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ORIGINAL PAPER

High throughput analysis of grape genetic diversity as a tool for germplasm collection management

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Abstract Using 20 SSR markers well scattered across the 19 grape chromosomes, we analyzed 4,370 accessions of the INRA grape repository at Vassal, mostly cultivars of *Vitis vinifera* subsp. *sativa* (3,727), but also accessions of *V. vinifera* subsp. *sylvestris* (80), interspecific hybrids (364), and rootstocks (199). The analysis revealed 2,836 SSR single profiles: 2,323 *sativa* cultivars, 72 wild individuals (*sylvestris*), 306 interspecific hybrids, and 135 rootstocks, corresponding to 2,739 different cultivars in all. A total of 524 alleles were detected, with a mean of 26.20 alleles per locus. For the 2,323 cultivars of *V. vinifera*, 338 alleles were detected with a mean of 16.9 alleles per locus. The mean genetic diversity (GDI) was 0.797 and the level of heterozygosity was 0.76, with broad variation from 0.20 to 1. Interspecific hybrids and rootstocks were more heterozygous and more diverse (GDI = 0.839 and 0.865, respectively) than *V. vinifera* cultivars (GDI = 0.769), *Vitis vinifera* subsp. *sylvestris* being the least divergent with GDI = 0.708. Principal coordinates analysis distinguished the four groups. Slight clonal polymorphism was detected. The limit between clonal variation and cultivar polymorphism was set at four allelic differences out of 40. SSR markers were useful as a complementary tool to traditional ampelography for cultivar identification. Finally, a set of nine SSR markers was defined that was sufficient to distinguish 99.8% of the analyzed accessions. This set is suitable for routine characterization and will be valuable for germplasm management.

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Introduction

In crop species, the genetic diversity conserved in germplasms is large, but only a small portion of the resources is used in practice. The majority of germplasms in the world are derived from seeds (Koo et al. 2004). For highly heterozygous plants, however, this method of conservation is not suitable, and they must be maintained in other forms. Perennial plants can be conserved in ex-situ field collections. The management of such collections becomes complex, however, when thousands of accessions are involved. Redundancy should be reduced to a minimum, homogeneity and "true to type" plant material must be ensured, and the introduction of new accessions optimized. Identification of the plant material is thus crucial and represents the first step in germplasm management.

Grapevine (*Vitis vinifera* L.) is very diverse, with 6,000–10,000 cultivars believed to exist in the world (Galet 2000), and many grape collections (http://www.vitaceae.org/index.php/Grape_Germplasm_Resources). This large diversity is mostly due to the long history of grapevine cultivation (McGovern 2003), and vegetative propagation,

which has enabled the conservation of cultivars over centuries. There is also a large diversity of complex *Vitis* hybrids and rootstocks (Galet 2000).

Ampelography has long been the single method used for identifying grape cultivars (Boursiquot and This 1996) but, as this process is carried out on adult plants, a long period is required before the identification of accessions can be completed. Since many synonyms or homonyms exist for cultivars (This et al. 2006), passport data are not always sufficient to certify identities and errors arise. In recent decades, new DNA-based methodologies were implemented, enabling easier and more accurate identification.

Several biochemical and molecular markers have been developed, such as isozymes, RFLP, and RAPD, but have proven to be unsuitable for management purposes in grape (Sefc et al. 2001). Rapid and robust systems of identification through the use of microsatellites have been proposed (Thomas and Scott 1993). These markers, based on the variation in numbers of a repeated element of 1–6 bases, have been used for identification purposes in many different species (Gupta and Varshney 2000) including grape (Thomas et al. 1993; Sefc et al. 2001). Such highly polymorphic markers are of particular interest since data can be compared between different laboratories through the use of a reference panel (This et al. 2004). Until recently, microsatellite markers were only used on relatively small sample sizes (165 cultivars by Sefc et al. 2000, 222 cultivars by Aradhya et al. 2003). In the last few years though, larger studies have been performed by Santana et al. (2010), Cipriani et al. (2010), and Ibanez et al. (2009) with the analysis of 425, 745 and 991 accessions, respectively.

The French INRA grape repository at Domaine de Vassal is the largest collection in the world, with a total of 7,500 accessions, more than 5,500 of which are *V. vinifera* cultivars (Marseillan plage, France, http://www1.montpellier.inra.fr/vassal/). The present project was launched to evaluate whether SSR markers could be effective for germplasm management of numerous accessions of *Vitis*, a genus with high cross-fertility (Levadoux et al. 1962), many close relatives (Bowers et al. 1999b; Di Vecchi-Staraz et al. 2007; Boursiquot et al. 2009), low clonal variation (Franks et al. 2002) and unconventionally defined cultivars (Boursiquot and This 2000). We analyzed 4,370 accessions representing different botanical levels (*Vitis vinifera* subsp *sativa*, *Vitis vinifera* subsp *sylvestris* and other *Vitis* species), and clonal diversity that may or may not be linked to morphological variation. We employed 20 SSR microsatellite markers to analyze the accessions, optimizing the analysis from the sampling stage to data management to facilitate the study of this large and diverse sample. We then evaluated the ability of SSR markers to differentiate grape cultivars, clones and interspecific material, and defined a minimal set of markers suitable for identity and homogeneity testing in the collection. In addition, we estimated the genetic diversity in a large sample of *V. vinifera* cultivars.

Materials and methods

Plant material and DNA extraction

We analyzed 4,370 accessions corresponding to 3,727 *Vitis vinifera* subsp. *sativa* accessions, 80 *Vitis vinifera* subsp. *sylvestris* individuals, 364 interspecific *Vitis* hybrid accessions used for fruit production and 199 *Vitis* rootstock accessions (Table 1); these four categories will be referred to hereafter as Sativa, Sylvestris, Hybrids, and Rootstocks, respectively. Five plants of each of these accessions are maintained on their own roots in sandy soil in a vineyard at the INRA "Domaine de Vassal" germplasm collection. Disks of 20 mm diameter made in young expanded leaves from one plant (40–50 mg of fresh material) were collected in spring and directly placed into 96-well microtube racks. Immediately after harvesting, samples were freeze-dried for 24 h at 0.370 mbar and −55°C. One 3-mm diameter iron bead was added to each microtube. Freeze-dried leaf tissues were ground twice for 1 min at 20 Hz. DNA was extracted according to Qiagen DNeasy plant mini kit protocol with minor modifications: addition of 1% w/v PVP-40 to the AP1 solution, addition of 180 µl AP2, and centrifugation at 6,000 rpm for 10 min (QIAGEN, Germany).

SSR amplification, multiplexing, sequencing conditions, and coding of alleles

A set of 20 SSR markers scattered throughout the genome was selected, providing high number of alleles. Of these markers, 11 were from previous studies (This et al. 2000, 2004) and 9 from a genetic map (Adam-Blondon et al. 2004), chosen according to their position and ease of genotyping (Table 2; Fig. 1). PCR and electrophoresis were performed as previously described by Adam-Blondon et al. (2004), with slight modifications: amplifications were carried out in a 20 µl reaction mix and PCR products were diluted 5 times for multiplex A and 10 times for multiplexes B and C. Different primer multiplexes and different amplification mixtures were tested. The final combination lead to eight PCR and three sequencing runs (Table 3). The separation of the fragments was carried out in an AB 3130 filled with POP 7 polymer (Applied Biosystems, CA). Peak sizes (decimal size) were determined using Genescan 3.7 software (Applied Biosystems), by comparing the data to an internal size marker (GENESCAN HD 400 ROX). Genotyper 2.5 software (Applied Biosystems) was used to round up the alleles. PCR for missing data were carried out in

Table 1 Distribution of the number of analyses according to the genetic origin of the material

	Total number of analyses	Number of single profiles	Number of different cultivars	Number of accessions from same cultivars	Number of cultivars assumed to be different	Number of accessions that underwent identification testing
Sativa	3,727	2,323	2,227	258	2,046	1,424
Sylvestris	80	72	72	1	79	79
Hybrids	364	306	306	23	244	96
Rootstocks	199	135	134	23	111	65
Total	4,370	2,836	2,739	305	2,401	1,664

Sativa = *Vitis vinifera* subsp. *sativa*, Sylvestris = *Vitis vinifera* subsp. *sylvestris,* Hybrids = complex interspecific *Vitis* hybrids used for fruit production, Rootstocks = *Vitis* hybrids of *Vitis* species used as rootstocks

simplex in order to obtain a better amplification product, and, whenever possible, were mixed before electrophoresis. In a few cases, no data was obtained despite three or four repetitions, due to multiband profiles or defective DNA.

Database development

A Microsoft Access® database was developed for management of the data produced during this project. It is connected to the online database used to manage the Vassal collection (http://bioweb.ensam.inra.fr/collections_vigne). For each analysis, the raw data, coded alleles, PCR amplification, date, run date, harvesting date, and the name of the person who performed each step were stored. Each data record is represented by a single code. In the code CRB03A01-1-a, for example, 03A01 is the DNA code corresponding to the plate number and the position in the rack, -1 is the SSR marker code, and -a represents the first analysis, -b the second (if relevant), and so on. DNA may have been extracted from a given plant several times, and analyzed with many markers; a marker may even have been amplified more than once for the same DNA. Each DNA code is linked to a single plant and location in the Vassal repository. Several queries were created to sort the alleles, check for duplicate analyses or missing data, as well as to compare and to identify samples.

Data checking

Chromatograms were read by two persons. Differences of 1 bp between alleles were checked by reamplification and reanalysis to determine whether a coding error had occured, or whether this was a real allele. Whenever present, replicates of the same accessions were compared. In cases of mismatch, data for both replicates were discarded and a new analysis performed on newly collected plant material. At the end of the study, 4–5 samples of each of the 50 initial runs were analyzed to verify the absence of a shift in the allele coding during the experiment. No coding differences were observed for the 244 alleles (out of 524 detected in this study) represented in this control panel. As further verification, allele frequencies were compared between each of the 50 runs performed and the global analysis. Apart from runs corresponding mainly to Sylvestris, Hybrids, or Rootstocks, no shift in allele size was revealed (data not shown).

Data analysis

Data analysis was performed using the "Excel Microsatellite Toolkit" (Park 2001). The number of alleles and allele frequencies were determined; the diversity index $Hep = \Sigma p_i^2$ (GDI; Weir 1989) and the discriminating power $D = 1 - \Sigma p_i^2$ (DP; Kloosterman et al. 1993) were then calculated from allele and genotype frequencies, respectively.

Simple matching distances (SMD, Bowcock et al. 1994) were calculated based on the 20 SSR markers and Principal coordinates analysis constructed using DARwin software (version 5.0.23; Perrier and Jacquemoud-Collet 2006). The probability of identity (PI) was computed using Famoz software (Gerber et al. 2003).

Results

Among the 4,370 accessions analyzed with 20 SSR markers, 2,836 single SSR_{20} profiles with a difference of at least one allele, or 2,739 profiles without clonal variation, were detected (Table 1). Despite the great care taken to reduce missing data (MD), about 0.89% of MD remained (Table 4). These represented 272 profiles (192 with 1 MD, 39 with 2 MD, 17 with 3 MD, 5 with 4 MD, 13 with 5–10 MD, 6 with 10–16 MD). Rootstocks and Hybrids presented slightly higher percentages of missing data than the other sources: 2.03% and 3.48%, respectively. The number of alleles and the distribution of Sativa and Sylvestris populations according to their geographic origin are presented in Table 5. Supplementary Table S1 shows the distribution of

Table 2 Names, linkage groups, primer sequences, references, and microsatellite sequences of the SSRs studied

SSR name	Linkage group	Forward primer	Reverse primer	Reference	Microsatellite core repeat
VMC1b11	8	CTTTGAAAATTCCTTCCGGGTT	TATTCAAAGCCACCCGTTCTCT	BV681754	(GA)n
VMC4f3	12	AAAGCACTATGGTGGGTGTAAA	TAACCAATACATGCATCAAGGA	Di Gaspero et al. (2000)	(CT)nTT (CT)n
VVIb01	2	TGACCCTCGACCTTAAAATCTT	TGGTGAGTGCAATGATAGTAGA	Merdinoglu et al. (2005)	(CT)n
VVIh54	13	CCGCACTTGTGTTGAATTTCAG	CAAACCGTTTTTACACCAGCAG	Merdinoglu et al. (2005)	(GA)n
VVIn16	18	ACCTCTATAAGATCCTAACCTG	AAGGGAGTGTGACTGATATTTC	Merdinoglu et al. (2005)	(CA)nCG (CA)n
VVIn73	17	TACTTCACCTAACAATACAGCT	AATACATAAGGTGAAGATGCCT	Merdinoglu et al. (2005)	(CA)n
VVIp31	19	TATCCAAGAGACAAATTCCCAC	TTCTCTTGTTTCCTGCAAATGG	Merdinoglu et al. (2005)	(GA)n
VVIp60	1	GGGGAATAACTAAATTGAGGAT	GTATGAATGCGGATAGTTTGTG	Merdinoglu et al. (2005)	(TG)nA(GT)n(GA)n
VVIq52	9	TAAAAGGATGGTAGATGACAGA	ACAGGAAAGTGTTCAATGGTTA	Merdinoglu et al. (2005)	(CT)n
VVIv37	10	GGTAGACCTTGAAATGAAGTAA	ATGCTGAAGTCACGTAATAGAA	Merdinoglu et al. (2005)	(TC)n(GT)n
VVIv67	15	TATAACTTCTCATAGGGTTTCC	TTGGAGTCCATCAAATTCATCT	Merdinoglu et al. (2005)	(CA)nAT(CA)n(GA)nTT(GA)n(AG)n
VVMD21	6	GGTTGTCTATGGAGTTGATGTTGC	GCTTCAGTAAAAAGGGATTGCG	Bowers et al. (1999a)	(CT)nGAGAAGG(A)n
VVMD24	14	GTGGATGATGGAGTAGTCACGC	GATTTTAGGTTCATGTTGGTGAAGG	Bowers et al. (1999a)	(CT)n
VVMD25	11	TTCCGTTAAAGCAAAAGAAAAAGG	TTGGATTTGAAATTTATTGAGGGG	Bowers et al. (1999a)	(CT)n
VVMD27	5	GTACCAGATCTGAATACATCCGTAAGT	ACGGGTATAGAGCAAACGGTGT	Bowers et al. (1999a)	(CT)n
VVMD28	3	AACAATTCAATGAAAAGAGAGAGAGAGA	TCATCAATTTCGTATCTCTATTTGCTG	Bowers et al. (1999a)	(CT)n
VVMD32	4	TATGATTTTTTAGGGGGGTGAGG	GGAAAGATGGGATGACTCGC	Bowers et al. (1999a)	(CT)n
VVMD5	16	CTAGAGCTACGCCAATCCAA	TATACCAAAAATCATATTCCTAAA	Bowers et al. (1996)	(CT)nAT(CT)nATAG(AT)n
VVMD7	7	AGAGTTGCGGAGAACAGGAT	CGAACCTTCACACGCTTGAT	Bowers et al. (1996)	(CT)n
VVS2	11	CAG CCC GTA AAT GTA TCC ATC	AAATTCAAAATTCTAATTCAACTGG	Thomas and Scott (1993)	(GA)n

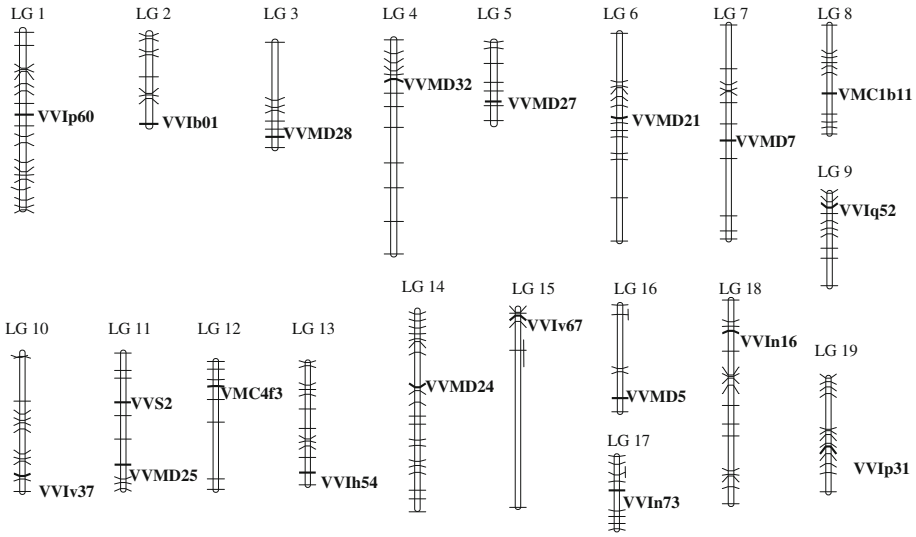

Fig. 1 Position of SSR loci on the consensus map of *Vitis vinifera* (Adam-Blondon et al. 2004; Doligez et al. 2006)

Table 3 PCR and multiplex conditions used

SSR marker	PCR multiplex[a]	Annealing T °C	Labeled primer concentration in pM	Run multiplex[b]	Dye	Size range in base pairs
VMC1b11	8	60	0.04	C	Fam	165–208
VMC4f3	8	60	0.05	C	Hex	156–230
VVIb01	7	56	0.08	C	Hex	278–318
VVIh54	6	56	0.04	C	Ned	139–187
VVIn16	3	56	0.04	A	Ned	141–175
VVIn73	7	56	0.04	C	Ned	254–269
VVIp31	4	56	0.04	B	Fam	158–210
VVIp60	2	56	0.08	A	Hex	291–348
VVIq52	6	56	0.05	C	Fam	71–89
VVIv37	4	56	0.08	B	Hex	141–181
VVIv67	1	56	0.08	A	Fam	305–386
VVMD21	2	56	0.05	A	Hex	218–267
VVMD24	5	56	0.065	B	Hex	200–226
VVMD25	7	56	0.1	C	Fam	234–272
VVMD27	2	56	0.065	A	Hex	172–218
VVMD28	1	56	0.1	A	Fam	214–282
VVMD32	5	56	0.065	B	Fam	217–289
VVMD5	3	56	0.065	A	Ned	216–287
VVMD7	5	56	0.08	B	Ned	231–268
VVS2	4	56	0.065	B	Ned	120–165

[a] SSR markers with the same number were amplified in a single PCR mix, all primers being pooled in the PCR mix

[b] SSR markers with the same letter were pooled and analyzed in the same sequencer run

allele frequencies and supplementary Table S2 the coded SSR data for the 47 cultivars used as references in This et al. (2004).

Genetic diversity

A total of 524 alleles (26.20 alleles on average; Table 4) were revealed, but this number fell to 414 and 380 when very rare alleles (i.e. those with frequencies lower than 0.05% or 0.1%) were removed. These numbers were also lower when considering Sativa, Sylvestris, Rootstocks, and Hybrids separately (Table 4). The overall diversity GDI was also high (0.797 ± 0.1046), but this included differences between Sativa, Sylvestris, Hybrids, and Rootstocks (Table 6, supplementary Table S3). The number of alleles per locus over the whole sample varied between 9 (VVIq52 and VVIn73) and 59 (VVIv67) in total, while GDI varied between 0.462 for VVIn73 and 0.903 for VVIp31.

Table 4 Number of alleles, number of genotypes, and percentage of missing data (MD) with regard to the material type, minima and maxima are indicated in *bold*

SSR marker	Total (2,836)			Sativa (2,323)			Sylvestris (72)			Hybrids (306)			Rootstocks (135)		
	Alleles	Genotypes	MD %	Alleles	Genotypes	MD %	Alleles	Genotypes	MD %	Alleles	Genotypes	MD %	Alleles	Genotypes	MD %
VMC1b11	19	108	0.60	15	71	0.43	9	20	1.39	14	60	1.31	18	57	1.48
VMC4f3	52	275	1.62	31	**162**	0.86	**18**	27	**4.17**	**40**	**137**	2.94	**39**	70	10.37
VVIb01	19	69	0.46	13	23	**0.13**	7	12	2.78	14	42	1.63	15	49	2.22
VVIh54	24	140	0.71	22	88	0.56	11	23	0	18	64	1.96	17	53	**0.74**
VVIn16	17	56	**0.32**	**6**	**17**	0.26	**5**	14	1.39	15	35	**0.33**	16	43	**0.74**
VVIn73	9	29	0.71	8	19	0.43	6	11	2.78	7	15	1.31	8	**12**	2.96
VVIp31	31	189	0.78	18	95	0.69	11	22	0	26	100	0.65	24	77	2.96
VVIp60	27	133	1.06	20	81	0.77	8	19	1.39	22	73	2.94	22	64	1.48
VVIq52	**9**	**26**	0.56	8	24	0.30	**5**	**8**	0	**6**	**12**	0.65	7	**12**	5.19
VVIv37	21	129	1.48	18	96	1.33	10	17	1.39	17	62	1.96	16	47	2.96
VVIv67	**59**	**285**	1.30	**36**	151	0.90	17	**33**	2.78	36	107	1.96	**39**	**91**	5.93
VVMD21	26	103	0.56	11	37	0.22	9	14	0	20	61	1.96	21	46	3.70
VVMD24	18	62	0.46	10	37	0.30	7	17	0	15	39	0.98	14	26	2.22
VVMD25	28	117	0.78	16	57	0.39	10	21	1.39	18	47	3.27	23	60	1.48
VVMD27	27	145	0.53	12	50	0.30	9	18	0	25	71	1.96	25	76	1.48
VVMD28	33	223	1.83	25	125	**1.51**	14	28	1.39	24	113	3.59	22	77	3.70
VVMD32	32	121	**2.22**	21	84	0.90	12	28	0	22	55	**6.89**	25	39	**15.56**
VVMD5	31	149	0.56	14	62	0.47	11	18	1.39	23	95	0.65	19	60	1.48
VVMD7	19	133	0.74	17	78	0.39	14	24	1.39	18	76	2.62	17	56	2.22
VVS2	23	138	0.56	17	94	0.52	10	21	0	20	82	0.98	19	59	**0.74**
Total	524	2,630		338	1,451		203	395		400	1,346		405	1,074	
Average	26.20	131.50	0.89	16.90	72.55	0.58	10.15	19.75	1.18	20.00	67.30	2.03	20.25	53.70	3.48
SD	12.17	70.96	0.58	7.90	42.19	0.15	3.51	7.31	1.17	8.01	31.94	1.90	8.47	21.00	3.83

Table 5 Geographical origin of Sativa and Sylvestris samples and corresponding number of alleles

Group name	Countries	Sativa sample		Sylvestris sample	
		Number of sample	Number of allele	Number of sample	Number of allele
Maghreb	Algeria, Morocco, Tunisia	88	217	16	125
Iberian Peninsula	Portugal, Spain	254	226	3	47
Western and Central Europe	Austria, Belgium, Czech republic, France, Germany, Netherlands, Slovakia, Switzerland, United Kingdom	744	277	50	186
Italian Peninsula	Italy	328	258		
Balkans	Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, Greece, Hungary, Romania, Yugoslavia	396	286	3	64
Russia and Ukraine	Moldova, Russia, Ukraine	107	207		
Eastern Mediterranean and Caucasus	Armenia, Azerbaijan, Egypt, Georgia, Israel, Lebanon, Syria, Turkey	155	215		
Middle and Far East	Afghanistan, China, India, Iran, Japan, Kazakhstan, Tajikistan, Turkmenistan, Uzbekistan, Yemen	90	216		
New World vineyards	Argentina, Australia, Chile, Mexico, Peru, South Africa, United States	115	228		
Non determined		46	185		

Hybrids and Rootstocks revealed the highest number of alleles (400 and 405, respectively) and the highest GDI (0.839 and 0.865, respectively), despite their relatively small sample size compared to the Sativa sample (GDI = 0.769). The Sylvestris sample presented only 203 alleles and the lowest GDI (0.708). For the Sativa sample, a total of 338 alleles were detected and the number of alleles per locus ranged from 6 (VVIn16) to 36 (VVIv67; Table 4).

The analysis revealed a high heterozygosity level ranging from 0.79 (Hybrids) to 0.62 (Sylvestris) with a mean of 0.76 and differences between loci (Table 6, supplementary Table S4). Individual heterozygosity ranged from 20% for Sativa cultivar Buckland Sweet Water (16 homozygote loci) to 100% for 18 accessions (20 heterozygous loci; supplementary Table S5). Sylvestris presented a shift towards lower degree of heterozygosity, and Hybrids and Rootstocks towards higher degree of heterozygosity (Fig. 2). Sixty-six percent of individuals in the collection had more than 15 heterozygous loci.

Simple matching pairwise distances (SMD) were computed for the 2,836 single profiles. The pairwise distance ranged from 0.2 to 1 and followed a normal curve that was slightly skewed towards large distances (Fig. 3). The mean distance between the Sativa individuals was 0.714, but was higher for Hybrids and Rootstocks (0.784 and 0.790, respectively).

The DP of each marker was calculated on 2,739 different profiles, i.e., those that differed at four or more loci. DP was 0.921 on average (SD = 0.073) with values ranged from 0.666 for VVIn73 to 0.981 for VVIp31 (Table 6 and supplementary Table S6); it was slightly lower for Sativa (between 0.574 for VVIn73 and 0.978 for VVIp31), and higher for Hybrids and Rootstocks.

To test the effectiveness of the markers in discriminating between interspecific material and/or subspecies, a Principal coordinates analysis was performed on the whole dataset (Fig. 4). The first two axes accounted for 2.9% and 2.67% of the total variation, respectively. The partitions between Hybrids and Rootstocks and between Sativa and Sylvestris were clear, despite the presence of overlapping zones.

Definition of a reduced set of marker

The SSR markers were very useful for identification purposes. Among the 1,664 accessions that underwent identification testing in the present study, 824 displayed a single pattern and were thus considered as original cultivars, while 840 corresponded to cultivars already identified and were thus defined as another accession of the same

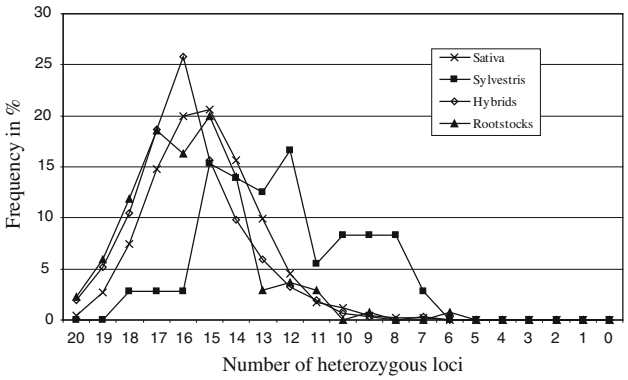

Fig. 2 Variation of the number of heterozygous loci per individual for each group calculated on single profile

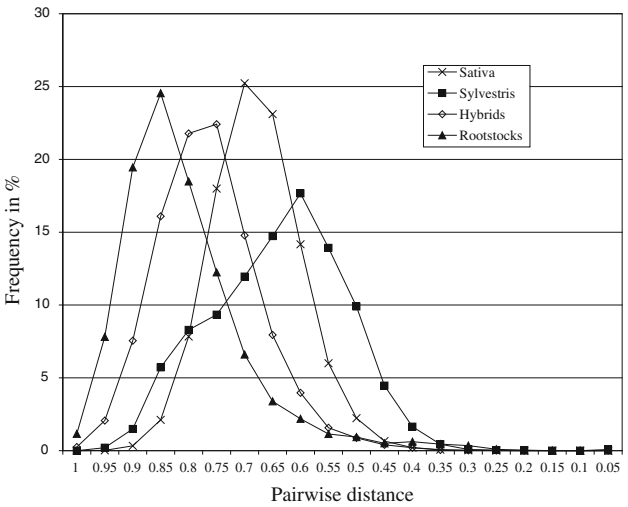

Fig. 3 Variation of pairwise distances calculated on single profile for each group

Table 6 Diversity index (GDI), frequency of heterozygosity and discriminating power (DP), value and standard deviation

	GDI	Frequency of heterozygosity	DP
Sativa	0.7689 ± 0.1207 (ab)	0.76 ± 0.12 (b)	0.9030 ± 0.0914 (ab)
Sylvestris	0.7075 ± 0.0879 (a)	0.62 ± 0.13 (a)	0.8547 ± 0.0725 (a)
Hybrids	0.8386 ± 0.0792 (bc)	0.79 ± 0.11 (b)	0.9414 ± 0.0523 (b)
Rootstocks	0.8650 ± 0.0910 (c)	0.78 ± 0.17 (b)	0.9444 ± 0.0568 (b)
Total	0.7969 ± 0.1046	0.76 ± 0.11	0.9211 ± 0.0733

ANOVA gives a significant *F*-test for each measured variable. Values with *different letters* in *parenthesis* are significantly different at $P < 0.005$ using multiple paired *t*-test

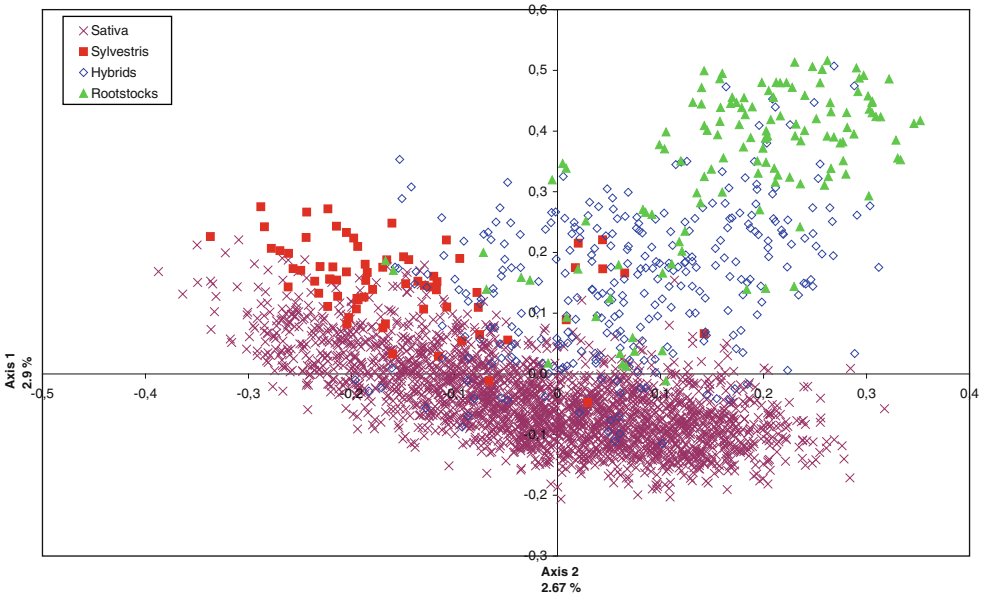

Fig. 4 Principal coordinates analysis constructed with 2,836 individuals using Darwin software

cultivar or synonyms. More interestingly, 1,050 cases of questionable synonymies were discovered or confirmed with the SSR analysis and validated at the morphological level.

Analysis using 20 SSRs may, however, be quite heavy for routine investigations. The PI was calculated for each marker in order to select a set of markers sufficient for the discrimination of the 2,739 cultivars. When arranged according to PI and cumulative PI, a set of eight markers (VVIp31, VVMD28, VVMD5, VVS2, VVIv37, VMC 1b11, VVMD27, VVMD32) was sufficient to identify all the cultivars (Table 7). For efficient, strong, and secure identification, analysis should, however, be performed using easy-to-score and reliable markers. Based on these limitations and the technical considerations for multiplexing, several of the SSR markers identified in this set were discarded and a second analysis was run. Another set of nine SSR markers was then defined (VVMD5, VVMD27, VVMD7, VVMD25, VVIh54, VVIp60, VVIn16, VVIb01, VVIq52; Table 8). Cumulative PI for this set reached 4.71 E-11. This minimal set of nine SSRs is now routinely used in our laboratory for identification purposes and for checking the homogeneity of the accessions.

Discrimination of *V. vinifera* subsp. *sativa*: cultivars versus clones

Another objective of this work was to test whether SSR markers could easily identify cultivars and clones when applied to a very large grape collection. With this objective in mind, we analyzed a number of cultivars that can be easily differentiated at the morphological level, clones of referenced cultivars, different accessions of the same cultivar and mutants considered as different cultivars (such as colored or aromatic mutants).

With the sole exception of the Aramon and (Petit Bouchet × Morrastel) no3 pair, which differed in only one allele, every single accession corresponding to a different cultivar diverged from the others by at least four alleles (SMD = 0.1). The next closest cultivars, differentiated by four alleles, were either self-pollinated progeny of the parental cultivar or pairs of full sibs (see supplementary Table S7).

Out of the 4,370 accessions analyzed, 1,631 corresponded to accessions of clonal origin or with similar names, and among these 484 were not discriminated. These corresponded to 305 accessions of the same cultivars (duplicates) and 179 known mutants of 86 referenced cultivars such as Pinot blanc, Pinot gris, Pinot teinturier, which are known mutants of Pinot noir or duplicate individuals of wild origin. The 97 cases of differentiated clones (5% of the clones, 2% of the total) revealed between 1 and 4 differences (supplementary Table S7). In some cases, we identified the series (i.e. the wild type and mutants with 1 or 2 differences) as, for example, Sultanine, Gora Chirine, and Sultanine Monococco. The only mutants distinguishable by four differences were Orbois blanc and Orbois rose. The molecular differences observed in this analysis were homozygote versus heterozygote differences (Grolleau noir is 360/360 whereas Grolleau gris is 360/371 at the VVIv67 locus) or size shifts in 1 allele (Pinot noir is 135/149 whereas Meunier is 126/135 at the VVS2 locus; Chasselas blanc is 163/180 whereas Chasselas Muscat is 180/192 at the VVIp31 locus; Carignan noir is 315/324 whereas Carignan blanc is 315/326 at the VVIp60 locus).

Table 7 Observed probability of identity calculated from 2,739 single accessions using Famoz software on 18 SSRs

SSR marker	Number of identical pairs of genotypes	Probability of identity	Cumulative probability of identity
VVIp31	68,553	0.017614	0.017614
VVMD28	1,937	0.019307	3.40 E−004
VVMD5	150	0.027619	9.39 E−006
VVS2	28	0.036684	3.45 E−007
VVIv37	14	0.038352	1.32 E−008
VMC1b11	5	0.040276	5.32 E−010
VVMD27	3	0.041122	2.19 E−011
VVMD32	2	0.041864	9.16 E−013
VVMD7	0	0.043336	3.97 E−014
VVMD25	0	0.065472	2.60 E−015
VVIh54	0	0.070242	1.83 E−016
VVIp60	0	0.073692	1.35 E−017
VVMD21	0	0.103113	1.39 E−018
VVMD24	0	0.114785	1.59 E−019
VVIn16	0	0.115865	1.85 E−020
VVIb01	0	0.133273	2.46 E−021
VVIq52	0	0.161869	3.98 E−022
VVIn73	0	0.314569	1.25 E−022

Table 8 Observed probability of identity calculated from 2,739 single accessions using Famoz software on nine SSRs

SSR marker	Number of identical pairs of genotypes	Observed probability of identity
VVMD5	106,545	0.027619
VVMD27	6,458	1.14 E−03
VVMD7	466	4.92 E−05
VVMD25	71	3.22 E−06
VVIh54	21	2.26 E−07
VVIp60	11	1.67 E−08
VVIn16	4	1.93 E−09
VVIb01	2	2.58 E−10
VVIq52	2	4.71 E−11

Discussion

The present work describes the SSR analysis of the INRA Vassal grape collection. The objectives were to estimate the usefulness of SSR markers as a management tool to complement morphological descriptions.

Development of a high throughput protocol

In order to allow the analysis of the grape collection, it was necessary to develop a high-throughput protocol, from plant harvesting to data storage and analysis. Two points are essential in this protocol: the standardization of plant harvesting (use of leave disks and 96-well microtube racks) and the optimization of the SSR analysis (design of multiplexes and use of capillary sequencers). The development of a relational database was also very helpful to manage the experiments and handling such a large amount of data.

Despite this high-throughput protocol, we reduced the percentage of MD to less than 1% by repeating certain experiments up to three times. However, a further reduction in this percentage would require an unreasonably high effort. Part of these MD corresponded to multiallelic genotypes, as already encountered in clonal variation (Franks et al. 2002) and analysis is underway to clarify such data. Other MD could correspond to homozygous null alleles, especially for those obtained with Rootstocks and Hybrids (for example, for VMC4f3 or VVMD32). The primers were designed on *Vitis vinifera* and the probability of null alleles is greater when they are used on other *Vitis* species and even outside the genus (Arnold et al. 2002). Finally, MD could correspond to low DNA quality, despite the fact that several DNA extractions were made. Markers with the highest percentage of MD were removed from the minimal set definition.

Use of SSR markers to differentiate subspecies or accessions of interspecific origin

Even though the analysis was mostly intended for the cultivated *Vitis vinifera*, we also included accessions of *Vitis vinifera* subsp. *sylvestris*, the wild relative of domesticated grape, as well as accessions issued from interspecific crosses.

The set of 20 SSR markers developed in this work clearly differentiated the Hybrids and, to an even greater extent, the Rootstocks. Indeed, few Rootstocks used worldwide have partly the *V. vinifera* genome, whereas all of the Hybrids, which were mostly created during the beginning of the twentieth century, contain a portion of it. Differentiation of the Sylvestris subspecies is less apparent, but the position of the main cluster of Sylvestris individuals at the periphery of the Sativa group nevertheless confirms the distinction of both compartments by SSR, as already stated by Grassi et al. (2003), and the status of two distinct subspecies. Interestingly, some accessions supposedly of wild origin clustered with the cultivated group, probably as a result of gene flow (Di Vecchi-Staraz et al. 2009).

How easily can SSRs distinguish clones and cultivars?

The SSR markers have often been considered very interesting at the cultivar level, since they can easily distinguish between different cultivars; however, they are less effective in differentiating clones (Sefc et al. 2000). In the present paper, we analyzed 4,370 accessions chosen to represent very different cultivars (genetic distance close to 1) and very close cultivars (siblings issued from the same crosses), as well as clones, mutants and different accessions of the same cultivar.

With the exception of two pairs attested by morphology, the cultivars showed at least four allelic differences, while clones showed fewer than four allelic differences. (Petit Bouschet × Morrastel) no3 cultivar is the result of a cross between Petit Bouschet and Aramon noir, and Petit Bouschet is a descendant of Aramon noir and Teinturier du Cher. Aramon is thus present twice in the parentage of (Petit Bouschet × Morrastel) no3, which explains their close relationship. Orbois blanc is related to either Gouais blanc or Savagnin blanc and is an old French cultivar with unexplained clonal variation.

Given that the diversity and discriminating power of the markers in the present analysis are similar to or greater than markers in other published works (Sefc et al. 2000; Aradhya et al. 2003; Martinez et al. 2006; Ibanez et al. 2009; Cipriani et al. 2010), and that we have analyzed a number of different cases, we believe that all existing cultivars could be discriminated using the 20 SSRs. Furthermore, additional markers have been used in specific cases (Di Vecchi-Staraz et al. 2007, 2009; Boursiquot et al. 2009) and conclusions were similar to those obtained with these 20 SSRs.

Would other markers be more appropriate than SSRs for cultivars and/or clonal distinction? Recent advances in marker technologies in grape have shed new light on the use of either longer SSR repeats (Cipriani et al. 2008, 2010) or SNP markers for cultivar identification (Vezzulli et al. 2008). Although longer repeats may be easier to read, the smaller number of alleles revealed would increase the total number of markers required. Indeed, Cipriani et al. (2010) detected 8.06 alleles with 34 SSRs in their sample of cultivated grape. Despite the great interest to use SNP markers for plant genetic analysis (Rafalski 2002), they still involve much work to develop and are more onerous to analyze than a well-optimized multiplexed SSR analysis. Nevertheless, Myles et al. (2010) developed a 9K genotyping SNP array on *Vitis* species, which could be used to rapidly genotype thousands of cultivars at a lower cost.

Although SSR markers are appropriate for cultivar identification, considering the low level of diversity between clones revealed with this technique (2% of the plants, 138 alleles concerned), SSR markers are definitely not well suited to clonal identification, as already mentioned (Imazio et al. 2002; Cipriani et al. 2010). Clonal variation associated with color differences can also be differentiated using the *VvMybA1* gene, a transcription factor involved in the qualitative and quantitative control of color (This et al. 2007), as recently carried out on a few dozen cultivars (Giannetto et al. 2008). Similarly, genes involved in the aroma traits (Duchêne et al. 2009) could also be used to distinguish aromatic mutants. Other methods based on transposable elements (Pelsy 2007), DNA methylation (Schellenbaum et al. 2008), copy number variation (CNV), grape genome sequencing (Jaillon et al. 2007), or next generation sequencing (NGS) could also be used.

A minimal set of SSR markers for routine analysis

We demonstrated that, apart from the Aramon/(Petit Bouschet × Morrastel) no3 and Muscat Ottonel/Muscat Saint-Laurent pairs, any cultivar can be distinguished from another of the 2,835 single SSR profiles analyzed in this study through the use of a set of nine easily multiplexed SSR markers. Since our sample contains the main cultivars grown in the world, most commercial issues should be covered. Although minimal, this set of markers nonetheless proved to be discriminatory, since cumulative PI (4.11 E-11) is still much greater than the value obtained with larger sets (Fernandez-Gonzalez et al. 2007; Zoghlami et al. 2009). However, endangered or important genetic resources may not be covered by this set and additional markers should be added to increase the confidence of the analysis in specific cases.

We have demonstrated that the SSR markers are very useful for germplasm management, as already observed in grape and many other species (Gupta and Varshney 2000; Dangl et al. 2001). Out of the 1,664 accessions not previously identified, half were identified with certainty to cultivars already existing in the collection, and the other half were considered as original cultivars. Comparisons of data should help us to identify accessions and synonyms. In

light of these results, international initiatives such as GRAPEGEN06, launched under the EU commission financial support (Maul and This 2008; Bacilieri 2007), should help to clarify identities.

Grape genetic diversity

This paper constitutes the largest analysis of genetic diversity in grape and confirms previous analyses suggesting that grape is a very diverse species (Martinez et al. 2006; Ibanez et al. 2009). Our analysis provides a broader, more global estimation of the genetic diversity: GDI on average is quite high for *V. vinifera* (0.769) and even higher for Rootstocks and Hybrids. It is as diverse as poplar (Smulders et al. 2008), rose (Esselink et al. 2003), wild populations of rice (Gao et al. 2006), and much more diverse than tomato (Ranc et al. 2008) or wheat (Peng et al. 2009). High genetic distance is a good indication that grape has been widely exchanged and crossed in order to increase its diversity level (This et al. 2006).

However, *V. vinifera* was found to be less diverse than Hybrids or Rootstocks, in accordance with previous observations (De Andrès et al. 2007). *V. vinifera* subsp. *sylvestris* is less diverse than the domesticated forms, which could be due to the scarcity of the endangered wild form, small natural populations and the small number of samples available in the collection.

This analysis also confirms the very high heterozygosity of grape, although a few cultivars with low heterozygosity can also be found. These could be of substantial interest to the grape genomic community, as already demonstrated by the sequencing of the near-homozygous PN40024 line (Jaillon et al. 2007).

The next steps will now involve parentage and structure analysis in *V. vinifera*, as already performed in rice (Allcochete et al. 2008) and maize (Vigouroux et al. 2008). As structure has been observed in the diversity of a core-collection derived from the INRA Vassal collection (Le Cunff et al. 2008), analysis should now be extended to the whole sample. Such studies are important to increase the usefulness of collections for genomic work through association analysis or the search for selective sweeps, in order to identify genes involved in quantitative traits.

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