Analysis of Grape *Vitis vinifera* L. DNA in Must Mixtures and Experimental Mixed Wines Using Microsatellite Markers

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Because wine quality highly relies on the varietal composition of the must, the development of methods allowing the authentication of varieties in musts and wines would be of great value as a guarantee of quality. Microsatellite markers have already been applied to the authentication of grape juices (Faria, M. A.; Magalhães, R.; Ferreira, M. A.; Meredith, C. P.; Ferreira Monteiro, F. *J. Agric. Food Chem.* **2000**, *48*, 1096−1100) and to the analysis of experimental wines (Siret, R.; Boursiquot, J. M.; Merle, M. H.; Cabanis, J. C.; This, P. *J. Agric Food Chem.* **2000**, *48*, 5035−5040). In the present paper, we accessed the usefulness of this technology for the analysis of must and wine mixtures. The detection limit of DNA mixtures was first estimated on DNA extracted from leaves: 4% of a foreign DNA can be detected. Analysis of must and wine mixtures (Chardonnay B/Clairette B and Syrah N/Grenache N) was performed on experimental fermentations. DNA was extracted along the fermentation process and analyzed using five microsatellite loci. The 70:30 (v/v) mixtures were successfully analyzed until the end of the fermentation. The applications of these results to commercial purposes are discussed.

KEYWORDS: Microsatellite; DNA; grape must; experimental wine mixture; varietal authentication

INTRODUCTION

Wine quality depends on the vinification process and the geographical origin of the grapes but also highly relies on the varietal composition of the must. This latter point is especially important for monovarietal wines for which only one cultivar is allowed. In the case of wines identified by an Appellation of Origin, only a limited number of cultivars can be used in order to guarantee a standardized quality. Because the final characteristics of wines can be modified if varieties of lower quality are employed, the development of methods allowing the authentication of varieties in musts and wines would therefore be of great value as a guarantee of quality and could be a useful tool in quality management.

Wine aromatic compounds, particularly monoterpenes, pigments, or other organic trace compounds, have been tested as indicators of the grape variety used to produce a given wine (*3−6*). Must and juice varietal differentiation has also been possible by means of isoenzyme patterns (*7, 8*) or by the use of

electrospray mass spectrometry for the determination of grape juice proteins (*9*). However, until now, no results have been available for wines.

Methods based on DNA typing were recently developed using the Polymerase Chain Reaction (PCR) technology. Microsatellite markers have been applied to the analysis of grape juices. These markers enabled the analysis of DNA in monovarietal juices (*1, 2*) as well as in must mixtures for combinations of up to five varieties (*1*), allowing the authentication of the cultivar(s). They also enabled the analysis of DNA during the fermentation process and in experimental monovarietal wines (*2*), but no attempts were made on mixed wines.

In the present paper, we assessed the usefulness of the microsatellite technology for the analysis of must mixtures during the fermentation process and for the analysis of wine mixtures. As a preliminary test, we analyzed leaf DNA mixtures with microsatellite markers in order to estimate the detection limit of a foreign DNA. Then, grape mixtures were made, and experimental fermentations were carried out. DNA was extracted during the fermentation process and analyzed using five microsatellite loci. Wine mixtures could be identified with this technique when 30% of a second cultivar was added. The applications of these results to commercial purposes are discussed.

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Table 1. Composition of the Must Mixtures Resulting from the Combination of Juices from the Four Cultivars (Expressed as Percent of the Final Volume)

cultivar	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
	must mixtures (v/v in %)									
Chardonnay B	90	70	50				100			
Clairette B	10	30	50					100		
Syrah N				90	70	50			100	
Grenache N				10	30	50				100

MATERIALS AND METHODS

Plant Material. Bunches (i.e., ~20 kg) of grapes of Chardonnay B, Clairette B, Grenache N, and Syrah N were collected at maturity from true to type vines grown at the Pech Rouge INRA experimental station (Gruissan, France). The plants were cultivated under the same conditions and following the standard practices for the area. Berries were transported to the laboratory without strict temperature control and stored at 4 °C prior to processing.

Young leaves were collected from the same cultivars at Domaine de Vassal INRA ampelographic collection (Marseillan, France) and stored at −80 °C until use.

Microvinification. The stems were removed after harvest, and the berries were crushed using a hydropneumatic press. Mixtures were made before the fermentation with Syrah N and Grenache N juices on the one hand and with Chardonnay B and Clairette B juices on the other hand, according to **Table 1** (mixtures M1−6). The four cultivars were also fermented as monovarietal wines (mixtures M7−10).

Juices from Grenache N and Syrah N were treated as red wines (i.e., with maceration of skins and seeds), whereas juices from Chardonnay B and Clairette B were treated as white wines (i.e., without maceration). For the red wines, juices with skins and seeds were homogenized and then mixtures were made according to **Table 1**. Juices (5−10 L) were fermented in 20 L polyethylene vials. Sulfur dioxide (SO$_2$) was added to stabilize the musts. Two grams of dehydrated yeast (*Saccharomyces bayanus*) was added to start the fermentation, conducted in a cellar at a temperature of 22 °C. The lengths of the fermentations varied from 8 days for the three Syrah N/Grenache N wine mixtures to 10 days for the three Chardonnay B/Clairette B wine mixtures. The monovarietal wines behaved identically. During the vinification process, density and temperature were monitored. At the end of the fermentations, analyses of the main wine characteristics were performed. Densities were <0.995 (20 °C), alcohol contents were >10.7 (% vol), and reduced sugar contents were <1 g/L for all wines, confirming the end of the fermentation process.

DNA Extractions. DNA extractions from leaves were performed according to the method of Loureiro et al. (*10*). For the extractions from wines, daily sampling (200 mL) was performed in each vial during 10 days for the Chardonnay B/Clairette B mixtures and during 8 days for the Syrah N/Grenache N mixtures. For the controls (monovarietal musts and wines) sampling was made at day 2 and at the last day of the fermentation. One hundred milliliters of the daily samples was centrifuged (10000*g* for 20 min at 4 °C). The pellets (solid parts in suspension) 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). DNA extractions were carried out according to the method of Siret et al. (*2*) with the following modifications. Linear polyacrylamide was added as carrier for alcohol DNA precipitations (*11*), and the final purifications were performed according to the protocol of the Dneasy Plant Minikit (Qiagen, Hilden, Germany). Seventy-five microliters of DNA solution was collected. DNA amounts, extracted from the second and last days of the fermentation, were estimated by visual comparison with known quantities of lambda DNA (Boehringer Mannheim GmBH, Mannheim, Germany) on 0.8% agarose gel stained with ethidium bromide.

Microsatellite Analysis. Mixtures of leaf DNA (**Table 2**) were analyzed using microsatellite loci VVMD5 (*12*), VVMD21, and VVMD32 (*13*). The amplifications were carried out according to the method of Loureiro et al. (*10*) using 25 ng of DNA mixtures (5 ng/μL) with the following modifications. The Gene Amp 9700 thermocycler (Applied Biosystems, Norwalk, CT) was programmed for one step of 3 min at 94 °C, followed by 45 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.

For the analysis of DNA extracted from musts and wines, amplifications were performed with 5 μL of the diluted DNA solution (one-fifth dilution) using the same amplification profile. Forty-five cycles were necessary to improve the signals after PCR. Microsatellite loci VVMD5, VVMD7 (*12*), VVMD21, VVMD24, and VVMD32 (*13*) were used.

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) after heating at 94 °C for 5 min. 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. Control amplifications for each microsatellite locus were performed with 25 ng of DNA extracted from leaves of the corresponding cultivar. Controls were loaded on the sequencing gel next to the corresponding samples. They enabled us to confirm the right genotype. To verify the absence of contamination in PCR reagents, amplifications with 5 μL of water, instead of DNA solution, were performed.

RESULTS

Preliminary Tests. Microsatellite markers used in this study were selected among 11 loci, on the basis of the quality of the profiles obtained with DNA extracted from leaves and on the basis of their ability to distinguish cultivars among a set of 110 cultivars (Siret et al., unpublished observations). Data obtained with five such loci for the four cultivars are presented in **Table 3**: lengths of microsatellite alleles are given in base pairs (bp). For the PCR amplifications, we used 45 cycles because tests have demonstrated that PCR with 45 cycles of amplification gave more intense profiles than PCR with 25 cycles (standard conditions) when low quantities of DNA were used.

The detection limit of DNA mixtures was first analyzed using locus VVMD32 (**Figure 1**). For this locus, Syrah N and Grenache N presented the same 240 bp allele, but the 250 and 272 bp alleles were, respectively, specific of Grenache N and Syrah N (**Table 3**). Amplification profiles obtained with Syrah N/Grenache N leaf DNA mixtures (**Table 2**) showed that the

Table 2. Composition of the DNA Mixtures Performed with DNA Extracted from Leaves (Expressed as Percent of the Final Volume)[a]

cultivar	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20
	DNA mixtures (v/v in %)																			
Chardonnay B	90	80	70	60	50						98	96	94	92	90					
Clairette B	10	20	30	40	50						2	4	6	8	10					
Syrah N						90	80	70	60	50						98	96	94	92	90
Grenache N						10	20	30	40	50						2	4	6	8	10

[a] DNA mixtures were adjusted to final concentrations of 5 ng/μL.

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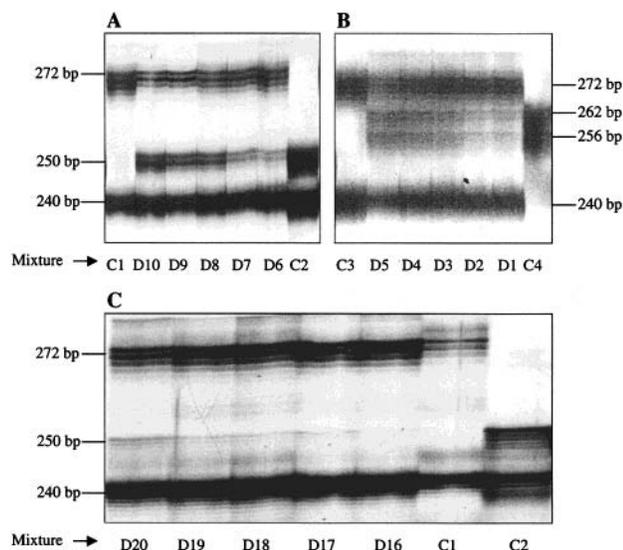
Siret et al.

Figure 1. Detection of leaf DNA mixtures using microsatellite locus VVMD32: amplification profiles with microsatellite locus VVMD32 using (A) 50:50 to 90:10 (v/v) Syrah N/Grenache N leaf DNA mixtures, (B) 50:50 to 90:10 (v/v) Chardonnay B/Clairette B leaf DNA mixtures, or (C) 90:10 to 98:2 (v/v) Syrah N/Grenache N leaf DNA mixtures. C1 and C2 are, respectively, Syrah N and Grenache N leaf DNA controls. C3 and C4 are, respectively, Chardonnay B and Clairette B leaf DNA controls. Codes refer to **Table 2**. Sizes of the alleles are indicated on the side.

Table 3. Microsatellite Profiles (Indicated as the Size of the Bands in Base Pairs) Revealed by PCR Amplification Using Loci VVMD5, VVMD7, VVMD21, VVMD24, and VVMD32 with DNA Extracted from Leaves

	microsatellite loci				
cultivar	VVMD5	VVMD7	VVMD21	VVMD24	VVMD32
Chardonnay B	234/238	240/244	247/247	209/217	240/272
Clairette B	226/232	240/250	247/247	209/213	256/262
Syrah N	226/232	240/240	246/266	209/215	240/272
Grenache N	226/240	240/244	241/247	211/217	240/250

250 bp allele of Grenache N was still amplified from D6 (90% of DNA from Syrah N and 10% of DNA from Grenache N) (**Figure 1A**). This test confirmed the capacity of microsatellite markers to discriminate, in the same PCR reaction, DNA from two different cultivars up to the proportion 90:10 (v/v). It was, however, possible to observe only one allele from Grenache N (i.e., the 250 bp allele) because the other was also present on the Syrah N profile. When the other mixture, Chardonnay B/Clairette B (**Figure 1B**), was analyzed, the two alleles of both cultivars (240/272 bp for Chardonnay B and 256/262 bp for Clairette B) were easily detected up to the proportion 90:10 (D1). When alleles of both cultivars are very close, such as in the case of the Chardonnay B/Clairette B mixture analyzed with VVMD5 (**Table 3**), again only one band was distinguished for Chardonnay B. It was indeed difficult to differentiate the 232 bp allele of Clairette B from the 234 bp allele of Chardonnay B, but the 226 bp allele of Clairette B was easily detected for the 90:10 mixture (data not shown).

We then evaluated the limit of detection of DNA mixtures when the proportion of foreign DNA fluctuated in the solution between 10 and 2%. For the DNA solution made of 96% of DNA from Syrah N and 4% of DNA from Grenache N (mixture D17), the Grenache N 250 bp characteristic allele was still amplified with VVMD32 microsatellite locus (**Figure 1C**). The

Table 4. Estimation of the DNA Concentration (in Nanograms per Microliter) Extracted from the Pellets on the Second and Last Days of the Fermentation for the Chardonnay B/Clairette B and Syrah N/Grenache N Mixtures

	must mixture (v/v)					
	90:10		70:30		50:50	
cultivar	day 2	last day	day 2	last day	day 2	last day
Chardonnay B/ Clairette B	10	<1	15	<1	15	1
Syrah N/ Grenache N	25	<1	25	<1	25	<1

detection limit was similar with both loci VVMD5 and VVMD21 and for the Chardonnay B/Clairette B mixtures.

Therefore, ∼4% of a foreign DNA can be differentiated using microsatellite technology on DNA extracted from leaves and with a final quantity of 25 ng of DNA per PCR reaction. These preliminary tests validated the usefulness of the microsatellite technology for the analysis of DNA mixture from two cultivars.

Extraction of DNA from Must. DNA extractions were performed only from the solid parts of must and wine mixtures. At the second day of fermentation, the extractions yielded ∼0.7−1 μg of DNA for the Chardonnay B/Clairette B must mixtures and ∼2 μg of DNA for the Syrah N/Grenache N must mixtures (**Table 4**). At the end of the fermentation process, DNA quantities were too low to be estimated by visual comparison, but DNA was nevertheless analyzed by microsatellite technology. Microsatellite profiles were obtained with all samples (**Figure 2**), confirming the presence of DNA.

Analysis of DNA and Mixtures Detection during Fermentation. Figure 2 presents the microsatellite profiles obtained with DNA extracted from the fermenting musts using VVMD32 locus. The specific alleles of Clairette B or Grenache N (the added cultivars) were distinguishable on the microsatellite profile for each mixture at the beginning of the fermentation (day 2), therefore confirming the possibility to analyze DNA and detect mixtures from nonfermenting must as previously reported (*1*). The intensity of Clairette B and Grenache N alleles increased when the proportion of these cultivars increased in the mixtures. The amplification was nevertheless possible with as low as 10% of the added variety.

During the fermentation process, with the reduction of the DNA quantity (*2*), the detection of the mixtures was more difficult. For the VVMD32 microsatellite locus, Chardonnay B specific alleles (240 and 272 base pairs) could be detected on all samples (**Figure 2A**). Clairette B specific alleles (256 and 262 base pairs) were detected until the fourth day of fermentation for M1, until the eighth day for M2, and until the last day for M3 (**Figure 2A**). For M3, the intensity of the amplification was very weak for days 8−10, but nevertheless the four bands were visible. For M2, we observed the disappearance of one allele from Clairette B at day 9 (**Figure 2A**), but a repetition of the amplification led to the amplification of both alleles. For Syrah N/Grenache N mixtures, similar results were obtained (**Figure 2B**), but in this case only one allele of Grenache N (250 bp) could be identified because the second one was in common with Syrah N. This allele was well amplified until the end of the fermentation for M5 and until the seventh day for M6. In the case of M4, the results were less consistent, but the amplification of a 250 bp allele could be observed faintly on days 4 and 7 (**Figure 2B**). For loci VVMD7, VVMD21, and VVMD24, similar results were obtained but the amplification signals were always weaker.
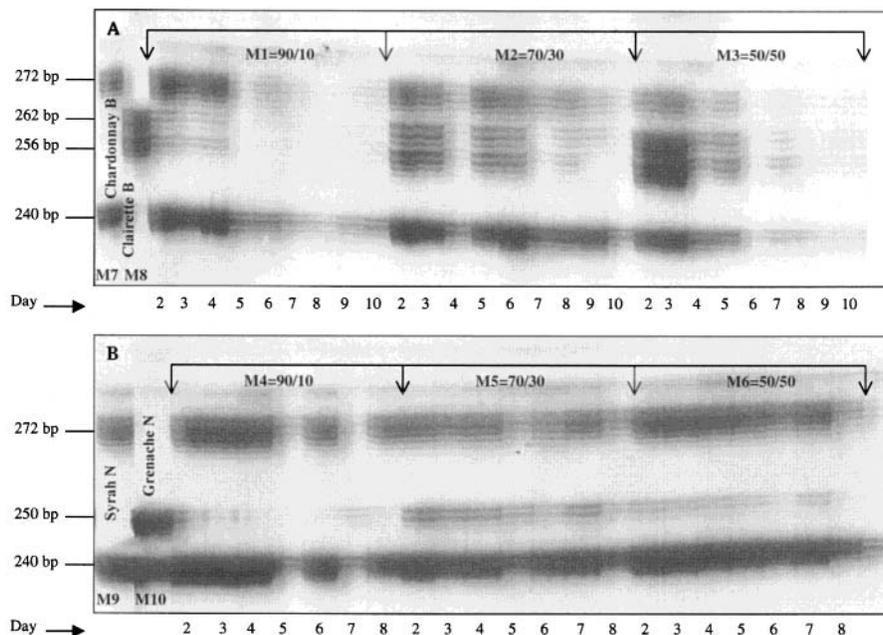
Figure 2. Detection of must and wine mixtures using microsatellite locus VVMD32: amplification profiles with microsatellite locus VVMD32 using DNA extracted from the pellets of (A) the three Chardonnay B/Clairette B and (B) the three Syrah N/Grenache N (**B**) mixtures, during the fermentation (in day). M7–M10 are amplifications performed with DNA extracted from the pellets of the monovarietal microvinifications, the second day of the fermentations. Codes refer to **Table 1**. Sizes of the alleles are indicated on the side.
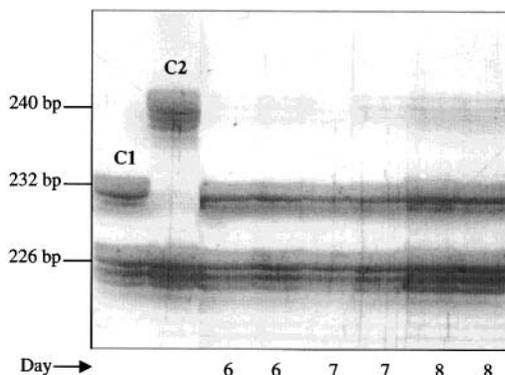

Figure 3. Confirmation of the characterization of 70:30% (v/v) mixtures with locus VVMD5: amplifications with DNA extracted from the pellets of the Syrah N/Grenache N M5 mixture, for the three last days of the fermentation process, were repeated twice. C1 and C2 are, respectively, Syrah N and Grenache N leaf DNA controls. Sizes of the alleles are indicated on the side.

Confirmation of the characterization of 70:30 mixtures was obtained with locus VVMD5; the allele(s) corresponding to the minor cultivar was (were) detected with mixtures M5 (**Figure 3**) and M2 (data not shown) until the last day. We thus demonstrated that mixtures could be clearly and repeatedly detected in musts and experimental wines when a foreign cultivar was present in the proportion of 30%.

DISCUSSION

In a previous study (*2*), we demonstrated that DNA could be analyzed from experimental wines until the last day of the fermentation process. It was confirmed in the present study. Our main objective, however, was to demonstrate that DNA mixtures could be detected in fermenting musts when DNA was less abundant and/or degraded (*2*). We tested the method on must mixtures containing ∼10% of an extra cultivar, because we demonstrated on DNA extracted from leaves that such

mixtures were clearly distinguished. Because of the possible decrease of the amplification profile intensity, we also checked other mixtures such as 70:30 and 50:50 (v/v).

As previously demonstrated by Faria et al. (*1*), the analysis of nonfermenting must mixtures was possible with the 90:10 (v/v) proportions and was of course easier when the proportion of the contaminant DNA was higher. In our case, mixtures were made of only two cultivars, but according to Faria et al. (*1*), the analysis could be extended to mixtures of five cultivars. These results could already be applied to the varietal authentication of commercial grape juices.

During the fermentation process, the distinction of the contaminant DNA was more difficult, and only in the case of the M5 mixture (70:30) were we able to repeatedly observe the allele of Grenache N (the added cultivar) in the final wine (**Figures 2B** and **3**). In the case of the M2 mixture, with locus VVMD32, only the 262 bp Clairette B specific allele was observed until the ninth day of the fermentation in the first analysis (**Figure 2A**), but repetitions of the amplifications led to the appearance of both alleles; this was confirmed with locus VVMD5. Because analysis of must and wine taken from fermentation tanks is now possible, concrete applications of this analysis to commercial cellars could now be considered.

The disappearance of the 256 bp Clairette B allele with M2 (**Figure 2A**) is due to a phenomenon called "allelic dropout" (*14*), which happens commonly when DNA is genotyped at a very low quantity. This kind of problem has already been encountered in molecular studies of ancient samples, forensic samples, and museum specimens and also in a microsatellite study of bonobo (*Pan pygmeus*) feces (*15*). In these cases the amount of DNA available for typing was very low and often in the picogram range. This type of error can be explained by sampling stochasticity. When template DNA is pipetted in a very dilute DNA extract, sometimes only one of the two alleles is amplified and detected, producing false homozygous (*14*). If only one allele of the contaminant DNA can be observed (i.e., the other one being common with the major DNA), allelic

dropout can thus limit the detection of the contaminant cultivar and repetitions of the amplification are necessary. When must and wine DNA is analyzed, it is thus also very important to choose the microsatellite loci so that both alleles of potential contaminant DNA could be visualized. In a study (*16*) we have demonstrated that only two microsatellite markers (VVMD28 and VVMD5) were necessary to identify the 44 main French cultivars and only three microsatellite markers (VVMD36, VVMD5, and VVMD32) to identify a sample of 110 cultivars including the most important European varieties, when the presence or absence of both alleles was taken into account. The discrimination power *D* (*17, 18*) can be considered to be a good estimator of the efficiency of a molecular marker. This parameter can be used to compare different types of markers even if only the allelic frequencies are known (*19, 20*). It also can be used to predict the efficiency of primers taken in combination and the risks of confusion due to the use of this combination. Other parameters consider the sizes of the alleles and the differences between alleles when two different individuals are considered, as, for example, $\delta\mu$ (*21*) or R_{ST} (*22*); therefore, a parameter similar to *D*, using the differences between alleles in the same individual, would be useful for the selection of loci.

Another kind of genotyping error could happen with microsatellites when amplification artifacts are produced. They could be misinterpreted as true alleles (*23*). With unknown must and wine mixtures, it would lead to false results. For commercial purposes, with unknown samples, repetitions of the analysis would therefore be required to minimize such risks.

Tests were also performed on the aqueous phase after centrifugation of the daily samples. Erratic and noninterpretable amplifications were obtained with these supernatants (data not shown). These results prefigured the difficulty of using the nuclear microsatellites for the analysis of commercial wines, because centrifugation likely reproduced the different treatments applied in wine-making.

Difficulty in the analysis of wines could also be explained by the quality of the grape DNA. The integrity of the DNA is indeed of crucial importance for PCR and determines the technique that can be applied, especially in food products (*24*). When degraded DNA is to be investigated, PCR amplification is often targeted at the mitochondrial (mt DNA) or chloroplast (cp DNA) genome, because of their relative abundance with respect to nuclear sequences. In addition, it has been suggested that mitochondrial or chloroplast DNA, being circular, has an intrinsically higher resistance, in particular against heat disintegration (*25*). Further investigations will be conducted to test the usefulness of cytoplasmic markers for the analysis of grape DNA in commercial wines.

ACKNOWLEDGMENT

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