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Detection of genetic diversity among clones of cv. Fortana (*Vitis vinifera* L.) by microsatellite DNA polymorphism analysis

by

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S u m m a r y : Allelic polymorphism at 6 microsatellite *loci* was analyzed in 5 clones of cv. Fortana, which had been divided into two morphological groups (CAB 2, CAB 13, CAB 14, and CAB 1, CAB 3, respectively) by discriminant multivariate analysis of leaf traits. Of the 6 microsatellite *loci,* 5 (VVS1, VVS2, VVS4, VVMD3, VVMD6) showed polymorphism between clones and one (VVMD7) gave identical DNA profiles. Two genotypic patterns were found: clones CAB 2, CAB 13 and CAB 14 appeared identical and distinct from clones CAB 1 and CAB 3, but all of them shared at least one of the two alleles for each *locus*, thus showing a certain degree of genetic relatedness. The slight morphological differences of the two Fortana clone groups could thus be related to the diversity of their genotypic profiles. Thus, the two clone groups may have originated not *via* vegetative propagation of a single individual but from seedlings, parents or siblings of this progenitor. The present investigation supports the assumptions of the polyclonal origin of cv. Fortana and concludes that the strict definition of cultivar, which admits of clonal origin only, is not altogether adequate for the old varieties of unknown origin. The Italian term "vitigno" and the French "cépage", which do not imply genetic uniformity, would appear more appropriate than cultivar to indicate a winegrape variety.

K e y w o r d s : grapevine, clone, clonal variability, cultivar, DNA, microsatellite analysis, simple sequence repeat, sequence tagged site, Vitis vinifera.

Introduction

The world wine industry is virtually characterized by vineyards with traditional, old cultivars perpetuated by vegetative propagation. Most of these cultivars are of unknown origin and can not be traced back to their progenitor but only to individual vines which represent the source plants of various clones. According to RIVES (1961, 1981), ROSELLI and SCARAMUZZI (1974), MULLINS and MEREDITH (1989), these source plants may have a dual origin: either a single seedling which produced *via* vegetative propagation different biotypes through somatic mutations, or more than one seedling, all with marked morphological uniformity deriving from the fact that the initial wild grape populations were rather isolated and hence closely related. As posited by RIVES (1961), it can be assumed that early grape growers planted vineyards with such material - genetically different yet morphologically very similar.

The polyclonal origin attributed to cultivars has represented for many years a fascinating assumption since there is no hard evidence to back it up. Current DNA analysis techniques based on molecular markers are capable of providing objective information about the potential genetic heterogeneity of clones within certain cultivars and hence of proving or disproving the polyclonal origin of such cultivars. Since the use of molecular markers is a recent development and only a limited number of new cultivars has been released in the 20th century *via* traditional breeding, the genetic improvement of old winegrape cultivars has so far mainly been based on the selection of superior clones without positive proof of their real genetic origin.

Clonal selection in Italy began in the 1960s, and about 400 clones belonging to roughly 110 cultivars have been registered in the National Catalogue. Clonal selection within a cultivar has provided evidence for differences in quantitative traits (e.g. yield, soluble solids) irrespective of morphological types (CALÒ *et al.* 1987; MANNINI 1995). Within some varieties it has also been possible to distinguish morphological types showing variations in quantitative characters (MANNINI *et. al.* 1987; BOURSIQUOT *et al.* 1989; SILVESTRONI *et al.* 1990; SCHNEIDER *et al.* 1991).

Clonal selection within cv. Fortana started in 1975 at the University of Bologna (INTRIERI *et al.* 1992) by exploring its growing sites, which are essentially restricted to the Modena and Ferrara Provinces of the Emilia-Romagna region. These investigations brought forth 5 virus-indexed biotypes, which were then separated into two groups by discriminant multivariate analysis of 24 leaf traits (SILVESTRONI *et al.* 1995). Although these two groups also exhibit slight differences as to berry size and date of budburst, they are morphologically quite similiar. Given the dual-origin proposition cited *supra*, it was assumed that these two morpho-types, which have always been considered as part of cv. Fortana, originated not *via* vegetative propagation of a single individual through subsequent accumulations of bud mutations but from the seedlings, par-

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ents or siblings of the progenitor (SILVESTRONI *et al.* 1995). To corroborate this assumption, further investigations on these Fortana clones were conducted by testing DNA polymorphism at 6 microsatellite *loci* since PCR (polymerase chain reaction)-based methods have proven to be a reliable tool for ascertaining the interrelationships of varieties *via* DNA fingerprinting of grapevine genotypes (SCOTT *et al.* 1993; THOMAS and SCOTT 1993; THOMAS *et al.* 1993; CIPRIANI *et al.* 1994; THOMAS *et al.* 1994; BOTTA *et al.* 1995; MULCHAY *et al.* 1995; VIGNANI *et al.* 1996).

Material and methods

Plant material: The clones and the basic traits of the two Fortana morphological types are listed in Tab. 1. Plant material was taken from the germplasm collection vineyard at the University of Bologna and DNA analyses were performed at the University of Siena.

DNA preparation and storage: Total DNA was extracted from young leaves by a modification of the method reported by MULCHAY *et al.* (1993). DNA was further purified by elution through a Chroma-spin 1000 column (Clontech Laboratories, Inc. Palo Alto, CA, USA) and quantified either by visual comparison with lambda DNA on ethidium bromide stained agarose gels or by fluorimetry. The DNA was stored at -20 °C in conventional TE (Tris 10 mM, EDTA 1 mM, pH 8.0).

Microsatellite *loci*: Of the 6 microsatellite *loci* analyzed, three (VVS1, VVS2 and VVS4) were characterized by THOMAS and SCOTT (1993); two (VVMD6 and VVMD7) were isolated by BOWERS *et al.* (1996) and the last one (VVMD3) was isolated in the laboratory of C. MEREDITH at the University of California Davis by VIGNANI and BOWERS (personal communication).

PCR amplification of microsatellite *loci*: DNA was diluted in water to a final concentration of 2.5 ng·µl^{-1} and stored at 4 °C for no more than two weeks. 4 µl of each dilution were added to 16 µl of the PCR reaction mix containing 2 µl of primer pair mix, [10 pmol·µl^{-1} each], 0.1 µl *Taq* DNA polymerase [5 units·µl^{-1}], PCR 1 x buffer ($MgCl_2$ 15 mM, KCl 50 mM, Tris HCl 10 mM pH 8.3, 0.001 % gelatin), 0.8 µl $MgCl_2$ [25 mM] and 1.6 µl dNTP mix [2.5 mM each] and sterile deionized water to reach the final volume of 16 µl. Each reaction was overlaid with one drop of mineral oil and briefly centrifuged at 14,000 rpm (30 s) in an Eppendorf-type minifuge. All amplifications were carried out with a Hybaid Omnigene thermal cycler running a program consisting of an initial denaturation step (5 min at 94 °C) and 40 cycles (of 92 °C, 1 min; 52 °C, 1 min; 72 °C, 2 min) followed by a 10-min elongation step at 72 °C. Before loading on acrylamide denaturing gels, 5 µl of each reaction mix were checked on agarose gels to verify quality and specificity of amplification.

Electrophoresis and silver staining: Denaturing buffer containing 10 mM NaOH, 0.05 % bromophenol blue and 0.05 % xylene cyanol were added to each sample to a final concentration of 75 % (v/v). Immediately before loading on gels, samples were denatured by heating at 72 °C for 3 min. After pre-running a 40 cm long sequencing gel (6 % polyacrylamide, 7 M urea) to reach 50 °C, 0.5 or 1 µl of the denatured sample was loaded onto the gel. Silver staining was performed using the Silver Promega Staining Kit (Promega Corp., Madison, WI, USA). Allele identification was performed as per the relative intensity of silver staining in replicate sequencing gels and the size of each allele was determined by comparison with a standard sequencing reaction (either M13 forward primer on pGEM, or T3 or T7 on pBluescript-SK were used).

Results and Discussion

The resulting data for the 6 *loci* analyzed for the 5 Fortana clones were reproducible. Five *loci* (VVS1, VVS2, VVS4, VVMD3, VVMD6) showed polymorphism between clones while one (VVMD7) had identical profiles for all the clones studied. Representative sequencing gels and the estimation of allele size are reported in the Figure; allele size per clone is also reported in Tab. 2.

Two genotypic patterns were found: CAB 2, CAB 13 and CAB 14 appeared to be identical and distinct from CAB 1 and CAB 3, which shared the same DNA profile. These results agree with previous morphology-based find-

Table 1

Denomination, geographical origin and different traits of the 5 Fortana clones distinguished into two morphological types (data reworked from INTRIERI *et al.* 1992 and SILVESTRONI *et al.* 1995)

Clone	Geographical origin of clone source plant	Morphological type	Berry size	Leaf upper lateral sinuses	Leaf petiolar sinus	Budbreak
CAB 2	Ferrara area	1	medium	deep	open	medium
CAB 13	Modena area	1	medium	deep	open	medium
CAB 14	Modena area	1	medium	deep	open	medium
CAB 1	Ferrara area	2	smaller	less deep	more open	earlier
CAB 3	Ferrara area	2	smaller	less deep	more open	earlier

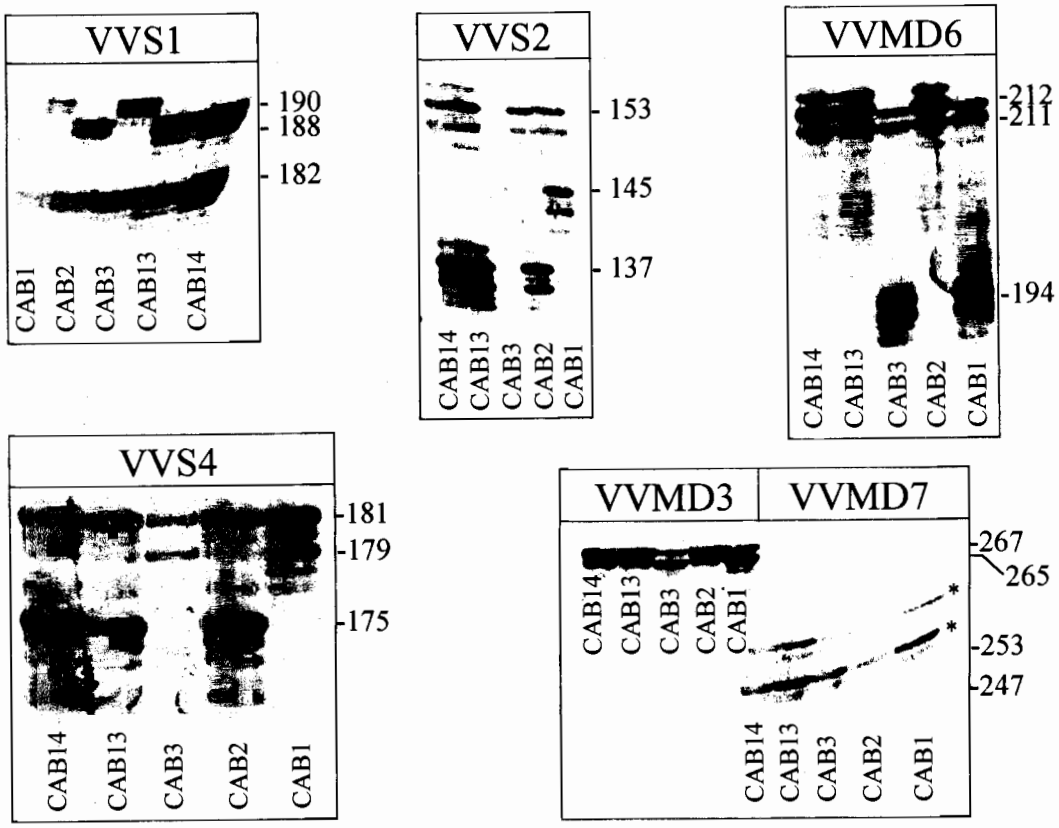

Figure: Genetic profiles of 5 Fortana clones obtained by PCR amplification of 6 microsatellite *loci* and subsequent electrophoresis of polyacrylamide gels and silver staining. Allele sizes are indicated on the right side of each gel.

Table 2

Allele sizes (bp) of 5 Fortana clones analysed at 6 microsatellite *loci*, previously distinguished into two morphological types

Clone	Morphological type	*Locus* VVS1	VVS2	VVS4	VVMD3	VVMD6	VVMD7
CAB 2	1	188 182	153 137	181 175	267	212 211	253 247
CAB 13	1	188 182	153 137	181 175	267	212 211	253 247
CAB 14	1	188 182	153 137	181 175	267	212 211	253 247
CAB 1	2	190 182	153 145	181 179	267 265	212 194	253 247
CAB 3	2	190 182	153 145	181 179	267 265	212 194	253 247
Polymorphism		yes	yes	yes	yes	yes	no

ings linking the resulting differences to the diversity of the genotypic profile of the two clone groups (Silvestroni *et al.* 1995). However, all the clones showed a certain degree of genetic relatedness since they shared at least one of the two alleles.

The data support the hypothesis that CAB 2, CAB 13 and CAB 14 originated from vegetative propagation of the same seedling, whereas CAB 1 and CAB 3 stemmed from another seedling genetically related to the former. The most likely proposition as to their relatedness is that these clone groups originated by vegetative propagation, the former springing from a single parent and the latter from one of its seedlings. In their description of Fortana, Cosmo and Polsinelli (1961) report that differences like berry size, bunch compactness and pedicel color have always been known by growers but note that these differences are minor; they ascribed them to site effects. Yet the Fortana clonal selection trials conducted by Silvestroni *et al.* (1990), whose design allowed for separation of environmental influences, showed little genetical variability as to bunch size and some leaf trait differences. However, in spite of these differences, Fortana clones are difficult to distinguish phenotypically without multivariate biometric assessment and, as previously reported, are cultivated by growers under an identical name and used to produce the same wine.

Given Fortana's polyclonal origin, it would be terminologically correct to assert that Fortana consists in fact of two distinct cultivars. Yet the term 'cultivar' is used in-

ternationally to identify cultivated plants that are readily distinguishable from others by their traits and that retain these distinctive features when they are vegetatively propagated. Since the two Fortana clonal groupings are not readily identifiable either by their morphological or quantitative traits, they should instead be considered as belonging to a single cultivar having two subgroups.

Given the assumptions of RIVES (1961), subsequently reiterated by ROSELLI and SCARAMUZZI (1974) and MULLINS and MEREDITH (1989), and in the light of recent DNA-based findings, a cultivar can be termed clonal when its biotypes derive from a single seedling through somatic mutations. While the slight changes in DNA sequences in these biotypes are not readily detectable with current DNA analysis methods, the clones of a clonal cultivar may be phenotypically differentiated from each other only by qualitative and/or quantitative traits, as for example those of cv. Pinot noir (SCOTT *et al.* 1993) and of Chardonnay (BOTTA *et al.* 1995). Yet it has to be noted that genetic differences in the DNA of *Vitis vinifera* cv. Seyval blanc regenerated from protoplasts (somaclones) have recently been detected through RAPD-PCR techniques (SCHNEIDER *et al.* 1996).

A cultivar can thus be termed polyclonal when its biotypes derive from more than one phenotipically similar and genetically related seedling. A number of clonal groups can originate from each of these individuals, each group deriving from somatic mutations of its vegetatively propagated mother plant and each one distinguishable from other groups by ampelometry and DNA detection techniques. An example of such a type is cv. Nebbiolo, which features differing morpho-types (SCHNEIDER *et al.* 1991) and differing genotypic patterns between some clones (BOTTA, personal communication). Within a single clonal group each clone may also be differentiated phenotypically but not easily genetically from the others by current techniques.

Today microsatellite DNA analysis makes it possible to investigate the degree of genetic relatedness of clones within a given variety and to discriminate easily clonal from polyclonal cultivars. In the light of our findings and of the above considerations, Fortana can be called polyclonal. This also implies that the strict definition of a winegrape cultivar as a group of individuals stemming from the vegetative propagation of a single mother plant can be applied with certainty to cultivars of known origin, i.e. only those that have been produced and cultivated in very recent times. The Italian term "vitigno" or the French "cépage", which eschews any connotations about origin and genetic uniformity (VIGNANI *et al.* 1996), would appear more appropriate than cultivar to designate winegrape varieties of unknown origin.

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