Theor Appl Genet (2000) 100:498–505

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Microsatellite variability in grapevine cultivars from different European regions and evaluation of assignment testing to assess the geographic origin of cultivars

Received: 10 April 1999 / Accepted: 25 May 1999

Abstract Nine microsatellite markers (VVMD5, VVMD7, VVS2, ssrVrZAG21, ssrVrZAG47, ssrVrZAG62, ssrVrZAG64, ssrVrZAG79 and ssrVrZAG83) were chosen for the analysis of marker information content, the genetic structure of grapevine cultivar gene pools, and differentiation among grapevines sampled from seven European vine-growing regions (Greece, Croatia, North Italy, Austria and Germany, France, Spain and Portugal). The markers were found to be highly informative in all cultivar groups and therefore constitute a useful set for the genetic characterization of European grapevines. Similar and high levels of genetic variability were detected in all investigated grapevine gene pools. Genetic differentiation among cultivars from different regions was significant, even in the case of adjacent groups such as the Spanish and Portuguese cultivars. No genetic differentiation could be detected between vines with blue and white grapes, indicating that they have undergone the processes of cultivar development jointly. The observed genetic differentiation among vine-growing regions suggested that cultivars could possibly be assigned to their regions of origin according to their genotypes. This might allow one to determine the geographical origin of cultivars with an unknown background. The assignment procedure proved to work for cultivars from the higher differentiated regions, as for example from Austria and Portugal.

Key words *Vitis* · Microsatellites · Genetic variation

Communicated by G. Wenzel

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Introduction

Microsatellites (SSRs) consist of tandemly repeated simple sequence motifs with a high variation in repeat number among individuals. This high level of polymorphism has made them invaluable markers for organisms where little information could be extracted from other marker types (Morgante and Olivieri 1993; Paetkau et al. 1995). Applications of microsatellite markers include individual or cultivar identification (e.g. Hokanson et al. 1998), parentage testing (e.g. Bowling et al. 1997), pedigree reconstruction (Sefc et al. 1998b) and studies of population structure (e.g. MacHugh et al. 1997; Petri et al. 1997).

In grapevines, one of the major applications of microsatellite markers is the identification and discrimination of cultivars in order to facilitate the management of cultivar collections and control the trade of plant material. So far, *Vitis* SSR primers have been developed by three groups (Thomas and Scott 1993; Bowers et al. 1996; Sefc et al. 1999). The usefulness of these markers has been assessed in samples of grapevine varieties cultivated in the vine-growing regions of Australia, California and Central Europe. However, due to the predominance of certain alleles, or the occurrence of null alleles

Table 1 Cultivars included in this study. The color of the berries is indicated as N (noir – blue berries), B (blanc – white berries) Rg (red) and Rs (rose)

Austrian/German		*Portuguese*		*Greek*		*Spanish*	
Blauburger	N	Alfrocheiro	N	Akominato	N	Airen	B
Blaufränkisch	N	Arinto de Bucelas	B	Aetonychi	N	Alarije	B
Elbling	B	Arinto no Douro	B	Agiorgitiko	N	Blanca Cayetana	B
Geißtutte	B	Avesso	B	Ákiki	Rs	Bobal	N
Goldburger	B	Azal Branco	B	Voidomato Lasithiou	N	Cariñena	N
Jubiläumsrebe	B	Baga	N	Dafnia	B	Garnacha	N
Königsast	B	Bical	B	Dermatas	B	Garnacha Tintorera	N
Müller Thurgau	B	Boal Cachudo	B	Eftakoilo	N	Godello	B
Neuburger	B	Boal Ratinho	B	Fokiano	N	Juan Ibañez	N
Orangetraube	B	Camarate	N	Karatsova Naousis	B	Malvar	B
Österreichisch Weiß	B	Esgana Cão	B	Kolokythas lefkos	B	Malvasia	B
Rheinriesling	B	Fernão Pires	B	Kotsifali	N	Mencia	N
Rotgipfler	B	Jaen	N	Krystalli	B	Merseguera	N
Schlagerblut	N	Negra Mole	N	Kritiko mavro	N	Monastrell	N
Silvaner Grün	B	Periquita	N	Liatiko	N	Ondarrabi Beltza	N
Veltliner Frührot	Rg	Rabo de Ovelha	B	Mantilaria	N	Palomino	B
Veltliner Grün	B	Ramisco	N	Mavrodaphni	N	Parellada	B
Veltliner Rot	Rs	Rufete	N	Merkouri	N	Tempranillo	N
Wildbacher Blau	N	Saborinho	N	Moschofilero	N	Verdejo	B
Zierfandler	Rs	Terrantêz	B	Nychato	B	Xarel Lo	B
		Touriga Nacional	N	Petrachladi		Zalema	B
French		Trajadura	B	Plyto	B		
Aligoté	B	Trincadeira Preta	N	Psevdosyriko	N	*Italian*	
Cabernet franc	N	Verdelho	B	Roditis kokkinos	Rs	Aglianico	N
Cabernet sauvignon	N	Vinhão	N	Romeiko	N	Albarola	B
Chardonnay	N	Viosinho	B	Romeiko Machaira	N	Avana’	N
Chenin blanc	B	Vital	B	Syriki	N	Avarengo	N
Cot	B			Theiako	B	Barbera	N
Gamay	N	*Croatian*		Thrapsathiri	B	Barbera bianca	B
Merlot	N	Babić	N	Tsaousi	B	Bonarda Piemontese	N
Pinot	B,N	Bogdanuša	B	Vidiano	B	Bosco	B
Sauvignon Blanc	B	Debit	B	Zakynthino	B	Brachetto d’Acqui	N
Semillon	B	Dolcin	B			Cortese	B
Shiraz	B	Gegić	B			Croatina	N
St. Laurent	N	Hravatica	N			Dolcetto	N
		Kraljevina Crvena	B			Erbaluce	B
		Lasina	N			Fiano	B
		Mirkovača	B			Grignolino	N
		Plavec Žuti	B			Lambrusca di Alessandria	N
		Plavina	N			Malvasia Casorzo	N
		Pošip	B			Malvasia Schierano	N
		Pošip Crni	N			Nebbiolo Lampia	N
		Ranfol	B			Neiret pinerolese	N
		Škrlet	B			Neretta cuneese	N
		Teran	N			Pelaverga Pagno	N
		Vela Pergolla	B			Quagliano	N
		Vrbnička Zlahtina	B			Refosco ped. rosso	N
		Žilavka	B			Sangiovese	N
						Teroldego	N
						Trebbiano toscano	B
						Uva rara	N
						Vermentino	B
						Vespolina	N

in some populations, the information content of a given marker may vary between the cultivars from different regions (Lopes et al. 1999). In the present work, we chose nine polymorphic and easily scorable markers among the published microsatellite sequences and compared their usefulness for the genotyping of grapevines from seven different vine-growing regions of Europe.

High genetic variability has so far been detected in grapevine cultivars from Central Europe (Sefc et al. 1999) and Portugal (Lopes et al. 1999). In the present study, we further characterize the levels of diversity within the grapevine samples from different areas.

Due to the exchange of grapevine cultivars between different vine-growing regions, the origin of cultivars is sometimes uncertain. Here, we endeavoured to include only cultivars which were reported to originate from the respective region, in order to prevent sampling of more-recently introduced cultivars. Based on these samples, we address the question of whether cultivars from different regions constitute distinct gene pools or whether

Table 2 Characterization of the nine microsatellite markers used in this study. The first three columns show observed heterozygosity (H_o), expected heterozygosity (H_e) and mean number of alleles (MNA) averaged over grapevine samples. Values in parentheses are standard deviations. Expected heterozygosity (H_e) and number of alleles (NA) for the total sample were calculated pooling all cultivars into one group

Locus	H_o	H_e	MNA	H_e total	NA total
VVS 2	0.831 (0.147)	0.777 (0.024)	7.7 (0.76)	0.836	13
VVMD 5	0.877 (0.058)	0.819 (0.018)	7.7 (0.76)	0.859	9
VVMD 7	0.776 (0.100)	0.719 (0.086)	7.3 (1.11)	0.798	12
ssrVrZAG 21	0.813 (0.117)	0.737 (0.059)	6.4 (1.27)	0.793	8
ssrVrZAG 47	0.846 (0.091)	0.770 (0.032)	6.7 (0.76)	0.828	9
ssrVrZAG 62	0.794 (0.103)	0.723 (0.073)	6.7 (1.25)	0.796	10
ssrVrZAG 64	0.904 (0.088)	0.794 (0.024)	6.7 (1.25)	0.822	11
ssrVrZAG 79	0.788 (0.074)	0.779 (0.050)	7.7 (1.38)	0.830	12
ssrVrZAG 83	0.781 (0.072)	0.677 (0.041)	4.0 (0.00)	0.710	4

gene flow mediated by cultivar exchange has been sufficient to homogenise the groups. Paetkau et al. (1995) developed a procedure in order to test whether differences between populations are large enough to make an individual's genotype characteristic of or even diagnostic for the population to which it belongs. The applicability of this test to specify the geographic origin of grapevine cultivars would be extremely interesting and is discussed in the present work.

Materials and Methods

Grapevine cultivars (Table 1) were sampled from Portugal (*n*=27; grapevine collections from the Estação Vitivinícola Nacional in Dois Portos and from Serviços Agrícolas in Biscoitos, Terceira), Italy (*n*=32; plant material was harvested from true-to-type individuals planted in collection fields in Piemonte, North West Italy, and available to the Centro per il Miglioramento Genetico e la Biologia della Vite – CNR, Torino, Italy), Croatia (*n*=19; collections of the Institute for Agriculture and Tourism in Porec and of the Faculty of Agriculture, Zagreb), Austria and Germany (*n*=20; collection of the HBLA and BA Klosterneuburg, Austria), France (*n*=13; collection of the HBLA and BA, Klosterneuburg, Austria), Greece (*n*=32; collections of the Laboratory of Plant Physiology and Biotechnology, University of Crete, and the Institute of Viticulture, Floriculture and Vegetable Crops, Heraklion, Crete) and Spain (*n*=21; *Vitis* germplasm bank of El Encín, Instituto Madrileño de Investigación Agraria y Alimentaria, Consejería de Economia y Empleo). DNA was extracted from leaf material according to the methods described by Thomas et al. (1993), Lodhi et al. (1994) and Lefort and Douglas (1999), or by using the QUIAGEN DNeasy Plant Mini Kit.

The cultivars were genotyped at the following microsatellite loci: VVS 2 (Thomas et al. 1994), VVMD 5 and VVMD 7 (Bowers et al. 1996), ssrVrZAG 21, ssrVrZAG 47, ssrVrZAG 62, ssrVrZAG 64, ssrVrZAG 79 and ssrVrZAG83 (Sefc et al. 1999). Six of the markers investigated here (VVS 2, VVMD 5, VVMD 7, ssrVrZAG 47, ssrVrZAG 62 and ssrVrZAG 79) have recently been chosen as a core set for the screening of grapevine collections in Europe covered by the GENRES#081 research project (see http://www.dainet.de/genres/vitis/vitis.htm). PCR and electrophoresis using the ALFexpress sequencer (Pharmacia Biotech, Vienna, Austria) were performed as described by Sefc et al. (1997).

A software programme was written to determine allele frequencies, the number of observed heterozygotes, expected heterozygosity (Nei 1973) and probability of identity (Paetkau et al. 1995), and to check for identical genotypes in different cultivars. Excess and deficiency of heterozygotes, deviations from Hardy Weinberg proportions, linkage disequilibrium, genic and genotypic differentiation were all tested using GENEPOP (Raymond and Rousset 1995). FSTAT (Goudet 1995) was employed to calculate and test F_{ST} values (Weir and Cockerham 1984), and Nei's D with standard deviations (1000 bootstraps) was calculated in MICROSAT (Minch 1997). Assignment tests (Paetkau et al. 1995) were performed using the program G-ASSIGN from the G-STAT package (Siegismund 1995).

Results and Discussion

Microsatellite polymorphism

Among several microsatellite markers which have been employed for the characterization of Central European grapevines (Sefc et al. 1998a, 1999), the nine most-informative ones were chosen in order to assess their value for the genotyping of grapevines from different European vine-growing regions. Cultivars were sampled from Greece, Croatia, Italy (mainly from the North West), Austria and Germany, France, Spain and Portugal. In total, 88 alleles were detected, giving a mean of 9.8 alleles per locus. The total number of alleles per locus varied between a broad range of from 4 to 13 alleles (Table 2). The expected heterozygosity over all cultivars was high and ranged from 0.710 (ssrVrZAG 83) to 0.859 (VVMD 5). At all loci, mean observed heterozygosity, averaged over samples, was higher than expected by the random union of gametes. The excess of heterozygotes was significant at ssrVrZAG 64 (P=0.0008) and ssrVrZAG 83 (P=0.0017).

One major application of microsatellite markers in viticulture is the identification of and the distinction between cultivars (Thomas et al. 1994; Bowers et al. 1996; Sefc et al. 1998a). Therefore, the potential of the markers to yield different genotypes for as many cultivars as possible is of great interest, and selection of the most-informative markers reduces the number of loci to be investigated for reliable cultivar distinction. In order to characterise the usefulness of the nine markers in different grapevine gene pools, the information content of each locus in each geographic group, and over all groups, was assessed as the probability of identical genotypes (PI) (Paetkau et al. 1995). When calculated across all cultivars, the PI was low for all loci (PI values from

Table 3 Probability of identity (PI) for each locus – population combination, for the total sample (all cultivars pooled into one group) and for the whole set of nine markers (cumulative PI)

Marker	Greek	Italian	Croatian	Austrian/ German	French	Portuguese	Spanish	Total sample
VVS2	0.13	0.12	0.14	0.16	0.19	0.12	0.13	0.09
VVMD5	0.08	0.11	0.12	0.10	0.11	0.11	0.13	0.07
VVMD7	0.14	0.11	0.27	0.10	0.24	0.17	0.21	0.11
VrZAG21	0.22	0.16	0.10	0.25	0.33	0.16	0.22	0.13
VrZAG47	0.11	0.13	0.19	0.18	0.15	0.17	0.13	0.10
VrZAG62	0.16	0.12	0.11	0.16	0.28	0.21	0.22	0.12
VrZAG64	0.20	0.10	0.13	0.16	0.13	0.11	0.14	0.10
VrZAG79	0.12	0.08	0.15	0.14	0.12	0.27	0.15	0.08
VrZAG83	0.31	0.27	0.36	0.25	0.27	0.22	0.25	0.24
Cumulative PI	4.0×10^{-8}	7.7×10^{-9}	6.5×10^{-8}	6.5×10^{-8}	2.5×10^{-7}	7.6×10^{-8}	1.1×10^{-8}	2.0×10^{-9}

Table 4 Genetic variability within the grapevine samples. The first column shows the sample size (*n*), followed by observed and expected heterozygosity (H_o and H_e) averaged over loci, the mean number of alleles (MNA) in the full-sized samples and in samples of size *n*=13, averaged over loci. Values in parentheses are standard deviations

Sample	*n*	H_o	H_e	MNA (full sample)	MNA (*n*=13)
Greek	32	0.809 (0.089)	0.758 (0.065)	7.3 (1.66)	6.1 (1.45)
Croatian	19	0.789 (0.123)	0.749 (0.078)	7.0 (1.50)	6.6 (1.42)
Italian	32	0.814 (0.062)	0.790 (0.049)	7.6 (1.74)	6.9 (1.53)
Austrian/German	20	0.841 (0.087)	0.763 (0.045)	6.4 (1.33)	6.2 (1.09)
French	13	0.858 (0.103)	0.727 (0.072)	5.8 (1.20)	5.8 (1.20)
Spanish	21	0.799 (0.114)	0.744 (0.062)	6.6 (1.13)	6.1 (0.93)
Portuguese	27	0.860 (0.111)	0.752 (0.055)	6.7 (1.32)	6.1 (1.27)

0.07 to 0.13) except for locus ssrVrZAG 83 (PI=0.24). The values of PI for each locus in each population and overall are shown in Table 3. The markers were not equally informative in all of the populations, as in some cases the information content of a locus is decreased in a certain population by the prevalence of one or two alleles. This is the case, for example, for locus VVMD 7 in the French and Croatian population, where one allele occurred at a frequency of over 50%. In the Portuguese cultivars, two alleles were predominant at locus ssrVrZAG 79, which increased the PI value to 0.27, while it ranged from 0.08 to 0.15 in the other populations. Cumulative probabilities to obtain identical genotypes from different cultivars at each of the nine microsatellite loci were in the order of 10^{-7} to 10^{-9}, which shows that the chosen marker set possesses high discriminative power in all of the investigated cultivar groups (the allelic profiles of all cultivars are listed at: http://www.boku.ac.at/zag/forsch/).

Comparison of genetic variability in different grapevine gene pools

In the next step, we attempted to describe and compare the genetic structures of the investigated grapevine gene pools.

A comparison of allelic data among the grapevine populations revealed similar levels of genetic variation for all groups (Table 4). The mean number of alleles (MNA) per population ranged from 5.8 (French) to 7.6 (Italian). As the lowest number of alleles was detected in the population with the smallest sample size and a positive correlation was observed between the MNA and the sample size of each population (r=0.830), even when the French sample was excluded (r=0.706), the MNA was also calculated for random samples of 13 cultivars per population (MacHugh et al. 1997). In the case of the French grapevines, the original 13 cultivars were used. After this correction, the allele numbers in the different populations varied over a narrower range than before (MNA from 5.8 to 6.9), but the Italian group was still the most variable one, and the French cultivars displayed the smallest number of alleles.

Gene diversities were high in all populations with values ranging from 0.727 to 0.790. The distribution of gene diversities among the populations was similar to that of the allele numbers: the differences between populations were low, the highest value was obtained for the Italian cultivars and the lowest variation was observed in the French sample. However, in none of the pairwise comparisons were the differences in variability measures between populations significant. The relatively low variability in the French group might be due to a sampling bias. However, when a search for possible pedigrees was conducted in a large number of French cultivars, a high proportion of putatively first-degree-related cultivars was found (Bowers et al. 1998), indicating a narrow ge-

Table 5 Genetic differentiation between grapevine samples. The lower triangle shows χ^2 values for the homogeneity of allele frequencies in pairwise comparisons (Inf.=infinity). The critical χ^2 value at P=0.05, df=18, is 28.87. Pairwise values for F_{ST} and standard deviations (in parentheses) are shown in the upper triangle

Sample	Greek	Croatian	Italian	Austrian/ German	French	Spanish	Portuguese
Greek		0.039 (0.008)	0.039 (0.011)	0.049 (0.012)	0.090 (0.015)	0.039 (0.013)	0.052 (0.011)
Croatian	82.629		0.026 (0.008)	0.044 (0.010)	0.077 (0.018)	0.066 (0.009)	0.060 (0.013)
Italian	Inf.	56.693		0.038 (0.009)	0.062 (0.015)	0.076 (0.014)	0.064 (0.014)
Austrian/German	Inf.	69.335	86.654		0.047 (0.012)	0.063 (0.017)	0.055 (0.013)
French	Inf.	Inf.	Inf.	73.547		0.070 (0.020)	0.052 (0.009)
Spanish	Inf.	Inf.	Inf.	Inf.	Inf.		0.020 (0.005)
Portuguese	Inf.	Inf.	Inf.	Inf.	90.435	55.358	

netic basis for these varieties. In contrast, no possible first-degree relationships could be detected among the Italian cultivars in this study.

In all populations, the mean number (averaged over loci) of heterozygous individuals was higher than expected, with a significant heterozygote excess in the French and the Portuguese grapevines (P=0.0036 and 0.0003, respectively). Testing across all loci and all populations resulted in a significant overall heterozygote excess (P=0.0004). However, a test for Hardy Weinberg equilibrium revealed no significant deviation across all populations (P=0.15), while the Portuguese grapevines were found to be in slight disequilibrium (P=0.05). In the French group, 100% heterozygosity was observed at loci ssrVrZAG 47 and ssrVrZAG 64. Only heterozygous cultivars were detected in the Portuguese population at loci VVS 2 and ssrVrZAG 64, and among the Spanish cultivars at VVS 2. In contrast, Croatian cultivars display a low level of heterozygosity at VVS 2, with an observed heterozygosity of 58% versus the expected 77%. This deviation has been found to be insignificant by an exact test using the Markov-chain method (P=0.07). However, the heterozygote deficiency could be indicative of the occurrence of non-amplifying alleles at this locus in the Croatian grapevines.

Whereas, on the whole, genotypic frequencies in the different populations stochastically follow the distribution expected according to Hardy Weinberg proportions, the assumptions underlying this model are certainly not fulfilled. Today's grapevine cultivars barely show the characteristics of naturally evolving populations. Genotypes have been maintained unchanged over hundreds of years by vegetative propagation, which led to highly overlapping generations. Additionally, natural conditions and agronomic use have imposed selection pressure on the genotypes. Prior to domestication, vine plants were dioecious and outbreeding and therefore attained a high level of heterozygosity. As a side effect, deleterious recessive traits accumulated in the genome (Olmo 1976) and, in consequence, a certain level of heterozygosity has become a vital condition for the plants. The selection for highly heterozygous plants was intensified in the course of the domestication and cultivation of grapevines, when the genotypes were evaluated according to their agronomic performance. Selfing of grapevines, and thereby decreasing heterozygosity, results in a substantial inbreeding depression within the offspring concerned. The excess of heterozygosity, which we observe in this study, is probably a consequence of both natural and human selection against homozygosity in grape plants.

Differentiation among grapevine cultivars originating from different regions and among berry color variants within regions

Based on the allele frequencies detected in the investigated grapevine samples, we addressed the question of whether cultivars in the different regions constitute genetically distinct gene pools. Allele frequencies among the samples were compared using the probability test for genic differentiation described by Raymond and Rousset (1995). Probabilities for differences in allele frequencies were pooled over all loci using Fisher's method and were highly significant for all pairwise comparisons (Table 5). Results for the distribution of genotype frequencies were closely correlated with the values for the allele frequencies, as the genotype composition approximately corresponds to the random union of the alleles according to their frequencies (see above). F_{ST} values (Weir and Cockerham 1984) were found to be significantly different from zero in all pairwise comparisons (Table 5). The 95% confidence intervals for the estimates of F_{ST} did not overlap zero, and probabilities P (F_{ST} not >0), calculated by permutting alleles, were below 0.001, except for the comparison between Spanish and Portuguese cultivars (P=0.002). Thus, detectable differentiation has occurred between all of the investigated grapevine samples, even in the case of the adjacent groups from Spain and Portugal or Italy and Croatia. According to these findings, each of the investigated cultivar samples constitutes an independent source of genetic variation, and therefore a valuable resource of genetic traits for grapevine breeders. The current tendency to plant successful and famous cultivars such as Cabernet Sauvignon or Chardonnay over all the vine-growing regions of the world should be balanced by the maintenance of the typical varieties of the diverse regions in order to prevent genetic erosion in this crop.

Table 6 Results of assignment testing. Cultivars belonging to the populations indicated in the first column were assigned to the seven different groups according to likelihood-ratio tests. Numbers show the percentage of individuals assigned to a given group, and values in parentheses give the percentage of individuals accepted in the respective group

Taken from:	Assigned to:						
	Greek	Croatian	Italian	Austrian/ German	French	Spanish	Portuguese
Greek	62.5 (87.5)	12.5 (46.9)	0 (9.4)	9.4 (28.1)	3.1 (6.2)	12.5 (34.4)	0 (15.6)
Croatian	5.3 (10.5)	68.4 (89.5)	15.8 (31.6)	5.3 (31.6)	5.3 (10.5)	0 (10.5)	0 (10.5)
Italian	3.2 (12.9)	12.9 (58.1)	45.2 (61.3)	12.9 (29.0)	19.4 (51.6)	6.5 (12.9)	0 (16.1)
Austrian/German	9.1 (18.2)	18.2 (27.3)	0 (9.1)	59.1 (72.7)	13.6 (36.4)	0 (0)	0 (4.5)
French	0 (0)	0 (0)	0 (0)	0 (20.0)	100 (100)	0 (6.7)	0 (0)
Spanish	4.8 (14.3)	0 (0)	0 (4.8)	0 (14.3)	28.6 (33.3)	57.1 (71.4)	9.5 (28.5)
Portuguese	3.7 (7.4)	0 (7.4)	0 (0)	0 (3.7)	29.6 (44.4)	11.1 (40.7)	55.6 (96.3)

In grapevines, several variants of berry coloring are known, ranging from blue via red to grey, green and yellow. In red and blue varieties, the anthocyanin content of the berries increases during veraison. Most of the genes involved in anthocyanin biosynthesis have already been characterised (Sparvoli et al. 1994). Cultivars may loose their ability to produce blue colored berries by mutations affecting genes in this pathway, and both blue and white variants are known for many of the old grape varieties such as Pinot. Furthermore, crosses between blue and white varieties can give rise to very successful cultivars, as for example Cabernet Sauvignon (Bowers and Meredith 1997). For these reasons, we would expect to find no genotypic differentiation between blue and white cultivars within a certain region. To evaluate this assumption, we separated the Greek, Italian, Croatian, French, Spanish and Portuguese groups according to their berry colour. In the Austrian/German group, the blue cultivars were strongly under-represented, and subdivision of the cultivars according to berry color would not have provided useful samples.

Allele frequencies were homogeneous among white and blue cultivars in the Greek, Spanish, Croatian and French samples. In the Portuguese and Italian grapevines, differentiation was detected among blue and white vines when testing for homogeneous allele distributions ($P<0.005$ and $P<0.03$, respectively). However, Nei's D between the white and blue varieties of these regions was not significantly different from zero, nor were pairwise F_{ST} values, as the 95% confidence intervals for both distance measures, determined by 1000 bootstraps over loci, overlapped zero.

Therefore, according to our expectation, white and blue cultivars are scarcely differentiated within their regions of origin, indicating that they have undergone the process of cultivar development jointly.

Assignment Tests

The highly significant results of the tests for allelic and genotypic differentiation among regions suggested that the genotype of a cultivar might be diagnostic of the population to which it belonged. This might allow tracing cultivars of unknown background back to their region of origin.

Assignment tests (Paetkau et al. 1995) are used to estimate the likelihood that an individual belongs to a given population by calculating the expected frequency of the observed genotype in each of the populations. The individual is then assigned to the population that has the highest likelihood of containing a member with the observed genotype. Rejection and acceptance of membership are based on a likelihood-ratio test (Siegismund 1995). First, we carried out the assignment test on the cultivars which had been used to build the different samples (Table 6). In order to account for deviations from Hardy Weinberg proportions at some of the loci, expected genotype frequencies were estimated based on Wright's fixation index F (Wright 1951).

On average, 66% of the cultivars were assigned to the population where they had been sampled from, and for 83% of these membership to their population of origin was accepted. The remaining cultivars were generally assigned to neighbouring populations, with the exception of a relatively high assignment rate of Greek cultivars to the Spanish population. A negative correlation was observed between the level of pairwise genetic differentiation between two regions and the number of incorrectly assigned cultivars. Exceptions were the high number of Spanish, Portuguese and Italian cultivars assigned to the French group despite their relatively large genetic distance from the French sample. The high number of cultivars assigned to the French group, which is represented by a rather small sample of 13 cultivars, may be an artefact caused by the test procedure. In order to eliminate the bias resulting from the inclusion of each individual's genotype in the allele distribution of its own sample, the genotype of the tested individual was added to each of the other samples prior to the calculation of genotype frequencies (Paetkau et al. 1995). This modification also prevents the rejection of an individual from a certain population because it carries a rare allele that has not been observed in that population (Siegismund 1995). However, the effect of the addition of one genotype to the estimated genotype frequencies of a sample increases

with decreasing size of the sample, which may cause a higher number of cultivars to be assigned to populations represented by a small sample (Simonsen et al. 1998).

We also used this test to assign cultivars of unknown origin to their corresponding populations. However, as already the assignment of cultivars of known origin has only partially been successful, the results have to be carefully interpreted. Nevertheless, some examples will be discussed:

(1) A synonym of the Italian cultivar Prosecco was found in the sample taken from Croatia (Maletić et al. 1999). The genotype of this cultivar was most likely to occur in the Croatian sample, while it was rejected from the Italian group ($P<0.005$). However, 13% and 15% of the Italian and Croatian cultivars were specified as belonging to the other group, respectively, which makes it impossible to distinguish between the membership to one or the other of these two neighbouring regions.
(2) In California, Zinfandel is one of the most famous of red wine varieties. This cultivar is also known in Italy as Primitivo and is considered to be either of Italian or Croatian origin. In the assignment test employed in this study, Zinfandel is accepted for membership in both Italian and Croatian grapevines, while it is rejected from the other regions.

No wrong assignments have occurred between the Croatian – Spanish, Croatian – Portuguese, Italian – Portuguese, Spanish – Austrian/German and Portuguese – Austrian/German cultivars (Table 6). Therefore, a distinction between cultivars originating, for example, either from Austria or from Portugal based on the assignment test should be reliable. One of the most-important red wine cultivars of Austria is called Blauer Portugieser (Blue Portuguese). Although the name of this cultivar points to a Portuguese origin, Blauer Portugieser is commonly believed to come from a certain Austrian region. However, discussions on this subject are still ongoing. In the assignment test, Blauer Portugieser was clearly rejected from all populations ($P<0.001$) except the Austrian/German group, which strongly supports the Austrian or German origin of this cultivar.

These results indicate that it may be possible to identify the origin of grapevines by their genotypes, provided that substantial differentiation has taken place between the populations in question. However, in the case of less-differentiated populations, a reliable assignment of genotypes to either one or the other of these populations could not be achieved in our study. For a successful assignment of a genotype of unknown origin to a population, it is important that the samples representing the populations are well-chosen and characteristic for the different regions. Perhaps the inclusion of a larger number of ancient cultivars would lead to better-defined reference samples and improve the resolution of the assignment test for grapevine cultivars.

Acknowledgements The experiments carried out in this work comply with the current laws of Austria.

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