Vitis 33, 211–215 (1994)

Grapevine fingerprinting using microsatellite repeats

by

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Summary: Five different microsatellite loci have been amplified through PCR (Polymerase Chain Reaction) in order to find polymorphisms useful for identification of 16 cvs and 42 clones or populations of *Vitis vinifera* L. No genetic variability was detected among clones and populations. All the cultivars have been identified, except those derived from bud mutations involving the colour of the berries, as the Pinot group (P. noir, P. blanc, P. gris), or those thought to be closely correlated or synonymous, such as 'Refosco di Faedis' and 'Refoscone'. One locus (VVS2) showed a high polymorphism with 12 alleles detected on high resolution agarose gel.

Key words: genetic markers, cultivar identification, PCR, Vitis vinifera L.

Introduction

Historically grape cultivars have been identified by comparison of morphological characters, a method based on the appearance of leaf, fruit, shoot tip and other organs. The expression of many of these traits are often affected by diseases or developmental and environmental factors. The morphological variabilities and the subjective evaluations made by man, limit ampelographic identification. Alternative biochemical approaches have been developed including phenolic or aromatic compounds analyses as well as serological analysis of pollen proteins (Samann and Wallace 1981). Isozymes have been used for cultivar identification in grapevines (Wolfe 1976, Stavrakakis and Loukas 1983, Subden *et al.* 1987, Bachmann and Blaich 1988, Benin *et al.* 1988, Parfitt and Arulsekar 1989, Walters *et al.* 1989) although problems associated with enzyme extraction, the general lack of discriminating enzyme systems and inconsistency in assaying enzymes have hampered the wide diffusion of the technique. Recently DNA analysis has presented a new approach for cultivar identification. RFLP (Restriction Fragment Length Polymorphism) markers (Striem *et al.* 1990, Bourquin *et al.* 1992, Bowers *et al.* 1993) as well as the generation of Random Amplified Polymorphic DNA (RAPDs) (Williams *et al.* 1990) by means of the PCR technology have been used in grapevine fingerprinting. The latter procedure has recently been used for the intraspecific characterization of *Vitis vinifera* (Jean-Jaques *et al.* 1993, Büscher *et al.* 1993, Gogorcena *et al.* 1993) and this is the method of choice when there is no previous knowledge about DNA sequences in the genome. Different classes of repetitive DNA sequences have been investigated in order to identify suitable markers (Thomas *et al.* 1993). The most informative or polymorphic markers appear to be the microsatellite repeats sequences. Microsatellite DNA consists of small repeated units, generally less than 4 nucleotides, which are present many times within a genome (Litt and Luty 1989, Tautz 1989). PCR primers designed to amplify such regions greatly increase the possibility of finding DNA polymorphisms (Morgante and Olivieri 1993).

Five primers amplifying 5 different loci in microsatellite regions in the grapevine genome have been recently discovered (Thomas and Scott 1993). A screening of 26 *V. vinifera* cultivars and 7 other species showed the markers were very informative for detecting high heterozygosity and high genetic variations between varieties. The present work aimed at: a) verifying the usefulness of PCR amplified microsatellites to detect the extent of genetic variability in *Vitis* using the 5 pairs of primers designed by Thomas and Scott (1993); b) improving the procedure described by the previous authors by eliminating the hazards linked to radioactive nucleotide labelling and the use of acrilamide on gels; c) checking the diversity within groups of closely related cultivars and clones of *V. vinifera*.

Material and methods

Plant material used for DNA extraction was obtained from a nursery (Vivai Cooperativi Rauscedo, Rauscedo, Italy) or from private farmers. Canes from each individual accession and cultivar (Tab. 1) were collected during the rest period in the winter and kept at 4 °C until used. Most cultivars were represented by selected clones. The cv. 'Ribolla' was represented by 2 recently selected clones and 5 locally known accessions. The cv. 'Verduzzo friulano' was only represented by unselected accessions. 'Refosco di Faedis' and ' Refoscone', which are recognised as belonging to the 'Refosco' family even if clearly distinguishable from other clones because of some morphological traits, were also represented by local accessions. The canes were forced in water and very young leaves collected and immediately used for DNA extraction or kept in a freezer at -80 °C. Three plants of 'Terrano' and 2 of 'Ribolla gialla Badia di Rosazzo' were selected to test the individual variability in all the five microsatellite loci.

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Table 1

List of cultivars, clones and accessions of *Vitis vinifera* used for DNA fingerprinting

Cultivar	Clone or accession
Cabernet franc	VCR 10, VCR 27, ENTAV 212.
Cabernet Sauvignon	R 5, ENTAV 338.
Carménère	VCR 9.
Chardonnay	R8, SMA 130, VCR 4, ENTAV 77, ENTAV 95.
Picolit	R 3.
Pinot blanc	VCR 1, VCR 5, VCR 7, VCR 9, ENTAV 54.
Pinot noir	VCR 4, INRA 113, INRA 115.
Pinot gris	VCR 5, VCR 7, ENTAV 49.
Refosco dal peduncolo rosso	ISVF 1, ISVF 4T.
Refosco di Faedis	
Refoscone	
Ribolla gialla	VCR 30, VCR 31, R. g. di Mernicco, R. g. Badia di Rosazzo, R. g. di Albana, R. verde di Albana, R. Battistig di Prepotto.
Ribolla nera	
Ribolla spizade	
Terrano	ISVF 2.
Verduzzo friulano	V. verde, V. dal peduncolo rosso, V. rosso, V. sanguigno, V. giallo di Ramandolo.

DNA extraction: DNA was extracted using the method of Doyle and Doyle (1990) with the following modifications: 1 g of leaves for each sample was frozen in liquid nitrogen, ground to fine powder and suspended in 6 ml of CTAB (hexadecyltrimethyl ammonium bromide) solution with the addition of 1% PVP 40 (polyvinylpyrrolidone) kept for 10 min at 65 °C. An equal volume of chloroform/isoamyl alcohol 24:1 was then added and the samples centrifuged for 20 min at 6000 *g*.

PCR protocols: Five pairs of primers, flanking the microsatellite DNA regions found in *Vitis* by THOMAS and SCOTT (1993) coded as VVS1, VVS2, VVS3, VVS4, VVS5 (THOMAS and SCOTT 1993) were synthesized (Genenco, Firenze, Italy) and utilized in order to amplify the repeat regions in the grapevine samples. Polymerase chain reactions (SAIKI *et al.* 1988) were carried out in 25 µl volume containing 24-200 ng of genomic DNA, 0.2 µM each primer, 200 µM each dNTP, 50 mM KCl, 10 mM Tris (pH 8.3), 0.01% gelatine, 2.5 mM $MgCl_2$ (1.5 mM with VVS3 primers) and 0.5 unit of Taq polymerase (Boehringer Mannheim, Germany). Samples were overloaded with 25 µl mineral oil. PCR reactions were carried out using a Perkin Elmer-Cetus Thermal Cycler 480 with the following profile: (i) 95 °C for 5 min per 1 cycle, (ii) 80 °C for 6 min per 1 cycle; (iii) 94 °C for 1 min, 50 °C for 50 s, 72 °C for 1 min per 26 cycles; (iiii) 72 °C for 7 min per 1 cycle. The polymerase was added during the 6 min period at 80 °C. The above annealing temperature (step iii) was increased to 55 °C using VVS3 primers (see results). 20 µl of each amplified sample was directly loaded into 4 % agarose gels (Metaphor FMC Bioproducts) in TBE buffer (SAMBROOK *et al.* 1989) containing 0.5 µg of ethidium bromide and electrophorised without any purification of the PCR mixture. The samples were run for 4-7 h at 3.5 V/cm.

Results

Amplification: All pairs of primers were successful in amplifying microsatellite repeats in the samples tested. The experiments were highly reproducible and no variability was detected among the 3 plants of 'Terrano' and the 2 plants of 'Ribolla gialla Badia di Rosazzo' tested. Amplified products were obtained both with 100 or 200 ng of genomic DNA but with the primers VVS1, VVS2, and VVS3, the lower concentration produced very faint bands on agarose gels. In contrast VVS5 primers were effective also with 24 and 50 ng of genomic DNA per reaction (the lowest concentrations tested). Some samples often gave faint amplified fragments with all the primers tested, probably due to the presence of contaminants inhibiting the PCR reactions. VVS3 primers amplified a non- specific fragment of about 450 bp with our standard PCR profile and it was far from the 100 - 200 bp, which is the range of fragment lengths reported by THOMAS and SCOTT (1993). Increasing the annealing temperature to 55 °C and lowering the Mg^{2+} concentration to 1.5 mM, caused more stringent amplification conditions and resulted in removing of the largest band.

In all cases bands were easily scorable and well separated one from the other ones.

Microsatellite polymorphism: All 5 microsatellite loci were found to be polymorphic. VVS2 (Fig. 1) and VVS5 (Fig. 2) were the most polymorphic loci and VVS3 the least (Tab. 2). The VVS1 amplified frag-

Fig. 1: VVS2 amplified microsatellite in 16 cultivars of *Vitis vinifera*. The first and last lanes are loaded with pBR322, Hae III digest, molecular marker. Lanes 2 - 13 represent 12 different groups of cultivars as listed in Tab. 2.

Table 2

Polymorphisms at the five microsatellite loci found among the grapevine cultivars tested

Locus	Pattern	Cultivars
VVS1	1	Chardonnay, Picolit, Pinot noir, P. gris, P. blanc, Ribolla spizade, Terrano;
	2	Cabernet franc, Cabernet Sauvignon, Carménère;
	3	Refoscone, Refosco di Faedis, Ribolle gialle [1];
	4	Refosco dal peduncolo rosso;
	5	Ribolla nera, Verduzzi [2];
VVS2	1	Pinot noir, Pinot blanc, Pinot gris;
	2	Chardonnay;
	3	Ribolla spizade;
	4	Ribolle gialle [1];
	5	Refoscone, Refosco di Faedis;
	6	Picolit;
	7	Terrano;
	8	Refosco dal peduncolo rosso;
	9	Ribolla nera;
	10	Verduzzi [2];
	11	Cabernet franc, Carménère;
	12	Cabernet Sauvignon;
VVS3	1	Chardonnay, Pinot noir, P. blanc, P. gris, Refosco dal peduncolo rosso, Ribolla nera, Ribolla spizade, Terrano, Cabernet franc, Cabernet Sauvignon, Carménère;
	2	Ribolle gialle [1], Refoscone, Refosco di Faedis;
	3	Verduzzi [2], Picolit;
VVS4	1	Ribolle gialle [1], Ribolla spizade, Refoscone, Refosco di Faedis, Picolit;
	2	Refosco dal peduncolo rosso;
	3	Ribolla nera, Cabernet franc, Cabernet Sauvignon, Carménère;
	4	Chardonnay, Pinot noir, Pinot blanc, Pinot gris, Terrano;
	5	Verduzzi [2];
VVS5	1	Pinot noir, Pinot blanc, Pinot gris;
	2	Chardonnay;
	3	Ribolla spizade;
	4	Ribolle gialle [1];
	5	Refosco di Faedis, Refoscone;
	6	Verduzzi [2];
	7	Picolit, Terrano;
	8	Refosco dal peduncolo rosso;
	9	Cabernet franc, Ribolla nera;
	10	Carménère;
	11	Cabernet Sauvignon.

[1] The name Ribolle gialle includes all the populations of Ribolla gialla included in Tab. 1.

[2] The name Verduzzi includes all the populations of Verduzzo presented in Tab. 1.

Fig. 2: VVS5 amplified microsatellite in 16 cultivars of *Vitis vinifera*. The first and last lanes are loaded with pBR322, Hae III digest, molecular marker. Lanes 2 - 12 represent 11 different groups of cultivars as listed in Tab. 2.

ments were approximately 200 - 184 bp long, VVS2 fragments ranged in size from 180 - 120 bp long, VVS3 fragments from 260 to 218 bp, VVS4 fragments were about 180 bp long with small differences between the visible bands, and VVS5 fragments were about 150–60 bp long. VVS5 primers probably either amplified 2 loci or a second non-specific product that had not been eliminated. The probable second locus was 60 bp long and it was not polymorphic but was present in all cultivars tested. If such a fragment is disregarded, the polymorphic VVS5 locus had fragments ranging from 150 to 80 bp.

The number of different alleles found was 5, 10, 3, 4, 7 for VVS1 - VVS5 respectively. The differences in length between the closest bands still distinguishable on agarose gel was estimated to be of 4 bp. The heterozygosity values ranged from 0 % in VVS3 locus to 81.2 % in VVS5 locus with intermediate values of 62.5 % in VVS1 and VVS4 loci, and 68.8 in VVS2 locus. VVS3 with our amplification conditions showed one band of different length for all

Table 3

Grouping of the tested grapevine cultivars and clones according to the five microsatellite loci

Group	Cultivar (clones)	patterns				
		a	b	c	d	e
1	Cabernet franc	2	11	1	3	9
2	Cabernet Sauvignon	2	12	1	3	11
3	Carménère	2	11	1	3	10
4	Chardonnay (all)	1	2	1	4	2
5	Picolit	1	6	3	1	7
6	Pinot noir, blanc, gris (all)	1	1	1	4	1
7	Refosco dal peduncolo rosso	4	8	1	2	8
8	Refosco di Faedis	3	5	2	1	5
9	Refoscone	3	5	2	1	5
10	Ribolla gialla	3	4	2	1	4
11	Ribolla nera	5	9	1	3	9
12	Ribolla spizade	1	3	1	1	3
13	Terrano	1	7	1	4	7
14	Verduzzo friulano	5	10	3	5	6

a = VVS1, b = VVS2, c = VVS3, d = VVS4, e = VVS5.

groups of cultivars (Tab. 2). Occasionally for less stringent conditions other faint bands appeared.

The cultivars were grouped using VVS2 and VVS5 primers respectively while the other loci were less informative (Tab. 3). It was never possible to distinguish the Pinot noir, blanc and gris on the basis of the pattern obtained (Tab. 3). The 'Refosco group' includes the cultivars: 'Refoscone', 'Refosco di Faedis', 'Refosco dal peduncolo rosso' and 'Terrano'. The first 2 for every locus showed the same electrophoretic profile. All populations of 'Ribolla' had a similar pattern for the 5 loci tested with the exception of 'Ribolla nera' and 'Ribolla Spizade' which were distinguishable from the other 'Ribolla' accessions. Using the primers for VVS2 and VVS5 loci, all cultivars showed a unique pattern except those of the Pinot group on one hand and 'Refosco di Faedis' and 'Refoscone' on the other.

Discussion

THOMAS and SCOTT (1993) proposed a protocol where PCR products were labelled during synthesis with ^{35}S dATP in a 10 µl PCR mix and the samples were loaded onto a (40 cm) long sequencing gel. The present work proves that amplified microsatellite repeats can be analyzed on high resolution agarose gel stained with ethidium bromide without the use of radioactive labelled material, allowing reliable grapevine cultivar fingerprinting to be performed in a greater number of laboratories. The possibility of distinguishing close bands was very high. In the 60 bp length range of the VVS2 amplified fragments 10 different alleles were found in the cultivars tested.

Most of the examined cultivars produced unique patterns, and this allowed the accurate and reliable identification of them among all cultivar tested. However the Pinot group remains the main exception, because the 3 cultivars tested, namely Pinot noir, Pinot blanc and Pinot gris, showed the same banding pattern on all 5 microsatellite sequences analysed. Recently BOWERS *et al.* (1993) have reported Pinot noir and Pinot gris as having indistinguishable banding patterns when checked for 38 fragments produced in an experiment of Southern blotting where 5 different cDNA fragments were used as probes. Pinot blanc and Pinot gris very likely originated as bud mutations from Pinot noir, the most ancient cultivar among the three. The differences are thought to be due to one or a few genes affecting the colour of the berry (GALET 1990).

Among the 'Refosco' group, a family of closely related genotypes widely grown in north-eastern Italy, 4 cultivars revealed 3 different patterns, with 'Refosco di Faedis' and 'Refoscone' sharing the same banding pattern in all DNA repeats. 'Refosco di Faedis' and 'Refoscone' are sometimes considered to be the same cultivar because of the difficulties distinguishing them on the basis of morphological characters (MARZOTTO 1923). Both are cultivated in the same area on the Faedis hills (Friuli, north-eastern, Italy). The 'Terrano'(also called 'Refosco d'Istria') which originated probably on the karstic hills of eastern Friuli-Venezia Giulia (Italy) or in the Istrian peninsula (Slovenia, Croatia) is considered to belong to the 'Refosco' group but with some particular characteristics. The molecular analysis based on microsatellite repeats confirmed the differentiation among the cultivars tested and strengthen the hypothesis of the homonymy for the 'Refosco di Faedis' and 'Refoscone'.

A limited area of eastern Friuli is the center of origin and the zone of greatest cultivation of the 'Ribolla' group. By molecular analysis 3 cultivars have been identified: 'Ribolla nera', 'Ribolla spizade' and the remaining 'Ribolla'. It was never possible to differentiate the 'Ribolla gialla' and 'Ribolla verde' populations and it seems that the unique diversity among them relies on some morphological trait. Probably the difference among those 'Ribolla' accessions is of the same order of magnitude as the difference among clones of a cultivar, and is not appreciated in this type of analysis. Cabernet franc and Carménère are close but different cultivars, as indicated in VVS5 locus, indirectly confirming the findings of CALÒ *et al.* (1991) who observed isozyme patterns.

All 5 amplified microsatellite repeats failed to separate clonal selections in all cultivars considered in the present study. It may be that clones are often selected on the base of phenotypic observations strongly affected by environment (disease load and others), as pointed out by THOMAS *et al.* (1993). Furthermore it could be expected that some clones differ from the others because of a few mutations. In this case specific markers linked with those mutations are necessary. As the grapevine DNA will be saturated with increasing numbers of markers, the separation of the true clones within a given cultivar will become more feasible.

Acknowledgement

This research was conducted with financial support of the Italian National Research Council (Consiglio Nazionale delle Ricerche), coordinated project 'Woody Plants Germplasm'.

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Received May 19, 1994