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Genotyping wine and table grape cultivars from Apulia (Southern Italy) using microsatellite markers

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Summary

Thirty-eight typical grapevine varieties of the Apulia region, Southern Italy, were genotyped at 6 microsatellite *loci* (VVS2, VVMD5, VVMD7, VrZAG47, VrZAG62, VrZAG79) with the aim to find synonymy and to confirm some pedigrees reported in literature. Microsatellites were amplified by PCR with ^{33}P-ATP labelled primers and alleles separated by electrophoresis using 6 % acrylamide sequencing gels.

The results confirm the high information level of the selected microsatellites. The number of alleles ranged from 7 to 11, producing up to 23 different combination patterns. The observed heterozygosity varied between 81.6 and 94.7 %, the discrimination power between 0.888 and 0.939, and the probability of identity was as low as 0.06-0.12. All cultivars of the study were discriminated from each other, except Regina (syn. Afuz Ali) and Mennavacca, which had the same profile. Finally, we were able to confirm the parentage of Victoria (Cardinal x Afuz Ali) and Matilde (Italia x Cardinal).

K e y w o r d s : microsatellites, wine grape cultivars, table grape cultivars, Apulia.

Introduction

The genetic pool of grapevines grown in the Bari and Foggia areas (Apulia region, Southern Italy) nowadays is a mixture of ancient and more recently introduced varieties. Before recent varieties will replace the ancient germplasm, it seems reasonable to make any effort to identify valuable genotypes and ancient, rare varieties, that could make the wine production of that area more typical.

Nowadays cultivars are identified by different molecular markers. Among them, microsatellites or SSRs (Simple Sequence Repeats) are becoming the markers of choice. They are short DNA sequences, 1-6 nucleotide-long, repeated several times at a given *locus* (Morgante and Olivieri 1993). Due to polymerase slippage during replication of the DNA sequence, the *locus* gave rise to alleles, which differ in length because of the different number of repeats of the core sequence. In plants, microsatellites were estimated to be as frequent as one in every 1-2.4 kbp in a set of species including *Arabidopsis thaliana*, rice, soybean, maize and wheat (Morgante *et al.* 2002). Many SSRs have already been isolated in grape and the high level of heterozygosity of the species (69-88 % according to Thomas and Scott 1993), which enhances the polymorphisms of SSR markers, led to solve many problems related to variety identification and pedigree analysis (Cipriani *et al*. 1994; Botta *et al.* 1995; Bowers *et al*. 1996; Meredith *et al*. 1996; Maletic *et al*. 1999; Sefc *et al*. 2000; Virk *et al*. 2000).

The aim of the present research was to evaluate the genetic diversity within the ancient grape germplasm still existing in Apulia and to study the relationship of this genetic pool with the international varieties cultivated in the same area. In cases where parent-offspring relationships were available from literature for cultivars included in the present study, the proposed pedigree was also checked.

Material and Methods

Thirty-eight varieties were analysed (Tab. 1). The material was sampled in commercial vineyards in the provinces of Bari and Foggia (Apulia). Cuttings of 2-3 buds each from individual vines were collected during winter and kept in jars with water until budburst. Young leaves were collected from the shoot tips and approximately 1 g was used for DNA extraction following the Doyle and Doyle method (1990) modified by Cipriani and Morgante (1993).

Six microsatellite *loci*, namely VVS2 (Thomas and Scott 1993), VVMD5, VVMD7 (Bowers *et al.* 1996), VrZAG47, VrZAG62, VrZAG79 (Sefc *et al.* 1999), were used in the analysis following the recommendations of the European GENRES 081 project, which is establishing the world's largest database of grape cultivars and which includes SSR markers among the descriptors (Dettweiler 1997).

PCR was performed in 10 µl of a mixture containing 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTP, 0.25 µM of each primer, 20 ng of template DNA, 0.5 U AmpliTaq™ Gold (Perkin Elmer, Norwalk, U.S.A.). One primer of each pair was labelled with γ^{33}P-ATP for 35 min at 37 °C using 1 µl reaction mix containing: 2.5 µM primer, 0.40 -0.52 pmol γ^{33}P-ATP at 2500 Ci/mmol, 70 mM Tris HCl, 5 mM $MgCl_2$, 0.5 mM DTT, and 0.4 U T4 polynucleotide kinase. PCR reactions were carried out using a Perkin Elmer 9700 thermocycler with the following temperature profiles:

loci VVS2, VrZAG47, VrZAG62, VrZAG79; (touch-down PCR), 95 °C for 10 min, (94 °C for 30 s, 65 °C (-1 °C for each cycle) for 30 s, 72 °C for 30 s) for 9 cycles; (94 °C for 45 s, 56 °C for 45 s, 72 °C for 45 s) for 21 cycles; 72 °C for

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Table 1

List of the cultivars included in this study

Cultivar	Berry color[a]
Local wine grape cultivars	
Aleatico	N
Barbarossa	RS
Bombino bianco clone R7	B
Bombino bianco di San Severo	B
Bombino nero	N
Negro amaro	N
Pagadebiti	B
Porcinale	N
Primitivo	N
Tuccanese	N
Uva di Troia clone R1	N
Verdeca	B
Local table grape cultivars	
Baresana	B
Corniola	B
Mennavacca	B
Moscato di Barletta	B
Moscato nero	N
Panse precoce	B
Prunesta	N
Regina nera	N
Uva Sacra	N
Wide-spread wine grape cultivars	
Chardonnay clone R3	B
Garganega	B
Lambrusco Maestri	N
Malvasia istriana clone R4	B
Montepulciano clone R7	N
Sangiovese clone R10	N
Sauvignon clone R8	B
Trebbiano toscano clone R4	B
Wide-spread table grape cultivars	
Cardinal	N
Italia	B
Matilde	B
Moscato d'Amburgo	N
Ohanez	B
Perla di Csaba	B
Red Globe	RG
Regina	B
Victoria	B

[a] N = black, B = white, RG = red, RS = rose.

7 min. *loci* VVMD5, VVMD7; (touch down PCR), 95 °C for 10 min, (94 °C for 30 s, 65 °C (-1 °C for cycle) for 30 s, 72 °C for 30 s) for 13 cycles; (94 °C for 45 s, 52 °C for 45 s, 72 °C for 45 s) for 17 cycles; 72 °C for 7 min.

The amplified product (10 µl) was mixed with 10 µl of loading dye, heated at 90 °C for 5 min; 5 µl where then loaded onto a 6 % polyacrylamide sequencing gel. Electrophoresis was carried out in a Hoefer™ SQ3 Sequencer (Pharmacia Biotech) using 0.6x TBE running buffer at 1500 V, 40-42 mA, 58 W. After drying the gel was exposed to Amersham Hyperfilm™ MP film for 1-7 d depending on the ^{33}P age. Allele length was evaluated by comparison with the sequence of the PUC18 plasmid, used as a reference ladder.

The information content of each microsatellite *locus* was calculated by two indices: the discrimination power $PD = 1 - \Sigma(p_i)^2$, and the probability of identity $PI = \Sigma p^4_i + \Sigma\Sigma(2p_ip_j)^2$, where p_i and p_j are the frequencies of *i* and *j* alleles, respectively. The PI index together with allelic frequency, expected and observed heterozygosity, and frequency of null alleles were calculated using the "Identity" software (WAGNER and SEFC 1999; http://www.boku.ac.at/zag/steink_ssr_frames-neu.htm).

Results and Discussion

The analyses of 38 grape cultivars confirmed the high information content and the discrimination power (D) of the microsatellites adopted, as was reported previously (SEFC *et al*. 1997, 1998; CRESPAN and MILANI 2001). The most informative *loci* were those isolated from the group of Vienna, namely VrZAG 47, VrZAG 62 and VrZAG 79, that showed 21-23 different allelic patterns (Tab. 2). D values of these markers were highest ranging from 0.931 to 0.939. D values of the remaining SSRs were lower but still ⊕ 0.888 in all cases.

The PI (probability of identity) value ranged between 0.06 and 0.12 and appeared to be inversely related to the D value. The PI index was very close to the threshold of 0.5 below which a microsatellite marker has been considered hyperpolymorphic in grape (SEFC *et al.* 2001).

The observed heterozygosity, that is the percentage of heterozygous individuals among all those examined, was between 81.6 % for the *loci* VVS2, VrZAG47, and VrZAG79, and 94.7 % for the *locus* VVMD5 (Tab. 2).

Considering the genetic pool, the probability of identifying individuals with the same profile at all *loci* was 6.76×10^{-7}. This low value can be explained by the high level of heterozygosity of the species, the high polymorphism of markers and the rather even distribution of allele frequencies (Figure). As a result, all cultivars of the study were separated from each other, except for two, Regina and Mennavacca, which showed the same alleles and therefore can be considered synonymous (Tab. 3). Interestingly, also Sangiovese and Tuccanese shared the same profile at all *loci* but one (VrZAG79). The cultivar Sangiovese is widespread in Italy, whereas Tuccanese has a limited spread in the Apulia area; the fact that these two names could refer to very close varieties was already observed locally (NOVELLO, pers. comm.), and it is now confirmed by the SSR analysis. Porcinale, or Porcinara, is reported to be a synonym of Negro amaro by some authors (BRUNI 1872; CALÒ *et al.* 2000): our results show that these two cultivars are different.

We also checked the proposed pedigree of the table grape cvs Victoria and Matilde. Victoria is a selection obtained by V. LEPÀDATU in 1980, by crossing Cardinal x Afuz Ali (the latter is a synonym of Regina) with the aim to reduce the yield instability of Romanian cultivars (LEPÀDATU and CONDEI 1984). Matilde was obtained in 1962 at the Istituto Sperimentale per la Frutticoltura in Rome by P. MANZO, who

Table 2

Genetic parameters of the 6 SSR *loci* analysed in 38 grape cultivars

Locus	No. of observed allelic patterns	No. of alleles	Allele size range (bp)	Expected hetero-zygosity (%)	Observed hetero-zygosity (%)	Frequency of null alleles (%)	D[a]	PI[b]
VVS2	16	8	135-153	78.9	81.6	-1.50	0.903	0.12
VVMD5	16	7	226-240	81.0	94.7	-7.59	0.888	0.11
VVMD7	19	10	233-263	80.9	89.5	-4.76	0.924	0.11
VrZAG47	21	9	153-172	84.0	81.6	+1.33	0.931	0.08
VrZAG62	22	11	187-206	86.6	92.1	-2.96	0.939	0.06
VrZAG79	23	10	237-259	82.0	81.6	+0.24	0.932	0.09

[a] D = Discrimination Power, [b] PI = Probability of Identity.

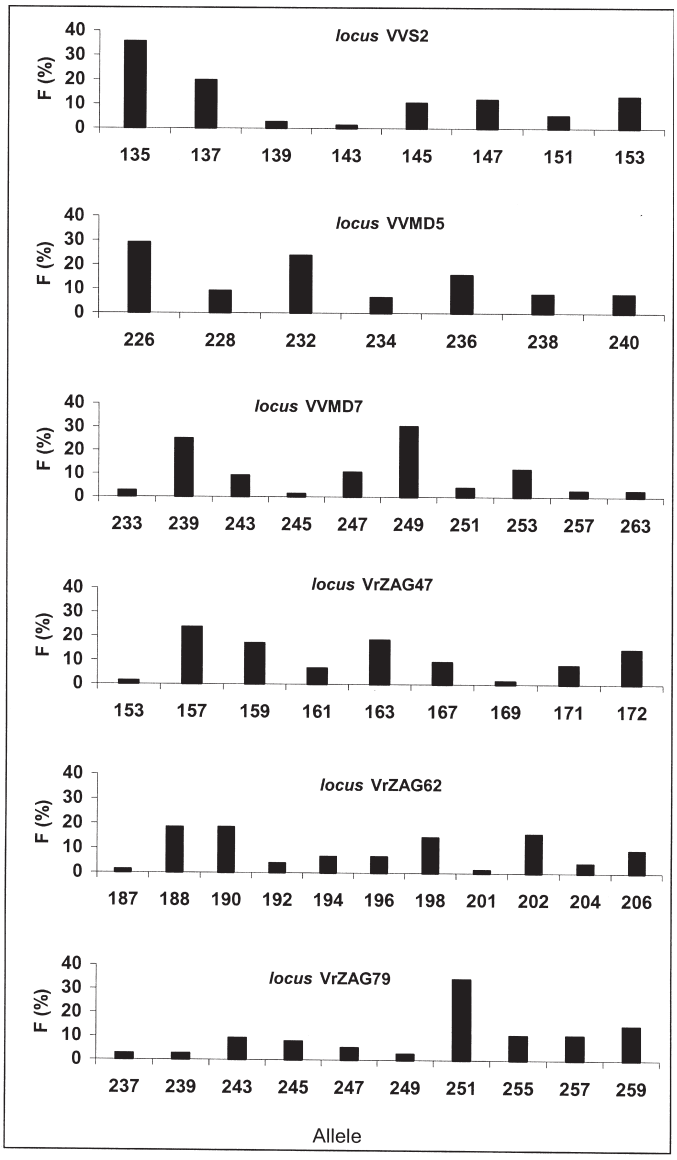

Figure: Allele frequency (F, %) at each of the 6 SSR *loci* analysed in the 38 grape cultivars of the study.

made crossings of Italia and Cardinal. The parentage analysis showed low likelihood ratios of the probability of the suggested parentage of both Victoria and Matilde (Tab. 4). In particular, the suggested parentage *vs.* the case in which one parent is a relative of the true parent is very low (8.26 and 7.40 in Cardinal x Afuz Ali; 1.13 and 6.86 in Italia x Cardinal):

Table 3

Allelic profile (allele size in bp) at 6 microsatellite *loci* of the 38 grape cultivars of the study

Cultivars	VVS2	VVMD5	VVMD7	VrZAG47	VrZAG62	VrZAG79
Local wine grape cvs						
Aleatico	135-137	226-228	239-249	157-171	188-198	249-255
Barbarossa	143-153	226-232	239-245	157-163	198-204	237-251
Bombino b. S. Severo	135-147	226-232	239-247	159-167	196-198	251-259
Bombino bianco R7	147-153	228-232	249-253	159-171	192-202	251-259
Bombino nero	135-147	226-228	239-253	159-159	187-201	255-259
Negro amaro	147-153	226-236	249-249	157-159	192-204	259-259
Pagadebiti	145-147	226-232	239-243	163-172	190-190	239-251
Porcinale	135-137	226-234	243-249	159-161	190-202	247-257
Primitivo	135-145	226-236	247-249	157-159	202-206	237-259
Tuccanese	135-135	226-236	239-263	157-163	196-198	245-249
Uva di Troia R1	145-153	226-232	243-253	167-171	190-202	251-251
Verdeca	135-147	232-240	239-239	157-167	190-198	243-251
Local table grape cvs						
Baresana	137-153	234-236	247-253	157-172	198-206	251-257
Corniola	135-153	234-240	233-249	161-172	188-206	251-257
Mennavacca	135-137	226-232	239-249	163-163	188-190	243-251
Moscato di Barletta	151-153	232-232	249-253	157-172	188-202	251-255
Moscato nero	135-137	226-228	239-249	157-172	188-198	249-255
Panse precoce	135-135	226-238	243-249	167-172	188-190	251-257
Prunesta	147-153	232-240	239-251	172-172	194-206	251-257
Regina nera	135-151	232-236	243-247	159-163	190-198	251-251
Uva Sacra	135-145	240-240	239-251	159-161	190-202	247-257
Wide-spread wine grape cvs						
Chardonnay R3	139-145	234-238	239-243	159-167	190-198	243-245
Garganega	135-145	226-232	249-253	157-172	202-202	251-251
Lambrusco Maestri	137-137	228-232	253-257	169-171	194-204	245-245
Malvasia istriana R4	145-147	226-240	239-253	157-157	198-202	243-251
Montepulciano R7	135-147	226-228	249-249	167-171	192-202	251-251
Sangiovese R10	135-135	226-236	239-263	157-163	196-198	243-259
Sauvignon R8	135-153	228-232	239-257	153-167	190-196	245-247
Trebbiano toscano R4	135-145	226-232	249-253	157-161	196-202	245-251
Wide-spread table grape cvs						
Cardinal	137-137	226-236	249-249	157-163	188-188	251-255
Italia	135-151	232-238	243-247	157-171	194-206	255-257
Matilde	135-137	236-238	247-249	157-163	188-194	255-255
Moscato d'Amburgo	137-151	232-238	247-249	157-163	188-194	239-255
Ohanez	135-139	234-236	233-251	161-172	202-206	251-257
Perla di Csaba	135-135	226-236	247-249	159-172	188-206	257-259
Red Globe	137-153	236-238	239-249	159-159	188-190	247-259
Regina	135-137	226-232	239-249	163-163	188-190	243-251
Victoria	137-137	226-236	239-249	163-163	188-190	243-251

this means that in the case of Matilde, *e.g.*, the probability that the second parent is not Cardinal but a relative of Cardinal, is 1.13 *vs.* 1, which is very high. Therefore we ascertained that 6 SSR *loci* are not sufficient for a reliable determination of the parentage.

Conclusions

The set of microsatellite *loci* used in this research proved to be a good tool to determine genetic identity of grapevine cultivars chosen for this experiment: its ability in discriminating cultivars is 4-fold higher than the 15,000 grapevine genotypes in the world. For a parentage analysis, a set of 6 microsatellites may not be sufficient; the number of micro-satellite *loci* to be analysed must be higher.

Table 4

Likelihood ratios of the probability of the suggested parentage of Victoria and Matilde *versus* other possibilities. Probability values were calculated from allele frequencies derived from our sample and from the 95 % upper confidence limits. The calculations are based on the data of 38 cultivars and 6 SSR *loci*

Cultivar	Suggested parents	Cumulative likelihood ratios of the suggested parentage (1) x (2) *versus*				
		X x Y[a,b]	(1) x X[a,c]	(1) x rel (2)[a,d]	(2) x X[a,c]	(2) x rel (1)[a,d]
Victoria	(1) Cardinal	4.40×10^4	4.25×10^2	8.26	2.30×10^2	7.40
	(2) Afuz Ali	(6.30×10^2)	(43.30)	(4.15)	(28.1)	(3.73)
Matilde	(1) Italia	5.60×10^4	1.07×10^3	1.13	4.43×10^2	6.86
	(2) Cardinal	(4.18×10^2)	(1.43×10^2)	(6.58)	(29.6)	(3.19)

[a] Values in parentheses are the cumulative likelihood ratios calculated with the 95 % upper confidence limits for the allele frequencies.
[b] X and Y are random unrelated cultivars.
[c] The identity of one of the suggested parents is assumed and the other parent is unknown.
[d] The identity of one of the suggested parents is assumed and the other parent is close relative to the second suggested parent.

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