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Molecular markers for establishing distinctness in vegetatively propagated crops: a case study in grapevine

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Abstract Distinctness, uniformity and stability (DUS) testing of varieties is usually required to apply for Plant Breeders' Rights. This exam is currently carried out using morphological traits, where the establishment of distinctness through a minimum distance is the key issue. In this study, the possibility of using microsatellite markers for establishing the minimum distance in a vegetatively propagated crop (grapevine) has been evaluated. A collection of 991 accessions have been studied with nine microsatellite markers and pair-wise compared, and the highest intra-variety distance and the lowest inter-variety distance determined. The collection included 489 different genotypes, and synonyms and sports. Average values for number of alleles per locus (19), Polymorphic Information Content (0.764) and heterozygosities observed (0.773) and expected (0.785) indicated the high level of polymorphism existing in grapevine. The maximum intra-variety variability found was one allele between two accessions of the same variety, of a total of 3,171 pair-wise comparisons. The minimum inter-variety variability found was two alleles between two pairs of varieties, of a total of 119,316 pair-wise comparisons. In base to these results, the minimum distance required to set distinctness in grapevine with the nine microsatellite markers used could be established in two alleles. General rules for the use of the system as a support for establishing distinctness in vegetatively propagated crops are discussed.

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Introduction

Vegetatively propagated crops have in common that their varieties are constituted by individuals that are genetically identical. In this way, each variety can be assimilated to an individual, what simplifies its analysis through molecular markers, in comparison with species where cultivars maintain certain intra-variety variability. In many crops, there is an increasing number of new varieties, produced in breeding centres worldwide. In a global world, the new varieties are quickly spread to other producer countries, where normally they have to be approved for their cultivation. Besides, breeders of these new varieties normally apply for plant breeders' rights (PBR, similar to a patent or to intellectual property rights) to recover their investments, and make their activity profitable. According to the 1991 Act of the International Union for the Protection of New Varieties of Plants (UPOV) Convention (http://www.upov.org/en/publications/conventions/1991/act1991.htm), a candidate variety has to comply with the requirements of novelty, distinctness, uniformity and stability, to be eligible for granting. A variety is considered distinct if it can be clearly distinguished from all the varieties of common knowledge; uniform if the number of off-types for the relevant characteristics does not exceed a threshold value; and stable if it keeps those relevant characteristics after reproduction. In the states that are party of the mentioned Act, these requirements are evaluated in a technical exam called distinctness, uniformity and stability (DUS) test (UPOV 2002). Even though there was no application for PBR, a new cultivar

has to pass a similar test to be approved for cultivation in many countries, i.e. to enter into the Commercial Varieties Registry. In the case of grapevine, PBR applies mainly to table grapes, because much less breeding efforts are being done on wine grapes.

The key issue in DUS testing is the establishment of distinctness. A variety may be considered to be clearly distinguishable if the difference in characteristics is consistent and clear (UPOV 2002). The 'clear distinctness' refers to the minimum distance that should exist between two plant varieties so that they are considered distinct, and provide a framework of protection around the granted variety. At the present time, DUS testing is done statutorily using almost exclusively morphology descriptors defined by UPOV (UPOV 2002). The candidate variety is pair-wise compared with all or a subset of the reference varieties, looking for a morphological 'clear difference'. This system presents two main limitations: morphology comparisons are expensive and time-consuming, and more important, morphology markers are sometimes subjective and/or non-definitive, due to the influence of the environment, and to a limited number of descriptors. Thus, it is important to develop more rapid and cost-effective testing procedures to improve the current testing systems. Nevertheless, the most extended opinion among breeders is that the use of molecular markers alone to establish distinctness would undermine the protection system, because it would dramatically reduce the minimum distance between varieties. Other possibilities under evaluation consider the use of molecular markers only as a complement, or a supplement, for DUS testing (Blouet et al. 2006). Different efforts have been done to assess molecular markers for DUS testing like microsatellites (Gunjaca et al. 2008; Kwon et al. 2005; Singh et al. 2004; Tommasini et al. 2003), AFLPs (De Riek et al. 2001) or both (Noli et al. 2008; Roldan-Ruiz et al. 2001). Nevertheless, at present, there exists no official way to establish the minimum distance using molecular markers.

Grapevine (*Vitis vinifera* L.) is one of the oldest cultures in the world. It is a diploid species ($2n = 38$), whose plants are woody, and its varieties are asexually multiplied through cuttings. A new variety usually arises from a sexual cross, where an embryo is produced, or sometimes from an established variety through different mechanisms, mainly the selection of natural somatic mutants. In this case, the new variety is called an essentially derived variety (EDV). There are thousands of varieties in the world (This et al. 2006) and many of them have been cultured for several centuries. Most are local varieties, and there are numerous synonyms (one variety having different names) and homonyms (different varieties having the same name) within and between countries. Morphological description of grapevine plants (called 'Ampelography') is a very complicated task, what requires skilled personnel. It is not unusual to find the same variety described unlikely by different experts, and so the problem in grapevine DUS testing is not to establish the distinctness mistakenly, rather than conclude wrongly non-distinctness.

Microsatellite markers have been extensively used in grapevine for different purposes: variety identification in collections, pedigree analysis, or genetic mapping (Sefc et al. 2001). As grapevine varieties are vegetatively multiplied, it is not expected to distinguish a variety and its EDVs through microsatellites, but this kind of markers still might play an important role supporting DUS testing. The purpose of this work was to evaluate the utility of a selected set of nine microsatellite markers for determining a minimum distance useful for establishing distinctness in grapevine. For that, a large number of accessions has been studied and pair-wise compared, and the highest intra-variety distance and the lowest inter-variety distance were determined. The procedure followed could be of general use for many vegetatively propagated crops.

Materials and methods

Plant material

Plant material consisted of 991 accessions of grapevine (Table S1, Supplementary material). The vast majority of them come from the collection of grapevine varieties of "El Encín" (BGVCAM). That material included a high number of the most cultivated grapevine varieties in the world, rootstock cultivars, species of non-vinifera *Vitis* genus and genus of *Vitaceae* family.

Microsatellite analysis

DNA extractions were done using Qiagen kits: DNeasy Plant Mini kit or DNeasy 96 Plant Kit. Two different plants per accession were studied, except when only one plant was available. Nine previously described nuclear microsatellite loci were used: VVMD5 (Bowers et al. 1996); VVMD27 and VVMD28 (Bowers et al. 1999); VVS2 (Thomas and Scott 1993); ssrVrZAG29, ssrVrZAG62, ssrVrZAG67, ssrVrZAG83 and ssrVrZAG112 (Sefc et al. 1999). A multiplex PCR with the nine markers was used. One primer of each pair was fluorescently labelled with Dye Phosphoramidites (6-FAM, HEX or TET). The separation of fragments and data analysis was carried out in an ABI PRISM 310, using TAMRA 500 as an internal marker and GeneScan software (Applied Biosystems, Foster City, CA) to size the fragments. PCR, electrophoresis, analysis and allele binning were done according to Ibáñez et al. (2009).

Several samples were amplified with a set of 20 microsatellites (16 different to the previously cited), which are

distributed in the 19 grapevine chromosomes, and have been used in previous works (Le Cunff et al. 2008): VVS2 (Thomas and Scott 1993); VVMD5, VVMD7, VVMD21, VVMD24, VVMD25, VVMD27, VVMD28, VVMD32 (Bowers et al. 1996, 1999); VMC4F3.1 (Di Gaspero et al. 2000); VVIN73, VVIP31, VVIP60, VVIQ52, VVIV37, VVIV67, VVIN16, VVIB01 and VVIH54 (Merdinoglu et al. 2005) and VMC1B11 (Welter et al. 2007). One primer of each pair was fluorescently labelled with Dye Phosphoramidites (6-FAM, HEX, TET, PET, VIC or NET). The separation of fragments and data analysis was carried out in an AB 3130, using LIZ 500 and GeneMapper 3.7 software (Applied Biosystems). The 20 STMS were amplified using two different multiplex PCR with 11 and 9 markers, respectively (Ibáñez et al. 2009).

Observed (H_o) and expected heterozygosity ($H_e = 1 - \sum p_i^2$; where p_i is the frequency of the i-allele), probability of identity ($PI = \sum p_i^4 + \sum [2p_i p_j]^2$; where p_i and p_j are the frequencies of the i- and j-alleles, respectively) and probability of null alleles ($r = [H_e - H_o]/[1 + H_e]$) were calculated using IDENTITY (Wagner and Sefc 1999) and Excel Microsatellite Toolkit (Park 2001).

Establishment of the minimum distance

In this study, a criterion to establish the minimum distance using molecular markers is proposed, consisting in the determination of (1) the highest 'intra-variety' variability, or maximum distance (measured in number of different alleles) between plants of the same variety; along this work, 'intra-variety' refers to all the plants originated from the same sexual embryo, including synonyms and EDVs; and (2) the lowest 'inter-variety' variability, or minimum distance (measured in number of different alleles) between plants of different varieties (excluding EDVs). If there is a clear border between those two values, an acceptable minimum distance using microsatellite markers could be established, with the exception of EDVs. A total of 991 accessions were pair-wise compared using the Excel Microsatellite Toolkit (Park 2001). In the first step, all those accessions that presented the same genotype for the nine microsatellite loci were further studied to determine if they were morphologically identical and/or well-known synonyms or sports. For that, morphology studies previously done in our Institute as well as bibliography were consulted (Branas and Truel 1965; Galet 2000; OIV 1987; Rodríguez Torres 2001). Matching varieties that could not be discarded in this way were analyzed with the set of 20 microsatellites. If there was a complete match, then they were considered the same variety or an EDV. The next steps were to search pairs of accessions with only one non-matching allele and pairs of accessions with two non-matching alleles, from a set of accessions where all the redundant genotypes had been eliminated. In these cases, the set of 20 microsatellites was always used to clarify samples identity. In base to previous studies, the accessions with differences of three or more alleles were directly considered as belonging to different varieties (Ibáñez et al. 2003).

Results

A system of nine microsatellites was used, including a multiplex PCR, a semiautomatic electrophoresis, detection and sizing procedure, as well as a binning method. The system was used to genotype 991 grapevine accessions that finally included 489 different genotypes (see below).

All the possible pair-wise comparisons were done between the 991 grapevine accessions. None full-matching for the nine microsatellites (18 alleles) analyzed was found for 352 accessions, representing thus unique genotypes in the whole collection. The other 639 accessions showed redundant genotypes for the nine microsatellites, i.e. genotypes repeated at least once, and involved 138 different genotypes (Table 1). The more numerous group with the same genotype was formed by 39 accessions, and corresponded to the cultivar 'Tempranillo' (Table 1, first row). In the other edge, there were 63 different genotypes, each represented by only two accessions (Table 1, last row).

All the accessions with redundant genotypes were further studied case by case to determine if the match between coincident accessions was by chance (i.e. different varieties with no differences for the microsatellite genotype), or if it was due to the fact that the two coincident accessions are from the same variety (absence of intra-variety variability). As many as 594 accessions could be classified as the same variety (same genotype and name) or synonym (same genotype, different name) or EDV (same genotype, different morphology) by looking at the literature. For instance, with the same microsatellite genotype than 'Garnacha Tinta' there were 32 accessions (Table 1, second row), including the presumed initial variety 'Garnacha Tinta' (hairless leaves, red berries) as well as several well-known EDVs like 'Garnacha Peluda' (hairy leaves), 'Garnacha Blanca' (white berries), and 'Garnacha Dorada' (golden berries). All these cases where enough information could be found to justify the matching genotypes were accepted as the same cultivar or synonymies or EDVs, and were not studied further. However, there were still doubts about the identity of 45 accessions, corresponding to 19 different genotypes (Table 2), because no information in relation with such matches could be found in the literature. These accessions were analyzed with the set of 20 microsatellites. In all the cases, the accessions that fully matched using the nine microsatellites, also totally matched with the

Table 1 Study of the redundancy in the collection of 991 accessions

Number of accessions per genotype	Number of different genotypes	Number of redundant accessions	Prime name[a]
39	1	38	Tempranillo
33	1	32	Garnacha Tinta
24	1	23	Eva
19	1	18	Palomino Fino
18	1	17	Jaen Blanco
18	1	17	Muscat of Alexandria
15	1	14	Alarije
14	1	13	Pedro Ximenes
13	1	12	Chasselas Blanc
13	1	12	Viura
12	1	11	Bobal
11	1	10	Airén
11	1	10	Muscat à Petits Grains Blancs
10	1	9	Alicante Henri Bouschet
10	1	9	Carignan Noir
8	1	7	Afus Ali
8	1	7	Ahmeur Bou Ahmeur
8	1	7	Alcañón
7	1	6	Monastrell
7	1	6	Pardillo
6	7	35	
5	5	20	
4	19	57	
3	24	48	
2	63	63	
Total			
639	138	501	

All the accessions were genotyped with the set of nine microsatellites and pair-wise compared. The different genotypes represented by at least two accessions are shown ordered by decreasing redundancy. In the upper part, containing the most redundant genotypes, the table shows for each genotype the number of accessions with such genotype (N), the number of redundant accessions ($N - 1$ per genotype), and the prime name of the cultivar. The lower part contains data for genotypes represented by six or less accessions, which have been grouped by redundancy (same number of accessions per genotype; e.g. there were seven different genotypes each represented by 6 accessions, or 63 different genotypes each represented by two accessions). In these rows with grouping data, the prime names have been omitted (there should be as many names as number of different genotypes)

[a] According to the Vitis International Variety Catalogue (http://www.vivc.bafz.de/index.php accessed on March 2009)

16 additional microsatellites (Table S2). In base to the results obtained for 25 different microsatellite markers, the conclusion was that all the coincident accessions that fully matched using the nine microsatellite markers belong to the same variety (or EDV).

These results were used to discard 501 redundant genotypes from the global set of 991 accessions. In the pair-wise comparisons of the 490 remaining genotypes, there was only a pair of accessions that matched for 17 of the 18 alleles: 'Chasselas Blanc' and 'Chasselas Gros Coulard' differed only in one allele at microsatellite ssrVrZAG83. Chasselas Blanc presented a heterozygotic genotype for that locus (192:201), that also appeared in other 12 accessions fully coincident with Chasselas Blanc (Table 1, row 9), whereas 'Chasselas Gros Coulard' presented only one peak in the electrophoretogram, what could correspond to a homozygotic genotype (192:192) or a heterozygotic genotype with a null allele (192:–). These varieties were not described like synonymies in the consulted bibliography. The two accessions were studied with the set of 20 microsatellites, and a full match with these additional microsatellites was obtained (Table 3A). The conclusion was that both are of the 'same' variety, and the genotype of 'Chasselas Gros Coulard' was discarded of the non-redundant set of accessions, which at this point included 489 accessions.

The next group of accessions studied differed in 2 of the 18 alleles compared. Only two cases were detected: 'Alphonse Lavallée' with 'Princeps', and 'Pizzutello Moscato Biondo' with 'Galletta Rosa'. In both cases, they differed in two alleles at two different microsatellites: ssrVrZAG83 and VVS2 in the first pair and ssrVrZAG112 and VVMD5 in the second one. Both pairs of varieties were not described like synonymies in the consulted bibliography. The amplification of 16 additional microsatellites revealed a total of 10 different alleles in eight loci in both cases (Table 3B). For this reason, the two pairs of varieties were considered different and kept in the non-redundant set of accessions. This set finally consisted in 489 genotypes, and was the basis for the study of the inter-variety variability.

Figure 1 shows the distribution of the distances (measured in number of different alleles) found in the pair-wise comparisons among the varieties of the final non-redundant set of accessions. The mean (12.4 alleles), mode (12) and median (12) distances are quite similar, and are around a 67% of the maximum distance (18 alleles). The shape of the curve is approximately normal, but slightly asymmetric. In the zone of highest similarity, the number of pairs of varieties decreases more rapidly and, for instance, there are only 8 pairs of accessions that differed in 3 alleles, and 43 pairs that differed in 4 alleles, compared with the 2,763 pairs that differed in all the 18 alleles.

The analysis of genetic and polymorphic parameters in the set of 489 accessions showed that the markers are very informative (Table 4). The number of alleles ranged from 15 to 23 (average 19.00), and eight out of the nine microsatellites showed PIC values above 0.7, while ssrVrZAG29 had the lowest value (0.36). Considering the complete set

Table 2 Nineteen different genotypes where two or more accessions fully matched for the set of nine microsatellites, and for which no information justifying such matches could be found in the literature

Genotype number	Accession 1	Accession 2	Accession 3	Accession 4
1	Agudelo	Chenin	–	–
2	Albarraz	Blanco Gordal	–	–
3	Albillo Blanco	Slavjanka	–	–
4	Aledo	Cherta	–	–
5	Apirena di Velletri	Kischmisch Ali Blanc	–	–
6	Baga	Diminitis	–	–
7	Beba	Blanca Superior para Parral	Tchaoutc	Valencí Blanco
8	Blanco de Mesa	Jerónimo de Tudela	Uva de Olaz	–
9	Bocalilla	Negra Rayada	Rollales Tinta	–
10	Caiño Portugues	Jaquez	Yaqui	–
11	Corinto Bianco	Don Bueno	Pedro Ximenez	–
12	De Rey	Forastera Blanca	–	–
13	Escañavella	Lanjarón Claro	–	–
14	Ferdinand de Lesseps	Pansá Blanca	–	–
15	Forcallat	Luisa Blanca	–	–
16	Garganega	Ugni Blanc	–	–
17	Japinkay	Palomino	Tempranillo de Granada	–
18	Moscatel	Romé	–	–
19	Pensal Blanco	Professor Aberson	–	–

Therefore they were analyzed with a set of 20 microsatellites, and again, all the accessions in the same row fully matched for this set of 20 microsatellites

of markers, the average observed (0.77) and expected (0.78) heterozygosities were very similar, and the total probability of identity was 6.93×10^{-12} for random varieties, and decreased to 1.83×10^{-4} for sibs.

Discussion

Individual microsatellite markers may differ in several characteristics, from stability to polymorphic information content, and thus the conclusions reached for a marker or set of markers can not be valid for another marker or set. In this work, one multiplex PCR of nine microsatellite markers was used to characterize grapevine varieties. The analysis of two plants per accession, which helped to minimize genotyping errors, the number of accessions (991) and of different genotypes (489) studied are enough as to consider the results obtained for this set of markers very significant. There is a published set of six microsatellites (This et al. 2004), that the *Organisation internationale de la vigne et du vin* (OIV) recently included in its descriptor list (OIV 2007). OIV descriptors are the most commonly used for grapevine out of the plant variety protection scope, where the use of UPOV descriptors is mandatory. This 'OIV set' of six markers presents two limitations: it is not decisive enough as to differentiate all the available varieties (Martín et al. 2003), and there are two pairs of markers in the same linkage groups (Riaz et al. 2004). Although the OIV set is useful to support variety identification, these two limitations might restrict its use to establish distinctness. For these reasons, we chose in 2001 the set used here, with more markers, publicly available, distributed in different linkage groups, highly polymorphic (except ssrVrZAG29) and able for multiplexing in one PCR. Four of the markers of the OIV set are included in the set studied here, while the other two were rejected because they are linked. The main constraint of these markers is that the repetitive units of the microsatellite sequences are 2 bp, what can cause some scoring difficulties in comparison with tetra- or pentanucleotide repeats. In grapevine, new markers with core repeats three to five nucleotides long have recently appeared, since the publishing of the whole genome sequence (Cipriani et al. 2008), although their use is still very limited. The set presented here includes microsatellites that are not difficult to score and have been already used to analyze more than 6,000 grapevine plants, including this study. Moreover, this set of markers has already been used to characterize the grapevine reference collection used for DUS testing by the Spanish Plant Variety Office.

The genetic parameters calculated in this work for the nine microsatellites confirmed that they are highly polymorphic (Table 4), with very high heterozygosities and

Table 3 Genotypes obtained with 25 loci microsatellite for the three pairs of accessions that matched for 17 of 18 alleles (A) or 16 of 18 alleles (B) with the nine initial microsatellites (upper part)

Microsatellite	A		B			
	Chasselas Blanc	Chasselas Gros Coulard	Alphonse Lavallée	Princeps	Galletta Rosa	Pizzutello Moscato Biondo
ssrVrZAG67	123:151	123:151	123:153	123:153	123:123	123:123
VVMD27	182:186	182:186	182:182	182:182	180:191	180:191
VVMD5	224:233	224:233	222:235	222:235	224:**227**	**222**:224
ssrVrZAG29	109:113	109:113	109:109	109:109	109:109	109:109
ssrVrZAG62	193:203	193:203	185:203	185:203	187:203	187:203
ssrVrZAG112	238:240	238:240	232:245	232:245	227:**236**	227:**227**
VVS2	130:140	130:140	130:**132**	132:**147**	130:134	130:134
ssrVrZAG83	192:**201**	192:**192**	195:**201**	**190**:195	190:195	190:195
VVMD28	216:266	216:266	242:242	242:242	234:242	234:242
VMC1B11	173:175	173:175	**167:175**	**173:185**	173:185	173:185
VVIB01	290:294	290:294	294:294	294:294	290:294	290:294
VVIH54	165:169	165:169	165:167	165:167	165:167	165:167
VVIP31	182:194	182:194	188:188	188:188	**188:190**	**182:192**
VVIP60	317:321	317:321	**321:327**	**317:317**	321:321	321:321
VVIQ52	84:88	84:88	**82**:84	84:**86**	**82:84**	**88:88**
VVMD7	236:244	236:244	246:252	246:252	240:246	240:246
VVMD24	208:212	208:212	208:212	208:212	208:208	208:208
VVMD25	239:253	239:253	**237**:253	**247**:253	**247**:253	**237**:253
VVIN73	263:263	263:263	256:263	256:263	263:263	263:263
VMC4F3.1	173:179	173:179	167:206	167:206	187:**206**	187:**203**
VVIN16	159:159	159:159	151:151	151:151	151:151	151:151
VVIV67	363:365	363:365	357:**372**	357:**389**	357:**365**	357:**375**
VVMD21	249:265	249:265	249:249	249:249	265:**265**	249:**265**
VVMD32	239:239	239:239	250:270	250:270	260:270	260:270
VVIV37	152:163	152:163	158:**163**	158:**171**	161:163	161:163

Allele sizes are given in base pairs. Alleles differing between the compared accessions are shown in bold

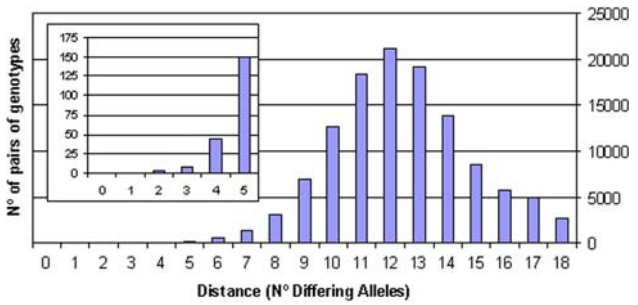

Fig. 1 Representation of the distances (measured in number of different alleles) for the 119,316 pair-wise comparisons among the 489 non-redundant genotypes with nine microsatellites. The small window is a zoom of the smallest distance zone

very low probabilities of identity, even for sibs. Besides, the results obtained with the set of 9 microsatellites were always confirmed by the set of 20, what supports their high resolution capacity. These characteristics, in addition to the multiplexed PCR, make of this set of nine microsatellites a very suitable system for the characterization of grapevine varieties. To facilitate their use, descriptors-like have been prepared in the manner of OIV descriptors for the five microsatellite markers not included in the OIV set (Supplementary Tables 3–7). These descriptors-like included a list of the alleles found as well as well-known reference varieties.

Regarding distinctness, the key issue is the establishment of the minimum distance. In terms of microsatellite markers, it is not expected to differentiate those varieties obtained through a somatic mutation, like EDVs. For those obtained through sexual reproduction (the vast majority), we considered adequate to set that minimum distance through the determination of the highest intra-variety variability (including EDVs), and of the lowest inter-variety variability (excluding EDVs). For that, 640 accessions were studied for the intra-variety variability, and 489 for the inter-variety variability.

In the case of accessions with coincident genotypes, the existence of previous information on their relationships, in addition to such full-matching with the nine microsatellites, was considered enough evidence as to consider them the 'same' variety. In the same way, a full-matching with the 25 microsatellites was considered itself a definitive burden for the same conclusion. This is broadly accepted in grapevine, and even certain EDVs have been discovered just because of their matching in a large number of microsatellites with another variety (Vargas et al. 2007). The fact that

Table 4 Genetic and polymorphic parameters obtained for the nine microsatellite markers used

Locus	NA	H_e	H_o	PIC	r
ssrVrZAG67	22	0.8361	0.7832	0.8163	0.0283
VVMD27	22	0.8438	0.8241	0.8233	0.0102
VVMD5	19	0.8746	0.8732	0.8604	0.0002
ssrVrZAG29	15	0.3755	0.3313	0.3572	0.0319
ssrVrZAG62	18	0.8174	0.8139	0.7963	0.0014
ssrVrZAG112	16	0.8154	0.8016	0.7915	0.0071
VVS2	21	0.8599	0.8773	0.8449	−0.0098
ssrVrZAG83	15	0.7652	0.7771	0.7259	−0.0072
VVMD28	23	0.8733	0.8732	0.8600	−0.0004
Average	19	0.785	0.773	0.764	

NA number of alleles; H_e expected heterozygosity; H_o observed heterozygosity; *PIC* polymorphic information content; *r* frequency of null alleles

the 45 accessions that could not be initially classified gave rise to the same result (full-matching), after analyzing the set of 20 microsatellites, supports the confidence on the set of nine markers, and subsequently, on the first discards.

The highest intra-variety variability found was one allele, and only in one pair of accessions, even though a large number of accessions were studied for 138 varieties (Table 1). These 138 varieties are quite diverse in their origin, use, phenology and morphology traits (Table S1, Supplementary material) (Galet 2000; Rodríguez Torres 2001).

It is difficult to determine beyond doubt, with our results, the origin of the difference found in Chasselas, but the genotype 192:201 appeared in 13 accessions, while 192:– only appeared in 'Chasselas Gros Coulard'. Thus, the most probable hypothesis is that 'Chasselas Gros Coulard' is a sport of 'Chasselas Blanc' where a somatic mutation in the 201 allele of ssrVrZAG83 to a null allele occurred. More mutations have been found at microsatellite loci in grapevine varieties: in 'White Riesling' (Regner et al. 2000), 'Black currant' (Ibáñez et al. 2000), 'Greco di Tufo', 'Muscat d'Alsace', 'Primitivo' (Crespan 2004) or 'Pinot' (Hocquigny et al. 2004). It is important to note that all these varieties with mutant alleles are very ancient (several centuries) and it is not expected that this phenomenon be frequent within the time of protection of a new variety (usually 30 years for a grapevine variety). Besides, not all the microsatellite markers are equally stable; in fact, some microsatellites have been proposed in grapevine for studying clonal variation because of their high intra-varietal variability (Regner et al. 2006). Considering the nine microsatellites used here, only one allele from VVS2 appeared mutated in some plants of different 'Pinot' varieties and in 'Greco di Tufo', in the literature cited above. In conclusion, the highest intra-variety variability found within this set of nine microsatellites was one allele, both in this work and in literature.

The inter-variety variability was studied in a non-redundant set containing 489 genotypes. This set contained very closely related varieties: parents, progenies, full sibs, half sibs, grandparents, etc. (Branas and Truel 1965; Galet 2000; Ibáñez et al. 2009). In a parentage analysis, as many as 730 compatible crosses were found among the 489 cultivars (data not shown). Nevertheless, in 99.96% of all the pair-wise comparisons (the way the DUS test is done) the number of non-matching alleles was five or more (Fig. 1). The zone of highest similarity is the most critical for distinctness (small graph in Fig. 1) and only in 10 cases out of 119,316, the difference between different varieties was smaller than four alleles.

In summary, the closest varieties differed in two alleles at two different microsatellites, while the highest difference found within a variety (and EDVs) was one allele. In base to these results, the minimum distance between grapevine varieties obtained through sexual reproduction can be established in two alleles for the set of nine microsatellites used in this work.

A similar approach was followed by Noli et al (2008), who assessed 98 microsatellite markers in 56 F_8 or F_9 lines or sub-lines, 2 BC_1 and 11 BC_3 of durum wheat to establish distinctness, and concluded that a set of 28 markers represented a useful pre-screening tool to identify the entry pairs sufficiently different (>12 polymorphisms) for which a field evaluation could be avoided. They set the number of polymorphisms needed (>12) based on the fact that between 0 and 11 microsatellite allele differences were found when compared morphologically indistinguishable lines.

Grapevine is a highly heterozygotic and polymorphic species (Velasco et al. 2007), and in that sense, it is very different to other crops like maize, pepper or rice, with a limited inter-variety variability. Singh et al. (2004) studied 23 rice genotypes with 55 microsatellite markers, 41 of them polymorphic, and found an average number of alleles per locus of 2.3, and an average PIC of 0.338. Gunjaca et al. (2008) studied 41 maize inbred lines with 28 microsatellite markers and found 4.4 alleles per locus. Kwon et al. (2005) studied 66 pepper varieties with 316 microsatellite markers, and only 27 were polymorphic, with an average number of 3.3 alleles per locus and an average PIC of 0.529. All these values are much lower than those obtained in this work, although the number of varieties studied here is also much higher.

Besides, grapevine has low intra-variety diversity, due to the way its varieties are multiplied. Rape is a primarily inbreeding species, but its out-crossing rate still produces variability within varieties, what oblige to analyze a high number of plants per variety. Tommasini et al. (2003)

studied 48 plants of each of 10 rape varieties using 15 microsatellites. They did not find difficulties to distinguish varieties, but in the assignment of individual plants to varieties, some plants were incorrectly assigned. In ryegrass, microsatellites also demonstrated their capacity to distinguish varieties, in spite of the variability among individuals within these genetically heterogeneous populations (Roldan-Ruiz et al. 2001). The case of rose is more similar to grapevine, as clear differences were found between intra-varietal (and EDVs) and inter-varietal genetic distances using AFLPs (Vosman et al. 2004). These clear differences were used to propose a threshold for establishing essential derivation, but the same threshold could be use for establishing distinctness in a similar way to that proposed here (excluding EDVs).

The most important issue considering the applicability of this method to the DUS testing is whether the conclusions reached in pair-wise comparisons using morphology and molecular markers would be always the same. In grapevine, it is not difficult to establish distinctness by means of morphology markers, and, as far as we know, there have not been any case where two grapevine varieties that were clearly different by means of these nine microsatellites (difference $\geq$ two alleles) were non-distinct by means of morphology. The problem use to be the opposite: many grapevine varieties considered different have been encountered to be the same (synonyms) after molecular marker analyses (Crespan et al. 2006; Ibáñez et al. 2009).

The study presented here allows the establishment of some general rules for the use of the set of nine microsatellites as a support for establishing distinctness in grapevine varieties, and, appropriately modified, to other vegetatively propagated crops. In summary, if a candidate variety is analyzed with these nine microsatellites and compared with a suitable reference database, three alternative types of results could be found: (1) full match with a variety: the candidate variety is of the same variety (non-distinctness) or is an EDV of the variety (distinctness). The final decision will rely on other characteristics (e.g. morphological, agronomical, etc.), but the advantage compared to the present situation is clear: only two or a few varieties have to be compared using morphological descriptors to reach a conclusion; (2) two or more different alleles with all the varieties compared: the candidate variety is a different variety, and distinctness could be established; (3) one different allele with any variety: this would be the 'shaded zone', and more microsatellites should be studied. Here, a set of 16 additional microsatellites, which are in use by different European laboratories (Le Cunff et al. 2008), are suggested. If there is a full match in the new microsatellites, the conclusion should be like the first case. Otherwise, it would be the second case.

In the practice, a more conservative approach could be followed. Given that the probability of finding two different varieties with only two or three non-matching alleles is so low (1.7×10^{-5} and 6.7×10^{-5}, respectively, based on the frequencies obtained, Fig. 1), and given that the probability of finding two different mutations in the microsatellites of one variety is also so low (6.79×10^{-9}, the mutation rate in grapevine per microsatellite is 8.24×10^{-5} after Crespan (2004)), the actual cases that may appear where the difference between two pair of varieties in comparison is two or three alleles will be very scarce. For that reason, it would be very low costly to include in the 'shaded zone' those cases where only two or three different alleles are found, and analyzing them with additional microsatellites. This conservative approach will contribute to avoid mistakes in particular cases where most similar varieties are being compared, but it would need a study similar to that presented here to establish a minimum distance for those additional markers. As an initial reference, in a pedigree study with 74 table grape varieties, the closest varieties differed in seven alleles for the 16 additional microsatellite markers used here (Ibáñez et al. 2009). A risk also exists that, increasing the number of markers, the probability of finding mutations, or intra-variety variability, increases as well.

In conclusion, no microsatellite system can be determinant for establishing non-distinctness, as an EDV will not be different of its initial variety in the microsatellite genotype, but the set of nine selected microsatellites described here can be decisive for establishing distinctness in grapevine from a two-allele difference. The minimum distance has to be established in a crop by crop basis, and it will depend essentially on the variability of the species, and the way of reproduction (van Eeuwijk and Baril 2001), but also on the concrete molecular markers used. The general procedure used to establish a minimum distance in grapevine by means of a set of nine microsatellites can be easily applied to other vegetatively propagated crops. Most of them have a low intra-variety diversity, while the inter-variety variability greatly differs between species. The level of that inter-variety diversity will determine the number of markers needed to find out the border between intra- and inter-variety diversity needed to establish the minimum distance and, consequently, distinctness.

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