# CHARACTERIZATION OF ENDANGERED GRAPEVINE CULTIVARS NATIVE TO CROATIA AND MONTENEGRO BY SSR AND S-SAP MARKERS


Žulj Mihaljević Maja[1], Šimon Silvio[1], Anhalt Ulrike[2], Forneck Astrid[2], Maraš Vesna[3], Pejić Ivan[1]

[1]University of Zagreb, Faculty of Agriculture, Department of Plant Breeding, Genetics and Biometrics, Svetošimunska 25, 10000 Zagreb, Croatia
[2]University of Natural Resources and Applied Life Sciences, Division of Viticulture and Pomology, Konrad Lorenz Strasse 24, 3430 Tulln a.d. Donau, Austria
[3]Plantaže A.D., U.I. Radomira Ivanovića br. 2 81000 Podgorica, Montenegro

## Introduction

In the agricultural sector of Croatia and Montenegro viticulture has a very long tradition and plays a significant role. Both countries base their development on sustainable agriculture and strong tourism so local autochthonous grapevine varieties and their products might contribute authenticity of the region. Besides modern international varieties and well established native varieties such as Vranac and Plavac mali, there are numerous autochthonous underutilized cultivars that need to be properly conserved and evaluated. They might have potential in production of original local wines and having valuable genes to be used in breeding programs.
DNA-based molecular markers have been widely used for characterization and estimation of genetic diversity and cultivar characterization. Microsatellites have become a favorite type of DNA marker for identification of grapevine cultivars, and their properties enable a wide range of applications from cultivar identification, determination of synonyms and homonyms to pedigree reconstruction and genome mapping (Sefc et al. 2009).
During this long process of vegetative propagation, mutations might have accumulated in some varieties resulting in different biotypes of the same cultivar (Meneghetti et al. 2011). Transposable elements like retrotransposons display a high degree of heterogenity and insertionaly polymorphism, both within and between species (Venturi et al. 2005). Consequently, a modified S-SAP (sequence-specific amplified polymorphism) method by Wegscheider et al. (2009) with universal primers for retrotransposons might be a method of choice to study the intravarietal diversity of the grape.
The objectives of the present study were (1) to collect underutilized and endangered cultivars in Croatia and Montenegro, (2) to define their reliable identification key by molecular markers (SSRs), (3) to determine synonyms, homonyms or misslabeled accessions by comparing genotypes with available database, as well as (4) to assess genetic variability among vines originating from the same cultivar (clonal variation).

## Materials, Methods and Results

Total of 200 accessions were selected during the field expeditions from 22 different locations in Croatia and Montenegro.
Genomic DNA was extracted from lyophilized leaves using E.Z.N.A. SP Plant DNA Kit (Omega Biotek, Doraville, USA) following the manufacturer's instructions with one modification, prolonging the incubation step to 20 min. Part of the DNA was used for DNA fingerprinting and other part for analysis of intra-varietal variability.

**SSR analysis**
Microsatellite analysis was performed by nine microsatellite (SSR) loci: VVS2 (Thomas and Scott 1993), VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32 ( Bowers *et al.* 1996, 1999), VrZAG62 and VrZAG79 (Sefc *et al.* 1999). This set of markers is highly polymorphic and has been used by the European GrapeGen06 consortium as the standard set for the screening of more than 4.000 accessions from different grapevine collections. PCR amplifications were carried out in Veriti$^{TM}$ Thermal Cycler (Applied Biosystems, Foster City, California, USA). Two multiplex PCR reactions were carried out for five and three of the analyzed SSRs and a singleplex for VVMD5. All forward primers were labeled to allow detection by using 6-FAM, VIC, PET, and NED fluorescent dyes. Multiplex PCR of SSR loci were suitably arranged based on expected allele lengths. The reactions were prepared in a final volume of 10 µL, containing 25 ng genomic DNA, 1 U Taq polymerase (Sigma-Aldrich, St.Louis, USA), 0.2 mM of each dNTP , 0.2µM of each forward and reverse primer for first multiplex reaction (VVS2, VVMD7, VVMD27 , ssrZAG62, ssrZAG79) and 0.3 µM of each forward and reverse primer for second multiplex reaction (VVMD25, VVMD28, VVMD32), 2X PCR buffer, 2.5 mM of $MgCl_2$ and 1X Q solution (Qiagen, Hilden, Germany). Singleplex was performed in a final volume of 10 µL containing 25 ng genomic DNA, 0.5 U Taq polymerase, 0.2 mM of each dNTP, 0.2µM of VVMD5 forward and reverse primers, 2X PCR buffer, 2.5 mM $MgCl_2$ and 1X Q solution. The following thermal cycling protocol was applied for all loci: 94°C·2min+35 x (94°C · 60s, 50°C · 60 s, 72°C · 60 s).

Amplified products were size-separated by capillary electrophoresis performed on ABI 3130 Genetic Analyzer together with GeneScan-500 $LIZ_{TM}$ standard internal weight marker using Performance Optimized Polymer 7 (Applied Biosystems). Labeled fragments were detected using GeneMapper software (Applied Biosystems).
Fragment (allele) sizing was standardized for all loci using a set of reference cultivars in order to achieve harmonization of results from this study with previously genotyped accessions from various projects, in first place with the results of GrapeGen06 project (Pejić et al., *in preparation*) and its SSR profiles stored in the European Vitis Database (http://www.eu-vitis.de/index.php).

**S-SAP protocol**
Eleven varieties (Magrovina, Draganela, Sansigot, Dišeća ranina, Jarbola, Sokol, Dobričić, Vranac, Grk, Kratošija and Čubrica) were chosen for S-SAP analysis, based on SSR results and number of accessions. DNA (13.5 µL) was digested with *Mse*I (Fermentas, St. Leon-Rot, Germany) in a total volume of 25 µL. The digestion was incubated for 2 hours at 65°C. Restricted DNA was further purified using the E.Z.N.A MicroElute DNA Clean-Up Kit (Omega Bio-Tek, USA). After purification, template DNA (25 µL) was prepared by adding 5 µL of a ligation mix (50 pmol *Mse*I adapter, 100 mM ATP, 10x T4 ligase buffer, and 1 U T4 Ligase (Fermentas, St. Leon-Rot, Germany), and was incubated overnight at room temperature (20°C). T4 ligase was inactivated by heating up to 65°C for 10 min. In the preamplification step, the primer M(0), homologous to the adapter sequence, was combined with one of six labeled (IRD700 and IRD800) universal retrotransposon primers: F0100, F0103, F0104, F0105, F0113, and F0117. The PCR reaction mixture contained 2.25 µL template DNA, 1.5 µM M(0), 1.5 µM transposon primer, 1x PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, and 1 U Taq DNA polymerase recombinant (Fermentas, St. Leon-Rot, Germany) in a final volume of 15 µL. The unselective PCR was conducted using the following program: 94°C · 60 s+ 26 x (94°C · 30 s, 56°C · 60 s, 72°C · 60 s) + 72°C · 6 min. The selective amplification was carried out in a total volume of 10 µL containing 1 µL preamplified DNA (diluted 1:10), 0.5 µM selective *Mse*I primer (M22, M23, M24, M25, M27) (Table 1), 0.5 µM transposon primer, 1x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.75 U Taq DNA polymerase recombinant (Fermentas, St. Leon-Rot, Germany) using the following cycle profile: 94°C · 60 s+ 12 x (94°C · 30 s, 65°C · 30 s, 72°C · 60 s) [annealing temperature was reduced by 0.7°C in each of the 12 cycles] + 26 x (94°C · 30 s, 56°C · 30 s, 72°C · 60 s) + 72°C · 6 min.

Bands were detected in a 6% polyacrylamide gel and visualized by the automated LI-COR NEN 4300 DNA annalyzer (LI-COR Biosciences, Bad Homburg, Germany). Genetic distance matrix was computed by the NTSYSpc version 2.10s software (MINCH 1997) based on DICE coefficient.

Table 1. SSR genotypes (nine loci) of the grapevine accessions used in this study

| Accession name | County of origin | No. of tested vines | Matching in EU-Vitis database ( variety name) | VVS2 | VVMD7 | VVMD27 | VrZag62 | VrZag79 | VVMD5 | VVD25 | VVMD28 | VVMD32 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Magrovina | CRO | 15 | Magrovina | 131 151 | 237 241 | 185 190 | 187 187 | 242 246 | 232 234 | 237 239 | 226 244 | 250 252 |
| Draganela | CRO | 15 | Draganela | 131 143 | 237 245 | 175 177 | 187 203 | 234 256 | 222 224 | 237 239 | 256 276 | 272 272 |
| Sansigot | CRO | 9 | Sansigot | 141 141 | 237 247 | 175 175 | 187 199 | 234 252 | 222 222 | 237 253 | 252 256 | 252 264 |
| Dišeća ranina | CRO | 15 | Dišeća ranina | 141 141 | 237 247 | 175 181 | 187 201 | 248 248 | 222 234 | 247 253 | 232 258 | 272 272 |
| Belina/Krstač | CRO/MNE | 7 | Apsimo prosotsanis | 141 141 | 237 247 | 175 177 | 187 203 | 234 256 | 237 242 | 247 253 | 232 244 | 250 264 |
| Belina | CRO | 2 | Slaviţă | 131 143 | 245 247 | 175 177 | 203 203 | 234 256 | 228 236 | 237 239 | 232 246 | 248 258 |
| Jarbola | CRO | 15 | Jarbola | 141 153 | 245 247 | 175 177 | 201 203 | 240 256 | 232 237 | 241 253 | 234 244 | 250 250 |
| Sokol | CRO | 15 | Sokol | 143 153 | 245 245 | 181 181 | 191 193 | 236 248 | 224 232 | 239 247 | 232 244 | 252 262 |
| Dobričić | CRO | 14 | Dobričić | 143 149 | 245 247 | 175 175 | 189 203 | 234 256 | 224 224 | 237 239 | 246 256 | 252 264 |
| Vranac / unknown | CRO/MNE | 19 | Vranac | 131 131 | 245 247 | 177 177 | 193 199 | 256 256 | 222 222 | 237 239 | 234 246 | 256 256 |
| Grk | CRO | 30 | Grk | 131 131 | 245 247 | 175 175 | 203 203 | 234 248 | 230 232 | 237 239 | 242 256 | 256 272 |
| Kratošija/Čubrica | MNE | 22 | Kratošija | 131 141 | 245 247 | 175 177 | 199 203 | 234 256 | 222 232 | 237 237 | 246 256 | 256 264 |
| Čubrica | MNE | 9 | Čubrica | 131 141 | 237 247 | 175 177 | 187 199 | 234 256 | 232 243 | 237 247 | 234 256 | 264 272 |
| Ružica | CRO | 1 | Pamidi | 133 141 | 237 237 | 179 185 | 187 187 | 242 250 | 222 222 | 247 253 | 246 256 | 252 256 |
| Krstač | MNE | 5 | Chaouch blanc | 133 149 | 245 247 | 175 179 | 187 203 | 244 246 | 224 234 | 247 253 | 236 256 | 252 272 |
| Krstač | MNE | 1 | Krstač | 131 137 | 237 237 | 181 181 | 187 195 | 248 256 | 228 237 | 237 237 | 242 256 | 240 256 |
| Krstač | MNE | 1 | unknown genotype | 131 137 | 247 247 | 181 181 | 193 203 | 240 240 | 222 236 | 247 253 | 232 244 | 250 264 |
| unknown | CRO | 1 | Moscato giallo | 131 141 | 237 247 | 175 175 | 185 185 | 246 252 | 224 237 | 239 253 | 234 244 | 258 272 |
| unknown | CRO | 1 | Škrlet | 131 131 | 247 253 | 181 189 | 199 203 | 248 248 | 222 224 | 239 253 | 246 266 | 252 272 |
| Dišeća ranina | CRO | 1 | Sauvignon | 131 149 | 237 255 | 171 185 | 187 193 | 242 244 | 224 228 | 239 247 | 232 234 | 240 256 |
| Dišeća ranina | CRO | 2 | Moslavac | 131 151 | 237 247 | 175 190 | 187 203 | 234 246 | 222 237 | 237 239 | 226 247 | 264 272 |

Totally, 21 different genotype out of 200 samples was identified (Table 1). Some accessions showed to be misslabeled (different genetic profile then presumed one). In most cases, accessions turned out to be true to type (like Magrovina, Draganela, Sansigot, Jarbola, Dobričić). For some varieties like Čubrica, based on multiple samples and ampelographic determination, we defined reliable identification key. Varieties like Magrovina, Sansigot, Draganela, Dišeća ranina, Čubrica, Vranac and Grk showed unique profiles not matching with any other genotype from database. On the contrary, supposedly autochthonous variety Sokol, turned out to be synonym for Luglienga bianca/Lignan blanc/ Frueher Leipziger and many other names in different countries (Picture 1). Subsequent survey of the literature (Bulić, S., 1949) revealed that this genotype was also grown in Croatia under the name 'Karmelitanka bijela'. Although this variety was once dispersed all across Croatia, it remained cultivated only its northern part.
We also identified two acessions that seem to be of Greek origin (Apsimo prosotsanis and Pamidi), not previously reported in Croatia. Two accessions named as Belina, also presumed to be rare autochthonous cultivars, showed the same profile like Romanian variety Slaviţă (it's name recalls to term 'Slavic' which could indicate it's Slavic origin). We have found homonymy in case of cultivar Krstač, i.e. several different genotypes having same name. Using a Vitis database and ampleographic records we were able to clarify the status of samples and establish true to type for 'Krstač'.

Picture 1. Bunch of Luglienga bianca (A), held at Institute of plant virology, Grugliasco unit (downloaded from The European *Vitis* Database) and bunch of Sokol (B).

Table 2. Detected polymorphism with six universal retrotransposon primers. Total number of markers and polymorphic markers for eleven *V. vinifera* cultivars.

| | Magrovina | Draganela | Sansigot | Dišeća ranina | Jarbola | Sokol | Dobričić | Vranac | Grk | Kratošija | Čubrica |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **No. Individuals** | 15 | 15 | 8 | 15 | 15 | 15 | 14 | 19 | 27 | 22 | 9 |
| **Total Polym. Marker** | 14 | 7 | 8 | 20 | 1 | 11 | 6 | 7 | 15 | 10 | 6 |
| **Specific Mutations[1]** | 7 | 1 | 5 | 15 | 1 | 4 | 6 | 5 | 9 | 9 | 3 |
| **Random Mutation[2]** | 7 | 6 | 3 | 5 | 0 | 7 | 0 | 2 | 6 | 1 | 3 |
| **Total Marker** | **322** | | | | | | | | | | |

[1] *Mutation in several clones of one cultivar*
[2] *Single mutation in only one clone*

Table 2. shows the results of S-SAP analysis for eleven varieties. Specific mutations that occur in all clones of one cultivar demonstrate the inter-varietal variability, and random mutations implicate the intra-varietal variability. Dišeća ranina showed to be genetically most heterogenous and variety Jarbola the least. Despite Dišeća ranina has very limited population, one of its distinctivness is female (functional) flower which is considered to be property of very old varieties. Low level of diversity among Jarbola vines might be due to sampled vines recently propagated from only few stock vines. Overall genetic similarity calculated from genetic distance matrix (data not shown), showed genetic similarity of 97.5%, as expected with closely related accessions.

## Conclusion

Genetic characterization of accessions from Croatia and Montenegro resulted in 21 different genotype. Several cases of synonymy and homonymy has been observed. Reliable identification key was determined or confirmed for most of accessions. S-SAP analysis revealed genetic variability between cultivars but as well within cultivars which provides insight in homogeneity of cultivars' population and indirectly poinst on capacity and necessity of clonal selection in the future.


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