Vitis vinifera Must Varietal Authentication Using Microsatellite DNA Analysis (SSR)

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A microsatellite DNA-based method for *Vitis vinifera* grape must authentication is presented. Five of the most important port wine producing grape cultivars (Tinta Roriz, Tinto Cão, Touriga Francesa, Touriga Nacional, and Tinta Barroca) were typed at four microsatellite loci described by Bowers et al. (*Genome* **1996**, *39*, 628−633) and Thomas and Scott (*Theor. Appl. Genet.* **1993**, *86*, 985−990). The corresponding 5 varietal musts and 26 must mixtures that result from the combination of the five varieties were also typed at the four loci. There were no differences between the corresponding leaf and varietal must profiles. All must combinations showed the expected band profiles corresponding to the sum of the varietal band profile components. Among the 26 must mixtures, 8 could be discriminated using the four loci.

Keywords: *Vitis vinifera; grape must; microsatellites; varietal characterization*

INTRODUCTION

In the European Union, wine is often identified by its Appellation of Origin (geographical origin), whereas law dictates must varietal composition. Moreover, in some countries, the predominant variety is itself the identity of the respective wine. This is mainly because grapevine varieties used to produce musts are of paramount importance for the final characteristics of wines. From an economical point of view, the fact that different varieties sometimes have far different economical values for wine-makers has induced the search for more accurate grapevine and must discrimination methods.

Although grapevine identification is sometimes a problem for wine-growers, when wine-makers face grape and must identification, increasing difficulties arise. Taking into account these facts, accurate identification of grapevine cultivars is crucial and the extrapolation of the grapevine identification methods into musts is particularly important.

The characterization and differentiation of musts have been based mostly on the analysis of chemical and biochemical parameters (Day et al., 1995; Forcen et al., 1992; Vasconcelos and Chaves das Neves, 1989). However, these methodologies require very time-consuming statistical approaches, and sometimes they do not answer to the botanical origin of the grapes used for must production. This is probably due to the differentiated expression of the biochemical parameters, which can be affected by both grapevine diseases and environmental factors. Must varietal differentiation has also been made by means of electrophoretic patterns of isoenzymes (González-Lara et al., 1989; Pueyo et al., 1993; Moreno-Arribas et al., 1999). The authors reported that it was possible to ascertain, in the majority of cases, the grape varieties from which the respective must originated, although no tests were performed in nonvarietal musts.

Some recently developed DNA typing PCR-based technologies offer an objective method for the characterization of grapevine cultivars. Among these, random amplified polymorphic DNA (RAPD) (Büscher et al., 1993; Grando et al., 1995; This et al., 1997) and simple sequence repeats (SSR) or microsatellite markers (Botta et al., 1993; Bowers and Meredith, 1997; Cipriani et al., 1994; Dolores-Loureiro et al., 1998; Grando and Frisinghelli, 1998; Magalhães et al., 1998; Sefc et al., 1998) achieved particular importance in grapevine varietal characterization.

Microsatellite markers have proved to be the superior marker for grapevine DNA typing as they are highly polymorphic, show a codominant mode of inheritance, and allow simple data interpretation (Thomas et al., 1994). Taking into account the above-mentioned considerations, microsatellite markers were those chosen for this work, in an attempt to apply this methodology in varietal characterization of currently used varieties for port wine production. In addition, the high level of polymorphism presented by this kind of marker does not imply the appearance of more than two amplified fragments per locus (if diploid plants are being analyzed), which is a simplifying factor when one is working with nonvarietal musts.

MATERIALS AND METHODS

Grapevine Material. Vegetal material, leaves, and musts from the varieties studied (Tinta Roriz, Tinto Cão, Touriga Francesa, Touriga Nacional, and Tinta Barroca) were obtained

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Table 1. List of Cultivars, Varietal Musts, and Must Mixtures Analyzed[a]

sample	constituent variety	type
R_L	Tinta Roriz	leaf
C_L	Tinto Cão	leaf
F_L	Touriga Francesa	leaf
N_L	Touriga Nacional	leaf
B_L	Tinta Barroca	leaf
R_M	Tinta Roriz	must
C_M	Tinto Cão	must
F_M	Touriga Francesa	must
N_M	Touriga Nacional	must
B_M	Tinta Barroca	must

sample	N	F	R	C	B	type
1	1/2	1/2				must mix
2	1/2		1/2			must mix
3	1/2			1/2		must mix
4	1/2				1/2	must mix
5		1/2	1/2			must mix
6		1/2		1/2		must mix
7		1/2			1/2	must mix
8			1/2	1/2		must mix
9			1/2		1/2	must mix
10				1/2	1/2	must mix
11	1/3	1/3	1/3			must mix
12	1/3	1/3		1/3		must mix
13	1/3	1/3			1/3	must mix
14	1/3		1/3	1/3		must mix
15	1/3		1/3		1/3	must mix
16	1/3			1/3	1/3	must mix
17		1/3	1/3	1/3		must mix
18		1/3	1/3		1/3	must mix
19		1/3		1/3	1/3	must mix
20			1/3	1/3	1/3	must mix
21	1/4	1/4	1/4	1/4		must mix
22	1/4	1/4	1/4		1/4	must mix
23	1/4	1/4		1/4	1/4	must mix
24	1/4		1/4	1/4	1/4	must mix
25		1/4	1/4	1/4	1/4	must mix
26	1/5	1/5	1/5	1/5	1/5	must mix

[a] Must mixtures were performed in equal parts as indicated.

from the germplasm collection maintained at Centro de Estudos Vitivinícolas do Douro, Régua, Portugal. Clean, young leaves were harvested and immediately stored in polypropylene sterile tubes containing 15× quantities of humidity indicator silica gel (Merck, Darmstadt, Germany) according to the procedure of Wang et al. (1996).

Varietal musts were obtained from grapes harvested in the same field as the leaves to ensure that the musts contain only one variety. All musts were prepared in a manual roller crusher and immediately frozen in plastic containers at −20 °C. At the laboratory 26 must mixtures were prepared in volumes of 100 mL to obtain homogeneous samples and to cover all possible combinations. Table 1 lists all of the analyzed samples.

DNA Extraction. DNA was extracted directly from dried leaves using a modification of the method described by Wang et al. (1996). Approximately 15 mg of dried plant tissue was quickly crushed together with 36 mg of polyvinylpyrrolidone (average molecular weight = 40000; Sigma, Steinheim, Germany) using a sterilized mortar and pestle. The resulting powder was transferred into 600 μL of extraction buffer [2% cetyltrimethylammonium bromide (CTAB), Sigma; 1.4 M NaCl, 10 mM Tris-HCl, pH 8, 20 mM EDTA, and 2% β-mercaptoethanol]. After 5 min of gentle agitation, the samples were incubated in a water bath at 65 °C during at least 60 min. One volume of chloroform/isoamyl alcohol (24:1) was added, and after 5 min of gentle agitation, the microtubes were centrifuged at 1200*g* for 5 min. The resulting supernatant was transferred into a clean microtube and the extraction with chloroform/isoamyl alcohol repeated. To the resulting aqueous phase was added 0.7 volume of ice-cold 2-propanol, and the tubes were incubated at −20 °C for at least 30 min. Tubes were then centrifuged at 12000*g* for 15 min, the liquid phase was discarded, and the resulting pellet was resuspended in 300 μL of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA). Half a volume of 5 M NaCl and, after homogenization, 2 volumes of ice-cold absolute ethanol were added. The tubes were centrifuged at 12000*g* for 15 min, the liquid phase was discarded, and the pellet was vacuum-dried at room temperature. Finally, the pellet was resuspended with 50 μL of diluted TE (0.1×) or sterile deionized water.

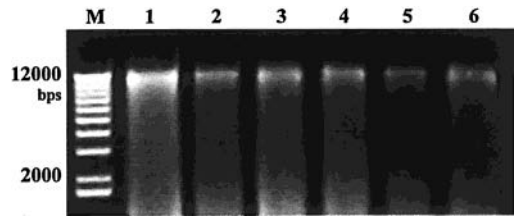

Figure 1. DNA extracted from grape musts. M, molecular weight marker; lanes 1−6, must extracts.

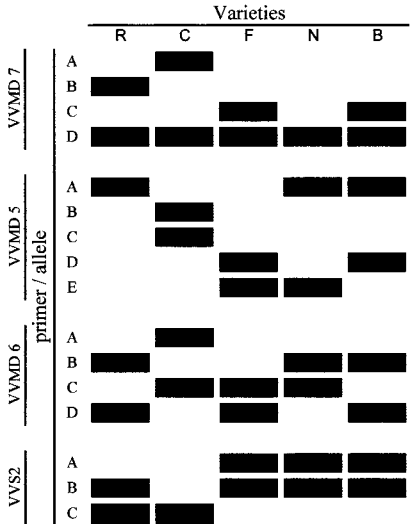

Figure 2. Genotypes for the five studied varieties at four SSR loci. R, Tinta Roriz; C, Tinto Cão; F, Touriga Francesa; N, Touriga Nacional; B, Tinta Barroca. Allele sizes are presented as letters.

Must DNA extractions were performed using basically the same protocol, although some modifications had to be implemented due to the sample nature. Two milliliters of homogenized must containing 2% β-mercaptoethanol was transferred into a 2 mL microtube, which was then centrifuged at 300*g* for 2 min. The supernatant was discarded and the resulting pellet washed two times with TE buffer. Immediately, 600 μL of the referred extraction buffer supplemented with 6% polyvinylpyrrolidone was added, and the extraction protocol proceeded as described above.

DNA quality was verified on an 0.8% agarose gel stained with ethidium bromide. DNA quantification was performed throughout by fluorometry using Hoechst 33258 (Merck).

Microsatellite Analysis. Four microsatellite loci were analyzed: VVMD5, VVMD6, VVMD7 (Bowers et al., 1996), and VVS2 (Thomas et al., 1993). PCR amplifications were performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA) in 20 μL reactions consisting of ≈10 ng of DNA template, 20 pmol of each primer, 200 μM each dNTP, 0.5 unit of *Taq* DNA polymerase (Life Technologies, Paisley, Scotland), 2 μL of 10× PCR buffer (provided with the polymerase), and 1.5 mM $MgCl_2$. The following amplification protocols were optimized for the analyzed loci. VVMD5: 94 °C for 2 min, 40 cycles of 92 °C for 30 s, 56 °C for 30 s, slope of 16 °C at 1 °C/s, 72 °C for 2 min, and 72 °C for 7 min. VVMD6 and VVMD7: 94 °C for 2 min, 40 cycles of 92 °C for 30 s, 52 °C for 30s, slope of 20 °C at 1 °C/s, 72 °C for 2 min, and 72 °C for 7 min. The chosen PCR protocol for VVS2 was as follows: 95 °C for 5 min, 10

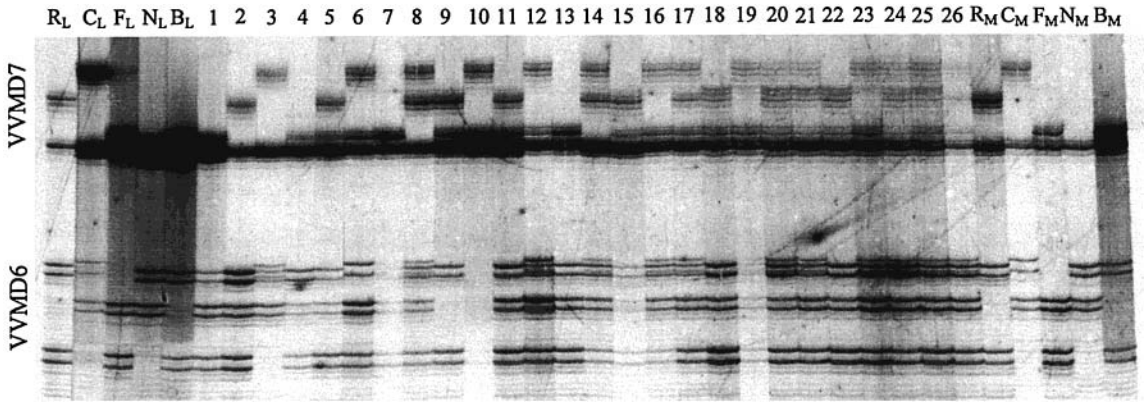

Figure 3. Silver-stained polyacrylamide gel of amplified products obtained with loci VVMD 7 and VVMD6. Presented samples are leaves, musts, and must mixtures.

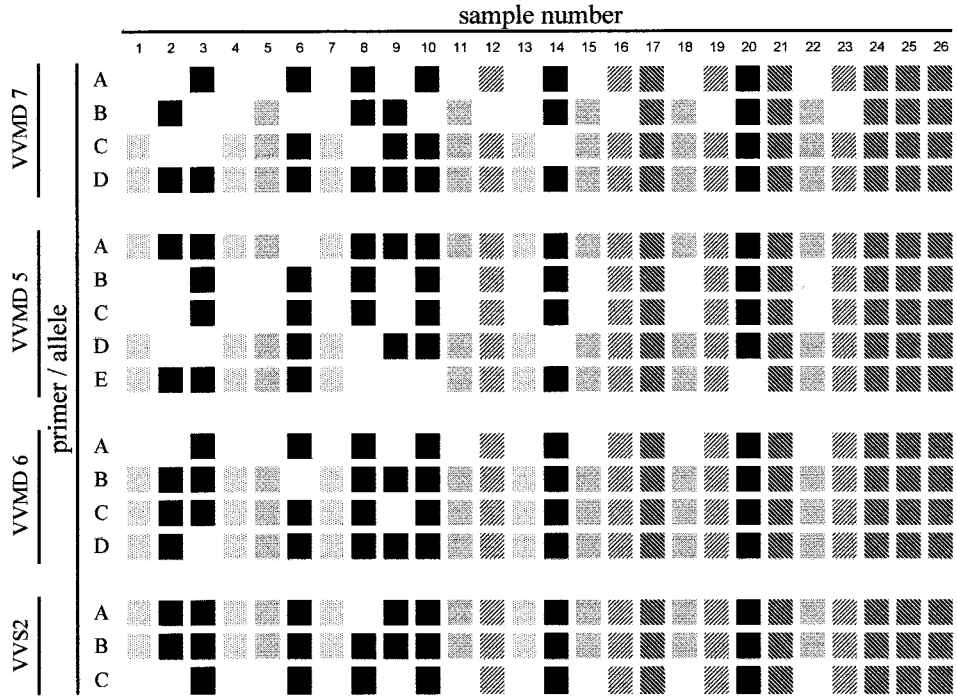

Figure 4. Must combination band profiles obtained with the four analyzed markers VVMD5, VVMD6, VVMD7, and VVS2. Black profiles indicate those that could be discriminated.

cycles of 50 °C for 15 s, 94 °C for 15 s, followed by 23 cycles of 50 °C for 15 s and 89 °C for 15 s (Sefc et al., 1998).

Gel Electrophoresis. To estimate amplification efficiency, 5 μL of the PCR reaction was run on a 2% agarose gel stained with ethidium bromide. Fifty microliters of denaturing dye solution (95% formamide, 0.5% bromophenol blue, 0.5% xylene cyanol) was added to the remaining 15 μL of the sample. Three to six microliters of this mixture was then denatured at 95 °C for 5 min and analyzed on 38.5 cm sequencing gel (6% acrylamide, 7.5 M urea in TBE). Electrophoresis was carried out at 35 mA for 2.5 h. The gel was silver stained according to the protocol described by Bassam et al. (1993).

RESULTS AND DISCUSSION

Leaf DNA extraction methodology was successfully applied to musts with some modifications as described under Materials and Methods. Figure 1 shows that nondegraded DNA was extracted from frozen musts in concentrations that ranged from 23 to 52 μg mL^{-1} (0.77−1.73 μg mL^{-1} of total must). Highly concentrated RNA is an amplification suppressor (Pikaart and Villeponteau, 1993). Therefore, in recalcitrant samples in which no amplification occurs, we performed RNA digestions resulting in an increased amplification efficiency.

The five studied grapevine varieties were genotyped at the four referred SSR loci, using leaf DNA extracts as templates. Figure 2 shows the obtained microsatellite profiles. All four microsatellite loci analyzed were found to be polymorphic in what concerns the five studied varieties. Although we found it necessary to analyze only the five most important varieties (among the recommended varieties for port wine production), in this study VVMD5 was the most polymorphic locus because all five varieties could be differentiated by genotyping only at this locus. These results obtained with primer VVMD5 make it a particularly useful locus as described by Bowers et al. (1996). In contrast, the least polymorphic locus was VVS2.

Figure 3 shows the polyacrylamide gel electrophoresis (PAGE) microsatellite DNA patterns obtained with loci VVMD6 and VVMD7 for all studied samples referred to in Table 1. Because allele sizes at these two loci do

not overlap, amplification products could be electrophoresed together. As expected, this methodology shows no differences between leaf and must DNA profiles. These facts led us to the conclusion that this molecular marker could be applied in detecting polymorphisms in varietal musts and achieve must DNA varietal characterization. Moreover, all must combinations show the expected band profiles corresponding to the sum of the varietal band profile components. Results obtained by genotyping must combinations at the four loci are schematically presented in Figure 4. Among the 26 must mixtures that result from the combination of the five varieties, eight could be discriminated (samples 2, 3, 6, 8−10, 14, and 20), and these are represented by the black band profiles in the diagram. From these eight discriminated combinations, six are included in the two-varieties combination group, two in the three-varieties group, and no discriminating profiles were found either in the four-varieties combination group or in the mixture of the five varieties. Due to the high level of polymorphism, VVMD5, by itself, provides complete discrimination of four combinations, whereas by the use of VVMD6 or VVMD7 only two mixtures are discriminated and the discrimination of only one combination is achieved by analyzing only the locus VVS2. Gray patterns presented in the diagram indicate groups of similar profiles that could not be completely discriminated because there are at least two identical profiles. Despite less information provided by these profiles, the probable number of constituent varieties can be assessed.

The growing number of microsatellite markers that can be used with this method will certainly increase the discriminating power in must varietal combinations (Bowers et al., 1999).

CONCLUSIONS

Microsatellite DNA analysis is a powerful, objective, and reliable technique for grapevine characterization. In this work the technique was successfully applied for the authentication of mono- and multivarietal musts. However, this method must rely on a *Vitis vinifera* microsatellite genotype database to allow the determination of not only the presence or absence of a certain variety but also the positive identification of others that may also exist in the must. This type of analysis becomes easier when one is working with musts originated from demarcated regions where only a few varieties are authorized for the production of quality wines. The presented methodology is particularly important when one needs to know if a must sample contains only one variety included in a group of possibilities. This kind of information achieves particular importance for the production of monovarietal wines.

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