Vitis **32,** 183–185 (1993)

Research Note

Molecular markers for grape characterization

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S u m m a r y : Five cultivars and 9 Pinot noir clones were used to test the usefulness of RFLP and RAPD markers and determine whether clonal selections could be differentiated.

K e y w o r d s : PCR, RFLP, RAPD, Vitis, molecular markers.

A b b r e v i a t i o n s : **PCR**: Polymerase chain reaction; **RFLP**: Restriction fragment length polymorphism; **RAPD**: Randomly amplified polymorphic DNA. **CTAB**: Alkyl trimethyl ammonium bromide; **TE**: Tris-EDTA (50 mM - 10 mM).

Materials and methods: Cabernet Sauvignon, Grey Riesling, Pinot blanc, Pinot gris, Primitivo di Giogia, and 9 Pinot noir clones were collected in both 1990 and 1991. 5 g fresh leaves were ground to a powder in liquid N_2. Frozen powdered tissue was quickly mixed with 65 °C 20 ml 2 x CTAB isolation buffer and the method of DOYLE and DOYLE (1987) used. Subsamples were purified by CsCl/ethidium bromide (1 g CsCl/1 ml TE) centrifugation at 65,000 rpm and 25 °C for 36 h (SAMBROOK *et al.* 1989).

A random genomic DNA library was constructed from Pinot noir. Total genomic DNA was isolated (BERNATZKY and TAKSLEY 1986) and digested with HindIII. 2 kb fragments were ligated into PUC 18 plasmids and transformed in DH-5 alpha *Escherichia coli* cells. Plasmids with grape inserts were selected on X-Gal substrate (SAMBROOK *et al.* 1989). Cloned fragments were isolated (MORELLE 1989) and screened for single or low copy sequences against EcoRV and HindIII digested Pinot noir total genomic DNA (LANDRY and MICHELMORE 1985). 5 µg total DNA were digested with 40 units EcoRI or HindIII for 5 h, electrophoresed in 0.8 % agarose and transferred onto Nytran membranes (S&S directions). Probes were ^{32}P labelled by random priming (FEINBERG and VOGELSTEIN 1983). Prehybridization and hybridization were done at 42 °C with 50 % formamide. Membranes were hybridized for 24 h and washed twice for 15 min with 2 x SSC (0.3 M NaCl, 0.003 M Na-citrate) 0.1 % SDS, at 25 °C, followed by two 15 min 1 x SSC, 0.5 % SDS washes at 40 °C, and 0.5 x SSC, 0.5 % SDS wash for 30 min at 65 °C, and exposed to X-ray film for 1-2 d at -70 °C with intensifying screens.

25 µl PCR DNA amplifications, containing 5 µl of DNA (1 ng/µl), 10 mM Tris-HCl pH = 8.3, 50 mM KCl, 0.001 % gelatin, 1.9 mM Mg_2Cl, 100 µM each of dNTP, 200 nM of Operon primers (O-02, O-05, and O-06) and 40 units/ml of Taq polymerase, and 1 drop of oil overlay. DNA was amplified with 45 cycles for 92 °C for 1 min; 35 °C for 1 min; and 72 °C for 2 min. Amplified DNA was extracted once with chloroform:isoamyl alcohol (24:1). DNA from 3 replicates of Primitivo di Giogia and Pinot noir cl. 15 were isolated using both CTAB and CsCl. Amplifications for Pinot gris, Pinot noir cl. 2A and 4, Primitivo di Giogia and Cabernet Sauvignon were done with 1990 and 1991 DNA. Amplification products were separated in a 1.5 % agarose gel at 55 V for 9 h.

Results and discussion: Significant polymorphisms were observed for probes pGAD10 and pGAD15 with EcoRI digestion. Most of the 65 probes did not show polymorphism. There was no polymorphism among Pinot noir clones. Pinot blanc, Grey Riesling and Cabernet Sauvignon were identical for pGAD10. Pinot gris differed from the other cultivars for both probes. RFLP analysis permitted differentiation of Cabernet Sauvignon, Pinot gris, and Pinot noir that isozyme techniques did not resolve (PARFITT and ARULSEKAR 1989). Grape cultivars showed a relatively low level of diversity when subjected to both isozyme and RFLP analysis.

RFLP analysis revealed polymorphism in grape DNA when minisatellite probes, such as M-13 were used (STRIEM *et al.* 1990; YAMAMOTO *et al.* 1991). RFLP analysis is apparently more efficient with multilocus probes and when hybrids expected to be polymorphic were analyzed.

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Figure shows polymorphic amplifications of grape total genomic DNA for PCR primer O-05 which produced 15 bands among the grape cultivars (O-02 and O-06 showed amplifications but no polymorphism). Nine bands were selected as markers for their consistency (5, 6, 7, 8, 9, 10, 11, 12 and 13), of which 5 were polymorphic (5, 6, 7, 11 and 12). Primitivo di Giogia, Grey Riesling, Pinot blanc and Cabernet Sauvignon could be uniquely separated. All Pinot noir clones and Pinot gris showed the same banding pattern. Pinot blanc was similar to Pinot noir with lower amplification of marker 12 in Pinot blanc.

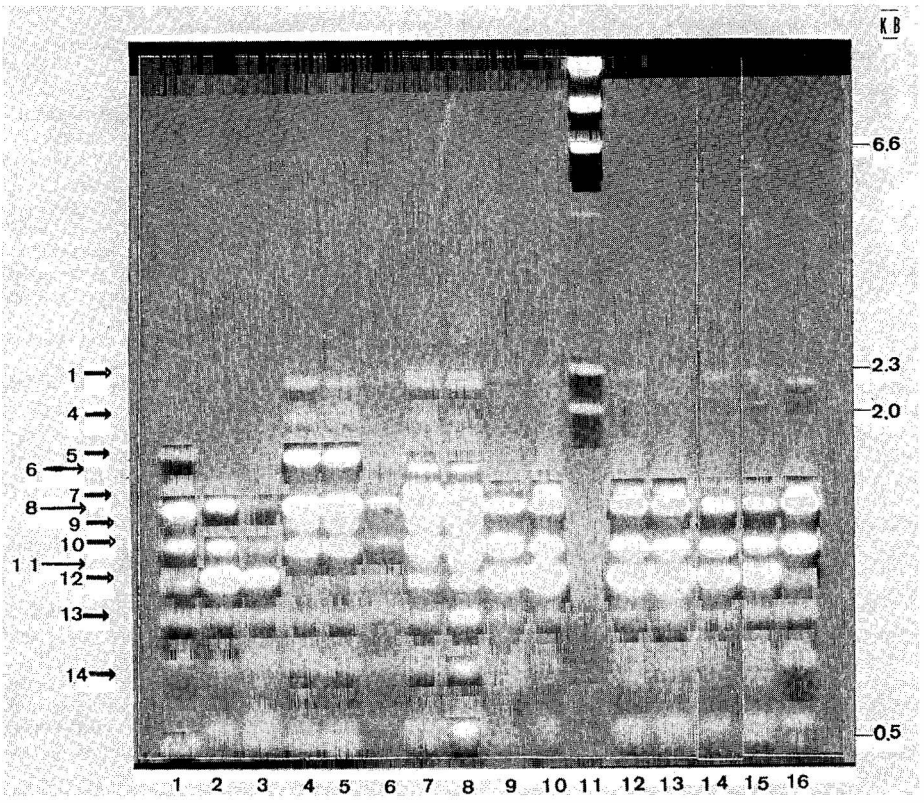

Figure: RAPD profiles by primer O-05. Lane (1) Grey Riesling; (2-3) Pinot noir clone 15 in 1990 CsCl and CTAB extracted DNA; (4-5) Cabernet Sauvignon in 1991 and 1990; (6-7-8) Primitivo di Giogia in 1990 CTAB DNA, 1991 and 1990 CsCl method; (9-10) Pinot noir clone 4 in 1991 and 1990; (11) Molecular size marker; (12-13) Pinot noir clone 2A in 1991 and 1990; (14-15) Pinot gris in 1991 and 1990; (16) Pinot blanc.

The PCR RAPD markers were potentially more useful than RFLPs, giving different patterns for all studied cultivars (except for Pinot noir clones) with only one RAPD marker. RAPDs avoided problems of low DNA concentrations and undigested DNA. For example, Primitivo di Giogia did not hybridize due to low DNA concentration. For RAPD analysis DNA digestion was not required and only small amounts of DNA were needed. Somewhat lower quality DNA can be used for RAPDs. Other experiments in our laboratory with apricot DNA suggest that polymorphism levels depends on both the primer and the species.

DNA amplifications for 1990 and 1991 were essentially identical. Bands with less than 800 bp (14) seem to be more variable. 2 kb sequences (bands 1-4) were poorly amplified, probably due to the large amount of substrate and more inefficient extension compared to smaller sequences. The reaction is competitive. Thus, high molecular weight bands should not be used for grape characterization.

Extracted CTAB-DNA gave the same, but less intense, bands than did CsCl purified DNA (Figure, lanes 3 and 6), probably due to errors in spectrophotometric measurement of the DNA used for amplification, caused by extraneous compounds. As a result, DNA concentrations may have been different than required for successful amplification. Fluorometric DNA measurement will give more precise estimates. Hu and Quirós (1991) did DNA amplifications using several isolation methods and reported no problem with amplification.

In conclusion, we suggest using multilocus probes for RFLP grape characterization rather than single copy probes which exhibit limited polymorphism. RAPD markers gave increased polymorphism in addition to avoiding technical problems of low DNA concentration and interferring substances. RAPD markers provided a consistent and rapid method for grape characterization.

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