

EVALUATION OF INTRAVARIETAL HOMOGENEITY WITHIN GRAPEVINE ROOTSTOCK CULTIVARS AS REVEALED BY SSR FINGERPRINTING

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Abstract

Traditional rootstock cultivars of importance for the Mid European viticulture originate mainly from Zsigmond Teleki's material developed at the end of the 19-th century. Nowadays most widespread varieties derived from Teleki's primary vine population could be denoted SO4, Kober 5BB, Teleki 5C and Kober 125AA. Recently SSR markers became one of the most suitable and reliable tools for cultivar identification, but some problems can appear if results obtained from two independent laboratories are compared. In order to overcome this problem, the system based on reference alleles was utilised in this work. Nine rootstock genotypes from 4 varieties collected in Czech Republic were genotyped by using 9 SSR loci and compared with published genotypes from Austria, Australia, France, Italy, Hungary and USA. The major SSR profile for all rootstocks was found by comparing obtained results of different laboratories. SO4 and 125AA with almost identical SSR profile for all genotypes appears as very stable. Contrarily, the analysis of T5C and Kober 5BB revealed a higher level of heterogeneity within genotypes belonging to these cultivars.

Keywords: SSR, *Vitis*, rootstock, variability, identification

Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important perennial crops in the world, widely cultivated in subtropical and temperate climate. The necessity to use rootstocks for grapevine cultivation was caused by the phylloxera, *Dactulosphaira vitifoliae* (Fitch) invasion occurred in European vineyards in the 60ies of the 19th century. Between 1885 and 1900, a significant effort to develop rootstock cultivars was made by European investigators after the discovery of the resistance of native American *Vitis* species to this insect (MILLARDET, 1885). These phylloxera-resistant species include *V. riparia*, *V. berlandieri*, and *V. rupestris*.

Due to the unsatisfying performance of the available phylloxera-resistant rootstocks on high lime content soil, the Hungarian farmer Zsigmond Teleki decided to breed his own suitable genotypes. He ordered seeds of rootstocks from Mr. Resseguier in France. In 1896 he obtained the material and sowed 10 kg of seeds originating from an open pollinated *Vitis berlandieri*. From 40.000 obtained seedlings, which demonstrated widely assorted morphological characters corresponding to different species of *Vitis* genus, Z. Teleki selected all seedlings resistant to fungal diseases, tolerant to high lime content and mainly with *V. berlandieri* x *V. riparia* morphological characters. Finally he divided the seedlings into 10 groups. Two of his groups, 8B and 5A, were marketed for quick propagation, but these were not the progeny of single plants. Later he selected T5C and T8B as single plants with defined traits from these pools. Further selection with Teleki's material was necessary and was performed by other breeders, as Fuhr and Rodrian for SO4 and Kober for K5BB and K125AA (BAKONYI and KOCSIS, 2004; MANTY 2005).

Ampelography based on morphological differences between the varieties is a traditional method for grape cultivar identification. However, the ampelographical description depends on environmental conditions, nutritional state and health status of the plant. Further, in case of grafted rootstocks only the lignified part and the roots remain for ampelographic evaluation.

In last 20 years a new alternative for cultivar identification based on genetic markers has arisen. Among them microsatellite markers have become the most suitable and reliable tool for cultivar identification. The main advantage of SSR markers is the high level of polymorphism in case of comparisons between different cultivars (THISet al. 2004), and conversely, its high stability if different clones of one cultivar were analysed (REGNER et al. 2000, IMAZIOet al. 2002). Thus, SSRs have been extensively exploited in a number of countries for identification of cultivars, characterization of grape genetic resources (FATAHiet al. 2003, HVARLEVAet al. 2004, GRANDOet al. 1998, MORAVCOVAet al. 2006), verification of synonyms or homonyms (FOSSATiet al. 2001; LABRA et al. 2001), parentage analysis (BOWERSet al. 1999, SEFCet al. 1998b) or mapping (ADAM-BLONDONet al. 2004, FISHERet al., 2004; RIAZ et al. 2004).

Mainly traditional cultivars belonging to the *V. vinifera* were genotyped by SSR markers. Data about rootstock genotyping are rare. Together with 47 *V. vinifera* cultivars SEFC et al. (1998a) genotyped 19 grape rootstock cultivars using 10 microsatellite markers. Four rootstocks included in an Iranian grapevine collection were characterized by SSR (FATAHiet al. 2003). Twenty rootstock accessions from Bulgaria and Cyprus were characterized by 14 microsatellite markers (DZHAMBAZOVA et al. 2007). Twenty-one rootstock accessions from Indian genepools were analyzed at seven grape SSR loci (UPADHYAYet al. 2007). However, no report analysing degree of polymorphism within clones of rootstock cultivars has been published yet. MORAVCOVA et al. (2006) suggest on the base of their results that Czech clones of Teleki 5C strongly differ at analysed loci from clones used within a project named „European Network for Grapevine Genetic Resources Conservation and Characterization” (<http://www.genres.de/vitis/>). This fact initialized our interest about variability of rootstocks used in different countries.

Material and methods

Plant material: Clones originating from Czech Republic were acquired from the Research Institute for Grapevine Breeding in Polesovice and by Ampelos, a breeding station for grapevine in Vrbovec. Each genotype was analysed twice using DNA originating from two different collections. In total 9 genotypes of Teleki 5C, SO4, Kober 5BB and Kober 125AA were analysed.

DNA extraction: DNA extractions were performed from 0.2 g of leaves by DNeasy Plant Mini Kit (Qiagen), according to manufacturer's instructions. The quality of the isolates was confirmed by electrophoresis on the 0.8 % agarose gel, quantity of DNA was measured on the base of fluorometric measurement with PicoGreen dye (Invitrogen/MolecularMicrosatellite loci: 9microsatellite loci were analysed for all 9 genotypes. Six of them (VVS2, VVMD5, VVMD7, VVMD27, VrZag62 and VrZag 79) were used and recommended for the cultivar identification task by the Genres 081 project (THISet al. 2004). In the meanwhile these SSR markers have been defined as genetic descriptors by the OIV (no. 801- 806). Other loci VVS4, VVMD31 and VVMD 28 were utilised and recommended for cultivar differentiation purposes by other authors (HINRICHSNet al. 2001, UPADHYAYet al. 2007, FOSSATI et al. 2001).

PCR protocol: Amplification was performed in reaction volume 25 μ l containing 20 ng of genomic DNA, 1 x buffer (10 mM Tris-HCl, pH8.8; 15 mM MgCl₂; 150 mM KCl and 0.1 % Triton X-100), 0.2 mM of each dNTP (Promega, USA), 0.2 μ M both primers and

0.5 U of Dynazyme II DNA polymerase (Finnzymes). Amplification was performed on T-gradient thermocycler (Biometra). After initial denaturation at 94 °C for 3 min., 40 cycles, consisting of : denaturing (30 sec. at 94 °C), annealing (45 sec. at 45- 55 °C, temperature varied for individual locus on the base of the thawing temperature proved by used primers), and extension (45 sec. at 72 °C), followed. The last cycle of the extension was prolonged to 9 minutes and the amplification products were then stored at 4 °C. Amplification was confirmed by running 20 μ l of the PCR product on the 1.5% agarose gel stained with ethidium bromide.

Allele sizing: DNA amplicons were analysed by the ABI PRISM 310 genetic analyser running Gene Scan Software (version 3.7, Applied Biosystems). Separation was performed in POP-4, a pre-formulated liquid polymer matrix, heated to 60°C. 0.5 – 2 μ l of amplified samples (according to signal intensity on control gel) were pooled together on the base of different fluorescence labels of primers (6-FAM, NED, JOE) to fully exercise detection labels on ABI 310. 1 μ l of the blended sample was mixed with 12 μ l of deionised formamide and 0.5 μ l DNA size standard (GeneScan 400 HD ROX, Applied Biosystems). The mixture was denaturated at 95 °C for 4 minutes and then immediately cooled on ice before loaded to the machine for separation.

Results and discussion

Comparison of absolute allele sizes: As mentioned by GRANDO and FRISINGHELLI (1998) and THISET *et al.* (2004), results differ in case of untreated allele sizes measured by different laboratories (Tab. 1). These discrepancies are caused by different laboratory equipment, individually adapted protocols and different technique of allele sizing at compared institutes. In general raw data could not be compared between different labs without correction and adaptations. Finally, a strategy for data comparison by means of reference to the selected alleles was used.

Transforming numerical data to reference codes: The system of reference alleles as genetic descriptors OIV 801- OIV 806 was utilised for evaluation of VVS2, VVMD5, VVMD7, VVMD27, VRZAG62 and VRZAG79 analysis results (http://www.eu-vitis.de/docs/descriptors/mcpd/OIV801_OIV806_5Juli2012.pdf). For the other 3 loci no common standard was defined up to the present time. Thus, for the loci VVS 4 and VVMD28 as well as VVMD 31 Dr. Regner have developed such a basepair ladder (personal communication, not published).

Small discrepancies within VVS2 and VVMD5 descriptor should be re-adjusted to evaluate the results logically. In case of K5BB is stated one more (false – n+22) allele for VVS2 descriptors. The situation is worse with T5C. This review shows that the sample of T5C used for GENRES project 081 was probably not true to type T5C. Evident differences of allele size of loci VVS2 and VVMD5 were found compared with results of other T5C genotypes. This genotype should be thus discarded from the set of reference cultivars.

Allele sizes increments were not evaluated as 2 bp, thus in contrast with dinucleotide nature of used SSR markers rarely happens. This variance can be the cause of many factors including different shape of stutter bands, extrabase additions that occur with some Taq polymerases (Brownstein *et al.* 1996) and different conditions for electrophoresis (used size marker, polymer and so on). Thus, 1bp shifts, which can be caused by above mentioned reasons in conjunction with typing discrepancies of individual evaluators, point mutations in the sequence outside the repeats or within irregular repeats, however, were ignored. Results in basepair lengths and neutral codes are available in Table 1.

Comparison of the SSR profiles

T5C : It is evident that the allelic profiles of Czech clones strongly differ from a group of genotypes from other countries. Czech analysed clones Po3/7 and 9/7 originate from selections carried out in Czech Republic long time ago. It is then possible to question the origin of material used already on the start of selection process.

SO4 : From a total of 9 genotypes all showed almost identical allelic profiles within all analysed loci. It is possible to find small differences in samples from Hungary (Jahnke *et al.*, 2011 and Vitis database) and Italy: smaller allele of VVS2. The question is whether allele “n+22” is a real allele, or “stutter” band of allele “n+24”. Based on this speculation, the results of analyses may have been the same within laboratories, but with different assessment of allelic profile. Sample from the USA also shows small (only one microsatellite repetition) variation within 3 alleles. Thus, intravarietal variability of SO4 rootstock is low and there is a high probability that the majority profile obtained by this comparison of data from different countries could be credible as a standard profile for this cultivar.

Kober 5BB : Group of 7 genotypes with identical SSR profile makes idea about majority profile which can be most probable for similar analysis of other clones registered under this cultivar. The K5BB clone137-Wu maintained in Czech institute differs slightly within three alleles, while the clone Po1/60 differs significantly from the majority profile for this cultivar.

Kober-125-AA : The distribution of alleles within compared genotypes of this cultivar is simply visible: all of them have the same SSR profiles, only the Hungarian clone GK49 is quite different.

Comparing of the results with data from the Vitis International Variety Catalogue (<http://www.vivc.bafz.de>) is not possible in the case of Teleki 5C and Kober 5BB rootstocks due to lack of their SSR profiles. Major SSR profiles obtained within this review for cultivars 125AA and SO4 are identical with allelic profile in international Vitis database, and so is the major profile of presented review.

Thus, it can be concluded that it is possible to define major SSR profiles for all compared cultivars, among which the SO4 and 125 AA cultivars with rare differences between genotypes appeared as the very stable.

The observed intravarietal variability could be based on several circumstances. First, the origin of individual cultivars is based on the selection by morphological traits which do not always allow a very precise definition. It was especially a problem in the case of first marketed population such as 8B or 5A, where different genotypes on the base of their morphological character were observed (BAKONYI and KOCSIS 2004). Further, there is the possibility of discrepancies within selection processes carried out by individual breeders. Possible misidentification during selection of clones could be a reason for further variations. Especially in the case of rootstocks, which are intensively propagated annually, it is possible to mention the danger of rapid spread of recently created mutations. The different SSR profile is only one indicator for such variation in the propagation material (HOCQUIGNY *et al.* 2004).

Conclusion

Reflecting all above mentioned facts it is strictly recommended to keep precise register of maintained rootstock cultivars with special emphasis on the name of the clone. Mistakes could easily be overlooked due to the narrow morphological character. Importance of the recommendation for accurate audit of clones strongly growing up in the light of recently observed decreasing of resistance against phylloxera in case of some T5C clones.

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Tab.1: Comparison of the results obtained in different laboratories. Numbers represent sizes of the alleles in base pairs (Czech clones and already published results from other countries). Relative allele sizes are expressed at code (“n+ number”). Transformation of the data was performed using methodology for OIV descriptors (European Vitis Database) when the Kober 5BB was used like reference cultivar. Green color marks major profile, orange marks differences

Teleki 5 C																			
samples from	clone ↓ / locus→	VVS2		VVS4		VVM5		VVM7		VVM27		VVM28		VVM31		VrZag 62		VrZag 79	
Czech rep. Baránková	Po 3/7-1	139 n+18	145 n+24	172 n+8	182 n+18	234 n+14	264 n+44	230 n+2	248 n+20	200 n+28	208 n+36	218 n	254 n+36	198 n+2	204 n+8	191 n+18	213 n+40	248 n+14	256 n+22
	9.7	139 n+18	147 n+26	175 n+10	183 n+18	234 n+14	264 n+44	228 n	262 n+34	200 n+28	204 n+32	218 n+2	254 n+38	199 n+2	205 n+8	191 n+18	209 n+36	249 n+14	253 n+18
USA LIN <i>et al.</i> , 1998		147 n+22	151 n+26			237 n+14	267 n+44	233 n	269 n+36				218 n	252 n+34	200 n+2	204 n+6			
Australia THOMAS <i>et al.</i> , 1994		145 n+22	149 n+26	177 n+10	187 n+20														
France THIS <i>et al.</i> , 2004		133 n+10	143 n+20			252 n+30	266 n+44	232 n	266 n+34	203 n+28	211 n+36					200 n+26	210 n+36	252 n+14	260 n+22
Austria SEFC <i>et al.</i> , 1998		144 n+22	148 n+26	176 n+10	186 n+20	234 n+14	264 n+44	228 n	262 n+34			216 n	252 n+36						
Hungary JAHNKE <i>et al.</i> , 2011	GK40, 42, E20	143 n+22	147 n+26					229 n	263 n+34			215 n	251 n+36						
	G46(64), WED(103) G41(74)	147 n+26	149 n+28					231 n+2	231 n+2			217 n+2	217 n+2						
Italy CRESPAN <i>et al.</i> , 2009		145 n+22	149 n+26			236 n+14	266 n+44	231 n	265 n+34	203 n+28	211 n+36					199 n+26	209 n+36	250 n+14	258 n+22
Major profile		n+22	n+26	n+10	n+20	n+14	n+44	n	n+34	n+28	n+36	n	n+36	n+2		n+26	n+36	n+14	n+22
SO 4																			
samples from	clone ↓ / locus→	VVS2		VVS4		VVM5		VVM7		VVM27		VVM28		VVM31		VrZag 62		VrZag 79	
Czech rep. Baránková	2 Wu	145 n+24	145 n+24	172 n+8	182 n+18	234 n+14	264 n+44	230 n+2	262 n+34	200 n+28	208 n+36	216 n	236 n+20	198 n+2	204 n+8	199 n+26	213 n+40	248 n+14	252 n+18
	Po/7-1	145 n+24	145 n+24	172 n+8	182 n+18	234 n+14	264 n+44	230 n+2	262 n+34	200 n+28	208 n+36	216 n	236 n+20	198 n+2	204 n+8	199 n+26	213 n+40	248 n+14	252 n+18
	Gm47	145 n+24	145 n+24	172 n+8	182 n+18	234 n+14	264 n+44	230 n+2	262 n+34	200 n+28	208 n+36	216 n	236 n+20	198 n+2	204 n+8	199 n+26	213 n+40	248 n+14	252 n+18
USA LIN <i>et al.</i> , 1998		147 n+22	147 n+22			237 n+14	267 n+44	235 n+2	269 n+36			218 n	238 n+20	200 n+2	206 n+8				
Australia THOMAS <i>et al.</i> , 1994		147 n+24	147 n+24	175 n+8	185 n+18														
Austria SEFC <i>et al.</i> , 1998		146 n+24	146 n+24	174 n+8	184 n+18	234 n+14	264 n+44	230 n+2	262 n+34			216 n	236 n+20						
Hungary JAHNKE <i>et al.</i> , 2011	(133)	143 n+22	145 n+24					231 n+2	263 n+34			215 n	235 n+20						
Italy CRESPAN <i>et al.</i> , 2009		145 n+22	147 n+24			236 n+14	266 n+44	233 n+2	265 n+34	203 n+28	211 n+36					199 n+26	213 n+40	250 n+14	254 n+18
Bulgaria Dzhambazova <i>et al.</i> , 2007		146 n+24	146 n+24			234 n+14	264 n+44	230 n+2	262 n+34	203 n+28	211 n+36					199 n+26	213 n+40		
Hungary Vitis database		121 n+22	124 n+24			218 n+14	224 n+44	229 n+2	235 n+34	171 n+28	175 n+36	215 n	219 n+20			172 n+26	178 n+40	234 n+14	238 n+18
Major profile		n+24	n+24	n+8	n+18	n+14	n+44	n+2	n+34	n+28	n+36	n	n+20	n+2	n+8	n+26	n+40	n+14	n+18
Kober 5 BB																			
samples from	clone ↓ / locus→	VVS2		VVS4		VVM5		VVM7		VVM27		VVM28		VVM31		VrZag 62		VrZag 79	
Czech rep. Baránková	137 Wu	139 n+18	147 n+26	174 n+10	182 n+18	234 n+14	264 n+44	230 n+2	262 n+34	188 n+16	208 n+36	216 n	254 n+38	198 n+2	204 n+8	199 n+26	213 n+40	248 n+14	252 n+18
	PO1/60	139 n+18	147 n+26	174 n+10	182 n+18	234 n+14	264 n+44	228 n	262 n+34	200 n+28	204 n+32	216 n	254 n+38	198 n+2	204 n+8	191 n+18	209 n+36	248 n+14	256 n+22
USA LIN <i>et al.</i> , 1998		143 n+18	151 n+26			237 n+14	267 n+44	235 n+2	269 n+36			218 n	252 n+34	200 n+2	206 n+8				
Australia THOMAS <i>et al.</i> , 1994		141 n+18	149 n+26	177 n+10	185 n+18														
France THIS <i>et al.</i> , 2004		141 n+18	149 n+26			236 n+14	264 n+44	234 n+2	266 n+34	191 n+16	211 n+36					200 n+26	214 n+40	252 n+14	260 n+22
Austria SEFC <i>et al.</i> , 1998		140 n+18	148 n+26	176 n+10	184 n+18	234 n+14	266 n+44	230 n+2	262 n+34			216 n	252 n+36						
Hungary JAHNKE <i>et al.</i> , 2011	GK14	139 n+18	147 n+26					231 n+2	263 n+34			215 n	251 n+36						
	GK13	143 n+22	147 n+26					229 n	263 n+34			215 n	251 n+36						
	CR2(18)	139 n+18	147 n+26					229 n	263 n+34			215 n	251 n+36						
Italy CRESPAN <i>et al.</i> , 2009		141 n+18	149 n+26			236 n+14	266 n+44	233 n+2	265 n+34	191 n+16	211 n+36					199 n+26	213 n+40	250 n+14	258 n+22
Bulgaria Dzhambazova <i>et al.</i> , 2007		140 n+18	148 n+26			234 n+14	264 n+44	230 n+2	262 n+34	191 n+16	211 n+36					199 n+26	213 n+40		
Major profile		n+18	n+26	n+10	n+18	n+14	n+44	n+2	n+34	n+16	n+36	n	n+36	n+2	n+8	n+26	n+40	n+14	n+22

125 AA																			
samples from	clone ↓ / locus→	VVS2		VVS4		VVMD5		VVMD7		VVMD27		VVMD28		VVMD31		VrZag 62		VrZag 79	
Czech rep. Baránková	3 Gm	139 n+18	145 n+24	174 n+10	182 n+18	224 n+4	264 n+44	228 n	248 n+20	200 n+28	208 n+36	216 n	254 n+38	198 n+2	202 n+6	191 n+18	209 n+36	248 n+14	256 n+22
	Po/3	139 n+18	145 n+24	174 n+10	182 n+18	224 n+4	264 n+44	228 n	248 n+20	200 n+28	208 n+36	216 n	254 n+38	198 n+2	202 n+6	191 n+18	209 n+36	248 n+14	256 n+22
USA LIN <i>et al.</i> , 1998		143 n+18	149 n+24			227 n+4	267 n+44	233 n	253 n+20			218 n	252 n+34	200 n+2	204 n+6				
Austria SEFC <i>et al.</i> , 1998		140 n+18	146 n+24	176 n+10	184 n+18	224 n+4	264 n+44	228 n	248 n+20			216 n	252 n+36						
Hungary JAHNKE <i>et al.</i> , 2011	GK46, (147)	139 n+18	145 n+24					229 n	249 n+20			215 n	251 n+36						
	GK49	149 n+28	149 n+28					229 n	229 n			215 n	269 n+54						
Hungary Vitis database		121 n+18	124 n+24			218 n+4	224 n+44	229 n	235 n+20	171 n+28	175 n+36	215 n	219 n+36			172 n+18	178 n+36	234 n+14	238 n+22
Major profile		n+18	n+24	n+10	n+18	n+4	n+44	n	n+20	n+28	n+36	n	n+36	n+2	n+6	n+18	n+36	n+14	n+22

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