* group.

Conclusions

This study provided the first molecular characterization of ancient grapevine cultivars grown in Serbia. Reference DNA profiles were generated for 12 varieties through a genotyping approach that included the 'core set' of 9 SSR markers chosen by the international grape community for genetic identification and further 13 common microsatellites to strengthen relationship analysis. Consistent matching with SSR markers of grapevines cultivated in neighbouring countries or maintained in European germplasm collections was found for most of the molecular profiles, suggesting possible synonyms. These included three Muscat-type cultivars which showed identical SSR profiles with true-to-type 'Moscato Giallo', 'Moscato Rosa' and 'Muscat fleur d'Oranger' varieties. When compared with germplasm representing the classical eco-geographic grouping of grape varieties, Serbian cultivars were divided into two distinct clusters. The Muscat-type cultivars were included within the *Convar orientalis subconvar caspica* while all other accessions were assigned to the group of *Convar pontica suconvar balcanica* in accordance with their origin.

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