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Diversity assessment of seedlings from self-pollinated Sangiovese grapevines by ampelography and microsatellite DNA analysis

by

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S u m m a r y : A population of *Vitis vinifera* L. seedlings deriving from a single self-pollinated Sangiovese vine were assayed for diversity by ampelographic and genetic techniques. After field-transplantation in 1987, the seedlings were initially screened in 1995. Twenty-four seedlings were of standard vigour and grape production. Woody cuttings from the 24 seedlings and the mother plant were self-rooted in 1995, and each vine was morphologically analysed and compared in 1997 using 31 descriptors of the ampelographic data sheet (OIV 1983) which are also recommended by UPOV for varietal identification - three for young shoots, 7 for shoots at bloom, 17 for adult leaves, one for flowers and 3 for berries. – In 1996 DNA was extracted from young apical leaves of the mother plant and the 24 seedlings. Ten molecular microsatellites, VVS1, VVS2, VVS5, VVS16, VVS29, VVMD5, VVMD6, VVMD7, VVMD17 and VVMD28, were used for progeny and mother plant comparison. The descriptor-based analysis showed that 12 of the 24 seedlings were morphologically similar amongst themselves and not different from the mother; the remaining 12 differing from each other and from the parent. The microsatellite analysis differentiated all 24 seedlings from the mother plant. Only two seedlings showed the same allele patterns at the 10 tested *loci*, although they differed morphologically. The results of both analyses indicate that self-pollination can generate phenotypically similar individuals that are difficult to distinguish morphologically, while their genetic polymorphism can easily be detected by microsatellite analysis. Thus it is possible, as suggested by RIVES (1961), that certain ancient cultivars comprise a number of clones that derive *via* vegetative propagation from closely related mother plants. Corroboration of the polyclonal origin in such cases can be performed by techniques combining morphological and molecular approaches.

K e y w o r d s : Vitis vinifera, mother plant, polyclonal origin, cultivar, clone.

Introduction

Vitis vinifera is considered to be a species originally comprising a series of cross-fertilized individuals held to be highly heterozygous, *i.e.* individuals marked by differing alleles at many gene *loci*. The resulting recombination of parental genes *via* sexual reproduction means that the offspring are likely to be genetically different or different from the parent. Yet progeny deriving from a cross of two differing genotypes or from self-pollination of a single parent may be so phenotypically similar to each other and/or to the parents (RIVES 1961) as to be mistaken at times for one of the parents (BRUNI 1960). BRUNI (1960, 1965) notes that some crosses of Sangiovese, Barbera, Malvasia toscana and Corniola were alike to their respective parental as to morphological and yield traits. Yet, among relatively recent studies on progeny from self-pollinated Sauvignon, it has been found that 40 % of the seedlings exhibit the same foliar morphology, which was attributed to the parent (BISSON 1986).

It can be argued that related seedlings which have a different genotype can be phenotypically similar, to the point of being considered as belonging to the same variety. Indeed, this is the assumption underpinning RIVES´ (1961) theory of the polyclonal origin of some ancient *V. vinifera* varieties, in which clonal variability may be derived as much from bud mutations occurring during vegetative propagation from a single progenitor as from mother plants deriving from different seedlings.

Recently published, initial data of a molecular investigation using 6 microsatellite *loci* on cv. Fortana corroborate this assumption (SILVESTRONI *et al.* 1997). The analyses showed two genetically differing groups of clones among the variety. Within each group the clones were genetically identical, and between groups the clones were closely related, sharing at least one allele at each *locus* and being similar as to morphological, yield and wine traits (SILVESTRONI *et al.* 1997). The use of microsatellites, initially employed for varietal characterization (THOMAS *et al.* 1994; BOTTA *et al.* 1995; BOWERS *et al.* 1996), has also proven helpful in detecting the possible polyclonal origin of some ancient cultivars. The results of a survey employing morphological and microsatellite markers on a model population of seedlings are herewith reported and discussed.

Material and Methods

P l a n t m a t e r i a l : The tested seedlings were derived from a single, self-pollinated mother plant of cv. Sangiovese (*Vitis vinifera* L.), the seeds being obtained in 1983 and ger-

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minated the next year. In March 1987 the bench-grown plantlets were transplanted to the field at the University of Bologna, Dipartimento di Colture Arboree; vines of standard vigour began cropping in 1990. From the surviving 43 seedlings (the original population comprised 59 seedlings), 24 were selected for testing as they showed no morphological or cropping anomalies. Woody cuttings of them and of the mother plant were self-rooted in 1995, subsequently transferred to bench conditions and grown so as to ensure maximum vegetative development.

Morphological analysis: In 1997 the self-rooted plants were morphologically tested (3-4 replicates per seedling) using 31 descriptors recommended by UPOV for varietal identification. The latter included 3 for young shoots (form, anthocyanin coloration, prostrate hair density); 6 for shoots at bloom (attitude, colour of the dorsal side of internodes, density of erect hairs on nodes, density of erect hairs on internodes, tendril distribution, tendril length); one for woody shoots (surface); 17 for adult leaves (size, blade shape, lobe number, anthocyanin coloration of the main veins on the upper side of blades, blistering of the upper side of blades, teeth shape, teeth length, length:base ratio of teeth, general shape of petiole sinus, shape of base of petiole sinus, particularities of petiole sinus, density of prostrate hairs between the veins on the lower blade side, density of erect hairs between the veins on the lower blade side, density of prostrate hairs on main veins on the lower blade side, density of erect hairs on main veins on the lower blade side, density of prostrate hairs on petiole, density of erect hairs on petiole); one for flowers (sex); and 3 for berries (shape, skin colour, flesh colour). Each descriptor was coded by extent of expression as per the OIV-UPOV data sheet (1983), which also includes recommendations as to how and when to collect the data. Note that tendril length expression was coded as per the Minimal Preliminary Description List edited by DETTWEILER (1996).

DNA extraction and microsatellite loci: Total DNA was extracted in 1996 from young leaves taken from each seedling and from the parent by the CTAB method after MULCAHY *et al.* (1993). Ten microsatellite *loci* – VVS1, VVS2, VVS5, VVS29 (THOMAS and SCOTT 1993), VVS16 (THOMAS, pers. comm.), VVMD5, VVMD6, VVMD7 (BOWERS *et al.* 1996), VVMD17 and VVMD28 (MEREDITH, pers. comm.) – were used for progeny and mother plant comparison. For each pair of primers one was labelled with fluorescent dye (THOMAS *et al.* 1994).

PCR amplification and microsatellite loci detection: DNA was quantified by fluorimetry and diluted in water to a final concentration of 12.5 $ng \cdot \mu l^{-1}$. The PCR was performed using a 20 µl mixture containing 50 ng genomic DNA, 1 U Taq DNA polymerase (Bresatec), 1x buffer (Bresatec), 2 mM of $MgCl_2$, 200 µM of dNTPs, and 0.5 µM of each primer.

All amplifications were carried out with a Hybaid Om-E thermal cycler running a program consisting of an initial denaturation step (3 min at 95 °C), and 30 cycles (denaturation, 45 s at 94 °C, annealing 30 s at 50 °C, extension 1 min at 72 °C) followed by a 7 min elongation step at 72 °C.

Mother plant samples were analysed after denaturation in an automated DNA sequencer apparatus (ABI) running GENESCAN software as reported by THOMAS *et al.* (1994) to check allele length (bp) at each *locus*.

For all seedlings, PCR samples with primers at VVS16 *locus* were analysed using the ABI apparatus to avoid identification errors because the two alleles differed by 6 bp only, while the analysis at the VVS1, VVS2, VVS5, VVS29, VVMD5, VVMD6, VVMD7, VVMD17 and VVMD28 *loci* were carried out in 6 % non-denaturing polyacrylamide gel since the two alleles, when present, differed by 9 bp or more. For this analysis 4 µl of the PCR sample was loaded with 2 µl of running dye onto the gel in 0.5 x TBE buffer. The samples were run for 30 min at 130 V and stained with ethidium bromide (0.5 $\mu g \cdot ml^{-1}$).

Results

Morphological analysis: The descriptor-based analysis showed that 12 seedlings did not differ from the mother plant (Tab. 1). The remaining 12 differed from the mother plant by one or more traits and were grouped as follows: 7 as blue-black berry skin and 5 as green-yellow berry skin, the differences from the mother plant being enumerated in Tabs. 2 and 3, respectively.

On the whole, differences were found for 11 of the 31 descriptors: the shoot-at-bloom descriptors differentiated accessions as to colour and tendril length; the foliar descriptors discriminated accessions by size, teeth length,

Table 1

Traits found to be common for a Sangiovese mother plant and 12 self-cross seedlings (A1, A2, A7, A8, A9, A11, A12, A14, A15, A16, A20, A21) as per 31 UPOV descriptors

1. Shoots and flowers
   - Open young shoot tip without anthocyanin coloration and with none or very sparse prostrate hairs. - Hermaphrodite flower. - Striate woody shoot surface. - Semi-erect shoot at bloom. - None or very sparse erect hairs on nodes and internodes. - Short tendrils with discontinuous distribution. - Green dorsal side of internodes.

2. Adult leaves
   - Medium size wedge-shaped leaf with 5 lobes. - Absent or very weak blistering of upper side. - Medium-long teeth with rectilinear sides. - High length:base teeth ratio. - Open petiole sinus with U-shaped base and no particularities.- Green main veins with none or very weak density of prostrate and erect hairs. - None or very weak density of erect and prostrate hairs between veins.

3. Leaf petioles
   - None or very sparse prostrate and erect hairs.

4. Berries
   - Roundish. - Blue-black skin. - Uncolored flesh.

Table 2

Differential traits of 7 seedlings with blue-black berry skin in comparison to the mother plant

Sangiovese mother plant	Seedlings
1. Short tendrils	Very short tendrils (A13, A26)
2. Short tendrils, none or very weak density of erect hairs on main veins, medium leaf size	Very short tendrils, weak density of erect hairs on main veins, medium-small leaf size (A6)
3. Short tendrils, none or very weak density of erect hairs on main veins, none or very weak density of prostrate hairs on main veins and between main veins	Very short tendrils, very weak density of erect hairs on main veins, weak density of prostrate hairs on main veins and between main veins (A18)
4. Short tendrils, none or very weak density of prostrate hairs on and between main veins, medium leaf size, medium-long teeth, high length:base teeth ratio	Very short tendrils, weak density of prostrate hairs on and between main veins, medium-small leaf size, medium teeth length, medium-high length:base teeth ratio (A27)
5. Medium leaf size	Large leaf size (A23)
6. Medium leaf size, medium-long teeth, short tendrils	Very large leaf size, long teeth, medium length tendrils (A17)

Table 3

Differential traits of 5 seedlings with green-yellow berry skin in comparison to the mother plant

Sangiovese mother plant	Seedlings
1. Blue-black berry skin	Green-yellow berry skin (A25)
2. Blue-black berry skin, short tendrils	Green-yellow berry skin, very short tendrils (A10)
3. Blue-black berry skin, short tendrils; medium leaf size	Green-yellow berry skin, very short tendrils, medium-small leaf size (A22)
4. Blue-black berry skin, green dorsal side of internodes, open petiole sinus	Green-yellow berry skin, red internodes dorsal side, slightly open petiole sinus (A24)
5. Blue-black berry skin, medium leaf size	Green-yellow berry skin, large leaf size (A19)

length:base ratio of teeth, general shape of petiole sinus, density of prostrate and erect hairs between veins and on main veins on the lower blade side; and the berry descriptors differentiated for skin colour. Note especially that no differences were found for traits of young shoot apex, tendril distribution, shoot attitude and hairs, leaf lobe number, blade shape, flower sex, berry shape and flesh colour.

Genetic analysis: Tab. 4 shows the results of the microsatellite survey for the mother plant and 12 morphologically similar seedlings, Tabs. 5 and 6 the survey for the mother plant and the 7 blue-black berry skin seedlings and the 5 green-yellow berry skin seedlings. Genotypes showing only one amplified fragment were considered as homozygotes.

The mother plant exhibited homozygosity at the *loci* VVS1, VVS2 and VVS29 and heterozygosity at VVS5, VVS16, VVMD5, VVMD6, VVMD7, VVMD17 and VVMD28. All seedlings differed from the mother plant. Seedlings A14 and A19, though clearly differing as to berry skin colour (the former blue-black, Tab. 4, and the latter green-yellow, Tab. 5), were identical as to allele length at all 10 *loci*.

Discussion

The descriptor-based ampelographic analysis showed differing degrees of uniformity between seedlings and/or the mother plant. Any environmental effects related to the

Table 4

Allele size at 10 microsatellite *loci* of Sangiovese and of the 12 seedlings which were morphologically indistinguishable from the parent

	Loci and allele size (bp)									
Individual	VVS1	VVS2	VVS5	VVS16	VVS29	VVMD5	VVMD6	VVMD7	VVMD17	VVMD28
Sangiovese	182	134	147 97	287 281	171	236 226	208 190	263 239	221 212	246 236
Seedling A 1	182	134	97	281	171	236 226	208	263 239	221	246 236
" A 2	182	134	147	287 281	171	236 226	208	263 239	212	246 236
" A 7	182	134	147	287 281	171	236 226	208 190	263 239	221	246 236
" A 8	182	134	97	281	171	236 226	190	239	212	246 236
" A 9	182	134	147 97	287 281	171	236 226	190	263 239	212	246 236
" A 11	182	134	97	287 281	171	236 226	208	263	221 212	246
" A 12	182	134	147	281	171	236 226	208	263	221	246
" A 14	182	134	97	287 281	171	226	208 190	263 239	221 212	246 236
" A 15	182	134	147 97	287 281	171	226	208 190	263 239	221 212	246
" A 16	182	134	147 97	287 281	171	226	208	263	221 212	246 236
" A 20	182	134	97	287 281	171	226	208 190	263 239	221	246 236
" A 21	182	134	147 97	287	171	236 226	190	239	221 212	236

Table 5

Allele size at 10 microsatellite *loci* of Sangiovese and of the 7 blue-black berry skin seedlings which were morphologically different from the parent

	Loci and allele size (bp)									
Individual	VVS1	VVS2	VVS5	VVS16	VVS29	VVMD5	VVMD6	VVMD7	VVMD17	VVMD28
Sangiovese	182	134	147 97	287 281	171	236 226	208 190	263 239	221 212	246 236
Seedling A 6	182	134	147	287 281	171	236 226	208	263 239	221 212	246 236
" A 13	182	134	147	287 281	171	226	208	263	221 212	246
" A 17	182	134	147 97	287 281	171	226	208 190	263 239	221 212	246 236
" A 18	182	134	147 97	287	171	226	208 190	263 239	221	246 236
" A 23	182	134	147 97	287 281	171	226	190	239	221	246
" A 26	182	134	147 97	287 281	171	226	190	263 239	221	246 236
" A 27	182	134	147 97	281	171	236 226	190	263 239	221	246 236

Table 6

Allele size at 10 microsatellite *loci* of Sangiovese and the 5 yellow-green berry skin seedlings

	Loci and allele size (bp)									
Individual	VVS1	VVS2	VVS5	VVS16	VVS29	VVMD5	VVMD6	VVMD7	VVMD17	VVMD28
Sangiovese	182	134	147 97	287 281	171	236 226	208 190	263 239	221 212	246 236
Seedling A 10	182	134	147	287 281	171	236 226	208 190	239	221	246 236
" A 19	182	134	97	287 281	171	226	208 190	263 239	221 212	246 236
" A 22	182	134	97	287 281	171	236	190	239	212	246
" A 24	182	134	147 97	287 281	171	236 226	208 190	263 239	212	246
" A 25	182	134	147	287 281	171	236 226	208 190	263 239	212	246 236

different traits, *e.g.* leaf size and tendril length, were held to be negligible because each individual was replicated and compared under the same environmental and cultural conditions. The overall results, in which some seedlings were not morphologically distinguishable from the mother plant, matched those reported for progeny from self-pollinated parent, see for example BISSON (1986). Yet provisional data (not reported) from the present study indicated that some individuals, which were indistinguishable from the mother plant, also had similar ripening date, and must traits (soluble solids, pH and total acidity).

These findings would indicate that there may have been analogous situations in the past, when the oldest winegrape varieties were first selected by men. It may even be assumed that the first growers occasionally established vineyards using genetically related individuals with similar morphological and yield traits which they considered as being the same variety. This could have happened especially in those restricted areas where wild vine populations deriving from either self-pollination or from the crossing of a few parents were most frequent.

It is thus possible, as RIVES (1961) suggests, that the heterogeneity found today within some old cultivars derives as much from bud mutations (not readily discernible by current genetic methods) as from their polyclonal origin. In the latter case (more than one mother plant), the origin of heterogeneity should be readily detectable by DNA typing systems, especially microsatellite sequence-tagged site markers. To be held as belonging to the same variety, the various clones making up said variety must have a very similar physiognomy as well as uniform quality traits and must technological characteristics. This is exemplified by the clones identified in the ancient cv. Fortana (SILVESTRONI *et al.* 1997).

One possible application today with anything but negligible implications is to create new progenies by self-pollinating a given variety. This would enable the selection and certification as clones of those individuals having similar morphological and berry quality traits as the parental. The advantages of such an approach which favours the segregation of alleles and increases the phenotypic variability of cultivars would include having a supply of plants that are both in a healthy state and are readily identifiable by DNA typing methods.

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References

BISSON, J.; 1986: Essay d'ampélographie génétiqué appliqué au Sauvignon autofécondé. Vignevini **13** (12), 63-65.

BOTTA, R.; SCOTT, N. S.; EYNARD, I.; THOMAS, M. R.; 1995: Evaluation of microsatellite sequence-tagged site markers for characterizing *Vitis vinifera* cultivars. Vitis **34,** 99-102.

BOWERS, J. E.; DANGL, G. S; VIGNANI, R.; MEREDITH, C. P.; 1996: Isolation and characterization of new polymorphic simple sequence repeat *loci* in grape (*Vitis vinifera* L.). Genome **39**, 628-633.

BRUNI, B; 1960: Contributo allo studio della filogenesi viticola. Atti dell'Accademia Italiana della Vite e del Vino. Vallecchi Ed. Vol. **XI**, 147-169.

- -; 1965: Miglioramento genetico delle viti mediante selezione da seme. Prove pratiche effettuate e risultati ottenuti. Atti dell'Accademia Italiana della Vite e del Vino. Vallecchi Ed. Vol. **XVII**, 171-188.

DETTWEILER, E.; 1996: Extrait du Code OIV caractères ampélographiques et de la Liste Minimale Provisoire. IV. Cours International de l'Ampélographie, 2-6 Septembre, Istituto Sperimentale per la Viticoltura di Conegliano.

MULCAHY, D. L.; SANSAVINI, S.; DOUGLAS, G. C.; LINSKENS, H. F.; G. BERGAMINI-MULCAHY; VIGNANI, R.: PANCALDI, M.; 1993: The use of random amplified polymorphic DNAs to fingerprint apple genotypes. Sci. Hort. **54**, 89-96.

O.I.V.; 1983: Code des caractères descriptifs des varietes et espèces de *Vitis*. OIV, Paris.

RIVES, M.; 1961: Bases génétiques de la sélection clonale chez la vigne. Ann. Amélior. Plantes, **11**, 337-348.

SILVESTRONI, O.; DI PIETRO, D.; INTRIERI, C.; VIGNANI, R.; FILIPPETTI, I.; DEL CASINO, C.; SCALI, M; CRESTI, M.; 1997: Detection of genetic diversity among clones of cv. Fortana (*Vitis vinifera* L.) by microsatellite DNA polymorphism analysis. Vitis **36**, 147-150.

THOMAS, M. R.; CAIN, P.; SCOTT, N. R.; 1994: DNA typing of grapevine: A universal methodology and database for describing cultivars and evaluating genetic relatedness. Plant Mol. Biol. **25**, 939-949.

- -; SCOTT, N. R; 1993: Microsatellite (MS) repeats in grapevine reveal DNA polymorphism when analyzed as sequence-tagged sites (STSs). Theoret. Appl. Genet. **86,** 985-990.

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