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PCR-RFLP analysis of *Vitis*, *Ampelopsis* and *Parthenocissus* and its application to the identification of rootstocks

by

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S u m m a r y : Methodologies based on the analysis of DNA polymorphism were recently described to identify and study grapevine rootstocks and cultivars. We report here rapid RFLP analysis of 17 *Vitis*, 3 *Ampelopsis* and 2 *Parthenocissus* DNAs amplified by PCR with primers based on sequences from previously defined polymorphic DNA fragments of *V. vinifera* cv. Chardonnay. These primers can be used as a tool for phylogenetic studies in the genus *Vitis* and more generally within the Vitaceae family. The DNAs of 22 rootstocks could be identified by PCR using 4 pairs of Chardonnay-derived primers combined with RFLP analysis. However, it was impossible to discriminate between 9 clones of the rootstock 3309 C. This technique is rapid and well reproducible.

K e y w o r d s : RFLP, Vitis, rootstock identification.

Introduction

The grapevines (*Vitis*) belong to the Vitaceae like their close relatives *Ampelopsis* Michaux and *Parthenocissus* Planchon (Emberger 1960; Negrul 1968; Galet 1992). *Vitis* is divided in two subsections: *Muscadinia* which is represented by three species, and *Euvitis* (85 species). A small number of these is of great agronomical importance (Scossiroli 1988; Galet 1988). The well-known *V. vinifera* L. is an Asian and European grapevine whose domestication by man began approximatively 6,000 years ago; its cultivars were propagated vegetatively (Levadoux 1956; Negrul 1968; Scossiroli 1988). Since the phylloxera invasion during the 19th century, the cultivars of *V. vinifera* must be grafted on selected *Vitis* hybrids which generally descend from crosses between american species (Galet 1988). To identify and study the more than 5,000 cultivars, ampelographers traditionally use qualitative or quantitative phenotypic characters also called ampelographical characters (Levadoux 1954; Galet 1988). However the ampelographical description may vary depending on environmental conditions: some plants like clones of the same cultivar are very similar, and some ampelographical characters may be observed only at a precise developmental stage of the plant, during a short period. To overcome these difficulties, some authors described methods for a rapid characterization using biochemical markers (Subden *et al.* 1987; Bénin *et al.* 1988; Tedesco *et al.* 1989). Although useful polymorphic isozyme patterns were described for different species and cultivars, the variations of the patterns due to environmental conditions or to the type of tissue analysed, and the small number of isozymes were an important hindrance for the development of this method (Stravrakakis and Loukas 1983; Subden *et al.* 1987). Other methods which are based on the analysis of DNA polymorphism do not have these disadvantages. DNA probes of other organisms have been used to analyse the restriction fragment length polymorphism (RFLP) of grapevine DNAs (Striem *et al.* 1990; Yamamoto *et al.* 1991; Sivolap *et al.* 1992). Repetitive DNA sequences (Thomas *et al.* 1993), unique or moderately repeated sequences of grapevine (Bourquin *et al.* 1991, 1992, 1993; Mauro *et al.* 1992) have been cloned and selected as RFLP probes which allowed identification and/or taxonomical study of various grapevine cultivars, rootstocks or species. A recently reported method based on random amplified polymorphic DNA (RAPD) by polymerase chain reaction (PCR) analysis allowed the detection of grapevine DNA polymorphism (Collins and Symons 1993).

We describe here another rapid method which analyses directly or with the aid of restriction enzymes the polymorphism of DNAs amplified by PCR using primers based on partially sequenced short *Pst*I DNA fragments of *V. vinifera* cv. Chardonnay. We have applied this technique to members of *Vitis*, *Ampelopsis* and *Parthenocissus* genera, and demonstrate its usefulness for the rapid identification of rootstocks.

Materials and methods

P l a n t m a t e r i a l s : Plants analysed are listed in Tab. 1 and 2.

D N A p r e p a r a t i o n : The DNAs of the first set of 17 plants (Tab. 2) were extracted and purified twice by ultracentrifugation in cesium chloride gradients as described by Bourquin *et al.* (1993).

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Table 1

Genera and species analysed in the experiments

Abbreviation	Genus	Species	Variety or cultivar
P. q. lac.	*Parthenocissus*	*quinquefolia*	*laciniata*
P. q. typ.	*P.*	*quinquefolia*	*typica*
A. c. ind.	*Ampelopsis*	*cordata*	*indivisa*
A. c. het.	*A.*	*cordata*	*heterophylla*
A. bre.	*A.*	*brevipedunculata*	
V. syl. m.	*Vitis*	*sylvestris* (male)	
V. syl. f.	*V.*	*sylvestris* (female)	
V. rub.	*V.*	*rubra*	
V. rip. M.	*V.*	*riparia*	Millardet
V. rip. G. M.	*V.*	*riparia*	Gloire de Montpellier
V. mon.	*V.*	*monticola*	Large Bell
V. doa.	*V.*	*doaniana*	
V. Lin.	*V.*	*Lincecumii*	
V. Dav.	*V.*	*Davidii*	
V. Lab.	*V.*	*Labrusca*	Isabelle
V. rup.	*V.*	*rupestris*	du Lot
V. Ber.	*V.*	*Berlandieri*	Thyers
V. amu.	*V.*	*amurensis*	
V. cin.	*V.*	*cinerea*	
V. cor.	*V.*	*cordifolia*	9 Couderc
V. bet.	*V.*	*betulifolia*	
V. ari.	*V.*	*arizonica*	

All plants were obtained from the ampelographical collections of INRA-Colmar, France

The DNAs of the second set of 19 plants (Tab. 2) and of the plants listed in Tab. 1 were extracted by the following rapid method: young leaves were collected in vineyards in July and frozen at -20 °C. A piece of 0.5 g of leaf tissue was placed in a plastic bag (7 cm x 7 cm) with one layer of Miracloth (Calbiochem) (5 cm x 5 cm). After addition of 1.5 ml of extraction buffer (0.44 M sucrose, 2.5 % (w/v) Ficoll 400, 5.0 % (w/v) Dextran T 40, 25 mM Tris-HCl (pH 8.0 at 25 °C)) the bag was sealed and put on a horizontal hard surface and the tissue was ground through the plastic with a manual grinder (Bioreba) (this tool is a sort of stamp equipped with ball bearings). The suspension was filtered through one layer of cotton gauze and centrifuged for 5 min at 350 *g* and 4 °C. The pellet containing the nuclei was resuspended in 1 ml of ice-cold deionized water. After a second centrifugation in the same conditions, the pellet was resuspended in 0.5 ml of ice-cold lysis buffer (0.2 M Tris-HCl (pH 8.0 at 25 °C), 1.0 M NaCl, 2 % (w/v) Sarkosyl) and put on ice for 10 min. The lysate was centrifuged for 5 min at 17,000 *g* and 4 °C. The supernatant (0.5 ml) was transferred into a clean tube and mixed with 0.1 volume of 3.0 M sodium acetate (pH 5.5 at 25 °C), followed by the addition of 0.6 volume of

Table 2

Grapevine rootstocks analysed in the experiments

Abbreviation	Cultivar	Clone number	*Vitis* species or hybrid [a]	Source [b]
rup. Lot	du Lot	110	*rupestris*	E [2]
rup. Lot	du Lot	110	"	I [1, 2]
rip. Gloire	Gloire de Montpellier	*	*riparia*	I [2]
101-14 Mgt	101-14 Millardet et Grasset	3	*rip.*x *rup.*	E [2]
3309 C.	3309 Couderc	111	*rip.* Z Couderc x *rup.* Martin	E [2]
3309 C.	3309 Couderc	*	"	I [1]
420 A Mgt	420 A Millardet et Grasset	241	*Berlandieri* x *rip.*	E [2]
420 A Mgt	420 A Millardet et Grasset	11	"	I [1]
161-49 C.	161-49 Couderc	197	"	E [2]
161-49 C.	161-49 Couderc	171	"	I [1]
5 BB	Teleki 5 BB	259	"	I [1, 2]
SO 4	SO 4	157	"	I [2]
SO 4	SO 4	102	"	I [1]
SO 4	SO 4	161	"	I [1]
SO 4	SO 4	5	"	I [1]
140 Ru.	140 Ruggeri	101	*Ber.* Résseguier N° 2 x *rup.* Lot	E [2]
140 Ru.	140 Ruggeri	101	"	I [1]
1103 P.	1103 Paulsen	113	"	E [2]
110 R	110 Richter	169	*Ber.* Résseguier x *rup.* Martin	I [2]
110 R	110 Richter	118	"	I [1]
44-53 Cl.	44-53 Castel	755	*rip.* Grand Glabre x 144 Malègue (144 Malègue: *cordifolia* x *rup.*)	E [2]
4010 Cl.	4010 Castel	243	*rip.* Grand glabre x A.R.G. 1 (A.R.G. 1: *vin.* Aramon x *rup.* Ganzin)	E [2]
196-17 Cl.	196-17 Castel	99	1203 Couderc x *rip.* Gloire (1203 Couderc: *vin.* Mourvèdre x *rup.*)	E [2]
196-17 Cl.	196-17 Castel	*	"	I [1]
Fercal	Fercal	242	(*Ber.* x Colombard) N° 1A x 333 E.M. (333 E. M.: *vin.* Cabernet-Sauvignon x *Ber.*)	E [2]
Fercal	Fercal	242	"	I [1]
LN 33	LN 33	D	1613 C. x *vin.* Thompson seedless (1613 Couderc: Solonis x Othello) (Othello: Clinton x *vin.* Black Hambourg) (Clinton: *Lab.* x *rip.*)	I [1]

[a] Galet 1990; [b] E = ENTAV-Le Grau du Roi, France, I = INRA-Colmar, France, [1, 2] = first and second set of experiments; * = unknown

isopropanol and precipitation at room temperature for 15 min. After centrifugation for 15 min at 17,000 *g*, the pellet was dried and redissolved in 20 µl of deionized water. 4 µl of ribonuclease A (0.1 mg/ml) DNase-free were added and the DNA solution was incubated for 30 min at 37 °C.

Primer construction: Four cloned *Pst* I DNA fragments of *V. vinifera* cv. Chardonnay were chosen among the most informative RFLP probes used in previous studies (BOURQUIN *et al.* 1991, 1992, 1993). Both extremities of each fragment were sequenced with the Multi-Pol sequencing kit (Stratagen) according to the supplier's instructions. Each pair of primers was deduced from the DNA sequences according to the rules given by INNIS and GELFAND (1990) and SAIKI (1990). The melting temperature of each primer was 68 °C. The main characteristics of each pair of primers were the following (sequence data not shown) (in the order: name, size of the first primer in nucleotides, size of the second primer, approximate size in base pairs of the fragment amplified from the original cloned *Pst* I fragment): PP122, 23-mer, 24-mer, 550 bp; PP307, 22-mer, 22-mer, 375 bp; PP746, 25-mer, 22mer, 280 bp; PP470, 26-mer, 24-mer, 420 bp.

PCR conditions: The amplification mixture consisted of 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25 °C), 0.1 % (v/v) Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dTTP, dCTP and dGTP, 0.25 µM of each primer, 10 ng template DNA / 0.1 ml, and 5 U Taq DNA polymerase (Promega) / 0.1 ml.The amplification mixture was overlaid with one volume of silicone oil 47 V 100 (Rhône-Poulenc). Amplification was carried out with a thermal cycler PHC-3 (Techne) programmed as follows: 5 cycles of 1 min at 93 °C, 2 min at 50 °C; 30 cycles of 20 s at 93 °C, 40 s at 50 °C; ending by 10 min at 72 °C. Total volume of reaction was 20 µl with PP 307, PP 746 and PP 470; 50 µl with PP 122.

RFLP analysis: After PCR amplification with PP 122, 40 µl of the reaction mixture were mixed with 4 µl of 3.0 M sodium acetate (pH 5.5 at 25 °C), followed by 2.5 volumes of 96 % ethanol and precipitated for 1 h at -20 °C. The DNA was pelleted by centrifugation for 15 min at 17,000 *g* and redissolved in 20 µl of deionized water. 6 µl of the amplified DNA were digested for 2 h at 37 °C in 15 µl with 5 U of a four-cutter enzyme: *Dde*I, *Hinf*I (New England Biolabs) or *Alu*I (BRL) according to the supplier's instructions. The reaction mixture was concentrated with two volumes of butanol before loading onto the gel.

Gel electrophoresis: PCR products (10 µl of the amplification mixture) were fractionated by vertical gel electrophoresis on a 5 % Long Ranger polyacrylamide gel (Serva) (140 mm x 150 mm x 1 mm), 1.2 x TBE (0.1 M Tris-borate, 0.1 M boric acid, 3 mM EDTA) in 0.6 x TBE electrophoresis buffer. The electrophoresis was stopped when the bromophenol blue marker reached the bottom of the gel.

For RFLP analysis, the total amount of each concentrated digestion was fractionated in the same conditions except for the electrophoresis which was stopped when the bromophenol blue marker was at 30 mm of the bottom of the gel. The gels were stained in ethidium bromide (1 µg/ml) during 15 min.

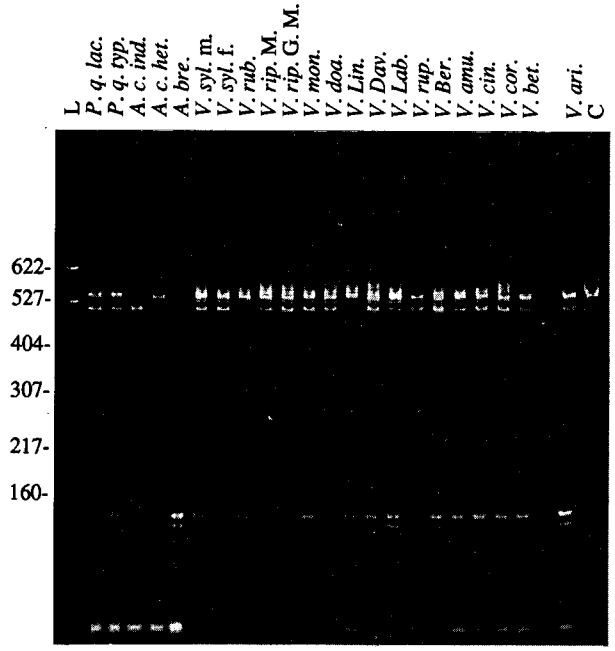
Results

RFLP analysis of DNAs of *Vitis*, *Ampelopsis* and *Parthenocissus* generated by PCR using a pair of Chardonnay primers: DNAs of the 17 *Vitis*, 3 *Ampelopsis* and 2 *Parthenocissus* species and varieties listed in Tab. 1 were obtained by the rapid isolation method and amplified by PCR with the pair of Chardonnay primers PP 122 (Fig. 1). Most samples show a strongly amplified DNA fragment of the same size as the amplified cloned DNA control. The three *Ampelopsis* species show a weak signal; the PCR products of both *A. cordata heterophylla* and *A. brevipedunculata* have been two-fold concentrated before loading. This low amplification may be due to some inhibitory effects of the DNA samples and/or to sequence differences between one or both primers and the corresponding *Ampelopsis* sequences. In some cases, e.g. both *V. riparia* cultivars, the intensity of the main fragment could be explained by the presence of other fragments of similar size. Three other amplified fragments of approximatively 500, 140 and 130 bp are more or less visible in all samples.

The PCR products were further analysed by *Alu*I, *Dde*I and *Hinf*I digestions (except for *V. rupestris*, *A. cordata heterophylla* and *A. brevipedunculata*). Ten to 11 informative or unique restriction fragments were generated in *Dde*I digestion, 7 to 8 by *Hinf*I digestion (data not shown). Among the 8 to 9 *Alu*I fragments, two are shared by both *V. sylvestris* samples and the Chardonnay control, and are absent in other samples (Fig. 2). Interestingly, the polymorphism did not allow to discriminate between the *Parthenocissus*, *V. riparia* and *V. sylvestris* pairs respectively. It should be noted that polyacrylamide gel electrophoresis permits the detection of very small size differences between restriction fragments (Fig. 2).

Identification of rootstocks by PCR-RFLP analysis: Thirty-five plants listed in Tab. 2 were analysed. In some cases, a hybrid is represented by two or more clones. The 20 hybrids and the two *Vitis* species examined here represent a large majority of the commercialized grapevine rootstocks.

Two sets of experiments were carried out independently. In the first set, 17 plants corresponding to 13 hybrids and *V. rupestris* of the INRA collection were analysed (Tab. 2); the DNA samples were prepared by the ultracentrifugation method. In the second set, 19 plants were analysed: 4 hybrids of the INRA collection, 15 hybrids, *V. riparia* and *V. rupestris* from the ENTAV collection (replaced by the *V. rupestris* from the INRA collec-



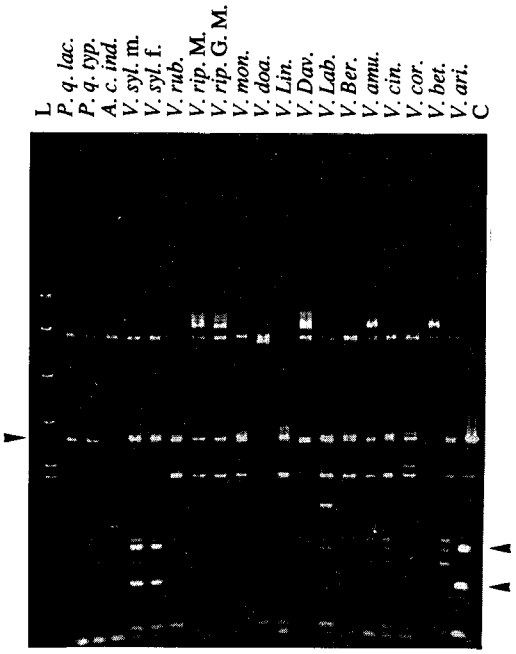


Fig. 1 (left): Patterns of *Vitis*, *Ampelopsis* and *Parthenocissus* DNAs amplified by PCR with the Chardonnay pair of primers PP 122. Abbr. are detailed in Tab. 1. Lane L: DNA marker (pBR 322 digested by *Msp*I; fragment size in base pairs). Lane C: DNA fragment amplified from the cloned *Pst*I Chardonnay fragment N_i 122 from which the primer sequences were derived (BOURQUIN *et al.* 1991).

Fig. 2 (right): *Alu*I digestion patterns of the PCR-amplified DNAs shown in Fig. I (*Dde*I and *Hinf*I digestion patterns not shown). Lane L as in Fig. 1. Lane C: *Alu*I digestion of the PCR-amplified DNA control. Arrow in the left margin indicates two informative fragments with a small size difference (approximatively 5 bp). Arrows in the right margin indicate two informative fragments which are shared only by both *V. sylvestris* samples and the Chardonnay control.

tion in the RFLP analyses) (Tab. 2); the DNA samples were prepared by the rapid isolation method (Materials and methods). Eleven hybrids or species were common to both sets of experiments. In each set, the four pairs of Chardonnay primers PP 307, PP 470, PP 746 and PP 122 were used separately in PCR amplifications. The four pairs of primers allowed the direct amplification of polymorphic DNA fragments. The most polymorphic patterns were obtained with PP 746 (Fig. 3) and PP 307 (data not shown). In both cases, four strongly amplified informative or unique DNA fragments were retained to identify the hybrids. Other too weak fragments were not used. The polymorphism obtained with PP 470 allowed identification of 3309 C, 101-14 Mgt and 1616 C. Finally, in the case of PP 122, the same type of pattern as for the *Vitis* species was observed except for 4010 Cl. which showed two additional fragments.

In both sets of experiments, the PCR products obtained with PP 122 were submitted to RFLP analysis with *Alu*I, *Dde*I and *Hinf*I. The three enzymes revealed an important polymorphism. Eight informative or unique *Hinf*I fragments were retained for identification; 7 fragments were retained in the case of *Alu*I (Fig. 4). The *Dde*I digestion pattern was used to separate 110 R and 1103 P. The same *Alu*I fragments of the Chardonnay control which are found in *V. sylvestris* are also present in 41 B Mgt and 4010 Cl., both related to *V. vinifera* (Tab. 2). Each of the 11 hybrids common to both sets of experiments showed the same patterns. Furthermore, we did not observe any polymorphism between the clones of each hybrid. These results demonstrate that the method is reproducible under our experimental conditions. The only exception was found in the

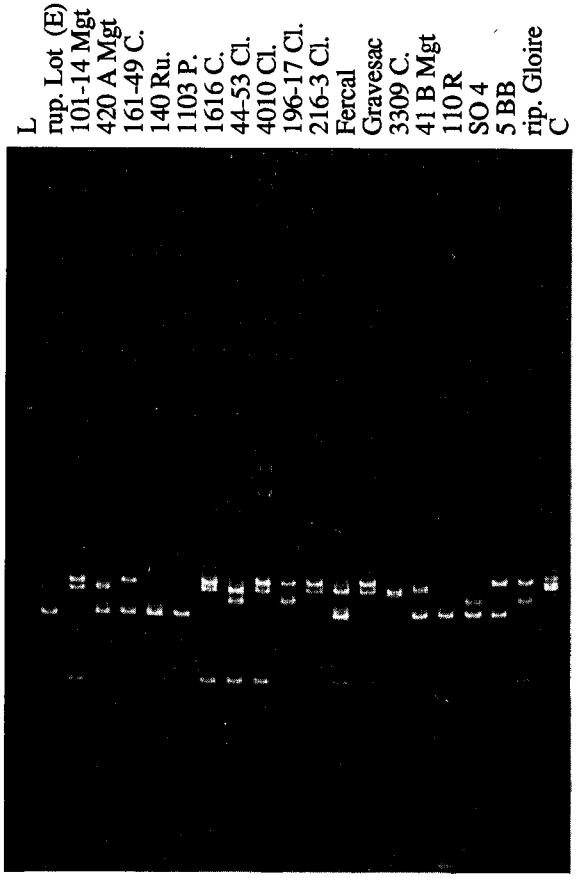


Fig. 3: PCR-amplified polymorphic DNA patterns of the rootstocks analysed in the second set of experiments obtained with the pair of Chardonnay primers PP 746. Abbreviations are detailed in Tab. 2. Lane L as in Fig. 1. Lane C: PCR control for the Chardonnay fragment N° 746.

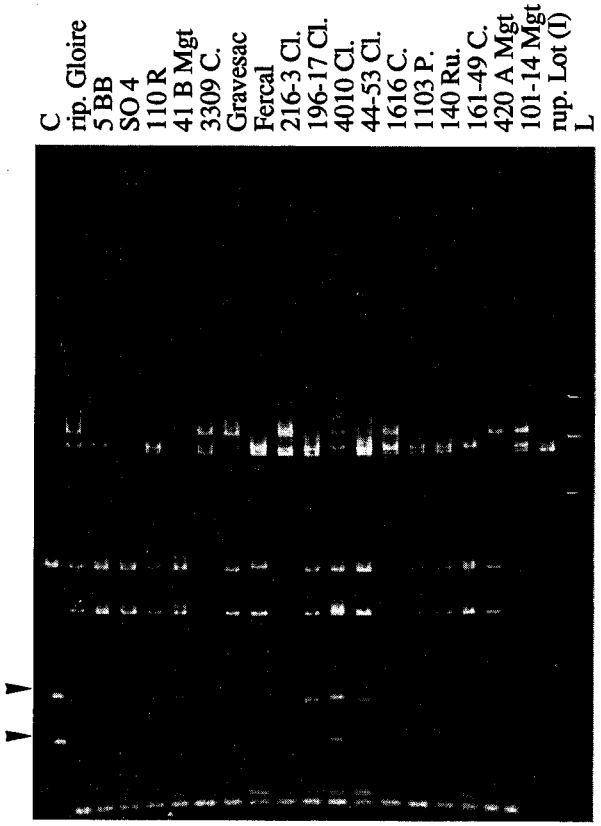


Fig. 4: *Alu*I digestion patterns of the PCR-amplified DNAs shown in Fig. 3 (except for *V. rupestris* which came from the INRA collection). Lane L as in Fig. 1. Lane C: *Alu*I digestion of the PCR-amplified DNA control (Fig. 3). Arrows in the left margin indicate the same two fragments as those described in Fig. 2 which are only shared by both 41 B Mgt and 4010 Cl. which are related to *V. vinifera,* and the Chardonnay control.

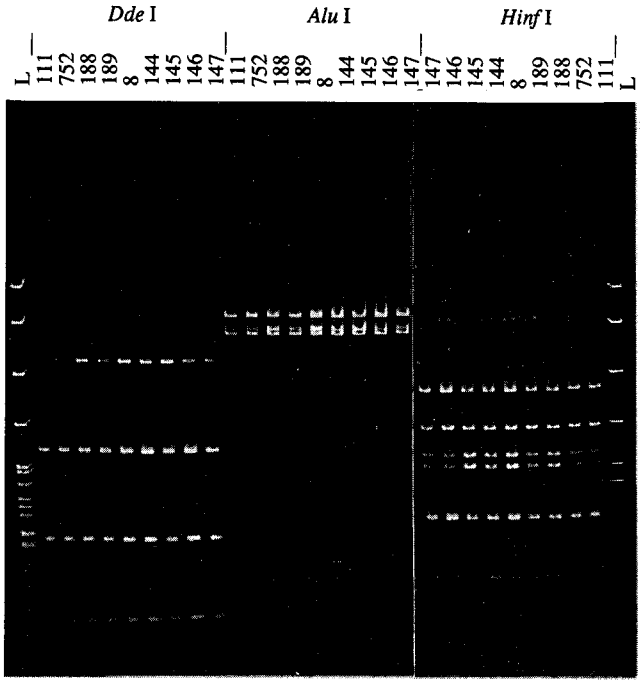


Fig. 5: RFLP patterns of the PCR-amplified DNAs of nine clones of 3309 C using the pair of primers PP 122 and the three enzymes *Dde*I, *Alu*I and *Hinf*I. Each clone number is indicated at the top of each lane. Lane L as Fig. 1.

analysis of *V. rupestris* with PP 122 and *Hinf*I, in which a restriction fragment which was clearly present in the first set was very weak in the second set (data not shown).

The results allow us to propose two protocols to identify each of the 22 rootstocks of this study.

The first solution combines the results obtained with PP 746 and those obtained by RFLP analysis with PP 122 and *Hinf*I (PP 122 / *Hinf*I). This allows the identification of 16 rootstocks. The three remaining couples (*V. rupestris* du Lot, Fercal), (101-14 Mgt, 1616 C) and (1103 P, 110 R) may be resolved in a second step with PP 122 / *Alu*I, PP 470 and PP 122 / *Dde*I, respectively. The second solution combines the results obtained with PP 307 and PP 122 / *Hinf*I. This allows the identification of 15 rootstocks. The three remaining groups (3309 C, 101-14 Mgt, 1616 C), (1103 P, 110 R) and (Vialla, 5BB) may be resolved in a second step by PP 470, PP 122 / *Dde*I and PP 122 / *Alu*I, respectively.

Comparison of nine clones of 3309 C : Nine clones of 3309 C were analysed in the same conditions as used for the hybrids with PP 307, PP 746, PP 122 / *Alu*I, PP 122 / *Dde*I and PP 122 / *Hinf*I. No polymorphism was detected (Fig. 5).

Discussion

In this study, we have demonstrated that pairs of primers based on DNA sequences of earlier characterized short *Pst*I DNA fragments of *V. vinifera* cv. Chardonnay can be used to amplify DNAs of species belonging to the three related genera *Vitis*, *Ampelopsis* and *Parthenocissus*. According to the results obtained with 15 species and 20 hybrids of *Vitis*, it should be possible to study the phylogeny within this genus, and to solve some long standing problems like the genotypic nature of Solonis (Galet 1988). It will be interesting to extend these investigations to other genera of Vitaceae. Eventually, this should be preceded by further selecting *Pst*I DNA fragments of Chardonnay first by DNA hybridizations, and second by detection of RFLPs. As shown here, the RFLP analyses of the amplified DNAs may be performed very quickly with exactly the same resolution as in previous studies (Bourquin *et al.* 1991, 1992, 1993). It will also be possible to sequence the amplified DNAs of strongly related plants like the clones or taxonomic groups in *V. vinifera* (Bourquin *et al.* 1992, 1993; Collins and Symons 1993). We have also demonstrated the value of this method to identify with an excellent reproducibility a large number of commercialized rootstocks either by direct amplification of polymorphic DNAs or by RFLP analysis of the amplified products. We have not investigated the causes of the polymorphism which may be numerous and more or less complex as for example insertions or deletions. Further development of this technique should be aimed at finding more discriminating pairs of primers, testing the reproducibility with very large groups of plants and adding more hybrids.

For identification, our method has several advantages over RAPD methodology and our previous RFLP studies: in particular, it is fast and cheap like RAPD, but the risks

to amplify DNAs from contaminating microorganisms are much lower than for RAPD, because of the high specificity of the primers and the corresponding stringent amplification conditions. The interpretation of the patterns is also facilitated by the smaller number of bands compared with the complex RAPD patterns (COLLINS and SYMONS 1993). However, the rapid differentiation of the numerous grapevine cultivars may require relatively complex patterns as generated by RAPD technique, but only if these patterns are reproducible. Finally, the two fragments described in PCR-RFLP analyses with *Alu*I / PP 122, and shared by 4010 Cl., 41 B Mgt, both *V. sylvestris* samples and the Chardonnay control, may be used to analyse the parental composition of some hybrids. More generally, the development of this methodology will facilitate genome studies and breeding programs in *Vitis*.

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