

Different DNA extraction methods can cause different AFLP profiles in grapevine (*Vitis vinifera* L.)

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Summary

Amplified fragment length polymorphism (AFLP) is widely used for DNA fingerprinting and it has been broadly applied in population genetics. Since it is based on restriction digestion and PCR-based amplification it can be influenced by different chemical compounds commonly found in the isolated DNA. DNA extraction procedures may alter the AFLP banding profiles through DNA quality. Hence the DNA extraction method is crucial to produce reproducible AFLP-banding profiles.

In this work two sets of AFLP analyses were performed on 62 Pinot noir, 6 Pinot blanc and 4 Pinot gris (*Vitis vinifera* L.) clones, and profiles obtained after three different DNA extraction methods were compared. AFLP profiles were different for the same genotypes due to the DNA extraction method used.

Key words: DNA extraction, *Vitis*, AFLP-PCR.

Introduction

Amplified fragment length polymorphism (AFLP) PCR techniques (Vos *et al.* 1995) are widely used for DNA fingerprinting. AFLP markers can be generated from DNA of any origin, therefore they have been used effectively in bacteria, fungi, animals and plants (MUELLER and LAREESA WOLFENBARGER 1999), including grapevine (*Vitis vinifera* L.) (*e.g.* CERVERA *et al.* 1998, 2000, GOTO-YAMAMOTO 2000, POPESCU *et al.* 2002, VIGNANI *et al.* 2002, FANIZZA *et al.* 2003, FORNECK 2005).

The quality of the extracted DNA and the method of extraction could affect the profiles obtained (JONES *et al.* 1997, REINEKE *et al.* 1998, BOITEUX *et al.* 1999), because several types of contaminants in the DNA can reduce the activity of restriction endonucleases, polymerases and ligases (SHIODA and MARAKAMI-MUOFUSHI 1987, DO and ADAMS 1991). A complete digestion of DNA is crucial for the accuracy of AFLP fingerprinting. It was found that in excess of restriction enzymes as applied in AFLP procedures, partial digestion may result from star activity in enzymes or contamination of DNA with negatively charged polysaccharides and phenols (DO and ADAMS 1991, DEMEKE and ADAMS 1992, LODHI *et al.* 1994), usually found in DNA extracted from *Vitis*

vinifera L. As an example for a polysaccharide heparin, occurring in animals, was found to inhibit *EcoRI* endonuclease cleavage of DNA at certain *EcoRI* sites (CHEN *et al.* 1990). Many factors inhibiting the PCR reaction were determined, including detergents, antibiotics, enzymes, polysaccharides, fats, proteins and other organic and inorganic chemical compounds (ROSSEN *et al.* 1992, WILSON 1997).

The quality of DNA depends on the extraction method used as well as on the additional purification steps. REINEKE *et al.* (1998) reported different AFLP profiles obtained from differently purified DNA from *Lymantria dispar* insects. Apart from the initial DNA extraction method, post extraction DNA purification steps may have additional impact on AFLP profiles. Since many innovative DNA extraction kits routinely apply column-based purification steps in the protocol (*e.g.* GREEN and THOMPSON 1999) this may be of relevance for further argumentation. As an example Zhang *et al.* (1999) reported variable AFLP fingerprints in *Rosa sp.* when using DNA isolated with two different methods (CTAB based and Qiagen DNeasy Plant Mini kit).

Several DNA extraction protocols are commonly used for fingerprinting in grapevine, mostly as modifications of the analog method. A similar extraction buffer based on Tris, EDTA and 2-mercaptoethanol (THOMAS *et al.* 1993) or with an addition of cetyltrimethylammonium bromide (CTAB) (*e.g.* DOYLE and DOYLE 1990, BOWERS *et al.* 1993, LODHI *et al.* 1994, WOLF *et al.* 1999, LABRA *et al.* 2001) is usually applied. A recent alternative to these methods is the column based Qiagen DNeasy Plant Mini Kit which yields sufficient good quality DNA; it has already been used for grapevine fingerprinting (*e.g.* POLLEFEYS and BOUSQUET 2003, ADAM-BLONDON *et al.* 2004, THIS *et al.* 2004).

Vitis vinifera ssp. and related species have been the subject of extensive genetic studies due to their worldwide cultivation and importance. Since AFLPs are frequently used to differentiate closely related genotypes, such as vegetatively propagated, identical “clones”, where the genetic polymorphism is low, it is important to be aware of possible modifying factors of any AFLP profile. If DNA extraction methods pose such selection pressure on data, this must be pointed out and in consequence corrected by aligning methods. The goal of our work was to compare AFLP results in closely related grapevine genotypes using three different DNA extraction methods and to detect the most reliable method for AFLP fingerprinting. We are reporting

the occurrence of variable AFLP profiles and statistic analyses in grapevine depending on the DNA extraction method used.

Material and Methods

Two individual analyses with different samples and different AFLP protocols were conducted in this work, further referred to AFLP analysis 1 and AFLP analysis 2.

AFLP analysis 1: Plant material and DNA extraction methods: Six clones of Pinot blanc (2-53Gm, 10-13Gm, 2-21Gm (Forschungsanstalt Geisenheim, Germany), D55, D57, and EA98-04 (Weinbauinstitut Freiburg, Germany)) and 4 clones of Pinot gris (D42, D53, FR52-121 (Weinbauinstitut Freiburg, Germany and H-1 (Hauser-Bühler, Vogtsburg-Bickensohl, Germany)) were analyzed in this work. Total DNA was isolated from young leaves (stored at -20 °C) using three different methods.

Method 1 was a modified CTAB method (with 6 % PVP) from Doyle and Doyle (1990). Samples were ground in liquid nitrogen and dispersed in 700 µl of extraction buffer (0.1 M Tris pH 8.0, 1.4 M NaCl, 2 % (w/v) cetyltrimethyl-ammonium bromide (CTAB), 0.2 % (v/v) 2-mercaptoethanol, 20 mM ethylenediaminetetraacetic acid (EDTA) and 6 % (w/v) polyvinylpyrrolidone (PVP)) and incubated at 65 °C for 30 min with occasional mixing by gentle tube inversion. Tubes were kept on ice, 700 µl of chloroform-isoamyl alcohol (24:1, v:v) was added and samples were shaken gently for 20 min, then centrifuged at 14,000 rpm for 8 min, 600 µl of aqueous phase was removed and 15 µl of RNase (10 mg·ml⁻¹) were added for a 30 min incubation-step at room temperature. 1/10 volume of 3M Na-acetate and 2/3 volumes of ice-cooled isopropanol were added and mixed by gentle inversion. Samples were stored at -20 °C for 20 min than centrifuged at 14,000 rpm for 10 min. The pellet was rinsed with 500 µl of 70 % ethanol, dried at 40 °C and resuspended in TE buffer.

Method 2, a modified protocol based on THOMAS *et al.* (1993), described in BÖHM (2000), did not contain CTAB in the extraction buffer. Two sets of ground samples (using liquid N₂) were dispersed in 1.2 ml of the extraction buffer "A" (0.2 M Tris HCl pH 8.0, 0.25 M NaCl, 0.1 % (v/v) 2-mercaptoethanol, 50 mM EDTA and 2.5 % (w/v) PVP), vortexed and centrifuged for 8 min at 14,000 rpm. The liquid phase was poured and the pellet resuspended in 0.8 ml of the extraction buffer "B" (0.2 M Tris HCl, pH 8.0, 0.5 M NaCl, 50 mM EDTA, 2.5 % (w/v) PVP, 3 % (w/v) Sarkosyl and 20 % (v/v) ethanol) and incubated for 30 min on 37 °C with occasional mixing by gentle tube inversion. An equal volume of chloroform-isoamyl alcohol (24:1, v:v) was added, mixed and centrifuged for 5 min at 14,000 rpm. This step was repeated twice, by collecting the aqueous phase (0.6 and 0.45 ml respectively) and adding one volume of chloroform-isoamyl alcohol (24:1, v:v). A total amount of 0.3 ml of the aqueous phase from the same two samples was pooled into one tube and 0.3 ml of isopropanol was added. After 10 min of centrifugation at 14,000 rpm the aqueous phase was poured and the pellet resuspended in 100 µl TE buffer. RNase was added following 15 min incubation at room temperature. 100 µl of 7.5 M ammonium acetate, pH 8, was added followed by centrifugation 2 min, 10,000 rpm. The aqueous phase (190 µl)

was collected in a new tube together with 190 µl of cold absolute ethanol and incubated for 10 min in the refrigerator, followed by centrifugation (10 min, 10,000 rpm), rinsing the pellet with 70 % ethanol, drying the pellet and resuspending it in 60 µl of TE buffer.

In method 3 Qiagen DNeasy Plant Mini kit was used for DNA extraction following the original procedure of the kit, supplemented by the manufacturer (Qiagen, Hilden, Germany).

DNA concentration was estimated by 1.5 % agarose gel electrophoresis using λ DNA (25, 50, and 100 ng·µl⁻¹).

AFLP protocol: AFLP analysis was performed according to Vos *et al.* (1995) with the modifications described below. Digestion was carried out in a final volume of 25 µl using the y⁺/Tango buffer with BSA (Fermentas, St. Leon-Rot, Germany), 45 U of *EcoRI*, and 3.6 U of *TruII* restriction enzymes (Fermentas, St. Leon-Rot, Germany) during 1.5 h at 37 °C followed by 2 h at 65 °C and 15 min at 85 °C. Ligation was done adding 5 µl of a mix containing 5 pmol of *EcoRI* adapter, 50 pmol of *MseI* adapter, 2 mM ATP, 5 U of T4 DNA ligase and ligation buffer (Fermentas, St. Leon-Rot, Germany). The ligation was incubated overnight at room temperature.

The first amplification was performed in a total volume of 20 µl using 3 µl of digested-ligated DNA template, 10 pmol of each primer, 2 mM of each dNTP, 3 mM MgCl₂, 0.3 U of *Taq* DNA polymerase recombinant (Invitrogen, Karlsruhe, Germany) and PCR buffer. The PCR amplifications were carried out applying the following PCR-steps: 94 °C·1 min⁻¹ + 26 x (94 °C·30 s⁻¹, 56 °C·1 min⁻¹, 72 °C·1 min⁻¹) + 72 °C·6 min⁻¹. The PCR products were diluted 1:20 and 2 µl were added in total volume of 20 µl PCR reaction containing 10 pmol of each primer, 2 mM of each dNTP, 3 mM MgCl₂, 0.5 U of *Taq* DNA polymerase recombinant (Invitrogen, Karlsruhe, Germany) and PCR buffer. The PCR program was a touchdown: 94 °C·min⁻¹ + 11 x (94 °C·30 s⁻¹, 65 °C·30 s⁻¹ (decreasing 0.8 °C every cycle), 72 °C·min⁻¹) + 26 x (94 °C·30 s⁻¹, 56 °C·30 s⁻¹, 72 °C·min⁻¹) + 72 °C·min⁻¹.

Four primer pairs were used in this analysis, chosen after screening among 16 pairs. One primer in a pair was marked with a fluorescent carbocyanine dye CyTM5 (MWG-Biotech AG, Ebersberg, Germany). The pairs were as follows: E10_{Cy5}-M16, E16-M17_{Cy5}, E(+0)-M8_{Cy5} and M8_{Cy5}-M17_{Cy5} (Tab. 1).

Table 1

Sequences of the primers used in this work

Primer	Sequence	Type
E(+0)	5'-GACTGCGTACCAATTC-3'	<i>EcoRI</i> primers
E7	5'-GACTGCGTACCAATTCATG-3'	
E10	5'-GACTGCGTACCAATTCACA-3'	
E15	5'-GACTGCGTACCAATTCAGG-3'	
E16	5'-GACTGCGTACCAATTCATC-3'	<i>MseI</i> primers
M8	5'-GATGAGTCCTGAGTAAATG-3'	
M16	5'-GATGAGTCCTGAGTAAATC-3'	
M17	5'-GATGAGTCCTGAGTAAAGT-3'	
M19	5'-GATGAGTCCTGAGTAACAG-3'	

The AFLP technique was confirmed for reproducibility by using standard control samples. Electrophoresis was done on 6 % acrylamide-bisacrylamide (19:1), 6.75 M urea and 0.6 x TBE gels running in 0.5 x TBE buffer on an automated analyzer (ALFexpress™ II DNA Analysis System, Amersham Biosciences, Freiburg, Germany). Bands were displayed and analyzed using Allele Locator 1.03 software (Amersham Biosciences, 1998).

The AFLP analysis 2 was done with 62 samples from Pinot noir clones (Tab. 2), using two DNA extraction methods, method 2 and method 3 described above. Digestion and amplification followed the methods described above with the exception that the primers were not fluorescently labeled; they were synthesized by Invitrogen, Karlsruhe, Germany. For the selective amplification three primer pairs were used: E7-M17, E15-M8 and E16-M19 (Tab. 1). The amplification products were separated on a 6 % polyacrylamide gel at 1600 V and silver stained as described in BASSAM and CAETANO-ANNOLES (1993).

Statistic analysis: The statistic analysis for both analyses was done using NTSYS-PC software, version 1.8

(ROHLF 1993). Dendrograms were constructed based on Simple Matching genetic distance and UPGMA clustering following the SAHN procedure (SNEATH and SOKAL 1973).

Results

AFLP analysis 1: All three DNA extraction methods used yielded sufficient DNA (method 1: mean 1.14 mg DNA, $\delta = 0.76$; method 2: mean 3.54 mg DNA, $\delta = 0.87$; method 3: mean 5 mg DNA, $\delta = 0$). The uniformity of DNA extracted was lowest in method 1 ranging from 0.44-2.75 mg DNA.

The number of total markers found (mean 112) and the degree of polymorphism (21.3 % average) was similar for all methods (Tab 3). Method 3 samples had 4.7 % of missing values in contrast to 2.1 % and 2.9 % for method 1 and method 2 respectively.

Each DNA extraction method produced a different AFLP-banding pattern for the very same genotype. This occurred also in the polymorphic 38 markers found indicating that no

Table 2
Samples used in the AFLP analysis 2

Sample number	Clone name (not official)	Clonal material sourced from	Sample number	Clone name (not official)	Location of clonal selection
52	We 815	Staatliche Lehr- und Versuchsanstalt Weinsberg, Germany	156	EA 86-10	Weinbauinstitut Freiburg, Germany
54	We 808		157	EA 86-13	
55	We 813		158	EA 88-17	
64	We M 242		159	EA 88-18	
68	We M 171		160	EA 88-19	
69	We M 1		161	Fr 52/86	
71	23		163	Fr 54-102	
72	Schneider	164	Fr 10		
80	18Gm	Forschungsanstalt Geisenheim, Germany	165	Fr 11	
81	20Gm		166	EA 86-3	
85	20-18Gm		185	EA 88-20	
86	1-36-4Gm		190	AT 89.01.25	Martin Auer, Hallau, Switzerland
87	1-1Gm		191	AT 89.04.06	
89	1-44Gm		193	AT 89.07.53	
90	1-58Gm		195	AT 89.09.07	
91	1-86Gm		203	A 87.21.07M	
93	2-4Gm		205	A 68.13.49	
94	2-9Gm		206	A 68.13.50	
95	2-10Gm		207	A 68.14.23	
96	2-6Gm		208	MII/FAW	
97	20-13Gm		209	M1/17/FAW	
100	20-20Gm		211	2/10 FAW	
101	20-26Gm	213	A.OBL.79.01.46		
102	20-27Gm				
103	4Gm	215	Pinot 115	Etablissement National Technique pour l' Amélioration de la Viticulture, France	
106	1Gm	217	Pinot 777		
140	108-8Gm	219	Pinot 28		
141	1-7-2Gm				
150	Fr 12L	Weinbauinstitut Freiburg, Germany	230	Frank 105 S	Reinhard Frank, Kenzingen, Germany
152	Fr 13L		232	Frank 105	
154	EA 79-82		233	F. Charisma	
155	EA 91-01		234	F. Classic	

Table 3

Number of total markers and percentage of polymorphic markers and missing values in AFLP analyses using three different DNA extraction methods

Trial	Markers and missing values	DNA extraction method		
		Method 1	Method 2	Method 3
AFLP analysis 1	Total markers	114	112	111
	Polymorphic markers	21.9 %	19.6 %	22.5 %
	Missing values	2.1 %	2.9 %	4.7 %
AFLP analysis 2	Total markers	-	125	133
	Polymorphic markers	-	48.8 %	24.1 %
	Missing values	-	9.9 %	1.8 %

polymorphic marker could be found without having an impact of the extraction method. For each extraction method statistical analysis was performed to assess genetic differences displayed in dendrograms. The total genetic variation differed among the three methods (SM coefficients) from 0.89 - 0.98 in method 1, 0.93 - 0.97 in method 2 and 0.90-0.99 in method 3 (Fig. 1). The altered amplification patterns, derived from alternative amplifications of random sequences within a genome, led to substantial differences within dendrograms (Fig. 1). For example clones 2-21Gm, D42 and H-1 are very close in method 3, but differentiated in the other two methods. In method 2 clones 10-13Gm and 2-21Gm are the closest in the dendrogram, but more differentiated in the other two methods, especially in method 1.

AFLP analysis 2: A pretest comparison among the two extraction methods used (with only few samples electrophoresed in the same gel) showed that method 3 had a better display of higher molecular weight bands and had more monomorphic bands (Fig. 2). Although the samples from method 2 for the pretest were stored at -20 °C we considered the differences may have occurred because of different DNA extraction methods applied.

Three primer pairs were used for the AFLP analysis of 62 Pinot noir clones. Statistical results between the extraction methods do not match in terms of number of polymorphic bands and missing values. Method 2 produced less markers (125) than method 3 (133), more polymorphic mark-

ers occurred in method 2 (61) than in method 3 (32) and 9.9 % of total bands were interpreted as missing values in method 2 (1.8 % in method 3). The missing values derived mostly from some samples having all faint or missing bands in some primer pairs.

A cluster analysis of the dataset was done. All samples with missing values in one or more primer pairs were excluded from the similarity analysis, decreasing the total number of samples to 47, but increasing the accuracy of the results. Method 3 had less polymorphism and many samples could not be differentiated. Still there were samples differently clustered and some samples could be referred as identical when using one DNA extraction method, and different when using another method (Fig. 3).

Discussion

It is commonly accepted that the AFLP method is reliable for phenetic distance analysis in grapevine (GOTOYAMAMOTO 2000, FANIZZA *et al.* 2003, FORNECK 2005), for differentiation of varieties (CERVERA *et al.* 1998, 2000, VIGNANI *et al.* 2002, FOSSATI *et al.* 2001), clones (CERVERA *et al.* 2002, IMAZIO *et al.* 2002, POPESCU *et al.* 2002), and sports (SCOTT *et al.* 2000). This suggests that confrontation of grapevine cultivars using the AFLP method is reliable as long as the DNA quality and purity remain constant.

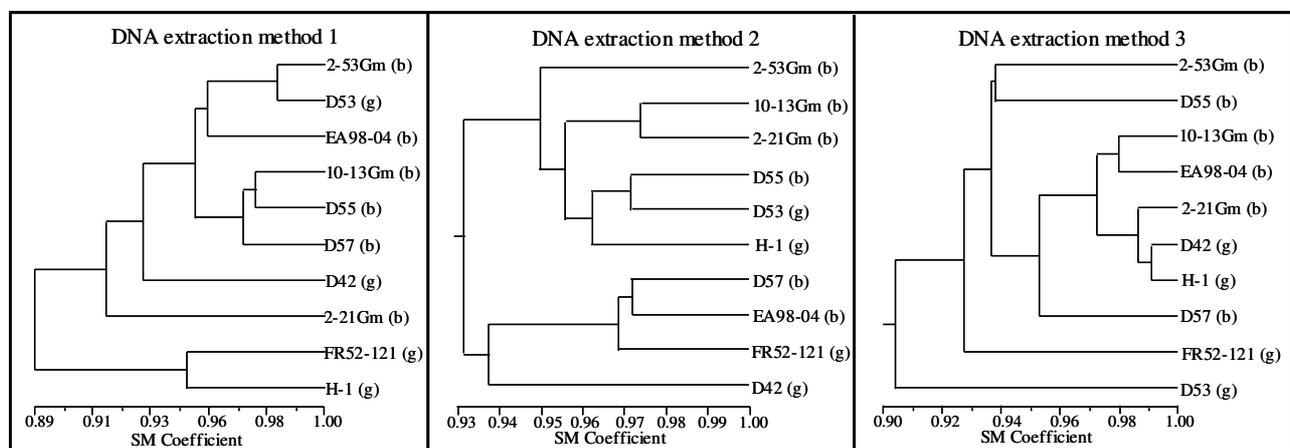


Fig. 1: Dendrograms based on Simple Matching genetic distance and UPGMA clustering for 10 Pinot clones using 3 the DNA extraction methods in AFLP analysis 1. (b) = Pinot blanc, (g) = Pinot gris.

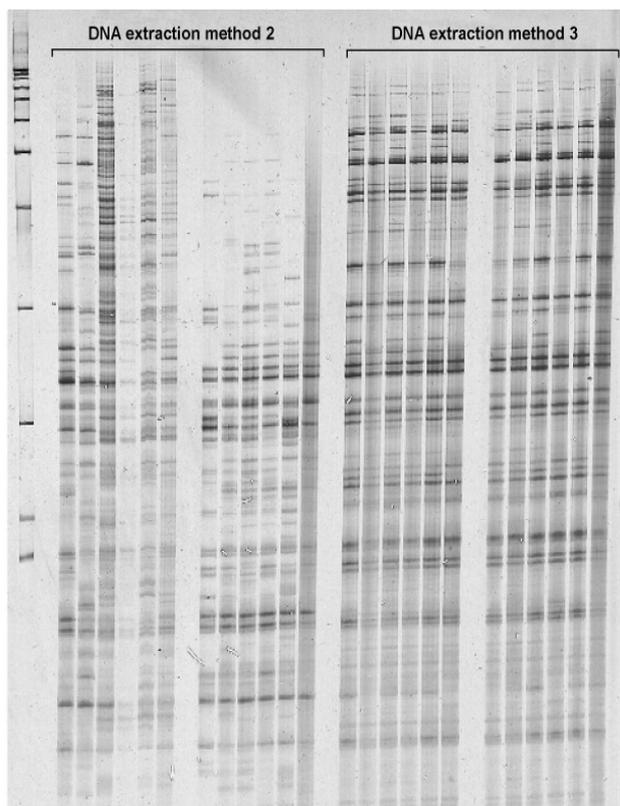


Fig. 2: Example of different AFLP profiles of Pinot noir clones using DNA extraction method 2 and method 3. Bands were displayed using silver staining.

The main prerequisite of restriction polymorphism methods is the complete DNA digestion. The DNA must be as pure as possible for a complete digestion. Since the relative proportions of affecting chemical components vary among cultivars, tissues, and even through seasons a “standard” needs to be found in terms of defining sample tissue and extraction methods. The digestion is usually assessed by gel electrophoresis. However, the critical amount of undigested DNA which could possibly alter the final AFLP results may not be visualized. A suitable method to check for small amounts of undigested DNA is an important issue in AFLP analyses.

In the AFLP analysis 1 only the polymorphic markers were different among extractions used. No monomorphic marker characteristic for one extraction was found (this could not be determined in the AFLP analysis 2 because the samples from two extraction methods were not run together on the gel). The polymorphism detected may be determined by the stable chemical compounds bound on specific sites of DNA making it uncleavable or stopping the PCR amplification at this specific sites. Since these polymorphic bands were reproducibly detected we opt for the occurrence of polymorphism due to DNA structures other than sequence differences or secondary structures such as methylation patterns. We strongly suggest that DNA structures interacting with chemical compounds may alter PCR-based restriction site amplification. Negatively charged polysaccharides and phenols in the DNA may cause partial digestion (Do and ADAMS 1991, DEMEKE and ADAMS 1992, LODHI *et al.* 1994) or

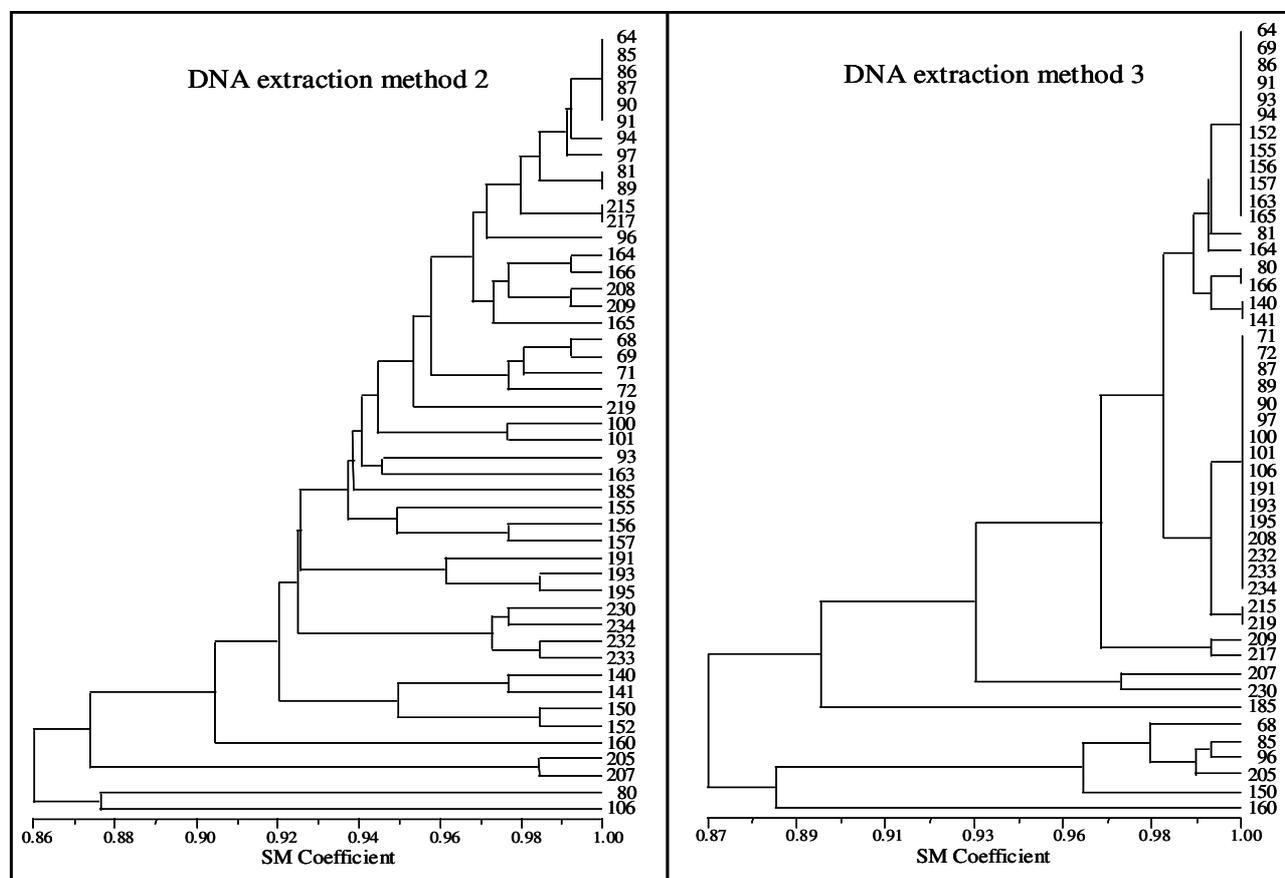


Fig. 3: Dendrograms based on Simple Matching genetic distance and UPGMA clustering in AFLP analysis 2 for 47 Pinot clones using two different DNA extraction methods. The dataset is based on three equal primer pairs.

PCR inhibition (KONJUL *et al.* 1999). Different extraction methods possibly can differently remove those compounds from the DNA. The high reproducibility of the AFLP and the insensitivity of the procedure to different laboratory conditions have been reported (JONES *et al.* 1997, HANSEN *et al.* 1999, BONIN *et al.* 2004) and we confirm these results for the case of identical DNA extraction methods. Our work shows that the display of some bands in the AFLP profile can be influenced by the DNA extraction method used, therefore combining samples with differently extracted DNA is not recommended. At that point we are unable to specify the reasons of the different results in our AFLP profiles, however, we point out the importance of the DNA extraction method. A top accuracy and fidelity of AFLP profiles is essential especially when fingerprinting closely related genotypes. Due to a higher genetic similarity all factors influencing the accuracy of the band display or inducing intra-genotype polymorphisms might have a bigger impact on final results, thus the whole fingerprinting procedure should be thoroughly standardized.

Different tissue types might have different AFLP profiles (BOITEUX *et al.* 1999, ARANZANA *et al.* 2001, ARNAU *et al.* 2002). This can be due to different degrees of DNA purity obtained from different tissues. Genetic variations due to chimeras might also occur, as was found in the SSR analysis of some grapevine cultivars by Riaz *et al.* (2002). Genetic differences were found among DNA extracted from the same type of tissue on the same plant using AFLP (STENKAMP in prep.) or SSRs (FRANKS *et al.* 2002).

Arnaud *et al.* (2002) found irreproducibility in the AFLP due to partial digestion, from tissues sampled in different periods of the growing season and from certain organs.

Another source of genetic variation of a genotype might be transposable elements. They are ubiquitous among all organisms analyzed so far and constitute a large part of plant genomes (KIDWELL and LISCH 1997, BENNETZEN 2000). Here we note that they can be activated in plants by stress (McCLINTOCK 1984, WESSLER 1996, CAPY *et al.* 2000) changing the original genome sequence. Although the influence of transposable elements was never considered in fingerprinting we think their activity might have repercussions on AFLP results hence the AFLP profile represents equally all parts of the genome analyzed.

Another issue in AFLP analysis is the subjectivity in annotating bands due to disparities in their intensity. BONIN *et al.* (2004) estimate that this error can be 2 % in AFLP analyses. Faint bands were considered as missing values, but the level of intensity between the selective amplification and the background noise is often difficult to standardize. Differences between band display methods might occur. We have compared the two methods used in this work with standard samples and we found no general differences (data not shown). Still it is possible for a band to be faint (annotated as missing value) in one display method and to be more intensive in other methods. This might be especially true for the fluorescent method as it seems to be more sensitive in displaying lower intensity bands. In our results (Tab. 3) a discrepancy in the percentage of missing values occurred between the two experiments. The percentage of polymorphic markers is generally higher in the AFLP experiment 2

than in the experiment 1, especially for the DNA extraction method 2 (48.8 %). The number of samples in the AFLP experiment 2 is bigger (62 vs. 10 samples in the AFLP experiment 1) increasing the chances to find polymorphic bands among the samples.

Thus, sampling should be standardized and more samples from the same plant should be verified for differences. Samples should be taken from healthy plants being not under extreme environmental conditions and pathogen free. To reduce statistical errors a larger number of polymorphic bands, excluding the ones containing any missing values, should be used for a better estimation of the genetic distances among genotypes, especially the closely related ones (FANIZZA *et al.* 2003). The fingerprinting procedure should be repeated from the first step. Special care should be taken to decrease human errors, especially the counting and typing of bands, which should be done by two different persons separately (BONIN *et al.* 2004).

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