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Repetitive DNA of grapevine: classes present and sequences suitable for cultivar identification

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Summary. Repetitive DNA sequences present in the grapevine genome were investigated as probes for distinguishing species and cultivars. Microsatellite sequences, minisatellite sequences, tandemly arrayed genes and highly repetitive grapevine sequences were studied. The relative abundance of microsatellite and minisatellite DNA in the genome varied with the repeat sequence and determined their usefulness in detecting RFLPs. Cloned *Vitis* ribosomal repeat units were characterised and showed length heterogeneity (9.14–12.15 kb) between and within species. A highly repetitive DNA sequence isolated from *V. vinifera* was found to be specific only to those species classified as *Euvitis*. DNA polymorphisms were found between *Vitis* species and between cultivars of *V. vinifera* with all classes of repeat DNA sequences studied. DNA sequences suitable for DNA fingerprinting gave genotype-specific patterns for all of the cultivars and species examined. The DNA polymorphisms detected indicates a moderate to high level of heterozygosity in grapevine cultivars.

Key words: DNA fingerprinting – Grapevine – RFLP – rDNA – Satellite DNA – *Vitis*

Introduction

Grapevine (*Vitis*) cultivation had its origins with the domestication of *V. vinifera* (L.) around 4000 BC (Olmo 1976). Today, cultivars of *V. vinifera* still represent the major grapevine cultivars grown worldwide for fruit and wine production, with *Euvitis* interspecific hybrids from recent breeding programmes restricted to particular regions (Dry and Gregory 1988). Grapevine cultivars have a long history of domestication, and the vegetatively propagated cultivars have spread throughout the world. This history has contributed to large lists of cultivar names, 14,000 (Alleweldt 1988)–24,000 (Viala and Vermorel 1909). However, it is generally believed that the actual number of cultivars is much lower, with figures of between 5,000–8,000 being suggested (Alleweldt 1988, Truel et al. 1980). With the difference between cultivars often being quite small it is easy to understand how over time they become wrongly identified and misnamed (McCarthy 1988). This confusion is compounded by the addition of the large number of clonal selections made from individual cultivars, often on performance rather than on morphological characters.

The identification of grapevines has traditionally relied on individuals skilled in the field of ampelography, with cultivars being distinguished on the basis of morphological characteristics, and there are a number of texts that describe quantitative ampelographic methods (e.g. Galet 1979). Nonetheless, the confusion surrounding the correct identification of cultivars would best be solved with a more objective means of distinguishing grapevine cultivars that is independent of the phenotypic characters used in ampelography. Molecular markers provide an alternative means of grapevine identification and could be used to investigate taxonomical relationships between *Vitis* species.

Isozymes have been investigated as molecular markers for the identification of grapevines (Parfitt and Arulsekar 1989; Walters et al. 1989; Weeden et al. 1988), but the limited number of isozyme systems available and their tissue-specific expression limit their wider adoption. DNA molecular markers are considered to be superior to

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isozymes for genetic identification and genome analysis because of the availability of a large number of potential polymorphic sequences or markers. DNA molecular markers, which are direct characters of the genotype and independent of the phenotype, may provide more objective descriptors for cultivar identification. They would also be directly applicable to breeding programmes and genome mapping with markers being linked to desirable traits, and to the isolation of specific genes. Such markers would be excellent descriptors for plant patents or plant variety rights submissions. Minisatellite DNA of both human and phage origin has been used as DNA molecular markers to produce DNA fingerprints of grapevines (Striem et al. 1990). These probes, which are used to demonstrate DNA polymorphism between grapevines, would appear to have limited application for routine grapevine identification as the multi-banding pattern and background hybridisation produce blots that are difficult to interpret.

Repetitive DNA sequences can be found clustered or dispersed throughout the nuclear genome of eukaryotes. Those DNA sequences repeated in groups or in 'tandem' are known as satellite DNA, of which two classes, microsatellite and minisatellite, are of interest for DNA fingerprinting studies. Repeats of simple sequences (Tautz and Renz 1984), known as microsatellites (Litt and Luty 1989), consist of a small repeat unit, generally less than four nucleotides, multiples of which may occur in any number of places in the genome but usually with an overall length of less than 100 bp. Minisatellites (Jeffreys et al. 1985; Nakamura et al. 1987) made up of larger repeating units produce regions up to 20 kb in length. Tandemly arrayed DNA sequences can also contain genes. The ribosomal RNA genes like the 5S repeat unit and the ribosomal DNA repeat unit containing the 18S, 25S and 5.8S genes are arranged in tandem and are highly repeated. Other types of repetitive DNA present in eukaryotic genomes include those clustered at centromeric or telomeric sites (Flavell 1986; John and Miklos 1979), with some having structural functions and some being dispersed throughout the genome like transposable genetic elements.

The study presented here investigated the different classes of repetitive DNA sequences in the grapevine genome and identified DNA sequences that would be suitable RFLP DNA markers for grapevine identification.

Materials and methods

Plant material used for nucleic acid extraction was obtained from the *Vitis* collections located at the CSIRO Division of Horticulture, Merbein, Victoria and the South Australia Department of Agriculture Nuriootpa Research and Advisory Centre, Nuriootpa, South Australia. Both collections have individual vine identification numbers as well as cultivar accession/import numbers that are available on request.

DNA extraction

Leaves (2 g) from individual plants were frozen in liquid nitrogen and ground to a fine powder, thawed and resuspended in 25 ml of buffer A [0.25 *M* NaCl, 0.2 *M* *TRIS*-Cl (pH 8.0), 50 m*M* EDTA, 0.1% v/v 2-mercaptoethanol, 2.5% w/v polyvinyl-pyrrolidone (MW 40,000)]. A crude nuclei pellet was obtained by centrifugation at 4,000 *g* for 10 min at 4 °C. The pellet was resuspended in 5 ml of extraction buffer B [0.5 *M* NaCl, 0.2 *M* *TRIS*-Cl (pH 8.0), 50 m*M* EDTA, 1% v/v 2-mercaptoethanol, 2.5% w/v polyvinyl-pyrolidone, 3% sarkosyl, 20% ethanol] and incubated at 37 °C for 30 min with occasional shaking. An equal volume of chloroform/isoamyl alcohol (24:1) was then mixed in by a brief vortex, and the phases were separated by centrifugation at 16,000 *g* for 10 min. The aqueous layer was collected, and 0.54 volume of isopropanol was added to precipitate the DNA. The DNA pellet obtained was resuspended in 600 µl TE containing 30 µg RNase A for 15 min and transferred to an eppendorf tube. Protein was removed by the addition of a half volume of 7.5 *M* ammonium acetate, followed by centrifugation and transfer of the supernatant to a new tube where the DNA was precipitated with a 0.54 volume of isopropanol. DNA was resuspended in 200 µl with yields between 25–150 µg DNA/g FW.

Construction and screening of a genomic library

DNA isolated from *V. vinifera* (L.) cv 'Sultana' was digested with the restriction enzyme *Taq*I, ligated into the *Acc*I-cut dephosphorylated (HK Phosphatase; Epicentre Technologies) vector pBluescript SK(+) (Stratagene) and used to transform competent Sure (Stratagene) *E. coli* cells. Colony lifts were done according to the procedure supplied with the nylon membrane (Bio-Rad). Oligonucleotide probes were hybridised to the membranes as described above to select colonies containing similar sequences. Plasmids selected in this way included pG7, p15M13 and p20M13. To select inserts representing highly repetitive *V. vinifera* DNA sequences, DNA from 'Sultana' was labelled by nick-translation (Bresatec kit) and used as a initial probe to select clones containing high-copy sequences. Spinach chloroplast DNA (cpDNA) was also labelled by nick-translation and used in a second screening to exclude clones containing plastid DNA. Plasmids containing high-copy 'Sultana' sequences were pt4, pt6, pt10, pt15 and pt16.

Cloning of ribosomal repeat units and copy number determination

DNA from *V. champini* and *V. vinifera* cv 'Cabernet Sauvignon' was digested with *Xba*I or *Hin*dIII and separated by electrophoresis on a 0.7% agarose gel; regions of the gel containing the ribosomal repeat unit were then identified by Southern analysis using the spinach rDNA clone (pSn31) (Scott and Timmis 1984). These regions were excised from the preparative gels, and the DNA was electroeluted, ligated to *Xba*I- or *Hin*dIII-cleaved pBluescript SK(+) and cloned as described above. The clones containing ribosomal repeat units were identified by colony hybridisation using random-primed pSn31 as a probe. Ribosomal DNA copy number was estimated by dot-blot analysis. A cloned 'Cabernet Sauvignon' rDNA repeat unit was dotted in a dilution series representing 500–10,000 copies. Genomic DNA was also dotted in a dilution series to represent various genome copy numbers. Genome copies per nanogram of genomic DNA were determined based on the calculated grapevine genome size (see below). Hybridisation of the cloned 'Cabernet Sauvignon' rDNA unit to the dot-blot allowed the matching of hybridisa-

tion intensities between samples and the calculation of rDNA copy number in a genome.

Southern blot analysis

Southern blot analysis on isolated DNA was performed essentially as described previously (Thomas et al. 1990) using 0.85% agarose gels and transfer to a Zeta-Probe (Bio-Rad) membrane. DNA inserts of plasmids were labelled by oligolabeling (Feinberg and Vogelstein 1983) using a kit (Bresatec) or by the polymerase chain reaction (PCR) (Schowalter and Sommer 1989) using standard 17 mer sequencing primers flanking the insert. The 20 µl of PCR mix contained 0.5 ng of plasmid, 1.25 U of *Taq* DNA polymerase (Promega), buffer (Promega), 25 pmol of each primer, 18.5 MBq alpha [^{32}P]dATP (III TBq/mmol), 200 µ*M* dGTP, dCTP and dTTP and 2.5 µ*M* dATP. After 5 min at 95 °C, 30 cycles of PCR were performed (denaturation: 1 min at 94 °C; annealing: 50 s at 50 °C; ramp: 1 min from 50°–72 °C; elongation: 1 min at 72 °C), followed by a 7-min elongation step using a Perkin-Elmer Cetus automated thermal cycler. Oligonucleotides synthesized with an Applied Biosystems 391 DNA synthesizer were radiolabelled with gamma [^{32}P]ATP (148 TBq/mmol) using a terminal kinasing kit (Bresatec). The hybridisation solution for oligonucleotide probes was 0.5 *M* Na_2HPO_4 (pH 7.2), 1 m*M* EDTA, 7% sodium dodecyl sulfate (SDS) and 10 µg/ml yeast tRNA. The standard hybridisation solution consisted of 0.25 *M* Na_2HPO_4 (pH 7.2), 1 m*M* EDTA and 7% SDS. The hybridisation temperature for oligonucleotides was determined by the formula $T_d = 2$ °C (number of A + T residues) + 4 °C (number of G + C residues) or by nearest-neighbour thermodynamic data (Rychlik and Rhoads 1989), and was 5 °C below T_d. The T_d values for individual oligonucleotides were: $(GT)_{10}$: 56 °C; $(GA)_{10}$: 50 °C; $(CAC)_5$: 45 °C; $(GACA)_4$: 48 °C; $(GATA)_4$: 40 °C; M13 tandem repeat (GAGGGTGGTGGCTCT): 50 °C; R2 (ATTTTGCCACTGAT): 38 °C; R7 (TCGGATCTGATTTC): 40 °C; and 14C2 (GGCAGGATTGAAGC): 44 °C (Vergnaud 1989). Membranes were washed for 15 min at room temperature in a 0.9 *M* NaCl and 90 m*M* trisodium citrate solution (6 × SSC), rinsed twice for 1 min in 6 × SSC at the temperature used for hybridization, and then exposed to Fuji RXG film. The human DNA probe pV47-2 (Longmire et al. 1990) and a potato cDNA probe pRS (I. Dry, unpublished) were hybridised and given a stringent wash (2 × SSC, 0.1% SDS) at 60 °C. The cloned spinach ribosomal repeat unit pSn31, *V. champini* large intergenic spacer region subclone pVc-A, pea 5S ribosomal repeat unit p5S1 (Ellis et al. 1988) and *V. vinifera* (L.) cv 'Sultana' genomic clones pG7, p15M13, p20M13, pt4, pt6, pt10, pt15 and pt16 were hybridised and given a stringent wash (2 × SSC, 0.1% SDS) at 65 °C. Membranes were given a final wash at room temperature in 0.1 × SSC and 0.1% SDS prior to exposure to film.

Quantitative microspectrofluorometry

Root tips from mung bean (*Vigna radiata* L.) seed germinated on damp filter paper, and *Vitis vinifera* (L.) cv 'Cabernet Sauvignon' cuttings grown under glasshouse conditions were fixed in 3:1 ethanol:acetic acid. These were taken back through an alcohol series to water and then softened and stained with DAPI (4'6-diamidino-2-phenylindole) as in Scott and Possingham (1980).

Microspectrofluorometric measurements of the cell nuclei were made with the preset value of the microscope photometer obtained using a uranyl glass standard (Lawrence and Possingham 1986). The 1C value of 0.5 pg DNA for *Vigna radiata* was used to calibrate grapevine nuclei values.

Results

The nucleus of the grapevine 'Cabernet Sauvignon' was found to have a 1C DNA value of 0.53 ± 0.028 pg. This represents a genome size of approximately 511 Mbp. Despite this relative small size repetitive DNA sequences were found to be well represented in the genome.

Microsatellite sequences

Genomic blots of 'Sultana', 'Cabernet Sauvignon' and *V. berlandieri* were hybridised with five oligonucleotide probes, $(GT)_{10}$, $(GA)_{10}$, $(CAC)_5$, $(GACA)_4$, and $(GATA)_4$ (Fig. 1), which are microsatellite sequences or simple sequence repeats previously found in eukaryotic genomes (Epplen 1988; Hamada et al. 1982; Tautz and Renz 1984). The relative abundance of these repeats in the grapevine genome varied depending on the repeat as shown by the time taken to expose the films. The dinucleotide repeats GA and GT are the most highly represented and mostly appear to be interspersed throughout the genome, although some discrete bands can be identified (Fig. 1). The hybridisation pattern produced was always a smear containing bands irrespective of the restriction enzyme used and was not improved with a stringent wash (30 min) of the membrane 10 °C above the hybridisation temperature. Increasing the number of nucleotides in the repeat (i.e. CAC, GATA, GACA; Fig. 1) produced a clearer banding pattern that in some cases could distinguish different cultivars and species, but associated with this was an increase in film exposure time due to the lower frequency of such repeats in the genome. A *V. vinifera* cv 'Sultana' genomic clone (pG7) selected from the genomic library with the oligonucleotide $(GATA)_4$ contained a $(GATA)_3$ repeat within a unique sequence. However, the hybridisation pattern produced with pG7 was not useful as a DNA fingerprinting probe as it identified its homologue and other conserved bands previously detected by the oligonucleotide $(GATA)_4$ (data not shown). The clone pRS is known to contain the trinucleotide motif CAA repeated frequently throughout the sequence (I. Dry, unpublished). This microsatellite repeat is also part of larger minisatellite type repeats within this clone. The repeat classes in this clone may be responsible for the ability of pRS to produce unique DNA fingerprints in a heterologous situation (Fig. 1).

Minisatellite sequences

The 15-bp oligonucleotide 'M13 tandem repeat' represents a single repeat unit from the M13 bacteriophage tandem repeat region that was identified by Vassart et al. (1987) as a useful probe for detecting hypervariable minisatellites in vertebrates. The hybridisation pattern shows that this oligonucleotide detects multiple loci and varia-

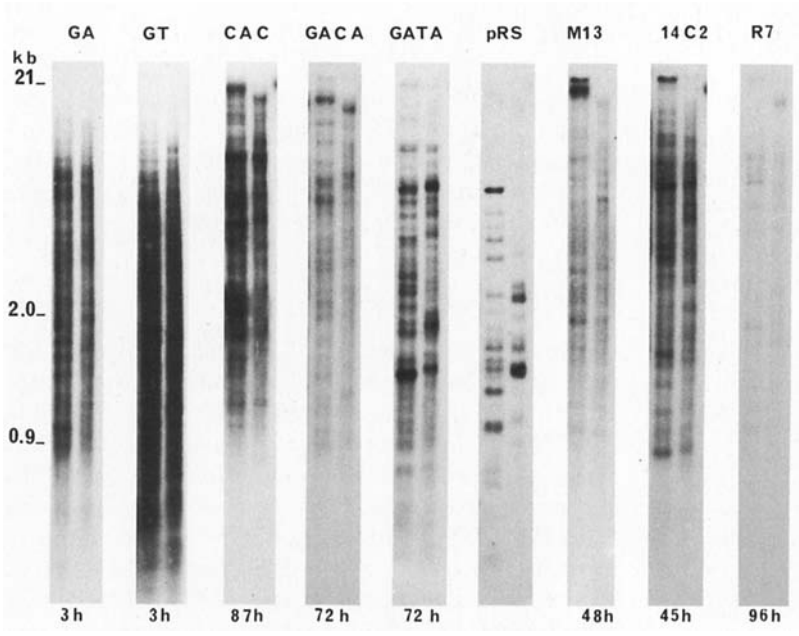

Fig. 1. Abundance of micro- and minisatellite repeats in the grapevine genome. *Dra*I-restricted *V. vinifera* cv 'Cabernet Sauvignon' (*left lanes*) or *V. berlandieri* DNA (*right lanes*) (2 μg per lane) was hybridised to the following oligonucleotide probes: *GA* $(GA)_{10}$, *GT* $(GT)_{10}$, *CAC* $(CAC)_5$, *GACA* $(GACA)_4$, *GATA* $(GATA)_4$; see materials and methods for description of *M13*, *14C2* and *R7*. Probe *pRS* is cloned DNA. End-labelled oligonucleotide probes had similar specific activity, and the film exposure time is in hours

tion in *Vitis* (Fig. 1), though the varying degrees of specificity and background hybridisation to sequences throughout the genome limits its usefulness as an objective measure of sequence variability for fingerprinting applications. The result appears to be similar to that reported by Striem et al. (1990), who used the whole M13 bacteriophage as probe. The human probe pV47-2, which was isolated from a human genomic library on the basis of its hybridisation to the M13 repeat (Longmire et al. 1990), detected a pattern in grapevines similar to the M13 tandem repeat oligonucleotide plus additional loci (results not shown). However, the pV47-2 probe also suffered from the same problem of background hybridisation to other genomic sequences. The grapevine minisatellite sequences detected by the M13 tandem repeat oligonucleotide (Fig. 1) and pV47-2 were frequently represented in the genome. Two 'Sultana' genomic clones (p15M13, p20M13) selected on the basis of their hybridisation to the M13 oligonucleotide were unsuitable for fingerprinting grapevine cultivars by detection of hypervariable minisatellite sequences as they detected very few polymorphisms between cultivars.

Polymers of random 14-bp oligonucleotides have been used successfully to detect human polymorphic minisatellite loci (Vergnaud 1989). One of these oligonucleotide sequences, 14C2, was capable of detecting polymorphic loci in grapevine (Fig. 1). The DNA sequences of the oligonucleotides R2 and R7 (see Materials and methods) were generated with a random number programme skewed to give a 38% G/C content representative of the average G/C content of plant nuclear DNA (Smillie and Scott 1969). Both R2 and R7 (Fig. 1) also detected polymorphic loci in grapevine though their abundance in the genome was less than that of 14C2, which was 57% G/C rich.

Repetitive tandemly arrayed genes

The spinach ribosomal DNA repeat unit (pSn31) was used to identify and clone *Vitis* ribosomal repeat units. Entire rDNA repeat units were cloned from genomic DNA of *V. champini* digested with *Xba*I and *V. vinifera* cv 'Cabernet Sauvignon' digested with *Xba*I or *Hin*dIII. Physical maps of the genes were constructed, and the intergenic spacer region and conserved coding regions of the genes were identified using pSn31 and wheat rDNA clones [pTA250.1, containing the full length intergenic spacer region and the 3′ and 5′ ends of the 25S and 18S rDNA; and pTA250.2, containing the 18S coding region, internally transcribed spacer region and the 25S coding region (Appels and Dvorák 1982)] as probes (Fig. 2). The clones pSn31 and pTA250.1 showed no homology with the *Bam*HI fragments containing the intergenic spacer region of individual *Vitis* rDNA clones (results not shown). In the case of *V. champini*, all of the 7 rDNA clones isolated were the same (9.14 kb) and corresponded to the major rDNA band in *Xba*I digests probed with pSn31. In the case of *V. vinifera* cv 'Cabernet Sauvignon', from 10 rDNA clones 4 different rDNA clones (pVvc-1, -2, -3 and -4 in Fig. 2) were identified that corresponded in size to the 4 major rDNA bands observed in *Xba*I digests probed with pSn31. The subclones pVc-A and pVvc-A from the intergenic spacer region of a *V. champini* (pVc) and *V. vinifera* unit (pVvc-3) (Fig. 2) were homologous and hybridised to the corresponding fragments in pVvc-1, -2 and -4, but not to any other region

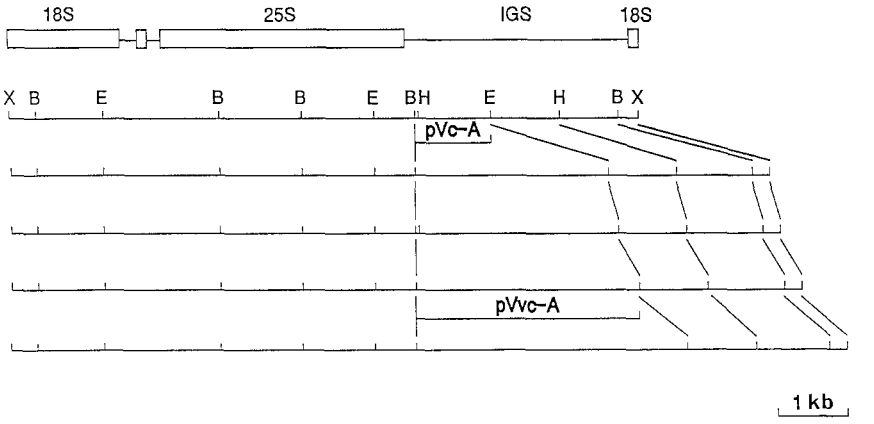

Fig. 2. Physical maps of the rDNA repeat units in *V. champini* (*pVc*) and *V. vinifera* cv 'Cabernet Sauvignon' (*pVvc-1,2,3,4*). The position of the 5.8S, 18S and 25S coding regions and intergenic spacer (IGS) region was deduced using the spinach (Sn31) and wheat (pTA250.1, pTA250.2) rDNA clones. The entire repeat unit is diagrammed pVc 9.14 kb, pVvc-1 11.05 kb, pVvc-2 11.2 kb, pVvc-3 11.5 kb, pVvc-4 12.15 kb. The fragments *pVc-A* and *pVvc-A* represent subclones. *E EcoRI*, *B BamHI*, *H HindIII*, *X XbaI*

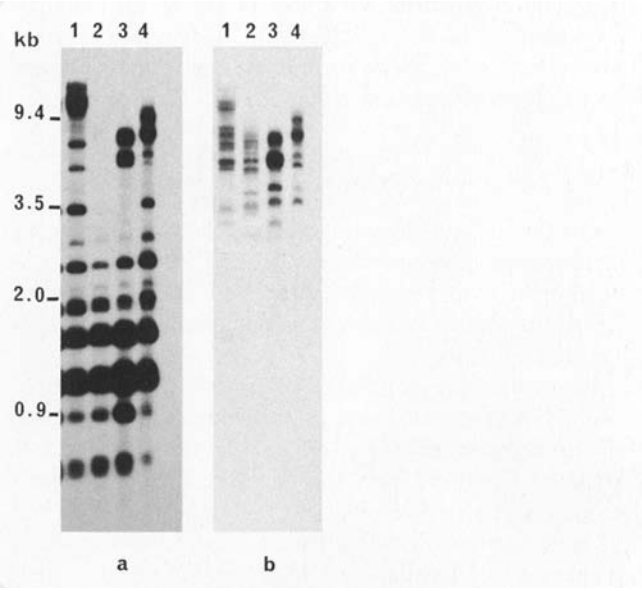

Fig. 3a, b. RFLP pattern of grapevine genomic DNA hybridised to the spinach ribosomal DNA repeat unit, pSn31 (**a**), and the grapevine spacer region, pVc-A (**b**). DNA was restricted with *RsaI*. Common bands between pSn31 and pVc-A are observed in *lanes 3 and 4*. *Lane 1* 'Chardonnay', *lane 2* 'Traminer', *lane 3* 'Villard Blanc', *lane 4* 'Villard Noir'

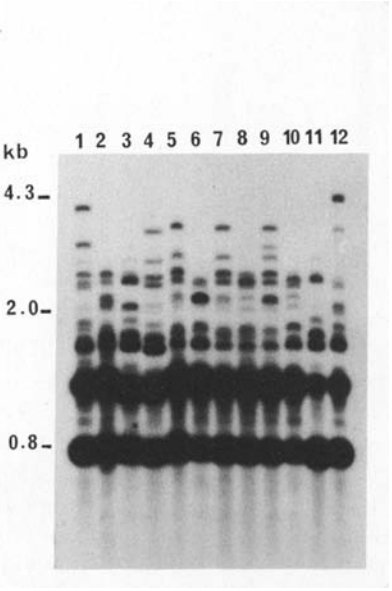

Fig. 4. Cultivar-specific RFLP patterns obtained with the 5S repeating unit p5Sl. Grapevine genomic DNA was restricted with *DraI*. *Lane 1* 'Sultana', *lane 2* 'Muscat Gordo Blanco', *lane 3* 'Grenache', *lane 4* 'Cabernet Sauvignon', *lane 5* 'Shiraz', *lane 6* 'Pinot Noir', *lane 7* 'Riesling', *lane 8* 'Semillon', *lane 9* 'Chardonnay', *lane 10* 'Traminer', *lane 11* 'Villard Blanc', *lane 12* 'Villard Noir'

in the rDNA clones. The copy number of the ribosomal repeat units in a diploid genome was estimated to be 1,500 and 2,500 for *V. champini* and *V. vinifera* cv 'Cabernet Sauvignon', respectively.

The spinach ribosomal DNA repeat unit (pSn31) containing the 18S, 25S, 5.8S genes and intergenic spacer regions detected polymorphisms between grapevines (Figs. 3, 6), although only the grapevine DNA fragments containing the conserved ribosomal DNA sequences hybridised to pSn31, as the large intergenic spacer region sequence from spinach is different to the grapevine spacer region. The intergenic spacer region from *V. champini*, pVc-A (Fig. 3), shows polymorphisms in bands specific to the DNA sequences present in the spacer region of grapevine ribosomal DNA. The common fragments identified by pSn31 and pVc-A (Fig. 3) suggest that the fragments contain both coding and intergenic spacer regions, while probe-specific bands represent fragments with either genes or spacer region only.

Another gene family tandemly arranged in repeating units that comprise gene and spacer region is the 5S rRNA gene. The pea 5S repeating unit (p5Sl) when hybridised to grapevine genomic blots also detected polymorphisms (Fig. 4). As with the ribosomal genes (Fig. 3), the hybridisation observed was likely due to those DNA fragments containing sequences of the conserved 5S rRNA gene (Gottlob-McHugh, et al. 1990), and the variability detected was probably due to variations in the sequence and length of the nonconserved spacer region as well as possible multiple sites in the genome.

Highly repetitive Vitis vinifera DNA

Five differently sized DNA clones representing highly repetitive sequences in the 'Sultana' genome were tested for their ability to distinguish differences within *Vitis*. Three of the probes (pt4, pt6, pt15) appeared to recognise highly repetitive elements in the genome (data not shown) that contained a conserved *Rsa*I site, thus reducing the repetitive element to its monomer or near monomer form. A ladder pattern, indicative of a tandem repeat, was produced with the restriction enzyme *Taq*I. However, this type of pattern produced by pt4, pt6, and pt15 showed few polymorphisms between cultivars when genomic DNA was digested with the enzymes *Bst*NI, *Rsa*I, *Taq*I and *Dra*I. Probe pt16 was capable of detecting differences between some cultivars and species, and *V. rotundifolia* was found to lack the highly repetitive pt16 sequence when compared to the other *Vitis* species tested (Fig. 5). This molecular discrimination of *V. rotundifolia* from other *Vitis* species correlates with the classification of *V. rotundifolia* in a separate section, *Muscadinia*, while the other *Vitis* species tested represent species from the section *Euvitis*.

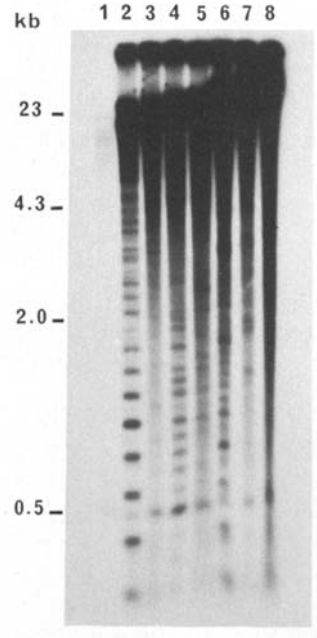

Fig. 5. Grapevine species probed with the highly repetitive DNA element pt16. DNA from each species (2 µg) was restricted with *Dra*I. *Lane 1* is *V. rotundifolia* representing the *Muscadinia* section. *Lanes 2–8* represent species from the *Euvitis* section: *lane 2 V. labrusca, lane 3 V. champini, lane 4 V. riparia, lane 5 V. rupestris, lane 6 V. amurensis, lane 7 V. berlandieri, lane 8 V. longii*

RFLP identification of grapevines with repetitive DNA

From the different repetitive DNA classes investigated three sequences were considered useful for RFLP identification of grapevines and were used further to detect DNA polymorphisms across a wider range of *Vitis* species and cultivars.

Enzymes such as *Eco*RI, *Pst*I and *Bam*HI cut grapevine DNA at a much lower frequency than was expected, and in some cases it was difficult to see any evidence at all of digestion from the ethidium bromide-stained gels. *Eco*RII also digested very little grapevine DNA, but its methylation-insensitive isoschizomer *Bst*NI digested grapevine DNA extensively (e.g. Fig. 6) indicating that grapevine DNA is extensively methylated. To overcome this problem the cytosine methylation-insensitive endo-

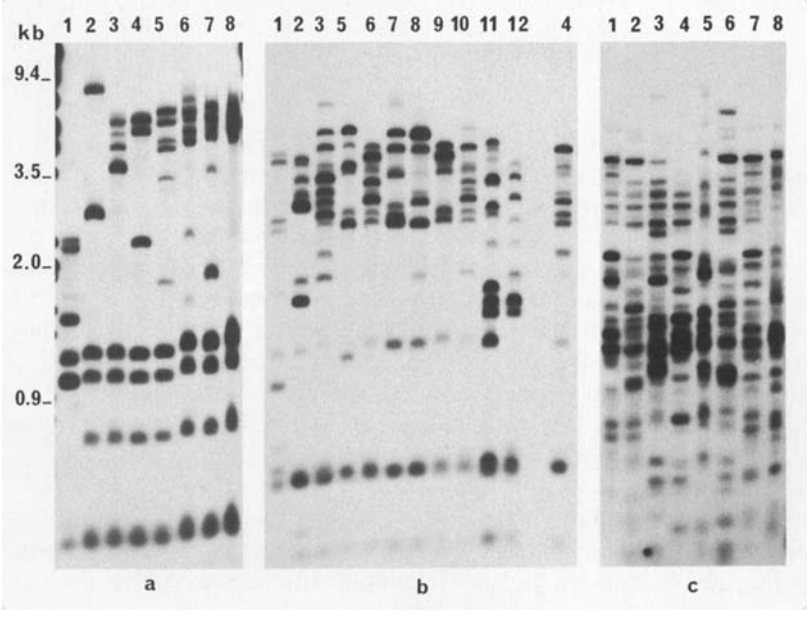

Fig. 6a–c. Grapevine DNA probed with pSn31 (*Bst*NI), pVc-A (*Taq*I) and pRS (*Dra*I). **a** Species were probed with pSn31; for lane identification see legend of Fig. 5. **b** cultivars were probed with pVc-A; see Fig. 3 for lane identification. **c** Cultivars from the *Cabernet* group were probed with pRS: *lane 1* 'Cabernet Franc', *lane 2* 'Cabernet Sauvignon', *lane 3* 'Fer Servadou', *lane 4* 'Petit Verdot', *lane 5* 'Malbec', *lane 6* 'Sauvignon Blanc', *lane 7* 'Merlot', *lane 8* 'Merlot Blanc'

nucleases *Bst*NI, *Rsa*I, *Taq*I and *Dra*I were used for the digestion of DNA samples. The best endonuclease/probe combinations for detecting differences at the DNA level between grapevine cultivars and species were *Dra*I/pRS, *Taq*I/pVc-A and *Bst*NI/pSn31. The three probes produced unique hybridisation patterns or DNA fingerprints for all of the grapevine species and cultivars tested (Fig. 6) including those *V. vinifera* cultivars grouped into the Cabernet class. Figure 6 shows only representative blots for the three probes though all three probes were equally capable of distinguishing *Vitis* species and cultivars. Of the 19 cultivars represented in Figs. 6 only 2 are known species hybrids, Villard Blanc and Villard Noir. The remaining 17, considered to be cultivars of *V. vinifera*, show significant genetic variation at the loci detected by the probes. The patterns produced by the probes pSn31 and pVc-A consist of fewer bands than that of pRS, making cultivar identification with these probes simpler. However, both pSn31 and pVc-A detect the same locus (loci), which is the ribosomal DNA repeat unit. The probe pRS hybridises to different regions of the genome to that of pSn31 and pVc-A and detects conserved bands (loci) as well as loci that are polymorphic. Despite the numerous bands that pRS detects, analysis of the less complex pattern found in the top half of the gel provides cultivar-specific patterns. From the results presented in Fig. 6 it would appear that the probes pSn31, pVc-A and pRS either alone or in combination will prove very useful for DNA identification of grapevine cultivars.

Discussion

Viticulture is substantially cultivar orientated for the production of wine and fresh or dried fruit. The difficulties encountered in accurate cultivar identification are due in part to the long history of viticulture, vegetative propagation of cultivars and the reliance on morphological characteristics for cultivar identification. It has been demonstrated in this study that the use of DNA molecular markers for grapevine identification represents an objective and viable alternative to ampelography. The restriction fragment length polymorphisms detected by the DNA probes in Fig. 6 indicate that even cultivars within *V. vinifera* display a high degree of genetic variation. The results establish a strong case for the adoption of DNA fingerprinting for cultivar identification in the viticulture industry to complement or replace existing identification procedures.

The ribosomal DNA repeat units from *V. champini* and *V. vinifera* cv 'Cabernet Sauvignon' were found to have different lengths, with different length classes present in 'Cabernet Sauvignon'. The lengths and the *Xba*I, *Eco*RI and *Bam*HI sites of the coding regions of all of the ribosomal DNA repeat unit clones were conserved, and these coding regions cross-hybridised with those of other plants (spinach, wheat), an observation that is consistent with the conservation or rRNA sequence across the plant kingdom. However, while the intergenic spacer regions within *Vitis* species were homologous, they showed no homology with those of spinach or wheat. This situation allowed an intergenic spacer region subclone (pVc-A) to be used as a probe for the detection of RFLPs different to those detected by pSn31. The *Vitis* DNA polymorphisms detected by the spinach ribosomal repeat unit (pSn31) and grapevine intergenic spacer region (pVc-A) appear to be largely due to DNA variation in the large intergenic spacer region uncovered by the restriction enzymes used for RFLP analysis, and this situation has been found for many plants (Rogers and Bendich 1987). The highly repetitive *Euvitis*-specific DNA sequence (pt16) isolated from *V. vinifera* cv 'Sultana' warrants further investigation in phylogenetic studies. Similar species-specific repetitive DNA sequences have been found to be useful in taxonomic studies (Crowhurst and Gardner 1991) as well as for the identification of parental chromosomes in interspecific hybrids (Itoh et al. 1991). Probe pt16 could be used as a molecular marker in the genetic analysis of hybrids generated between *V. vinifera* and *V. rotundifolia*. These hybrids have been reported to have a high potential for grapevine improvement by combining the fruit quality of *V. vinifera* with the disease resistance of *V. rotundifolia* (Olmo 1986). Genetic relationships within *Vitis* can also be studied at the DNA level. Our study suggests there is an opportunity to investigate phylogenetic relationships in grapevines on an objective basis at the molecular level.

The separation of the cultivars of *V. vinifera* achieved in these reports, together with the lack of separation of clonal selections (results not presented), suggests that many of the established grapevine cultivars are the result of crosses. While we have, thus far, been unable to separate varietal clones of grapevines with this technology, it may be that some of clonal selection is based on a selection for disease load rather than on genetic or somatic variation (N. Habili, M.R. Thomas and N.S. Scott, unpublished results).

The oligonucleotide probes representing micro- and minisatellite sequences did not appear suitable for adoption as probes for grapevine DNA fingerprinting using Southern blot technology despite the ability of some to detect RFLPs. The data presented here indicate that like other eukaryotes (Epplen 1988; Hamada et al. 1982; Tautz and Renz 1984) the grapevine genome is rich in microsatellite sequences and some of them individually are likely to show polymorphisms. This possibility is currently being investigated using such microsatellite sequences to detect polymorphisms by the sequence tagged site approach as suggested by Beckmann and Soller

(1990). Both this approach and that described in these results are expected to yield techniques for both grapevine cultivar identification and grapevine breeding.

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