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Microsatellite genotyping of old Slovenian grapevine varieties (*Vitis vinifera* L.) of the Primorje (coastal) winegrowing region

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Summary

Thirty-three grapevine varieties from Slovenia were genotyped at 21 microsatellite loci in order accurately to identify varieties and to evaluate their synonyms and homonyms, including varieties cultivated in neighbouring countries. Among Slovenian varieties some previously assumed synonyms were confirmed and some new ones were discovered: 'Poljšakica Drnovk' = 'Istrska Malvazija', 'Pikolit Italy' = 'Pikolit Vienna', 'Vitovska grganja' = 'Racuk' and 'Prosecco' = 'Glera' = 'Števerjana'. Types of Zelen ('Zelen Pokov', 'Zelen 66' and 'Zelen 2.4') were distinct at several microsatellite loci so can only be considered to be homonyms. Two 'Picolit' types were considered to be 'true-to-type' on the basis of comparison with 'Picolit' clones from Italy. Synonymy between 'Heunisch' and 'Belina' was not confirmed in our study, since 'Belina Pleterje' differentiated from 'Heunisch weiss' at 13 out of 19 loci. Comparison of 'Vitovska grganja' from Slovenia with 'Vitouska' from Italy also showed dissimilarities at the majority of the analysed loci.

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

Introduction

Varied climatic and geological conditions have contributed to the great diversity of *Vitis vinifera* L. varieties in Slovenia. Around 50 well-known and rare varieties exist in Slovenia today, the majority of them being listed in the International List of Vine Varieties and Their Synonyms (OIV 2005). Although morphological and agronomical descriptions of the majority of autochthonous varieties exist, this is mostly unexplored plant material, and some of these varieties survived only in less productive vineyards or in germplasm collections. In 1980 we started systematically to collect our old and/or neglected varieties and set up four grapevine collections which include old varieties and clonal candidates. One of the problems in the management of these germplasm collections is the use of synonymic and homonymic designations, which are not completely reliable mainly because of inadequate documentation and poor preservation of historical facts related to grape growing and trade. The identification of plant material by ampelographic methods often results in misinterpretations (Dettweiller 1993). In contrast, DNA-based markers are independent of environmental factors and are therefore more reliable for variety identification (Botta *et al.* 1995). In the last decade, more than 60 SSR (Short Sequence Repeat) primers from the genomic libraries of *Vitis vinifera* L. have been developed and used for identification purposes (Thomas and Scott 1993, Bowers *et al.* 1996, 1999, Sefc *et al.* 1999, Lefort *et al.* 2001).

The aim of our work was to genotype old Slovenian grapevine varieties using 21 SSR markers in order to examine their synonyms and homonyms, also in comparison with varieties cultivated in neighbouring countries. Additionally, genetic variability was assessed among analysed varieties.

Material and Methods

P l a n t m a t e r i a l : The study was carried out on 32 old Slovenian grapevine varieties plus 'Chardonnay', 'Pinot noir', 'Cabernet-Sauvignon', 'Sultanine', 'Touriga Nacional' and 'Barbera' as reference varieties and *Vitis rupestris* L. as an out-group variety (Table). The vines are grown in grapevine germplasm collections in the Primorje winegrowing region (collection vineyards: Nova Gorica, Vipava and Goriška Brda).

D N A i s o l a t i o n : DNA was extracted from young leaves of shoot tips of individual vines using the modified CTAB method described by Kump and Javornik (1996).

M i c r o s a t e l l i t e a n a l y s i s : The old Slovenian grapevines, the reference variety 'Chardonnay' and *Vitis rupestris* L. were genotyped at 21 microsatellite loci: VVS2 (Thomas and Scott 1993); VVMD5, VVMD6, VVMD7, VVMD8 (Bowers *et al.* 1996); VVMD17, VVMD24, VVMD25, VVMD27, VVMD31, VVMD32, VVMD36 (Bowers *et al*. 1999); ssrVrZAG21, ssrVrZAG47, ssrVrZAG62, ssrVrZAG67, ssrVrZAG79, ssrVrZAG83 and ssrVrZAG112 (Sefc *et al.* 1999); ssrVvUch11 and ssrVvUch29 (Lefort *et al.* 2001). Five other reference varieties 'Pinot noir', 'Cabernet-Sauvignon', 'Sultanine', 'Touriga Nacional' and 'Barbera' were genotyped at 8 SSR loci (Table). The choice of these loci made the results comparable to the grapevine genotyping results of neighbouring countries and allows the integration of the results into the European grapevine database. The high number of SSR loci was useful in comparing allelic profiles of synonymic varieties. The data for comparison were obtained from dif-

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T a b l e

Grapevine genotypes at twenty-one SSR loci represented by allele sizes in base pairs (bp)

Name	Rebula	Belina Pleterje	Laški Rizling	Pinela	Zelen 66	Zelen Pokov	Zelen 2.4	Zelenika	Poljšakica Lože	Poljšakica Drnovk	Planika	Bela Glera	Briška Glera	Guštana	Vitovska.Grganja	Pergolin	Danijela	Vrtovka	Dišečka	Dolga Petlja	Volovnik	Racuk	Cividin	Zunek	Rečigla	Števerjana	Klarnca	Pikolit Vienna	Pikolit Italy	Vitis rupestris	Rdeča žlahtnina	Prosecco	Istrska Malvazija	Chardonnay	Pinot noir	Cabernet-Sauvignon	Sultanine	Touriga Nacional	Barbera
ssrVrZAG112	240	240	230	234	230	242	234	234	234	234	234	234	242	234	230	234	234	234	242	234	234	230	234	234	234	242	234	234	234	242	240	242	234	240					
ssrVrZAG112	240	242	234	242	238	262	242	242	234	240	242	262	262	234	234	234	240	242	242	238	234	234	240	238	242	262	242	242	242	250	242	262	240	240					
ssrVrZAG21	206	200	190	204	200	190	204	206	204	194	200	200	190	190	200	206	190	204	200	206	204	200	194	202	200	190	206	194	194	204	200	190	194	200					
ssrVrZAG21	206	206	206	206	206	206	206	206	214	202	206	206	200	202	206	214	206	206	202	206	214	206	206	206	206	200	206	200	200	208	206	200	202	206					
ssrVrZAG47	164	158	164	158	168	162	158	160	158	158	158	158	158	164	160	160	164	158	160	160	158	160	160	158	160	158	168	160	160	184	164	158	158	160					
ssrVrZAG47	164	160	168	160	172	172	160	160	160	158	158	158	172	164	172	172	168	168	172	172	160	172	164	162	172	172	172	164	164	186	168	172	158	168					
ZAG62	188	196	194	204	194	188	204	188	196	188	188	188	188	192	194	188	194	200	192	204	188	194	196	190	200	188	188	192	192	196	194	188	188	188	188	188	188	188	192
ZAG62	200	204	196	204	204	194	204	204	204	188	200	196	204	194	204	196	196	204	204	204	204	204	202	196	204	204	200	204	204	196	204	204	188	196	194	194	188	194	200
ssrVrZAG67	128	140	140	134	150	150	134	134	140	134	134	134	150	128	140	140	128	134	150	140	150	140	156	128	140	150	140	140	140	158	128	150	134	140					
ssrVrZAG67	160	160	140	140	150	160	140	140	150	140	150	150	150	128	150	150	128	140	156	156	150	150	164	140	156	150	160	156	156	158	154	150	140	154					
ZAG79	240	238	252	244	246	250	244	238	252	238	244	252	250	240	244	250	240	252	250	242	252	244	244	244	250	250	252	240	240	260	252	250	238	244	240	248	248	246	244
ZAG79	252	260	252	252	258	252	252	260	252	256	260	260	260	252	252	260	252	252	260	242	260	252	260	248	260	260	252	260	260	264	260	260	256	246	246	248	260	246	260
ssrVrZAG83	188	188	188	188	188	190	188	188	190	188	188	188	188	188	188	194	190	190	188	190	190	188	194	190	190	188	190	194	194	186	190	188	188	188					
ssrVrZAG83	188	188	194	190	188	200	188	188	194	194	194	194	194	188	194	194	194	200	194	194	194	194	200	194	194	194	200	194	194	216	200	194	194	200					
ssrVvUch 11	246	246	246	242	242	242	242	242	242	242	242	242	242	242	242	238	248	242	242	248	242	242	244	248	242	242	240	248	248	244	242	242	242	248					
ssrVvUch 11	248	248	248	262	246	262	262	248	248	248	262	246	246	248	262	242	262	244	262	262	248	262	248	262	246	246	244	264	264	252	244	246	248	262					
ssrVvUch 29	211	211	213	297	211	209	297	297	211	215	211	211	309	211	211	211	289	211	207	207	215	211	211	211	207	309	211	213	213	285	289	309	215	289					
ssrVvUch 29	289	297	289	309	211	211	309	297	289	297	309	309	309	289	297	289	289	297	211	289	289	297	289	211	297	309	309	289	289	295	309	309	297	297					
VVMD17	220	222	222	212	220	220	212	220	222	220	222	222	222	222	222	220	220	220	220	220	222	222	212	220	220	222	222	212	212	220	212	222	220	220					
VVMD17	220	222	222	220	220	220	220	220	222	220	222	222	222	224	222	220	224	220	220	220	222	222	220	222	220	222	222	220	220	220	212	222	220	222					
VVMD24	212	208	212	208	208	212	208	208	206	208	208	208	212	208	208	208	208	212	208	208	206	208	208	208	208	212	208	206	206	206	208	212	208	208					
VVMD24	214	216	216	216	216	212	216	208	208	210	212	216	216	208	208	216	208	216	216	216	208	208	216	208	216	216	212	216	216	206	212	216	210	216					
VVMD25	243	241	257	241	243	241	241	241	243	257	241	241	241	243	241	241	241	241	243	257	241	241	257	243	257	241	241	241	241	239	243	241	257	241	241	241	241	251	241
VVMD25	251	241	271	251	257	257	251	257	265	257	245	243	245	251	257	257	251	251	265	257	265	257	259	243	265	245	257	257	257	239	257	245	257	257	251	251	251	257	257
VVMD27	186	180	186	180	190	184	180	182	180	180	180	180	180	186	182	182	186	180	182	182	180	182	182	180	182	180	190	182	182	206	186	180	180	182	186	176	182	182	186
VVMD27	186	182	190	182	194	194	182	182	180	180	180	180	194	186	194	194	190	190	194	194	182	194	186	184	194	194	194	186	186	208	190	194	180	190	190	190	195	190	190
VVMD31	208	210	210	214	212	216	212	210	210	202	212	210	210	216	202	210	214	208	208	208	210	202	210	210	208	210	208	202	202	210	210	210	202	212					
VVMD31	208	210	214	214	212	216	214	212	214	208	214	214	214	216	212	210	216	212	210	214	214	212	214	210	210	214	210	214	214	216	214	214	208	214					
VVMD32	271	263	239	255	263	239	255	249	251	255	251	251	261	251	251	251	251	255	249	271	251	251	239	261	261	261	255	239	239	233	239	261	255	239	239	239	249	239	251
VVMD32	271	271	271	271	271	271	271	271	255	271	263	263	263	261	271	271	271	271	261	271	255	271	271	271	271	263	271	249	249	235	239	263	271	271	271	239	249	271	271
VVMD36	242	262	252	252	252	250	252	262	252	252	252	252	250	262	252	242	250	252	252	252	242	252	252	252	252	250	250	252	252	238	262	250	252	252					
VVMD36	252	262	262	274	252	252	274	274	262	262	252	252	252	296	274	250	296	252	296	252	262	274	252	252	296	252	250	252	252	238	294	252	262	274					
VVMD5	226	232	224	230	224	224	232	234	238	220	224	224	224	226	224	224	226	224	224	232	224	224	236	224	226	224	224	230	230	234	226	224	220	232	225	229	232	223	223
VVMD5	226	244	236	232	224	224	232	238	238	238	238	244	244	234	238	224	236	232	232	232	238	238	236	238	232	244	226	236	236	234	234	244	238	236	236	238	232	234	223
VVMD6	198	188	198	208	188	198	208	208	188	188	208	188	206	208	188	206	206	206	188	188	208	188	206	188	188	206	206	198	198	204	198	206	188	198					
VVMD6	206	208	206	208	208	206	208	208	208	206	208	206	208	208	198	208	208	208	206	208	208	198	208	206	206	208	206	208	208	214	206	208	206	208					
VVMD7	243	245	247	247	247	239	247	239	239	239	239	239	239	247	239	239	247	247	239	239	239	239	247	239	249	239	239	247	247	257	239	239	239	239	239	239	239	239	249
VVMD7	249	247	257	249	247	257	249	249	247	239	253	239	247	247	255	243	247	249	247	249	247	255	261	247	249	247	249	247	247	261	247	247	239	243	243	239	253	239	253
VVMD8	140	140	142	140	142	142	140	140	154	142	142	142	142	142	154	142	140	142	140	142	154	154	142	154	140	142	142	140	140	170	142	142	142	140					
VVMD8	142	140	146	142	154	142	142	140	154	154	154	154	146	166	156	142	164	154	142	154	154	156	142	154	142	146	154	166	166	170	142	146	154	146					
VVS2	143	131	133	131	141	131	131	131	143	141	141	141	131	143	131	131	131	131	149	141	131	131	139	131	141	131	137	133	133	135	131	131	141	135	135	137	143	141	131
VVS2	149	131	149	131	151	133	131	141	149	141	143	143	141	153	141	141	153	137	151	141	149	141	141	135	149	141	141	137	137	135	141	141	141	141	149	149	149	149	133

ferent publications, in which reference varieties were not always used, but normalization and comparison was possible on the basis of difference between two alleles.

P C R c o n d i t i o n s : In a total volume of 10 µl the PCR reaction mixture contained 20 ng of genomic DNA, 1 x PCR buffer (Fermentas), 0.2 mM of each dNTP's (Sigma), 2 mM $MgCl_2$ (Fermentas), 0.5 µM of each primer and 0.25 U of *Taq* DNA polymerase (Fermentas). One of the primers for each locus was labelled with fluorescent Cy-5 dye for fluorescent detection (IDT lnc., BioSciences). The amplification of microsatellite loci was performed in a Whatman Biometra T-Gradient thermocycler with the following steps: hot start for 5 minutes at 95 °C; 26-40 cycles of denaturation at 94 °C for 30-45 s, annealing at 50-58 °C for 30-45 s and an extension step at 72 °C for 90 s. Reactions were completed by incubating at 72°C for 8 min.

After PCR optimization and amplification of individual loci, the amplification products were separated on 6 % polyacrylamide, 7 M urea and 1 x TBE gels, running in 0.5 x TBE buffer on an ALFexpress DNA automated sequencer (GE Healthcare). The allele sizes were analyzed with AlleleLocator 1.03 software (GE Healthcare). Alleles were precisely sized against an ALFexpress sizer 50-500 (GE Healthcare) and by internal DNA standards of different sizes amplified from plasmid.

S t a t i s t i c a l a n a l y s i s : Expected heterozygosity (He; Nei 1978), observed heterozygosity (Ho) and probability of identity (PI; Paetkau *et al.* 1995) were calculated using Identity 1.0 software (Wagner and Sefc 1999). This program was also used to detect identical genotypes. Genetic distances between varieties were calculated in Microsat (Minch 1997) as 1- proportion of shared alleles.

Results and Discussion

Allele sizes for each accession are reported in the table. A total of 169 alleles were detected, with an average of 8 alleles per locus. Thirty-seven alleles from 18 loci were variety specific, but 55 % of these alleles were specific for *Vitis rupestris* L. The average expected heterozygosity (0.779) was slightly higher than observed heterozygosity (0.767). This may be due to the ancient common origin of most accessions, which had not been subjected to a high degree of human selection, which acts against homozygosity (Sefc *et al*. 2000). The product of the PI values across all loci was 2.33×10^{-18}, thus demonstrating the high distinguishing capacity of these markers. The average similarity of all varieties is 34 % of shared alleles, which is close to the average similarity observed for mid-European cultivars (40 %, Sefc *et al.* 1998).

Groups of cultivars with similar names or identical allelic profiles were investigated in order to assess their relationship. The variety 'Picolit' is represented in Slovenia by two distinct, morphologically different types 'Picolit Italia' and 'Picolit Wienna'. Microsatellite markers revealed no differences at 21 loci. Both our samples of 'Picolit' variety were further compared with 'Picolit' clones from Italy (Zulini *et al.* 2005), revealing the same allelic profiles at all 7 SSR loci and they can therefore be considered to be 'true-to-type' 'Picolit'.

The synonymy found between 'Vitovska grganja' and 'Racuk' could not yet be confirmed, despite obtaining identical allelic patterns, because an accurate morphological characterization of 'Racuk' is still lacking. 'Vitovska grganja' is an old grape variety cultivated in Slovenia in winegrowing districts of the Vipava valley and the Karst and also in the north-east part of Italy, where it was an important variety in the past. The allelic profiles of our 'Vitovska grganja' were further compared with the Italian variety 'Vitouska', recently published by Crespan *et al.* (2007). Dissimilarity was revealed at 14 out of 16 loci, but the two varieties share at least one allele per locus at 15 loci, suggesting that they might be in a parent/progeny relationship.

Varieties 'Prosecco, 'Briška Glera' and 'Števerjana' form another group of synonyms. 'Prosecco' and 'Glera' had already been shown to be synonyms on the basis of morphological descriptors and isoenzyme analysis (Rojc 1995), while 'Števerjana' has not previously been considered to be their synonym. The comparison of 16 SSR loci of our 'Prosecco' with Italian 'Prosecco tondo', which was recently analysed by Crespan *et al.* (2007), revealed no differences. On the basis of this comparison, it can be said that 'Prosecco' = 'Prosecco tondo' = 'Glera' but, according to Crespan *et al.* (2007), the synonym 'Glera' is mainly applied to 'Prosecco lungo' and less frequently to 'Prosecco tondo'.

Comparison between the two Gleras ('Briška Glera' and 'Bela Glera') included in our analysis revealed differences at 16 out of 21 loci, so they are considered homonyms. They also differ in the shape and compactness of the grape cluster, as well as in the white hairiness of the lower part of the leaf, which is expressed only in 'Bela Glera' ('White Glera').

Two varieties, 'Poljšakica Drnovk' and 'Poljšakica Lože', which were expected to have the same genetic profile, were different at many analysed microsatellite loci. Moreover, 'Poljšakica Drnovk' resulted in the same allelic pattern as 'Istrska Malvazija'. It therefore seems that the wrong name has been assigned to this variety in the Goriška Brda collection vineyard, where it is cultivated.

The synonymy of 'Heunisch' = 'Ranfol' = 'Belina', which was first mentioned by Goethe (1887), was also analysed. Comparing 19 SSR loci of 'Belina Pleterje' with 'Heunisch weiss' (Regner *et al.* 2000), discrepancies were found at 13 loci, but the two varieties share one common allele at all 13 loci. Goethe (1887) described a range of varieties named Belina, differing in the pilose and whiteness of the underside of the leaf. Furthermore, the variety 'Belina Pleterje' was compared to the synonymic variety 'Ranfol Bijeli' from Croatia using microsatellite data of Maletić *et al*. (1999). Comparison of 8 SSR loci resulted in the same allelic profiles, except at VVMD7, where a triallelic profile was observed in 'Belina Pleterje' (234, 246, 248) instead of the usual diallelic profile observed in the Croatian 'Ranfol Bijeli' (236, 246). These triallelic genotypes may be periclinal chimeras, in which two cell layers,

L1 and L2, of the plant meristem are genetically different (Hocquigny *et al.* 2004). Mutations in relationship to locus VVMD7 have also been previously reported (Ibanez *et al.* 2000**;** Hocquigny *et al.* 2004).

The SSR profiles of different types of Zelen ('Zelen Pokov', 'Zelen 66' and 'Zelen 2.4') were compared. They showed differences at several microsatellite loci and it can thus be concluded that Zelen varieties from the Primorje winegrowing region are a heterogeneous group consisting of several genotypes.

Among 32 Slovenian grapevine accessions genotyped at the 21 microsatellite markers, 23 displayed different allelic profiles. In pairwise comparison, the highest genetic dissimilarity (86 %) was found between 'Rebula' – 'Volovnik', 'Dolga Petlja' – 'Guštana' and 'Klarnca' – 'Pikolit'. Other studied varieties also showed great differences on the basis of the microsatellie markers used, so it can be concluded that Slovenia has a diverse gene pool of *Vitis vinifera* L. varieties, which was also confirmed by their morphological descriptions (Tomažič and Korošec-Koruza, 1997).

In conclusion, microsatellites are very powerful means of identifying synonyms in germplasm collections, thereby allowing the removal of duplicates. The 32 varieties investigated in this study are only a small selection of the varieties grown in Slovenia. However, within this limited selection, we have begun the evaluation of rare and neglected grapevine varieties. The genetic characterization of a large number of varieties will contribute to improved organization of grapevine collections and the possibility of identifying and exchanging true-to-type material. This work will allow the integration of the obtained allele data into the European grapevine genetic resource database defined by the European project GrapeGen06.

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