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ORIGINAL PAPER

An extensive study of the genetic diversity within seven French wine grape variety collections

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Abstract The process of vegetative propagation used to multiply grapevine varieties produces, in most cases, clones genetically identical to the parental plant. Nevertheless, spontaneous somatic mutations can occur in the regenerative cells that give rise to the clones, leading to consider varieties as populations of clones that conform to a panel of phenotypic traits. Using two sets of nuclear microsatellite markers, the present work aimed at evaluating and comparing the intravarietal genetic diversity within seven wine grape varieties: Cabernet franc, Cabernet Sauvignon, Chenin blanc, Grolleau, Pinot noir, Riesling, Savagnin, comprising a total number of 344 accessions of certified clones and introductions preserved in French repositories. Ten accessions resulted in being either self-progeny, possible offspring of the expected variety or misclassified varieties. Out of the 334 remaining accessions, 83 displayed genotypes different from the varietal reference, i.e., the microsatellite profile shared by the larger number of accessions. They showed a similarity value ranging from 0.923 to 0.992, and thus were considered as polymorphic monozygotic clones. The fraction of polymorphic clones ranged from 2 to 75% depending on the variety and the set of markers, the widest clonal diversity being observed within the Savagnin. Among the 83 polymorphic clones, 29 had unique genotype making them distinguishable; others were classified in 21 groups sharing the same genotype. All microsatellite markers were not equally efficient to show diversity within clone collections and a standard set of five microsatellite markers (VMC3a9, VMC5g7, VVS2, VVMD30, and VVMD 32) relevant to reveal clonal polymorphism is proposed.

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Introduction

Grapevine is one of the oldest agricultural crops cultivated to produce table fruits, dry fruits, juice and wine. The number of different varieties or cépages held in germplasm collections around the world is estimated to be approximately 10,000 (Alleweldt and Dettweiler 1994). Among them only few hundreds are cultivated for commercial wine production (Truel et al. 1980). This great diversity probably originated from several mechanisms: multiple

domestication events from wild vines, the old practice of growing seedling from spontaneous or controlled crossing and to a lesser extent conventional breeding during the last century. Thanks to molecular markers, the pedigree of few varieties has been reconstituted. For example, Cabernet Sauvignon is an offspring of Cabernet franc and Sauvignon blanc (Bowers and Meredith 1997), Chardonnay and Gamay are two offspring of Pinot and Gouais (Bowers et al. 1999a) and three varieties from the Aosta Valley (Italy) are related as well, Cornalin du Valais being an offspring of Petit Rouge and Mayolet (Vouillamoz et al. 2003). The selected germplasms were asexually propagated, in some cases over centuries, yielding clones genetically identical to the parental plant as long as spontaneous mutations did not arise. Thus, traditionally, clones are attributed to a variety on the basis of several common ampelographic traits. Nevertheless, phenotypic variations between clones of a same variety are often observed and accepted. Such variation can be due to somatic mutations spontaneously occurring in the regenerative cells that gave rise to the clones. Polymorphism within varieties was also proposed to be due to polyclonality meaning that more than one seedling, marked with morphological uniformity, could give rise to a same grapevine variety (Rives 1961). For example, Fortana, which comprises clones sharing common morphological traits and uses but two distinct genotypic patterns, was thought to have a polyzygotic origin (Silvestroni et al. 1997).

Over the years, varieties are therefore constituted of clones with homogeneous phenotypic characters but that can show minor differences. Vine growers and winemakers have identified and propagated clones with particular agronomic traits that may include color or flavor variation, early or late ripening, or limited productivity. However, when clones of the same variety are different enough to be grown for different wine production they are considered as different cultivars (Boursiquot and This 1999) (Table 1). To improve vineyard, clonal selections are conducted by exploiting intravarietal diversity. The first selection was made in Germany based on visual selection and lead to the certification of the first elite clones in 1896 (Huglin and Schneider 1998). In France, clonal selection began in the 1960s, with both sanitary and genetic objectives. Each clone is selected through a process based on the evaluation of the viticultural characteristics and sanitary status of the best existing plants for each variety. This rigorous selection can last more than a decade to obtain a reduced assortment of clones for each variety, each registered under a unique certification number. Most of the French vineyards are currently planted with certified clones (Anonymous 2007a). However, exclusively planting a small number of elite clones threatens clonal diversity in the vineyard. To preserve the diversity that was present in ancient grapevine plots, prospecting has been carried out in France since the 1970s and regional repositories have been established. Repositories exist in France for at least 85 varieties, gathering a total of more than 14,000 introductions (Audeguin et al. 1998). In addition to saving clonal diversity, these collections are a resource to select and certify new clones as well as to understand the mechanisms of clonal diversification within each variety. The management of these collections requires tools to avoid redundancy, to track introductions that were wrongly assigned to a variety and to assist clone selection.

Table 1 Different levels of *Vitis vinifera* L. classification according to Boursiquot and This (1999)

Classification	Example	Reproduction mode (diversity origin)
Species	*Vitis vinifera*	Sexual reproduction (recombination)
Sub-species	*sativa*, *silvestris*	
Variety or cépage	Chenin, Pinot, Savagnin	
Cultivar	Pinot noir, Pinot blanc, Pinot gris Savagnin blanc, Savagnin rose, Gewurztraminer	Vegetative propagation (mutations)
Clone or introduction	CTPS.220, 293.Ang.0.1415	

Somatic variation contributes to the genetic evolution of higher plants, particularly in vegetatively propagated crop species (D'Amato 1997). Molecular basis of spontaneous mechanisms that promote the genome plasticity of clones is poorly understood (Arencibia et al. 2005; Breto et al. 2001). Nevertheless, the identification of certified clones in fruits species requires the application of fast and reliable techniques able to detect somatic mutations. Microsatellite (SSR) markers, whose polymorphism reflects changes in the number of repeats in each genotype are currently the preferred technique for molecular characterization of plant species because of their high level of polymorphism and the fact that they are mostly codominant markers (Wünsch and Hormaza 2002). The high reproducibility of these markers compared to others allows confident exchanges and standardization of protocols among laboratories. While microsatellite markers failed to distinguish bud sports from their progenitors in apple (Gianfranceschi et al. 1998; Hokanson et al. 1998), pear (Yamamoto et al. 2001), clementines (Breto et al. 2001) or peach (Cipriani et al. 1999), they have revealed allelic diversity among clones of grapevine varieties such as Chardonnay (Riaz et al. 2002), Pinot (Franks et al. 2002; Hocquigny et al. 2004; Riaz et al. 2002), Cabernet Sauvignon (Moncada et al. 2006) or Tannat (González-Techera 2004). As the level of molecular polymorphism between these clones is very low, these varieties have a monozygotic origin and comprise only clones derived through repeated vegetative propagation cycles, initially started from a unique single seedling that is

the common ancestor plant (Hocquigny et al. 2004). Moreover, thanks to the codominant determinism of microsatellite markers, appearance of triple-allele genotypes have been associated with periclinal chimeras, in which two genetically different cell layers can account for the maintenance of three alleles at a locus in an individual. Periclinal chimeras are relatively stable and can be vegetatively propagated. Thus, mutations associated with this type of cellular organization contribute to the diversity of species multiplied by vegetative propagation. Indeed, genetic chimerism has been demonstrated in wine grape varieties such as Chardonnay through regeneration of somatic embryos from the cell layers of the shoot meristem (Bertsch et al. 2005). Chimerism leads to the leaf hairiness variations of Pinot meunier (Boss and Thomas 2002; Franks et al. 2002) as well as to the specific color of Pinot gris (Hocquigny et al. 2004) and Malian, a bronze-berried bud sport of Cabernet Sauvignon (Walker et al. 2006).

Previous studies explored genetic diversity within varieties by analyzing clones that had most of the time diverse geographical origins. Considering only varieties analyzed in the present study, a set of 24 clones of Traminer, synonymous of Savagnin, from different countries and of different types revealed a total identity for all nine microsatellite markers tested (Imazio et al. 2002). Identification of ten clones of White Riesling chosen in collections maintained in Austria and Germany was carried out using 40 microsatellite markers, among them five displayed polymorphism identifying seven genotypes (Regner et al. 2000). Twenty-five Pinot clones from California, Australia and Europe were analyzed with 100 microsatellite markers allowing 12 clones to be uniquely distinguished (Riaz et al. 2002). Finally, 66 Cabernet Sauvignon clones from 7 countries studied with 84 microsatellite markers enabled the tracing of geographical dispersal of this variety (Moncada et al. 2006). The work presented in this paper aimed at assessing and structuring the intravarietal diversity level at the regional level within seven collections of accessions belonging to seven wine grape varieties with two common sets of nuclear microsatellite markers. These collections comprised certified clones or numerous plants brought in French repositories, after prospecting for new individuals between 1972 and 2001 in the French regions where these varieties are traditionally planted. Thus, this extensive genotyping study gives a first insight into the diversity level of several varieties in different French vineyards at the time of prospecting. For the first time, diversity within the varieties Cabernet franc, Chenin and Grolleau is reported. In addition, this study includes Savagnin clones classified as three distinct cultivars in the French official catalogue and grown to produce either aromatic or neutral wines (Duchêne et al. 2009; Pacottet 1903): Gewurztraminer (pink skin berries/aromatic wine), Savagnin rose (pink skin berries/neutral wine) and Savagnin blanc, also known as Traminer, (white skin berries/neutral wine) (Anonymous 2007b). We evaluated the relevance of microsatellite markers to unambiguously distinguish individual clones within each collection as well as to efficiently identify accessions erroneously attributed to a collection.

Materials and methods

Plant material

The plant material consisted of 344 accessions belonging to 7 wine grape varieties: Cabernet franc, Cabernet Sauvignon, Chenin blanc, Grolleau, Pinot noir, Riesling, and Savagnin. Each variety was represented by a random collection of certified clones preserved in the French national repository (ENTAV, Le Grau du Roi, France) (Supplementary Table 1) and by accessions recovered from field selections and maintained in different germplasm repositories at the Institut National de la Recherche Agronomique (INRA, France): Angers, Bordeaux, and Colmar (Supplementary Table 2). The germplasm repositories were constituted between 1972 and 2001 (Table 2).

DNA extraction

Young expanded leaves of shoot tips of 344 individual field-grown were ground into fine powder with liquid nitrogen. Total DNA was extracted with the Qiagen DNeasy TM Plant mini-kit (Qiagen, Hilden, Germany), as described by the supplier.

Microsatellite analysis

Two sets of nuclear microsatellite markers randomly chosen were used. Set A comprising 12 markers was used to analyze Cabernet franc, Chenin blanc and Grolleau clones and set B of 30 markers to analyze Cabernet Sauvignon, Pinot noir, Riesling and Savagnin. Eight markers were common to both sets (Table 3). Markers of the two sets were previously mapped and are scattered on 9 and 18 of the 19 grapevine reference linkage groups, respectively (Doligez et al. 2006). Microsatellite markers were amplified in multiplex using one 6-FAM, HEX or NED fluorophore-labeled primer (PE Applied Biosystems, Warrington, UK). PCR amplifications were carried out according to Hocquigny et al. (2004). PCR fragments were resolved on an automated 310C ABI PRISM DNA sequencer (PE Applied Biosystems, Foster City, CA), and sized with an ROX-labeled internal standard (50–654 bp) (PE Applied Biosystems, Foster City, CA). Microsatellite alleles were scored using GenScan (version 3.1) and Genotyper (version 2.5.2) software

Table 2 For each variety, number and type of accessions in study

Variety	Type	Number of accessions			Microsatellite set	Area of prospection in France	Year of repository planting
		Certified clones	Introductions	Total			
Cabernet franc		1	18	19	A	Anjou-Touraine	2001
Cabernet sauvignon		1	22	23	B	Bordelais	1987
Chenin		2	22	24	A	Anjou-Touraine	1983–1998
Grolleau		2	52	54	A	Anjou-Touraine	1996
Pinot noir		27	101	128	B	North East	1980–1982
Riesling		3	44	47	B	Alsace	1976–1978
	Gewurztraminer	3	16	19	B	Alsace	
Savagnins	Savagnin rose	1	4	5	B	Alsace	1972–1974
	Savagnin blanc	3	22	25	B	Alsace	
Total number of clones		43	301	344			

The seven collections comprised accessions that were either certified clones or introductions. Introductions corresponded to vines collected in the field in different areas in France by visual inspection and placed in repositories in order to preserve diversity within each variety. Each accession has been genotyped with microsatellite markers belonging to set A or B

(PE Applied Biosystems, Foster City, CA). All markers amplified one locus except VMC1e11 that should amplify two loci, one with an almost invariant allele sized 180 bp, and a second one with alleles sized from 190 to 204 bp. At each locus, a genotype displaying a single allele was considered as homozygous, and a genotype showing two or more alleles as heterozygous, without taking into account that amplification of a single allele could also result from a heterozygous locus with a null allele. In the end, each allele was scored in a binary table, where 0 and 1 correspond to the absence and presence, respectively. To confirm variant genotypes, DNA from the same extract was amplified twice.

Phylogenetic analysis

Genetic similarities between pairs of accessions were measured by the DICE similarity coefficient based on the proportion of shared alleles (Dice 1945; Nei and Li 1979) with SIMQUAL module of NTSYSpc program (version 2.02i) (Rohlf 1997) from the binary matrix. For Savagnin clones, a phylogenic analysis was done using the most-parsimonious tree search performed with Phylo-win (Galtier et al. 1996). Internal support was assessed using 500 bootstrap replicates. Bootstrap support is indicated as percentage of trees containing that group of clones. Percentages of less than 40% were not reported.

Results

Almost all accessions have a monozygotic origin

Fifty-eight genotypes were found in the 7 collections totaling 344 accessions, the number of genotypes detected in each collection ranging from 3 to 17 (Table 4). The reference genotype was determined as that shared by the majority of the accessions of a collection even if some loci displayed three alleles (Supplementary Table 3 a–g). Thus, the reference genotype may differ from the expected ancestral genotype when pedigree could have been reconstituted. The proportion of accessions sharing the reference genotype depended on the collection and on the set of markers: using microsatellite set A, it ranged from 92.6% for Grolleau to 75% for Cabernet franc and using microsatellite set B, it ranged from 85.1% for Riesling to 24.5% for Savagnin. Heterozygosity level of the reference genotype was determined as the fraction of loci amplifying more than one allele. This level ranged from 0.58 for Cabernet franc to 0.75 for Chenin blanc and Grolleau and from 0.70 for Savagnin to 0.87 for Pinot noir and Riesling in the sets A and B, respectively (Table 4).

Fifty-one variant genotypes that differed from the seven reference genotype were classified in three classes (Table 4): the first class grouped genotypes that differ at a limited number of loci, mainly by addition of a new allele to the reference genotype; the second class, genotypes that had a heterozygosity level lower than those of the reference genotype and did not displayed new alleles; and the third class, variant genotypes that displayed new alleles for most of the markers.

Forty-three class 1 genotypes were recorded, displayed by 83 accessions (Table 4). Although differing from the reference genotype for 1–6 markers, these variant genotypes showed a genetic similarity ranging from 0.923 to 0.992. Altogether, 41 new allelic combinations were scored at individual loci, among them 23 resulting from the addition of new alleles to the allelic combination of the reference genotype, thus, leading to triple-allele and sometimes four-allele combinations. In each collection, we

Table 3 The two sets of microsatellite markers used in this study

	Reference	Microsatellite motif	Linkage group	Marker sets	
				A	B
VMC1e11	BV681759	TC	16	X	X
VMC2a7	BV722616	TC	18		X
VMC2a12	BV722635	GA	11	X	X
VMC2h3	BV722674	GA	17		X
VMC3a9	BV722646	TC	17	X	X
VMC3b7-2	BV722638	GA	19		X
VMC3b12	BV722656	GA	13		X
VMC3g8-2	BV722645	GA	9		X
VMC4d9-2	BV722745	TC	15		X
VMC5g7	BV722848	GA	2	X	X
VMC5h11	BV722726	TC	19		X
VMC6c10	BV208996	GA	14		X
VMC7f2	BV005171	TC	18		X
VMC8a7	BV722699	TC	1	X	
VMC8f10	BV722704	TC	3		X
VMC8g6	BV209027	$(TC)_xCC(TC)_y$	12		X
VrZAG25	BV722839	GA	10	X	
VrZAG30	UniSTS:502640	GA	6		X
VrZAG62[a]	BV722852	CT	7	X	X
VrZAG67	BV722854	TA	10		X
VrZAG79[a]	BV722842	GA	5	X	X
VrZAG93	BV722844	GA	2	X	
VVMD24	UniSTS:502541	CT	14		X
VVMD25	UniSTS:502515	CT	11		X
VVMD27[a]	UniSTS:502597	CT	5		X
VVMD28	UniSTS:502465	CT	3		X
VVMD30	UniSTS:502635	CT	15	X	
VVMD31	UniSTS:502488	CT	7		X
VVMD32	UniSTS:502471	CT	4		X
VVMD36	UniSTS:502591	CT	3		X
VVMD5[a]	UniSTS:502567	$(CT)_xAT(CT)_yATAG(AT)_z$	16		X
VVMD7[a]	UniSTS:502486	CT	7		X
VVS2[a]	G64021	CT	11	X	X
VVS29	BV722863	GT	1	X	X

Linkage groups according to the reference map of Doligez et al. 2006

Locus reference: NCBI nucleotide, UniSTS otherwise

[a] Markers of the standard set of microsatellites (This et al. 2004)

estimated that all accessions displaying the reference genotype and class 1 variants had a monozygotic origin, i.e., no genes were reshuffled by meiotic recombination events. All certified clones were monozygotic. Among the 334 monozygotic accessions identified in the 7 collections, 29 had a unique genotype making them distinguishable. The others sharing the same genotype were classified in 21 groups comprising from 106 to 2 accessions.

Comparing the intravarietal diversity of variant clones studied with microsatellite set A, only one variant was identified among the 51 (1.9%) Grolleau clones (Supplementary Table 3d) while 4 variants were scored among the 18 (21.1%) Cabernet franc (Supplementary Table 3a) and the 22 (16.7%) Chenin blanc (Supplementary Table 3c) clones. For clones studied with microsatellite set B, only class 1 variants were identified within the Riesling (Supplementary Table 3f) and Savagnin (Supplementary Table 3g) collections while variants of the 3 classes were identified within the Pinot noir collection (Supplementary Table 3e). Five variants (10.6%) were scored among the 47 Riesling clones, 13 variants (56.5%) among the 22 Cabernet Sauvignon clones, and 19 variants (14.8%)

Table 4 Global analysis of the microsatellite genotyping of the accessions representing the seven grapevine varieties

	Varieties							Total
	Cabernet franc	Cabernet sauvignon	Chenin blanc	Grolleau	Pinot noir	Riesling	Savagnins	
Total number of accessions	19	23	24	54	128	47	49	344
Number of microsatellite markers	12	30	12	12	30	30	30	
Total number of genotypes	6	6	7	3	15	4	17	58
Number of accessions displaying the reference genotype	15	10	19	50	106	40	12	252
Percentage of accessions displaying the reference genotype	75.0	41.7	76.0	92.6	82.8	85.1	24.5	
Number of heterozygous loci in the reference genotype	7	24	9	9	26	26	21	
Total number of variants	5	14	6	4	22	3	37	91
Class 1 variants								
Number of variant clones	4	13	4	1	19	5	37	83
Percentage of variant clones	21.1	56.5	16.7	1.9	14.8	10.6	75.5	
Heterozygosity level	0.58	0.80	0.75	0.75	0.87	0.87	0.70	
Number of variant genotypes	4	4	4	1	11	3	16	43
Number of polymorphic loci	4	3	3	1	6	4	6	
Number of new allelic combinations	4	5	4	1	10	4	13	41
Number of tri- or tetra-allele combinations	1	7	2	1	9	2	4	26
Number of distinguishable clones	4	2	4	1	8	2	8	29
Genetic similarity values	0.923–0.976	0.977–0.992	0.962–0.978	0.978	0.971–0.991	0.967–0.991	0.960–0.991	
Total number of monozygotic clones	18	22	22	51	125	47	49	334
Class 2 variants								
Number of variant accessions	1	0	0	0	1	0	0	2
Number of new homozygotic loci	3	–	–	–	9	–	–	
Heterozygosity level	0.33	–	–	–	0.57	–	–	
Genetic similarity values	0.904	–	–	–	0.900	–	–	
Class 3 variants								
Number of variant accessions	0	1	2	3	2	0	0	8
Number of polymorphic loci	–	24	11	7	18–16	–	–	
Heterozygosity level	–	0.67	0.67–0.83	0.83	0.67–0.73	–	–	
Genetic similarity values		0.521	0.511–0.483	0.659	0.715–0.730	–	–	
Number of different genotypes	–	1	2	1	2	–	–	6
Total number of misclassified introductions	1	1	2	3	3	0	0	

Class 1 variants correspond to monozygotic clones which genotype differed from the reference genotype; class 2 variants to self-offspring of the expected variety; and class 3 to clones belonging to other varieties. Both, class 2 and class 3 variants corresponded to introductions that were misclassified within the grapevine varieties studied

among the 125 Pinot noir clones. Thirty-seven Savagnin clones among the 49 studied (75.5%) were polymorphic. Savagnin reference genotype and genotype 17 as well were shared by clones classified as Gewurztraminer, Savagnin rose or Savagnin, while genotype 9 was only displayed by Savagnin blanc and Gewurztraminer clones. In total, the 25 Savagnin blanc, 5 Savagnin rose and the 19 Gewurztraminer accessions were distributed in 13, 2 and 7 distinct genotypes, respectively, among which 8 genotypes were represented by a unique accession. The phylogenic analysis showed that Savagnin blanc clones were distributed in 13 genotypes scattered in all clusters. In contrast, for Gewurztraminer clones, six genotypes were grouped into a cluster of seven, among which is found the reference genotype, the seventh genotype (17) being attributed to a distinct cluster (Fig. 1).

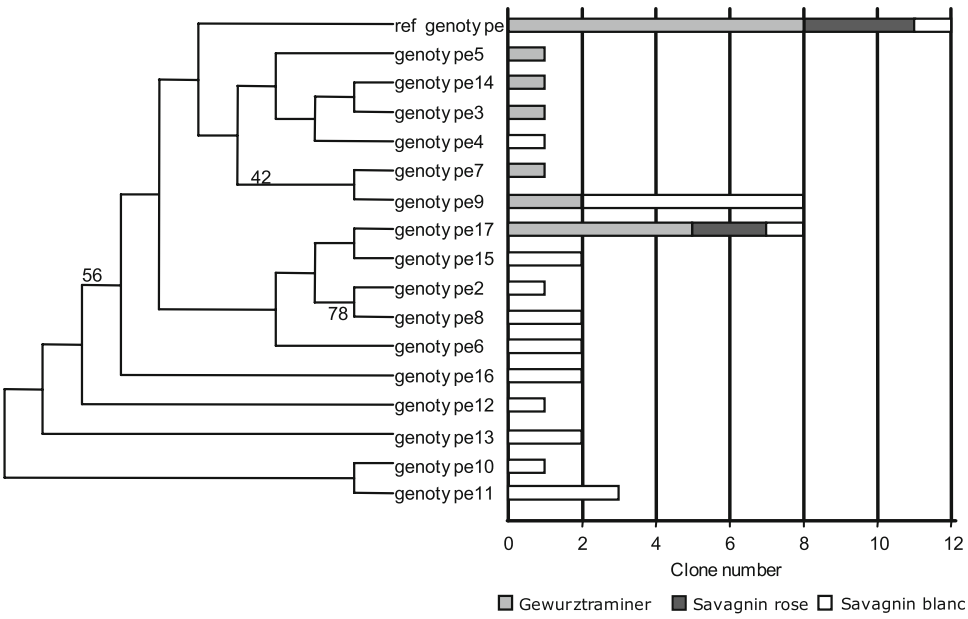

Fig. 1 The most parsimonious tree estimating the genetic relatedness among Savagnin clones analyzed with 30 microsatellite markers and their distribution according to their genotype

Variability of microsatellite markers in monozygotic variants

A limited number of microsatellite markers revealed polymorphism among monozygotic clones, 3 markers (VMC3a9, VMC5g7 and VVS2) generated variant genotypes in 4 collections while 18 markers did not reveal any variability, in particular VMC1e11, VMC2a12, VrZAG62 and VVS29 common to both sets of markers. Several other markers revealed variant genotypes in one, two or three collections (Table 5).

Thirty-eight new alleles were scored in the 41 allelic combinations specific to variant monozygotic clones. Compared to the reference genotype alleles the nearest in length, 12 new alleles (31.6%) showed a size smaller than the reference allele while 26 (68.4%) a larger one. However, it is not possible to know which original allele mutated, particularly when a new allele is added to the reference genotype. The size variation scaled from a −18 to +35 bp, 10 of the variant alleles (26.3%) differing by addition of 2 bp corresponding to an increase of one repeat of the core microsatellite sequence (Fig. 2).

Misclassified introductions

One Cabernet franc and one Pinot noir accession displayed class 2 genotypes further referred as genotypes A. At several loci, they showed, respectively, 3 and 9 homozygous loci that were heterozygous in the corresponding reference genotype. Thus, the heterozygosity levels of Cabernet franc and Pinot noir genotypes A were 0.33 and 0.57, respectively, instead of 0.58 and 0.87 for the corresponding reference genotypes (Table 4). Moreover, genotypes A did not display any allele that was not previously amplified in the reference genotype. From these data, these variant accessions were assumed to be self-offspring of Cabernet franc and of Pinot noir, respectively, instead of being true clones.

The remaining eight variant accessions belonging to the four repositories Cabernet Sauvignon, Chenin blanc, Grolleau and Pinot noir showed six distinct genotypes (genotypes B) that differed from their respective reference genotypes by new alleles at most of the loci, leading to genetic similarities lower than 0.73. Comparing genotype B of Cabernet Sauvignon to the reference genotype of Cabernet franc at the 8 common loci revealed identical allelic combinations at all loci. Studying 164 European genotypes of grapevine at 9 microsatellite loci, Sefc et al. (2000) concluded that the probabilities to observe the same genotype for two varieties at the same locus ranged from 0.07 to 0.24 according to the locus. In our case, if we admit the highest value of 0.24 for all the tested loci, the likelihood that two individuals with the same genotype belong to two different varieties at 8 microsatellite loci is 1.1×10^{-5}. This low probability supports the assignment of this Cabernet Sauvignon variant to the variety Cabernet franc. Genotypes B1 and B2 of Chenin blanc and genotype B of Grolleau displayed at least one new allele compared to the reference genotype for 9, 10 and 7 microsatellite loci out of the 12 analyzed, respectively. Their similarities with the reference genotype ranged from 0.483 to 0.659. These variants could be assigned to varieties different from the expected one (Laucou et al., unpublished results). Chenin blanc genotype B1 was shown to be Arbois, a variety that

Table 5 Polymorphic microsatellite markers in the seven clone collections

	Varieties							Total
	CF	CS	CH	GR	PN	RI	SA	
VMC1e11	0	0	0	0	0	0	0	0
VMC2a7		0			0	0	1	1
VMC2a12	0	0	0	0	0	0	0	0
VMC2h3		0			0	1	0	1
VMC3a9	1	1	1	0	1	0	0	4
VMC3b7-2		0			0	0	0	0
VMC3b12		0			0	0	0	0
VMC3g8-2		0			0	0	1	1
VMC4d9-2		0			0	0	0	0
VMC5g7	0	1	1	0	1	0	1	4
VMC5h11		0			0	0	0	0
VMC6c10		0			0	1	0	1
VMC7f2		0			0	0	0	0
VMC8a7	0	0	0	0				0
VMC8f10		0			0	0	0	0
VMC8g6		0			1	0	0	1
VrZAG25	0	0	0	0				0
VrZAG30		0			0	0	0	0
VrZAG62	0	0	0	0	0	0	0	0
VrZAG67		0			0	0	0	0
VrZAG79	0	0	0	0	1	0	0	1
VrZAG93	1	0	0	0				1
VVMD24		0			0	0	0	0
VVMD25		0			0	0	0	0
VVMD27		0			0	0	1	1
VVMD28		0			0	0	0	0
VVMD30	1	0	1	1				3
VVMD31		1			0	0	0	1
VVMD32		0			1	1	0	2
VVMD36		0			0	0	0	0
VVMD5		0			0	0	0	0
VVMD7		0			0	0	1	1
VVS2	1	0	0	0	1	1	1	4
VVS29	0	0	0	0	0	0	0	0
Total	4	3	3	1	6	4	6	

0: no polymorphism; 1: at least one polymorphic genotype revealed by the marker

CF Cabernet franc, *CS* Cabernet Sauvignon, *CH* Chenin blanc, *GR* Grolleau, *PN* Pinot noir, *RI* Riesling, *SA* Savagnin

belongs to the Messiles sortotype as Chenin blanc, while genotype B2 was shown to be Aligoté, a variety classified in the Gouais sortotype (Bisson 2001). In the same way, Grolleau genotype B was shown to be Abondance.

Finally, Pinot noir genotypes B1 and B2 displayed allelic combinations different from the reference profile at 18 and 16 loci out of the 30 scored, respectively. In both cases allelic combinations comprised at least one Pinot allele. However, as the similarity values of these 2 variants were 0.715 and 0.730, respectively, these plants were most probably two unknown varieties related to Pinot.

Discussion

Misidentified introductions

From this extensive study on the molecular variability inside 344 accessions belonging to 7 varieties, the first conclusion is that within each varietal collection almost all plants are true clones having a monozygotic origin. However, several introductions from field selection based on visual ampelographic inspection in accordance with descriptors of the corresponding variety displayed genotypes distinct from the reference genotype. They differed either by an increase of homozygosity level or by the appearance of unexpected alleles at many loci. Thus, microsatellite markers allowed these accessions of different sexual origin to be clearly detected and considered as misidentified introductions in germplasm repositories. This result agrees with the proposition of using a set of six standard microsatellite markers (VVMD5, VVMD7, VVMD27, VVS2, VrZAG62, and VrZAG79) to identify grapevine varieties (This et al. 2004). In our study, these six markers were polymorphic for the Cabernet Sauvignon genotype B, five of them for the Pinot noir genotype B1 and four for genotype B2 while VVS2, VrZAG62, and VrZAG79 were polymorphic for the Chenin blanc genotypes B1 and B2 and Grolleau genotype B. In the collections we studied, misidentified introductions were either self-progeny of the expected variety, related seedlings or other varieties so alike that they have been mistaken one for the other. Cases of polymorphism accounting for polyzygoticity were previously reported for wine grape varieties such as Sangiovese (Staraz et al. 2007; Vignani et al. 2002) and Fortana, for which clones deriving from two different related seedlings were difficult to be phenotypically distinguished, and thus identified by the same name and used to produce the same wine (Silvestroni et al. 1997). The advent of molecular fingerprinting that clearly identifies clones versus non-clones tends to disprove the polyclonality's theory (Rives 1961) and leads to consider most varieties as being constituted of only monozygotic clones. As a matter of fact, prospecting for high clone diversity exposes to the risk of attributing clones by mistake to the expected variety as it was observed in the Chenin blanc, Grolleau and Pinot collections. In that case, microsatellite genotyping confirms it usefulness for detecting introduction errors in clone collections and nurseries.

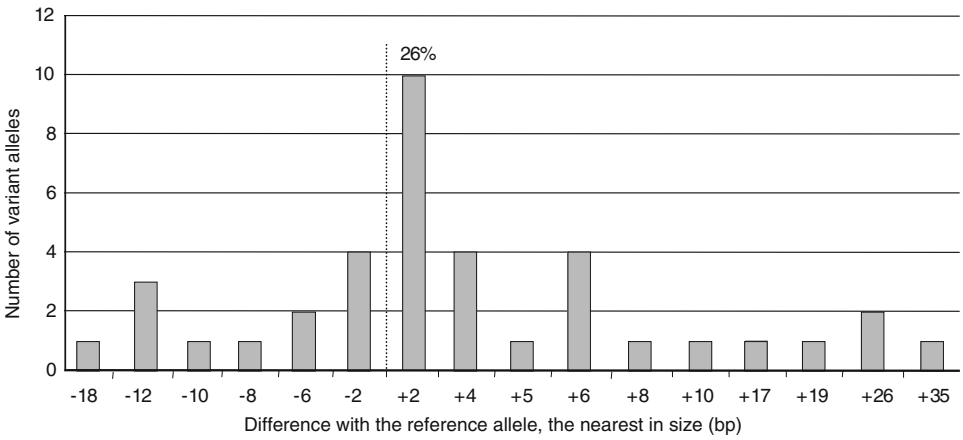

Fig. 2 Size variation of polymorphic alleles from monozygotic clones compared to the reference genotype allele the nearest in size represented by the *dotted line*

Monozygotic clones

Using certified clones as reference, the main point is that most of introductions preserved in French germplasm collections are monozygotic clones derived by vegetative propagation from a single selected vine. Determination of the reference genotype for a variety can be deduced from the progenitor genotypes when pedigree data are available (Bowers and Meredith 1997; Bowers et al. 1999a). Thus, grapevine being a diploid species the genotype of original seedlings at each locus should be either homozygous or heterozygous. In this study, Cabernet Sauvignon, being a progeny of Cabernet franc and Sauvignon blanc (Bowers and Meredith 1997), shared one allele with Cabernet franc at all eight common markers as expected. However, the Cabernet Sauvignon SSR profile we propose as reference was a variant of the ancestral seedling genotype, we chose it even though it displayed triple-allele loci, because this pattern was shared by the largest number of clones we analyzed.

Based on the reference genotype, the heterozygosity level of true clones belonging to the seven varieties ranged from 0.58 for Cabernet franc to 0.87 for Pinot noir and Riesling, confirming that grapevine is mainly a heterozygous species (Table 4). These values are in accordance with the average heterozygosity level of 0.77 given for grapevine (Aradhya et al. 2003), ranging from 0.47 for Tannat to 0.80 for Chardonnay (González-Techera et al. 2004). This high heterozygosity level facilitates the identification of accessions resulting from self-pollination. However, the estimation of the heterozygosity level greatly depends on the varieties considered as well as on the set of microsatellite markers used and gains in accuracy are expected when studying a high number of loci and varieties.

Using microsatellite markers, we have identified variants among monozygotic clones. Most of the new alleles characterizing these variants had a size longer than the nearest reference allele, one-fourth of them by an increase of 2 bp that most likely corresponds to one di-nucleotide repeat. This result is in accordance with the polymorphism of microsatellite markers previously described within the grape varieties Muscat d'Alsace, Greco di Tufo and Primitivo (Crespan 2004). The addition of one repeat unit probably arose by slippage of the DNA polymerase during replication. Slippage seems to play a major role in the generation of mutations in microsatellites (Eisen 1999). This mutation mechanism has the particularity to generate homoplasy, i.e., two alleles identical in size but resulting from independent events. The new alleles were in most cases associated with the two alleles of reference genotype leading to triple-allele genotypes characterizing periclinal chimeras (Franks et al. 2002; Hocquigny et al. 2004; Riaz et al. 2002). Such variability was already observed for Pinot meunier (Boss and Thomas 2002) and Pinot gris (Hocquigny et al. 2004), Cabernet Sauvignon (Moncada et al. 2006) or Chardonnay (Riaz et al. 2002). The multiple occurrences of clones displaying at least one triple-allele genotype at one locus that was observed within all varieties strengthens the prevalence of chimerism for the clonal diversification of grapevine varieties.

Limits of microsatellite markers for clone distinction

Altogether, only 29 among the 334 monozygotic clones could be unambiguously distinguished by a specific genotype, the others formed 21 distinct groups of clones sharing the same genotypes. Concerning microsatellite markers, they were not equally efficient to reveal diversity in all clone collections (Table 5). In our study, markers VMC3a9, VMC5g7 and VVS2 were the most efficient in revealing polymorphism in four of the collections. Both VMC5g7 and VVS2 confirmed their relevance for clone identification. VMC5g7 was previously shown polymorphic in Chardonnay (Riaz et al. 2002) and Carmenère clones (Moncada and Hinrichsen 2007) and VVS2 in Pinot

meunier (Franks et al. 2002), Pinot gris (Hocquigny et al. 2004) and Greco di Tufo clones (Crespan 2004). Regarding other loci, VVMD32 had previously revealed polymorphism in Muscat d'Alsace and Pinot gris clones (Crespan 2004; Hocquigny et al. 2004) as well as VVMD7 in Carmenère clones (Moncada and Hinrichsen 2007) and in two accessions of Black Currant and Mavri Corinthiaki that were shown to be synonymous table grape varieties (Ibanez et al. 2000). Based on these results we propose a standard set of five microsatellite markers comprising VMC3a9, VMC5g7 and VVS2 as well as VVMD30 and VVMD 32 that should be relevant to reveal clonal polymorphism and useful to assist in selecting and identifying clones. These five markers are independently scattered on five linkage groups and all of them are based on (CT) di-nucleotide repeats (Table 3). In fact, among the 37 markers tested, 35 were based on (CT) di-nucleotide resulting from the common use of (GA) probes to fish them (Bowers et al. 1996, 1999b; Sefc et al. 1999). Only VrZag 67 and VVS29, which did not reveal clonal variation, were not based on the (CT) motif (Table 5). It confirms that the (CT) motif is susceptible for slipped-strand mispairing during DNA replication, the process most likely leading to microsatellite allele length polymorphism (Levinson and Gutman 1987). In the present case of polymorphism between clones, this mechanism should have arisen during DNA replication prior to mitosis.

Comparison of the collections of four varieties studied with 30 SSR

No species except grapevine have so many varieties reported to be vegetatively propagated over very long period of time, thus grapevine is a great model to evaluate genome plasticity. The four collections we studied with 30 SSR were constituted between 1972 for Savagnin and 1987 for Cabernet Sauvignon by prospecting for vine diversity within ancient plots. Thus, this genotyping study revealed the diversity level of these varieties in the different French vineyards at the time of prospecting. Comparing diversity within the Savagnin and Riesling collections constituted at the same period and comprising a similar number of clones, the number of variants is much larger in the Savagnin than in the Riesling collection. Wide clonal diversity for any variety would, in the first place, depend on the age of the variety, meaning that the more ancient the variety the longer it would have been accumulating mutations. Attesting the age of a variety is still very difficult. Pinot is thought to be among the most ancient varieties. At the time of the Roman conquest the Roman agricultural writer Columella described a variety present in Burgundy in terms that are consistent with the properties of Pinot noir (Viala and Vermorel 1901–1910). Moreover, Pinot and Savagnin show primitive morphological characteristics analogous to those of the wild-type ssp. *silvestris*, and are thus considered as "archaic" varieties (Levadoux 1956). However, despite their phenotypic diversity we showed in this study that 84.8% of the Pinot noir clones collected in North East of France displayed the same genotype with 30 microsatellite markers while Hocquigny et al. (2004) found a lower value of 65% analyzing with 50 microsatellite markers a collection of 145 Pinot clones belonging to five cultivars. As a comparison, only 24.5% of the 49 Savagnin clones belonging to three cultivars displayed the same genotype at 30 microsatellite loci. Thus, a wide clonal diversity may reflect the interest of vine growers and winemakers to select over long period of time clones with particular characteristics such as color and flavor as observed for Savagnin. The clonal variability could also be related to the cultivation extension and cultural practices that would favor the chance of spontaneous field mutations appearance. In addition, different growers managing practices operating informal "clonal selection" for centuries would have increased this chance. Finally, some varieties could have a stronger proclivity toward spontaneous mutations in the vineyard owing to a particular genetic background.

The case of the Savagnin clones

The Savagnin clones showed the widest diversity, nevertheless no clear-cut genetic grouping corresponding to the ampelographic classification was observed. Reference genotype of Savagnin was shared by clones classified in all the three cultivars Gewurztraminer, Savagnin rose and Savagnin blanc, in spite of their differences regarding berry skin color and aromatic expression (Duchêne et al. 2009; Pacottet 1903). However, our data showed a wider genetic diversity within the set of 25 Savagnin blanc than within the set of 19 clones of Gewurztraminer, which displayed 13 and 7 genotypes, respectively. Such diversity is reported here for the first time. It contrasts with a previous analysis carried out on 24 Traminer, a synonymous of Savagnin, clones of different provenance and types, which revealed a total identity for all of the nine microsatellite markers tested, among them VVS2 polymorphic in our study (Imazio et al. 2002). For grape berry color variation, previous investigations have associated the lack of anthocyanin pigment in grapevine with the insertion of *Gret1*, a retrotransposon 10,422 bp long of the grapevine genome, in the promoter region of *VvmybA1*, a gene encoding a transcription factor inducing the anthocyanin pathway (Kobayashi et al. 2004). Walker et al. analyzed 55 white grape varieties at locus *VvmybA1* and showed that they were all homozygous for *Gret1* insertion preventing anthocyanin biosynthesis (Walker et al. 2007). Unfortunately, Savagnin blanc was not tested in this study, but this

variety is expected to be homozygous for the *Gret1* insertion as well. In contrast, a pink-skinned Gewurztraminer clone displayed a 44-bp-long insertion followed by two single nucleotide polymorphisms upstream from the promoter region of *VvmybA1* instead of the full *Gret1* insertion (This et al. 2007). This shorter insertion could probably allow the partial expression of the *VvmybA1* gene and thus the pigmentation of the clone. As Savagnin blanc, Savagnin rose and Gewurztraminer are monozygotic clones, we proposed that the pink-skinned clones of Savagnin could be bud sports from a white-skinned clone, by imperfect excision of *Gret1*. The flavor trait specific to Gewurztraminer that has never been recorded for a white clone could have been acquired by somatic mutation from a pink-skinned clone in a second step. Despite the lack of robustness of the clustering analysis, it seems that this sequence of mutational events could have happened at least twice. Since clones of the three cultivars can have a same genotype with the markers used, it is more likely that the studied markers are unlinked to the somatic mutations leading to the specific phenotypes.

Toward new types of molecular markers adapted to clone identification

Microsatellite markers showed their limit to help clonal selection and identification. Thus, to achieve this goal new type of markers should be developed to detect natural somatic mutations. Few examples of mutations leading to clonal diversity are reported so far. Large deletions were shown to differentiate the black-skinned Pinot noir (Yakushiji et al. 2006) and Cabernet Sauvignon from their respective white-skinned bud sports Pinot blanc and Shalistin (Walker et al. 2006). Almost all colored varieties including Pinot noir and Cabernet Sauvignon are heterozygous for the *Gret1* insertion in the promoter of the *VvmybA1* gene, the red functional allele being dominant over the white one. The phenotypic polymorphism between black-skinned and white-skinned derived cultivars results from a deletion in the functional red allele at *VvmybA1* locus (Yakushiji et al. 2006) which is >260 Kb long in Shalistin (Walker et al. 2006). Other molecular mechanisms possibly leading to clonal variations can be investigated, in particular, the contribution of transposable elements. Transposable elements are mobile genomic entities able to insert new copies around the host genome. Only three retrotransposons were characterized before the release of the grapevine genome sequence: *Vine-1* (Verriès et al. 2000), *Tvv1* (Pelsy and Merdinoglu 2002) and *Gret1* (Kobayashi et al. 2004). *Vine-1* sequence-specific amplified polymorphism (S-SAP) has been investigated within six clones of Pinot and three clones of Traminer; while few polymorphisms could characterize each of the three Traminer clones, the six Pinot clones could not be distinguished in spite of their different color phenotypes (Labra et al. 2004). Assessing this technique using data sequence of the transposable element families recently identified in the grapevine genome could reveal those involved in somatic mutations and thus the most powerful for clone fingerprinting (Benjak et al. 2008; Moisy et al. 2008). Finally, the sequences of the grapevine genome now available (The French–Italian Public Consortium for Grapevine Genome Characterization 2007; Velasco et al. 2007) provide a framework to rapidly characterize all types of polymorphism that arose during vegetative propagation by comparing sequences of different clones belonging to a same variety. Based on such exhaustive information, new specific tools should be developed to deeply analyzing grapevine germplasm collections.

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