

Evaluation of microsatellite sequence-tagged site markers for characterizing *Vitis vinifera* cultivars

by

R. BOTTA¹⁾, N. S. SCOTT²⁾, I. EYNARD¹⁾*) and M. R. THOMAS²⁾

¹⁾ Centro di Studio per il Miglioramento Genetico e la Biologia della Vite, CNR, Torino, Italia

²⁾ CSIRO Division of Horticulture, Adelaide, Australia

S u m m a r y : Twenty four cultivars and clones from Italian germplasm collections were DNA typed for the four sequence-tagged microsatellite sites VVS1-FP, VVS2-JOE, VVS5-FP and VVS29-TAM. The analysis was performed using primers labelled with different fluorochromes and using a GENESCAN apparatus. The results were compared with data obtained for samples from Australian collections. By comparing the combined genotype of the studied loci all the cultivars could be singularly distinguished except Favorita, Pigato and Vermentino, thus supporting the hypothesis that all three are the same cultivar. Cultivars common to Italian and Australian germplasm collections were found to have the same genotype indicating that the study of microsatellites as sequence-tagged site (STS) markers is a suitable universal system for worldwide grapevine cultivar identification. Application of the semi-automated GENESCAN system made the analysis of microsatellite STS markers fast and reproducible between laboratories.

K e y w o r d s : grapevine, cultivar identification, DNA, microsatellite analysis, STMS, molecular markers, database.

Introduction

The requirement for true to type plant material is necessary not only for breeding purposes but also for the planting of vineyards within the EEC (Reg. no 3800/81 and following modifications). In addition, *Vitis vinifera* cultivars have been estimated to number 5000-8000 (TRUEL *et al.* 1980; ALLEWELDT 1988) including common and rare cultivars. Because this germplasm is spread over a number of different international collections it is essential for the future maintenance and classification of the collections that past identification mistakes and cases of synonymy are identified and corrected. Biochemical methods for grapevine cultivar identification have been developed to complement and assist ampelographic identification with DNA methods providing an objective means for determining the genotype of a cultivar.

The ideal requirements for a molecular identification system are: ease of methodology, reproducibility of the analysis within and between laboratories, highly polymorphic markers and simple interpretation of the data. At present few molecular identification systems satisfy all of these requirements. Isozyme analysis has been used (WOLFE 1976; BACHMANN 1989; CALÒ *et al.* 1989) but recently molecular DNA markers have been considered preferable as the marker is a direct indicator of genotype and avoids problems associated with environmental influences, physiological factors and developmental and tissue specific expression. Recently a number of different DNA typing markers have been investigated for grapevine identification including RFLP markers (BLAICH 1989; STRIEM *et al.* 1990; BOURQUIN *et al.* 1992; BOWERS *et al.* 1993; THOMAS *et al.* 1993), RAPD markers (JEAN-JAQUES *et al.* 1993; GOGORCENA *et al.* 1993; BÜSCHER *et al.* 1993; BÜSCHER *et al.* 1994) and

microsatellite sequence-tagged site (STMS) markers (THOMAS and SCOTT 1993; THOMAS *et al.* 1994; BOWERS and MEREDITH 1994).

RAPD markers have been tried for grapevine cultivar identification using a wide range of short primers (JEAN-JAQUES *et al.* 1993; GOGORCENA *et al.* 1993; BÜSCHER *et al.* 1993) but the results obtained from the different laboratories were variable with no agreement on the critical conditions required for the reproducibility of the results. Microsatellite DNA is interspersed in eukaryotic genomes and are regions of up to 100 bp repetitions of 1-4 nucleotide units. Microsatellites, studied as sequence-tagged sites (STSs), have a codominant mode of inheritance and have been suggested to be the marker of choice for both cultivar identification and breeding. It has been demonstrated that grapevine STMS markers are highly polymorphic and suitable for cultivar identification (THOMAS and SCOTT 1993; THOMAS *et al.* 1994).

This study investigates the usefulness and reproducibility of STMS marker analysis and compares the DNA typing results of cultivars common to both the Australian germplasm collections and Northern Italy collections. In addition, DNA profile data of cultivars grown in Northern Italy and internationally popular French cultivars were collected and added to the international grapevine DNA database maintained at CSIRO Division of Horticulture.

Materials and methods

Leaf samples were harvested from 21 cultivars and 2 clones of Dolcetto, Grignolino and Chardonnay. Leaves were sampled from the collection fields of the "Centro per il Miglioramento Genetico e la Biologia della Vite - CNR"

Correspondence to: Dr. R. BOTTA, Centro di Studio per il Miglioramento Genetico e la Biologia della Vite, CNR, Via Pietro Giuria 15, I-10126 Torino, Italy. — Contribution no 300 of Centro Miglioramento Genetico e Biologia della Vite.

*) deceased in February 1993

located in Grugliasco and Chieri (Torino - Italy). The samples were stored at -70°C and transported in dry ice from Italy to Australia for DNA extraction. 2 g of tissue were ground in liquid nitrogen to a fine powder and the DNA was extracted following the procedure described by THOMAS *et al.* (1993) using a TRIS-EDTA-NaCl buffer containing 3% Sarkosyl and 20% ethanol. After purification, the DNA was finally suspended in 200 μl Tris-EDTA buffer with an average yield of 25 $\mu\text{g/g}$ tissue FW.

The samples were DNA typed at the 4 STMS loci VVS1-FP, VVS2-JOE, VVS5-FP, VVS29-TAM identified and described by THOMAS and SCOTT (1993) and by THOMAS *et al.* (1994). The oligonucleotide primers flanking the microsatellite sequences were used for PCR amplification. One primer of each pair had an attached fluorochrome in order to obtain fluorescent PCR products labelled with one of the following dyes: blue (FluorePrime, Pharmacia), green (JOE, Applied Biosystems Instruments) or yellow (TAMRA, ABI). The PCR was performed using 20 μl of a mixture containing 50 ng DNA, 1.25 U of Taq DNA polymerase (Promega), reaction buffer (Promega, 50 mM KCl, 1.5 mM MgCl_2 , 10 mM Tris-HCl, pH 9 and 0.1% Triton X-100), 0.5 μM of each primer and 200 μM of each dNTP. PCR conditions were: 3 min at 95°C , then 26 cycles of denaturation (45 s at 94°C), annealing (30 s at 50°C) and extension (1 min 30 s at 72°C); a final elongation step was done at 72°C for 7 min. The reaction was performed using a Corbett FTS-1 fast thermal cyclor.

For the gel analysis 0.5 μl of each PCR sample was used as described by THOMAS *et al.* (1994). For each cultivar, the loci VVS1-FP, VVS2-JOE and VVS29-TAM were mixed together and analysed in the same lane since they were labelled with different fluorochromes. The locus VVS5-FP was analysed separately. All samples loaded on a gel also contained a standard DNA size marker labelled with a red fluorescent dye (ROX, ABI). Samples were analysed, after denaturation in 3 μl formamide at 94°C for 3 min, on a sequencing gel (6% polyacrylamide 19:1, 8.3 M urea, 1x TBE buffer) in an automated Applied Biosystems 373A DNA sequencing apparatus using GENESCAN software. The data obtained was collated and transferred into the grapevine DNA database.

Results and discussion

The GENESCAN system collects data from each gel and stores it in a digital format for computer analysis. A digital image of the gel is generated in addition to electrophoretograms for each locus and tabulated data that includes assigned sizes, in base pairs (bp), for each allele. The Figure is an example of electrophoretogram profiles and tabulated data obtained for the loci VVS1-FP, VVS2-JOE and VVS29-TAM of Erbaluce. Each DNA band (allele) on a gel is represented by a peak in the electrophoretogram. Selection of a peak within an electrophoretogram highlights the tabulated data for that allele, in the example (Figure) the VVS2-JOE (VS2) allele selected has a size of 151 bp.

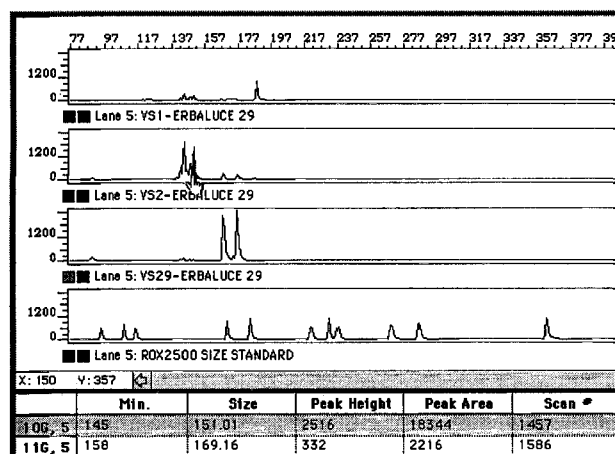


Figure: Electrophoretogram profiles and tabulated GENESCAN data for the cultivar Erbaluce. The information was collected from a single gel lane. The top profile is for the locus VVS1-FP the next is the locus VVS2-JOE followed by the VVS29-TAM locus and lastly the profile of the size standard. The values across the top profile represent the size in base pairs. The second peak (allele) for the VVS2-JOE locus has been selected (shown by the hand icon) and the relevant data is automatically highlighted in the table at the bottom. An allele size of 151.01 bp is displayed for this allele which becomes 151 bp when rounded to whole base pairs.

Tab. 1 shows the DNA profile data of cultivars collected from germplasm collections in Italy. For each VVS locus the size of alleles are recorded according to THOMAS *et al.* (1994). The combined genotype across all loci represents the particular DNA profile of a cultivar, for example, the DNA profile of Albarola is 190:181 (VVS1-FP), 155:133 (VVS2-JOE), 146:97 (VVS5-FP) and 171:- (VVS29-TAM). All the 21 cultivars studied for the 4 loci could be singularly distinguished from the others with the exception of Favorita, Vermentino and Pigato which had the same alleles for the analysed loci (Tab. 1). Favorita is grown in Piemonte, Pigato is from Liguria and Vermentino is cultivated in Liguria, Sardinia, Toscana and Corsica; the 3 cultivars are considered by SCHNEIDER and MANNINI (1990) being the same cultivar and the results of the DNA analysis support this hypothesis.

The DNA profile data obtained for the same cultivars typed from Italy and from Australia were consistent (Tab. 2) and confirmed the usefulness and reproducibility of STMS markers for cultivar identification. Similarly, the clones studied Dolcetto, Grignolino and Chardonnay had the same alleles for all 4 loci (Tab. 2), demonstrating that the technique accurately confirms the identity of cultivars but is in general not suitable for characterizing clonal differences (THOMAS and SCOTT 1993). It should be emphasised that the genetic data for the Australian plants were collected during 1992 and early 1993 and entered into the DNA database while the plants grown in Italy were analysed during November 1993. Thus the information in Tab. 2 illustrates a number of important advantages of the system; first - clones grown in different environments have the same DNA profile, second - different DNA samples from the same cultivar produce the same DNA profile and third - clones of the same cultivar do not have to be compared on

Table 1
DNA profiles of cultivars from Italian collections

CLONE	CULTIVAR	DNA No.	VVS1-FP		VVS2-JOE		VVS5-FP		VVS29-TAM	
			alleles	alleles	alleles	alleles	alleles	alleles		
CVT 18/CNR/CHIERI (TO)	Bosco	IT1	181	-	135	133	146	110	171	-
CVT AT 261/CNR/CHIERI (TO)	Grignolino	IT2	190	-	135	133	121	97	179	171
STD/CNR/CHIERI (TO)	Croatina	IT3	181	-	151	139	110	97	181	171
STD/CNR/CHIERI (TO)	Cortese	IT4	181	-	151	133	146	120	181	171
CVT TO 29/CNR/CHIERI (TO)	Erbaluce	IT5	190	-	151	145	97	-	179	171
CN 111/CNR/CHIERI (TO)	Nebbiolo	IT6	190	187	155	-	146	110	179	171
CVT 3/CNR/CHIERI (TO)	Albarola	IT7	190	181	155	133	146	97	171	-
CVT AT 424/CNR/CHIERI (TO)	Barbera	IT8	190	183	135	133	120	90	171	-
CVT 154/CNR/CHIERI (TO)	Freisa	IT9	190	187	155	133	103	97	179	171
CVT CN 16/CNR/CHIERI (TO)	Moscato	IT10	181	-	133	-	110	-	171	-
CVT CN 19/CNR/CHIERI (TO)	Arneis	IT11	190	183	135	-	121	97	171	-
CVT AL 275/CNR/CHIERI (TO)	Dolcetto	IT12	190	181	143	139	110	97	171	-
CVT AT 159/CNR/CHIERI (TO)	Malvasia Casorzo	IT13	181	-	151	133	121	101	179	171
CN 69/CNR/CHIERI (TO)	Dolcetto	IT14	190	181	143	139	110	97	171	-
CVT 84/CNR/CHIERI (TO)	Vermentino	IT15	190	-	151	133	118	90	171	-
CVT 105/CNR/CHIERI (TO)	Favorita	IT16	190	-	151	133	118	90	171	-
CVT 121/CNR/CHIERI (TO)	Pigato	IT18	190	-	151	133	118	90	171	-
STD/CNR/CHIERI (TO)	Vespolina	IT19	190	183	155	143	110	97	179	-
CVT AT 275/CNR/CHIERI (TO)	Grignolino	IT17	190	-	135	133	121	97	179	171
STD/CNR/GRUGLIASCO (TO)	Cabernet Sauvignon	IT20	181	-	151	139	123	103	181	179
STD/CNR/GRUGLIASCO (TO)	Merlot	IT21	190	181	151	139	146	121	181	175
STD/CNR/GRUGLIASCO (TO)	Chardonnay	IT22	190	183	143	137	146	90	179	171
STD/CNR/GRUGLIASCO (TO)	Chardonnay	IT23	190	183	143	137	146	90	179	171
STD/CNR/GRUGLIASCO (TO)	Pinot Noir	IT24	190	183	151	137	146	121	179	171

Table 2
Comparison of clones grown in Italy and Australia

CLONE	CULTIVAR	DNA No.*	VVS1-FP		VVS2-JOE		VVS5-FP		VVS29-TAM	
			alleles	alleles	alleles	alleles	alleles	alleles		
FVF6V4/VX/UCD HT119	Barbera	C383	190	183	135	133	120	90	171	-
CVT AT 424/CNR/CHIERI (TO)	Barbera	IT8	190	183	135	133	120	90	171	-
FVG9V3/VX/UCD	Cabernet Sauvignon	C198	181	-	151	139	123	103	181	179
STD/CNR/GRUGLIASCO (TO)	Cabernet Sauvignon	IT20	181	-	151	139	123	103	181	179
FV110V5/CX/UCD	Chardonnay	C13	190	183	143	137	146	90	179	171
STD/CNR/GRUGLIASCO (TO)	Chardonnay	IT22	190	183	143	137	146	90	179	171
STD/CNR/GRUGLIASCO (TO)	Chardonnay	IT23	190	183	143	137	146	90	179	171
FVD5V12A/VX/UCD	Pinot Noir	C27	190	183	151	137	146	121	179	171
STD/CNR/GRUGLIASCO (TO)	Pinot Noir	IT24	190	183	151	137	146	121	179	171
NFD3V14/VX/UCD	Merlot	C17	190	181	151	139	146	121	181	175
STD/CNR/GRUGLIASCO (TO)	Merlot	IT21	190	181	151	139	146	121	181	175
CVT AL 275/CNR/CHIERI (TO)	Dolcetto	IT12	190	181	143	139	110	97	171	-
CN 69/CNR/CHIERI (TO)	Dolcetto	IT14	190	181	143	139	110	97	171	-
CVT AT261/CNR/CHIERI (TO)	Grignolino	IT2	190	-	135	133	121	97	179	171
CVT AT 275/CNR/CHIERI (TO)	Grignolino	IT17	190	-	135	133	121	97	179	171

* DNA nos starting with "C" represent plants grown in Australia while DNA nos starting with "IT" refer to plants grown in Italy.

the same gel as the information in the database is suitable for inter-gel comparisons.

The combined use of grapevine STMS markers and a semi-automated analysis system like GENESCAN satisfies many of the requirements for the ideal molecular identification system. The methodology is quick and uncomplicated, cultivar DNA profiles are reproducible within and between laboratories, grapevine STMS markers are highly polymorphic and being single locus markers the data interpretation is simple. The reproducibility of the grapevine STMS-GENESCAN system also makes the method particularly attractive for building a common international

grapevine DNA database accessible to all institutions involved in grapevine identification. However, at the moment, the high cost of the instrument makes it unaffordable for many laboratories. An option for laboratories wishing to use GENESCAN for DNA typing, but not wanting to purchase a machine, may be to use machines in other laboratories that are able to offer such a service. Alternative methods of STMS analysis have been tried and successfully used but they are more time consuming than GENESCAN and require an appropriate size standard to determine the size of the alleles if the information is to be added to the international databank.

Acknowledgements

This research was carried out as part of a joint CNR/CSIRO research collaborative project titled "DNA fingerprinting of grapevine and other horticultural species". We thank P. CAIN for technical assistance.

References

- ALLEWELDT, G.; 1988: The genetic resources of *Vitis*. Federal Research Centre for Grape Breeding, Geilweilerhof, Germany.
- BACHMANN, O.; 1989: Isoenzymes as a tool for grape cultivar identification. *Riv. Viticolt. Enol.* **42** (1), 27-31.
- BLAICH, R.; 1989: The analysis of restriction fragment length polymorphism as a tool for the differentiation of grapevine cultivars. *Riv. Viticolt. Enol.* **42** (1), 33-35.
- BOURQUIN, J. C.; TOURNIER, P.; OTTEN, L.; WALTER, B.; 1992: Identification of sixteen grapevine rootstocks by RFLP and RFLP analysis of nuclear DNA extracted from the wood. *Vitis* **31**, 157-162.
- BOWERS, J. E.; BANDMAN, E. B.; MEREDITH, C. P.; 1993: DNA fingerprint characterization of some wine grape cultivars. *Amer. J. Enol. Viticult.* **44**, 266-274.
- ; MEREDITH, C. P.; 1994: Microsatellite length polymorphism within ancient wine grape cultivars (*Vitis vinifera* L.). II Intern. Conf. Plant Genome, 24-27 January 1994, San Diego, CA, USA.
- 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.
- ; --; BLAICH, R.; 1993: Identification of grapevine cultivars by DNA analyses: Pitfalls of Random Amplified Polymorphic DNA techniques using 10mer primers. *Vitis* **32**, 187-188.
- CALÒ, A.; COSTACURTA, A.; PALUDETTI, G.; CALÒ, G.; ARULSEKAR, S.; PARFITT, D.; 1989: The use of isozyme markers to characterize grape cultivars. *Riv. Viticolt. Enol.* **42** (1), 15-22.
- GOGORCENA, Y.; ARULSEKAR, S.; DANDEKAR, A. M.; PARFITT, D. E.; 1993: Molecular markers for grape characterization. *Vitis* **32**, 183-185.
- JEAN-JAQUES, I.; DEFONTAINE, A.; HALLET, J. N.; 1993: Characterization of *Vitis vinifera* cultivars by Random Amplified Polymorphic DNA markers. *Vitis* **32**, 189-190.
- SCHNEIDER, A.; MANNINI, F.; 1990: Indagine comparativa su "Vermentino", "Pigato" e "Favorita" in Piemonte e Liguria. *L'Informatore Agrario* **46** (8), 103-108.
- STRIEM, M. J.; SPIEGEL-ROY, P.; BEN-HAYYIM, G.; BECKMANN, J.; GIDONI, D.; 1990: Genomic DNA fingerprinting of *Vitis vinifera* by the use of multi-loci probes. *Vitis* **29**, 223-227.
- 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.
- ; MATSUMOTO, S.; CAIN, P.; SCOTT, N. S.; 1993: Repetitive DNA of grapevine: classes present and sequences suitable for cultivar identification. *Theoret. Appl. Genet.* **86**, 173-180.
- ; SCOTT, N. S.; 1993: Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence tagged sites (STSs). *Theoret. Appl. Genet.* **86**, 985-990.
- TRUEL, P.; RENNES, C.; DOMERGUE, P.; 1980: Identification in collections of grapevine. *Proc. 3rd Intern. Symp. Grape Breed. Davis, CA, USA*, 78-86.
- WOLFE, W.H.; 1976: Identification of grape varieties by isozyme banding patterns. *Amer. J. Enol. Viticult.* **27**, 68-73.

Received August 28, 1994