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

by

R. Botta[1]), N. S. Scott[2]), I. Eynard[1])*) and M. R. Thomas[2])

[1]) Centro di Studio per il Miglioramento Genetico e la Biologia della Vite, CNR, Torino, Italia
[2]) CSIRO Division of Horticulture, Adelaide, Australia

Summary: 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.

Key words: 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

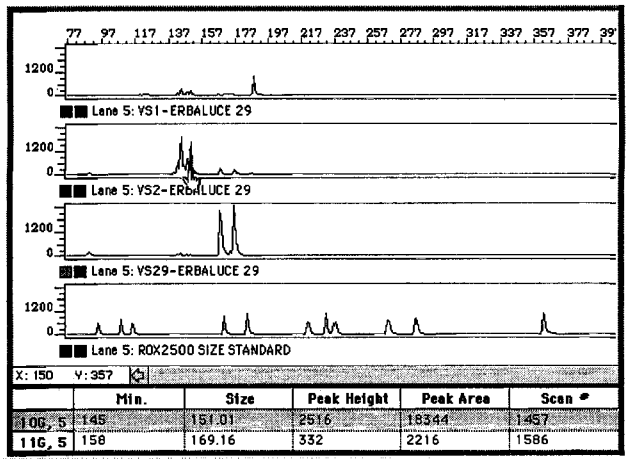
located in Grugliasco and Chieri (Torino - Italy). The samples were stored at -70 °C and transported in dry ice from Italy to Australia for DNA extraction. 2 g of tissue were ground in liquid nitrogen to a fine powder and the DNA was extracted following the procedure described by THOMAS *et al.* (1993) 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 µg/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 cycler.

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.




| | Min. | Size | Peak Height | Peak Area | Scan # |
|---|---|---|---|---|---|
| 106, 5 | 145 | 151.01 | 2516 | 16344 | 1457 |
| 116, 5 | 158 | 169.16 | 332 | 2216 | 1586 |

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. Similarily, 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 alleles | | VVS2-JOE alleles | | VVS5-FP alleles | | VVS29-TAM 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 alleles | | VVS2-JOE alleles | | VVS5-FP alleles | | VVS29-TAM 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 |
| FVI10V5/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.

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## 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 lenght 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.

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