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Genetic characterization of grapevine (*Vitis vinifera* L.) cultivars from Castilla La Mancha (Spain) using microsatellite markers

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Summary

A total of 73 accessions of *Vitis vinifera* L., corresponding to local grape cultivars from Castilla La Mancha (Spain) mostly collected in the districts around the Serranía de Cuenca, were analyzed with 12 microsatellite markers in order to ascertain identity and to detect synonymy and homonymy.

The allelic pattern of the 73 accessions belonged to 39 different cultivars: 23 coincided with those of known grape cultivars and 16 were different such as 'Flamenca', 'Churriago', 'Pintailla', and 'Gallera Negra'. Homonymous designations were also detected like 'Coloraillo', 'Moravia Dulce' and 'Botón de Gallo' and synonymous names such as 'Garnacha' and 'Tinto Basto', 'Machina', 'Tortosi' and 'Rojal' as well as 'Moravio' and 'Bobal'.

K e y w o r d s : microsatellite, *Vitis vinifera* L., synonymous, grapevine.

Introduction

Today, vineyards in Spain are mainly concentrated in the central part of the country. Castilla-La Mancha is the vine growing region, with almost 600,000 ha, representing 50 % of the total vine growing area in Spain and around 7 % of the world. Viticulture is therefore a crucial sector for this region.

The legacy of grape varieties in the region is relatively unknown and comprises a few dozen varieties, most subject to selective pressure. Only some selective cultivars, many of them foreign, benefit from the restructuring of vineyards and often at the cost of autochthonous, minority varieties. Some of them, whose surfaces have continually diminished in recent decades, may soon be on the verge of extinction, and are already affected by an important loss of genetic diversity.

In view of the need to learn more about the wealth of this heritage, to clarify once and for all the synonyms and homonyms affecting some of its components and, ultimately, to establish an "autochthonous" ampelographic collection in the field, in 2004 there was started a process of territorial exploration, identification and characterization of plant material.

In the last 20 years, methods for identifying grapevine cultivars using molecular markers have been established. Simple sequence repeats (SSRs), also known as microsatellites, have so far proven to be the most useful technology for the genetic identification of grapevine varieties and evaluation of genetic diversity (Thomas and Scott 1993, Sefc *et al.* 2001).

The aim of this study was to characterize 73 autochthonous grapevine accessions grown in Castilla La Mancha, collected up to 2005, by analyzing 12 microsatellite regions, in order to establish a germplasm bank of the different varieties cultivated in the region.

Material and Methods

The 73 accessions used in this study and the local denomination are shown in Tab. 1. Some were authorized or recommended varieties in Castilla La Mancha and the rest were unknown and/or appeared with the local denomination. The Figure shows the provinces of Castilla La Mancha and the prospected zones. Most accessions were collected from regions within the territory bordering the Serrania of Cuenca (mountain range), where grapevines are marginal crops often grown on plurivarietal plots. Two internationally known cultivars, 'Cabernet Sauvignon' and 'Chardonnay', were also included to compare allele size results with those of other laboratories.

D N A e x t r a c t i o n : DNA was extracted from leaves or roots from *Vitis vinifera* by the CTAB method according to Steenkamp *et al.* (1994), adapted for small volumes.

M i c r o s a t e l l i t e a n a l y s i s : 12 microsatellite loci were selected. The 6 core loci, as per the recommendation of the EU project Genres081 (This *et al.* 2004) were: VVS2 (Thomas and Scott 1993), VVMD5, VVMD7 (Bowers *et al.* 1996), VVMD27 (Bowers *et al.* 1999), VrZAG62 and VrZAG79 (Sefc *et al.* 1999). Samples were also analyzed at 6 additional loci, ssrVrZAG67, ssrVrZAG64, ssrVrZAG83 (SEFC *et al.* 1999), VVMD21, VVMD28, VVMD36 (Bowers *et al.* 1999). The forward primer from each pair was fluorescently labelled to allow detection. 6-FAM (blue), VIC (green), PET (red) and NED (yellow) (Applied Biosystems) were used.

Three previously optimized multiplex PCR reactions were performed to obtain a similar quantity of amplifications for all the microsatellite markers. PCR reactions were carried out in the GeneAmp®PCR System 9700 thermocycler (PE Applied biosystems), in 10 µl of a mixture containing 20 ng DNA, 0.2 U Taq DNA polymerase (Biotools),

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Table 1

Genotypes of the 73 analyzed accessions. Allele sizes are expressed as base pairs

Cultivars	Accessions	VVS2		VVMD5		VVMD7		VVMD 21		VVMD27		VVMD 28		VVMD 36		ZAG62		ZAG 64		ZAG 67		ZAG79		ZAG 83	
Airen	Airen (61) Malvar (3) Blanca (18)	143	145	222	230	240	250	198	204	177	190	232	242	263	283	187	199	133	139	128	146	246	258	193	197
Alarije	Aris (27) Torrontes (33)	143	145	230	232	236	236	202	204	181	190	232	256	271	283	185	187	133	135	128	152	250	256	193	197
Albillo Mayor	Albillo (31, 65)	143	145	228	232	236	250	188	202	179	190	232	256	271	283	185	199	137	156	122	136	250	256	197	197
Alcañon	Bobal Blanca (23)	133	145	230	234	236	236	200	204	177	190	232	242	265	283	185	187	133	145	128	161	250	256	193	197
Ariño	Unknown (1)	143	151	222	230	236	246	198	202	175	190	226	256	259	259	187	203	137	139	122	148	256	260	191	193
Beba	Botón de Gallo (4) Teta de Vaca (8) Uva de planta (19)	135	143	232	236	240	246	198	204	177	185	242	256	259	259	187	203	133	156	128	136	242	246	197	197
Bobal	Bobal (56), Moravio (55)	145	147	224	230	236	240	188	204	177	185	232	258	259	265	187	187	139	156	136	148	242	246	197	197
Brujidera	Colgadera (7) Crujidera (6) Moravia Dulce (16,50) Rucial (48)	143	145	224	228	236	240	202	212	179	190	242	252	249	265	187	191	135	139	152	157	246	256	191	197
Coloraillo	Coloraillo (38)	143	145	224	232	236	236	204	212	179	179	252	256	249	259	187	195	135	139	146	152	246	250	197	197
Corazón de Cabrito	Gordera Manchega (5, 63) Gordera (30) Gordal (34) Rompetinajas (67)	133	145	230	234	236	236	188	204	177	181	232	256	259	265	187	195	139	160	130	148	236	246	193	197
Cinsaut	Botón de Gallo (62)	133	133	222	222	240	244	198	200	175	177	226	232	249	271	187	203	156	156	136	136	254	258	193	197
Garnacha	Garnacha (26), Tinto Basto (42)	137	145	222	236	236	240	200	202	190	190	242	242	261	265	187	187	133	139	128	146	256	256	191	193
Malvar	Malvar (43)	143	145	232	236	236	240	202	204	175	190	256	256	259	271	185	187	135	139	148	152	250	256	193	197
Montua	Chelva (73)	143	151	230	234	240	250	202	204	177	181	232	258	265	283	187	187	133	139	128	144	246	256	197	197
Moravia Agria	Moravia Agria (40,47,49)	145	151	224	232	236	250	200	200	175	175	256	258	265	271	187	193	137	156	122	136	250	250	193	197
Moscatel Grano Menudo	Moscatel Grano Menudo (70,72)	133	133	224	232	230	246	204	204	175	190	244	266	239	259	185	195	137	156	122	136	250	254	191	191
Pardillo	Blanca Pequeña (9) Marisancho (17)	145	157	232	236	236	246	200	204	181	190	232	256	271	283	185	193	139	139	146	157	250	256	197	203
Planta fina	Pasera (24, 71) Coloraillo (11,12)	143	145	224	236	236	240	204	204	175	190	246	256	259	259	185	187	139	139	146	148	250	256	197	197
Rojal	Machina (45) Rojal (58, 66) Tortosi (68)	137	145	224	230	236	240	200	204	181	190	234	242	249	261	187	187	133	139	128	146	246	256	193	197
Tardana	Tardana (10, 21)	137	149	222	224	236	236	200	204	175	190	234	242	249	261	187	187	135	139	122	146	246	256	191	193
Tempranillo	Negra (28)	143	145	232	232	236	250	202	204	179	179	256	256	259	271	195	199	137	139	122	146	246	250	197	197
Teta de vaca	Botón de Gallo (20) De la Panga (2)	135	147	228	234	236	246	198	212	179	190	246	252	249	259	191	203	135	156	136	152	246	256	197	203
Tinto Velasco	Frasco (57) Granadera (15)	133	133	228	234	230	250	200	202	175	181	246	258	259	265	199	203	135	139	146	152	236	250	197	197
Genotype 1	Churriago (13) Tinto de Villar de Olalla (29)	145	145	224	232	240	246	204	212	179	190	242	256	265	271	191	193	139	139	146	157	256	256	197	197

Tab. I continued

Cultivars	Accessions	VVS2		VVMD5		VVMD7		VVMD 21		VVMD27		VVMD 28		VVMD 36		ZAG62		ZAG 64		ZAG 67		ZAG79		ZAG 83	
Genotype 2	Tinto fino (14)	147	157	224	232	236	236	200	204	177	181	234	242	249	259	185	187	139	139	146	157	246	250	197	203
Genotype 3	Botón de Gallo (22) Botón de Gallo (25, 69) Gallera Dorada (51)	133	135	222	228	236	246	188	212	181	181	232	256	271	271	185	187	133	135	128	152	242	250	191	197
Genotype 4		135	143	228	232	236	240	188	212	177	179	232	234	245	283	187	203	139	156	146	152	246	248	197	197
Genotype 5	Moscatel (32, 41) Gordera Roja (35)	133	143	224	232	246	248	188	198	175	185	242	256	249	259	185	203	135	156	122	136	242	254	191	197
Genotype 6	Gordera Negra (54)	135	143	230	234	240	240	200	204	190	190	246	256	259	259	187	187	135	139	122	148	256	256	193	197
Genotype 7	Teta de Vaca (36)	137	145	234	236	240	240	198	204	177	179	232	246	259	283	187	187	133	135	122	128	246	246	193	197
Genotype 8	Unknown (37)	135	135	228	234	236	246	188	198	185	185	242	246	259	265	193	201	133	135	122	128	246	256	193	203
Genotype 9	Moravia Dulce (39)	143	145	224	232	236	240	202	212	179	190	242	256	259	265	191	195	139	139	146	157	246	256	191	197
Genotype 10	Coloraillo (44)	145	157	228	236	236	240	204	212	181	190	242	256	265	271	185	191	135	139	146	152	250	256	191	197
Genotype 11	Pintailla (46)	135	145	222	236	236	236	202	204	177	177	234	256	259	271	187	187	133	139	128	148	246	246	193	197
Genotype 12	Gallera Negra (52)	133	143	232	232	236	236	188	204	179	185	256	256	259	271	185	187	139	156	136	146	242	250	197	197
Genotype 13	Coloraillo (53)	143	147	230	242	236	240	202	212	175	190	234	256	245	259	187	203	133	135	128	152	248	256	193	197
Genotype 14	Unknown (59)	143	157	222	232	236	244	200	212	175	181	234	256	259	271	185	203	135	139	146	152	250	250	197	203
Genotype 15	Unknown (60)	137	145	230	232	240	246	198	204	181	190	246	256	249	249	187	203	133	133	128	128	236	256	193	197
Genotype 16	Flamenca (64)	133	145	224	228	230	240	204	212	175	190	242	266	259	265	191	195	135	156	136	152	254	256	191	197
Cabernet sauvignon	Clone 15	139	151	228	236	236	236	198	204	171	185	232	234	249	259	187	193	135	156	122	136	246	246	203	203
Chardonnay	Clone 96	137	143	230	234	236	240	198	204	177	185	216	226	249	271	187	195	156	160	136	150	242	244	191	203

Figure: Map of Castilla La Mancha and location of the prospected zones. Provinces. Cuenca (Cu), Albacete (Ab), Ciudad Real (Cr), Guadalajara (Gu) and Toledo (To). Zones: A, Sacedón (Gu); B, Arrancacepas (Cu); C, Campillo de Altobuey (Cu); D, Villaverde y Pasaconsol (Cu); E, Aliaguilla (Cu); F, Casillas De Ranera (Cu); G, Ribatajada (Cu); H, Villar De Olalla (Cu); I, Madrigueras (Ab); J, Dos Barrios (To); K, Casasimarro (Cu); L, Villagarcía Del Llano (Ab); M. Cañaveras (Cu); N, San Clemente (Cu),O. Casas de Fernando Alonso (Cu), P. Casas de Haro (Cu). Q. Tomelloso (Cr).

200 µM of each dNTPs, 1x reaction buffer, 1.5 mM of $MgCl_2$, and different amounts of each primer pairs depending on the set (in set A, 0.1 µM of each VrZAG62 primer, 0.2 µM of each primer of the three primer pairs VVMD7, VVMD27 and VrZAG79, 0.3 µM of each VVS2 primer; in set B, 0.1 µM of each VVMD28 primer and 0.3 µM of each VVMD5 primer; and in set C 0.1 µM of each primer of ZAG83 and VVMD21, 0.2 µM of ZAG67, VrZAG64 and 0.3 µM of VVMD36). PCR conditions were 95 ºC for 12 min, 10 cycles of 15 s at 94 ºC, 15 s at 55 ºC and 15 s at 72 ºC, followed by 20 cycles of 15s at 89 ºC, 15s at 55 ºC and 15s at 72 ºC, and a final extension of 30 min at 72ºC. PCR amplifications were separated using capillary electrophoresis, and analysis of fluorescence with an ABIPRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Fluorescently labelled fragments were detected and sized using Genemapper software (Applied Biosystems). Genescan-500 LIZ™ (Applied Biosystems) was used as internal standard to assign sizes to DNA fragments.

Data analysis: The genotypes of all accessions in this study were tested against the database containing genotypes of Spanish varieties of grapevine (Martin *et al.* 2003, Ibañez *et al.* 2003) and other European varieties (Sefc *et al.* 2000). The number of alleles, the allele frequencies, the expected and observed heterozygosity, the probability of identity and the probability of null alleles were calculated using Identity 1.0 software (Wagner and Sefc 1999).

Results and Discussion

The 12 microsatellite loci chosen for this study discriminated 39 different genotypes in 73 analysed cultivars.

Microsatellite results, expressed as allele size in base pairs, are presented in Tab. 1. 'Chardonnay' and 'Cabernet Sauvignon' were used as references, in order to compare the obtained data with other existing microsatellite libraries.

Only the 39 different genotypes obtained were used for the calculation of genetics parameters (Tab. 2) in order to avoid overestimation. A total of 93 alleles, ranging from 10 in ZAG67 and VVMD28 and 4 in ZAG83, were detected with an average of 8 alleles per locus. The most frequent allele was ZAG83-197, which showed a frequency up to 50 % and 13 alleles were unique.

Table 2

Number of alleles (AO), expected heterozygosity (He), observed heterozygosity (Ho), Frequency of null alleles (r), and PI, Probability of identity of 12 SSR loci studied in 39 genotypes obtained from 73 cultivars

Locus	AO	He	Ho	r	PI
VVS2	9	0.815	0.872	-0.032	0.104
VVMD5	8	0.845	0.923	-0.042	0.080
VVMD7	7	0.702	0.744	-0.025	0.218
VVMD27	6	0.808	0.795	0.007	0.117
ZAG62	8	0.749	0.795	-0.027	0.141
ZAG79	9	0.783	0.821	-0.021	0.142
ZAG 67	10	0.853	0.949	-0.052	0.074
ZAG 64	7	0.759	0.821	-0.035	0.164
ZAG 83	4	0.596	0.692	-0.061	0.314
VVMD 21	6	0.798	0.923	-0.070	0.121
VVMD 28	10	0.815	0.897	-0.045	0.099
VVMD 36	9	0.800	0.846	-0.026	0.117
TOTAL	93				2.29E-11
MEAN	7.75	0.777	0.840	-0.036	0.141

The expected heterozygosity (gene diversity) ranged from 0.596 at locus ZAG83 to 0.853 at locus ZAG67, with a mean value 0.777. The observed heterozygosity varied between 0.692 at loci ZAG83 and ZAG62 and 0.949 at locus ZAG67. For all loci, Ho was higher than He, and the probability of null alleles was always negative, except for VVMD27, and very close to 0, indicating the low probability of null alleles at all studied loci. Samples in which only one single allele per locus was detected were considered as homozygous genotypes instead of heterozygous with a null allele.

The most informative locus for the studied set of accessions was ZAG67, with a probability of identity (Probability of obtaining identical profiles) of 0.074, and least informative locus was ZAG83 with PI. 0.314.

The 12 microsatellite loci used reflected a high discrimination power (99.998 %) and a low probability that two randomly chosen individuals had identical genotypes using the 12 loci (PI. 2.29 10-11). Thus, cultivars with identical genotypes were considered synonymous. This confirmed the suitability of the system for genetic identification. The number of primers sufficient for reliable varietal identification depends on the nature and discriminating power of each primer (Tessier *et al.* 1999). Normally 6 primer pairs are sufficient for differentiating between genotypes (Zulini *et al.* 2002, This *et al.* 2004), but closely related cultivars require larger numbers of pairs (Meredith 1999). In this case, the 6 couples of primers recommended by the GENRES081 project would be sufficient for differentiating the 39 genotypes obtained.

Tinto Velasco appears removed from the remaining genotypes, with 28 % shared alleles and 'Brujidera' and 'Moravia Dulce' (Genotype 9) are the closest genotypes among all analyzed and shared 75 % of alleles studied.

Cultivar identification: Microsatellite analysis enabled the identification of unknown cultivars (1) which is 'Ariño' in comparison with existing SSR-markers databases. However, the other three unknown accessions are 3 misnamed genotypes. Synonymy detection: In comparison with existing SSR-marker database synonymous accessions could be assigned (Tab. 1). Homonymy detection: Owing to differing genetic profiles for 16 of the analyzed accessions homonymy was detected, namely 'Coloraillo' (44, 53 and 11, 12), 'Moravia Dulce' (39), 'Moscatel' (32, 41), 'Botón de Gallo' (4, 20, 22, 25, 62 and 69), 'Teta de Vaca' (8) and 'Torrontes' (33). 16 unique genetic profiles were detected that probably corresponded to varieties not described previously (genotypes 1 to 16 in Tab. 1). In the future, these new genotypes will have to be described ampelographically and, where possible, named accordingly. Four genotypes called 'Coloraillo' were differentiated, from the true 'Coloraillo' (accession 38) described by Martin *et al.* (2003). Accessions 11 and 12 were synonyms of 'Rojal' and the other two (44 and 53) are two different genotypes. 'Botón de Gallo' showed the same phenomenon. Five different genotypes were among the 6 studied accessions, three (22, 25 and 69) with two different genotypes, accession (62) was 'Cinsaut', accession 20 and 'De la Panga' (2) were identified as 'Teta de Vaca' and accession (4), 'Teta de Vaca' (8) and 'Uva de Planta' (19) were 'Beba'. 'Moravia Dulce' displayed the largest number of synonymous: some were previously known, such as 'Brujidera' and 'Crujidera', and others were new, such as 'Colgadera' and 'Rucial'. Accession 39 turned out to be a homonym from the previous group. 'Moravio' was a synonym of 'Bobal' and not of 'Moravia'. 'Tinto Fino' has always been considered a synonym of 'Tempranillo', 'Cencibel' or 'Tinto del País', but in this case, this accession was a different genotype; hence, it was a misnamed accession. 'Tinto Basto' (authorized variety in Castilla La Mancha) is a synonym of 'Garnacha'. 'Torrontes' (33) was confirmed to be a synonym of 'Alarije' and 'Aris', contrasting with the 'Torrontes' described in the bibliography.

'Malvar' is sometimes confused with 'Airén' (Ibañez *et al.* 2003), but is clearly separable by microsatellite analysis; hence, accession 3 is considered to be a misnamed genotype and not a homonym of 'Malvar' (43). 'Bobal Blanca' is a synonym of 'Alcañon'. This variation is not due to berry color mutation such as in the 'Garnacha Blanca', 'Peluda', 'Gris' and 'Tinta' varieties (Ibañez *et al.* 2003 and Martin *et al.* 2003), whose differences could not be detected by microsatellite analysis. In this case, they had different genotypes and only shared 42 % of the studied alleles with 'Bobal Tinta'.

In the cases of 'Gallera Negra', 'Gallera Dorada', 'Gordera Manchega' and 'Gordera Negra/Roja', despite withdrawals in the same geographical area and similar names, the phenotypic and genetic variations were very high, sharing only 46 % and 42 % of the studied alleles, respectively; Hence, they surely had different origins.

It is important to highlight that the genotype obtained for 'Moscatel' (32, 41) did not coincide with any of those described by Crespan *et al.* (2001), for 64 accessions of Moscatel, and shared 50 % alleles with 'Moscatel de Grano Menudo' which is closer to Genotype 16 that also has Muscat flavour.

The results obtained show that the differentiation of certain Castilla La Mancha cultivars with microsatellite markers is feasible. This study allowed us to clarify some synonyms, homonyms and misnaming. 16 genotypes of unknown identity have been found. Therefore, this is the first step in the process of establishing a collection of varieties of grapevine, using the region as a reference and where most of the cultivars are perfectly identified and catalogued.

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