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Application of microsatellite markers to parentage studies in grapevine

by

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S u m m a r y : The use of microsatellites in genetic analysis does not only allow differentiation but also identification and parentage analysis of grapevine cultivars. Many of the cultivars which are of great economic importance, like Cabernet Sauvignon, have been selected and propagated centuries ago and often lack reliable documentation about their origins. In our study, 51 grapevine cultivars were genotyped at 24 microsatellite loci and searched for possible parent-offspring combinations. Our data confirm the origin of Cabernet Sauvignon from a cross between Cabernet franc and Sauvignon blanc. Furthermore we proved the parentage of the cultivars Neuburger (Silvaner x Veltliner rot), Blauburger (Portugieser blau x Blaufränkisch), Zweigelt (Blaufränkisch x St. Laurent) and Müller-Thurgau (Rheinriesling x Chasselas de Courtillier) at 24 SSR loci.

K e y w o r d s : grapevine, parentage analysis, microsatellites, simple sequence repeats.

A b b r e v i a t i o n s : SSR = simple sequence repeats, cpDNA = chloroplast DNA.

Introduction

The world's collections of grape plant material are estimated to contain about 5,000 different cultivars (ALLEWELDT 1988) and the number of cultivar names worldwide in use is even larger. Most of the cultivars which are of economic importance today have been known for centuries. However, until the last century, breeding steps have not been documented and therefore the origins of many grapevine cultivars are unknown. Two concerns are therefore of major interest to grapevine breeders and wine producers: (1) to find a reliable method to identify grapevine cultivars and to distinguish between different cultivars in order to redress definition errors and double designations; (2) to understand the genetic events which led to today's cultivar range.

The demands for reliable cultivar identification and parentage analysis are met by the microsatellite (or SSR) markers. The application of microsatellite markers to vine genotyping has been described by THOMAS and SCOTT (1993), THOMAS *et al.* (1994), CIPRIANI *et al.* (1994), BOTTA *et al.* (1995), BOWERS *et al.* (1996) and BOWERS and MEREDITH (1997). Microsatellites are tandemly arranged repeats of short nucleotide sequences, which are spread all over the genome of most eukaryotes. Due to the high variability of the repeat number, each individual may hold a unique fingerprint. The inheritance of microsatellite alleles from one generation to the other follows the codominant Mendelian manner. Therefore, the study of microsatellite polymorphisms allows the understanding of family structures and genetic histories.

THOMAS *et al.* (1994) have already applied microsatellite analysis to parentage studies in Australian grapevine cultivars. Further studies on the parentage of European cultivars using microsatellite markers have been undertaken by REGNER *et al.* (1996) and recently by BOWERS and MEREDITH (1997), demonstrating the origin of Cabernet Sauvignon.

The present work shows the results of parentage analysis among 51 grapevine varieties, including the parentages of Müller-Thurgau and Neuburger and the confirmation of the origins of Cabernet Sauvignon, Blauburger and Zweigelt.

Materials and methods

The plant material used in this study was obtained both from field and in vitro collections of the Höhere Bundeslehranstalt und Bundesamt für Wein- und Obstbau, Klosterneuburg, Austria (Tab. 1). Leaves from field plants were harvested during 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).

The cultivars were analyzed at the following 24 microsatellite loci: VVS1, VVS2, VVS3, VVS4 (THOMAS and SCOTT 1993), VVS29 (THOMAS, personal communication), VVMD5, VVMD7 (BOWERS *et al.* 1996), VVMD28, VVMD32, VVMD36 (BOWERS and MEREDITH, personal communication), and microsatellite loci recently developed in our laboratory from a library of *Vitis riparia* (publication in preparation): ssrVrZAG 7, ssrVrZAG 12, ssrVrZAG 15, ssrVrZAG 21, ssrVrZAG 25, ssrVrZAG 29, ssrVrZAG 30, ssrVrZAG 47, ssrVrZAG 62, ssrVrZAG 64, ssrVrZAG 67, ssrVrZAG 79, ssrVrZAG 83 and ssrVrZAG 112.

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 polymerase and reaction buffer (10 mM Tris pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X 100; PCR machine: Hybaid Omnigene). One primer of each pair was labelled

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Table 1

Genotyped grapevine cultivars

Andre	Pinot gris
Bianca	Pinot noir
Blauburger	Portugieser blau
Blaufränkisch	Portugieser grün
Bouvier	Rathay
Cabernet franc	Rheinriesling
Cabernet Sauvignon	Roesler
Chardonnay	Rotgipfler
Chasselas de Courtillier	Sämling 88
Furmint	Sangiovese
Goldburger	Sauvignon blanc
Gutedel rot	Semillon
Gutedel weiß	Silvaner grün
Grenache	Silvaner rot
Heunisch	St. Laurent
Jubiläumsrebe	Sultanina
Königin der Weingärten	Traminer
Lambrusco di Sorbara	Veltliner braun
Morillon	Veltliner frührot
Merlot	Veltliner grün
Müller-Thurgau	Veltliner rot
Muskat Ottonel	Welschriesling
Neuburger	Wildbacher blau
Österreichisch weiß	Zierfandler
Perle von Csaba	Zweigelt
Pinot blanc	

with the fluorescent Cy-5 dye to enable detection of the fragments in the Alf express automated sequencing system (Pharmacia Biotech, Vienna, Austria).

A two-step PCR protocol (SMITH *et al.* 1995) was chosen for the amplification of all loci but one (VVS 4): 95 °C for 5 min, 10 cycles of 50 °C (58 °C for ssrVrZAG 64, 45 °C for ssrVrZAG 67) for 15 s, 94 °C for 15 s, followed by 23 cycles of 50 °C (58 °C for ssrVrZAG 64, 45 °C for ssrVrZAG 67) for 15 s and 89 °C for 15 s. Final extension was avoided by transferring the reaction tubes to 4 °C immediately. The two-step protocol did not work for locus VVS 4 and therefore the following protocol was used for amplification: 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 µl were mixed with equal volumes of loading buffer (Formamide containing 5 $mg \cdot ml^{-1}$ Dextran blue) and 1 µl of each Cy-5-labelled size markers. Size markers were produced in our laboratory by PCR amplification of fragments with sizes of 100, 125, 150, 175, 200, 225, 250, 275 and 300 bp from the plasmid pUC 18. Samples were denatured at 95 °C for 2-3 min and analyzed on a sequencing gel (6 % acrylamide, 1x TBE buffer, 7 M Urea) in an automated sequencing apparatus (Alf express, Pharmacia Biotech, Vienna, Austria). Fragment lengths were estimated with the help of the internal size standards by Fragment Manager software (Pharmacia). With a self-written software, the allelic profiles within all permutations of 3 cultivars among the 51 varieties were compared and possible parents-offspring combinations were identified. For the resulting cultivar combinations, cumulative likelihood ratios were calculated from the relative allele frequencies and their 95% upper confidence limits analogous to the method described by BOWERS and MEREDITH (1997) to indicate the probability of the corresponding parentage case. Relative allele frequencies and their 95 % upper confidence limits were estimated from 45 cultivars, as we included only one of genotypically identical types of cultivars in the calculation. One locus (ssrVrZAG 12) was discarded from the statistical analysis, as no relative allele frequencies could be calculated due to the presence of null alleles at this locus.

Results and Discussion

Data obtained from the analysis of 51 *Vitis vinifera* cultivars of international and Austrian interest genotyped at 24 SSR loci in order to reveal and verify crossing events support the origin of Cabernet Sauvignon from a crossing between Cabernet franc and Sauvignon blanc (Tab. 2 A). Cabernet Sauvignon has its origin in the French Bordeaux region and is now one of the most important red wine cultivars of the world. A connection to Cabernet franc was suspected due to the similarities in morphological characteristics and the flavour of the wine (VIALA and VERMOREL 1901). However, the much higher content of phenolic and tannic compounds of the Cabernet Sauvignon wines could not be aligned to the other Cabernet variety. Some flavour components attached to the taste of Sauvignon wines also appear in Cabernet Sauvignon (BRANAS 1980). From the ampelographic point of view, Sauvignon blanc shows the strongest similarity to Cabernet Sauvignon outside the Cabernet family (VIALA and VERMOREL 1901). Although similarities of Cabernet Sauvignon to the two other cultivars have been known for a long time, it was not possible up to now to uncover their genetic relationship. Only very recently a paper proposing the descent of Cabernet Sauvignon from the two mentioned varieties based on microsatellite data was published (BOWERS and MEREDITH 1997). As our study includes 15 new microsatellite loci, our data provide strong support for this hypothesis. Together with the 30 microsatellite loci used by BOWERS and MEREDITH (1997), 45 microsatellite loci now indicate this parentage. Further evidence is gained by the extension of the number of grapevine varieties analyzed, as our collection contains 38 varieties which are not included by BOWERS and MEREDITH (1997) (Tab. 1). No other cultivar in our collection could possibly be a parent of Cabernet Sauvignon. Cumulative likelihood ratios (Tab. 3) were calculated from the 14 newly analyzed loci (VVS 3 and all ssrVrZAG loci except ssrVrZAG 12): (1) The likelihood ratio of the probability that Cabernet franc and Sauvignon blanc are the parents of Cabernet Sauvignon versus the probability that two random cultivars are the parents is 4.2×10^6 :1. (2) The likelihood of Cabernet franc and Sauvignon blanc being the parents is 8.1×10^4 higher than

Table 2

Genotypes of cultivars involved in parentage cases. Numbers represent allele lengths in basepairs. Locus ssrVrZAG 12 contains a series of null alleles, as was proven in segregation analysis. Therefore genotypes displaying only one allele at this locus may be heterozygous with one null allele

	A			B			C			D			2 E		
Locus	Sauvignon blanc	Cabernet Sauvignon	Cabernet franc	Silvaner	Neuburger	Veltliner	Portugieser rot	Blau-burger	Blau-fränkisch	St. Laurent	Zweigelt	Blau-fränkisch	Rhein-riesling	Müller-Thurgau	Chasselas de Courtillier
VVS1	180:189	180:180	180:180	179:189	179:189	189:189	179:180	179:189	189:189	182:189	189:189	189:189	189:189	182:189	182:189
VVS2	132:150	138:150	138:146	150:152	130:150	130:132	142:150	142:150	142:142	136:150	136:142	142:142	142:150	142:150	150:154
VVS3	212:218	212:218	212:212	218:218	218:218	212:218	218:218	218:218	218:218	212:260	212:218	218:218	212:218	218:218	218:218
VVS4	167:168	167:174	166:174	167:167	167:167	167:174	167:174	167:174	167:174	166:172	166:167	167:174	167:167	167:172	167:172
VVS29	168:176	176:178	172:178	168:176	168:168	168:168	168:168	168:168	168:168	168:176	168:176	168:168	168:176	176:176	168:176
VVMD5	226:230	230:238	224:238	224:230	224:238	238:244	224:230	230:238	224:238	226:226	224:226	224:238	224:232	224:226	226:234
VVMD7	236:254	236:236	236:260	240:244	244:250	236:250	240:252	240:246	236:246	236:254	236:236	236:246	246:254	244:254	240:244
VVMD28	234:236	234:236	228:236	228:236	228:246	246:268	228:260	228:246	246:246	234:236	234:246	246:246	228:234	234:244	218:244
VVMD32	239:239	239:239	239:257	271:271	263:271	253:263	251:271	271:271	249:271	261:271	249:261	249:271	251:272	251:251	251:261
VVMD36	262:294	252:262	252:252	262:274	262:274	262:262	262:274	264:274	262:264	252:252	252:262	262:264	252:262	252:262	252:262
ssrVrZAG 7	155:155	155:155	108:155	155:155	155:155	155:157	155:155	155:155	155:155	151:157	155:157	155:155	155:155	155:155	155:155
ssrVrZAG 12	153:0	153:0	153:0	158:0	153:158	153:0	153:0	158:0	153:158	153:0	153:158	153:158	172:0	0:0	0:0
ssrVrZAG 15	165:165	163:165	163:181	165:165	165:165	165:173	165:165	165:165	165:165	175:177	165:175	165:165	165:165	165:165	165:175
ssrVrZAG 21	204:206	200:206	190:200	200:206	200:206	200:204	200:206	206:206	202:206	200:206	202:206	202:206	202:206	202:202	202:206
ssrVrZAG 25	236:245	225:236	225:225	236:245	236:238	225:238	225:236	225:236	225:225	225:236	225:236	225:225	225:225	225:225	225:225
ssrVrZAG 29	112:116	112:112	112:112	112:116	112:112	112:112	114:116	112:116	112:112	112:116	112:116	112:112	112:116	112:116	112:116
ssrVrZAG 30	149:151	149:151	149:151	149:149	149:149	143:149	149:149	149:149	147:149	149:151	147:151	147:149	147:151	147:149	149:149
ssrVrZAG 47	153:167	153:167	159:167	167:112	167:172	161:172	159:172	157:159	157:172	163:167	157:163	157:172	159:167	159:159	159:167
ssrVrZAG 62	187:193	187:193	193:203	187:203	191:203	191:195	187:203	187:203	193:203	193:193	193:193	193:203	193:203	193:193	187:193
ssrVrZAG 64	139:143	139:159	157:159	139:143	139:143	143:163	139:163	139:159	139:159	139:163	139:159	139:159	137:159	137:163	159:163
ssrVrZAG 67	126:149	126:139	139:139	126:159	126:149	149:149	126:132	126:139	139:149	126:152	126:139	139:149	139:152	149:152	139:149
ssrVrZAG 79	244:246	246:246	246:258	248:250	250:250	250:250	248:258	236:258	236:250	238:246	236:238	236:250	242:244	242:244	244:258
ssrVrZAG 83	190:200	200:200	194:200	188:190	188:190	188:190	190:194	188:190	188:194	188:194	194:194	188:194	188:194	194:200	188:200
ssrVrZAG 112	234:240	229:234	229:242	238:240	234:238	234:234	229:240	229:242	234:242	229:242	242:242	234:242	240:242	229:240	229:240

Table 3

Likelihood ratios of the probability of the suggested parentages of Cabernet Sauvignon, Neuburger, Blauburger, Zweigelt and Müller-Thurgau versus other possibilities. Probability values were calculated from allele frequencies derived from our sample and from the 95 % upper confidence limits. The calculations are based on the data of 45 cultivars and 14 microsatellite loci (VVS 3 and all ssrVrZAG loci except ssrVrZAG 12) for Cabernet Sauvignon and 45 cultivars and 23 microsatellite loci for Neuburger, Blauburger, Zweigelt and Müller-Thurgau as described in Materials and methods. The order of the parents in the table does not indicate the actual direction of the cross

Cultivar	Suggested parents	Cumulative likelihood ratios of the suggested parentage (1) x (2) versus				
		X x Y[a,b]	(1) x X[a,c]	(1) x rel (2)[a,d]	(2) x X[a,c]	(2) x rel (1)[a,d]
Cabernet Sauvignon	(1) Cabernet franc (2) Sauvignon blanc	4.2×10^6 (1835.8)	8137.9 (198.6)	22.1 (6.4)	3.7×10^4 (708.9)	33.7 (10.4)
Neuburger	(1) Silvaner (2) Veltliner rot	2.0×10^{13} (2.4×10^7)	4.3×10^8 (2.7×10^5)	299.4 (41.0)	2.5×10^7 (5.1×10^4)	508.3 (70.9)
Blauburger	(1) Portugieser (2) Blaufränkisch	1.3×10^{12} (3.0×10^6)	2.4×10^7 (4.0×10^4)	306.7 (42.4)	1.0×10^8 (1.6×10^5)	1075.4 (137.3)
Zweigelt	(1) St. Laurent (2) Blaufränkisch	1.5×10^{14} (7.5×10^7)	1.4×10^8 (1.9×10^5)	610.0 (84.6)	2.5×10^{10} (7.5×10^6)	2648.8 (292.3)
Müller-Thurgau	(1) Rheinriesling (2) Chasselas de Courtillier	8.2×10^{13} (7.3×10^7)	2.1×10^8 (1.8×10^5)	1031.1 (132.8)	2.3×10^8 (2.0×10^5)	870.8 (104.8)

[a] Values in parentheses are the cumulative likelihood ratios calculated with the 95 % upper confidence limits for the allele frequencies.
[b] X and Y are random unrelated cultivars.
[c] The identity of one of the suggested parents is assumed and the other parent is unknown.
[d] The identity of one of the suggested parents is assumed and the other parent is a close relative to the second suggested parent.

the likelihood of Cabernet Sauvignon being an offspring of Sauvignon blanc and any random cultivar and (3) 3.7×10^4 times higher than the likelihood of a cross between Cabernet franc and any random cultivar. (4) The parentage of Cabernet franc and Sauvignon blanc is 22 times more probable than a cross between Sauvignon blanc and a close relative to Cabernet franc and (5) 34 times more likely than a cross between Cabernet franc and a close relative of Sauvignon blanc.

The corresponding values derived from the allele frequencies at 24 loci in a collection of partly different cultivars by BOWERS and MEREDITH (1997) are 1.5×10^{14}, 1.6×10^{11}, 3.7×10^7, 3989 and 576. Combining these values yields very high levels of probability of the origin of Cabernet Sauvignon from a cross between Cabernet franc and Sauvignon blanc.

Although the cultivars involved in the parentage of Cabernet Sauvignon are now identified, the direction of the cross is still unknown. Since the chloroplast (cp) genome is usually maternally inherited in angiosperms, polymorphisms in the cpDNA of the parents can reveal the female partner of a cross. To that aim, 20 SSR primer pairs of cpDNA for *Pinus thunbergii* (VENDRAMIN *et al.* 1996), the largest set of chloroplast SSR primers available, were tested on the 3 grapevine cultivars. PCR reactions were carried out according to VENDRAMIN *et al.* (1996). For 7 primer pairs (Pt9383, Pt15169, Pt36480, Pt48210, Pt87268, Pt100783), PCR products of expected size were obtained, but no polymorphism was detected (data not shown). Therefore, the question of the direction of the cross cannot be answered so far.

REGNER *et al.* (1996) proposed parentages of Neuburger, Blauburger, Zweigelt and Müller-Thurgau based on the data of 6-8 microsatellite markers. Our data from 18 additional microsatellite loci only partly support the results obtained by the small amount of markers used.

The cv. Neuburger has its geographic origin in the Austrian Wachau region and was thought to descend from a natural cross between Pinot blanc and Silvaner. The results obtained by REGNER *et al.* (1996) at 7 microsatellite loci exclude Pinot blanc from the parentage of Neuburger and suggest a cross between Veltliner rot and Silvaner. This assumption is now supported by the data of 18 additional loci and a second clone of Neuburger (Tab. 2 B). Cumulative likelihood ratios were calculated from the relative allele frequencies of 23 microsatellite loci and yielded high levels of probability (Tab. 3).

Blauburger (Portugieser blau x Blaufränkisch) and Zweigelt (Blaufränkisch x St. Laurent) are red wine cultivars bred in Klosterneuburg around 1920. The breeding records of the two varieties are still available and are in good agreement with the results of the microsatellite analysis (Tab. 2 C and D). Tab. 3 shows the corresponding likelihood ratios. Zweigelt, bred by Prof. Zweigelt in Klosterneuburg, is the

most popular red wine variety in Austria and economically also the most important one of the newly developed cultivars for red wine production in Europe.

The parentage of the variety Müller-Thurgau has always been a matter of discussion, although documentation exists that indicates its origin from a cross between Rheinriesling and Silvaner achieved by Prof. Müller about 100 years ago. However, the involvement of Silvaner in this cross has been doubted (BREIDER 1952) and DNA analysis finally excluded Silvaner as a possible parent of Müller-Thurgau (BÜSCHER *et al.* 1994, THOMAS *et al.* 1994, REGNER *et al.* 1996). Based on the results obtained with 8 SSR loci, REGNER *et al.* (1996) suggested the origin of Müller-Thurgau in a cross between Rheinriesling and Gutedel weiß. However, during the analysis of further 16 SSR markers we found several deviations in the allele lengths between Müller-Thurgau and Gutedel (Chasselas white). We analysed different types of the Gutedel or Chasselas group and found one cultivar whose allelic profile in combination with that of Rheinriesling could produce the genotype of Müller-Thurgau: Müller-Thurgau appears to be a descendant from a cross between Rheinriesling and Chasselas de Courtillier (Tab. 2 E). The parentage is supported by high likelihood ratio values (Tab. 3).

In the present work, parentage analysis of grapevine cultivars with the help of microsatellite markers (1) supports the breeding records of Blauburger and Zweigelt, (2) rejects the common assumptions on the origins of Müller-Thurgau and Neuburger and (3) confirms the recently reported origin of Cabernet Sauvignon.

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