Vitis 39 (3), 103–107 (2000)

Differentiation and identification of White Riesling clones by genetic markers

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Summary

Three different marker systems were used to genotype 10 clones of the grapevine cultivar White Riesling. All clones could be differentiated by means of genetic polymorphism gained by RAPD, SSR or Inter-SSR markers. While RAPD profile lacks stability for an identificational approach, individual SSR and Inter-SSR alleles could be retrieved within samples of the same clone. The polymorphic DNA fragments confirm the genetic variability within a traditional grapevine cultivar and the reproducibility of some of these markers allows the identification of clones. Since SSR and Inter-SSR markers show high stability when comparing data from different laboratories these methods are appropriate to establish data bases for the characterization of clonal grapevine material.

K e y w o r d s : grapevine, RAPD, SSR, Inter-SSR, DNA.

Introduction

For a long time it has been accepted that genetic mutations are the basis of clonal differences. After the discovery of graft-transmissible diseases and endophytes, phytopathological and ecological aspects are also considered to be responsible for different behaviour of individual plants of the same cultivar. Differences between clonal material remaining after cultivation in tissue culture and elimination of viruses by thermotherapy (Mannini *et al.* 1997) again indicate the more important influence of the genetic basis.

Today growers prefer to cultivate clonal material of traditional cultivars instead of mass-propagated grapevines. Due to differences in the viticultural as well as the sensorial behaviour, scientists tried to find markers which are linked to individual clones and which are stable during propagation. Since ampelographic descriptions or chemical analyses could not be applied to characterize the differences between clones or to identify them, legal claims of breeders to protect clones could not be applied.

White Riesling originating from the Rhine valley area is nowadays cultivated on more than 67 000 ha worldwide (Galet 1990). Riesling was mentioned for the first time in 1493 (Ambrosi *et al.* 1994) and therefore more than 500 years of its propagation have passed. The long period of Riesling cultivation was sufficient for varietal diversification into individual genotypes. Hence the importance of clonal selection (Schöffling and Stellmach 1993) to maintain yield and sensory quality of this cultivar has been recognized. Numerous comparative trials resulted in more than 80 Riesling clones registered in Germany. The most prominent clone which is also cultivated in several other European countries and overseas, is doubtless Gm 239 developed by the breeding department of the Forschungsanstalt Geisenheim.

Due to the stability of polymorphism, SSR markers are favoured to identify cultivars, but that should not exclude their use for clonal differentiation. Molecular markers for grapevine genotyping have been available for several years. Whereas AFLP (Zabeau and Vos 1993; Sensi *et al.* 1996), RFLP (Bourquin *et al.* 1995; Bowers and Meredith 1996) and RAPD (Regner and Messner 1993; Grando *et al.* 1995) markers are useful tools to differentiate genotypes and to detect polymorphism, SSR markers are preferably suitable to identify cultivars (Thomas and Scott 1993; Regner *et al.* 1996).

Moreover SSR markers are inherited in a codominant way and therefore they also meet the demands of parentage analysis and detection of incrossing events. Genetic fingerprinting of more than 1200 grapevine genotypes enables us to identify most unknown cultivars. In the frame of this work we have tried to find SSR loci with different alleles within one cultivar. Furthermore our aim was to show that clones differ in their genetic profiles and that it is possible to identify them by genetic markers.

Material and Methods

The plant material was taken from the collection of the Höhere Bundeslehranstalt und Bundesamt (HBLAuBA) für Wein- und Obstbau Klosterneuburg, Austria. The Forschungsanstalt Geisenheim, Germany, kindly provided the Riesling clones 239, 239-20, 239-12, 237-20, 110-11, and 198-30 for comparative field trials. Samples for clonal identification were collected from these plants. The clones Kl 20, Kl 23, Kl 1/6 were selected by the breeding department of the HBLAuBA Klosterneuburg while the clone TR 356 originated from Bad Kreuznach, Germany. Independent samples to evaluate the reproducibility of different markers were obtained from the same plantations but from different vines. Since the number of samples per clone was limited, a general identification of a clone was not possible due to the unknown stability of the clonal polymorphism.

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DNA was extracted from young leaves according to the protocol of THOMAS *et al.* (1993) modified by REGNER *et al.* (1998).

The clones were analysed with 47 SSR, 68 RAPD and 5 Inter-SSR markers. The VVS markers were developed by THOMAS and SCOTT (1993) and the VVMD markers by BOWERS *et al.* (1996) as well as by BOWERS *et al.* (1999). The VRZAG (SEFC *et al.* 1999) and VRG (REGNER *et al.* unpubl.) markers were obtained from investigations on simple sequence repeats of *Vitis riparia*.

The amplification of the SSR loci was performed by following the procedure of SMITH *et al.* (1995) using only two-step cycles. The general PCR protocol applied for these studies was 2 min denaturation at 94 °C and 35 cycles with annealing phase for 30 s between 45 and 55 °C and denaturation for 15 s at 92 °C. The annealing temperature for each locus was set according to the original protocol. A final extension of the fragments was performed at 72 °C for 5 min. Due to the different size range of the involved loci, multiplex analysis of PCR fragments was feasible. The alleles of at least three loci were separated on a sequencing gel. The solution for PCR amplification contained 20 µl of the buffer solution consisting of 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH = 8.8, 1.5 mM $MgCl_2$, 0.01 % Tween 20, 0.1 mM each dNTP (GenXpress, Vienna) 0.2 µM primer, 0.7 U Biotherm Taq DNA polymerase (GenXpress, Vienna) and 50 ng genomic DNA of grapevine.

Yield of DNA fragments was estimated by running an aliquot of the sample on a 2 % agarose gel stained with ethidiumbromide. The samples were denaturated by heating together with formamide and loaded together with a size standard (Genescan 350 Tamra, Appl. Biosys.) onto a 6 % polyacrylamid gel. Detection of the SSR fragments labelled with 6-FAM, HEX and TET was carried out by an automated sequencer (ABI 373, Perkin-Elmer, Vienna). Labelling with different fluorescent colouring agents facilitated the application of multiplex PCR.

RAPD analysis was carried out using the same clones; therefore they are verified as true to cultivar by SSR profiles. Decamer oligonucleotides were obtained from Operon Technologies, Alameda, USA (kit B1-20, C1-20, D1-20, E13) and Metabion GmbH, Martinsried, Germany (GTO: -3,-4,-5, M10, O5, O19, Q5). Amplification was performed in 20 µl of the buffer solution, which consisted of 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH = 8.8, 1.5 mM $MgCl_2$, 0.01 % Tween 20, 0.1 mM each dNTP (GenXpress, Vienna) 0.2 µM primer, 1 Unit Biotherm Taq DNA polymerase (GenXpress, Vienna), and 20 ng genomic DNA of grapevine.

An Omnigene (Hybaid, GB) thermocycler processed 40 cycles of 30 s at 92 °C, 90 s at 38 °C and 60 s at 72 °C. The arbitrarily amplified fragments were separated on a 2 % agarose gel and detected by ethidiumbromide staining. Documentation was done by taking Polaroid photographs.

For the application of Inter-SSR markers (FANG and ROOSE 1997) the following primers were used: GK1: (GA)8 CTC, GK2: (CA)8 T and GK3: (GCT) (AGT)(GCT) (CA)7, GK4: TGA (CT)8, GK5: GG(CT)8. These markers were used as single markers for amplification of an unknown region between two SSR loci with the same annealing site. The PCR protocol and the reaction solution were performed according to the SSR procedure. The samples were separated either on agarose gel stained with ethidiumbromide or on sequencing gel and silver stained.

Results and Discussion

Ten clones of Riesling (239 Gm, 239-12 Gm, 239-20 Gm, 237-20 Gm, 110-11 Gm, 198-30 Gm, TR 356, Kl 20, Kl 23, Kl 1/6) were analysed by genetic markers for detecting polymorphism to differentiate these genotypes. Since SSR markers are considered as one of the most useful marker classes, we genotyped all clones with about 40 SSR markers. Some data, especially the polymorphic ones, are shown in Tab. 1. Hence at the loci VVMD6, VRZAG12, VRG1, VRG2, VRG3 we detected polymorphism within the cultivar White Riesling. These polymorphisms were verified by a second analysis and an independent sample of the clone was used for identification. The second sample, however, was prepared also from the same plantation. We avoided taking material from growers outside due to the high risk of receiving material that was not well-defined. It is not surprising to find genetic differences in morphologically different genotypes, however, genetic differences at SSR loci can be considered to be very rare. Nevertheless they are very useful markers and their polymorphism could directly be used for clonal identification. The most frequently found polymorphism was the occurrence of null alleles. It is assumed that the loss of an annealing site is caused by some kind of mutation. As we lost the admittance to the locus we could not gain any additional information about the changes, but additional information about the sequence of the null allele could be obtained by using degenerate primers. All null alleles of the different Riesling clones were confirmed by the second independent sample of the same clone.

SSR markers however are stable and easily reproducible even in different laboratories and therefore characterization of clones should be possible. Further studies will be necessary to comment on the stability of the clonal polymorphism within vegetatively propagated clonal material from different places .

Similar deviations of SSR alleles from the profile of a cultivar were observed by analysing clonal material of Merlot and Grüner Veltliner. The null alleles were even stable when decreasing the annealing temperature from 50 to 47 °C. Hence these markers are useful to establish a database for clonal identification.

More polymorphism was gained by applying RAPD technique to the same 10 clones of White Riesling. Since we were aware of the restricted reproducibility of RAPD we used only polymorphic fragments of high intensity and moderate size between 100 and 3000 bp. Despite these restrictions we were able to find a primer which resulted in an individual profile for almost every clone and even for the subclones of 239 Gm (Tab. 2). Only the clone Kl 20

Table 1

Allele length at several SSR loci developed from 10 clones of White Riesling

Locus	239 Gm	Kl 23	Kl 20	Kl 1/6	TR 356	239/20 Gm	239/12 Gm	237/20 Gm	110/11 Gm	198-30 Gm
VVS 1	189:189	189:189	189:189	189:189	189:189	189:189	189:189	189:189	189:189	189:189
VVS 2	142:150	142:150	142:150	142:150	142:150	142:150	142:150	142:150	142:150	142:150
VVS 3	212:218	212:218	212:218	212:218	212:218	212:218	212:218	212:218	212:218	212:218
VVS 4	167:167	167:167	167:167	167:167	167:167	167:167	167:167	167:167	167:167	167:167
VVS 29	168:176	168:176	168:176	168:176	168:176	168:176	168:176	168:176	168:176	168:176
VVMD 5	224:232	224:232	224:232	224:232	224:232	224:232	224:232	224:232	224:232	224:232
VVMD 6	208:210	208:210	208:210	208:210	208:210	208:210	208:210	*210*	*208*	*210*
VVMD 7	246:254	246:254	246:254	246:254	246:254	246:254	246:254	246:254	246:254	246:254
VVMD 8	140:144	140:144	140:144	140:144	140:144	140:144	140:144	140:144	140:144	140:144
VVMD 14	228	228	228	228	228	228	228	228	228	228
VVMD 17	220:220	220:220	220:220	220:220	220:220	220:220	220:220	220:220	220:220	220:220
VVMD 21	248	248	248	248	248	248	248	248	248	248
VVMD 24	207:215	207:215	207:215	207:215	207:215	207:215	207:215	207:215	207:215	207:215
VVMD 25	250:256	250:256	250:256	250:256	250:256	250:256	250:256	250:256	250:256	250:256
VVMD 26	251	251	251	251	251	251	251	251	251	251
VVMD 27	180:188	180:188	180:188	180:188	180:188	180:188	180:188	180:188	180:188	180:188
VVMD 28	228:234	228:234	228:234	228:234	228:234	228:234	228:234	228:234	228:234	228:234
VVMD 31	203:213	203:213	203:213	203:213	203:213	203:213	203:213	203:213	203:213	203:213
VVMD 32	251:271	251:271	251:271	251:271	251:271	251:271	251:271	251:271	251:271	251:271
VVMD 36	252:262	252:262	252:262	252:262	252:262	252:262	252:262	252:262	252:262	252:262
VRZAG 7	155:155	155:155	155:155	155:155	155:155	155:155	155:155	155:155	155:155	155:155
VRZAG 12	154-173	*154*	154-173	154-173	*154*	154-173	154-173	154-173	154-173	154-173
VRZAG 15	165:165	165:165	165:165	165:165	165:165	165:165	165:165	165:165	165:165	165:165
VRZAG 21	202:206	202:206	202:206	202:206	202:206	202:206	202:206	202:206	202:206	202:206
VRZAG 25	225:225	225:225	225:225	225:225	225:225	225:225	225:225	225:225	225:225	225:225
VRZAG 29	112:116	112:116	112:116	112:116	112:116	112:116	112:116	112:116	112:116	112:116
VRZAG 30	147:151	147:151	147:151	147:151	147:151	147:151	147:151	147:151	147:151	147:151
VRZAG 47	159:167	159:167	159:167	159:167	159:167	159:167	159:167	159:167	159:167	159:167
VRZAG 62	193:203	193:203	193:203	193:203	193:203	193:203	193:203	193:203	193:203	193:203
VRZAG 64	137:159	137:159	137:159	137:159	137:159	137:159	137:159	137:159	137:159	137:159
VRZAG 67	139:152	139:152	139:152	139:152	139:152	139:152	139:152	139:152	139:152	139:152
VRZAG 79	242:244	242:244	242:244	242:244	242:244	242:244	242:244	242:244	242:244	242:244
VRZAG 83	188:194	188:194	188:194	188:194	188:194	188:194	188:194	188:194	188:194	188:194
VRZAG 93	188	188	188	188	188	188	188	188	188	188
VRZAG 112	240:242	240:242	240:242	240:242	240:242	240:242	240:242	240:242	240:242	240:242
VRG 1	226	226	226	226	226	226	226	*226: 228*	226	226
VRG 2	157	157	*157:167*	*108:157*	157	157	157	157	157	*157:167*
VRG 3	214	214	214	214	214	214	214	214	214	*198:214*
VRG 4	193	193	*150:193*	*150:193*	193	193	193	193	193	193
VRG 5	167-193	167-193	167-193	167-193	167-193	167-193	167-193	167-193	167-193	167-193

could not be differentiated from all other clones by applying a single RAPD marker, but by combining several markers it was possible to get an individual profile for even this clone. On the other hand the possibility to differentiate the clone 239 Gm from the subclones 239-17 Gm and 239-20 Gm showed us that genetic stability cannot overcome several years of intense propagation. Both subclones were selected from 239 Gm vines. Furthermore using RAPD primer B13 we also differentiated these subclones 239-17 Gm and 239-20 Gm from their "mother" clone 239 Gm. In fact by reproducing the profile of a clone with the second independent sample we observed changes in fragment pattern. In our analyses we did not lose the main bands for differentiation but faint bands easily appeared or disap-

Table 2

RAPD markers appropriated to identify a specific clone of White Riesling. Only the clone Kl 20 required several markers for differentiation to all other clones

Clone	239 Gm	Kl 23	Kl 20	Kl 1/6	TR 356	239/20 Gm	239/12 Gm	237/20 Gm	110/11 Gm	198/30 Gm
Primer	B-8, B-13	B-7, B-17	-	B-13	B-11, GT-03	B-13	B-13	B-4	B-2	D1, B-3, B-5

peared with increasing sample number. Since we were aware of the limited reproducibility of these RAPD results, we avoided running the analyses under other conditions like infrastructure, polymerase and procedures. Furthermore not all independent samples of a clone matched the same profile. We also observed genetic instability within a clone by using RAPD markers. The bands differing the subclones from their mother clone can be especially regarded as unstable. According to our experience, RAPD is not an appropriate tool for identification but for differentiation and could be used for generating polymorphism. Restricted recognition of DNA patterns was feasible by using internal standards and comparison of profiles with that of an unknown sample. The Figure shows the identification of a second vine sample of Riesling clone 198-30 by a homologous RAPD pattern generated with the marker B3. For general identification it would be necessary to transform the polymorphic RAPD DNA into stable SCAR markers.

It is possible that RAPD markers will reflect more than the genome of the grapevine. DNA of endophytes and other foreign organisms theoretically might be prepared with grapevine DNA. On the other hand if there are traces of foreign DNA, only faint bands could be derived from that DNA. The genetic profile corresponds to the clonal material with or without impurities and will even be transmitted by propagation. Nevertheless RAPD markers represent a very efficient method to differentiate clones in the laboratory. In other institutes the use of RAPD failed to differentiate clones or sports of a classic cultivar like Burgundy (YE *et al.* 1997). The main reasons may have been low genetic variability within the material, too little or unsuitable markers and technological parameters such as the annealing temperature.

Polymorphic DNA was also gained by using Inter-SSR markers. While SSR markers show only sparse polymorphism, Inter-SSR markers offer several polymorphisms even with one marker due to the numerous fragments (Tab. 3). With some markers, distinction of too many fragments could make the interpretation of the profiles difficult. The reproducibility is given and therefore Inter-SSR can be used for clonal identification.

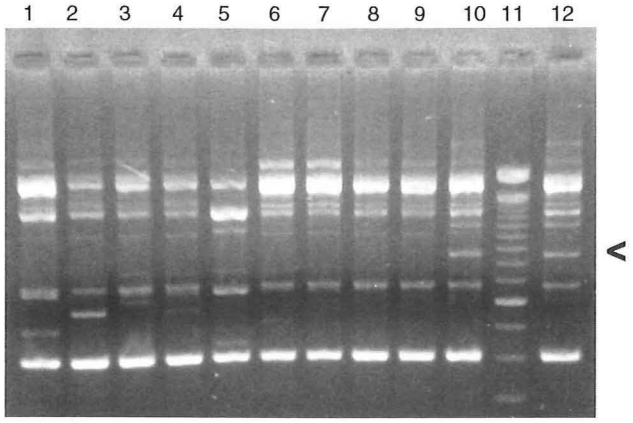

Figure: RAPD profiles separated on agarose gel stained by ethidiumbromide. DNA of 10 White Riesling clones (lane **1**: 239 Gm, **2**: Kl 23, **3**: Kl 20, **4**: Kl 1/6, **5**: TR 356, **6**: 239-20 Gm, **7**: 239-12 Gm, **8**: 237-20 Gm, **9**: 110-11 Gm, **10**: 198-30 Gm, **11**: 100 bp length marker, **12**: second sample of 198-30 Gm) was amplified with the oligonucleotide B3. This primer may be useful for recognition of the clone 198-30 Gm. The bar indicates the DNA fragment used for clone 198-30 Gm differentiation.

With regard to reproducibility, this paper is in agreement with MORENO *et al.* (1998) but disagrees in detecting intervarietal polymorphism.

All these methods offer some help to identify specific genotypes within one cultivar. The differences in the reproducibility of the results are shown in Tab. 4. For conclusion we would define markers with high polymorphism within a cultivar to be more unstable than less polymorphic ones. Nevertheless preference of marker class depends on the aim of the study.

Despite the improved methods for identification there is no chance for legislation (BECHER, pers. comm.) to protect clones within the same legal frame as new varieties. According to the UPOV rules uniformity and stability are prerequisites for variety protection, but clones do not fulfill

Table 3

Polymorphism gained by Inter-SSR marker GK5. The clones Kl 20 and Kl1/6 are lacking one of the main band (480 bp) and 237-20 Gm is lacking a 780 bp fragment of the Riesling profile gained with all other clones. Length of fragments was estimated running a 50 bp ladder

Length of fragments	239 Gm	Kl 23	Kl 20	Kl 1/6	TR 356	239-20 Gm	239-12 Gm	237-20 Gm	110-11 Gm	198-30 Gm
240 bp	+	+	+	+	+	+	+	+	+	+
280 bp	+	+	+	+	+	+	+	+	+	+
310 bp	+	+	+	+	+	+	+	+	+	+
330 bp	+	+	+	+	+	+	+	+	+	+
400 bp	+	+	+	+	+	+	+	+	+	+
480 bp	+	+	-	-	+	+	+	+	+	+
550 bp	+	+	+	+	+	+	+	+	+	+
620 bp	+	+	+	+	+	+	+	+	+	+
690 bp	+	+	+	+	+	+	+	+	+	+
780 bp	+	+	+	+	+	+	+	-	+	+

Table 4

Reproducibility of polymorphism with the different methods in different samples of the same clone was classified as: + high reproducible, + - not full reproducible, - low reproducible

Method	239 Gm	Kl 23	Kl 20	Kl 1/6	TR 356	239-20 Gm	239-12 Gm	237-20 Gm	110-11 Gm	198-30 Gm
SSR	+	+	+	+	+	+	+	+	+	+
RAPD	+ -	+ -	+ -	+ -	+ -	-	-	+ -	+ -	+ -
INTER-SSR	+	+	+	+	+	+	+	+	+	+

these criteria. Nevertheless differentiation or even identification of clonal grapevine material are essential to determine distinctness and to detect wrong designations.

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Received January 3, 2000