Vitis 39 (3), 109–114 (2000)

Characterization of genetic variation within table grape varieties (*Vitis vinifera* L.) based on AFLP markers

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Summary

AFLPs were used to characterize a collection of 35 table grape varieties. A single AFLP reaction with a single combination of selective primers yielded an average of 50 polymorphic and scorable amplified bands in this collection and was enough to unequivocally identify each analyzed variety. Genetic similarity between different varieties was estimated, based on the results obtained with two primer combinations, to be between 0.65 and 0.90, while sibling varieties derived from the same cross showed a genetic similarity higher than 0.80. The analysis of several samples belonging to the same varieties, yet from different origins, showed stability of the AFLP patterns as to unequivocally and reproducibly identify them. Furthermore, some specific primer combinations detected a low level of variation that could be due to somatic variation and would be useful in clonal identification.

K e y w o r d s : DNA fingerprinting, molecular markers, variety identification, clone identification, table grape.

Introduction

Identification of grapevine varieties has always been a concern of viticultural research. In wine producing areas there is a need for variety standardization, because varietal identification is often obscured by ambiguous or inaccurate ancestral denominations. Regarding table grapes, the development of new and highly productive varieties requires accurate identification methods for product certification and property protection. As an alternative to traditional ampelography, isozyme analysis was, as for other crops, one of the first methods available (Wolfe *et al.* 1976; Subden *et al.* 1987; Benin *et al.* 1998; Weeden *et al.* 1998). Despite the reproducibility and relative simplicity of this approach, its level of resolution turned out to be insufficient to distinguish between closely related grapevine varieties. Molecular techniques, based on the analysis of variability at the level of nucleic acids, have brought to the field useful new genetic markers for variety identification. At present, it is possible to identify most grape varieties by RFLPs (Bourquin *et al.* 1993; Bowers and Meredith 1996), RAPDs (Collins and Symons 1993; Jean-Jaques *et al.* 1993; Grando *et al.* 1995; Moreno *et al.* 1995), microsatellites (Thomas and Scott 1993; Thomas *et al.* 1994; Bowers *et al.* 1996), ISTRs (Sensi *et al.* 1996), and AFLPs (Sensi *et al.* 1996; Cervera *et al.* 1998). Microsatellite markers have been widely and successfully used for genetic characterization of grapevine varieties due to the simplicity of the assay and their high PIC (polymorphism information content) (Powell *et al.* 1996). Nowadays, a set of SSRs markers are internationally used for genetic identification of grapevine varieties (Sefc *et al.* 1998; Lopes *et al.* 1999), and many more are being developed by an international consortium. These SSRs will also be useful as anchors to identify homologous linkage groups among genetic linkage maps of *Vitis*. However, due to their low multiplex ratio, SSRs are not the best markers to saturate specific map chromosomal regions or to search for somatic variation that could allow the identification of selected clones.

AFLP [Amplified Fragment Length Polymorphism (Vos *et al.* 1995)] based on the selective amplification of DNA restriction fragments, has proven to be a powerful technique in genetic analysis. AFLPs are characterized by a high multiplex ratio, allowing the analysis of more than 100 amplified fragments per primer combination. AFLPs were first used in comparison with ISTRs to distinguish varieties within the group of Sangiovese-related grapevines (Sensi *et al.* 1996). Authors detected more polymorphism with ISTRs than with AFLPs and concluded that ISTRs were more informative. However, Cervera *et al.* (1998) showed that AFLP can detect high levels of polymorphism and characterized a large collection of grapevine accessions with only two primer combinations. In fact, AFLP can be a powerful technique to identify variety-specific polymorphic fragments and to rapidly analyze the genetic similarities among accessions belonging to different grapevine varieties.

The aim of this work was to evaluate the usefulness of AFLP for the characterization of variation in table grape varieties and its use in their identification. Table grape varieties are commonly produced by crossing known varieties, which can derive from complex pedigrees, and selecting the best F_1 hybrids. Given the existing genetic variation in grapes, the identification of table grape varieties based on AFLP markers should not pose major problems. How-

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ever, it was important to know the minimal requirements to achieve accurate identification and to analyze the existence of intersample variation that could hamper the resolution of the identification method. Some of the traditional varieties have been improved by clonal selection during the last decades and are still the subject of clonal selection programs. Therefore, it was also important to test whether AFLP could be used as a tool to distinguish among the different selected clones. With these goals we analyzed a collection of 35 table grape varieties that had previously been analyzed with other markers (SÁNCHEZ-ESCRIBANO 1998) as well as 19 independent samples belonging to three of these varieties.

Material and Methods

Plant material and DNA extraction: Thirty-five table grape varieties of *Vitis vinifera* were used in this study (see. Fig. 2). The study also included samples corresponding to 5 plants each of cv. Flame Seedless and Italia, 9 samples of cv. Napoleon, derived from clonal selection programs, and one sample, Richter 110 (*Vitis berlandieri* x *V. rupestris*), commonly used as rootstock, that was used as a reference outgroup in the analysis. In total, 55 genotypes were analyzed. Representative individuals of these varieties are kept in the collection of CIDA, Murcia (Spain). Total genomic DNA was isolated from young frozen leaves from plants grown in the collection or from grapevines sampled in the fields, as described by TORRES *et al.* (1993).

AFLP analysis was performed according to VOS *et al.* (1995) with slight modifications described by CERVERA *et al.* (1998). Preamplification was carried out using *Eco*RI +A / *Mse*I +C primers. Two primer combinations were used for selective amplification: 2 *Eco*RI (+ACC, +ACT) / *Mse*I +CAT and 2 *Eco*RI (+ACC, +ACT) / *Mse*I +CTG. DNA amplified fragments were separated on 4.5 % acrylamide/bisacrylamide 19:1, 7.5 M urea and 1x TBE gels. In order to test the presence of somatic variation two additional primer combinations were used to analyze 19 genotypes (5, 9 and 5 plants of cvs Flame, Napoleon and Italia): 2 *Eco*RI (+ACC, +ACT) / *Mse*I +CTC and 2 *Eco*RI (+ACC, +ACT) / *Mse*I +CTT.

Table 1

Polymorphic fragments detected with the primer combinations used in the analysis of the collection of table grape varieties

Primer combination	Total fragments	Polymorphic fragments	% polymorphic fragments
2 E (+ACC, +ACT)/M + CAT	129	78	60.4
2 E (+ACC, +ACT)/M +CTG	120	47	39.1
Total	249	125	50.2

Data analysis: Scorable bands within fingerprints were scored as 1 and their absence as 0. Estimates of genetic similarity (GS) between pairs were based on the number of shared amplification products according to Dice coefficient (SNEATH and SOKAL 1973). Genetic similarities among genotypes were represented in a dendrogram based on the unweighted pair-group method of arithmetic averages (UPGMA). This analysis was performed using the NTSYS-PC software package, version 1.8 (ROHLF 1993). Good-fit of cluster analysis (between the similarity matrix and the dendrogram obtained) was measured by calculating the cophenetic correlation between the similarity matrix and the cophenetic matrix.

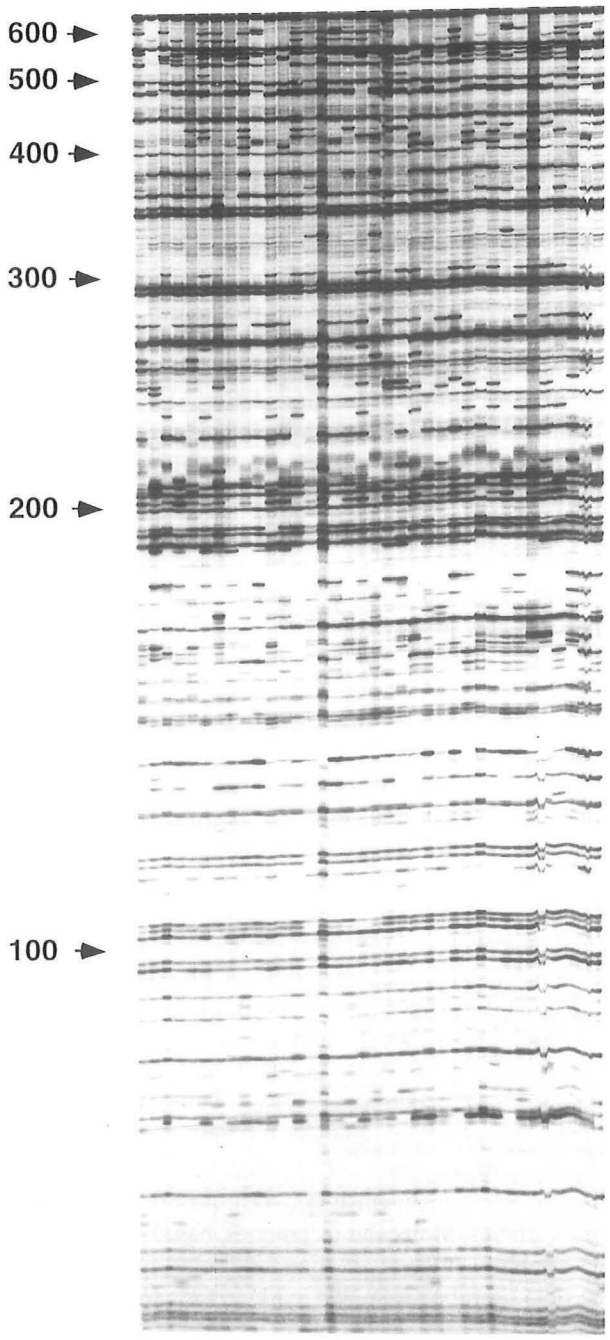

Fig. 1: AFLP analysis of 35 table grapevine varieties. The DNA fingerprint was generated with primer combination 2 *Eco*RI (+ACC, +ACT) / *Mse*I +CAT. The arrows indicate the size marker positions.

Results

Four different primer combinations were used for the identification of the table grape varieties. Selection of these primer combinations was based on previous analyses on wine grape varieties (CERVERA *et al.* 1998). The first two primer combinations (2 E (+ACC, +ACT) / M +CAT and 2 E (+ACC, +ACT) / M +CTG) corresponded to the same primers used in wine grape identification (CERVERA *et al.* 1998) and allowed the identification of 129 and 120 total bands, respectively, in the table grape varieties. Out of these, 78 and 47 respectively, *i.e.* ca. 50 % of the total bands showed clear polymorphism (Tab. 1), and were scored for their presence or absence in the 35 table grape accessions analyzed. AFLP patterns shown for each variety for primer combination 2 E (+ACC, +ACT) / M +CAT are illustrated in Fig. 1, and were repeatedly found in different experiments using different plants belonging to the same accession (see below).

Genetic similarity among the different accessions, based on the presence or absence of amplified fragments, was calculated independently for each primer combination. Each combination alone was enough to unambiguously distinguish between the 35 table grape varieties. Based on the genetic similarity data obtained with the two primer combinations used in this study, table grape varieties were grouped in clusters, as shown in Fig. 2. The rootstock Richter 110 was used as an outgroup. The cophenetic correlation between the similarity matrix and the cophenetic matrix was high (0.89, p=0.002) indicating a good fit of the cluster analysis. Most of the accessions showed similarities between 0.65 and 0.80, and a closer analysis of the dendrogram indicated that accessions typically considered as different varieties show similarities higher than 0.60, generally in the range 0.65 - 0.90. Varieties like Alphonse Lavallée and Michel Palieri, Cardinal and Matilde, Ruby Seedless and Emerald Seedless, Donna Maria and Muscat of Alexandria, Dawn Seedless, Flame Seedless and Perlette, or Thompson Seedless and Rutilia, showed GS values over 0.80 that could indicate a closer family relationship. In fact, investigation of the pedigrees of these varieties explains in most cases these higher GS values. In this way, Alphonse Lavallée is an F_1 from Bellino and Lady Downes (WAGNER and TRUEL 1998) while Michel Palieri was derived from a cross between a sibling of Alphonse Lavallée and Red Málaga (WAGNER and TRUEL, 1998). Matilde was selected in the F_1 progeny derived from a cross between Cardinal and Italia (WAGNER and TRUEL 1998). Dawn Seedless was selected in the F_1 progeny from a cross between Gold and Perlette (WAGNER and TRUEL 1998), and Rutilia was selected in the F_1 progeny derived from a cross between Aramon and Sultanina (a synonym of Thompson Seedless) (WAGNER and TRUEL 1998). Furthermore, Emerald Seedless and Ruby Seedless are siblings selected from the same F_1 progeny (MANZO and TAMPONI 1997), and Donna Maria is derived from Muscat of Setubal (WAGNER and TRUEL 1998), likely more related to Muscat of Alexandria than to other varieties.

Given the possibility of studying sequence variation at a large number of genomic sites, we wondered about the frequency of variation among grapevines of the same variety or among their derived clonal selections. A very high level of variation could hamper varietal identification. On the other hand, some level of detectable and stable genetic variation could be very useful to distinguish between clonal selections. Therefore, we analyzed different samples of three well-known table grape varieties with a total of 4 primer combinations, allowing us to score variation for

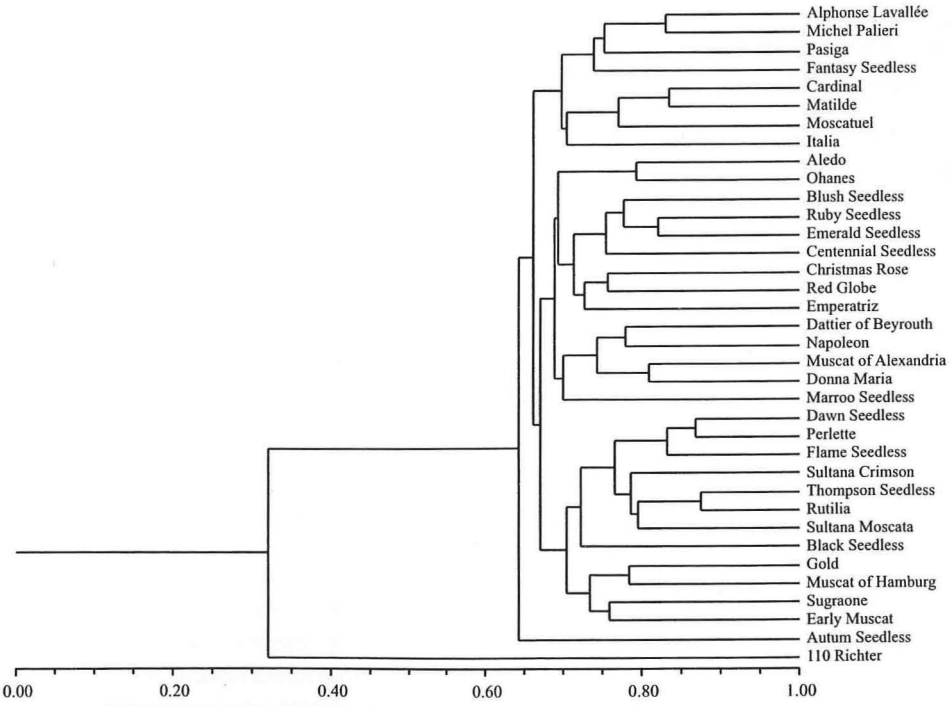

Fig. 2: Dendrogram representing the genetic similarity among table grapevine varieties. The dendrogram was established using the UPGMA clustering method on the Dice estimates of genetic similarities based on AFLP analysis with two primer combinations.

Table 2

Polymorphic fragments detected with 4 primer combinations used in the analysis of somatic variation

Primer combination	Total fragments	Polymorphic fragments	Intravarietal polymorphic fragments	% polymorphic fragments	% intravarietal polymorphic fragments
2 E (+ACC, +ACT)/M +CAT	103	43	0	41.7	0
2 E (+ACC, +ACT)/M +CTG	101	21	1 (Flame Seedless)	20.7	0.99
2 E (+ACC, +ACT)/M +CTC	121	27	0	22.3	0
2 E (+ACC, +ACT)/M +CTT	115	45	3 Flame Seedless 2 Napoleon 4 Flame Seedless & Napoleon	39.1	7.8
Total	440	136	10	30.9	2.2

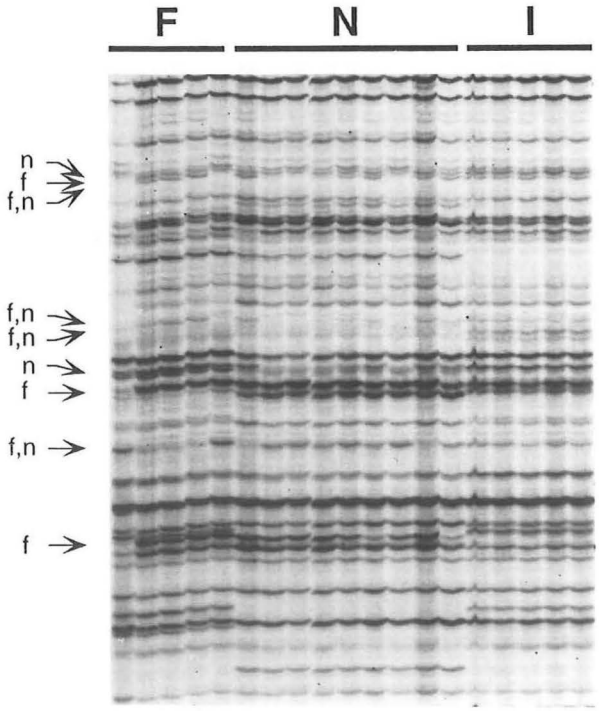

Fig. 3: Polymorphisms for AFLP fingerprints among plants of the same variety. F=Flame Seedless, N=Napoleon, I=Italia. The DNA fingerprint was generated with primer combination 2 *Eco*RI (+ACC, +ACT) / *Mse*I +CTT. Arrows indicate the polymorphisms detected for the presence or absence of specific amplified bands in either Flame Seedless (f) and/or Napoleon (n) samples.

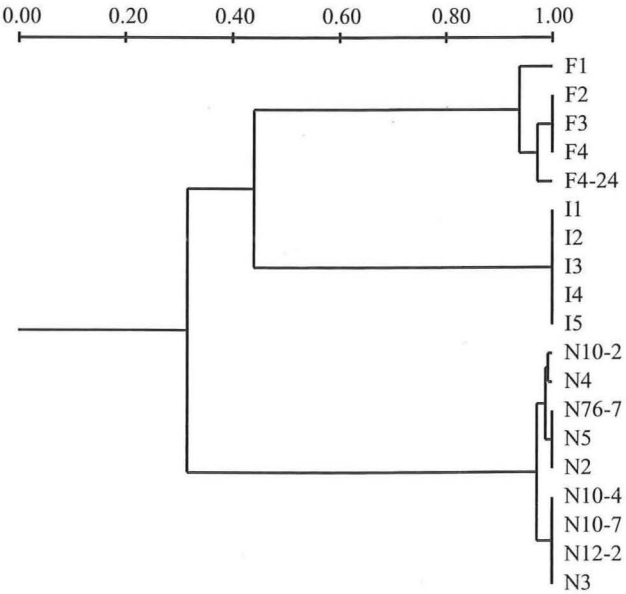

Fig. 4: Dendrogram representing the genetic similarity among plants of three different grapevine varieties. F = Flame Seedless, N=Napoleon, I=Italia. The dendrogram was established using the UPGMA clustering method on the Dice estimates of genetic similarities based on AFLP analysis with 4 primer combinations.

136 polymorphic bands (Tab. 2). The results corresponding to polymorphism detected by each primer combination were very variable. Some primer combinations (first and third on Tab. 2) did not detect any variation among samples, while the two other primer combinations identified variation among the samples. Sequence variation detected with combination 2E (+ACC, +ACT) / M +CTT allowed distinction of a higher number of plants within varieties Flame and Napoleon, while no difference could be found among different plants of Italia (Tab. 2, Fig. 3). The genetic similarities estimated from the clonal selections with all 4 primer combinations are shown in Fig. 4. For 5 samples belonging to variety Italia, collected in different fields of the same growing area (Southeastern Spain), no difference was found. This was different for plants corresponding to Flame Seedless. In a similar screening of different fields, two of the samples showed a fingerprint slightly different from the rest (Fig. 4). Finally, some of the 9 samples analyzed within Napoleon were known to be derived from clonal selections performed in 1978, when two viticulturally different clones, N10-7 and N76-7, were selected (CARREÑO 1998). The cluster analysis of the Napoleon samples distinguished between two sets of plants which were related to those two clonal selections.

Discussion

Ampelographic markers as well as different types of molecular markers have been used in genetic identification of grapevine varieties with different levels of success. In this study we show that AFLPs, with specific conditions for selective amplification, can yield a high number of polymorphic bands per primer combination, being highly efficient in the distinction of table grape varieties. In fact, only one primer combination is enough to characterize the collection of 35 varieties from CIDA (Centro de Investigación y Desarrollo Agroalimentario). These results are not surprising since other molecular markers like microsatellites (SÁNCHEZ-ESCRIBANO 1999) have also been shown to be useful for the distinction of these table grape varieties. However, the complexity of the analysis itself precludes its general use for varietal identification in many laboratories.

The values of genetic similarity found between different table grape varieties were in the range of 0.6 - 0.9, very similar to the values obtained when comparing wine grape varieties (CERVERA *et al.* 1998). Knowing the family relationships of some of the varieties, we observed that those with closer genetic relationships like parents and offspring (*e.g.* Cardinal and Matilde) or siblings derived from the same cross (*e.g.* Ruby Seedless and Emerald Seedless) showed similarity values higher than 0.8. Considering these results, the high values of genetic similarity found between varieties like Ohanes and Aledo of unknown origin could indicate that they are also parentally related. In fact, both varieties are natives of the same area in Southern Spain. Although, due to their dominant behavior, AFLP markers are less informative than codominant markers to analyze parentage studies, this analysis provides, in one or two reactions, a general view of the genetic similarities between different varieties, representing useful information to design new crosses for breeding purpose.

SSRs have become generally accepted as molecular markers for genetic identification of grapevine varieties. However, it is possible to foresee that future automated identification and certification of varieties and clones will require a development of methods based simply on the presence or absence of amplified products without knowing their size. AFLP could be used to screen hundreds or thousands of genomic sites and identify specific polymorphisms only present or absent in a given variety. These polymorphisms could be converted into single easily detectable markers, *e.g.* SCARs (PARAN and MICHELMORE 1993) allowing the rapid certification of a given variety by a simple presence/absence PCR test. Using only two AFLP primer combinations we have been able to identify variety-specific polymorphic amplified bands for 7 of the 35 characterized varieties including Fantasy Seedless, Italia, Marroo Seedless, Matilde, Ohanes, Sugraone, and Blush Seedless; this demonstrates the capabilities of this method to detect variety-specific polymorphisms.

AFLP analyses of different samples belonging to the same variety demonstrate, on one side, the high reproducibility of these molecular markers that, in the case of Italia, give the same pattern of presence or absence for a total of 217 amplified bands in 5 different plant samples from different fields. On the other side, the possible use of AFLPs to distinguish between different clones of a variety is suggested by the analysis of the Napoleon samples. Napoleon grapevines grown in the region of Murcia are mostly derived from two clonal selections supplied to the growers in 1978 (CARREÑO 1998). The AFLP analysis allows to discriminate between these two clonal selections and to assign the samples collected in the field to one or the other selection. Finally, variation found in Flame Seedless is more difficult to explain due to the lack of information on the origin of the plants used by the growers. The highly similar values among these plants (GS 0.9) do not support the existence of different sibling F_1 clones and could simply indicate a higher rate of somatic variation within this variety (SÁNCHEZ-EZCRIBANO 1998). These results demonstrate the high resolving power of AFLP and the additional information they can provide on the genetic similarity of each variety. Similarly to the discussion mentioned above on the development of variety-specific markers, AFLPs could also provide a tool to develop molecular methods for clonal identification.

Acknowledgements

The authors thank JUAN CARREÑO and ADRIÁN MARTÍNEZ for the collection of grapevines and fruitful discussions all along the development of the investigation; CARLOS MALPICA for critical reading of the manuscript; JAVIER PAZ-ARES, ANTONIO LEYVA and EMILIO RODRIGUEZ-CEREZO for sharing some of the laboratory facilities, and ANGEL SANZ for the photographic work. M.T.C. was funded by a postdoctoral fellowship from Comunidad Autónoma de Madrid and E.S-E. was funded by a predoctoral fellowship from INIA. This research was funded in part by the projects INIA SC94-092, PETRI 95-0282-CT and CM 07B-0010-1997. Support to the research activity at the Centro Nacional de Biotecnología is provided by a specific agreement CSIC-INIA.

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Received April 25, 2000