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ORIGINAL PAPER

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Evidence on the evolution of polymorphism of microsatellite markers in varieties of *Vitis vinifera* L.

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Abstract This paper contributes a description of four mutations in microsatellite allele-length found within strains of each of the varieties known as Muscat d'Alsace, Greco di Tufo, Primitivo and Corvina veronese: the comparison of microsatellite sequences of wild and mutated strains showed in all cases an increase of one or more repeats of the core GA sequence. Whereas studies on animals reported mutations which originated in gametes, in grapevines somatic cell-mutations take place and are eventually fixed and transmitted to new individuals through vegetative propagation. The mutations detected in 1998 were found again 3 years later in seven out of eight plants. A chimeric situation was clearly noted and resolved in three of the four cases described. Considerations about the genesis of microsatellite alleles and the reliability of fingerprinting with these markers are provided.

Introduction

Grapevine clones have been propagated agamically for centuries, to the point that ancient genotypes are recognisable in varieties still cultivated like the Muscats, Malvasias and Pinots. Vegetative propagation allows the transmission of unvaried plant characteristics to the material propagated. It is also known that, the older a vine variety is, the greater is the probability of finding different individual plants. When such differences have a genetic basis, clonal selection becomes an effective tool in grapevine improvement.

In recent years, microsatellite markers have been largely used for grapevine identification, because they are supposed to provide unique molecular-fingerprinting patterns, typical of each vine variety (Thomas and Scott 1993; Cipriani et al. 1994). Within a single variety, other tools, such as AFLP markers (Cervera et al. 1998), provide a better resolution. Recently intra-varietal differences have also been described using microsatellites. For example, Regner et al. (2000), working on a set of clones of White Riesling, reported differences in the presence/absence of some alleles at particular microsatellite loci.

This paper contributes a description of several cases of mutation in microsatellite allele-length found within strains of each of the varieties known as Muscat d'Alsace, Greco di Tufo, Primitivo and Corvina veronese. More than one accession of each cultivar was available and this allowed the comparison of the prevailing microsatellite allele with its mutated version. Prevailing and variant alleles were sequenced to assess the nature of the mutation. The stability of the mutated allele over time was also verified, where possible, considering original and mutated stock plants.

Materials and methods

Plant material

Plant materials were from the Spresiano (Treviso, Italy) and Susegana (Treviso, Italy) collections of the Istituto Sperimentale per la Viticoltura. A sample of Muscat d'Alsace blanc (the synonym of Moscato bianco or Muscat blanc à petits grains, Crespan and Milani 2001) was provided by Dr. M. Stefanini of the Istituto Agrario di S. Michele all'Adige (Trento, Italy) and one of Muscat d'Alsace rosé by Dr. J. M. Boursiquot of INRA (Montpellier, France). The number of accessions analysed were four for Muscat d'Alsace, six for Greco di Tufo, five for Primitivo and three for Corvina veronese (Table 1). The identification of prevailing (non-mutated) microsatellite alleles at the loci studied (see later) was carried out on a number of grapevine varieties, in part reported by Crespan and Milani (2001) and summarised in Table 1.

DNA analysis

DNA was extracted from young leaflets according to Dellaporta et al. (1983) as modified by Crespan et al. (1999). The PCR reaction-mixture (25 μl final volume) contained 20 ng of total DNA, 0.5 U

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Table 1 Microsatellite data at 16 loci for Greco di Tufo and Primitivo and probability of identical genotypes (PI)

Microsatellite loci	Number of tested cultivars	Size (bp) of alleles recorded for				PI
		Greco di Tufo		Primitivo		
VVS1	144	190	188	190	188	0.264582
VVS2	295	155	133	143	133	0.083278
VVS29	65	171	171	179	171	0.38526
VVMD5	295	232	228	236	226	0.071937
VVMD7	295	249	247	249	247	0.10495
VVMD8	65	141	141	157	141	0.215988
VVMD27	280	189	185	181	179	0.091482
VVMD28	134	239	231	261	251	0.058755
VVMD32	66	253	253	265	257	0.087616
VRZAG 21	223	202	190	206	200	0.136759
VRZAG 62	292	203	201	203	199	0.088159
VRZAG 64	217	143	137	163	159	0.097153
VRZAG 79	287	250	242	258	236	0.077163
ISV2	110	165	157	165	141	0.08998
ISV3	83	145	133	139	139	0.334381
ISV4	93	177	169	177	177	0.155552
						Cumulated over all loci 2.76×10^{-15}

of *Taq* DNA polymerase with the relative reaction buffer (Polymed, Florence, Italy), 1.5 mM of $MgCl_2$, 200 μM of each dNTP and 20 pmol of each primer. The PCR was performed in a PTC-100 thermal cycler (MJ Research, Mass., USA) with a two-step protocol (Sefc et al. 1997): 5 min at 95°C, 10 cycles of 50°C for 15 s and 94°C for 15 s, followed by 23 cycles of 50°C for 15 s and 89°C for 15 s, and a final step of 8°C for at least 10 min to stop the reaction. Five microlitres of the PCR product were tested on 2% agarose gel. On the basis of signal intensity, 1–2 μl of amplified DNA were used for electrophoresis. Samples were denatured at 94°C for 3 min in a buffer containing formamide and loaded on to a sequencing gel (5% polyacrylamide, 1×TBE, urea 7 M). Amplification products of cultivars with alleles of known molecular size were used as references for allele sizing. Bands were revealed by silver staining, dipping the glass plate with the gel into various solutions as follows: 20 min in 10% acetic acid; four rinses of 5 min each with distilled water; 20 min in 0.2% $AgNO_3$; a brief rinse of 10 s with distilled water, and finally 5–10 min in 3% NaOH and 0.05% formaldehyde, until bands appeared.

The polymorphic loci considered in this study were VVMD32 (Bowers et al. 1999), VVS2 (Thomas and Scott 1993), VVMD7 (Bowers et al. 1996) and ISV8 (microsatellite locus VMC 6e4, obtained by the Istituto Sperimentale per la Viticoltura as a member of the Vitis Microsatellite Consortium).

When mutations in amplification product-length were found, the analysis was repeated: (1) on the same DNA sample, (2) on a new DNA sample extracted from the mutated strain, and (3) where possible, on DNA extracted from other plants obtained through vegetative propagation from the original accession. As Table 1 shows, controls were carried out in different years.

Fragment sequencing

Fragments were isolated from the polyacrylamide gel after silver staining, and the band was cut with a scalpel and dipped in 10 μl of TE overnight. Two microliters of this solution was used to re-amplify the sample, and PCR products were sequenced with a multicapillary sequencer (Applied Biosystem 3100 or 3700), following standard procedures.

Statistical analysis of microsatellite data

The freeware program "Identity 1.0" by Wagner and Sefc (1999) (http://www.boku.ac.at/zag/forsch/MANUAL.rtf) was used to calculate the probability of identity (PI) as indicated by Paetkau et al. (1995). PI measures the probability for identical genotypes at a single locus, and cumulated over the considered loci, by multiplication, shows the probability that two different varieties share the same genotype by chance. PI was computed by elaborating microsatellite data available at the Istituto Sperimentale per la Viticoltura for a variable number of different varieties, depending on the locus (Table 1).

Results

Identification of mutated clones

Molecular fingerprinting supports the assignment of the clones under study to the varieties Moscato bianco, Greco di Tufo, Primitivo and Corvina veronese. All plants described below showing mutations in amplified product length were fingerprinted with at least 16 highly polymorphic microsatellite loci for a precise molecular identification, and revealed a pattern typical of the variety to which they belong. Molecular data of Muscat d'Alsace and Corvina veronese were published in Crespan and Milani (2001); those of Greco di Tufo and Primitivo are reported in Table 1.

The statistical elaboration of microsatellite data with the Identity 1.0 software (Wagner and Sefc 1999) clearly shows that the probability of identity over all the 16 loci listed in Table 1, i.e. the probability that two different varieties share the same pattern by chance, is very low (2.76×10^{-15}).

Mutations and their stability

Table 2 lists, case by case in bold-type, the length of microsatellite mutated alleles.

Four accessions of Muscat d'Alsace (blanc, rouge, rosé) were analysed: two were from the Istituto Sperimentale per la Viticoltura collections and the others were supplied by the Istituto Agrario of S. Michele all'Adige

Table 2 Accessions studied, their origin and allelic state at selected microsatellite loci

Accession	Source (and collection)	Year of DNA extraction	Length (bp) of prevailing alleles		Length (bp) of mutated allele	Number of GA repeats[a]
Muscat d'Alsace (blanc)	Colmar, France (Susegana, Italy)	2000	273	265		22
Muscat d'Alsace (rouge)	Colmar, France (Susegana, Italy)	1999	273	265		22
Muscat d'Alsace (blanc)	Istituto Agrario of S.Michele all'Adige (TN) (same Institute)	2000	273	265		22
Muscat d'Alsace (rosé)	INRA Domaine de Vassal (France) (same Institute)	2000	273	265		22
Muscat d'Alsace (blanc)	Colmar, France (Susegana, Italy)	1999, 2000	273		**267**	23
Greco di Tufo	Istituto Tecnico Agrario of Avellino, Italy (Spresiano, Italy)	1998	155	133		24
Greco di Tufo	Melfi, Potenza, Italy (Spresiano, Italy)	1998	155	133		24
Greco di Tufo	Calabria, Italy (Spresiano, Italy)	1998	155	133		24
Greco di Tufo	Az. Mastroberardino, Italy (Spresiano, Italy)	1998	155	133		24
Greco di Tufo	Az. Feudo S. Gregorio, Italy (Spresiano, Italy)	1999	155	133		24
Greco di Tufo	Collection ISV Turi (Spresiano, Italy)					
plant 1		1998		133	**157**	25
		2001	155	133		24
plant 2		1998, 2001		133	**157**	25
plant 3		1998, 2001		133	**157**	25
plant 4		1998, 2001		133	**157**	25
plant 5		1998, 2001		133	**157**	25
Primitivo	Crispiano, Taranto, Italy (Spresiano, Italy)	1998	249	247		16
Primitivo	Copertino, Lecce, Italy (Spresiano, Italy)	1998	249	247		16
Primitivo	Sava, Taranto, Italy (Spresiano, Italy)	1998	249	247		16
Primitivo	Gioia del Colle, Bari, Italy (Spresiano, Italy)	1998	249	247		16
Primitivo	Collection ISV Turi (Spresiano, Italy)					
plant 1		2001	249	247		16
plant 2		2001		247	**251**	17
plant 3		1998, 2001		247	**251**	17
Corvina veronese	S. Floriano (Verona), Italy, clone 7	1999, 2001	142	132		21
Corvina veronese	S. Floriano (Verona), Italy, clone 48	1999, 2001	142	132		21
Corvina veronese	S. Floriano (Verona), Italy, clone 146	1999, 2001		132	**148**	24

[a] The number refers to the mutated allele and to the prevailing allele from which the mutation originated

(Trento, Italy) and by INRA (France). The four accessions turned out to have a similar genetic situation at the VVMD32 locus, where two alleles were always amplified: one of 273 and a second of 265 bp. A mutation was found at this locus in a plant of Muscat d'Alsace blanc, where a change from 265 to 267 bp was observed. In a following year the new 267 bp allele was found again when amplifying the DNA extracted from the same plant.

Six accessions of the variety Greco di Tufo were considered for the genetic state at the microsatellite locus VVS2. Five of them were all identical to each other in showing two alleles of 155 and 133 bp. A new allele of the locus VVS2 was present in the sixth accession of Greco di Tufo obtained from the Turi collection of Istituto Sperimentale per la Viticoltura. The new VVS2 allele was 157 bp long deriving from a mutation of the 155 bp allele. For the Greco di Tufo from Turi, the original material consisted of five plants which were propagated by cuttings. The plants deriving from each original plant were kept separated (plants 1 to 5 in Table 2) and one for each original plant was chosen randomly and tested. The analyses carried out on DNAs extracted in 1998 indicated that the mutant allele was present in all these plants. The analyses repeated in 2001, extracting DNA from the same five plants, confirmed the mutation in four individuals out of five. In this one case the mutated allele was no longer present when newly sampled leaflets were used for DNA extraction (Table 2, plant number 1). To explain this finding, among possible hypotheses back-mutation cannot be excluded, but it seems much likely that in the presence of a chimeric state of this Greco di Tufo accession the wild-type cells were the ones giving rise to the sampled shoot (see discussion).

Table 3 Frequency of mutated alleles detectable at microsatellite loci

Origin of data	No of varieties considered	No of plants controlled	Total no. of microsatellite loci	No of mutated alleles found	Frequency of mutants per plant analysed per marker locus
This paper and data of [a]	37	216	899	31	7.982×10^{-5}
Other data present in literature	4	8	49	4	0,005
Cumulative data	41	224	948	35	8.241×10^{-5}

[a] Riaz et al. 2002, Franks et al. 2002

Five accessions of Primitivo were considered. The first four listed in Table 2 all had alleles of 247 and 249 bp at the microsatellite locus VVMD7. A mutation was found at the same locus in a plant of Primitivo deriving from the accession from Turi (Table 2, plant number 3), which showed an extra allele of 251 bp, while other plants of the same accession had alleles of 247 and 249 bp. The mutation was found again when amplifying the DNA extracted 3 years later. A further plant of Primitivo from Turi, independent from the first one, was found with the mutation (Table 2, plant number 2).

Three accessions of Corvina veronese were considered (clones 7, 48 and 142). Clones 7 and 48 showed at the ISV8 locus the amplification of two alleles of 142 and 132 bp. Clone 142 had a mutation at the same locus with an allele of 148 bp instead of 142 bp. Analyses repeated on the same clone 2 years later confirmed the presence of the mutated allele.

The comparison of microsatellite sequences of wild and mutated strains showed in all cases an increase of one or more repeats of the core GA sequence (Table 2).

Frequency of mutations at the microsatellite loci

A computation of the frequency of mutations at the microsatellite loci in grape is shown in Table 3. Data available until now from present paper, and from literature, were used: 41 cultivars, each one represented by at least two plants, were sampled for a total of 224 individuals; 948 microsatellite loci were analysed and 35 mutations were found, with a frequency of 8.24×10^{-5}.

Chimerism

It is difficult to evaluate a chimeric state at microsatellite loci on silver-stained plates, especially if the differences in length of the amplified products are small, i.e. of the order of 2 bp. In fact, silver staining displays for each allele a set of different fragments produced by PCR amplification resulting in a complex profile. When a plate is to be read, only the bands with the stronger signal are considered for one set. Nevertheless a chimeric situation was clearly noted and resolved in three of the four cases of mutation described in this paper.

In Fig. 1, for example, the polymorphism found in Greco di Tufo at the VVS2 locus (lanes 1 and 2) and in Corvina veronese at the ISV8 locus (lanes 3 and 4) is shown. The comparison of bands allow us to suppose the presence of a third allele of 155 bp in lane 2 (VVS2 mutated plant), having an intensity comparable with that of 157 bp allele. Also in lane 4 (ISV8 mutated plant), even if the signal intensity is not the same, the presence of a third band of 142 bp may be observed with a stronger signal emerging under a set of bands with decreasing intensity.

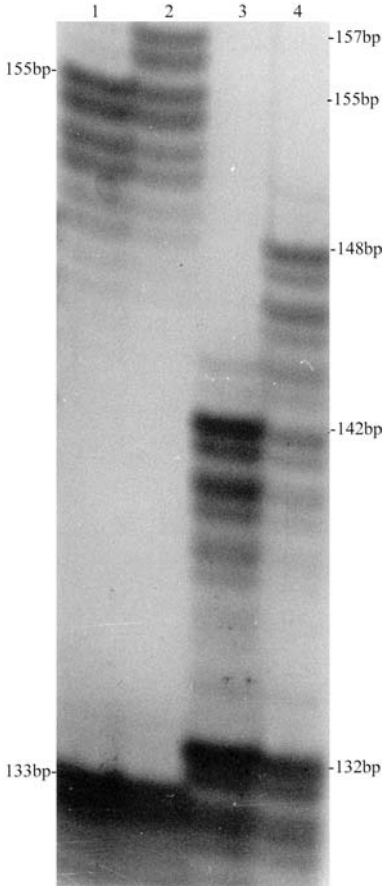

Fig. 1 Microsatellite polymorphism. Greco di Tufo at the VVS2 locus: *lane 1*, predominant alleles of 155 and 133 bp; *lane 2*, mutated plant with three alleles of 157, 155 and 133 bp; Corvina veronese with polymorphism at the ISV8 locus: *lane 3*, predominant alleles of 142 and 132 bp; *lane 4*, mutated plant with alleles of 148 and 132 bp

Discussion

DNA regions containing microsatellites are considered mutational "hot spots": the high molecular polymorphism noted for these loci is due to differences in the lengths of DNA-amplified alleles deriving essentially from a variable number of microsatellite repeat units. Two mutational mechanisms have been hypothesised to explain how microsatellites mutate: (1) polymerase slippage or slipped strand mispairing, and (2) unequal crossing-over. A number of models for microsatellite evolution have been proposed, models which are useful in interpreting data related to the genetic structure of species and of populations (Valdes et al. 1993; Di Rienzo et al. 1994; Feldman et al. 1997; Goldstein and Pollok 1997; Kruglyak et al. 1998). However, the ongoing gathering of experimental data still shows how complex is the phenomenon of allele generation at microsatellite loci (Li et al. 2002).

The molecular data provided by this paper, together with few other results available for grapevine, represent an experimental contribution to the debate on the evolution of microsatellite polymorphism. Four mutations are reported: three increase the DNA sequence by one repeat, whereas the mutation at the locus ISV8 represents an increase of three repeats. The data of Franks et al. (2002) can be added to our case-study: two of the mutated alleles displayed analogous length increases (from 186 bp to 188 bp in the locus VVS19 for Primitivo di Gioia; and from 121 to 123 bp in the locus VVS5 for Pinot Meunier). For that study, sequencing results confirmed the addition of one repeat unit. Riaz et al. (2002) drew similar conclusions for cases identified by analysing Pinot nero and Chardonnay clones. Modification of the number of repeats is therefore a frequent and ongoing process and, when variations are in the order of 2 to 6 bp, as in the cases described here, it tends to be unidirectional and the length of the original allele increases by the addition of one or more units. Ibañez et al. (2000), working on two accessions of grapevines well-known to be synonyms, Black Currant and Mavri Corinthiaki, verified a difference in length of two nucleotides in one of the alleles of the locus VVMD7. Unfortunately, only two accessions were compared, so it was not possible to check if the mutated allele was the shorter or longer one. The same reasoning applies to the case described by Lefort and Roubelakis-Angelakis (2001), who were able to distinguish Kolokythas lefkos from its synonym Roditis lefkos based on microsatellite locus VRZAG 64, for which alleles of 139 bp, and of 139 and 137 bp, were found, respectively. These authors also distinguished Romeiko mavro from its synonym Tsardana considering the alleles at the locus VVMD7, which were of 236 and 246 bp, and of 236 and 244 bp, respectively.

Mutations similar to those we have described, that is prone to expansion, have been observed in different animal and plant species (Rubinsztein et al. 1995; Primmer et al. 1996, 1998; Ramakrishna et al. 1998; Karhu et al. 2000; Zhu et al. 2000; Neff and Gross 2001). However, whereas studies on animals reported mutations which originated in gametes, in grapevines somatic cell-mutations take place and are eventually fixed and transmitted to new individuals through vegetative propagation.

Mutations of the one-core repeat type appear to be most frequent in *Vitis vinifera* L. The slippage of more than one repeat, even if less common, may also occur as a result of a single event (Primmer et al. 1996, 1998; Di Rienzo et al. 1994): in all probability the Corvina veronese mutation (+6 bp at the locus ISV8) did not pass through intermediate stages represented by alleles of 144 and 146 bp.

As correctly observed by Primmer et al. (1998), it may be an arbitrary choice "to decide" which allele mutated. Nevertheless, for grape it is possible to spot the mutant allele in different ways, either if the mutated state is present with a second or a third allele (the case of chimerism). In the first instance, the mutated plant is compared with a series of other accessions where the lengths of alleles have been determined, and thus it could be verified which allele was substituted by the new one. In the second situation, two cell lines of the mutated plant can be analysed separately. Molecular chimeras present in leaves, which derive from the activity of two distinct meristem ontogenetic layers (L1 and L2), are much less evident when considering stems and roots, which derive mainly from the L2 layer. Moreover, it is possible to separate artificially the two cell lines in vitro, providing later-on evidence on the layer of origin of the mutant allele. In the case of Pinot Meunier harbouring an additional allele of 129 bp at the microsatellite VVS2 locus, together with the alleles of 138 and 153 bp (Franks et al. 2002), it was concluded that the new allele derived from a large deletion of the longer one, since the L2 layer (alleles of 129 and 138 bp) was separated from L1 (alleles of 138 and 153 bp) through in vitro culture.

On the whole, the grapevine data, including those of the present paper, indicate the occurrence of two kinds of mutations: variations in the number of repeat units (referable to the step-wise mutation model), mainly prone to increasing, and large deletions (Riaz et al. 2002); both forms may co-exist in the same variety and at the same locus.

Mutation stability

Mutations at microsatellite loci may take place in both gametes and somatic cells. Probably this type of mutation occurs fairly frequently and may, or not, be included in vegetatively propagated material depending on the state and position of the mutated cell: if the mutation occurs in a meristematic cell, it has the potential to extend to the tissues deriving from that cell, and to remain stable over the years in at least one layer of the same plant and in its clones after propagation. A confirmation is given in the paper by Franks et al. (2002) on Pinot Meunier, which has retained a chimeric mutation at locus VVS19 for

centuries. A chimeric situation can be envisaged, as a matter of fact, to be the starting point for the origin of a new allele (Franks et al. 2002; Hocquigny et al. 2002; Riaz et al. 2002). This chimeric state is reflected in the finding of three instead of two different alleles in the majority of the mutated plants considered (Fig. 1).

In the cases presented in this paper, the mutations detected in 1998 were found again 3 years later in seven out of eight plants, the only one not confirmed being a sample from plant number 1 of Greco di Tufo (Table 2). As the mutation found in Greco di Tufo is the best investigated case, one may suppose from the collected data that the mutation is periclinal, since it was generally conserved in time and after propagation. So, a possible explanation for this particular case may be that: (1) Greco di Tufo from Turi has a chimeric state at the locus VVS2, and (2) the shoot sampled in 2001 has been developed from an adventitious bud generated from cells deriving from a single-cell layer due to layer invasion, and therefore showing the allelic state of only one layer (Spena and Salamini 1995).

Validity of microsatellite genotyping and importance of analysing different accessions of the same variety

These experimental data show that mutation at microsatellite loci occurs in very different vine varieties: therefore, this should be considered a general phenomenon that occurs by chance and is relatively frequent in plant genomes. This means that a reliable genotypic fingerprinting based on microsatellite markers requires the analysis of different accessions for the same cultivar, especially in the case of ancient varieties in which the likelihood that mutations are accumulated and fixed is quite high. When differences are found, the dominant allele should be taken as a reference, considering the divergent one as a mutant form which has not to be considered a proof of varietal contamination. The frequency of mutations at microsatellite sites is affected by some important factors, when considering vegetatively propagated plants, such as grapevine cultivars: obviously, the likelihood to find these mutations is directly proportional to the age of the variety, its spreading and therefore the number of individuals, besides the number of microsatellite loci analysed. As already thoroughly explained by Franks et al. (2002), it will be a reliable historical documentation integrated by a congruent dataset to establish the correct assignment of varieties in cases of discrepancies in molecular data. A simple suggestion is also to approach the use of molecular markers in varietal fingerprinting by choosing polymorphic loci less prone to mutate, as is the case for SNPs or AFLPs which are based on the molecular variability present at a single or at few nucleotide positions.

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