Toward the Authentication of Varietal Wines by the Analysis of Grape (*Vitis vinifera* L.) Residual DNA in Must and Wine Using Microsatellite Markers

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In an attempt to develop a technique for the identification of grape cultivars in commercial wines, a method for the extraction of DNA from must and experimental wines was adapted and optimal PCR conditions for the amplification of this DNA were established. DNA was analyzed during the fermentation process for six cultivars (Chardonnay, Clairette blanche, Grenache noir, Merlot, Muscat blanc à petits grains, and Syrah). The extractions were performed on solid parts in suspension as well as on the aqueous fraction. Expected profiles for these cultivars were obtained with DNA extracted from the solid parts during all of the fermentation process and for the wine. The analysis of DNA extracted from aqueous fractions was less reproducible, and microsatellite amplifications were obtained only in the case of Clairette blanche, Merlot, and Syrah wines. Results demonstrate that the purification process is adequate for the analysis but that the DNA concentration represents the main limiting factor. Technical improvements of the method are discussed.

Keywords: *Microsatellite; DNA; grape must; monovarietal wine; varietal characterization*

INTRODUCTION

Monovarietal wines represent a type of product showing a great development worldwide. This kind of wine requires true identity of the grapes used for its production in order to meet regulations and to guarantee quality for the consumer. The quality of the final product greatly depends on the origin and the identity of the cultivar.

Identification of the grapevine cultivars or analysis of the geographical origin of the grape from the must or the wine was attempted using different techniques such as PAGE analysis of the native must proteins (Moreno-Arribas et al., 1999), thermal ionization mass spectrometry (Horn et al., 1993), or ^{2}H NMR spectroscopy (Day et al., 1995). DNA marker technologies have been applied to the analysis of complex products such as authentication of seafood products (Bossier, 1999), in which degradation is likely to occur. These technologies could be applied to the authentication of wine because DNA marker analysis, when applied to DNA extracted from the plant, leads to the unambiguous identification of grape cultivars, as previously reported (Thomas et al., 1994; Tessier et al., 1999).

Grapevine DNA present in the grape juice originates mainly from the berries. These fruits are rich in polysaccharides, tannins, and polyphenols (Cabanis et al., 1999) and contain DNA suitable for analysis (Sefc et al., 1998a). During the fermentation process, the berries undergo extensive transformations and biochemical modifications [for a review see: Flanzy (1999)]. After fermentation, wine is further processed (Escudier et al., 1999), and many treatments (fining and filtration) likely affect the DNA content of the wine.

No report on the authentication of wine by the analysis of DNA is currently available. The microsatellite technique is by far the most promising for this purpose because microsatellite markers are highly polymorphic (Jarne and Lagoda, 1996) and species-specific with some homologies between only closely related species (Bruford and Wayne, 1993; Pépin et al., 1995). This technology requires a very small quantity of DNA (10−25 ng per reaction in standard conditions). Microsatellite markers have been developed for grapevine (Thomas and Scott, 1993; Bowers et al., 1996; Sefc et al., 1999) and have been extensively used for identification of cultivars or the reconstitution of pedigrees (Thomas et al., 1994; Botta et al., 1995; Bowers and Meredith, 1997; Lin and Walker, 1998; Loureiro et al., 1998; Sefc et al., 1998b; Bowers et al., 1999a). The main limiting factors for the analysis of wine could likely be the low quantity of DNA present in solution and/or the possible degradation of DNA during the fermentation and aging process. Another limiting factor could be the complexity of the wine (Flanzy, 1999), containing in particular potential inhibitors of the PCR such as tannins and polyphenols (Ogunjimi and Choudary, 1999) or polysaccharides (Jobes et al., 1995).

To demonstrate the presence of DNA in monovarietal wine and its analysis for authentication purposes, DNA

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was analyzed during an experimental fermentation process using grapes from six cultivars (Chardonnay, Clairette blanche, Grenache noir, Merlot, Muscat blanc à petits grains, and Syrah), among the most important cultivars worldwide. We adapted DNA extraction and PCR procedures for the characterization of musts and experimental wines. DNA was extracted from microvinifications and DNA analysis carried out on samples taken daily until the end of the vinification, using four microsatellite loci. Analyses were performed both on solid phase still present in the wine (as total DNA potentially present in a nonpurified product) and in the aqueous phase (similar to a more elaborated product).

MATERIALS AND METHODS

Plant Material. Bunches (i.e., ~20 kg) from Chardonnay, Clairette blanche, Grenache noir, Merlot, Muscat blanc à petits grains, and Syrah grapes were collected at maturity from true to type vines grown at Pech Rouge INRA station (Gruissan, France). The plants have been cultivated under the same conditions and following standard practices for the area.

Leaves from the same cultivars were collected at an early stage from plants cultivated at Domaine de Vassal ampelographic collection and stored at −80 °C until use.

Microvinification. After harvest, the stems were removed and the grapes were crushed using a hydropneumatic press. The juice from each cultivar was collected in 20 L polyethylene vials. Sulfur dioxide (SO_2) was added to a final concentration of 40 mg/L to stabilize the juice. Two grams of dehydrated yeast (*Saccharomyces bayanus*) was added to start the fermentation, conducted in a cellar at a temperature of 22 °C. Juices from Grenache noir, Merlot, and Syrah were treated as red wines (i.e., with maceration of skins and seeds), whereas juices from Chardonnay, Clairette blanche, and Muscat blanc à petits grains were treated as white wines (i.e., without maceration). Temperature and density of the solutions were monitored. Physical and biochemical analyses of the resulting wines were performed at the end of the vinification. Before the transfer into bottles, sulfur dioxide was added to a final concentration of 100 mg/L.

DNA Extractions. Extractions of DNA from leaves were performed according to the method of Loureiro et al. (1998). For must and wine, samples of 300−400 mL were performed every day in each vial. The DNA extractions were carried out with 100 mL of the daily samples and were repeated twice in some cases, whereas the remaining 100 or 200 mL was stored at −20 °C. To recover the solid parts in suspension, 100 mL of the solution was centrifuged (10000*g* for 20 min at 4 °C). The supernatant (aqueous fraction) and the pellet (solid parts) were stored separately at −20 °C until use.

Several extraction techniques have been used as described by Steenkamp et al. (1994), Kim et al. (1997), and Loureiro et al. (1998), as well as several DNA extraction kits (QIAprep Spin Miniprep, DNeasy Plant MiniKit, and QIAamp Tissue Kit, Qiagen, Germany). We also combined several of these techniques to develop the following method.

The solid parts (pellet) were resuspended in 20 mL of TEX buffer (1 M Tris-HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, 3% CTAB, and 1% β-mercaptoethanol). After homogenization by vortexing, the tube was incubated for 60 min at 65 °C under constant agitation. One volume of chloroform/isoamyl alcohol (24:1) was added to the tube, which was then agitated. After centrifugation at 5000*g* for 20 min at 4 °C, the upper aqueous phase was transferred into another tube and 0.1 volume of 10% CTAB was added. A second chloroform/isoamyl alcohol extraction was then performed. After centrifugation, the upper aqueous phase was transferred into a new tube and the nucleic acids were precipitated for 30 min at −70 °C using 1 volume of cold 2-propanol and centrifuged at 15000*g* for 20 min at 4 °C. The pellet was dissolved in 0.5 mL of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

Forty microliters of proteinase K (20 mg/mL, Sigma) was added and incubated for 30 min at 50 °C. After one phenol/chloroform (25:25) and one chloroform/isoamyl alcohol (24:1) extraction, the supernatant was transferred into another tube and nucleic acids were precipitated using 2 volumes of cold 95% ethanol in the presence of 2.5 M ammonium acetate. After centrifugation at 15000*g* for 30 min at 4 °C, the pellet was resuspended in 250 μL of Qiagen P1 buffer and processed according to the protocol of the Qiaprep Spin Miniprep kit (Qiagen, Hilden, Germany). Fifty microliters of DNA solution was then collected. The amount of DNA was estimated on 0.8% agarose gel stained with ethidium bromide and by visual comparison with known quantities of lambda DNA (Boehringer Mannheim GmbH, Mannheim, Germany).

DNA extraction from the aqueous phase required a precipitation step. The supernatant was thawed at room temperature, and the nucleic acids were precipitated at −70 °C for 1 h using 1 volume of cold 2-propanol in the presence of 0.3 M sodium acetate. DNA was pelleted by centrifugation at 15000*g* for 30 min at 4 °C, resuspended in TEX buffer, and processed as the solid parts.

Microsatellite Amplification. Analyses of microsatellite loci VVMD5 (Bowers et al., 1996) and VVMD27, VVMD32, and VVMD36 (Bowers et al., 1999b) with DNA extracted from leaves were carried out according to the method of Loureiro et al. (1998) with the following modifications. The Gene Amp 9700 (Perkin-Elmer, Norwalk, CT) thermocycler was programmed for one step of 3 min at 94 °C, followed by 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, and a final step of 6 min at 72 °C. The DNA quantity for standard reactions was 25 ng, but concentrations ranging from 25 ng to 0.1 pg were tested to establish the lowest DNA quantity required for amplification.

For the analysis of DNA extracted from must and wine, the same cycles were applied. Single-step (40 or 50 cycles) and duplicative PCR techniques (25 cycles followed by 40 cycles) were tested. In the latter case, 5 μL of the first PCR reaction was transferred into the second PCR reaction.

After amplification, an equal volume of loading buffer (95% formamide, 0.5% bromophenol blue, 0.5% xylene cyanole, and 10 mM EDTA) was added to each sample. Two microliters of each sample was then loaded on a sequencing gel (6% acrylamide gel, 7.5 M urea in 1× TBE). Electrophoresis was carried out at 65 W for 3 h. The gel was then stained according to the protocol of the Promega silver staining kit (Promega, Madison, WI).

Data Analysis. For each of the six cultivars from which grapes were harvested, control reactions for each microsatellite locus have been performed. These controls consisted of PCR amplifications from 25 ng of DNA extracted from leaves of the corresponding cultivar. They enabled us to confirm the amplifications from the must or the wine, that is, to compare the sizes of the amplified fragments.

RESULTS

Microvinifications. During the vinification process, temperature and density were monitored. Figure 1 presents the evolution of both temperature (Figure 1A) and density (Figure 1B) for each of the six microvinifications. The length of the fermentations varied from 5 days for Chardonnay, showing the lowest density at crushing ($D = 1.076$), to 15 days for Grenache noir and Muscat blanc à petits grains. For the latter, due to the slow fermentation process, the addition of another 2 g of yeast was necessary at day 7 (arrow in Figure 1). At the end of the fermentations, before the second addition of SO_2 and the transfer into bottles, analyses of the characteristics of the wines were performed. Reducing sugar content was <1.5 g/L, and alcohol content varied from 10.1% for Chardonnay to 13.6% for Grenache noir.

Adaptation of the Methods for the Analysis of Musts and Wines. Different DNA extraction procedures have been tested with the aqueous phase (Steenkamp et al., 1994; Kim et al., 1997) as well as several

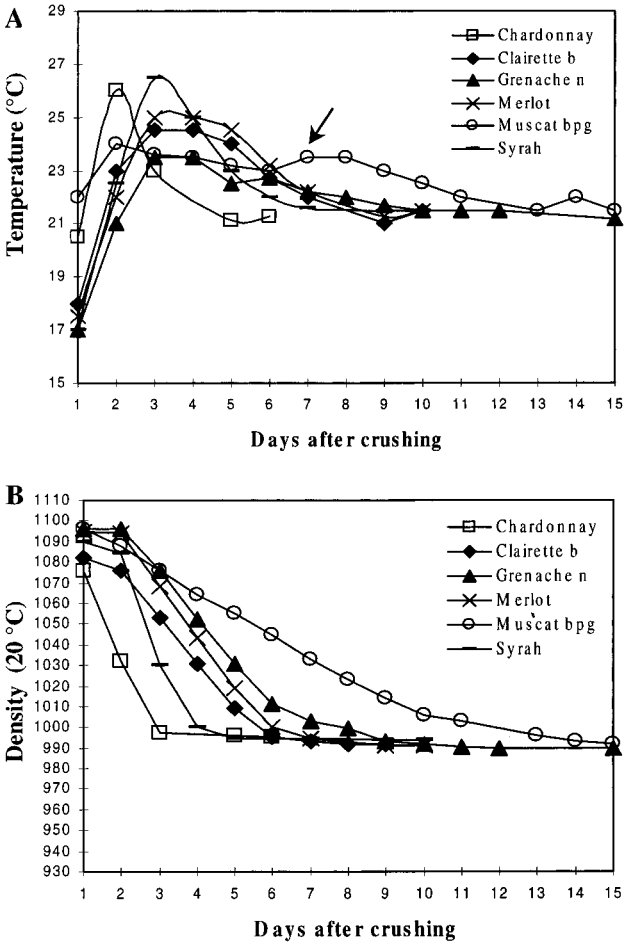

Figure 1. Evolution of temperature (A) and density (B) of the solution during the vinification process (the arrow indicates additional yeasting).

DNA extraction kits (QIAprep Spin Miniprep, DNeasy Plant MiniKit, and QIAamp Tissue Kit, Qiagen). PCR amplifications with microsatellite in these cases produced very faint and nonreproducible patterns. We therefore decided to develop a new extraction procedure by combining classic protocols and column purification process. In parallel, modifications of the standard conditions used for microsatellite analysis (Loureiro et al., 1998) have been tested (see Materials and Methods). Forty cycles of amplification yielded better amplification profiles than the other conditions. Moreover, single-step PCR amplification was preferred over duplicative PCR strategies because the latter are often unreliable and can increase contamination problems.

The microsatellite loci used in this study were selected among 11 loci, on the basis of the quality of the profiles obtained on DNA extracted from leaves and their ability to distinguish cultivars among a set of 110 cultivars (Siret et al., unpublished observations). The lengths of the microsatellite alleles for the six cultivars are presented in Table 1. At least two loci are necessary to distinguish these cultivars on the basis of the analysis of leaves DNA. Furthermore, we evaluated the lowest DNA quantity required for amplification. In our conditions, amplifications with VVMD5 and VVMD32 loci were obtained with DNA quantities as low as 1 pg per tube. For such quantities, amplification profiles showing only one band were sometimes obtained (data not shown).

Analysis of DNA from Must. The weight of the solid parts in solution was measured at the beginning of the fermentation. It varies among cultivars from 6.1 to 1.89 g/100 mL of solution (Table 2). DNA extractions performed from the solid parts, at the beginning of the fermentation, yielded 250−500 ng of DNA for Clairette blanche and Syrah. For the other cultivars, DNA quantity was too low to be estimated by ethidium bromide staining (Figure 2). Microsatellite profiles were, however, obtained with all samples (Figure 3). They were identical to the profiles of the controls (Figure 3, compare day 1 and C lanes). DNA extracted from the supernatant (day 2) was analyzed as well. Due to the low quantity, this DNA could not be quantified by ethidium bromide staining, but 50 μL of DNA solution, sufficient for ~10 PCR amplifications, was collected from the QIAprep Spin column. In this case, amplifications were obtained only for Clairette blanche, Syrah, and Merlot musts, with the expected profiles (Figure 4, lanes a, c, and e, respectively).

Table 1. Microsatellite Profiles (Indicated as the Size of the Bands in bp) Revealed by PCR Amplification Using VVMD5, VVMD27, VVMD32, and VVMD36 Loci, with DNA Extracted from Leaves for Six Cultivars

	microsatellite loci			
cultivar	VVMD5	VVMD27	VVMD32	VVMD36
Chardonnay	234/238	181/189	240/272	253/275
Clairette blanche	226/232	179/191	256/262	253/275
Grenache noir	226/240	194/194	240/250	265/269
Merlot	226/236	189/191	240/240	253/253
Muscat blanc à petits grains	228/236	179/194	264/272	243/263
Syrah	226/232	189/191	240/272	253/295

Table 2. Evolution of the Weight of the Solid Parts in Solution (Pellet) during the Fermentation in Grams per 100 mL

	cultivar					
day	Chardonnay	Clairette blanche	Grenache noir	Merlot	Muscat blanc à petits grains	Syrah
1	1.93	3.83	3.11	1.89	6.10	4.79
2	4.11	3.10	1.48	2.43	4.43	2.75
3	2.92	2.61	1.97	2.37	4.06	3.55
4	DM[a]	2.98	1.95	2.37	3.41	1.91
5	1.25	2.40	1.26	2.66	2.95	1.53
6		2.06	1.85	1.70	2.02	DM
7		DM	1.55	1.47	1.57	1.02
8		DM	1.15	DM	1.67	DM
9		1.36	1.09	1.38	DM	0.79
10			DM		1.70	
11			0.70		DM	
12					DM	
13					2.00	
14					DM	
15					1.78	

[a] DM, data missing.

Analysis of DNA during Fermentation. For the six fermenting musts, the weight of the solid parts decreased during the fermentation process but was never <0.7 g/100 mL of solution (Table 2). DNA was extracted from daily samples for the six fermentations from the solid parts and the aqueous fraction. In the pellet, the quantity of DNA extracted increased during the beginning of the fermentation and then rapidly decreased. At the end of the fermentation, DNA was not visible on agarose gel after staining with ethidium bromide (Figure 2). PCR amplification with the four microsatellite loci was possible for each fermenting must, but some differences appeared according to the length of the fermentation and also according to the primers. Figure 3 presents microsatellite profiles obtained with DNA extracted from the solid parts of the fermenting musts for each cultivar using VVMD5 and VVMD32 primers. For most fermenting musts, ampli-

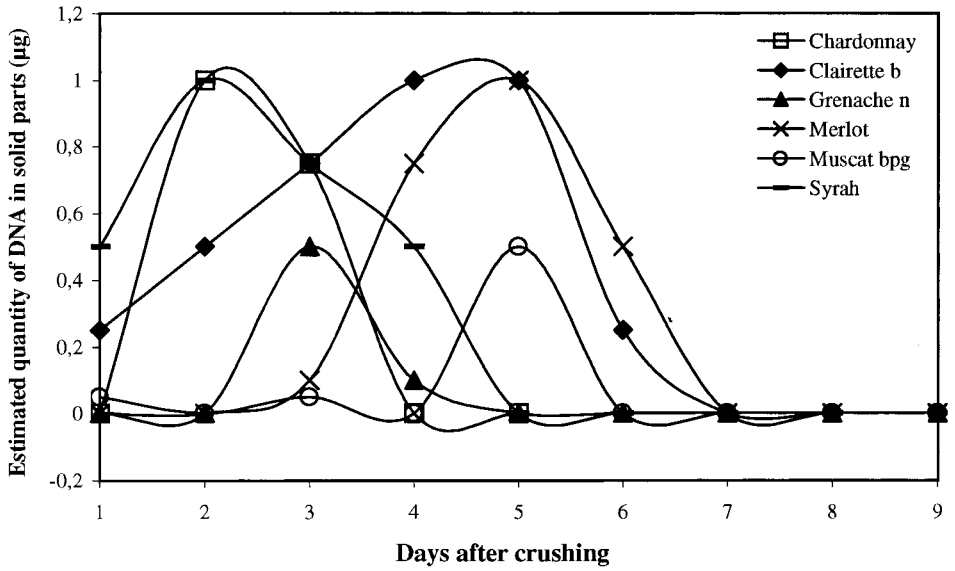

Figure 2. Evolution of the DNA quantity in the solid parts, in micrograms per 100 mL of must, during the vinification process. DNA quantity was estimated on 0.8% agarose gel stained with ethidium bromide by visual comparison with known quantities of lambda DNA.

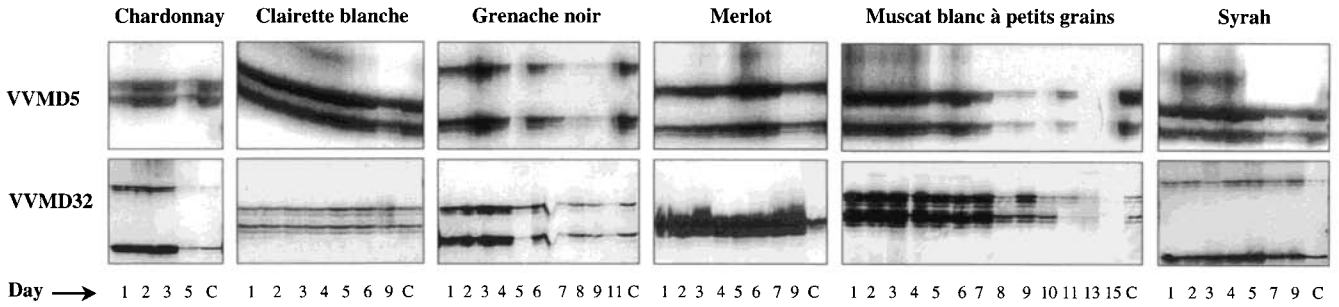

Figure 3. PCR amplification profiles obtained with microsatellite loci VVMD5 and VVMD32 using DNA extracted from the solid parts (pellet) of the fermenting must, at different days of the fermentation process for the six cultivars. Lane C is the control reaction using DNA extracted from leaves from the corresponding cultivar. The scales are not relative among patterns; that is, bands of the same size for different cultivars do not necessarily correspond.

fications were possible with DNA extracted until the end of the fermentation, but the intensity of the band was weaker for the last day. In the case of Muscat blanc à petits grains, the intensity of the amplification decreased with DNA extracted after 7 days of fermentation, but amplification was still possible with DNA extracted until day 13 for VVMD32 and with DNA extracted until day 11 for VVMD5 (Figure 3). For primers VVMD27 and VVMD36, similar results were obtained but the amplification signals were always weaker.

With DNA extracted from the aqueous fraction, amplifications were first tested with DNA extracted at day 2 and at the last day of the fermentation for Muscat blanc à petits grains and for each daily sample for Chardonnay, Grenache noir, and Merlot fermenting musts. Amplifications (Figure 4) were successful only for Merlot fermenting must at days 2 and 3 of the fermentation. Amplifications were then tested with DNA extracted from the aqueous fraction at day 2 and at the last day of the fermentation for Clairette blanche and Syrah fermenting musts and were successful (Figure 4). To extend the analysis for these two cultivars and confirm the results, a repetition of the extractions was performed with each sample for both fermenting musts. The expected profiles were obtained with DNA extracted from Clairette blanche fermenting must at days 2 and 3 only and with Syrah fermenting must at

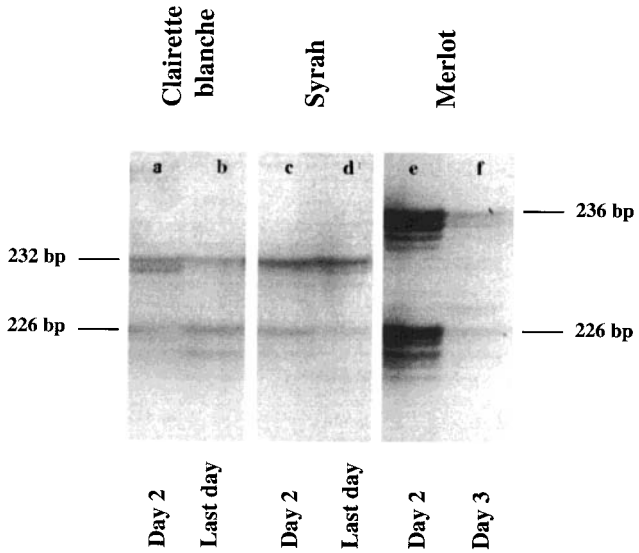

Figure 4. PCR amplification profiles obtained with microsatellite locus VVMD5 using DNA extracted from the aqueous phase (supernatant) during the fermentation process, at days 2 and 9 (last day) for Clairette blanche and Syrah fermenting musts and at days 2 and 3 for Merlot fermenting must. The sizes of the bands are indicated according to Table 1.

days 1, 2, and 9 with weaker intensity for the latter (data not shown). Amplifications with DNA extracted

from Clairette blanche fermenting must at days 4−6 and 9 (last day) of the fermentation yielded profiles with only one band (data not shown).

DISCUSSION

This paper presents the first report of the successful extraction and analysis of DNA from must and experimental wine using molecular techniques.

No data were available on the presence of DNA either in fermenting must or in commercial wine, so we started this work by the analysis of experimental wines produced under controlled conditions. The objective was to demonstrate that DNA was still present in the must and was not degraded during the fermentation process.

Extraction and PCR analysis of the DNA were successful from the must (day 1), for which amplifications with DNA extracted from the solid parts always yielded bands matching the profiles of the control cultivars. Microsatellite loci VVMD5 and VVMD32 are sufficient for the differentiation of at least the six tested cultivars. Two microsatellite loci are also necessary for the discrimination of the 44 main cultivars grown in France (Siret et al., unpublished observations). For the identification of additional cultivars, more loci would be necessary as developed by Tessier et al. (1999). Because 50 μL of DNA solution, sufficient for 10 PCR reactions, was obtained, it is therefore now possible to identify any cultivar used for monovarietal wine in France from the analysis of the must.

At the beginning of the fermentation, the quantity of DNA extracted from the pellet increased, whereas the pellet weight decreased slowly, probably due to structural changes of the tissue integrity. In fact, DNA extraction protocol did not involve grinding of the pellet under liquid nitrogen; therefore, more DNA was recovered when the cells were degraded. After the middle of the fermentation, the DNA quantity decreased dramatically in the pellet along with its weight. DNA was not visible on agarose gel after staining with ethidium bromide at the end of the fermentation, but this method is not very sensitive. PCR amplification was possible with all pellets, demonstrating the presence of DNA, except after day 13 with Muscat blanc à petits grains fermentation. However, the whole experiment was designed to conserve the same conditions for all of the fermentations. We therefore chose to work with a constant starting volume of 100 mL regardless of the cultivar. This volume proved to be sufficient for the analysis of the wine from most cultivars but should probably be increased in the case of Muscat blanc à petits grains.

Considering the analysis of the liquid phase, enough DNA for PCR amplification was recovered for at least two of the six cultivars at the end of fermentation. However, DNA amplifications were more erratic and dependent on the cultivar. In some cases, only one allele was obtained. The main reason was probably the low quantity of DNA present in solution. Indeed, these results are consistent with those also obtained when using 1 pg of DNA extracted from leaves. Because the DNA quantity of the genome was estimated to be ~1.00 pg/2C for *Vitis vinifera* (Lodhi and Reisch, 1995), 1 pg of DNA would therefore correspond to less than one copy of each allele. It would then lead to nonreproducible amplifications of only one band. When successful, the amplifications yielded, however, patterns matching the profiles for the control cultivars obtained with DNA extracted from leaves. There was therefore no inference of contaminant DNAs (yeast, bacterium), and the degradation of DNA, if occurring, was not severe enough to prevent amplification as reported for ancient DNA extracted from archeological samples (Yang et al., 1997). No differences were observed in the amplifications between red and white wines. Most of the DNA analyzed in the solution probably originated from the berry flesh and not from the seeds and the skins. Because the berries are of maternal origin only (Pratt, 1972), DNA present in the wine should therefore match the profile of the maternal cultivar, with no contaminants from paternal origin, which would occur with the seeds.

In this study, experimental and nontreated wines showing density, reducing sugars, and alcohol values consistent with commercial wines (Cabanis et al., 1999) were analyzed. The reason for the choice of this kind of wine was to certify the identity of the cultivars used in their making and to avoid any extra removal of DNA likely to occur with additional treatments. Taking into account that DNA was still present in the liquid phase after centrifugation at 10000*g* for 30 min, one might suppose that it would still be present in commercial wine. However, the DNA concentration is very low, and results were highly dependent on the cultivar. To successfully characterize commercial wines, some improvements of the technique would be necessary. On the one hand, the volume of the solution could be increased. Higher volumes would, however, require additional steps such as concentration of the solution. The volume of the samples in our experiments did not allow us to perform such implements. On the other hand, other markers could be tested. The microsatellite loci used in this study were selected on the quality of their profiles with DNA extracted from leaves. The performances of the four microsatellites tested were different, loci VVMD5 and VVMD32 yielding more intense amplifications. Other grapevine microsatellite loci have been published (Bowers et al., 1996, 1999b; Sefc et al., 1999), and ~100 microsatellites loci are under development through an international consortium. Selection of the most powerful primers could now be achieved. More sensitive detection systems such as automatic sequencers could also be tested. Furthermore, nuclear microsatellites are single-copy markers. On the contrary, mitochondrial or chloroplast genomes are highly repeated in the cell (Leaver and Gray, 1982; Lewin, 1987). PCR analysis of organelle DNA has been reported using genes or noncoding regions (Dumolin-Lapegue et al., 1997; Marchelli et al., 1998) or microsatellite sequences (Powell et al., 1995). This type of marker could lower the detection limit for the analysis of DNA in wine. Polymorphism revealed with such markers and their ability to identify grape cultivars remain, however, to be assessed as well as specificity for grapevine DNA.

In conclusion, the DNA extraction procedure was adequate for the purification of the DNA from possible inhibitors that may be present in the must or wine. We have demonstrated that DNA is present in wines at the end of the fermentation, in solid particles as well as in solution in some cases. This DNA could be analyzed using the microsatellite technique even if improvements of the detection technique should be still developed to characterize commercial wines.

ABBREVIATIONS USED

bp, base pair; bpg, blanc à petits grains; CTAB, cetyltrimethylammonium bromide; D, density; DNA,

deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid disodium salt; PCR, Polymerase Chain Reaction; PAGE, polyacrylamide gel electrophoresis; TBE, Tris−borate−EDTA buffer; TEX, extraction buffer.

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