

Analysis of a spontaneous mutant and selected clones of cv. Italia (*Vitis vinifera*) by AFLP markers

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Summary

A spontaneously formed mutant and selected clones (from clonal selection) of the table grape cv. Italia (*Vitis vinifera*) were evaluated with regard to the possible use of molecular markers for grapevine clone differentiation. The identified off-type grapevine, which presents a mutated branch and a normal one on the same plant, removes any doubt as to its origin and allows a better evaluation of the suitability of molecular markers for the differentiation of grape clones. AFLPs were used as molecular markers because a large number of *loci* can be screened in a single assay, which is useful for any study on genotype relationships when a large number of bands (variables) is required. Different primer combinations (49) produced 3880 scorable AFLP bands but none showed any polymorphism among clones. Nevertheless it is suggested to use both AFLP and morphological markers for the differentiation of grapevine clones. The AFLPs would confirm the high level of DNA similarity among the suspected clones while morphological characters would allow to verify, through appropriate field experimental designs, the reliability of the phenotypic differences detected among grape clones.

Key words: molecular marker, clones, off-type grapevine, table grape.

Introduction

The discrimination of clones, sports and other types of propagation material is very important *e.g.* for germplasm maintenance, breeding and certification. Morphological characters have been used for clonal selection and variety differentiation in *Vitis vinifera*. This approach requires repeated measurements and appropriate field experimental designs because of the environmental variation.

Molecular markers have been used successfully for genetic diversity studies, paternity analyses, as well as cultivar differentiation in grapes and other fruit species. In *Vitis* some authors have tried to differentiate very closely related material (clones) by molecular markers with contrasting results. Some authors (GOGORCENA *et al.* 1993; VIGNANI *et al.* 1996; CERVERA *et al.* 1998; LOUREIRO *et al.* 1998; MORENO *et al.* 1998; YE *et al.* 1998; DANGL *et al.* 2001) failed to obtain differences among grape clones using RAPD, SSR and ISSR markers while others (SCHNEIDER *et al.* 1996; SENSI *et al.* 1996; CERVERA *et al.* 1998; REGNER *et al.* 2000; SCOTT *et al.* 2001)

were able to discriminate grape clones using RAPD, SSR and AFLP markers. These different results might be due to different causes and in particular to the unknown origin of clones. There is no clear evidence on the origin of a clone within a variety because most sports are not reported until selective pruning and propagation have isolated them. The safest way to demonstrate the origin of a new clone is by the coexistence of the original variety and the bud sport on the same vine.

A large number of DNA fragments (or variables) is essential for the differentiation of varieties or related genotypes (clones). The recent development of the amplified fragment length polymorphism (AFLP) approach (Vos *et al.* 1995), which enables simultaneous analysis of a large number of marker *loci* throughout the genome, appears to be remarkably powerful. This research has been carried out to evaluate the possibility to differentiate grapevine clones by AFLP markers analyzing material derived from a known mutant and selected clones.

Material and Methods

An off-type grapevine of the cv. Italia (*Vitis vinifera*) was taken into consideration because it presented a mutated branch and a normal one on the same plant, which gave clear evidence of the origin of the new clone. This vine was called to our attention by a table grape grower in the area of Bari (Southern Italy) and it has been under our observation for over 3 years now. Three other clones of the cv. Italia from a clonal selection, not carried out by the authors, were also included in this analysis for some different cluster characteristics (Tab. 1). The data of the selected clones, planted in a randomized block design (3 blocks with 8 replications each) for registration procedures, were provided by the Department Protezione delle Piante (University of Bari). The data relative to the spontaneous mutant have been taken for 3 years from the whole original plant, which allows to evaluate the differences between the mutated branch and non-mutated one. Thus no confidence limits have been reported in Tab. 1. A possible cytochimeric (polyploid) nature of the mutant was determined by measuring stomata size, seed size (dry weight (at 80 °C) of 100 seeds) and berry size (mean berry weight of a cluster) indicators of tetraploidy in *Vitis* (OURECKY *et al.* 1967), and by counting the chromosome number of root tips of seedlings derived from the mutant clusters.

Table 1

Morphological differences of selected clones and of a spontaneous mutant of the cv. Italia. (All morphological data refer to clone vs. original cv. Italia)

Clone	Characters
Bicchieri (Red Italia)	Red berry vs. white berry
LaNotte (Elongate Italia)	Berry length (3 cm vs. 2.5 cm)
Dipinto (Early Italia)	Harvesting time (2 weeks earlier)
Mutated branch of Italia	Cluster weight (1,600 g vs. 650 g) Berry weight (7.1 g vs. 11.2 g) Time of flowering (May 28 vs. June 20) Sugar content (11 °B vs. 14 °B)

Total DNA was isolated from young leaves as described by BOWERS *et al.* (1993) with a CTAB buffer (3 % CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 0.5 % v/v β -mercaptoethanol). AFLP analysis was performed according to Vos *et al.* (1995): the DNAs (1200 ng per sample) were digested using 7 U of *MseI* and *EcoRI* restriction enzymes, ligated to *EcoRI* (5 pmol) and *MseI* (50 pmol) adapters by adding 1 U of T4 DNA ligase (MBI Fermentas) and 12 nmol of ATP, in a 60 μ l final volume of digestion-ligation buffer (50 mM Tris-HCl, 50 mM MgAc, 250 mM KAc, pH 7.5), during 2 h at 16 and 37 °C. 5 μ l of 1:10 aliquots were pre-amplified in 25 μ l reactions with 0.048 mM of each dNTP, 2.5 mM MgCl₂, 1 U of *Taq* DNA polymerase, in 1x Magnesium-Free buffer (Promega), 50 ng and 75 ng of primers with one 'selective' nucleotide, respectively, *MseI* primer (5'-GATGAGTCCTGAGTAA-3' plus C or G or A) and *EcoRI* primer (5'-CGACTGCGTACCAATTC-3' plus A or G). The 25 amplification cycles were: 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min. *EcoRI* primers were labelled with γ -[³³P]-ATP. Then 3.2 μ l of 1:45 diluted aliquots of the pre-amplifications were amplified with 49 primer combinations, extended 4 to 6 selective nucleotides altogether (Tab. 2): the first 11 cycles with an annealing ramp of -0.7 °C per cycle, starting at: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min, followed by 24 standard cycles (94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min). The 9.6 μ l reaction contained 0.25 U of *Taq* DNA polymerase, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 3.2 ng and 32 ng of *EcoRI* and *MseI* primers. Amplified fragments were separated on 5 % polyacrylamide denaturing gels, which were dried and laid onto x-ray films.

Results and Discussion

Several observations of qualitative characters of leaves (general form, sinuses, dentation, color, surface, indument, texture), shoot (color, internode length) did not reveal any difference between the clones vs. the original cv. Italia; in Tab. 1 only the cluster characters (three-year mean), which

Table 2

Primer combinations used for AFLP analysis

<i>MseI</i> primers	<i>EcoRI</i> primers
ACC	GC
ACT	GC
CAC	AAC, AAG, ACA, ACT, ATA, ATC, ATG, ATT
CAT	AAC, AAG, ACA, ACT, ATA, ATC, ATG, ATT
CCA	ACT, AT, ATA, ATC, ATT
CT	AT
CTC	AAC, AAG, ACA, ACT, AT, ATA, ATC, ATG, ATT
CTG	AAC, AAG, ACA, ACT, AT, ATA, ATC, ATG, ATT
GA	AAC, ACA
GGC	AAC, ACA, ATA, AAC, ACA, ATA, ATG, ATT

showed distinct morphological differences, are reported. The clones named Bicchieri, Dipinto and LaNotte, from clonal selection, differed from the original Italia table grape only with regard to the red berry color (Bicchieri), early ripening (Dipinto) and berry length (LaNotte). These clones are being evaluated for their sanitary status (virus); it was excluded that the red berry color could be attributed to virus infection.

There is no doubt about the spontaneous mutant of the cv. Italia, which presented a mutated branch and a normal one on the same plant. The mutated branch shows morphological differences (Tab. 1) in comparison with the normal one; it differs particularly in the cluster size (Fig. 1), flowering time and ripening time; all the differences were recorded in a three-year period. In *Vitis* the origin of large-clustered grapevines is usually traceable to a single mutated bud on an otherwise normal vine (OLMO 1935). Spontaneous mutations in *Vitis* have been described by several authors (OLMO 1935; SNYDER and HARMON 1935; SCHERZ 1940; EINSET and PRATT 1954; BREIDER 1962; THOMPSON and OLMO 1963;

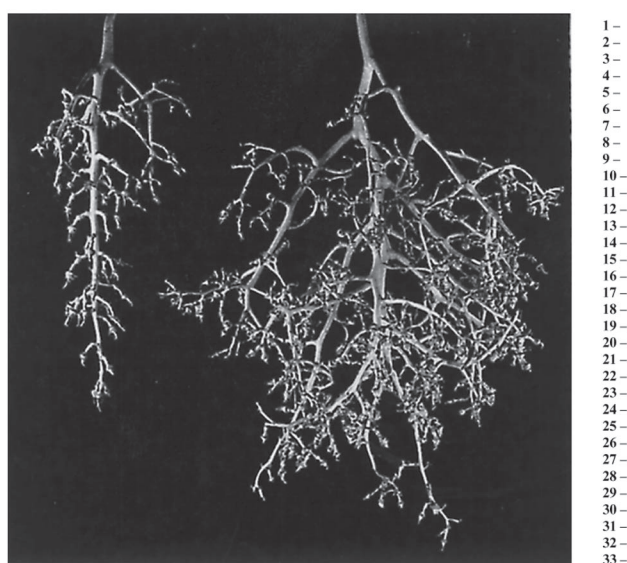


Fig. 1: Rachis from a normal (left) and a mutated (right) branch of cv. Italia. Scale on the right in cm.

OURECKY *et al.* 1967); in most of these the cytochimeric (polyploid) nature was observed while in some it was not. Due to the incomplete knowledge of the histogenic ontogeny of chimeras, we have limited the analysis to exclude the cytochimeric (polyploid) nature of our mutant by measuring stomata size, seed size, berry size (indicators of tetraploidy in *Vitis*, OURECKY *et al.* 1967) of the mutated branch *vs.* the normal one of the cv. Italia: stoma length 27.8 μm *vs.* 28.0 μm , stoma width 19.1 μm *vs.* 19.0 μm ; 100-seed weight 3.7 g *vs.* 4.2 g; berry weight 7 g *vs.* 11 g; in addition root tips of plantlets, from seeds of the clusters of the mutated and normal branch, presented a diploid chromosome number ($2n = 38$, data not shown). These data exclude any cytochimeric (polyploid) nature of the mutated branch of the cv. Italia. On the contrary, to demonstrate the chimeric nature of a clone due to gene mutation, a complex analysis, at molecular and morphological level, would be necessary to verify the presence of the mutated allele; this would require high investment not justifiable for the purpose of clone differentiation.

A practical and realistic solution would be to verify the high similarity among clones at the molecular level, and to verify their phenotypic differences. For this purpose the knowledge of the origin of the mutant (clone) and the use of molecular markers, which allow the screening of a large number of *loci* in a single assay, become important. In our work, the existence of a mutated branch and a normal one on the same vine of the cv. Italia removes any doubt as to the origin of the new clone while the AFLP markers allow the detection of a large number of DNA fragments. The AFLP patterns of the grapevine clones taken into consideration are shown for some primer combinations (Fig. 2); in this figure two different grape cultivars, as an 'unrelated group', and an additional replicated sample from the mutated branch were also included as control. All 49 primer combinations (Tab. 2) produced 3880 scorable AFLP bands but none showed any polymorphism among the clones of the cv. Italia. With some primer combinations, occasionally a single polymorphic band was detected among some clones; this was not reproduced when those primer combinations were replicated. In our study, the AFLP reproducibility was about 99.6 %, which indicates that AFLP markers have not only a high reproducibility but also that they are suitable for any study on the relationships among genotypes when a large number of bands is required.

Our result on the differentiation of the clones of the cv. Italia contrasts with that of SCOTT *et al.* (2001), who found two out of 3000 AFLP bands to distinguish an early mutant of cv. Flame Seedless. On the other hand, CERVERA *et al.* (1998), in differentiating grape clones by AFLP, were successful with some varieties but not with others. Recently FRANKS *et al.* (2002) and RIAZ *et al.* (2002) were able to differentiate some chimeric clones but not all clones within varieties by SSR. These different findings might be due to several factors, *e.g.* different markers, different primer combinations, different varieties.

The lack of detection of DNA differences among the cv. Italia clones might be due to the small fraction of the genome explored even though a large number of AFLP fragments were analyzed. To explore a larger portion of the ge-

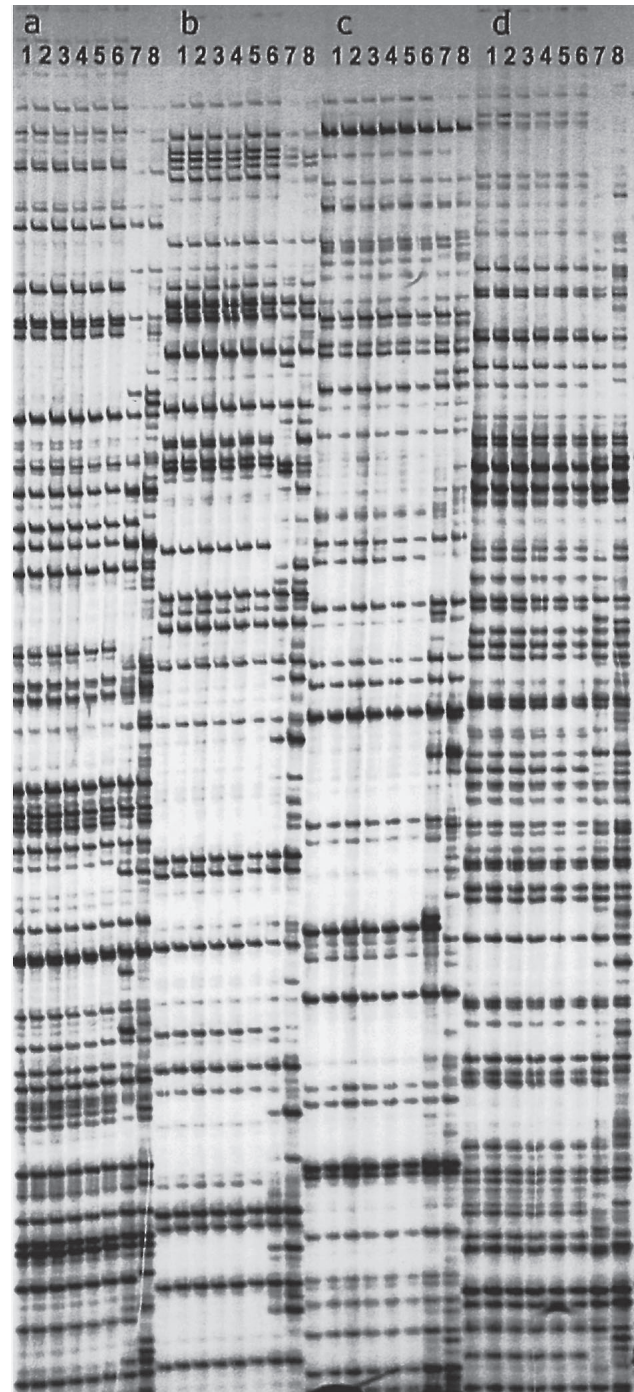


Fig. 2: AFLP profiles of cv. Italia clones and two wine grape cultivars. Sample numbers refer to: 1 LaNotte, 2 Bicchieri, 3 Dipinto, 4 Italia mutated branch, 5 Italia normal branch, 6 Italia mutated branch, 7 Malvasia Nera (wine grape), 8 Greco di Tufo (wine grape). Panels a to c are different primer combinations: a) MseI(+CTC)-EcoRI(+ATG). b) MseI(+CTG)-EcoRI(+ATG). c) MseI(+CAC)-EcoRI(+ATG). d) MseI(+CAT)-EcoRI(+ATG). For details see Material and Methods.

nome more primer combinations could be assayed but it would require higher cost, not justifiable for clone differentiation; moreover the mutation might be restricted to a very small region of the genome or might involve a point mutation in a DNA regulatory sequence, which might be difficult to detect by AFLP or other similar techniques.

The use of both molecular and morphological markers for the differentiation of grape clones could be suggested.

Molecular markers can point out a high level of DNA similarity among differing phenotypes (putative clones); this would give a preliminary indication on the presence of suspected clones. Morphological characters, with an appropriate field experimental design, would allow to verify the reliability of the phenotypic differences detected among clones. On the other hand, using only morphological characters, the analysis would not be complete because of the possibility of a non-clonal origin of the differing phenotype. Thus the use of both, molecular markers with an accessible cost, and morphological characters with an appropriate field experimental design, would nowadays be a useful and realistic solution for the differentiation of grapevine clones.

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