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Genotyping of grapevine and rootstock cultivars using microsatellite markers

by

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S u m m a r y : Sixty-six grapevine and rootstock cultivars from an Austrian germplasm collection were genotyped using the following 10 microsatellite loci: VVS1, VVS2, VVS3, VVS4 (THOMAS and SCOTT 1993), VVS29 (THOMAS, pers. comm.), VVMD5, VVMD7 (BOWERS *et al.* 1996), VVMD28, VVMD32 and VVMD36 (BOWERS and MEREDITH, pers. comm.). All cultivars except those which are thought to be closely related (e.g. Portugieser blau and Portugieser grün) provided unique allelic profiles. A phenogram based on pairwise similarity values revealed the separation of rootstock cultivars from the *Vitis vinifera* varieties. The probability for the presence of null alleles was estimated from heterozygote deficiencies and null alleles were statistically excluded at 9 of the 10 loci. In order to demonstrate the distinctive power of the microsatellite markers investigated, gene diversity (GD) values were calculated. For both grapevine and rootstock cultivars we estimated a GD range from 0.70 to 0.91, while GD values for grapevines only range from 0.52 to 0.87 and values for rootstocks from 0.29 to 0.86.

K e y w o r d s : Vitis, microsatellites, simple sequence repeats, genotyping, identification.

A b b r e v i a t i o n : GD = gene diversity.

Introduction

The increasing international trade of grapevine and rootstock plant material as well as of wine necessitates a reliable identification of genotypes. Ampelographic methods and isozyme analysis fail to meet the demand since too few distinguishing features are available and vines are exposed to environmental influences (PARFITT and ARULSEKAR 1989; WALTERS *et al.* 1989; SCHNEIDER 1996). In contrast, DNA-based methods are independent of environmental factors including the season, as DNA can be isolated from woody material (BOURQUIN *et al.* 1992). Both RFLP (STRIEM *et al.* 1990; BOURQUIN *et al.* 1992, 1993, 1995; MAURO *et al.* 1992; BOWERS *et al.* 1993; GUERRA and MEREDITH 1995) and RAPD (COLLINS and SYMONS 1993; GOGORCENA *et al.* 1993; JEAN-JAQUES *et al.* 1993; REGNER and MESSNER 1993; TSCHAMMER and ZYPRIAN 1994) proved to distinguish between cultivars. However, the interpretation of RFLP patterns is often difficult (STRIEM *et al.* 1990), whereas the difficulty to standardize the RAPD procedure due to differences of DNA quality and primer concentrations, DNA polymerases and thermocyclers (BÜSCHER *et al.* 1993; MCPEARSON *et al.* 1993) impedes a comparison of results between laboratories. In recent years, microsatellite markers were recommended for vine genotyping (THOMAS and SCOTT 1993; THOMAS *et al.* 1994; CIPRIANI *et al.* 1994; BOWERS *et al.* 1996; REGNER *et al.* 1996). The combination of data of several highly polymorphic microsatellite loci results in individual allelic profiles enabling the distinction of cultivars, while their codominant manner of inheritance allows parentage analysis (THOMAS *et al.* 1994, BOWERS and MEREDITH 1997, SEFC *et al.* 1997). The consistency of the results over time, different laboratories and analysis systems supports the establishment of a worldwide genotyping database (BOTTA *et al.* 1995). In this work, we report the characterization of 66 *Vitis vinifera* and rootstock cultivars used in Austrian viticulture by microsatellite allele lengths.

Materials and methods

Plant material was obtained from field and *in vitro* collections of the Höhere Bundeslehranstalt und Bundesamt für Wein- und Obstbau (HBLA u. BA) Klosterneuburg, Austria. Leaves from field plants were harvested in spring and summer and stored at -20 °C. DNA was extracted from 2 g of leaf tissue following the procedure described by THOMAS *et al.* (1993).

47 *Vitis vinifera* and 19 rootstock varieties (Tab. 1) were typed at the following 10 SSR loci: VVS1, VVS2, VVS3, VVS4 (THOMAS and SCOTT 1993), VVS29 (THOMAS, pers. comm.), VVMD5, VVMD7 (BOWERS *et al.* 1996), VVMD28, VVMD32 and VVMD36 (BOWERS and MEREDITH, pers. comm.). PCR was performed in 20 µl of a mixture containing 50 ng DNA, 1 µM of each primer, 100 µM of each dNTP, 1U Taq DNA polymerase in reaction buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X 100). One primer of each pair was labelled with the fluorescent Cy-5 dye to enable detection of the fragments in the Alf express automated sequencing system (Pharmacia Biotech, Vienna).

The following PCR protocol (SMITH *et al.* 1995) was chosen for the amplification of 9 of the loci: 95 °C for 5 min, 10 cycles of 50 °C for 15 s, 94 °C for 15 s, followed by 23 cycles of 50 °C for 15 s and 89 °C for 15 s. Final extension was

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Table 1

Genotypes of the grapevine and rootstock cultivars at 10 microsatellite loci

Grapevine cultivar	VVS 1	VVS 2	VVS 3	VVS 4	VVS 29	VVMD 5	VVMD 7	VVMD 28	VVMD 32	VVMD 36
Andre	189:189	142:150	212:218	170:172	168:168	226:238	246:254	234:246	271:271	262:262
Bianca	180:193	132:150	210:218	166:172	168:178	226:234	240:248	218:236	254:271	252:268
Blauburger	179:189	142:150	218:218	167:174	168:168	230:238	240:246	228:246	271:271	264:274
Blaufränkisch	189:189	142:142	218:218	167:174	168:168	224:238	236:246	246:246	249:271	262:264
Bouvier	182:189	132:150	212:218	167:172	168:176	226:226	240:240	218:268	271:271	252:262
Cabernet franc	180:180	138:146	212:212	166:174	172:178	224:238	236:260	228:236	239:257	252:252
Cabernet Sauvignon	180:180	138:150	212:218	167:174	176:178	230:238	236:236	234:236	239:239	252:262
Chardonnay	182:189	136:142	212:218	167:172	168:176	232:236	236:240	218:228	239:271	252:274
Furmint	179:189	132:152	214:218	168:174	168:176	224:238	236:246	228:248	263:271	252:274
Goldburger	182:182	134:142	212:212	167:167	176:176	236:236	236:244	258:268	239:239	252:262
Gutedel weiß, rot	182:189	132:142	212:218	167:167	176:178	226:234	236:244	218:268	239:239	262:262
Jubiläumsrebe	179:180	150:150	212:218	174:174	168:176	230:238	240:244	228:236	251:271	262:274
Königin der Weingärten	180:180	132:134	212:218	167:168	168:176	224:234	244:246	234:268	271:271	262:274
Lambrusco di Sorbara	187:189	134:154	212:218	168:168	168:176	226:230	250:254	234:264	249:251	252:268
Merlot	180:189	138:150	212:218	167:174	172:178	224:234	236:244	228:234	239:239	252:252
Müller-Thurgau	182:189	142:150	218:218	167:172	176:176	224:226	244:254	234:244	251:251	252:262
Muskat Ottonel	180:189	132:142	218:218	167:167	168:176	224:226	236:240	258:268	239:271	262:274
Neuburger	179:189	130:150	218:218	167:167	168:168	224:238	244:250	228:246	263:271	262:274
Österreichisch weiß	179:189	142:152	214:218	167:174	168:176	224:232	236:244	228:248	271:271	262:274
Perle von Csaba	180:189	132:154	212:218	167:167	176:176	234:234	244:246	218:268	271:271	262:294
Pinot blanc, noir, gris	182:189	136:150	212:218	167:172	168:176	226:236	236:240	218:236	239:271	252:252
Portugieser, blau, grün	179:180	142:150	218:218	167:174	168:168	224:230	240:252	228:260	251:271	262:274
Rathay	189:189	136:150	218:218	166:174	168:168	224:230	236:240	228:246	271:271	264:274
Rheinriesling, Riesling rot	189:189	142:150	212:218	167:167	168:176	224:232	246:254	228:234	251:271	252:262
Rösler	189:195	136:142	212:218	167:167	168:176	226:242	236:236	234:238	249:249	238:252
Rotgipfler	189:189	132:150	212:218	167:174	168:168	230:244	236:254	236:268	263:271	252:262
Sauvignon blanc	180:189	132:150	212:218	167:168	168:176	226:230	236:254	234:236	239:239	262:294
Seifert	182:189	142:150	212:218	166:167	168:176	230:242	236:246	228:238	271:271	238:274
Semillon	180:189	132:132	212:218	166:167	168:168	234:236	236:254	234:244	239:271	262:262
Silvaner grün, rot	179:189	150:152	218:218	167:167	168:176	224:230	240:244	228:236	271:271	262:274
Sultanina	180:187	144:150	212:218	174:174	168:176	232:232	236:250	218:244	249:249	248:266
Traminer	161:189	150:150	212:218	167:174	168:168	230:236	240:254	234:236	239:271	252:262
Veltliner braun	182:189	136:150	212:218	167:167	168:176	224:226	236:254	228:268	239:251	262:262
Veltliner grün	161:180	132:150	212:212	166:174	168:168	230:230	244:254	234:248	240:256	252:262
Veltliner frührot	179:189	132:150	212:218	167:174	168:176	230:238	244:250	236:268	263:271	262:262
Veltliner rot	189:189	130:132	212:218	167:174	168:168	238:244	236:250	246:268	253:263	262:262
Welschriesling	182:189	134:150	212:218	167:167	176:176	224:236	244:254	246:258	239:271	252:262
Wildbacher blau	189:189	142:150	212:218	167:172	168:176	226:238	236:236	236:246	249:271	252:262
Zierfandler	161:189	132:132	218:218	167:168	168:168	238:238	240:250	234:246	263:271	262:286
Zweigelt	189:189	136:142	212:218	166:167	168:176	224:226	236:236	234:246	249:261	252:262

avoided by transferring the reaction tubes to 4 °C immediately after the last cycle. One locus (VVS4) did not respond to the above protocol and was amplified using the following program: 94 °C for 1 min, 35 cycles of 50 °C for 1 min, 72 °C for 1 min, 92 °C for 30 s, then 72 °C for 1 min.

To estimate the DNA concentration, 12 µl of the PCR reaction mixture was run on a 2 % agarose gel and stained with ethidium bromide. Depending on the intensity of the signal, 0.5 to 6.0 µl were mixed with equal volumes of loading buffer (Formamide containing 5 mg·ml^{-1} Dextran blau) and 1 µl of the corresponding Cy-5 labelled size standards (Pharmacia Biotech, Vienna). Samples were denatured at 95 °C for 2-3 min and analysed on a sequencing gel (6 % acrylamide, 1x TBE buffer, 7M Urea) in an automated sequencing appa-

Table 1, continued

Rootstock cultivar	VVS 1	VVS 2	VVS 3	VVS 4	VVS 29	VVMD 5	VVMD 7	VVMD 28	VVMD 32	VVMD 36
41 B	182:196	134:142	214:218	167:174	172:178	224:226	228:236	242:268	239:254	246:262
Binova	182:194	146:146	210:214	174:184	172:172	234:264	230:262	216:236	234:259	238:238
Cosmo 10	193:194	146:146	210:214	176:186	172:172	234:264	230:262	216:236	236:259	238:238
Cosmo 2	193:194	144:148	210:214	174:184	172:172	234:264	248:248	244:252	234:259	250:250
Couderc 1616	193:193	138:140	210:210	174:190	172:180	262:266	236:246	242:250	244:244	238:238
Couderc 3309	186:186	122:160	210:210	184:186	172:176	250:262	242:256	240:246	236:236	238:238
Fercal	192:194	142:142	210:214	174:178	172:172	234:262	248:248	220:244	236:243	238:238
Geisenheim 26	189:193	134:142	210:218	167:190	168:172	234:264	244:248	236:242	236:251	254:294
Kober 5 BB	193:194	140:148	210:214	176:184	172:172	234:264	230:262	216:252	234:259	238:248
Kober 125 AA	182:194	140:146	210:214	176:184	172:172	224:264	228:248	216:252	234:259	238:248
Reckendorf 7	193:194	140:148	210:214	174:184	172:172	234:264	262:262	216:236	236:259	238:238
Ruggeri 140	193:194	136:142	210:214	174:184	172:176	244:266	228:254	234:242	234:251	238:238
Ruggeri 225	193:193	140:148	210:214	176:184	172:172	234:264	230:262	216:252	234:259	238:248
SO 4	182:194	146:146	210:214	174:184	172:172	234:264	230:262	216:236	234:259	238:238
Teleki 5 C	182:194	144:148	210:214	176:186	172:172	234:264	228:262	216:252	236:259	238:248
Teleki 8 B	193:194	140:146	210:214	174:184	172:172	224:264	230:262	216:252	234:259	238:238
Vitis Berlandieri Planchon	193:193	138:140	210:212	174:190	172:172	250:262	252:252	236:250	236:244	238:238
V. riparia portalis	182:193	140:144	210:210	184:186	172:172	264:264	248:262	216:244	234:236	238:250
V. rupestris du Lot	193:193	136:136	210:210	172:184	172:186	234:266	254:258	220:242	234:236	238:238

ratus (ALFexpress, Pharmacia Biotech, Vienna). Fragment lengths were estimated with the help of the internal size standards by Fragment Manager software (Pharmacia). The genetic distances between the cultivars were calculated as [-ln (proportion shared alleles)] using the microsatellite distance program (Microsat) by E. MINCH (http://lotka.stanford.edu/microsat.html). This program performs pairwise comparisons of all individuals tested. For each pair, the proportion of shared alleles is determined and this information is transformed into a pairwise distance matrix. From this matrix, a phenogram was drawn using the programs Kitsch (included in the PHYLIP package by J. FELSENSTEIN, 1989) and Treeview (PAGE 1996).

Results and Discussion

Fourty-seven *Vitis vinifera* and 19 rootstock cultivars were analysed at the 10 microsatellite loci mentioned in Material and methods. 90 % of the tested varieties yielded unique allelic profiles (Tab. 1).

The phenogram shown in Fig. 1 clusters the cultivars according to their microsatellite allele based pairwise similarities calculated as proportion-shared alleles. In *V. vinifera*, no allelic difference was detected within the following groups: Silvaner rot and S. grün, Portugieser blau and P. grün, Gutedel rot and G. weiß, Rheinriesling and Riesling rot, Chardonnay and Morillon and the Pinot family (Pinot noir, P. blanc and P. gris). The Pinot types are considered to differ only by a few point mutations affecting berry colour (GALET 1990). This might also be the case with the Silvaner, Riesling, Portugieser and Gutedel types. Therefore, genotypic uniformity should be expected in the remaining genome. Morillon and Chardonnay, however, were clearly differentiated by RAPD analysis (TSCHAMMER and ZYPRIAN 1994). The rootstock Binova is a selected clone of SO 4 and both cultivars share the same allelic profile. The inability to detect genetic differences between clones and types of the same cultivar by microsatellite markers has also been reported by THOMAS *et al.* (1994). The hybrids of *V. riparia* and *V. Berlandieri* (SO 4, Binova, Cosmo 10, R 7, Kober 5 BB, Ruggeri 225, Teleki 8 B, Kober 125 AA, Teleki 5 C and Cosmo 2) are joined into a cluster.

On the whole, the rootstock cultivars are separated from the *V. vinifera* individuals. However, the rootstocks Geisenheim 26 (Trollinger x *V. riparia*) and 41 B (Gutedel x *V. Berlandieri*) are grouped among the *V. vinifera* cultivars. This may be explained as follows: Since we included a large number of genotypes of *V. vinifera*, we were able to detect a wide range of the *V. vinifera* alleles. In contrast, we characterized only a few rootstock genotypes and hence detected only a small portion of the actual allele range. Therefore, alleles of the hybrids originating from *V. vinifera* find the matching alleles in our *V. vinifera* set, while not all alleles originating from the rootstock parents are present in our genotypes. Thus, in this study, the hybrids Geisenheim 26 and 41 B share more alleles with the *V. vinifera* cultivars than with the rootstocks, which puts them in the *V. vinifera* section.

In contrast to the large distance between the rootstocks and *V. vinifera,* the cultivars within both groups display a considerable degree of similarity. This is reflected by the fact that the average proportion of shared alleles is 40 % within the *V. vinifera* cultivars and 43 % within the rootstocks. Besides, one third of the *V. vinifera* cultivar pairs have more than half of their alleles in common. Therefore the branch pattern cannot be used to draw conclusions with

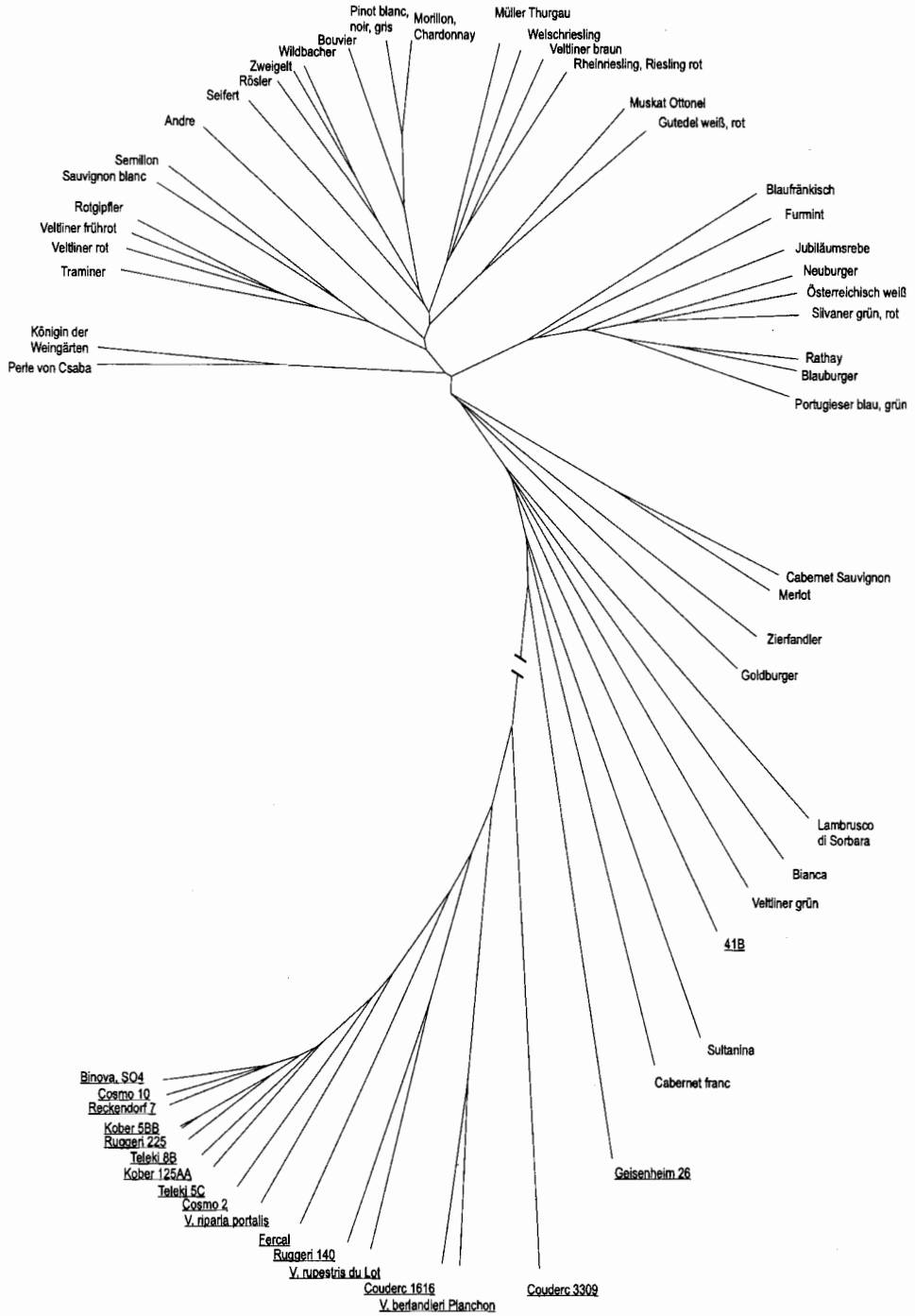

Fig. 1: Phenogram based on a genetic distance matrix. The stretch connecting rootstocks and *Vitis vinifera* was shortened to one third. The names of the rootstock cultivars are underlined.

regard to the degree of kinship between the cultivars and clusters illustrate similarity rather than kinship. Nevertheless, in several cases offsprings are grouped close to one of their parents, as are Rathay to Blauburger, Königin der Weingärten to Perle von Csaba, Blauburger to Portugieser blau, Silvaner to Österreichisch weiß (SEFC *et al.* 1998), Neuburger to Silvaner (REGNER *et al.* 1996; SEFC *et al.* 1997), Rotgipfler and Veltliner frührot to Veltliner rot (SEFC *et al.* 1998). In contrast, the genetic distances between Welschriesling and Goldburger or between Gutedel and 41 B are comparatively large, though they are parent and offspring as well, while other closely joined cultivars are not known to be genetically related.

Locus specific deviations of allele length estimates have been observed comparing the allele sizes detected by silver staining (BOWERS *et al.* 1996) and the size estimates obtained with the ABI/GENESCAN system (THOMAS *et al.* 1994). Fragments detected in the ALFexpress system differed from the results in the above mentioned systems by plus 1 to minus 4 bp. Since Taq DNA polymerase has a tendency to add additional non-templated nucleotides (HU 1993), we used the two step PCR protocol to prevent this activity (SMITH *et al.* 1995). This effect has not been considered in former vine SSR genotyping approaches and therefore further differences in allele sizes may occur apart from separation system derived deviations. At one locus (VVS 4), the two step

Table 2

The estimated frequency of null alleles within the *Vitis vinifera* individuals at 10 microsatellite loci

Locus	VVS1	VVS2	VVS3	VVS4	VVS29	VVMD5	VVMD7	VVMD28	VVMD32	VVMD36
Frequency of null alleles	-0.014	-0.039	-0.121	-0.009	-0.037	0.015	-0.045	-0.056	0.084	-0.024

protocol did not suppress the nucleotide addition entirely. To avoid ambiguity, amplification of this locus was performed under conditions allowing the constant addition of an extra nucleotide. Fragment lengths registered at this locus are therefore one bp larger than the true allele.

Cultivars displaying only one allele per locus were generally not assumed to harbour null alleles. Within the 40 different *V. vinifera* genotypes, the frequency of null alleles (r) was calculated using the expression presented by BROOKFIELD (1996): $r = (H_e-H_o)/(1+H_e)$. H_e and H_o are expected and observed heterozygosity values, respectively. The values for r (Tab. 2) were negative for all loci except for VVMD 5 (r = 0.015) and VVMD 32 (r = 0.084). This algorithm has been developed for panmictic, natural populations. Therefore, a positive value for r does not necessarily imply the presence of null alleles in the *V. vinifera* set. Apart from a null allele series, constraints resulting from breeding techniques may be responsible for heterozygote deficiencies. A positive value for r in our study solely indicates the possible presence of null alleles. The 19 rootstock individuals represent just a small part of the genetic range of the rootstock species and are still more exposed to breeding constraints than the *V. vinifera* population. Therefore, no realistic estimates of allele frequencies can be calculated from our rootstock sample and neither deficiency of heterozygotes nor the probability of null alleles are assessable.

Gene diversity was calculated as $1-\Sigma p_i^2$ (NEI 1973) to indicate the polymorphism and the distinctive power of each locus. p_i denotes the frequency of allele i in the cultivars. The resulting diversities within the total sample range from 0.7 at VVS 3 and VVS 29 to 0.9 at VVMD 28. Gene diversities among *V. vinifera* on the one hand and the rootstock cultivars on the other hand illustrate the applicability of the loci to each of these two groups (Tab. 3).

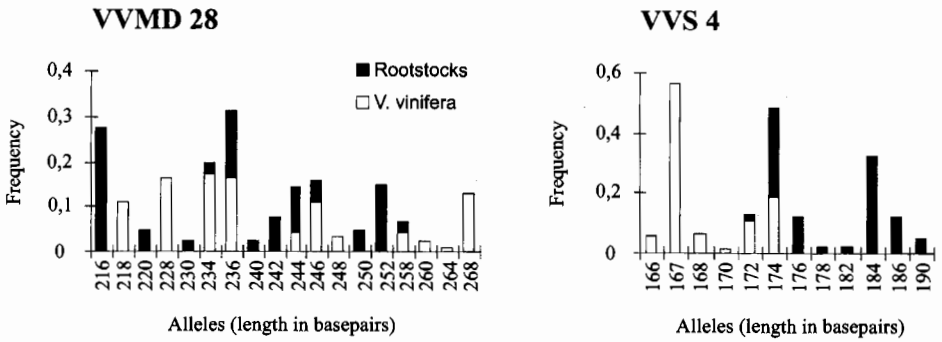

Fig. 2: Distribution and relative frequency of microsatellite alleles at the loci VVMD 28 and VVS 4 in *Vitis vinifera* and in rootstock cultivars.

Table 3

Gene diversity values of 10 microsatellite loci in all *Vitis* cultivars studied and in subsamples consisting of *Vitis vinifera* or rootstock cultivars

Locus	VVS1	VVS2	VVS3	VVS4	VVS29	VVMD5	VVMD7	VVMD28	VVMD32	VVMD36
V. vinifera and rootstock cultivars	0.80	0.87	0.70	0.77	0.70	0.89	0.87	0.91	0.83	0.80
V. vinifera cultivars	0.68	0.81	0.52	0.67	0.55	0.86	0.80	0.87	0.73	0.71
Rootstock cultivars	0.71	0.86	0.54	0.79	0.29	0.78	0.84	0.85	0.78	0.48

From all alleles detected at the 10 loci, one third is specific to *V. vinifera*, one third occurs only in the rootstock species and one third is shared between both groups. Fig. 2 shows a comparison between the frequency distribution of alleles in the *V. vinifera* and in the rootstock cultivars. As at VVMD 28, at most loci the alleles either appear in both rootstocks and *V. vinifera* cultivars or the allele size ranges of both groups overlap. VVS 4, VVMD 5 and VVMD 36, however, exhibit two distinct allele size distributions with only few alleles common to both groups. Interspecific hybrids between *V. vinifera* and the rootstock species were excluded from this calculation.

The results obtained so far show that a distinction of the majority of the Austrian cultivars with microsatellite markers is feasible. The data collection will be supplemented with the genetic profiles of further cultivars and information from more microsatellite loci for still indistinguishable cultivars. In future, questionable identities of trade or breeding material can be investigated by comparing their genetic profile with the reference profiles in the database.

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