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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.

**Key words:** *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 (DETT-

WEILLER 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

**Plant material:** 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).

**DNA isolation:** DNA was extracted from young leaves of shoot tips of individual vines using the modified CTAB method described by KUMP and JAVORNIK (1996).

**Microsatellite analysis:** 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-



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

**PCR conditions:** In a total volume of 10  $\mu$ l 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<sub>2</sub> (Fermentas), 0.5  $\mu$ M 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 Inc., 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.

**Statistical analysis:** 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  $2.33 \times 10^{-18}$ , 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 Vienna'. 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 CRESPIAN *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 (ROJČ 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 CRESPIAN *et al.* (2007), revealed no differences. On the basis of this comparison, it can be said that 'Prosecco' = 'Prosecco tondo' = 'Glera' but, according to CRESPIAN *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 microsatellite 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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