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# Characterization of RAPD markers in *Vitis*

by

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S u m m a r y : A study was initiated to investigate the possibility of using RAPD markers in related populations of *Vitis*. We also sought to design primers that could amplify translation initiation sites (Kozak sequence) as a mean to maximize the production of RAPD markers from single copy DNA sequences in the genome. RAPD bands were labeled and used as probes on blots with either genomic DNA or RAPD products from cvs Aurore, Cayuga White, Horizon and Illinois 547-1. Reamplification of excised RAPD products produced either several bands of smaller size, a single band of smaller size or a single band of the same size as the original band. Among 16 probes hybridized to genomic DNA blots, three probes, including one from the Kozak primer amplification, hybridized to 1-2 bands, 5 probes hybridized to 3-8 bands and 8, including two from a Kozak primer reaction, to more than 10 bands on the genomic DNA blots. Twelve RAPD bands were also probed on RAPD blots derived from the RAPD reaction that produced each probe. Three of those probes hybridized to 1-2 bands, 8 hybridized to 3-8 and one hybridized to more than 10 bands indicating the presence of probe sequences in more than one RAPD band as amplified with the same primers. This result and the observations on reamplification of RAPD bands support the hypothesis that some of the longer RAPD fragments harbor internal priming sites that are either not amplified unless the reaction mixture is saturated with longer products or are not present as frequently as the longer sites. RAPD DNA probes from one primer also hybridized to the RAPD products of other primers indicating amplification from the same sequence but different sized repetitive DNA. RAPD reactions were also run with 16 primers on parental DNA of 2 crosses used in genetic mapping (Cayuga White x Aurore and Horizon x Illinois 547-1). These reactions generated 140 bands; 100 bands were shared by both populations, including 47 polymorphic bands. Ten polymorphic bands in Cayuga White x Aurore and 22 in Horizon x Illinois 547-1 were population specific. The RAPD analysis as well as hybridization of RAPD markers to the genomic blots suggest that linkage analysis could be used in related segregating populations with carefully chosen markers. Tagging single copy regions with Kozak-sequence-derived primers may be possible, but the low number of probes tested and lack of DNA sequence information prevents any definite conclusions.

K e y w o r d s : chemiluminescent, hybridization, internal priming sites, Kozak, Southern.

## Introduction

Since first reported, random amplified polymorphic DNA (RAPD) markers (Welsh and McClelland 1990, Williams *et al.* 1990) have been used in numerous scientific studies in plants and animals (del Tufo and Tingey 1994). RAPD markers are generated by the amplification of target DNA with a single primer of arbitrary sequence without prior knowledge of DNA sequences. RAPD markers have been very useful, especially in constructing genetic maps (Carlson *et al.* 1991, Reiter *et al.* 1992, Williams *et al.* 1993, Hemmat *et al.* 1994) as well as taxonomic and phylogenetic studies (Bachmann 1992, Williams and St 1993). Each primer, which is a short-sequence oligonucleotide, can amplify one to several DNA bands based on the presence or absence of annealing sites. This phenomenon, though useful for developing and saturating genetic maps, can also complicate the analysis (Riedy *et al.* 1992). RAPD markers are detected as present/absent, thus are dominant as compared to co-dominant RFLP and isozyme markers (Rafalski and Tingey 1993, Williams *et al.* 1993).

The dominant nature of RAPD markers poses a limitation in $F_2$ populations as heterozygous *loci* cannot be differentiated from homozygous *loci* (Devos and Gale 1992, Kazan *et al.* 1993). Also the presence of several such dominant markers in a region may make it difficult to estimate gene order (Paran and Michelmore 1993). This could be overcome by several different strategies: sequence tagged sites (STSs) which are characterized by short sequence single copy DNA as amplified in PCR; sequence characterized amplified regions (SCARs) (Olson *et al.* 1989) designed by sequencing the flanking region of a RAPD marker and then picking a pair of primers to amplify the precise sequence in PCR (Paran and Michelmore 1993); cleavage amplified polymorphic sequences (CAPS), in which PCR-amplified regions from parental and progeny DNA are restricted with endonucleases and homozygotes differentiated from heterozygotes based on the restriction implied in all these methods (Akopyanz *et al.* 1992); and amplification fragment length polymorphisms (AFLPs) where DNA is restricted, modified with linkers and amplified in PCR with oligonucleotides (Zabeau and Vos 1992).

Due to the dominant nature, RAPDs show certain limitations. RAPD markers are thought to be population-specific. Genetic maps developed in one cross could not be used readily for another cross in *Stylosanthes* (Kazan *et al.* 1993). The usage of RAPD markers in genetic analyses is

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further limited since plants contain a high proportion of non-coding DNA, as found in grape (> 95 %) by LODHI and REISCH (1995). These markers may only be used for genetic analysis in the population where they were originally amplified. Another shortcoming of RAPD markers is that bands may be amplified from repetitive DNA (WILLIAMS *et al.* 1990, DEVOS and GALE 1992, WILLIAMS *et al.* 1993) and even from chloroplast or mitochondrial DNA (THORMANN and OSBORN 1992, KAZAN *et al.* 1993). Moreover, inconsistency in the presence or absence of a RAPD band in several rootstocks of grape was attributed to the staining of RAPD gels with ethidium bromide as confirmed by Southern hybridization (XU *et al.* 1995) or due to variation in the amount of amplification from different sites. Amplification from repetitive and non-nuclear DNA may be avoided by designing primers homologous to DNA sequences frequently found in the coding regions. This strategy may be equivalent to tagging unknown genes. The Kozak sequence, GCCGCCPuCCAUGG, was found to be a consensus sequence for translation initiation in higher eukaryotes (KOZAK 1984, 1987). Additionally, presence of a purine at -3 was the most conserved nucleotide in eukaryotes (HEIDECKER and MESSING 1986, PALUH *et al.* 1988). Similarly, G at the +4 position was necessary for efficient translation (KOZAK 1986). Based on this information, we envisioned that NNNNNNPuNNAUGG could be used to design primers for amplification of genomic DNA in PCR. These primers would be designed in such a way that the most conserved sequences are included in each primer and less conserved sequences are varied to amplify DNA segments adjacent to varying Kozak sequences. Due to high GC composition, the probability of targeting exons is enhanced as MONTERO *et al.* (1990) reported 20 % more GC content in exons than introns.

The objective of the present study was to explore the possibility of using RAPD markers in populations other than the one in which they were developed. We, therefore, studied the nature of RAPD markers in *Vitis* and designed primers that we hoped would target DNA from the more highly conserved regions of structural genes.

## Materials and methods

Plant material: All PCR amplification and Southern hybridization data were collected on genomic DNA from the parents of two interspecific hybrid populations, (Cayuga White x Aurore and Horizon x Illinois (Ill.) 547-1) as well as *Vitis vinifera* cv. Cabernet Sauvignon. Horizon and Cayuga White are siblings of *V. vinifera*, *V. labrusca*, *V. rupestris* and *V. aestivalis* ancestry; Aurore is a complex hybrid of *V. vinifera*, *V. rupestris* and *V. aestivalis;* and Ill. 547-1 is a cross between *V. rupestris* and *V. cinerea*

RAPD reactions: DNA was extracted from leaves following the protocol of LODHI *et al.* (1994) and RAPD reactions were carried out as described by LODHI *et al.* (1995) except that three different types of primers and primer combinations were used (Tab. 1):

1) Primers designed from the Kozak sequence (Kozak primers) were used singly to a final concentration of 0.2 µM;
2) RAPD primers were used singly to a final concentration of 0.2 µM;
3) RAPD primers were used in combination with Kozak primers (0.1 µM each).

Table 1

Primer sequences used in RAPD reactions

| Name | Sequence (5'÷3') |
|---|---|
| Kozak | |
| K1 | GCG ACC ATG G |
| K2 | CAG GCC ATG G |
| K3 | GCC ATG GAC G |
| K4 | GAT GGT ACC G |
| K5 | CGC AGG ATG G |
| K6 | CGA TGA CTG G |
| K7 | GGG ATG GCT G |
| K8 | CCC ATG GGT G |
| Others | |
| B356 | GCG GCC CTC T |
| B379 | GGG CTA GGG T |
| B388 | CGG TCG CGT C |
| B389 | CGC CCG CAG T |
| B391 | GCG AAC CTC G |
| GTO4 | GTG GTT GCG A |
| GY107 | GTT CAG GGC T GTT TAT AG |
| OPD-8 | GTG TGC CCC A |
| P33 | GTA AAA CGA C GGC CAG T |
| P35 | TGC GCA ACG T TGT TG |
| P210 | AAA TGC GGC A |
| P232 | CCG CTT GTT G |
| P250 | TGA GCT CCG T |
| P382 | GAA CCG GAT C |
| P437 | CGG ATC GAC A |
| P443 | GCC GTG ATA G |
| S34 | GAT AGC CGA C |
| S69 | CAT CGA ACC G |

Amplification products were electrophoresed in a 2 % agarose gel. Polymorphic bands were excised from the gel and (the agarose) was chopped into smaller pieces in 100 µl $H_2O$ and refrigerated at 4 °C until used (for long term storage, the tubes were kept at -20 °C). Excised bands were immersed in water for 5-6 h and reamplified with the same primers as were used originally for amplification. In cases where more than one band was observed after reamplification, the amplification was repeated at least once with one of the reamplified product, to be used as a probe.

Southern blotting: About 12 µg genomic DNA from Cayuga White, Aurore, Horizon and Ill 547-1 was restricted with 40 to 50 units of either *EcoR* I, *Hind* III, *BamH* I or *EcoR* V (Promega, WI, USA). The digestion was carried out at 37 °C for a minimum of 6 h and electrophoresed on 0.8 % agarose at 15 mA. The gel was exposed to UV (λ=302 nm) for 3 min for depurination, denatured in 1.5 M NaCl plus 0.5 M NaOH with constant shaking for 1 h and neutralized in 1.5 M NaCl plus 1 M Tris-HCl, pH 7.4 for 10 min. DNA was transferred to a MagnaGraph (MSI,

Westborough, MA) nylon membrane by wet blotting as described by SAMBROOK *et al.* (1989). After DNA transfer, the membrane was exposed to UV for 2 min for cross linking, soaked in 5X SSC (750 mM NaCl, 75 mM sodium citrate; pH 7.0) and stored wet at 4 °C until used.

Hybridization. Probe labeling: Simultaneous amplification and labeling of the selected band was done by PCR (CELEDA 1992). An aliquot of 2-3 µl from the gel with the RAPD band was added to the other components of the RAPD reaction. For labeling, the dNTPs consisted each of 0.12 mM dATP, dCTP, dGTP, 0.08 mM dTTP and 0.02 mM of alkali labile DIG-11-dUTP (Cat. # 1573 152, Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) in a final volume of 30 µl. Amplification was carried out in a 30-cycle reaction, each cycle at 94 °C for 60 s, 50 °C for 45 s and 72 °C for 105 s in a PTC-100 thermocycler (MJ Res. Corp., Watertown, MA, USA). An aliquot of 5 µl of the amplified reaction mixture was run on a 2 % agarose gel to confirm whether a single product was produced and whether the probe was labeled. The probes varied between 200 and 800 bp (Tab. 2). Labeling of DIG-dUTP from the labeling mixture was estimated with the Genius 1 Labeling and Detection Kit (Cat. # 1093 657, Boehringer Mannheim).

Probe hybridization: Prehybridization and hybridization with the labeled probe was carried out in a Mini Hybridization Oven (Cat. # H 9300, Hybaid, Peddington, Middlesex, UK) in hybridization tubes. Membranes were prehybridized in 15 ml hybridization solution (5X SSC, 1.0 % w/v Blocking reagent (Cat. # 1096 176, Boehringer Mannheim), 0.1 % N-lauroylsarcosine and 0.02 % sodium dodecyl sulfate). Additionally, 300 µl sonicated and autoclaved calf thymus DNA (5 mg·ml$^{-1}$) was boiled for 10 min and added to the prehybridization solution. Prehybridization, with constant rotation of the tubes, was done at 68 °C for 5-6 h with new membranes and 2-3 h in case of used membranes.

The probe (25-50 ng) was denatured for 5-7 min at 100 °C, mixed with the prehybridization solution and incubated at 68 °C for 10-15 h with constant rotation of the tubes. Following incubation, the membranes were washed twice with 2X SSC plus 0.1 % SDS for 5 min, each at 68 °C, and twice with 0.1X SSC plus 0.1 % SDS for 15 min, each at 37 °C. Each membrane was hybridized 3-5 times with different probes. Hybridized probes were stripped from membranes by incubation at 37 °C in alkaline probe-stripping solution (0.2 N NaOH, 0.1 % SDS) for 30 min. Membranes were rinsed in 2X SSC and stored at 4 °C.

Chemiluminescent detection: For washing, blocking, antibody binding and exposure to x-ray film, the procedure of VAILLANCOURT *et al.* (1992) was fol-

Table 2

Detection of hybridization pattern observed with probes amplified and labeled with digoxigenin in RAPD reactions, on membranes carrying restricted genomic DNA of cvs Aurore, Cayuga White, Horizon, Illinois 547-1 and Cabernet Sauvignon

| Probe | | Target | Number of bands hybridized[b] | | | |
|---|---|---|---|---|---|---|
| Name | Size (bp) | DNA[a] | *ER*I | *H*III | *BH*I | *ER*V |
| B356 #30[c] | 290 | A,C,H,I | 4 | 4 | - | 4 |
| B356 #232[c] | 480 | A,C,H,I,CS | >10 | >10 | - | - |
| B379 #31[c] | 410 | A,C,H,I | 2 | 3 | 5 | - |
| B388 #238[c] | 800 | A,C,H,I,CS | 2 | 1 | - | - |
| B388 #248[c] | 600 | A,C,H,I,CS | >10 | >10 | - | - |
| B388 #359B[c] | 500 | A,C,H,I,CS | >10 | >10 | - | - |
| B389 #305[d] | 590 | A,C,H,I,CS | >10 | >10 | - | - |
| B391 #57[c] | 290 | A,C,H,I | 1 | 1 | 1 | - |
| GTO4 #12[c] | 700 | A,C | >10 | >10 | >10 | >10 |
| K1 #02[c] | 500 | A,C | >10 | >10 | >10 | >10 |
| K5 #128[d] | 400 | A,C | 0 | 0 | 2 | - |
| K6 #202[d] | 200 | A,C,H,I,CS | >10 | >10 | - | >10 |
| OPD-8 #01[d] | 750 | A,C | - | 4 | 8 | - |
| P33 #03[c] | 430 | A,C | >10 | >10 | - | >10 |
| P35 #242[c] | 380 | A,C,H,I,CS | 4 | 1 | - | - |
| P443 #6[c] | 400 | A,C,H,I | 3 | 3 | 1 | - |

[a] Probes were hybridized to restricted and immobilized DNA from: A = Aurore, C = Cayuga White, H = Horizon, I = Illinois 547-1 or CS = Cabernet Sauvignon. A, C, H and I are interspecific hybrids and CS is a cultivar of *Vitis vinifera*. First part of the probe name indicates the primer name which was used to amplify the genomic DNA in a RAPD reaction and the last part is the probe number.

[b] Restriction enzymes used for genomic DNA digestion of A, C, H, I and CS: *ER*I, *EcoR* I; *H*III, *Hind* III; *BH*I, *BamH* I; and *ER*V, *EcoR* V. Dashes (-) in the table indicate that a particular enzyme was not used for the restriction of genomic DNA.

[c] RAPD bands excised from the amplification of Aurore DNA.

[d] RAPD bands excised from the amplification of Cayuga White DNA.

lowed except that 2 % blocking reagent was used instead of Carnation nonfat dried milk powder in the blocking buffer.

### Results and Discussion

Probing genomic DNA: Sixteen RAPD bands excised from amplifications of Aurore and Cayuga White DNA, were used as probes on restricted genomic DNA of Aurore, Cayuga White, Horizon, Ill 547-1 and Cabernet Sauvignon (Tab. 2). Eight of those probes hybridized to repetitive DNA (>10 bands) (Fig. 1 a), three probes to single copy DNA (1-2 bands) (Fig. 1 c) and five to low copy DNA (3-8 bands) (Fig. 1 d) of the genotypes included in the study. Williams *et al.* (1990) reported a higher percentage of single copy DNA markers (6 out of 11) and lower for repetitive DNA (5 out of 11) in soybean. In *Fusarium solani* f. sp. *cucurbitae* only one RAPD band out of 7 hybridized to low copy repetitive DNA and 6 were identified as single copy DNA (Ross *et al.* 1991). Total genomic DNA content of soybean (2.31 pg/2C; Arumuganathan and Earle 1991) is more than double that of grape (1.00 pg/2C; Lodhi and Reisch 1995). It is, therefore, expected that soybean contains more repetitive DNA than grape (Flavell 1985, Cullis 1990, Lapitan 1992) which would increase the probability of these sequences being picked up by arbitrary primers. This assumption is supported by the observation that one RAPD band hybridized to several RAPD products in Southern hybridization on RAPD blots in grape (the present study) and soybean (Williams *et al.* 1990). The hybridization pattern indicates that the probe was amplified from repetitive DNA, therefore, hybridized to the same DNA on the genomic blots. Amplification of a certain sequence is based on the sequence of a single oligonucleotide used as a primer as well as on the GC content. Increased amplification of repetitive sequences was unexpected as GC rich primers were used for amplification. One possible explanation is that microsatellite regions in *Vitis* are rich in (GT)n, (GA)n, (CAC)n, (GACA)n and (GATA) (Thomas *et al.* 1993) whereas the same regions in soybean are high in (AT)n or (TAT)n repeats (Morgante and Olivieri 1993). The probability of amplification of these repetitive sequences with high GC primers will be higher in grapes as compared to soybean. Fungi generally lack the abundance of non-coding DNA, therefore, most of the RAPD products would be amplified from single copy DNA.

No obvious difference was found in the hybridization pattern of RAPD probes excised from RAPD amplification with arbitrary or Kozak primers (Tab. 2). Three RAPD products of Kozak primers were used as probes; one hybridized to single copy DNA on the genomic blots of Aurore and Cayuga White whereas the other two hybridized to repetitive DNA. The Kozak primers used for amplification in this experiment did not reliably amplify coding region sequences. Further testing of Kozak primer amplified sequences should be pursued to determine their utility in targeting single copy sequences.

Probe P35 #242 hybridized to a single band on genomic DNA restricted with *EcoR* I while it hybridized to 4 bands when digested with *Hind* (Fig. 1 b). This indicated the presence of 3 *Hind* sites in the single *locus* characterized by *EcoR* I. Similarly, restriction of genomic DNA with different enzymes resulted in a single band of different sizes when probed with B391 #57 (Fig. 1 c). The presence of restriction sites within a *locus* could be of significance for gene mapping and cloning by chromosome walking.

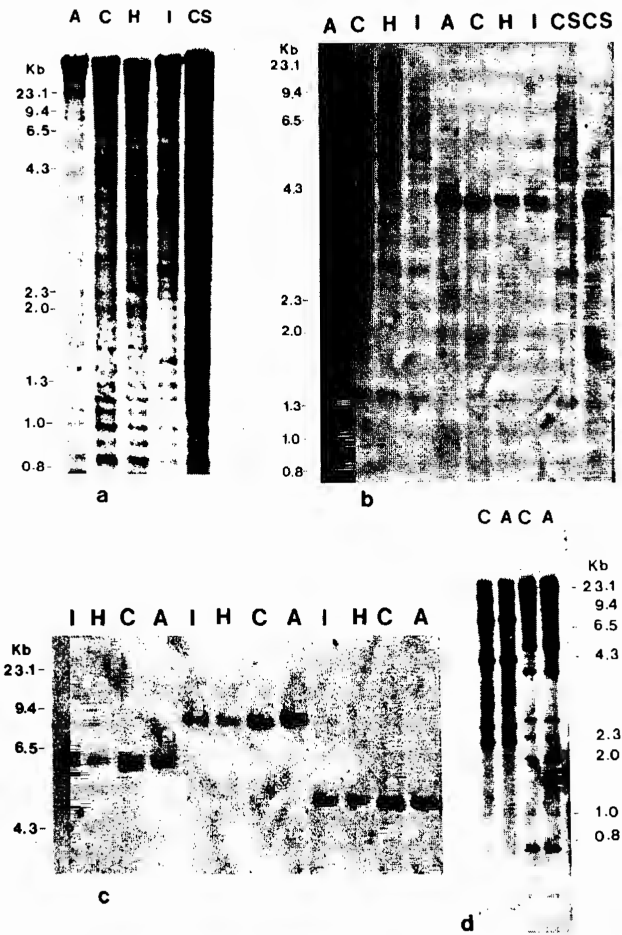


Fig. 1: Fluoretograms of southern hybridization of RAPD DNA probes on genomic DNA digested with different restriction enzymes. RAPD bands were excised from agarose gel, re-amplified, labeled with alkali labile DIG-11-dUTP and detected with a chemiluminescent method as described in Materials and Methods. The numbers on left or right of the figures indicate the DNA size markers in kilobases produced with either a mixture of λ DNA/*Hind* III and ØX174 RF/*Hae* III or λ DNA/*Hind* III alone. Letters A, C, H, I and CS are abbreviated for genomic DNA of Aurore, Cayuga White, Horizon, Illinois 547-1 and Cabernet Sauvignon, respectively.
**a**: Repetitive DNA pattern as detected by hybridization of probe B389 #305 (590 bp) with genomic DNA of CS, I, H, C and A digested by *EcoR* I in lanes 1-5 from right to left; **b**: Variation in hybridization pattern of probe P35 #242 (380 bp) with genomic DNA restricted with *Eco* RI (lanes 1-4 and 9, from left to right) and *Hind* III (lanes 5-8 and 10); **c**: Hybridization of probe B391 #57 (410 bp) to a single band on genomic DNA blots containing A, C, H and I. DNA was digested with *EcoR* I (lanes 1-4), *Hind* III (lanes 5-8) and *BamH* I (lanes 9-12); **d**: Hybridization pattern of low copy DNA observed with probe OPD-8 #01 (750 bp) and genomic DNA of A and C digested with *EcoR* I (lanes 1-2) and *Hind* III (lanes 3-4).

Probing amplified DNA: Twelve probes from RAPD bands were hybridized to RAPD blots containing amplification products from Aurore, Cayuga White, Horizon and Ill. 547-1 (Tab. 3). These probes were derived from

Table 3

Detection of number of bands and variation of hybridization pattern as observed with probes amplified and digoxigenin labeled with 10-18 bases long oligonucleotides in RAPD reactions, on mem-branes carrying DNA previously amplified in RAPD reactions with the same primers as used for the probes

| Probe | | Number of bands hybridized[a] | | | | Poly-morphism[b] | |
|---|---|---|---|---|---|---|---|
| Name | Size (bp) | A | C | H | I | CxA | HxI |
| B388 #359B[c] | 490 | 2 | 3 | 3 | 2 | Yes | Yes |
| B389 #305[d] | 590 | 0 | 1 | 1 | 1 | Yes | No |
| GY107 #216[c] | 350 | >10 | 2 | 1 | 2 | Yes | Yes |
| K1 #201[d] | 390 | 0 | 2 | 2 | 2 | No | Yes |
| K5 #237[d] | 800 | 5 | 5 | 3 | 4 | Yes | Yes |
| K6 #202[d] | 300 | 4 | 4 | 4 | 7 | No | Yes |
| P35 #353[d] | 280 | 3 | 1 | 1 | 1 | Yes | No |
| P210 #304[c] | 990 | 6 | 5 | 5 | 2 | Yes | Yes |
| P250 #302[c] | 280 | 5 | 5 | 5 | 5 | No | Yes |
| P437 #301[c] | 710 | 1 | 0 | 0 | 0 | Yes | No |
| S34 #303[d] | 580 | 2 | 4 | 6 | 4 | Yes | Yes |
| S69 #214[c] | 250 | 4 | 5 | 5 | 7 | Yes | Yes |

[a] Hybridization of the RAPD DNA probes with the bands amplified in RAPD reactions from genomic DNA of: A = Aurore, C = Cayuga White, H = Horizon and I = Illinois 547-1.
[b] Polymorphisms detected with RAPD DNA probes on the genomic DNA amplified in RAPD reactions with the patental DNA of CxA = Cayuga White x Aurore and HxI = Horizon x Illinois 547-1.
[c] RAPD bands excised from the amplification of Aurore DNA.
[d] RAPD bands excised from the amplification of Cayuga White DNA.

bands polymorphic between Aurore and Cayuga White, reamplified, and labeled with DIG-11-UTPs. Probes K1 #201 and B389 #305 hybridized to amplified DNA of all cultivars except that B389 #305 did not hybridize to any band on Aurore thus indicating that B389 #305 was polymorphic between Aurore and Cayuga White while it was monomorphic in Horizon and Ill. 547-1. This could be because the probes were prepared from Cayuga White. Two probes, P437 #301 and P35 #353, hybridized to one band, probes K6 #202, S69 #214, S34 #303, P210 #304 and B388 #359B to 3-10 bands and probes K5 #237 and P250 #302 to >10 bands. Probe GY107 #216, excised from an Aurore DNA amplification, hybridized to >10 bands on RAPD blots of Aurore and to a single band in the other 3 cultivars. In cases where one probe hybridized to >1 RAPD band, the hybridized bands included the one that was used to develop the probe.

RAPD products excised for labeling were reamplified at least two times to make sure that the probe consisted of only one sequence. Probes P437 #301 and B388 #359B produced 2 and 3 bands, respectively, on reamplification of a single band even though highly stringent conditions were used. P437 #301 was produced from Cayuga White DNA and 710 bp long. The first reamplification produced two bands: 710 and 470 bp. Further reamplification of 710 bp band resulted in 3 bands, 470, 385 and 200 bp while the 470 bp band produced 2 bands: 385 and 200 bp. To test whether all these products were produced from a single *locus*, the 710 bp band was labeled and probed on the RAPD blots generated with primer P437. The probe hybridized to one band, implying that the smaller bands were produced from multiple internal priming sites. Probe B388 #359B, 710 bp, generated three products on reamplification, 490, 300 and 190 bp. Reamplification of thc 490 bp band rcsulted in a single band which hybridized to 2-3 bands on a RAPD DNA blot indicating the presence of multiple copies of the amplified products.

Probes K6 #202, GY107 #216, K5 #237, P250 #302, and B389 #305 produced a single but smaller DNA product on reamplification. TINKER *et al.* (1993) found that 5 out of 8 RAPD bands hybridized to more than one band that were slightly shorter in size than the original band in barley. Other probes, K1 #201, S69 #214, S34 #303, P210 #304 and P35 #353 produced a single band on reamplification and mostly hybridized to 2-7 RAPD products on the RAPD blots. Similar results have been noted by other researchers (personal communications), in *Pinus radiata*, *Drosophila* and *Mycobacterium avium* by CHARLIE BELL, LOUIS VAN DE ZANDE and DONNA JENSEN respectively. In our experiment, the initial RAPD reaction was carried out at a low stringency annealing temperature, 35 °C, while the annealing temperature was 55 °C in subsequent reamplification and labeling steps. RAPD reactions are typically carried out with a single primer. It has been observed that DNA could be amplified with just one annealing site, with one partial complementation out of two required (PARKS *et al.* 1991, VENUGOPAL *et al.* 1993). Increasing the annealing temperature would result in the amplification from annealing sites with high complemen-tation. Internal priming sites may be present in an amplified product. TINKER *et al.* (1993) suggested that products from these internal sites were not produced since these might be weak priming sites and would not be amplified unless the longer fragments become abundant in the reaction mixture. These products were not visible with ethidium bromide staining. In our case we used a higher temperature for reamplification as compared to their experiment where a consistent annealing temperature was used. When a higher temperature is used, the products are produced from rare internal priming sites with more intensity. Some evidence for the presence of internal priming sites is provided by linkage analysis (LODHI *et al.* 1995). In some linkage groups of *Vitis* several markers generated by a single primer have been mapped in one linkage group. Some show strong linkages (< 5 cM), others cosegregate and still others are > 20 cM apart on the same linkage group. The present data is insufficient to prove the validity of the hypothesis that RAPD amplification can take place from internal priming sites of a fragment. Analysis of additional markers along with DNA sequencing and cloning information is needed.

Two RAPD DNA probes, S69 #214 and K6 #202, were also hybridized on the RAPD blots of genomic DNA of Aurore, Cayuga White, Horizon and Ill 547-1, amplified with different primers besides their own amplification products (Fig. 2 a and b). Probe S69 was hybridized on RAPD blots of genomic DNA with primers S34, S62, B388, OP20 and P232 and probe K6 #202 on K1, K4, K5, P35,

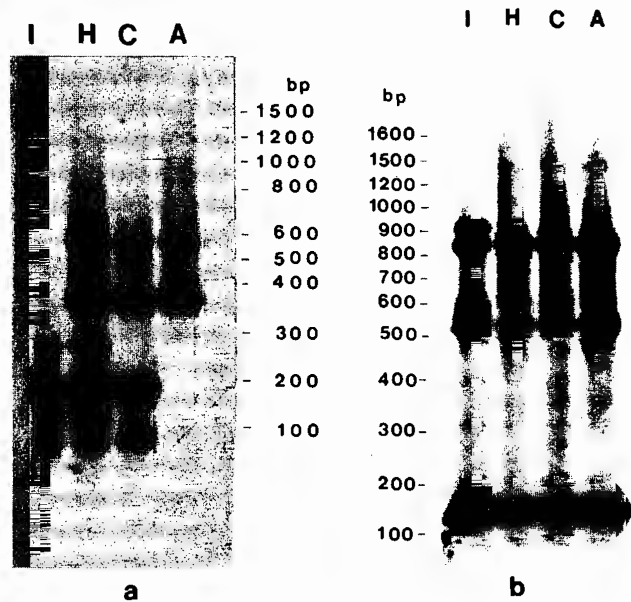


Fig. 2: Fluoretograms of the hybridization of RAPD DNA probes labeled with alkali labile digoxigenin-11-dUTP. RAPD blots were prepared by transferring the products of RAPD reactions of A (Aurore), C (Cayuga White), H (Horizon) and I (Illinois 547-1), to membranes from agarose gels. The hybridization products were visualized by chemiluminescent techniques. The numbers on the side of the figures are DNA size markers observed with 100 bp DNA ladder (Gibco-BRL).

**a**: Hybridization of probe S34 #303 (580 bp) on RAPD products amplified with primer S34; **b**: Hybridization of probe K6 #202 (200 bp) on RAPD products amplified with primer K6.

P210 and P382. Probe S69 #214 hybridized to 4-7 bands of the RAPD blot of S69 as well as 3-4 bands and 5-6 bands on RAPD blots of B388 and OP20, respectively. No hybridization was seen with the products of other primers. The K6 #202 hybridized to 4-7 bands on the K6 DNA blot as well as to 5-7 bands on the K4 blot. This probe did not hybridize to blots with RAPD products of other primers. Hybridized products on each primer blot differed in size. This phenomenon indicated that the RAPD bands from S69 and K6 were repetitive in nature and hybridized to the products of amplification from other primers. Moreover, some primers with different sequences amplify similar products from repetitive genomic DNA indicating the presence of different primer annealing sites in or at the end of the interspersed repetitive DNA, each amplifying from a different site with the same repeat sequence. This observation is supported by linkage analysis of *Vitis* of markers produced from primers B388, OP20 and S69 (LODHI *et al.* 1995). Markers produced with OP20, and B388 (OP20bb and B388g) map (8.0 cM apart) at linkage group E of the Cayuga White map. Similarly, two other markers of primers S69 and B388 (S69d and HB388s) are mapped on the same linkage group (group V).

Use of primer combinations: Arbitrary primers were also used in combination with Kozak primers and the RAPD phenotypes were compared in Aurore, Cayuga White, Horizon and Ill. 547-1 (Tab. 4). Several such *loci*, generated with a primer combination, have already been mapped in *Vitis* (LODHI *et al.* 1995). Though no hybridization and DNA sequencing was done with these bands, they could be useful in understanding the nature of amplification of such *loci*. They could also be used to understand whether amplification takes place from a single *locus* with internal priming sites or several *loci* amplifying independently. Linkage analysis of these *loci* suggests that amplification produces bands other than the ones amplified either with the arbitrary or Kozak primers used alone. Several *loci* amplified with primer pairs were unlinked or showed weak linkage with other *loci* amplified with one of the primers and mapped to a different linkage group (Lodhi *et al.* 1995).

Use of RAPDs in related populations: To explore the possibilities of using RAPD markers developed in a single population for use on other populations, RAPD reactions were carried out on the parental DNA of two populations that were used for genetic mapping. Tab. 4 shows the similarities observed between the phenotypes of RAPD bands in these two populations amplified by 16 primers. A total of 140 bands were produced in RAPD reactions; 100 of these were common to both populations and 47 were polymorphic in one or both of the populations. Ten polymorphic bands were observed only in Aurore x Cayuga White and 22 in Horizon x Ill. 547-1. From these observations it could be anticipated that genetic maps could be used in different segregating populations, though in this case Cayuga White and Horizon

Table 4

Summary of the RAPD amplification of the parental DNA of two populations, Cayuga White x Aurore and Horizon x Illinois 547-1

| Primer | Total no. of bands produced | Bands present in both sets of parents | | No. of polymorphic bands present only in[c] | |
|---|---|---|---|---|---|
| | | All[a] | Poly[b] | C x A | H x I |
| B388 | 10 | 5 | 4 | 0 | 3 |
| B389 | 6 | 3 | 2 | 0 | 3 |
| GY105 | 18 | 13 | 6 | 2 | 3 |
| GY107 | 17 | 15 | 5 | 1 | 2 |
| K1 | 4 | 4 | 2 | 0 | 0 |
| K4 | 6 | 1 | 0 | 1 | 2 |
| K5 | 10 | 10 | 3 | 0 | 2 |
| K6 | 10 | 7 | 4 | 2 | 2 |
| P35 | 4 | 2 | 2 | 0 | 0 |
| P210 | 10 | 4 | 3 | 1 | 2 |
| P250 | 5 | 2 | 1 | 1 | 0 |
| P232 | 6 | 5 | 2 | 1 | 0 |
| P437 | 10 | 9 | 2 | 1 | 0 |
| S34 | 11 | 10 | 7 | 0 | 1 |
| S62 | 5 | 3 | 1 | 0 | 1 |
| S69 | 8 | 7 | 4 | 0 | 1 |
| Total | 140 | 100 | 47 | 10 | 22 |

[a] RAPD bands of similar size observed in Aurore and/or Cayuga White and Horizon and/or Illinois 547-1 (polymorphic and monomorphic).
[b] RAPD bands present either in Aurore or Cayuga White as well as in Horizon or Illinois 547-1 (only polymorphic).
[c] RAPD bands from column 2, 3 and 4 polymorphic only in C x A or H x I.

were siblings, thus the two populations tested were related. Polymorphic bands specific for the new population could be added to the existing map by linkage analysis. This is partially supported by the hybridization of the RAPD markers on the genomic as well as RAPD blots (Figs. 1 and 2). However, DNA fragments migrating to the same positions on gels and scored identically could be unrelated DNA sequences (THORMANN and OSBORN 1992; XU *et al.* 1995). More work, for example, sequencing DNA of the fragments of similar size should be done to test this hypothesis. This approach of using markers developed in different populations can save time and unnecessary repetition of laboratory experiments. Also a similar approach could be used with 3-4 segregating populations and a more general map could be developed with the polymorphic *loci* common to all the populations. A RAPD reactions run with 4 Kozak primers (Tab. 4) on Cayuga White, Aurore, Horizon and Ill. 547-1 genomic DNA produced 30 bands including 10 that were polymorphic and population specific.

We have provided some evidence that RAPD amplification takes place in the single copy as well as repetitive regions of the grape genome. Also internal priming sites may be present in the amplified RAPD product that result in the amplification of smaller products on reamplification. The results are inconclusive without DNA sequence analysis. Genetic maps developed for a population could be used (with caution) in related populations by screening the primers on parental DNA. The results have been partially confirmed by Southern hybridization of RAPD products on genomic DNA and RAPD blots.

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