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Grapevine microsatellite repeats: Isolation, characterisation and use for genotyping of grape germplasm from Southern Italy

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Summary

New microsatellite repeat markers from *Vitis vinifera* L. are described. Of the designed primer pairs, 7 were able to amplify homologous loci in 5 genetically diverse *Vitis* genotypes. The 7 markers developed in this study and 6 additional ones were used to fingerprint grapevine germplasm from southern Italy. Microsatellite analysis allowed almost all the analysed genotypes to be differentiated and uncovered two mislabeled cultivars (Castiglione and Marsigliana nera). No clonal differences were detected. Genetic relationships between the Italian cultivars were also investigated and a considerable degree of similarity was found.

K e y w o r d s : cultivar identification, DNA, microsatellites, SSR.

Introduction

Winemakers and viticulturists of the Calabria region of southern Italy have an increasing interest in the exploitation of local grapevine varieties. This interest, along with the need for conservation of the genetic variability of Italian varieties, has resulted in a strong interest in grapevine cultivar identification not only by traditional morphological criteria, but also on the basis of DNA analysis. Ampelography is not always a reliable method for identifying *V. vinifera* cultivars (BOWERS *et al*. 1993). The emergence of new PCR-based molecular markers (microsatellites, RAPDs, AFLPs), that are independent of the phenotypic traits used in ampelography, provide an alternative means for grapevine identification. In recent years microsatellite markers (also known as SSRs; simple sequence repeats) have become popular for genetic analysis as they are highly polymorphic, show a co-dominant mode of inheritance, allow simple data interpretation and are amenable to automation (THOMAS and SCOTT 1993; THOMAS *et al.* 1994). All these positive features make microsatellites suitable markers in several applications such as cultivar identification, determination of genetic relatedness, diagnosis for traits of economic value, assessment of genetic diversity contained within a collection (HOKANSON *et al.* 1998) and genetic mapping studies (WEBER 1990). The co-dominant mode of inheritance of microsatellites not only simplifies analysis but also facilitates the transfer of markers between genetic maps derived from different crosses. In spite of the many advantages of microsatellite markers, only a small number is available for many crop species. The situation is different for mammalian maps where it has been reported that 5264 and 7377 microsatellite loci have been used to make high density maps in human (DIB *et al*. 1996) and in mouse (DIETRICH *et al*. 1996), respectively. The process of discovering and characterising informative microsatellite loci is usually both laborious and expensive. Genomic libraries have to be established and screened with a particular repeat motif. The generation of each marker requires sequencing of a particular clone, design and synthesis of a suitable pair of primers and performing a polymerase chain reaction in order to test the putative marker for its polymorphic behavior. However, the introduction of procedures such as automatic sequencing and library enrichment (KIJAS *et al.* 1994, EDWARDS *et al.* 1996) place these markers within the reach of plant geneticists (GUPTA *et al*. 1996).

The research presented here describes the characterisation of new grapevine microsatellite markers and their utilisation for determining the identification and genetic relatedness of 17 cultivars predominantly grown in Calabria and Sicily.

Material and Methods

I s o l a t i o n o f n e w g r a p e v i n e m i c r o s a t e l l i t e m a r k e r s : Forty-four clones from a microsatellite enriched library of *Vitis vinifera* L. cv. Shiraz were grown overnight at 37 °C on Terrific Broth (TB) enriched with 100 $\mu g \cdot ml^{-1}$ ampicillin. Plasmid DNA was isolated using the BRESA-SpinTM Plasmid Mini Kit (BresaGen Pty Ltd) following manufacturer's instructions. The inserts contained in the plasmid were sequenced on an Automated DNA Sequencer model 373A (Applied Biosystems, Inc.).

Oligonucleotides complementary to the flanking regions of the repeats were designed to produce PCR products ranging from 80 to 300 bp in length. The forward primer of each primer pair was labeled with a fluorescent dye using the following Applied Systems fluorochromes: 6-FAM (blue), HEX (yellow), and TET (green). Primers were synthesized by GIBCO Life Technologies.

M i c r o s a t e l l i t e a n a l y s i s . P l a n t m a t e r i a l : Young leaves, not yet fully expanded, were sampled from the collection of the "Istituto Sperimentale per la Viticoltura"

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of Conegliano Veneto and of the University of Reggio Calabria (Italy).

Genomic DNA isolation: Genomic DNA was extracted from leaf material as described in LODHI *et al.* (1994). A phenol-chloroform extraction was then performed and the DNA was precipitated with the addition of 0.5 volumes of 7.5 M ammonium acetate and 0.6 volumes of isopropanol. The DNA pellet was washed with 70 % ethanol, dried in a rotary evaporator and resuspended in 100-200 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

Polymerase Chain Reaction: Prior to testing each primer pair for amplification across a large range of genotypes, experiments were conducted on a selected number of genotypes to optimise the reaction conditions. To test each primer set for successful amplification of target genomic sequence, DNA from three *Vitis vinifera* cultivars (Cabernet Sauvignon, Riesling and Sultana), one *Muscadinia rotundifolia* (*Vitis rotundifolia*) genotype and one rootstock (*Vitis riparia*) were used in a series of PCR experiments.

PCRs were performed in a 25 µl volume containing 25 ng of genomic DNA, 1U of Taq DNA polymerase (GIBCO Life Technologies), 1x buffer (GIBCO Life Technologies), 2.5 mM $MgCl_2$, 0.2 µM of each primer, 200 µM dNTPs. Reactions were carried out in a thermal cycler (Hybaid Omn-E) using the following program: 1 min at 94 °C, 1 min at 61 °C, 1 min at 73 °C for 35 cycles. For primer pairs where the primer with the lowest melting temperature was below 60 °C the PCR tests were carried out at an annealing temperature of 51 °C. Those primer pairs that failed to generate products at 61 °C were retested at 51 °C.

In addition to the 7 SSR markers developed in this present study, three SSR markers were from THOMAS and SCOTT (1993), one from THOMAS *et al.* (1994), one from BOWERS *et al.* (1996) and one previously unpublished is described here for the first time. SSR primer designation, sequences and references are summarized in Tab. 1. For these loci, PCRs were performed in a 10 µl volume containing 50 ng of genomic DNA, 1U of Taq DNA polymerase (GIBCO Life Technologies), 1x buffer (GIBCO Life Technologies), 2 mM $MgCl_2$, 0.125 µM of each primers, 200 µM dNTPs. Reactions were carried out in a thermal cycler (Hybaid Omn-E) using the following program: 3 min at 95 °C, 45 s at 94 °C, 30 s at 50 °C, 1 min at 72 °C for 30 cycles followed by a 7 min elongation step.

Fragment detection: PCR products were diluted 20 times with water. 1-3 µl of the diluted samples were mixed with 0.5 µl of a size standard (GENESCAN-350 TAMRA; Applied Biosystems Inc.) and 1.5 µl of formamide. Prior to loading, samples were denaturated at 96 °C for 2.5 min and immediately placed on ice. The PCR products were separated on a sequencing gel (6 % polyacrylamide, 7 M urea, 1x TBE). Gels were run for 12 h on an automated DNA sequencer (model 373A, Applied Biosystems Inc.). The fluorescently labelled amplified fragments were detected and sized using GENESCAN Analysis 2.1 software supplied by Applied Biosystems Inc.

Genetic diversity and discrimination power: The level of genetic variation present at a particular locus was expressed in terms of gene diversity observed within the study population. Gene diversity was calculated as $1-\Sigma\ p_i^2$ (NEI 1973), where p_i denotes the frequency of allele i in the cultivars. In order to compute gene diversity, genotypes showing only a single amplified allele were assumed to be homozygous for that allele. Since possible heterozygous plants with a null allele are not distinguished from homozygotes, gene diversity values may be an underestimate.

The discrimination power at a locus, which provides an estimate of the probability that two randomly sampled cultivars would be differentiated by their allelic profile, was calculated with the formula $1-\Sigma\ (P_i)^2$, where P_i represents the frequency of each genotype (KLOOSTERMAN *et al.* 1993).

Phylogenetic analysis: Genetic relationships among the 17 cultivars were investigated using the GENEDIST program from the PHYLIP (Phylogeny Inference Package) program package (Version 3.57c) developed by

Table 1

Microsatellite primer designation, sequences and references

Primer*	Sequence (5' to 3')	Reference
VVS1A	ACAATTGGAAACCGCGTGGAG	THOMAS and SCOTT (1993)
VVS1B	CTTCTCAATGATATCTAAAACCATG	
VVS2A	CAGCCCGTAAATGTATCCATC	THOMAS and SCOTT (1993)
VVS2B	AAATTCAAAATTCTAATTCAACTGG	
VVS5A	ATTGATTTATCAAACACCTTCTACAT	THOMAS and SCOTT (1993)
VVS5B	TAGAAAGATGGAAGGAATGGTGAT	
VVS16A	TCAAACTATTATTCAAACCAAAGTAC	this paper
VVS16B	TCGATTTCAACAAATTTAGAAATA	
VVS29A	CCCCAAGGCTCTGAAAACAAT	THOMAS *et al.* (1994)
VVS29B	TGCAAAGCAAATAAAGCTTCCA	
VVMD7A	AGAGTTGCGGAGAACAGGAT	BOWERS *et al.* (1996)
VVMD7B	CGAACCTTCACACGCTTGAT	

* Each primer pair consists of an A and B primer.

JOSEPH FELSENSTEIN. GENEDIST computes genetic distances from gene frequencies using Nei's genetic distance measure (NEI 1972) and this has previously been shown to be suitable for analysis of microsatellite data (TAKEZAKI and NEI 1996). The pairwise distance matrix generated by the program GENEDIST was used to infer a phylogenetic tree using the distance matrix program NEIGHBOR also from the PHYLIP package. Bootstrap analysis was used to determine the significance of the phylogenetic prediction results.

Results and Discussion

New grapevine microsatellite markers were obtained from 44 clones isolated from a library enriched for microsatellite sequences. Attrition rates of the microsatellite clones are shown in Tab. 2 with only a portion of them found to be suitable for primer design and PCR testing. Sequence analysis revealed 13 (29.5 %) contained repetitive arrays with suitable flanking regions for primer design. Eleven (25.0 %) of the sequenced clones were rejected due to the lack of microsatellite inserts in the sequenced region (500 bp from either side). For the remaining 20 (45.5 %), primer pairs flanking the microsatellite motifs could not be designed due to

Table 2

Attrition rate of microsatellite clones

	Clones	Total (%)
Clones sequenced	44	100.0
Clones rejected	31	70.5
too close to end	11	25.0
not containing SSRs	20	45.5
Useful clones	13	29.5
Clones with primers designed	13	29.5
primers that gave PCR products	9	20.5
primers that gave no PCR products	4	9.5

the location of the repeat too close to vector sequence. The grapevine microsatellite repeats were classified as perfect, imperfect and compound according to WEBER (1990). Nine of the microsatellite sequences were perfect with no interruption to the core-motif repeat sequence, three were imperfect, having interruptions within the tandem array and one was compound. All of the identified repeats were dinucleotide with the exception of VMC7h2 which was a trinucleotide. The longest tandem array sequenced was a perfect repeat with a total of 41 units, the smallest of them contained 9 repeat units. The SSR locus names and the description of the repeat motifs are listed in Tab. 3. The number of compound repeats observed (1/13) was in agreement with other studies. In humans 11 % (12/111) of sequenced dinucleotide repeats were compound (WEBER 1990) and lower proportions have been observed within plants. Only 7 % (2/30) of di- and trinucleotide soybean repeats were compound (AKKAYA *et al*. 1995) while none was found during development of 30 *Arabidopsi*s dinucleotide markers (BELL and ECKER 1994).

Primer design and testing: Conversion of sequenced clones containing microsatellite DNA into sequence-tagged site markers involved the design of primers flanking the microsatellite repeats such that the microsatellite repeat and flanking DNA region between the primers could be amplified in a PCR reaction. The primers were designed so that the size of amplification products was kept between 80-300 bp to allow accurate sizing of PCR products. The primers were between 16 and 24 bases in length. Primer sequences and annealing temperatures are shown in Tab. 3.

Nine (69 %) of the primer pairs designed from grape microsatellite sequences produced PCR products while the remaining 4 primer pairs failed to give any amplified products even after repeated attempts using less stringent annealing temperatures (Tab. 2). Primer failure may be caused by differences in the sequenced clone compared to the genomic sequence if the primer amplified the target region from the library clone but not from genomic DNA. This was not the case for two primer pairs (VMC7a3 and VMC7g6) that were unable even to amplify the insert when the original clone was supplied as template. More likely, this result was caused by poor quality sequence in the region where primers were designed. Where PCR products were successfully amplified, the length of observed products were the same as predicted on the basis of the sequence data.

Length polymorphism of microsatellites: Testing of the new markers against different grapevine genotypes found that many PCR fragments were occasionally produced by two SSR markers (VMC7a4 and VMC7f6), as observed by others (LITT 1992). This problem can be due to "polymerase slippage" which can sometimes be minimised by increasing annealing temperatures for a given pair primer or by reducing the number of amplification cycles (LIN and WALKER 1998) or it can be due to amplification of multiple loci. In the case of the markers VNMC7a4 and VMC7f6 this problem persisted even after modification of PCR conditions suggesting that amplification of multiple loci was the problem. This prevented their further use for genetic analysis.

The remaining 7 microsatellite loci were tested to verify their ability to amplify homologous loci in 5 genetically diverse genotypes (*Vitis vinifera:* Cabernet Sauvignon, Riesling, Sultana; *Muscadinia rotundifolia*, and *Vitis riparia*). DNA fragments were amplified from all of the *Vitis* genomes indicating that a high level of sequence conservation is present within the priming sites in each of these genotypes (Tab. 4). Even *M. rotundifolia*, which is considered to belong to a separate genus (SMALL 1903) has sufficient DNA homology at the primer sites to allow efficient amplification of the analysed microsatellite sites. This agrees with results from THOMAS and SCOTT (1993).

No homozygous null alleles were detected among the cultivars at the tested loci (Tabs 4 and 5). This finding is in agreement with THOMAS *et al.* (1994) who observed only three homozygous null genotypes from more than 450 STS assays typed at 7 loci suggesting that a low frequency of null alleles exists in the *V. vinifera* and hybrid cultivar popu-

Table 3

Classification and primer sequences for new microsatellite loci

Locus	Repeated motif	Repetition type	Primer sequences 5'- 3' (A & B primers for each locus)	Amplification	Annealing temp. (°C)
VMC7a3	$(TC)_{41}$	perfect	ATGTTTATTGGGGGAA ACTATAGGGAAGGGTCTC	No	-
VMC7a4	$(CT)_{30}$	perfect	TAAGGTGGATTAGTTTTGGGTC AAACTCCAAACGATCTGATTCT	Yes	51
VMC7b1	$(TC)_{22}$	perfect	CACGCAATCTCTCATTTCACAAA TGGTTTAGGTGACCCAACCTTTA	Yes	61
VMC7b2	$(TC)_{19}$	perfect	CTTGAATAACAGTCGAAAGTCC TCAAAGTTTTAAATCAACCCAC	No	-
VMC7c3	$(TC)_{15}CC(TC)_{8}$	imperfect	CTTTGGAGAGTTTCCAGAGGTA ACTGCTCTAACAGTCCTTTGCT	Yes	61
VMC7f1	$(TC)_{13}$	perfect	TAACTCAACCTCTTTTTGCTGC GTATTGCTAACAAAGTCAGACCAC	No	-
VMC7f2	$(CT)_{9}$	perfect	AAGAAAGTTTGCAGTTTATGGTG AAGATGACAATAGCGAGAGAGAA	Yes	61
VMC7f6	$(GA)_{14}$	perfect	ATTGCTTCCAAAAAGAGA ACCCAAACCCAAATAGAT	Yes	51
VMC7g3	$(CT)_{3}AGA(TC)_{16}$	compound	TTACTAGTGCTGTCCTGCTCCA TGCTTCCTCTCTTTCAACTTTCA	Yes	61
VMC7g5	$(CT)_{20}$	perfect	TGCTTCCTCTCTTTCAACTTTCA CCCACCTTCATCTCACAGATTCA	Yes	61
VMC7g6	$(CT)_{12}CCC(CT)_{8}$	imperfect	TTTCAGGGTTGTTGTCCCATTG CATGCCTCGCTAACGAAGT	No	-
VMC7h2	$(TCT)_{11}$	perfect	CCACCAATACATACAGAGGGAC GTCCAATCAGGACCGATGAGAA	Yes	61
VMC7h3	$(GA)_{3}AAA(GA)_{26}$	imperfect	TCAGATATTGAAGAACACCACA ACTAGAAAATGCACAATCTCCC	Yes	61

Table 4

Allele sizes measured at the 7 new microsatellite loci

Locus	Cabernet Sauvignon		*Muscadinia rotundifolia*		Riesling		Sultana		*Vitis riparia*	
VMC7b1	215	239	220	244	234	244	217	244	215	-
VMC7c3	92	-	103	108	92	-	92	102	92	-
VMC7f2	200	202	195	197	202	206	200	202	200	202
VMC7g3	120	136	118	124	120	142	120	126	120	124
VMC7g5	171	184	180	190	150	184	165	-	165	169
VMC7h2	121	133	121	-	133	-	121	-	121	130
VMC7h3	136	-	125	136	134	-	136	-	125	136

lation. Likewise, no null alleles were observed by LIN and WALKER (1998) in their study of 58 rootstocks. Allele sizes measured at each locus are presented in Tab. 4. The longest fragment observed was 244 bp while the shortest fragment amplified from any of the genotypes was 92 bp. The most polymorphic marker was VCM7g5 with 7 different length alleles, while loci VCM7h2 and VMC7h3 only had three different length alleles present across all the genotypes tested.

In the present study, sizing of the PCR products was done by real-time laser scanning with an automated DNA sequencing device using GENESCAN software. Application of the semi-automated GENESCAN system makes the analysis of microsatellite markers fast and reproducible between laboratories (BOTTA *et al*. 1995) and an internal size standard makes this detection system highly accurate.

Cultivar identification: The high degree of

Table 5

DNA profiles of 17 Italian cultivars analysed at 13 microsatellite loci

Cultivar	VVS1		VVS2		VVS5		VVS16		VVS29		VVMD7		VMC7b1		VMC7c3		VMC7f2		VMC7g3		VMC7g5		VMC7h2		VMC7h3	
Mantonico pizzutella	182	-	134	153	118	-	246	287	172	-	246	248	214	244	104	-	200	206	119	-	165	-	121	130	124	136
Mantonico vera	182	-	134	153	118	-	246	287	172	-	246	248	214	244	104	-	200	206	119	-	165	-	121	130	124	136
Castiglione	182	189	134	145	111	118	287	-	171	-	246	262	214	244	92	103	202	206	119	133	169	187	121	124	124	136
Marsigliana nera	182	189	134	145	111	118	287	-	171	-	246	262	214	244	92	103	202	206	119	133	169	187	121	124	124	136
Gaglioppo	182	189	134	153	89	97	287	-	171	-	238	262	216	244	92	103	206	-	119	141	169	-	121	130	124	134
Guardavalle	182	191	147	153	116	124	287	-	171	-	238	252	216	-	128	130	202	-	119	-	169	-	121	-	134	147
Calabrese	189	-	145	153	97	-	266	284	171	181	246	262	238	244	92	103	206	-	119	131	157	165	124	135	134	140
Magliocco canino	182	189	134	-	89	114	287	-	171	-	238	-	214	244	92	103	200	202	119	133	165	-	124	130	124	136
Nerello mascalese	182	191	134	145	89	97	281	-	171	-	238	248	216	244	92	94	200	202	119	141	169	187	121	130	124	134
Pecorello	182	189	134	153	118	-	266	287	171	179	248	262	214	244	92	103	202	-	119	-	165	169	121	-	124	-
Perricone	165	182	134	-	118	-	287	-	171	-	238	-	228	246	92	103	202	-	119	129	165	-	130	-	124	136
Moscatello nero	166	182	134	-	116	-	287	-	171	-	238	248	234	-	103	-	200	202	119	131	165	169	130	-	134	136
Moscatello bianco	182	-	134	-	111	-	253	287	171	-	232	248	234	-	104	-	200	-	119	-	165	-	130	-	136	162
Greco nero	182	-	134	145	118	-	229	266	171	-	248	-	244	-	92	103	202	206	119	-	165	169	121	-	124	134
Greco bianco	182	189	134	145	97	111	287	-	171	-	238	248	226	244	104	-	200	206	119	-	165	187	121	130	134	140
Greco bianco di B.	181	191	145	-	89	-	275	284	171	-	242	248	214	244	92	94	200	202	119	-	165	169	124	130	134	-
Zibibbo	167	182	134	152	85	89	287	293	171	-	248	250	214	234	104	-	200	208	119	-	165	-	121	130	134	136

polymorphism detected by microsatellite markers provides a very efficient system for cultivar identification. Seventeen grape cultivars grown in Calabria and Sicily were analysed at 13 microsatellite loci in order to determine genetic identities, estimate genetic diversity and identify genetic relationships. All of the cultivars analysed could be discriminated unambiguously from the others with the exception of two pairs; Mantonico pizzutella and Mantonico vera, and Castiglione and Marsigliana nera which had identical alleles across all tested loci. Tab. 5 shows the allele lengths observed at the microsatellite loci investigated. Mantonico vera and Mantonico pizzutella are considered by ROCCO ZAPPIA (pers. comm.) to be clones of cv. Mantonico and the results of the DNA analysis support this hypothesis. The inability to detect genetic differences between clones of the same cultivar by microsatellite markers has also been reported by THOMAS *et al.* (1994) and BOTTA *et al*. (1995). Regarding Castiglione and Marsigliana nera, ampelographic descriptions (ANONYMOUS 1988; ANTONACCI and PLACCO 1993) indicate that they are different cultivars, but since they were genetically identical at 13 loci it appears that the analysed samples belong to the same cultivar. An error may have occurred at some stage in the past during propagation, planting or documentation. In order to ascertain which of the cultivars the molecular profile belongs to, young leaves of Castiglione sampled from the collection of the University of Reggio Calabria were analysed. The alleles observed at each locus were identical to the ones previously found, which suggests that the molecular genotype is representative of Castiglione. Zibibbo is considered a synonym of Muscat of Alexandria (BOTTA *et al.* 1999) however the DNA profile does not match that of the DNA profile of Muscat of Alexandria from the CSIRO grapevine DNA database (data not presented). Further research is required to determine the origin of this Zibibbo clone.

Many grapevine cultivars are difficult to differentiate on the basis of phenotypic characters, and difficulties in identification are compounded by extensive synonymy (MULLINS *et al*. 1992). MAZZEI and ZAPPALÀ (1964) reported that in Nicastro, Sambiase and S. Eufemia Lametia the cv. Greco nero is known with the names of Marcigliana or Marsigliana. However, ANTONACCI and PLACCO (1997) reported that these two cultivars, or vitigni, are erroneously considered synonymous and are often confused in the vineyards at the tirrenic coast in the Catanzaro province. Incorrect identification of grapevine cultivars and mixed plantings are not uncommon in viticulture and a germplasm study of *Vitis* cultivars and species held at the Plant Genetic Resources Unit of USDA-ARS (Cornell University) detected wrongly identified grape species (*V. vulpina* and *V. cinerea*) using SSRs (LAMBOY and ALPHA 1998). Microsatellite markers appear to be useful tools for genetic resources management since they allow accurate identification of germplasm and enable the discovery and discarding of duplicates. Ideally, germplasm collections should exhibit minimal redundancy with regard to genotypes to be most efficiently managed and utilized. Maintaining long-lived woody perennials such as *Malus* is estimated to cost US$ 50-75 per accession per year, so identifying and eliminating redundancy in such collections is a priority (FORSLINE and WAY 1993). A similar priority exists for grapevine germplasm collections, however because of clonal differences within cultivars, DNA analysis should not be the only criterion for discarding plant material.

All the cultivars typed had two alleles or one allele. No homozygous null alleles were detected. The number of alleles detected per locus ranged from 4 for VVS29, VMC7f2, VMC7g5 and VMC7h2, to 9 for VVS16, with a mean value of 6 per locus (Tab. 6). This value is low when compared to the grape SSR literature with reported values of 8.4, 17.2 and 27.6 alleles per locus (THOMAS and SCOTT 1993; LAMBOY and ALPHA 1998; LIN and WALKER 1998). This discrepancy can be explained by the smaller number of genotypes analysed in our study that reduced the probability of finding a higher allelic diversity as well as their closer genetic relatedness (see below).

Genetic diversity and phylogenetic relatedness: The genetic diversity within the popula-

Table 6

Genetic diversity and discrimination power of microsatellite loci

Locus	Allele number	Range size	Observed alleles									Genetic diversity	Discrimination power
VVS1	7	165 -191	165	166	167	181	182	189	191			0.57	0.80
VVS2	6	134 -157	134	145	147	152	153	157				0.60	0.75
VVS5	8	89 -124	85	89	97	111	114	116	118	124		0.52	0.88
VVS16	9	229 -293	229	246	253	266	275	281	284	287	293	0.53	0.74
VVS29	4	171 -181	171	172	179	181						0.31	0.40
VVMD7	8	232 -262	232	238	242	246	248	250	252	262		0.79	0.90
VMC7b1	8	214 -246	214	216	226	228	234	238	244	246		0.77	0.80
VMC7c3	6	94 -104	92	94	103	104	128	130				0.76	0.68
VMC7f2	4	200 -208	200	202	206	208						0.69	0.83
VMC7g3	5	119 -141	119	129	131	133	141					0.40	0.66
VMC7g5	4	157 -187	157	165	169	187						0.65	0.82
VMC7h2	4	121 -135	121	124	130	135						0.67	0.78
VMC7h3	6	124 -162	124	134	136	140	147	162				0.77	0.81

tion samples ranged from 0.31 at locus VVS29 to 0.79 at locus VVMD7 (Tab. 6). Sixty-five percent of the tested cultivars could be distinguished using the single marker VVMD7. The discrimination power at individual loci in the sample ranged from 0.4 at VVS29 to 0.9 at VVMD7 (Tab. 6). Assessment of genetic diversity is very important in plant breeding as only those loci for which the parents carry different alleles contribute to the genetic variance of the cross. If breeders could predict quantitative genetic parameters such as heterosis or progeny variance, the efficiency of breeding programs could be increased by concentrating the efforts on the most promising crosses (Bohn *et al.* 1999). Bowers *et al.* (1999) have suggested that heterosis may have played a significant role in the emergence of successful wine grapes like Chardonnay and Cabernet Sauvignon. Gouais blanc and Pinot, the putative parents of Chardonnay, are genetically dissimilar, sharing only 20 out of 64 alleles at 32 loci studied. Likewise the parents of Cabernet Sauvignon (Cabernet Franc and Sauvignon blanc) are also genetically dissimilar, sharing only 12 of 56 alleles at 28 loci.

The results of the phylogenetic analysis are reported in the Figure. Bootstrap values at a branching point denote the number of bootstrap trees comprising a cluster of the same composition. The higher values indicate the most reliable branches of the tree. The cultivars analysed in this study displayed a considerable degree of similarity. In fact the percentage of shared alleles between Zibibbo and Gaglioppo, that are localized at the edges of the phylogenetic tree, is 35 %. Unfortunately, the lack of information about the pedigree of these cultivars makes it hard to give an accurate interpretation of the phenogram. Although names may suggest a common origin, the cultivars Greco nero, and Greco bianco di Bianco belong to a different cluster to the cultivar Greco bianco. It is important to underline that the results of the phylogenetic analysis cannot be used to draw conclusions with regard to the degree of kinship between the cultivars since clusters illustrate similarity rather than kinship. Nevertheless, Sefc *et al.* (1998) found that in several cases offsprings are grouped close to one of their parents. DNA sequence analysis of microsatellite loci and of their flanking regions could provide further and more accurate information about phylogenetic affinities among species or between them. Recent reports have shown unexpected complexities in the mutational process of microsatellites. Allelic difference can involve not only the number of SSRs, but also different kinds of "interruptions" within a tandem-repeat array (Urquhart *et al.* 1993; Garza *et al.* 1995; Angers and Bernatchez 1997) as well as nucleotide substitutions and insertion/deletions in regions flanking the repeat motifs (Garza *et al.* 1995; Grimaldi and Crouau-Roy 1997). Moreover alleles belonging to the same size class can include alleles identical by descent (homology) and alleles that have achieved the same length via convergent evolutionary events (homoplasy) (Estoup *et al.* 1995). This indicates that microsatellite alleles of identical length can be members of different genealogical clades. Caution should therefore be exercised if investigating relatedness solely on the basis of allele length.

Conclusions

This study has dealt with the identification and characterization of 7 new grapevine microsatellite STS markers and confirmed the informativeness of the marker type within the *Vitis* genus. The present research has allowed us to assign a unique genetic DNA profile to nearly all the Calabrian cultivars analysed and to reveal the genetic relationship between them. Moreover this study has dealt with the uncovering of two mislabeled cultivars. The results of the genealogical analysis revealed interesting genetic similarities between the analysed cultivars and raise new questions concerning their origin. However, the phenogram cannot be used to draw definite conclusions about the level of kinship

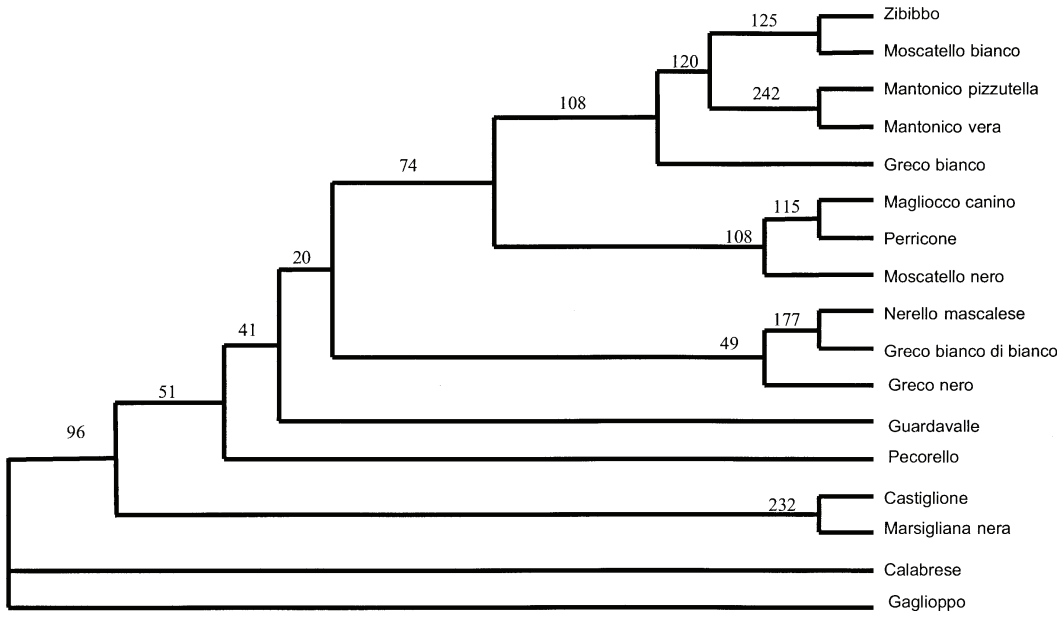

Figure: A Neighbor-joining tree of 17 Italian genotypes. Numbers at nodes indicate the number of times the grouping occurred from 250 bootstrap replication.

between them. A deeper analysis of the nucleotide sequences of the microsatellite loci and of their flanking regions and a better knowledge of their evolutionary processes appear necessary to achieve more information about the origins and relatedness of the analysed cultivars.

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References

AKKAYA, M. S.; SHOEMAKER, R. C.; SPECHT, J. E.; BHAGWAT, A. A.; CREGAN, P. B.; 1995: Integration of simple sequence repeat DNA markers into a soybean linkage map. Crop Science **35**, 1439-1445.

ANGERS, B.; BERNATCHEZ, L.; 1997: Complex evolution of a salmonid microsatellite locus and its consequences in inferring allelic divergence from size information. Mol. Biol. Evol. **14**, 230-238.

ANONYMOUS; 1988: Catalogo Nazionale delle Varietà di Viti. Ed.: Min. Agric. For., Ist. Sperim. Vitic., Conegliano, Italia.

ANTONACCI, D.; PLACCO, L.; 1993: Marsigliana. Vignevini **9**, 57-61.

- -; - -; 1997: Vitigni di qualità di antica coltivazione nell'Italia meridionale: i vitigni "Greci" nella viticoltura calabrese. Vignevini (7/8), 3-8.

BELL, C. J.; ECKER, J. R.; 1994: Assignement of 30 microsatellite loci to the linkage map of *Arabidopsis*. Genomics **19**, 137-144.

BOHN, M.; UTZ, H. F.; MELCHINGER, A. E.; 1999: Genetic similarities among winter wheat cultivars determined on the basis of RFLPs, AFLPs, and SSRs and their use for predicting progeny variance. Crop Science **39**, 228-237.

BOTTA, R.; AKKAK, A.; SCHNEIDER, A.; 1999: Caratterizzazione di Moscati in collezione a Canelli mediante marcatori molecolari: Analisi di sinonimie, omonimie e relazioni di parentela. Quad. Vitic. Enol. Univ. Torino **23**, 33-44.

- -; 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.; BANDAMAN, E. B.; MEREDITH, C. P; 1993: DNA fingerprinting characterization of some wine grape cultivars. Am. J. Enol. Vitic. **44**, 266-174.

- -; BOURSIQUOT, J. M.; THIS, P.; CHU, K.; JOHANSSON, H.; MEREDITH, C. P.; 1999: Historical genetics: The parentage of Chardonnay, Gamay, and other wine grapes of northeastern France. Science **285**, 1562-1565.

- -; 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.

DIB, C.; FAURE, S.; FIZAMES, C.; SAMSON, D.; DROUOT, N.; VIGNAL, A.; MILLASSEAU, P.; MARC, S.; HAZAN, J.; SEBOUN, E.; LATHROP, M.; GYAPAY, G.; MORISSETTE, J.; WEISSENBACH, J.; 1996: A comprehensive genetic map of the human genome based on 5,264 microsatellites. Nature **380**, 152-154.

DIETRICH, W. F.; MILLER, J.; STEEN, R.; MERCHANT, M. A.; DAMRON-BOLES, D.; HUSAIN, Z.; DREDGE, R.; DALY, M. J.; INGALLS, K. A.; O'CONNOR, T. J.; EVANS, C. A.; DEANGELIS, M. M.; LEVINSON, D. M.; KRUGLYAK, L.; GOODMAN, N.; COPELAND, N. G.; JENKINS, N. A.; HAWKINS, T. L.; STEIN, L.; PAGE, D. C.; LANDER, E. S.; 1996: A comprehensive genetic map of the mouse genome. Nature **380,** 149-152.

EDWARDS, K. J.; BARKER, J. H. A.; DALY, A.; JONES, C.; KARP, A.; 1996: Microsatellite libraries enriched for several microsatellite sequences in plants. BioTechniques **18**, 21-24.

ESTOUP, A.; TAILLIEZ, C.; CORNUET, J. M.; SOLIGNAC, M.; 1995: Size homoplasy and mutational processes of interrupted microsatellites in two bee species, *Apis mellifera* and *Bombus terrestris* (Apidae). Mol. Biol. Evol. **12**, 1074-1084.

FORSLINE, P. L.; WAY, R. D.; 1993: Apple accessions of low priority targeted for removal from the National Plant Germplasm System. Fruit Var. J. **47**, 204-214.

GARZA, J. C.; SLATKIN, M.; FREIMER, N. B.; 1995: Microsatellite allele frequencies in humans and chimpanzees, with implications for constraints on allele size. Mol. Biol. Evol. **12**,594-603.

GRIMALDI, M. C.; CROUAU-ROY, B.; 1997: Microsatellite allelic homoplasy due to variable flanking sequences. J. Mol. Evol. **44**, 336-340.

GUPTA, P. K.; BALYAN, I. S.; SHARMA, P. C.; RAMESH, B.; 1996: Microsatellites in plants, a new class of molecular markers. Curr. Sci. **70**, 45-54.

HOKANSON, S. C.; SZEWC-MCFADDEN, A. K.; LAMBOY, W. F.; MCFERSON, J. R.; 1998: Microsatellite (SSR) markers reveal genetic identities, genetic diversity and relationships in a *Malus* x *domestica* Borkh. core subset collection. Theor. Appl. Genet. **97**, 671-683.

KIJAS, J. M. H.; FOWLER, J. C. S.; GARBETT, C. A.; THOMAS, M. R.; 1994: Enrichment of microsatellites from the citrus genome using biotinylated oligonucleotide sequences bound to streptavidin coated magnetic particles. Biotechniques **16**, 656-662.

KLOOSTERMAN, A. D.; BUDOWLE, B.; DASELAAR, P.; 1993: PCR-amplification and detection of the human D1S80 VNTR locus. Int. J. Leg. Med. **105**, 257-264.

LAMBOY, W. F.; ALPHA, C. G.; 1998: Using simple sequence repeats (SSRs) for DNA fingerprinting germplasm accessions of grape (*Vitis* L.) species. J. Am. Soc. Hort. Sci. **123**, 182-188.

LIN, H.; WALKER, A.; 1998: Identifying grape rootstocks with Simple Sequence Repeat (SSR) DNA markers. Am. J. Enol. Vitic. **49**, 403-407.

LITT M.; 1992: PCR of TG microsatellites. In: M. J. MCPHERSON, P. QUIRKE, G. R. TAYLOR (Eds.): PCR, A Practical Approach, 85-99. Oxford University Press.

LODHI, M. A.; YE, G. N.; WEEDEN, N. F.; REISCH, B. I.; 1994: A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. Plant Mol. Biol. Reptr. **12**, 6-13.

MAZZEI, A.; ZAPPALÀ, A.; 1964: Greco Nero. Ministero Agricoltura e Foreste - Principali Vitigni da Vino Coltivati in Italia. Vol. III 23.

MULLINS, M. G.; BOUQUET, A.; WILLIAMS, L. E.; 1992: Biology of the Grapevine. Cambridge Univ. Press, Cambridge, UK.

NEI M.; 1972: Genetic distances between populations. Am. Nat. **106**, 283-292.

- -; 1973: Analysis of gene diversity in subdivided populations. Proc. Nat. Acad. Sci. USA **70**, 3321-3323.

SEFC, K. M.; REGNER, F.; GLOSSL, J.; STEINKELLNER, H.; 1998: Genotyping of grapevine and rootstock cultivars using microsatellite markers. Vitis **37**, 15-20.

SMALL, J. K.; 1903: Flora of the Southeastern United States. New York.

TAKEZAKI, N.; NEI, M.; 1996: Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. Genetics **144**, 389-399.

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

- -; SCOTT, N. S.; 1993: Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs). Theor. Appl. Genet. **86**, 985-990.

URQUHART, A.; KIMPTON, C. P.; GILL, P.; 1993: Sequence variability of the tetranucleotide repeat of the human beta-actin related pseudogene H-beta-Ac-psi-2 (ACTBP2) locus. Hum. Genet. **92**, 637-638.

VIGNANI, R.; BOWERS, J. E.; MEREDITH, C. P.; 1996: Microsatellite DNA polymorphism analysis of clones of *Vitis vinifera* "Sangiovese". Sci. Hortic. **65**, 163-169.

WEBER, J. L. 1990: Informativeness of human (dC-DA)n. (dG-dT)n polymorphism. Genomics **7**, 524-530.

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