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Grape microsatellite markers: Sizing of DNA alleles and genotype analysis of some grapevine cultivars

by

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Summary: Capillary electrophoresis fluorescence-based technology and fragment sizing software were applied in order to type grape cultivars at microsatellite loci described by THOMAS and SCOTT (1993) and BOWERS *et al.* (1996). Whereas the data set was closely consistent with already known genotypes for the same cultivars, estimates of allele length differed remarkably from those reported. The pedigree of the Riesling crosses Bacchus, Optima, Müller-Thurgau, Scheurebe, Ehrenfelser, Kerner, Rotberger, Rieslaner and Incrocio Manzoni 6.0.13 were investigated, and Mendelian inheritance of automatically sized alleles proved to be easily scorable. In addition, ancient cultivars of the vine-growing area of Trentino were characterized at microsatellite loci. Genotypes of Marzemino, Teroldego, Lagrein, Lambrusco a foglia frastagliata and Groppello gentile proved to share more than 50 % of alleles.

Key words: DNA typing, automated sizing, microsatellites, parentage analysis, indigenous grapevines.

Introduction

DNA markers which are direct characteristics of the genotype and which are independent of the phenotype, provided a wealth of polymorphisms, enabling the identification of cultivars and the construction of saturated genetic maps in many higher plants.

Owing to the relative simplicity of the DNA amplification approach when compared to RFLP (Restriction Fragment Length Polymorphism) technology, PCR-based marker tools such as RAPD (Random Amplified Polymorphic DNA) and STS (Sequence Tagged Sites) have recently been preferred also by grape research groups for genome studies (THOMAS *et al.* 1994, LODHI *et al.* 1995, XU *et al.* 1995).

Microsatellite sequences have become the genetic markers of choice in mammalian and plant systems due to their abundance, high degree of polymorphism and amenability to automation (WEBER and MAY 1989). The co-dominant mode of inheritance of microsatellites not only simplifies analysis but also facilitates the transfer of markers between maps derived from different crosses.

The development of grapevine genome microsatellite markers was first reported by THOMAS and SCOTT (1993) and then by BOWERS *et al.* (1996) which referred to STMS (Sequence Tagged Microsatellite Sites) and to SSR (Simple Sequence Repeats), respectively.These studies described a large number of alleles, as well as a very high level of heterozygosity in grapes, which provides a considerable resolving power for the accurate separation of cultivars. Moreover, the primers for STMS analysis which derived from regions flanking the repeated sequences of *V. vinifera* DNA, successfully amplified polymorphic alleles from other related *Vitis* species.

The high level of genetic diversity in grapes which are found world-wide, the interest in evaluating genetic relatedness within the *Vitis* genus, and the need for variety recognizing systems useful for certifying the identity of grape cultivars encourage an attempt to integrate and exploit the genotype data which are revealed at microsatellite loci in grapes.

Microsatellite markers are easily shared by the communication of primer sequences, but results are expressed as relative allele lengths and allele size estimates are affected by the different methods of determination (WEBER 1990, SMITH 1995). We here compare a first set of grapevine genotypes identified at 9 previously described microsatellite loci using capillary electrophoresis fluorescence-based technology and fragment sizing software. Parentage analyzes of some Riesling crosses, and the genotypes of ancient grapevine cultivars still grown in our region (Trentino, Italy), are also included in this study to verify the identification of common alleles and to increase information for allelic frequency estimates in *V. vinifera.*

Material and methods

Grape leaves used for DNA extraction were obtained from the *Vitis* collection held by the Istituto Agrario at San Michele all'Adige, Trento, Italy. Studies were carried out on the wine cultivars listed in Tab. 1. As a different method for the sizing of DNA fragments was used in this study, a number of varieties were analyzed in order to identify allele classes reported at the same loci by other authors: these were Cabernet franc, Cabernet Sauvignon, Chardonnay, Chenin blanc, Gewürztraminer, Merlot, Müller-Thurgau, Pinot blanc, Riesling, Semillon, Shiraz and Silvaner. In order to follow pedigree relationships with microsatellite markers, cultivars from 9 Riesling crosses, including the parents, were examined. Genotype similarity at microsatellite loci was evaluated for 9 varieties (e.g., Lambrusco f.f. (a foglia frastagliata)) belonging to local ancient germplasm. After extraction and

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Table 1

Vitis vinifera cultivars analyzed in this study

Cultivar	Parents
Cabernet franc	
Cabernet Sauvignon	
Chardonnay	
Chasselas de Courtillier	
Chenin blanc	
Gewürztraminer	
Merlot	
Pinot blanc	
Riesling	
Semillon	
Shiraz	
Silvaner	
Müller-Thurgau	Riesling x Chasselas de Courtillier
Ehrenfelser	Riesling x Silvaner
Scheurebe	Silvaner x Riesling
Rieslaner	Silvaner x Riesling
Bacchus	(Silv. x Riesl.) x Müller-Thurgau
Optima	(Silv. x Riesl.) x Müller-Thurgau
Kerner	Schiava grossa x Riesling
Rotberger	Schiava grossa x Riesling
Incrocio Manzoni 6.0.13	Riesling x Pinot blanc
Corvina veronese	
Groppello gentile	
Lagrein	
Lambrusco f.f.	
Marzemino	
Nosiola	
Portugieser	
Schiava grossa	
Teroldego	

RNase A digestion (DOYLE and DOYLE 1991) 100-120 ng of genomic DNA was added to a PCR mix containing 0.25 µM of each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$ (2 mM with VVMD5 and VVMD8 primers), 0.5 U AmpliTaq Gold and 1X Gene Amp PCR Buffer II (Perkin Elmer), in 25 µl volume. Primer sequences used are those designed by THOMAS and SCOTT (1993) and BOWERS *et al.* (1996). One oligonucleotide of each primer pair was fluorescently labeled with Dye Phosphoramidities (Abi Prism). TET (green) was attached to VVS1 and VVS3 primers, 6-FAM (blue) was used to label VVS2, VVS5 and VVMD7 primers and HEX (yellow) was attached to VVS4, VVMD5, VVMD6 and VVMD8 primers.

PCR was performed using a Gene Amp PCR System 9600 (Perkin Elmer) under the following conditions: 12 min at 95 °C, then 30 cycles of denaturation (45 s at 94 °C), annealing (30 s at 51 °C; 54 °C in PCR with VVS1 and VVS3 primers), extension (1 min 30 s at 72 °C) and a final elongation step at 72 °C for 7 min. Amplifications were confirmed by running 7 µl of the PCR product on 2 % agarose gel. DNA fragments were then analyzed on the ABI Prism 310 Genetic Analyzer running GeneScan Software (2.1) through GA 310 POP-4, a pre-formulated liquid polymer matrix, heated at 60 °C. Before injection, 0.4-1.5 µl of well defined PCR samples were pooled together and mixed with 12 µl of deionized formamide and 0.5 µl of red fluorescently labeled DNA size standard (GeneScan 500 TAMRA), and were then denaturated for 2 min at 95 °C. To allow detection of the 400 bp peak of the size standard, the data collection time was set to 24 min. Among analysis parameters, the Local Southern Method was chosen for size calling.

Results and Discussion

Automated sizing of microsatellite alleles: The genotypes at VVS1, VVS2, VVS4, VVS5, VVMD5, VVMD6, VVMD7 and VVMD8 loci proved to be highly consistent with those reported by THOMAS *et al.* (1994) and BOWERS *et al.* (1996) for the same *V. vinifera* cultivars (Tab. 2). VVS3 alleles were not compared, as difficulties in the amplification of two different DNA products from described heterozygous genotypes were often observed at this locus. Standard deviations lower than 0.2 bp were calculated for data obtained running the same capillary, whereas differences in size of around 1 bp sometimes resulted between capillaries (data not shown). For this reason, the sizing of alleles at VVS4 and VVMD6 loci required more care, since microsatellite lengths can differ by as little as 1 bp. Assigned DNA fragment sizes differed remarkably from those obtained in other laboratories for the same alleles detected on acrylamide or agarose gels, the apparent fragment length always proving to be shorter in our case (Tab. 3). This did not affect results, as alleles were easily identified by comparing genotype data sets. Base pairs offset showed a locus dependence, and for some loci this varied in function of allele sizes. However, a constant difference in size was observed for all allele classes at VVS1, VVS4 and VVMD5 loci. Such differently estimated sizes of alleles can result by the extreme denaturing electrophoretic conditions we ran as DNA fragments were separated at 60 °C through a polymer matrix containing 8 M urea and additional denaturant agents.

Parentage analysis of Riesling crosses: Microsatellite repeats developed in the grapevine genome showed Mendelian segregation, indicating that markers are heritable and reliable for genetic analysis or for the examination of pedigrees. Nine cultivars and their parental lines were characterized at microsatellite loci. Genotypes are reported in Tab. 2. Three pedigrees showed inconsistencies with the parental genotypes. The lines generating Müller-Thurgau have been discussed more than once on the basis of molecular marker findings (BÜSCHER *et al.* 1994, THOMAS *et al.* 1994, REGNER *et al.* 1996). Genotypes at 8 microsatellite loci excluded the cultivar Silvaner as a parent, not only for Müller-Thurgau but also for Scheurebe and Ehrenfelser, whereas Riesling can be confirmed as a parent in all the crosses investigated in this study. Silvaner was verified in Rieslaner parentage, as well as in that of Optima and Bacchus. It is interesting to note that both Optima and Bacchus genotypes displayed alleles belonging to two different Silvaner

Table 2

Allele sizes (bp) of cultivars typed at microsatellite loci in order to analyze parentage of Riesling crosses and evaluate genetic diversity among ancient grapevines related to the Trentino growing area

	Locus															
Cultivar	VVS1		VVS2		VVS4		VVS5		VVMD5		VVMD6		VVMD7		VVMD8	
Riesling	186	-	139	148	166	-	83	98	222	230	204	206	247	255	137	141
Silvaner	176	186	148	150	166	-	123	144	222	228	187	204	241	245	135	137
Müller-Thurgau	179	186	139	148	166	171	83	107	222	224	197	204	245	255	137	141
Chasselas de C.	179	186	148	152	166	171	107	144	224	232	197	206	241	245	137	152
Pinot b.	179	186	133	148	166	171	119	144	224	234	197	-	237	241	135	137
Ehrenfelser	186	-	139	148	166	171	98	107	222	224	187	204	237	255	137	141
Scheurebe	186	-	139	148	166	-	98	107	222	234	204	206	245	247	141	162
Rieslaner	186	-	148	-	166	-	98	144	222	-	204	206	241	247	137	-
Bacchus	186	-	148	-	166	171	107	123	222	224	204	206	241	245	137	141
Optima	179	186	148	-	166	-	83	123	222	-	197	206	245	247	141	-
Kerner	177	186	148	152	166	-	98	107	222	232	204	206	245	255	137	162
Rotberger	177	186	131	148	166	-	83	107	230	234	204	-	245	255	137	152
Incrocio Manzoni	186	-	148	-	166	-	83	144	222	224	197	206	241	247	137	-
Corvina veronese	158	184	148	152	168	173	107	144	228	236	206	-	237	-	135	162
Groppello gentile	184	186	129	152	168	173	107	144	222	-	187	197	245	251	135	137
Lagrein	177	186	133	152	167	173	107	144	224	234	197	206	237	245	135	152
Lambrusco f.f.	177	186	129	152	168	173	98	144	222	224	197	206	245	247	135	-
Marzemino	177	186	129	-	166	173	107	144	222	228	197	206	237	260	135	137
Nosiola	184	-	129	152	167	173	92	144	222	224	206	-	245	-	137	-
Portugieser	176	-	139	148	167	173	96	123	222	228	187	204	241	253	137	-
Schiava grossa	177	186	131	152	166	-	107	-	232	234	204	206	245	-	152	162
Teroldego	177	179	133	152	171	173	107	144	222	224	197	206	237	245	135	-

types: the "wrong" one related to the Müller-Thurgau case and the cultivar currently known as Silvaner. Genotyping pedigree at newly developed microsatellite repeat loci, SEFC *et al.* (1997) put Chasselas de Courtillier forward as the second parent of Müller-Thurgau. We also defined allele lengths at VVS1, VVS2, VVS4, VVS5, VVMD5, VVMD6, VVMD7 and VVMD8 loci for the Chasselas de Courtillier genome, and found results consistent with this suggestion.

Kerner and Rotberger genotypes are both explained by the segregation of Riesling and Schiava grossa alleles, as well as Incrocio Manzoni 6.0.13, which had alleles in common with the known parents.The pedigree of Incrocio Manzoni 6.0.13 has been discussed on a number of occasions, and the self pollination of Riesling or a cross between Riesling and Chardonnay were suggested instead. Microsatellite and RAPD genotypes verified Incrocio Manzoni 6.0.13 as a cross of Riesling x Pinot (GRANDO *et al.* 1997) but could not identify the male parent Pinot blanc, since the marker system does not show polymorphisms within the Pinot family.

Microsatellite polymorphisms in indigenous wine cultivars: DNA typing the wine grape varieties related to our region, a number of different allele length classes, ranging from 4 at the VVMD6 and VVMD8 loci to 7 at the VVMD7 locus, were detected (Tab. 2). All loci, except for VVS2, represented more than 60 % of the allelic

Table 3

Range of allele lengths estimated in this study and base pairs (bp) offsets when compared to sizes reported by THOMAS and SCOTT (1994) at VVS loci and BOWERS *et al.* (1996) at VVMD loci

Locus	Size range (bp)	Offset (bp)
VVS1	158 - 186	-4
VVS2	121	-5
	129 - 141	-4
	144 - 152	-3
VVS4	165 - 175	-2
VVS5	83 - 85	-7
	92 - 98	-5
	107 - 115	-3
	119 - 144	-2
VVMD5	222 - 242	-4
VVMD6	187	-7
	197 - 206	-8
VVMD7	231 - 255	-2
	260	-3
VVMD8	135 - 141	-6
	152 - 162	-5

Table 4

Similarity values (%) for pairwise comparison of grapevine genotypes at 8 microsatellite loci

1	Lambrusco f.f.	-							
2	Teroldego	63	-						
3	Marzemino	56	56	-					
4	Lagrein	63	75	56	-				
5	Groppello gentile	56	50	56	50	-			
6	Nosiola	50	44	38	44	50	-		
7	Schiava grossa	31	31	31	50	25	19	-	
8	Portugieser	13	38	25	13	25	25	6	-
9	Corvina veronese	38	44	44	44	44	31	25	19	-
		1	2	3	4	5	6	7	8	9

variation drawn by THOMAS and SCOTT (1993) and BOWERS *et al.* (1996) when surveying international *V. vinifera* cultivars.

Indigenous grapevines tested revealed unique genotypes at microsatellite loci, but also shared many common alleles. Even if there were enough DNA polymorphisms to type these cultivars, high allele frequencies (28-44 %) were estimated for one or more length classes at each locus (cases in which only one microsatellite was detected were scored as heterozygous genotypes having a null allele). VVS2 - 152 bp, VVS4 - 173 bp, VVS5 - 144 bp, VVMD5 - 222 bp, VVMD6 - 206 bp, VVMD7 - 245 bp and VVMD8 - 135 bp were the most prevalent DNA fragments. Similarity values among genotypes were calculated as the percentage of common alleles shared by each genotype pair. The wine grape cultivars typically related to the Trentino growing area, i.e. Lambrusco f.f., Teroldego, Marzemino, Lagrein and Groppello gentile, proved to be clustered by a genotype similarity ranging from 50 to 75 % (Tab. 4).

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References

BOWERS, J. E.; DANGL, G. S.; VIGNANI, R.; MEREDITH, C. P.; 1996: Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). Genome **39**, 628-633.

BÜSCHER, N.; ZYPRIAN, E.; BACHMANN, O.; BLAICH, R.; 1994: On the origin of the grapevine variety Müller-Thurgau as investigated by the inheritance of random amplified polymorphic DNA (RAPD). Vitis **33**, 15-17.

DOYLE, J. J.; DOYLE , J. L.; 1990: Isolation of plant DNA from fresh tissue. Focus **12**, 13-15.

GRANDO, M. S.; FRISINGHELLI, C.; MALOSSINI, U.; 1997: Analisi molecolare dei parentali dell'Incrocio Manzoni 6-0-13. L'Enotecnico **12**, 89-92.

LODHI, M. A.; DALY, M. J.; YE, G. N.; WEEDEN, N. F.; REISCH, B. I.; 1995: A molecular marker based linkage map of *Vitis*. Genome **38**, 786-794.

REGNER, F.; STEINKELLNER, H.; TURETSCHEK, E.; STADLHUBER, A.; GLÖSSL, J.; 1996: Genetische Charakterisierung von Rebsorten (*Vitis vinifera*) durch Mikrosatelliten-Analyse. Mitt. Klosterneuburg **46**, 52-60.

SEFC, K.; STEINKELLNER, H.; WAGNER, H. W.; GLÖSSL, J.; REGNER, F.; 1997: Application of microsatellite markers to parentage studies in grapevine. Vitis **36** (4), 179-183.

SMITH, R. N.; 1995: Accurate size comparison of short tandem repeat alleles amplified by PCR. BioTechniques **18**, 122-128.

THOMAS, M. R.; CAIN, P.; SCOTT, N .S.; 1994: DNA typing of grapevines: A universal methodology and database for describing cultivars and evaluating genetic relatedness. Plant Mol. Biol. **25**, 939-949.

- -; SCOTT, N. S.; 1993: Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs). Theor. Appl. Genet. **86**, 985-990.

WEBER, J. L.; 1990: Informativeness of human $(dC\text{-}dA)_n\cdot(dG\text{-}dT)_n$ polymorphisms. Genomics **7**, 524-530.

- -; MAY, P.E.; 1989: Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Amer. J. Human. Genet. **44**, 338-396.

XU, H.; WILSON, D. J.; 1995: Sequence-specific polymerase chain reaction markers derived from randomly amplified polymorphic DNA markers for fingerprinting grape (*Vitis*) rootstocks. J. Amer. Soc. Hort. Sci. **120**, 714-720.

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