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Molecular characterization of officially registered Sangiovese clones and of other Sangiovese-like biotypes in Tuscany, Corsica and Emilia-Romagna

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Summary

The present study was designed (1) to identify and determine the origin of the genetic variability *via* SSR and AFLP within a group of 39 Sangiovese clones officially listed in the National Grapevine Registry, (2) to pinpoint varietal differences and potential family relations among 34 Sangiovese-like biotypes, via the SSR markers. Most biotypes are regarded as Sangiovese but sometimes are known under different names. In both studies the reference standard was the registered Sangiovese clone SG 12T.

No polymorphism was found among the officially listed 39 clones analysed at 6 microsatellite loci. This enabled us to confirm their origin from a single mother plant, thereby supporting the view that any morphological or qualitative differences may be the result of propagation-related mutagenic events. A subsequent AFLP analysis of 26 of the 39 clones showed polymorphic bands in three of them (two identical) that may correspond to a mutagenic event.

Assays with SSR markers on 34 Sangiovese-like biotypes collected in Tuscany, Corsica and Emilia-Romagna showed that 28 are identical with the reference Sangiovese clone SG 12T, while the remaining 6 (Sangiovese 1, Sangiovese 6, Morellino, Poverina, Sangiovese forte and Brunellone) are genetically different from SG 12T and among one another so that no direct family relations could be established.

K e y w o r d s : *Vitis vinifera*, Sangiovese, microsatellite (SSR), amplified fragment length polymorphism (AFLP), cultivar, clone.

Introduction

Certain grapevines evince intra-cultivar variability that, if environmental and viral causes are ruled out, may be ascribed either to bud mutations that arose during agamic propagation from a single mother plant, or to agamic multiplication, with or without mutagenic events from seed-derived, closely related and morphologically similar individuals (Rives 1961). In the first instance, current DNA assays using microsatellites (SSRs) do not appear to be capable to detect any genetic differences arising from bud mutations in the course of agamic descendance from a single mother plant (Thomas and Scott 1993, Bowers *et al.* 1996, Silvestroni *et al*. 1997, Sefc *et al.* 1998). The only exceptions reported involve differences among biotypes of the Pinot group imputable to periclinal chimeras and shown to have more than two alleles at a single SSR locus (Franks *et al*. 2002). While only a few specific cases are reported in literature, genetic differences among mutated biotypes deriving from a single mother plant have been found using AFLPs (amplified fragment length polymorphisms), which are potentially better suited for this purpose (Cervera *et al*. 1998, Scott *et al*. 2000, Bellin *et al*. 2001). In the second instance of variability arising from a few closely related, seed-derived individuals, the inter-biotype genetic differences are readily discernible via several kinds of markers. Of these, SSRs are particularly useful because of their polymorphic and co-dominant traits and because they can determine the dimension in base pairs of alleles at each locus both, in cultivar identification (Thomas *et al.* 1994, Bowers *et al*. 1996, Filippetti *et al*. 2001) and in studying inter-varietal relations (Bowers and Meredith 1997, Sefc *et al.* 1998).

The present study investigates the genetic basis of variability of a number of registered Sangiovese clones and attempts to establish the identity or the varietal diversity and any kinship relations in a group of randomly collected but as yet not definitively named grape accessions.

Material and Methods

The first investigation was carried out on 39 Sangiovese clones listed in the National Grapevine Variety Registry (Tab. 1), and the second on 34 new accessions largely thought to belong to cv. Sangiovese but locally known to have different names (*e.g.* Morellino, Chiantino, Nielluccio). These 34 Sangiovese-like accessions were found during ampelographic surveys conducted in Tuscany, Corsica and Emilia-Romagna (Tab. 2). The registered Sangiovese SG 12T clone was need as reference in both assays.

Young leaves of individual vines were taken from the variety collections at the nursery Vivai Cooperativi in Rauscedo and at the Departments of Horticulture and of Fruit Tree and Woody Plant Science, Universities of Florence and Bologna. Their DNA was extracted with the DNeasy® Plant Mini Kit (Qiagen, Milan) and quantified with an ND-100 spectrophotometer (Nanodrop, Wilmington, DE). All the samples in both investigations were assayed using

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Table 1

List and provenance of registered Sangiovese clones assayed by SSRs. Clones denoted with an asterisk were also analysed by AFLPs

Registered clones	Area of origin
Chianti classico 2000/1(*)	Castellina in Chianti, Siena - Tuscany
Chianti classico 2000/2 (*)	Barberino Val D'Elsa, Florence- Tuscany
Chianti classico 2000/3 (*)	Barberino Val D'Elsa, Florence- Tuscany
Chianti classico 2000/4 (*)	Gaiole in Chianti, Siena - Tuscany
SG Rauscedo 10 (*)	Antella, Florence- Tuscany
SG VCR 4 (*)	Antella, Florence- Tuscany
SG VCR 30 (*)	Mercatale Val di Pesa, Florence- Tuscany
Montalcino 42 (*)	Montalcino, Siena - Tuscany
SG VCR 5 (*)	Montalcino , Siena - Tuscany
SG VCR 6 (*)	Montalcino, Siena - Tuscany
SG VCR 103 (*)	Montalcino, Siena - Tuscany
SG VCR 102 (*)	Montalcino, Siena - Tuscany
SG BF 10 (*)	Montalcino, Siena - Tuscany
SG BF 30 (*)	Montalcino, Siena - Tuscany
TIN-10 (*)	Montalcino, Siena - Tuscany
TIN-50 (*)	Montalcino, Siena - Tuscany
JANUS-10 (*)	Montalcino, Siena - Tuscany
JANUS-20 (*)	Montalcino, Siena - Tuscany
B-BS-11 (*)	Montalcino, Siena - Tuscany
SG Rauscedo 24 (*)	Predappio, Forli-Cesena - Emilia-Romagna
SG VCR 19 (*)	Predappio, Forli-Cesena - Emilia-Romagna
SG VCR 23 (*)	Vecchiazzano, Forli-Cesena - Emilia-Romagna
SG VCR 16 (*)	Vecchiazzano, Forli-Cesena - Emilia-Romagna
SG 2T (*)	Savignano sul Rubicone, Forli-Cesena - Emilia-Romagna
SG 4T (*)	Savignano sul Rubicone, Forli-Cesena - Emilia-Romagna
SG 12T (*)	Predappio, Forli-Cesena - Emilia-Romagna
U.S. FI-PI 3	Lamole, Florence- Tuscany
U.S. FI-PI 172	Lamole, Florence- Tuscany
Peccioli 1	Peccioli, Pisa - Tuscany
SS-FP-A5-48	Lamole, Florence- Tuscany
CSV-AP-SG5	Valle, Macerata - Marche
AP-SG 1	Cossignano, Ascoli Piceno - Marche
AP-SG 2	Cossignano, Ascoli Piceno - Marche
UBA 74/C	Crispiano, Taranto - Apulia
UBA 79/C	Poliporo, Matera - Basilicata
UBA 63/F	Metaponto, Matera - Basilicata
FEDITH 20-CH	Gaiole in Chianti, Siena - Tuscany
FEDITH 21-CH	Gaiole in Chianti, Siena - Tuscany
FEDITH 22-CH	Gaiole in Chianti, Siena - Tuscany

the 6 microsatellite markers VVMD5, VVMD6, VVMD7 (BOWERS *et al*. 1996), VVMD17, VVMD25 (BOWERS *et al*. 1999) and VVS16 (Thomas *et al*. 1994). The amplification mix included 100 ng of DNA in a solution (20 µl) containing Taq DNA buffer (1X), $MgCl_2$ (2 mM), dNTPs (0.2 mM each), Taq (0.75 U) and 2 primers (0.5 mM each). The amplification products were subjected to electrophoresis on polyacrylamide gel (5 % in acrylamide, 7 M urea) and staining by silver nitrate 1 ‰ w/v. The allelic dimensions were directly assayed on each gel in comparison to a ladder of 25 and 100 base pairs and reference Sangiovese clone SG 12T.

In the first investigation part of the registered clones (Tab. 1) were also assayed with AFLPs with some of the most polymorphic primers according to the protocol of VOS *et al*. (1995). Each DNA sample (500 ng) was mixed in RL Buffer 5X (10X One Phor All, 20 $mg \cdot ml^{-1}$ BSA, DTT 1M) and 5 units of Eco RI and 5 units of MseI (for a total volume of 40 µl) and digested for 90 min at 37 °C. The solution for the ligase reaction (10 µl of RL Buffer 5X, Eco RI adapter 5 $pmol \cdot \mu l^{-1}$, MseI adapter 5 $pmol \cdot \mu l^{-1}$, ATP 10 mM, 1 unit T4 ligase) was added immediately to the restriction products and was run for 4 h at 37 °C. The ligase solution (5 µl)

Table 2

List and provenance of the Sangiovese-like accessions collected in Tuscany, Corsica and Emilia-Romagna

	Province of origin		Province of origin
Tuscany		Emilia-Romagna	
Morellino di Scansano	Grosseto	Sangiovese 1	Forlì and Cesena
Morellino	Grosseto	Sangiovese 3	Forlì and Cesena
Brunellone	Grosseto	Sangiovese 5	Forlì and Cesena
Brunelletto	Grosseto	Sangiovese 6	Forlì and Cesena
Vigna maggio SG 1	Florence and Siena	Sangiovese 9	Bologna
Cacchiano SG 5	Florence and Siena	Sangiovese10	Forlì and Cesena
SG Liliano 1	Florence and Siena	Sangiovese 11	Forlì and Cesena
Poverina	Florence and Siena	Sangiovese 13	Forlì and Cesena
Sangiovese forte	Florence and Siena	Sangiovese14	Forlì and Cesena
Chiantino	Florence and Siena	Sangiovese15	Forlì and Cesena
Sangiovese grosso	Florence and Siena	Sangiovese 16	Forlì and Cesena
Sangiovese polveroso	Florence and Siena	Sangiovese 19	Forlì and Cesena
Prugnolo gentile	Siena	Sangiovese 44	Forlì and Cesena
Corsica		Sangiovese 50	Forlì and Cesena
Nielluccio 904	Bastia	Sangiovese 60	Ravenna
Nielluccio 902	Bastia	Sangiovese 61	Ravenna
		Sangiovese 62	Ravenna
		Sangiovese 63	Ravenna
		Sangiovese 64	Ravenna

was diluted 10 times and added to a solution of 15 µl of master mix for pre-amplification (buffer 10X, dNTPs 10 mM), 0.5 mM Eco RI primer (Eco RI adapter + 1N), 0.5 mM MseI primer (MseI adapter + 1N) and 1 unit Taq (Amersham Biosciences, UK); the subsequent PCR profile had 24 cycles, each including denaturation (94 °C for 30 s), pairing (56 °C for 30 s) and final extension (72 °C for 60 s). The pre-amplification products were checked on agar gel at 1 % w/v, diluted 30 times and amplified (5 µl diluted pre-amplification product + 15 µl master mix) with 9 primer combinations having three selective bases (Tab. 3).

PCR analysis was performed with the following cycle profile: a 30 s denaturation step at 94 °C; a 30 s annealing step at 65 °C and a 1 min extension step at 72 °C. The annealing temperature in the next 12 cycles was subsequently reduced by 0.7 °C for each cycle, then continued at 56 °C for the remaining 24 cycles.

The amplification products were separated on a 5 % polyacrylamide gel and stained with silver nitrate (1 ‰ w/v); 5 accessions of the clone Sangiovese SG 12T and the cv. Albana were used as controls. The absence or presence of bands was visually checked.

Results and Discussion

Registered Sangiovese clones

SSR assay: Among the 39 clones as well as between them and Sangiovese SG 12T no polymorphism was found (Tab. 4). Their uniformity indicated that they all belong to cv. Sangiovese and any chance that individuals among them derived from different mother plants can be ruled out. Indeed, according to Sefc *et al*. (1999, 2000), the theoretical probability that two biotypes, which proved to be identical at 5 SSR *loci*, do not belong to the same variety is about 10^{-5} and drops even further to about 10^{-9} if there are 9 non-polymorphic loci.

AFLP analysis: In this assay, performed on 26 clones (see Tab. 1), we found genetic variations that remained undetected in the former test. Chianti Classico 2000/1,

Table 3

Primer combinations used for AFLP analysis.

Primer	MseI-CCT	MseI-CTG	MseI-CAT	MseI-CGA	MseI-CGG
Eco RI–ACT	X	X	X	X	X
Eco RI–ACC	X	X	X		
Eco RI–ACG	X				

Table 4

Results from 39 registered Sangiovese clones assayed at 6 SSR loci and the reference Sangiovese SG 12T. All alleles are shared. Numerical values are allelic lengths of base pairs

Locus	Reference Sangiovese SG 12T	Chianti classico 2000/1, Chianti classico 2000/2, Chianti classico 2000/3, Chianti classico 2000/4, SG Rauscedo 10, SG VCR 4, SG VCR 30, U.S. FI-PI 3, U.S. FI-PI 172, Peccioli 1, SS-FP-A5-48, Montalcino 42, SG VCR 5, SG VCR 6, SG VCR 102, SG VCR 103, SG BF 10, SG BF 30, TIN-10, TIN-50, JANUS-10, JANUS-20, B-BS-11,SG Rauscedo 24, SG VCR 19, SG VCR 23, SG VCR 16, SG 2T, SG 4T, SG 12T, CSV-AP-SG5, AP-SG 1, AP-SG 2, UBA 74/C, UBA 79/C, UBA 63/F.
VVMD5	224-234	224-234
VVMD6	190-208	190-208
VVMD7	239-263	239-263
VVMD17	212-221	212-221
VVMD25	242-242	242-242
VVS16	281-287	281-287

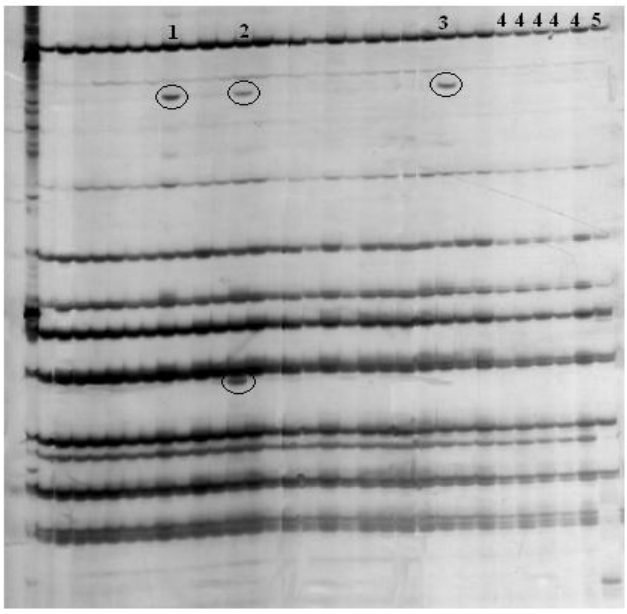

Fig. 1: AFLP assay combining primers Eco RI-ACC and MseI-CTG on 26 out of the 39 registered clones listed in Tab. 1. The numbers on top denote: (**1**) Sangiovese Chianti Classico 2001/1; (**2**) Sangiovese VCR5; (**3**) Sangiovese Chianti Classico 2001/2; (**4**) control Sangiovese SG 12T (5 repeats); (**5**) cv. Albana (outside control). The polymorphic bands detected in clones as per numbers 1, 2 and 3 are circled.

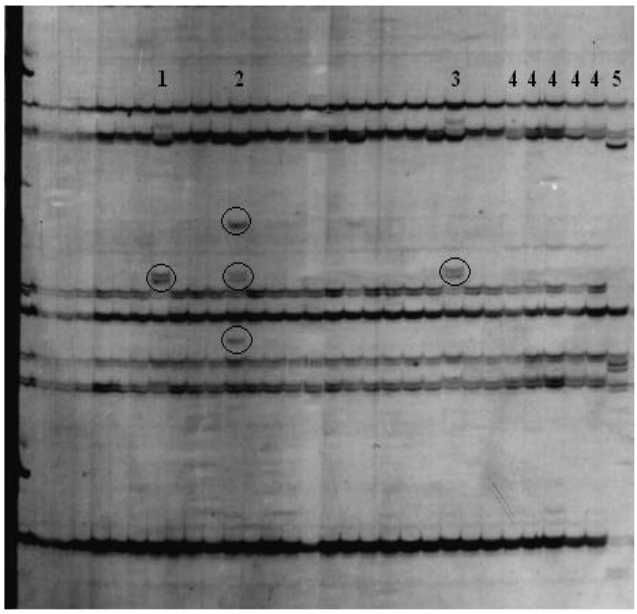

Fig. 2: AFLP assay combining primers Eco RI-ACT and MseI-CCT on 26 out of the 39 registered clones listed in Tab. 1. For details see Fig. 1.

Chianti Classico 2000/2 and the VCR 5 showed polymorphic bands for two primer combinations. The two first clones were identical (Figs 1 and 2). The runs were repeated several times and the results appeared perfectly reproducible.

Sangiovese-like biotypes of Tuscany, Corsica and Emilia-Romagna

SSR analysis: For the SSR runs on the 34 accessions assumed to be cv. Sangiovese 6 markers were used and 28 biotypes identical to Sangiovese SG 12T were detected (Tab. 5). The remaining 6 accessions, Morellino, Sangiovese forte, Brunellone and Poverina (from Tuscany) and Sangiovese 1 and 6 (from Emilia Romagna), proved to be genetically different from each other and from the reference Sangiovese SG 12T (Tab. 5). The results indicate that the Tuscan Morellino shares 7 out of 12 alleles with SG 12T and both Sangiovese forte and Brunellone 8 out of 12, but each one has a locus with no common alleles with SG 12T. Poverina shares 7 out of 8 alleles with SG 12T, although the 4 loci analyzed were not sufficient to draw definite conclusions. Sangiovese 1 shared only 4 out of 12 alleles with SG 12T and Sangiovese 6 only 4 out of 10, and both had two loci with no alleles in common with SG 12T. In case of a close genetical relationship between two individuals, like parent-offspring or brother-brother, there must be at least one common allele at each locus. Hence, it seems evident that for 5 out of these 6 biotypes (Poverina was uncertain) there is no direct kinship among them and cv. Sangiovese.

Table 5

Results of SSR assays at 6 loci of 34 Sangiovese-like accessions and the reference Sangiovese SG 12T. Numerical values are allelic lengths of base pairs. Shared alleles are marked in bold

Accessions	Loci and allele size (bp)					
	VVMD5	VVMD6	VVMD7	VVMD17	VVMD25	VVS16
Referent Sangiovese SG 12T	224-234	190-208	239-263	212-221	242-242	281-287
Tuscany Morellino di Scansano, Brunelletto, Vigna maggio SG 1, Cacchiano SG 5, SG Liliano 1, Chiantino, Sangiovese grosso, Sangiovese polveroso, Prugnolo gentile Corsica Nielluccio 904, Nielluccio 902 Emilia-Romagna Sangiovese 3, Sangiovese 5, Sangiovese 9, Sangiovese 10, Sangiovese 11, Sangiovese 13, Sangiovese 14, Sangiovese 15, Sangiovese 16, Sangiovese 19, Sangiovese 44, Sangiovese 50, Sangiovese 60, Sangiovese 61, Sangiovese 62, Sangiovese 63, Sangiovese 64	**224-234**	**190-208**	**239-263**	**212-221**	**242-242**	**281-287**
Morellino (Tuscany)	**224-234**	190-190	248-263	**212-221**	240-240	287-287
Sangiovese forte (Tuscany)	**224-234**	**190-208**	239-248	**212-221**	242-261	284-284
Brunellone (Tuscany)	**224-234**	208-210	246-263	**212-221**	**242-242**	284-284
Poverina (Tuscany)	**224-234**	**190-208**	248-263	**212-221**	-	-
Sangiovese 1 (Emilia-Romagna)	224-224	208-208	248-248	221-221	240-240	287-287
Sangiovese 6 (Emilia-Romagna)	224-224	190-208	248-248	221-221	240-240	-

Conclusion

The overall results of the SSR analysis showed that the examined 39 registered Sangiovese clones are derived from a single mother plant. AFLP analysis on 26 of these clones showed DNA differences in three of them and for two primer combinations (see Figs. 1 and 2). This finding has to be taken as an isolated case and it is in accordance with similar findings in literature (Franks *et al*. 2002, Cervera *et al*. 1998, Scott *et al*. 2000, Bellin *et al*. 2001). Yet these differences, presumable due to spot mutations, indicate that the clones Sangiovese VCR5, Sangiovese Chianti Classico 2000/1 and Sangiovese Chianti Classico 2001/2, the latter two undistinguishable from each other, can be unequivocally identified .

The SSR assays on the 34 Sangiovese-like accessions of hitherto undefined identity indicate that 28 belong to cv. Sangiovese. However their agronomic value could only be demonstrated in field tests run under the protocol for clonal selection currently employed in Italy. Excluding Poverina, the remaining 5 (Morellino, Sangiovese forte, Brunellone, Sangiovese 1, Sangiovese 6) differ from each other and are not closely related to cv. Sangiovese. They might be biotypes of unknown origin grown locally or misnamed varieties. In the first case if their field performance deemed promising, they might be added to the National Grapevine Variety Registry after description of their ampelographic and oenological profile.

Since the homonyms and synonyms are unfortunately still rather common in the European ampelographic heritage, the data reported for the Sangiovese-like clones may help to reduce confusion in the regulatory provisions for the EU's Denomination of Origin directive.

A final implicit issue involves whether a new accession with a substantial morphological and oenological similarity to a reference cultivar and genetical related to it (*i.e.* siblings or parents offspring) should be considered as part of the intra-varietal variability of that given cultivar and registered as its 'clone' (Rives 1961).

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