Theor Appl Genet (2004) 109: 1448–1458
DOI 10.1007/s00122-004-1760-3

ORIGINAL PAPER

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Development of a standard set of microsatellite reference alleles for identification of grape cultivars

Received: 9 July 2003 / Accepted: 16 June 2004 / Published online: 30 September 2004

Abstract In order to investigate the comparability of microsatellite profiles obtained in different laboratories, ten partners in seven countries analyzed 46 grape cultivars at six loci (VVMD5, VVMD7, VVMD27, VVS2, VrZAG62, and VrZAG79). No effort was made to standardize equipment or protocols. Although some partners obtained very similar results, in other cases different absolute allele sizes and, sometimes, different relative allele sizes were obtained. A strategy for data comparison by means of reference to the alleles detected in well-known cultivars was proposed. For each marker, each allele was designated by a code based on the name of the reference cultivar carrying that allele. Thirty-three cultivars, representing from 13 to 23 alleles per marker, were chosen as references. After the raw data obtained by the different partners were coded, more than 97% of the data were in agreement. Minor discrepancies were attributed to errors, suboptimal amplification and visualization, and misscoring of heterozygous versus homozygous allele pairs. We have shown that coded microsatellite data produced in different laboratories with different protocols and conditions can be compared, and that it is suitable for the identification and SSR allele characterization of cultivars. It is proposed that the six markers employed here, already widely used, be adopted as a minimal standard marker set for future

Communicated by C. Möllers

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00122-004-1760-3

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grapevine cultivar analyses, and that additional cultivars be characterized by means of the coded reference alleles presented here. The complete database is available at http://www.genres.de/eccdb/vitis/. Cuttings of the 33 reference cultivars are available on request from the Institut National de la Recherche Agronomique Vassal collection (didier.vares@ensam.inra.fr).

Introduction

The genus *Vitis* L. is diverse, comprising 40–60 species in Asia, about 25 in North America and a single European species—*Vitis vinifera* L. This last one is the main species cultivated today, while the other *Vitis* species are used mostly for breeding rootstocks and fungus-resistant scion cultivars. In *V. vinifera* L., around 6,000 cultivars are estimated to exist (Alleweldt and Dettweiler 1994), of which less than 400 are of commercial importance (Galet 2000). Therefore, today most of the genetic resources of *V. vinifera* L. are maintained in germplasm collections.

Grapevines have been cultivated for about 5,000 years (Zohary and Hopf 2000). The ease of vegetative propagation has favored widespread diffusion of many cultivars to diverse regions of the world (Dion 1977; Fregoni 1991). As a consequence, some cultivars now have up to 100 synonyms, and numerous homonyms also exist (http://www.genres.de/idb/vitis/). Because accurate identification of accessions is a basic requirement for the rational management and use of germplasm, the clarification of synonymy, homonymy, and misnaming is a significant problem in the 130 grapevine collections that exist worldwide (Dettweiler et al. 2000a).

The identification of grape cultivars has traditionally been based on ampelography (from the Greek *ampelos*—grapevine and *graphos*—description), which is the analysis and comparison of morphological characters of leaves, shoot tips, fruit clusters, and berries (Galet 1991; Boursiquot and This 1996; IPGRI UPOV OIV 1997; Galet 2000). Expertise in ampelography, however, is restricted to a small and declining number of specialists. Additionally, the expression of morphological characters is influenced by environmental factors, individual plant biology, and life history. Furthermore, juvenile plants are nearly impossible to identify because within 4 or 5 years, they do not exhibit the typical morphological traits of adult plants. Some genetically related cultivars are morphologically very similar and difficult to differentiate by visual comparison (Aradhya et al. 2003). On the other hand, intravarietal clones can differ considerably in phenotype even though they have virtually identical DNA profiles (Vignani et al. 1996; Franks et al. 2002; Riaz et al. 2002).

To surmount these limitations, molecular markers have been used to differentiate, characterize, and identify grapevine accessions. RFLP (Striem et al. 1990; Bourquin et al. 1993; Bowers and Meredith 1996), RAPD (Grando et al. 1995; Loureiro et al. 1998; Ye et al. 1998; Tessier et al. 1999), AFLP (Sensi et al. 1997; Cervera et al. 1998), and microsatellite markers (Botta et al. 1995; Lin and Walker 1998; Sefc et al. 2000; Aradhya et al. 2003) have all proven useful. Microsatellite markers are favored, however, because of their combination of polymorphism, reproducibility, and their codominant nature (Sefc et al. 2001).

GENRES081 was a European Union research project focused on the compilation, standardization, and exchange of information concerning grapevine genetic resources (Dettweiler et al. 2000b; This and Dettweiler 2003; http://www.genres.de/vitis/). The partners in the project, representing the major European grapevine collections, set as an objective the development of a central European database containing reference microsatellite profiles for true-to-type identification of grapevine accessions. However, as demonstrated in tomato (Bredemeijer et al. 2002) and wheat (Röder et al. 2002), for such a database to be useful to diverse laboratories employing differing equipment and methods, alleles must be standardized. Thus, the main objectives of the present study were: (1) to compare different methods of microsatellite DNA profiling for reproducibility among the partners and (2) to standardize allele scoring by defining reference alleles. Six informative markers were selected, and all participants analyzed identical DNA samples. Because of the diversity of laboratory equipment and protocols, no standardization of PCR protocols was attempted. A set of coded alleles based on well-known reference cultivars was developed that facilitates data comparison among laboratories and will permit the development of a common international database.

Materials and methods

Cultivars

A total of 47 grapevine accessions were analyzed, corresponding to 46 different cultivars ('Merlot' was represented twice since material from this cultivars originated from two different germplasm collections, Table 1). For the names of the cultivars, "N," "B," and "RG" refer to berry skin color—black, white and red, respectively. The number and choice of cultivars analyzed reflect the evolution of the microsatellite project within GENRES081 over the course of three international workshops held in 1998, 1999, and 2001 (http://www.genres.de/vitis).

The first round of analyses was limited to five regionally important cultivars in order to obtain preliminary information on data consistency (Table 1; analysis group 1). These cultivars were expected to be quite distinct in allelic profile because of their different geographic origins.

In an effort to reach better agreement, in a second round, ten well-known cultivars were analyzed (Table 1; analysis group 2). Cultivars from the first round were also analyzed in round 2.

Table 1 Grapevine cultivar accessions analyzed in this study. Trueness-to-type was defined by ampelography

Analysis group[a]	Cultivar name[b]	Code	WIEWS[c] institute code	True to type?	Reference pool	Accession number
1	Furmint B		DEU098	Yes	No	52-09-034
1	Merlot N	ME	DEU098	Yes	Yes	52-07-045
1	Touriga nacional N		DEU098	Yes	No	52-10-006
1	Trebbiano toscano B		DEU098	No	No	52-10-019
1,2	Sultanina gigas B	SU	DEU05	Yes	Yes	10|14
2	Barbera N	BA	ITA360	Yes	Yes	CVT424
2	Cabernet franc N	CF	FRA139	Yes	Yes	324Mtp39
2	Cabernet-Sauvignon N	CS	ITA362	Yes	Yes	304
2	Chardonnay B	CH	ITA339	Yes	Yes	–
2	Merlot N	ME	ITA339	Yes	Yes	–
2	Muscat à petits grains blancs B	MU	FRA139	Yes	Yes	555Mtp22
2	Pinot noir N	PI	ITA362	Yes	Yes	1560
2	Silvaner B	SI	AUT024	Yes	Yes	IV-7-12
2	Traminer rot RG	TR	DEU098	Yes	Yes	52-03-007
3	Admirable de Courtiller B		FRA139	Yes	No	814Mtp1
3	Agiorgitiko N		FRA139	Yes	No	1816Mtp2
3	Alvarelhao N	AL	FRA139	Yes	Yes	1481Mtp2
3	Carignan N		FRA139	Yes	No	18Mtp8
3	Castel 216-3[d]		FRA139	Yes	No	9017Mtp3
3	Couderc 1616[d]	16C	FRA139	Yes	Yes	9039Mtp1
3	Couderc 3309[d]	33C	FRA139	Yes	Yes	9043Mtp4
3	Fercal[d]	FE	FRA139	Yes	Yes	9219Mtp2
3	Goethe 9[d,e]	GO	FRA139	No	Yes	9000Mtp537
3	Hans RG		FRA139	Yes	No	1595Mtp1
3	Jacquez N[e]	JA	FRA139	No	Yes	5000Mtp69
3	Kober 5 BB[d]		FRA139	Yes	No	9171Mtp1
3	Madeleine royale B	MAR	FRA139	Yes	Yes	653Mtp1
3	Malègue 44–53[d]	4MA	FRA139	Yes	Yes	9081Mtp3
3	Mancin N	MAN	FRA139	Yes	Yes	1216Mtp1
3	Mauzac B	MAU	FRA139	Yes	Yes	443Mtp14
3	Mavrodaphni N		FRA139	Yes	No	1800Mtp3
3	Millardet et Grasset 101-14 N[d]	1MG	FRA139	Yes	Yes	9095Mtp1
3	Millardet et Grasset 420A[d]	4MG	FRA139	Yes	Yes	9122Mtp3
3	Mourvèdre N		FRA139	Yes	No	64Mtp2
3	Muscat of Alexandria B		FRA139	Yes	No	308Mtp2
3	Paulsen 1103[d]		FRA139	Yes	No	9003Mtp1
3	Portugieser blau N	PO	FRA139	Yes	Yes	450Mtp1
3	Richter 110[d]	11R	FRA139	Yes	Yes	9159Mtp2
3	Richter 99[d]	99R	FRA139	Yes	Yes	9157Mtp3
3	Romorantin B	RO	FRA139	Yes	Yes	304Mtp8
3	Rondinella N		FRA139	Yes	No	1295Mtp1
3	Ruggeri 140[d]		FRA139	Yes	No	9001Mtp1
3	Salvador (=Seibel 128)	SAL	FRA139	Yes	Yes	5026Mtp4
3	Schwarzmann	SCH	FRA139	Yes	Yes	9221Mtp1
3	Teleki 5 C[d]	5C	FRA139	Yes	Yes	9179Mtp3
3	Veltliner rot RG	VE	FRA139	Yes	Yes	284Mtp4
3	Vialla N[d]	VIA	FRA139	Yes	Yes	9005Mtp1
3	Vital B	VI	FRA139	Yes	Yes	2103Mtp1

[a]Refers to round of analysis (see text).
[b]Berry skin color: *B* White, *N* black, *RG* red
[c]*WIEWS* World Information and Early Warning System, http://apps3.fao.org/wiews/institute_query.htm
[d]Rootstock cultivars
[e]Although the identity of these accessions is not yet confirmed, we kept them in the reference pool since the material can be obtained from the Institut National de la Recherche Agronomique (INRA) Vassal collection

In a third round designed to complete the allelic ladders for the six microsatellite loci, 34 additional cultivars, including 15 rootstock cultivars, were selected (Table 1; analysis group 3). This group was chosen after consulting three existing grapevine microsatellite databases in order to maximize allele diversity (Sefc et al. 2000; E. Zyprian and C. Meredith, unpublished data). Whenever possible, well-known and widely distributed cultivars were chosen in order to maximize the value of this work to the scientific community.

Plant material

To prevent confusion concerning trueness-to-type of the analyzed accessions (Dettweiler et al. 2000a), it was important to insure a single source for each sample in the analysis, particularly in the second and third round. Trueness-to-type was defined by comparing morphological descriptions and photographs to the documentation of morphological characters in the literature. For each accession used, fresh young leaves or late-winter cuttings of true-to-type vines were collected from only one germplasm collection (Table 1). For the first and second rounds, DNA was extracted from leaves by single partners and distributed to all the others. For the third round, cuttings were collected from the Institut National de la Recherche Agronomique Vassal domain and sent to each partner for local DNA extraction. DNA from these cuttings was sent to the Meredith laboratory in the United States because of quarantine restrictions.

Marker selection

In 1998, VVS, VVMD, and VrZAG markers were the most widely used grapevine microsatellite markers (Cipriani et al. 1994; Thomas et al. 1994; Botta et al. 1995; Bowers et al. 1996; Regner et al. 1996). Based on the experience of the project partners, six polymorphic markers were chosen: VVS2 (Thomas and Scott 1993), VVMD5, VVMD7 (Bowers et al. 1996), VVMD27 (Bowers et al. 1999b), VrZAG62, and VrZAG79 (Sefc et al. 1999).

DNA extraction

DNA was isolated from fine powdered leaf or cambium tissues frozen in liquid nitrogen and ground in a mortar. Partners (partner no. according to title page) used a number of different DNA extraction protocols, including those of Doyle and Doyle (1990) with an additional RNAse A-digestion step (partner 5), Doyle and Doyle (1990) modified by Cipriani et al. (1994) (partner 10), Thomas et al. (1993) (partner 3), Thomas et al. (1993) without the initial step (partner 8), Crespan et al. (1999) (partner 6), Ferreira Monteiro et al. (2000) (partner 9), or according to the protocol for DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) (partner 2, partner 4), or the latter with addition of 1% Polyvinylpyrrolidone 40 (Sigma-Aldrich, Dearborn, Mich., USA) to AP1-buffer and doubling the amount of AE-buffer for elution (partner 1). Specific conditions are available on request from this@ensam.inra.fr.

PCR conditions

According to each partner's preferences for different *Taq* DNA polymerases and thermal cyclers, various individual strategies for optimization and generalization of PCR conditions were employed (specific conditions are available on request from this@ensam.inra.fr). PCR reaction mixes and cycling strategies differed widely, but there was a tendency towards hot-start PCR. Most partners preferred a three-step cycling routine, but partners 6, 7, and 8 used differing two-step PCR regimes. Partner 10 performed a touchdown PCR, differentiated as two separate protocols: (1) for VVS2, VrZAG62, VrZAG79, touchdown PCR of −1°C/cycle from 65 to 56°C and (2) for VVMD5, VVMD7, VVMD27, touchdown PCR of −1°C/cycle from 65 to 52°C. Some partners generalized primer-specific annealing temperatures to reduce the number of cycling programs.

Microsatellite analysis

Different systems for fragment differentiation and allele size determination were also employed. Six partners separated amplification products manually by high-voltage electrophoresis on vertical, denaturing polyacrylamide sequencing gels, and four partners utilized single capillary or gel-based electrophoresis systems for automatic fragment separation in ABI Prism Genetic Analyzers (Applied Biosystems, Foster City, Calif., USA). Precise conditions are available on request from this@ensam.inra.fr.

Manual analysis

Polyacrylamide gels (5–6% polyacrylamide, 7–8 M urea) were prepared according to standard protocols (Sambrook et al. 1989). Before loading, samples were denatured for 2–3 min at 94–95°C in solutions containing balanced amounts of PCR amplification products in a buffer of formamide or FBX-marker (100 µl 1 M NaOH, 400 µl H_2O, 9.5 ml formamide, 50 mg Bromophenol Blue, 50 mg xylene cyanol). After electrophoresis, bands were visualized by a silver-staining procedure, according to the protocol provided with the Silver Sequence DNA Sequencing System (Promega, Madison, Wis., USA). Partner 6 performed silver staining according to Crespan and Milani (2001), following Bassam et al. (1991) and Tixier et al. (1997), with the exception of using NaOH instead of $NaCO_3$. Partner 10 used $\gamma[^{33}P]$-ATP-labeled primers and detection of PCR fragments on autoradiographic film after 1–7 days of exposure to fragment radiation.

Automatic analysis

For automatic electrophoresis (single capillary on ABI 310 or gels on ABI 373 and 377), the amplification product was added to 12–20 µl of deionized formamide. Adapted amounts of denatured PCR fragments labeled with fluorescent dye phosphoramidites were loaded, separated on the capillaries or the gels, and detected by the system's laser. Fluorescent emission was analyzed by GeneScan software, version 2.1 (Applied Biosystems), using internal-lane size standards (ROX or TAMRA) and the system's local southern method for automatic size calling of peak positions.

Fragment-size determination and data analysis

Fragment-size determination for manual analysis was adjusted by the use of PCR fragments of previously analyzed cultivars as internal size standards, in addition to commercial weight markers. Once the fragment sizes of reference alleles had been determined, partners 2 and 6 used exclusively self-made primer-specific allelic ladders.

The automatic peak labeling of the ABI Prism Genetic Analyzers needed some additional control by visual inspection of individual peak positions, rounding up or down the decimal places to reach integer allele numbers. Previously labeled peak profiles of reference

cultivars aided decision making. Partner 4 applied the algorithm of Ghosh et al. (1997), using the common average value of identical fragments to direct the algorithmic rounding of decimal variations into same integer numbers except for VVMD27, which was rounded manually because some of its alleles differ only by 1 bp.

Polymorphism information content (PIC) and discrimination power were calculated according to Lamboy and Alpha (1998).

Results and discussion

Although a considerable amount of microsatellite data has been published for grape, interlaboratory variations have made comparisons of results difficult, more particularly because grapevine is highly heterozygous. In this study, we have compared microsatellite data for 46 different cultivars with six microsatellite markers among ten different laboratories and implemented a reference allele system for uniform grapevine microsatellite analysis.

All data from this work, including detailed data from each partner are available upon request to this@ensam.inra.fr or at http://www.genres.de/eccdb/vitis/

Comparison of allele sizes

Comparison of the allele sizes obtained by the different laboratories for each DNA sample for the six microsatellite markers produced no satisfactory agreement (Table 2) as it has been previously reported for other crops (Haberl and Tautz 1999). Although some partners obtained similar results, in other cases the raw data for identical alleles differed by as much as 5 bp (e.g., VVMD5 or VVS2, Table 2). The magnitude of the difference varied by marker (e.g., no more than 3-bp differences for VVMD7), but in general, the raw data (without any transformation) could not be compared. In order to increase the general applicability of this work, we had previously decided not to standardize the protocols. Each of the ten partners analyzed the same DNA with its own laboratory equipment and individually adapted protocols, so some of the differences may have been the consequence of the different protocols. No clear relationship could be found, however, between any protocol step and the fragment size. Automatic sequencing does tend to give smaller fragment sizes, but comparable sizes were obtained with both automated and manual techniques. Since direct comparison of profiles was impossible without an additional harmonization procedure, we tested several methods, for comparing data between laboratories.

Transforming numerical data

The relative differences between the two fragments of an allele pair were first considered. The data were coded as size differences between the smallest allele observed in the sample (n) and size of the allele of the cultivar. For example for partner 1, 'Mourvèdre' was 134/152 for VVS2, and was coded n + 10/n + 28 (Table 3) since the smallest allele, n, was 124. With this method, results from the different laboratories were more consistent. But discrepancies still could not be avoided for all the markers, and shifts in the relative difference between the alleles occurred (Table 3). The differences were mostly the result of the rounding methods. The simple mathematical algorithms employed in the automatic scoring of peak sizes can produce artificial shifts by automatically

Table 2 Examples of profiles obtained by the ten partners for two cultivars displaying identical coded data among the partners. Allele sizes are in base pairs

Marker	Most frequent profile		Other profiles							
			1		2		3		4	
VVMD05										
Mourvèdre	226	240	223	237	225	239	228	242		
Malegue 44–53	252	264	249	261	251	263	254	266		
No. of partners (size dif.)[a]	5		2 (−3)		2 (−1)		1 (+2)			
VVMD7										
Mourvèdre	249	249	247	247	248	248	250	250		
Malegue 44–53	233	239	231	237	232	238	234	250		
No.of partners (size dif.)	6		1 (−2)		2 (−1)		1 (+1)			
VVS2										
Mourvèdre	133	151	130	149	133	152	134	152	135	153
Malegue 44–53	139	145	136	142	139	146	140	146	141	147
No.of partners (size dif.)	4		2 (−3)		1 (0/+1)		2 (+1)		1 (+2)	
VrZAG62										
Mourvèdre	189	205	187	203	188	204	189	205	190	206
Malegue 44–53	175	180	174	178	175	180	177	181	178	184
No. of partners (size dif.)	3		2 (−1/−2)		3 (−1/0)		1 (0/+1/+2)		1 (+3/+1)	

[a] *size dif*. Size difference, expressed by comparison to the most frequent genotype

Table 3 Comparison of profiles at locus VVS2 after codification, using relative size differences between smallest allele detected in the sample (*n*) and size of the allele of the cultivar

Cultivar	Profiles[a] no. (number of partners)			
	Number 1 (5)		Number 2 (2)	
Barbera	n + 10	n + 12	n + 10	n + 12
Cabernet-Sauvignon	n + 6	n + 18	n + 6	n + 19
Chardonnay	n + 4	n + 10	n + 4	n + 10
Couderc 3309	n	n	n	n
Furmint	n	n + 20	n	n + 21
Mourvèdre	n + 10	n + 28	n + 10	n + 29
Pinot noir	n + 4	n + 18	n + 4	n + 19
Sultanina	n + 12	n + 18	n + 12	n + 19
Silvaner	n + 18	n + 20	n + 19	n + 21
Touriga national	n + 10	n + 18	n + 10	n + 19
Traminer rot	n + 18	n + 18	n + 19	n + 19

[a]Profiles 1 and 2 correspond to the different results obtained by five and two partners, respectively

rounding up. Furthermore, this strategy depends on the size and composition of the sample: according to the sample analyzed by different laboratories, the smallest allele observed (n) might vary, and comparison of data between laboratories would be difficult. This strongly argues against this strategy. Thus it was decided to concentrate on the development of a standardized coding procedure. For each SSR marker, the profiles of the 46 cultivars were compared and PCR fragments (alleles) were sorted according to their lengths. We thus selected one PCR fragment of each size represented in order to cover the entire size range of detected fragments. These selected fragments were designated as reference alleles and assigned code names based on the cultivar in which they were observed. If the fragment were the shorter or the longer for this cultivar, it was designated "1" or "2," respectively. For example, the shorter fragment of 'Cabernet-Sauvignon' was assigned the code CS1. The cultivars selected as references, including both scion and rootstock cultivars, are presented with their codes in Table 1. For most of the reference alleles, there was a consensus between the partners. For VVMD27, the data were identical. In very few cases (2.4%) some discrepancies arose (i.e., one partner found one or several additional reference alleles), particularly for VrZAG62, VrZAG79, and VVMD7, but we discarded them as genotyping errors. The use of these cultivar-specific fragments as size standards produced a homogenous coding system, comprising a relatively complete allelic ladder for each of the six microsatellite loci (Table 4).

The number of reference alleles among the 33 reference cultivars ranged from 13 for VrZAG79 to 23 for VVMD27 (Table 4). The relative difference in size between the shortest and the longest alleles ranged from 26 bp for VrZAG79 to 46 bp for VVMD5 and VrZAG62. For VVMD7, VVMD27, VVS2, and VrZAG79, the allelic ladders were almost complete, with nearly every expected size increment observed, whereas more gaps were observed for VVMD5 and VrZAG62, notably for the larger allele sizes. A few of these missing

Table 4 List of the reference alleles for each of the six loci, with a general indication of their size. The size is also given as relative size to n. The codes are as indicated in Table 1. Numbers after the codes refers to shortest (1) or longest (2) allele of the reference cultivar. The numbers are given as indication and correspond to the most common size among the partners. Rootstocks alleles are shown in *italics*

VVMD5			VVMD7			VVMD27			VVS2			VrZAG62			VrZAG79		
222	n	AL1	232	n	*FE1*	175	n	CS1	123	n	*33C1*	174	n	*1MG1*	238	n	RO1
226	n + 4	CF1	234	n + 2	MU1	179	n + 4	MU1	125	n + 2	*VIA1*	175	n + 1	*4MA1*	240	n + 2	PI1
228	n + 6	MU1	236	n + 4	VIA1	181	n + 6	CF1	127	n + 4	*4MG1*	180	n + 6	*4MA2*	244	n + 6	CH1
230	n + 8	MAU1	238	n + 6	JA1[a]	183	n + 8	*FE1*	129	n + 6	RO1	182	n + 8	*33C1*	246	n + 8	CH2
232	n + 10	TR1	240	n + 8	CF1	185	n + 10	PI1	131	n + 8	VE1	184	n + 10	*FE1*	248	n + 10	CF1
234	n + 12	CH1	244	n + 12	TR1	186	n + 11	*GO1*[a]	133	n + 10	BA1	186	n + 12	MU1	250	n + 12	SI1
236	n + 14	MU2	246	n + 14	*33C1*	187	n + 12	*VIA1*	135	n + 12	BA2	188	n + 14	CH1	252	n + 14	TR2
238	n + 16	CH2	248	n + 16	ME2	189	n + 14	CS2	137	n + 14	CH1	190	n + 16	*33C2*	254	n + 16	VI2
240	n + 18	CF2	250	n + 18	MU2	191	n + 16	ME2	139	n + 16	CF1	192	n + 18	VE1	256	n + 18	MU2
244	n + 22	JA2[a]	252	n + 20	*FE2*	193	n + 18	*4MG1*	141	n + 18	*G02*[a]	194	n + 20	CF1	258	n + 20	*4MA1*
246	n + 24	VE2	254	n + 22	SU2	194	n + 19	MU2	143	n + 20	CH2	196	n + 22	CH2	260	n + 22	CF2
252	n + 30	*33C1*	256	n + 24	PO2	195	n + 20	*16C1*	145	n + 22	SU1	198	n + 24	JA2[a]	262	n + 24	*4MA2*
256	n + 34	*1MG1*	258	n + 26	TR2	197	n + 22	*1MG1*	147	n + 24	CF2	200	n + 26	*5C1*	264	n + 26	*99R2*
262	n + 40	*GO1*[a]	260	n + 28	*33C2*	201	n + 26	SAL2	149	n + 26	*99R2*	202	n + 28	SCH2			
264	n + 42	*33C2*	262	n + 30	*99R2*	203	n + 28	*5C1*	151	n + 28	SI1	204	n + 30	CF2			
266	n + 44	*1MG2*	264	n + 32	CF2	205	n + 30	*4MA1*	153	n + 30	SI2	210	n + 36	*5C2*			
268	n + 46	*11R2*	266	n + 34	*5C1*	207	n + 32	*1MG2*	155	n + 32	MAR2	214	n + 40	*11R2*			
						209	n + 34	*VIA2*	157	n + 34	MAN2	220	n + 46	*FE2*			
						211	n + 36	*16C2*	161	n + 38	*33C2*						
						213	n + 38	SCH2									
						215	n + 40	*4MA2*									
						217	n + 42	*4MG2*									
						219	n + 44	*GO2*[a]									

[a]Although the trueness-to-type of these cultivars is not confirmed, since the material can be obtained from the INRA Vassal collection, we kept them as references

alleles were observed, but synonymy of cultivar names could not be resolved. By consequence, no true-to-type accession could be proposed as a reference.

In most cases, allele size increments were 2 bp, in accordance with the dinucleotide nature of these markers (Thomas and Scott 1993; Bowers et al. 1996, 1999b; Sefc et al. 1999). For VVMD27 and VrZAG62, however, a few 1-bp increments were repeatedly recorded (n + 11 and n + 19 for VVMD27 and n + 1 for VrZAG62). Such fragment patterns might be interpreted as stutter or as the extra base additions that occur with some *Taq* polymerases (Brownstein et al. 1996), or they might even be completely ignored. In our case, however, comparison of data over laboratories confirmed these differences.

Special attention was given to the selection of the reference cultivars. Preference was given to well-known cultivars, except for a few alleles that were detected only in rare cultivars. The total number of cultivars in the reference set was minimized by selecting cultivars useful for more than one locus. In order to fulfill these criteria and to cover the complete range of alleles, ten well-known cultivars ['Barbera', 'Cabernet-Sauvignon', 'Cabernet franc', 'Chardonnay', 'Merlot', 'Muscat à petits grains blancs', 'Pinot noir', 'Sultanina' (or 'Thompson seedless'), 'Silvaner', and 'Traminer rot' (or 'Gewürztraminer")] were initially selected. Seven well-known rootstocks ('Couderc 1616', 'Couderc 3309', 'Millardet et Grasset 101-14', 'Millardet et Grasset 420A', 'Richter 99', 'Richter 110', and 'Teleki 5C') were also added to the set. The inclusion of rootstock cultivars into the analysis was necessary, not only because of their general importance in grapevine cultivation, but also because of the high number of exclusive alleles not found among *V. vinifera* cultivars. The rootstocks frequently displayed characteristic allele clusters that were either smaller (e.g., VrZAG62) or larger (e.g., VVMD5 and VVMD27) compared to those that were detected in *V. vinifera* cultivars. In order to fill most of the remaining gaps, a few less well-known scion cultivars were added too. The 33 reference cultivars are available upon request to didier.vares@ensam.inra.fr.

The extension of the allele ranges for these markers will also be of great importance for the analysis of other *Vitis* species and interspecific hybrids. For example, in a work on 105 accessions of 16 American *Vitis* species (found in the pedigrees of almost every known hybrid), 25 and 23 alleles were observed for VVS2 and VVMD7, respectively (Lamboy and Alpha 1998). The allele ranges, however, were very similar compared to our work. Very few rare alleles (three for VVMD7, five for VVS2), present in a few species only (*V. Vulpina* L., *V. palmata* Vahl, *V. Piasezkii* Maxim., *V. arizonica* Engelm.), were outside the size range represented in our reference set.

Comparison of coded data

For each cultivar and each marker, the data were coded according to the defined reference alleles and compared. Complete data are available on request to this@ensam.inra.fr or at http://www.genres.de/eccdb/vitis/.

Compared to the raw data (Table 2) and relative allele size differences (Table 3), coding of the numerical data allowed the immediate and direct comparison of data. In fact, coded data were easy to compare and, except for a few discrepancies, were identical for all partners. For four cultivars ('Hans RG', 'Malegue 44–53', 'Mancin N', and 'Mourvèdre N'), the data were completely identical. For the others, in general only one partner showed discrepancies for one or two loci. The "true" allele was thus deduced from the data that were identical among most of the partners. The discrepancies were then calculated from these "true" profiles (Table 5). Excluding missing data, 97.5% of the alleles (4,487 alleles out of 4,600) were completely identical among partners (Table 6). Data homogeneity was especially high for VVS2, VVMD27, and VVMD7 (98.8, 98.6, and 98.0%, respectively). On the other hand, the results for VrZAG62 and VrZAG79 were in agreement for 96.4% and 96.2% of the alleles, respectively.

VVMD5, VVMD7, and VVMD27 are robust markers with stable, clear fragment patterns. VVS2 was easy to score since all alleles are represented in the reference set. On the other hand, VrZAG62 and VrZAG79 could produce interpretation problems because of their tendency to produce unclear banding patterns or stutter. Three types of discrepancies were observed. In a few cases, the data were very different. These discrepancies most probably correspond to typing errors. Most differences were consistent 1- or 2-bp shifts for several alleles, either in the standards or in the analyzed cultivars. Finally, some discrepancies occurred in the case of single alleles that are only 2 bp apart.

Data discrepancies due to shifts are difficult to explain. Many were avoided by covering almost the complete spectrum of alleles detected for each of the loci. In any case, they showed the necessity to optimize PCR conditions and visualization techniques in order to avoid conditions that can lead to stutter or fluctuating fragment patterns (Hu 1993; Smith et al. 1995). A good strategy is the general use of a hot-start *Taq*, the optimization of annealing temperatures for each primer pair, and the use of cultivar-specific, coded reference fragments as universal size standards. Using pigtail primers (Bredemeijer et al. 2002; Röder et al. 2002) can also help to circumvent these problems.

Discrepancies in single alleles only 2 bp apart are easier to explain than to avoid. They are likely due to misinterpretation of homozygous versus heterozygous state of cultivars when the alleles are only 2 bp apart (one microsatellite repeat unit), and the microsatellite locus also shows either extra base additions or stutters. Heterozygosity in grape is very high and extra difficulties have been described when working with heterozygous plants (Vosman et al. 2001). No easy way to reduce this type of error can be proposed. Automatic sequencing seems less subject to these errors but they arise nevertheless.

Table 5 Summarized coded microsatellite profiles for 46 grapevine cultivars uniformly coded by the PCR fragments of selected reference cultivars

Cultivars	VVMD5		VVMD7		VVMD27		VVS2		VrZAG62		VrZAG79	
Admirable de Courtiller	CF1	MU2	CF1	TR1	PI1	MU2	BA1	CH1	CH1	CF1	TR2	4MA1
Agiorgitiko	TR1	CF2	TR1	MU2	CS1	PI1	CH2	SU1	5C1	SCH2	CF1	CF1
Alvarelhao	AL1	CF1	CF1	CF1	PI1	CS2	BA1	SI1	CH1	CF1	TR2	CF2
Barbera[a]	CF1	CF1	MU2	SU2	PI1	CS2	BA1	BA2	VE1	5C1	CH1	CF2
Cabernet franc[a]	CF1	CF2	CF1	CF2	CF1	CS2	CF1	CF2	CF1	CF2	CF1	CF2
Cabernet-Sauvignon[a]	TR1	CF2	CF1	CF1	CS1	CS2	CF1	SI1	CH1	CF1	CF1	CF1
Carignan	CF1	MU1	CF1	CF1	CF1	PI1	CH2	SU1	MU1	CH1	TR2	CF2
Castel 216-3	MU2	11R2	FE2	99R2	1MG2	16C2	CH1	16C2	33C2	CH2	MU2	99R2
Chardonnay[a]	CH1	CH2	CF1	TR1	CF1	CS2	CH1	CH2	CH1	CH2	CH1	CH2
Couderc 1616[a]	33C2	11R2	CF1	FE2	16C1	16C2	CF1	16C2	33C2	33C2	MU2	4MA1
Couderc 3309[a]	33C1	33C2	33C1	33C2	PI1	16C2	33C1	33C2	33C1	33C2	MU2	4MA1
Fercal[a]	MU2	33C2	FE1	FE2	FE1	CS2	CH2	CH2	FE1	FE2	CH2	4MA1
Furmint	CF1	CF2	CF1	MU2	MU1	MU2	BA1	SI2	CH1	CF2	RO1	SI1
Goethe 9[ab]	GO1	33C2	FE2	5C1	GO1	G02	CH1	16C2	SCH2	SCH2	4MA2	4MA2
Hans	CH1	VE2	MU2	SU2	FE1	CS2	VE1	BA1	VE1	CF2	TR2	TR2
Jacquez[ab]	MU1	JA2	JA1	CF1	MU1	CS2	CF1	CH2	MU1	JA2	SI1	SI1
Kober 5BB	MU2	1MG2	MU1	5C1	ME2	16C2	16C2	99R2	5C1	11R2	TR2	CF2
Madeleine Royale[a]	MU1	MU2	TR1	ME2	CF1	CS2	SI1	MAR2	CH1	CF1	CH2	CF2
Malegue 44-53[a]	33C1	33C2	MU1	CF1	4MA1	4MA2	CF1	SU1	4MA1	4MA2	4MA1	4MA2
Mancin[a]	TR1	CH2	CF1	CF1	CS1	CS2	CF1	MAN2	CH1	CF1	CF1	TR2
Mauzac[a]	MAU1	TR1	CF1	MU2	PI1	ME2	BA1	SI1	CH1	5C1	TR2	TR2
Mavrodaphni	CF1	TR1	CF1	CF1	FE1	CS2	CH2	CH2	MU1	CH1	CH1	CH2
Merlot[a]	CF1	MU2	CF1	ME2	CS2	ME2	CF1	SI1	CF1	CF1	CF2	CF2
Millardet de Gt.101-14[a]	1MG1	1MG2	TR1	FE2	1MG1	1MG2	BA1	CH2	1MG1	33C2	MU2	4MA1
Millardet de Gt. 420A[a]	CH2	33C2	FE1	CF2	4MG1	4MG2	4MG1	CH1	33C2	CH2	MU2	MU2
Mourvedre	CF1	CF2	MU2	MU2	MU1	CS2	BA1	SI1	CH1	CF2	TR2	4MA2
Muscat à p.g. blancs[a]	MU1	MU2	MU1	MU2	MU1	MU2	BA1	BA1	MU1	CH2	TR2	MU2
Muscat of Alexandria	MU1	TR1	MU2	FE2	MU1	MU2	BA1	99R2	MU1	CF2	CF1	MU2
Paulsen1103	MU2	MU2	MU1	TR2	5C1	1MG2	CH1	CF2	CH2	11R2	TR2	99R2
Pinot noir[a]	MU1	CH2	CF1	TR1	PI1	CS2	CH1	SI1	CH1	CF1	PI1	CH2
Portugieser[a]	CF1	TR1	TR1	PO2	CF1	MU2	CH2	SI1	CH1	CF2	SI1	CF2
Richter 110[a]	CH1	11R2	FE1	TR2	CS2	4MA1	CH1	CH2	CH2	11R2	CH1	CF2
Richter 99[a]	MU2	MU2	FE1	99R2	ME2	1MG2	CH1	99R2	CH2	5C2	TR2	99R2
Romorantin[a]	CH1	CH2	TR1	MU2	MU1	CS2	RO1	BA1	CH1	CF2	RO1	CH2
Rondinella	CF1	TR1	CF1	CF1	MU1	CS2	CH2	SI1	CH1	CF1	CF1	TR2
Ruggeri 140	VE2	11R2	FE1	TR2	CS2	4MA1	CH1	CH2	CH2	11R2	CH1	CF2
Salvador[a]	CF1	33C1	ME2	FE2	CF1	SAL2	BA1	BA1	CF1	CF1	CH1	CH2
Schwarzmann[a]	33C1	1MG2	FE2	5C1	1MG2	SCH2	CH1	SU1	5C1	SCH2	MU2	4MA1
Silvaner[a]	CF1	TR1	TR1	ME2	CS2	MU2	SI1	SI2	CH1	CF2	SI1	TR2
Sultanina[a]	CH1	CH1	CF1	SU2	CF1	MU2	SU1	SI1	CH1	CH1	CF1	CF2
Teleki 5C	33C1	1MG2	FE1	5C1	5C1	16C2	BA1	CH2	5C1	5C2	TR2	CF2
Touriga national	CF1	MU2	CF1	CF1	CF1	CS2	CH2	SI1	CH1	CF1	CH2	CH2
Traminer rot[a]	TR1	CH2	TR1	TR2	CS2	CS2	SI1	SI1	CH1	CF1	CH2	TR2
Veltliner rot[a]	CF2	VE2	CF1	SU2	FE1	MU2	VE1	BA1	VE1	CH2	TR2	TR2
Vialla[a]	1MG2	1MG2	VIA1	FE2	VIA1	VIA2	VIA1	BA2	SCH2	SCH2	SI1	MU2
Vital[a]	AL1	CF2	CF1	CF1	CF1	MU2	SU1	SI1	CH1	CH1	CF1	VI2

[a]Belongs to the reference pool
[b]Although the trueness-to-type of these cultivars is not confirmed, since the material can be obtained from the INRA Vassal collection, we kept them as references

The percentage of consistency reported here is similar to that reported for tomato (Bredemeijer et al. 2002). In comparing data between only two laboratories, the authors reported 97.3% concordance, slightly less than the 99.5% reported for wheat (Röder et al. 2002) with generalized PCR conditions.

Since the illustrated method enabled easy comparisons among the partners, we propose that other labs utilizing grapevine SSR fingerprints convert to the code. This could be achieved by identifying the size of the reference alleles in their database, and converting all data sharing the same size, using the proposed codes (Table 4). An access database was developed in order to

Table 6 Consistency of the data over the partners. Each allele for the 44 cultivars analyzed in round two and three was compared between each partners

Loci	Percentage of concordant data[a]
VVMD5	97.2
VVMD7	98.0
VVDM27	98.6
VVS2	98.8
VrZAG62	96.4
VrZAG79	96.2
Mean over loci	97.5

[a]Incorrect data/correct data

help this conversion and is available on request to this@ensam.inra.fr.

Development of the database

The data for the 46 analyzed cultivars were verified by seven to ten partners, so the database developed in this study is very strong. It also includes several major cultivars that are grown worldwide and will be highly useful as multiconfirmed reference for identification purposes.

The six selected microsatellite markers are suitable for grapevine cultivar characterization because of their high degree of allelic polymorphism (PIC varies from 0.86 for VVMD7 to 0.91 for VVMD5 in our study) and high discrimination power (ranging from 0.95 for VrZAG62 to 1 for VVS2). Because they are already in wide use, these six markers should be recommended generally as the minimal standard marker set for future grapevine-cultivar analyses. That would facilitate the creation of uniform, easily comparable data catalogs for the comparative identification of unknown or unconfirmed accessions in international grapevine germplasm collections. In all cases, the six markers turned out to be sufficient to differentiate each of the 46 cultivars in this study by a clear, individual allelic profile. As few as two markers (e.g., VVMD27 and VVMD5) were sufficient for the discrimination of the cultivars, but more loci were chosen in order to increase the polymorphism and thus reduce the probability of false identity. Since this project began, the number of available grape microsatellite markers has rapidly increased to about 400. Not all those markers are necessary for identification, and there is no question that not all these markers can be standardized by a comparative international research effort. But for the purpose of general data comparability across laboratories, every scientist working with additional microsatellite markers would be advised to select only clearly expressed and highly polymorphic markers and to define a set of cultivar-based reference alleles that represent the allelic ladder as completely as is practical. This would enable others to easily convert the data into comparable data catalogs.

Conclusion

The objective of the study was to test the general comparability and reproducibility of microsatellite data produced by different laboratories under varying local conditions. If the characterization of grapevine cultivars by microsatellite fragment analysis works universally and independently from analysis systems and laboratory equipment, each analysis of identical DNA samples must produce identical allelic profiles.

In the present study, our only requirement was the analysis of identical samples, in order to avoid problems of sample identity as observed by Röder et al. (2002) and Bredemeijer et al. (2002). DNA from 47 samples was then analyzed with six microsatellite markers. To allow comparability of the data among the partners, the data were coded according to reference alleles from 33 cultivars. To reduce discrepancies due to stutter and fluctuating fragment patterns, the optimization of protocols individually adapted to local laboratory equipment must be completed for each individual marker.

The most frequent alleles for all six markers can be provided by a set of 17 cultivars as follows: 'Barbera', 'Cabernet-Sauvignon', 'Cabernet franc', 'Chardonnay', 'Merlot', 'Muscat à petits grains blancs', 'Pinot noir', 'Sultanina', 'Silvaner', 'Traminer rot', 'Couderc 1616', 'Couderc 3309', 'Millardet et Grasset 101-14', 'Millardet et Grasset 420A', 'Richter 99', 'Richter 110', and 'Teleki 5C'. For applied viticulture studies, fundamental grapevine biology research and for management of grapevine germplasm, the accurate identification of cultivars is essential. The establishment and feeding of a uniform database with confirmed microsatellite profiles for true-to-type grapevine cultivar would support better and more rationalized management of grapevine collections.

Although the 46 cultivars employed in this study represent a small proportion of total cultivars, they nevertheless were chosen in order to be highly diverse. A total of 107 alleles were detected with the six markers (from 13 to 23 alleles each) with a mean value of 17.8 alleles per locus. This value is very high compared to other data published on grape. Bowers et al. (1999a) detected a mean of 11 alleles per locus with 350 French cultivars, and Sefc et al. (2000) detected a mean of 9.8 alleles per locus with a set of 164 cultivars. This number is, however, in the same range as was observed with a set of 58 rootstocks and one *V. vinifera* cultivar by Lin and Walker (1998) since they detected a mean of 17.6 alleles per locus. Our results were achieved by combining *V. vinifera* L. cultivars with interspecific hybrid rootstock cultivars.

The cultivars analyzed in this study include some of the world's major wine cultivars (e.g., 'Cabernet-Sauvignon', 'Chardonnay', 'Merlot'), an internationally important table grape and raisin cultivar ('Sultanina'), and some of the most important and widely grown rootstocks. Thus, the results presented here are likely to be of value in all grape-growing regions of the world.

We have used in this work only six loci, not all of which are localized on different linkage groups (Riaz et al. 2003). Complete and irrefutable identification will often require a larger number of loci. However, the main goal of this work was to facilitate the comparison of grape microsatellite data among laboratories. In doing so, we have thus established the foundation for an international grape-cultivar database that will benefit the entire grape community.

We have demonstrated the usefulness of the coding procedure. We encourage other communities working on genetic resources to develop a similar method of defining common and unique reference allele set (representing a large range of alleles and a ladder as complete as possible) for several SSR loci. This set of

reference alleles can then be used in order to code the data from several laboratories and would enable a very easy comparison of data and/or genetic resources.

Acknowledgements This work was supported in part by a European Union research grant (GENRES081) for the analysis and management of grapevine genetic resources throughout Europe. The authors wish also to thanks Peter Isaac, Christopher Owens and Peter Cousins for useful comments. The authors are grateful to Pr. Bruce Reisch for further comments, which helped improve this paper.

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