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Identification and molecular biodiversity of autochthonous grapevine cultivars in the 'Comarca del Bierzo', León, Spain

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Summary

A prospecting work at the 'Comarca del Bierzo' in Spain has been carried out in order to evaluate the existing biodiversity and detect the neglected and endangered minor grapevine cultivars of the zone. As a result, a total of 79 different accessions were collected and studied using STMS markers for preliminary variety identification, that was confirmed on the base of ampelographic observations. Several synonymies and homonymies were detected. A total of 33 different cultivars were identified. Comparison of the genotypes, allelic frequencies and allelic sizes are presented. Conservation of the endangered cultivars is highly recommended.

K e y w o r d s : ampelography, microsatellites, synonymies, *Vitis vinifera*.

Introduction

The 'Comarca del Bierzo', located in the northwest of the León province in Spain is an area of vineyards for obtention of VQPRD (Vin de Qualité Produit dans des Régions Déterminées), and corresponds to the Apellation of Origin 'D.O. Bierzo'. García de los Salmones (1914) mentioned about 30 white and 30 red cultivars existing in this area, although probably including also a certain number of synonymies. The 'D.O. Bierzo', only includes four white, 'Godello', 'Doña Blanca', 'Malvasía' and 'Palomino', and two red, 'Mencía' and 'Garnacha Tintorera' cultivars (Ortiz and Villaseca 2004). This fact leads to the reduction of the cultivars. As a consequence, an intense erosion of grapevine germplasm exists, with a high risk of extinction of old autochthonous cultivars. During the last years a prospecting study in the search of endangered plant material has been carried out.

STMS (sequence-tagged microsatellite site) markers were used in order to identify the collected cultivars, since they have a high level of reproducibility, and are adequate for the genotyping of grapevine cultivars (Sefc *et al.* 1998, Martín *et al.* 2003). Complementary ampelographic observations were made to confirm the obtained results.

The objective of the present study was to collect minor and endangered cultivars in the 'D.O. Bierzo', identify them and analyze their variability. The detection of synonymies among the studied cultivars was based on the molecular characterization and ampelographic observations, taking some previous studies into account (Martín *et al.* 2003, Pinto-Carnide *et al.* 2003).

Material and Methods

A broad survey of the 'Comarca del Bierzo' was carried out in order to study the existing variability in the area. The plants selected were either supposed being different cultivars or plants of the same cultivated variety in order to assess their true to typeness. Twenty five plants ('unknown' in Tab. 1) were sampled because they were different of the predominant variety in the plot and were not previously identified as being an already known variety. Tab. 1 lists the 79 accessions that were sampled at different plots, with the indication of the berry color as well as the local name of the variety.

Samples of young fresh leaves were collected in the field and kept at -80 ºC until analyzed. For the molecular characterization, DNA extraction was carried out by using the MasterPure™ Plant Leaf DNA Purification Kit (Epicentre Technologies, Madison, USA). The following six STMS loci were used: VVS2 (Thomas and Scott 1993), VVMD5 and VVMD7 (Bowers *et al.* 1996), and ssrVrZAG47, ssrVrZAG62 and ssrVrZAG79 (Sefc *et al.* 1999), under the conditions detailed in a previous work (Martín *et al.* 2003). Polymorphism of the amplified products was detected in an automated DNA sequencer ABI PRISM model 310 (PE Applied Biosystems). As a result of the analysis, genotypes for each cultivar were obtained for the studied loci.

Microsatellite genotypes were compared with some of the available databases (Sefc *et al.* 1998, http://www.genres.de/CF/eccdb/vitis, Martín *et al.* 2003) by using reference standard cultivars and comparing the relative allele sizes for the different STMS loci.

In order to confirm the provisionally identified cultivars on the base of the molecular results, ampelographic observations were made in the field at different times of the year. The minimal list of descriptors for germplasm collections was used (OIV 1984) (data not presented).

For the study of the molecular biodiversity, the allele and genotype frequencies were obtained by counting and direct calculation. The observed heterozygosity H_o was calculated as the ratio between heterozygote genotypes and

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Table 1

Size of the alleles for the six analyzed microsatellite loci in the different cultivars

Variety[1]	Local names[2]	C[3]	N[4]	VVS2		VVMD5		VVMD7		ZAG47		ZAG62		ZAG79		Observations
Albarín Negro (Alfrocheiro Preto)	Estaladiña	N	2	140	150	222	234	251	255	155	165	187	199	249	249	'Bruñal' in 'Arribes de Duero'
Allarén	Allarén	B	2	140	142	234	236	237	255	165	171	187	187	249	255	Cultivated in 'Galicia'
Aramon	Aramon; Gran Negro	N	4	140	148	230	230	231	241	157	171	187	195	241	255	
Brancellao	Unknown	N	1	130	150	218	222	237	237	161	165	187	193	249	257	
Cabernet Sauvignon	Cabernet	N	1	136	150	228	236	237	237	151	165	187	193	245	245	
Cagarrizo	Unknown	B	1	130	150	228	236	241	245	161	165	193	203	243	249	
Chasselas Cioutat	Unknown	B	1	130	140	224	232	237	245	161	165	193	203	249	257	
De Rey	Palomino Macho	B	1	130	140	222	230	245	247	155	157	187	193	241	247	
Doña Blanca (Malvasía Blanca; Moza Fresca)	Unknown	B	1	134	150	218	230	237	247	157	157	185	203	245	245	Cultivated in 'Galicia'
Garnacha Tinta	Unknown	N	1	134	142	222	236	237	241	171	171	187	187	255	255	
Garnacha Tintorera (Alicante Bouschet)	Alicante Tinta	N	4	130	142	222	234	237	241	157	171	187	187	241	255	
Garnacho (Morrastel Bouschet)	Unknown	N	1	136	150	222	230	237	241	157	159	187	187	241	257	
Godello	Godello	B	2	150	156	222	234	237	241	161	165	185	187	249	249	
Malvasía Riojana (Alarije)	Unknown	B	1	140	142	230	232	237	237	161	171	185	187	249	255	
Mandón	Valenciana Tinta	N	1	140	150	222	236	237	237	159	171	185	187	255	257	Cultivated in 'Galicia'
Mencía	Mencía Pajaral	N	3	142	150	222	232	247	255	157	165	187	193	245	249	Cultivated in 'Galicia' and 'Castilla y León'
Merenzao	Merenzao; Estaladiña; Ojos de Sapo; Bastardo; Pan y Carne	N	8	140	150	234	234	237	255	151	165	187	187	243	245	Cultivated in 'Galicia'
Merlot	Unknown	N	1	136	150	222	232	237	245	165	167	193	193	257	257	
Monastrell	Unknown	N	1	130	150	222	236	247	247	155	165	187	203	249	259	
Moscatel de Grano Menudo	Moscatel Rubio	B	1	130	130	224	232	231	247	155	171	185	195	249	253	
Mouratón (Juan García)	Aramon	N	5	134	150	230	234	247	255	157	165	187	203	245	249	
Negreda	Negreda	N	3	134	150	222	228	237	261	157	161	187	193	245	257	
Negrón de Aldán (Petit Bouschet)	Tinta Aragonesa	N	2	130	150	230	234	237	241	157	165	187	195	241	243	
Palomino	Unknown	B	2	130	142	224	236	237	247	161	171	187	193	249	255	
Pan y Carne (Merenzao Negreda)	Pan y Carne; Estaladiña; Aragonés; Rastralleira	N	4	140	150	222	234	251	255	157	165	193	199	243	259	
Picapoll	Unknown	B	1	134	150	222	228	237	247	155	167	185	203	249	249	
Picapoll Rosa (Alsacia)	Alsacia	R	4	134	150	222	228	237	247	155	167	185	203	249	249	
Prieto Picudo Blanco	Mando	B	1	140	156	232	236	237	237	155	161	185	185	249	249	
Río Abaixo	Río Abaixo	B	4	130	142	230	236	241	255	157	165	187	193	241	245	
Sumoll	Rabo de Ovella	N	4	130	142	222	236	241	247	155	157	185	187	245	259	
Tempranillo	Unknown	N	5	140	142	232	232	237	251	159	159	195	199	245	249	
Temprano Blanco (Chasselas Doré)	Cáselas; Pixa de Galo	B	5	130	140	224	232	237	245	161	165	193	203	249	257	
Temprano Colorado (Chasselas Rose)	Alsacia	R	1	130	140	224	232	237	245	161	165	193	203	249	257	

[1] Grapevine variety. Synonymies within parenthesis. [2] Names given by the growers of the area. [3] Color of the berry: B = white; N = black; R = rose. [4] Number of accessions analyzed of this

total analyzed genotypes for each locus, and the expected heterozygosity H_e as $(1 - \sum P_i^2)$, p_i being the frequency of each allele for each locus (Nei 1987). The effective number of alleles, ENA, was estimated as $(1/\sum P_i^2)$, according to Kimura and Crow (1964). These calculations were carried out with the software package POPGENE (Yeh *et al.* 1997). The polymorphism information content, PIC, which is the probability that an individual is informative with respect to the segregation of its inherited alleles, was calculated according to Botstein *et al.* (1980). The discrimination power, D, estimates the probability to distinguish randomly sampled accessions with the microsatellite results (Lamboy and Alpha 1998). It was calculated for each locus from the probability of coincidence, C, D being (1 - C). C is the probability that two cultivars match by chance in one locus $(C = \sum P_i^2)$, P_i representing the frequency of different genotypes observed at that locus. The discrimination power for all loci combined, D_T, was calculated as $D_T = 1 - C_T$ where $C_T = \prod C_m$ for each of the m = 6 loci.

Results

Allele sizes of genotypes obtained for the six STMS loci analyzed are shown in Tab. 1. Differences in at least one of the loci occurred in all cases except for the two cultivars 'Picapoll' and 'Picapoll Rosa', and the three 'Chasselas' ('Chasselas Cioutat', 'Chasselas Doré' and 'Chasselas Rose'), in a total of 30 different allelic profiles. Twenty four out of the 30 different STMS profiles were already known and published elsewhere (Martín *et al.* 2003, http://www.genres.de/CF/eccdb/vitis/), whereas the profiles of 'Aramon', 'Cagarrizo', 'Mandón', 'Negreda', 'Pan y Carne', and 'Río Abaixo' are described for the first time. The identity of the varieties in most of the cases was confirmed by ampelographic observations compared to previous descriptions (Chomé *et al.* 2003, and unpublished results). With respect to those cultivars with the same microsatellite profiles: 'Picapoll' and 'Picapoll Rosa' differed in the color of the berry, as well as 'Chasselas Doré' and 'Chasselas Rose'; 'Chasselas Cioutat' differed from 'Chasselas Doré' in the shape of the leaves, being deeply divided in the first one. Six cultivars with specific microsatellite profiles were not previously ampelographically described: 'Aramon', 'Cagarrizo', 'Mandón', 'Negreda', 'Pan y Carne', and 'Río Abaixo'. Preliminary results of the ampelographic description has confirmed that they correspond to minor, not previously characterized cultivars.

All of the unknown accessions were identified, and many cases of synonymies were detected in the studied accessions, as shown in Tab. 1. The number of accessions that correspond to the same cultivar ranges from 1 up to 8 for 'Merenzao'.

Frequencies of the alleles for each of the microsatellites are shown in Tab. 2. The total number of alleles was 47 and the number of alleles per locus ranged from 6 to 9, with a mean of 7.8 alleles per locus. For each of the loci there was at least one allele with a frequency higher than 0.20, two for VVS2 and ssrVrZAG62. In VVMD7 and ssrVrZAG62, the alleles 237 and 187 respectively, represent around 40% of the total number. Conversely, 13 alleles showed a frequency below 5 %.

With respect to the observed genotypes (Tab. 3) there was only one case in ssrVrZAG62 where an observed genotype, CB, had a frequency higher than 0.20. The number of observed genotypes ranges from 13 to 20, with an average of 16.3 and a total of 98.

The observed heterozygosity (H_o) was always higher than the expected (H_e), except in the case of ssrVrZAG79 (Tab. 4). The values of H_o ranged from 72.3 % in ssrVrZAG79 to 97 % in VVS2, with a mean value of 85.4 %. The discrimination power of all loci combined was very high, reaching a value over 99.9 %.

Discussion

All the cultivars included in the study have specific microsatellite profiles, with the following exceptions: 'Picapoll' and 'Alsacia', the last one probably being a mutation that only affected berry color, and the three 'Chasselas' ('Chasselas Cioutat', 'Chasselas Doré' and 'Chasselas

Table 2

Frequencies of the alleles at the studied microsatellite loci (unique alleles in bold numbers)

	Microsatellite loci											
	VVS2		VVMD5		VVMD7		ssrVrZAG47		ssrVrZAG62		ssrVrZAG79	
	Allelic size	Frequency	Allelic size	Frequency	Allelic size	Frequency	Allelic size	Frequency	Allelic size	Frequency	Allelic size	Frequency
A	130	0.212	218	0.030	231	0.030	151	0.030	185	0.152	241	0.091
B	134	0.091	222	0.242	237	0.409	155	0.121	187	0.394	243	0.061
C	136	0.045	224	0.076	241	0.136	157	0.197	193	0.212	245	0.167
D	140	0.197	228	0.076	245	0.091	159	0.061	195	0.061	**247**	0.015
E	142	0.136	230	0.136	247	0.167	161	0.152	199	0.045	249	0.348
F	**148**	0.015	232	0.152	251	0.045	165	0.258	203	0.136	**253**	0.015
G	150	0.273	234	0.136	255	0.106	167	0.045			255	0.121
H	156	0.030	236	0.152	**261**	0.015	171	0.136			257	0.136
I											259	0.045

Table 3

Genotype observed (GO) and genotype frequencies (GF) of the six STMS loci in the 33 different cultivars. GO combinations of the two alleles according to the left column in Tab. 3

VVS2		VVMD5		VVMD7		ssrVrZAG47		ssrVrZAG62		ssrVrZAG79	
GO	GF	GO	GF	GO	GF	GO	GF	GO	GF	GO	GF
AA	0.030	BA	0.030	BB	0.152	CB	0.061	AA	0.030	BA	0.030
EA	0.121	DB	0.091	CA	0.030	CC	0.030	BA	0.121	CA	0.030
FA	0.121	EA	0.030	CB	0.152	DC	0.030	BB	0.152	CB	0.030
FC	0.030	EB	0.061	DB	0.121	DD	0.030	CB	**0.212**	CC	0.061
FE	0.091	EE	0.030	DC	0.030	EB	0.030	CC	0.030	DA	0.030
GE	0.030	FB	0.061	EA	0.030	EC	0.030	DA	0.030	EB	0.030
HA	0.121	FC	0.121	EB	0.121	GA	0.061	DB	0.061	EC	0.091
HC	0.152	FE	0.030	EC	0.030	GB	0.061	EB	0.030	EE	0.152
HD	0.091	FF	0.030	ED	0.030	GC	0.152	EC	0.030	FE	0.030
HE	0.121	GB	0.121	EE	0.030	GE	**0.182**	ED	0.030	GA	0.061
HF	0.030	GE	0.061	FB	0.030	HB	0.061	FA	0.091	GE	0.091
IE	0.030	GG	0.030	GB	0.061	HG	0.030	FB	0.061	GG	0.030
IH	0.030	HB	0.121	GC	0.030	IB	0.030	FC	0.121	HA	0.030
		HC	0.030	GE	0.061	IC	0.061			HC	0.030
		HD	0.061	GF	0.061	ID	0.030			HE	0.121
		HE	0.030	HB	0.030	IE	0.061			HG	0.030
		HF	0.030			IG	0.030			HH	0.030
		HG	0.030			II	0.030			IB	0.030
										IC	0.030
										IE	0.030

Table 4

Number of observed genotypes G_o, number of possible genotypes (G_p), effective number of alleles, ENA, observed H_o and expected H_e heterozygosity, polymorphism information content, PIC, probability of coincidence, C, and discrimination power, D, with six STMS loci in the analyzed cultivars

Locus	G_o (G_p)	ENA	H_o	H_e	PIC	C	D
VVS2	13 (45)	5.31	0.970	0.824	0.786	0.104	0.896
VVMD5	18 (36)	6.48	0.909	0.859	0.827	0.076	0.924
VVMD7	16 (36)	4.23	0.818	0.775	0.737	0.095	0.905
ssrVrZAG47	18 (45)	5.95	0.909	0.845	0.811	0.087	0.913
ssrVrZAG62	13 (21)	4.04	0.788	0.764	0.718	0.118	0.882
ssrVrZAG79	20 (45)	5.08	0.723	0.815	0.780	0.074	0.926
Mean	- -	5.18	0.854	0.814	0.777	-	-
Cumulative	- -	-	-	-	-	0.575×10^{-6}	0.99999943

Rose'), the first one with divided leaves and the last with pink berries. Consequently, the discrimination power of the STMS is markedly high, although some cultivars with different morphological characteristics like berry color can not be distinguished by these molecular markers, as previously mentioned by other authors (SEFC *et al.* 1998, LOPES *et al.* 1999). The character of divided leaves in 'Chasselas Cioutat' could also be due to a somatic mutation.

With respect to the molecular variability detected, the most frequent alleles were the same of the publication of MARTÍN *et al.* (2003) in a study of 176 Spanish cultivars, except in the case of ssrVrZAG47 locus that has the highest frequency for the allele 165 (0.258) instead of the allele 157 (0.219) in the mentioned study; the percentage of alleles with a frequency f > 0.20 was higher than in the same study, 17 % vs. 9 %, and the percentage of relatively infrequent (f < 0.05) alleles was lower, 23.4 % vs. 53 %. The average number of alleles per locus was 7.8. There were four unique alleles, with sizes 148 (VVS2), 261 (VVMD7), and 247 and 253 (ssrVrZAG79). Moreover, the number of observed genotypes was lower in the present work than in the survey of MARTÍN *et al.* (2003), 98 vs. 199, probably as a consequence of the lower number of cultivars studied.

The locus ssrVrZAG62 presented the most frequent genotype, 187/193, followed by ssrVrZAG47, 161/165. For each locus, the number of unique genotypes varied from 6 to 14 (0.030 frequency), the highest number for ssrVrZAG79, like in MARTÍN *et al.* (2003). The cultivars with higher number of unique genotypes were 'Moscatel de Grano Menudo' with five of the six loci, and 'Tempranillo' and 'Prieto Picudo Blanco' with four.

The observed heterozygosity was always higher than the expected one except for the locus ssrVrZAG79. The excess of heterozygotes might be due to the selection for yield and quality as previously reported by Lopes *et al.* (1999). Assuming homozygosity instead of heterozygosity with a null allele for ssrVrZAG79 when detecting only one allele per locus, might be the reason for the difference, considering that this locus has a higher number of homozygotic accessions.

In the view of our results the most informative STMS locus was VVMD5 with a PIC of 82.7 %, a ENA of 6.48 and a probability of coincidence of 0.076, and the least informative ssrVrZAG62 with a PIC of 71.8 %, a ENA of 4.04 and a probability of coincidence of 0.118. Other studies with the same set of STMS loci arrived at the conclusion that VVMD5 is the most informative (Lopes *et al.* 1999, Sánchez-Escribano *et al.* 1999, Sefc *et al.* 2000, Martín *et al.* 2003).

To complete the discussion, the studied cultivars have been grouped according to the color of the berries, since the interest of this characteristic from the agronomical and the commercial viewpoints.

Fifteen cultivars with red berries and non pigmented pulp were identified in different plots. 'Mencía' (Chomé *et al.* 2003), has certain intravarietal variability. Some clones of the called 'Mencía Pajaral' were collected, that showed shorter internodes and more marked and serrated teeth, although ampelographic and molecular characterization identified them as the same 'Mencía' cultivar. Several accessions of 'Merenzao' (Chomé *et al.* 2003) received different homonymies, like 'Estaladiña' and 'Pan y Carne'. 'Mouratón', is known as 'Juan García' in the Arribes del Duero region. 'Mandón' also grown in some plantations of the same region (Rubio *et al.* 2005) was collected with the local name of 'Valenciana Tinta'. 'Brancellao'(Chomé *et al.* 2003) is a minor cultivar. 'Monastrell' (Chomé *et al.* 2003) is grown in many of the grapevine regions of Spain. Only some isolated plants of 'Aramon', or 'Aramon black' were detected. 'Albarín Negro', that is 'Alfrocheiro Preto' (Portugal) was detected under the homonymie of 'Estaladiña'. 'Pan y Carne' is a variety mentioned by García de los Salmones (1914) as existing in León province. At present it is almost extinct. In some plots it was also found under the homonymous name of 'Estaladiña'. 'Negreda' is a variety not previously described, that was detected in several places in the region. It might correspond to the cultivar with this name mentioned by García de los Salmones (1914) in Galicia and in León. Several plots had plants of the cultivars 'Tempranillo', grown in many D.O. of Spain and 'Sumoll' grown in some localities in the Catalonian region, and collected as 'Rabo de Ovella', that should be considered as a misname since it is not the Portuguese variety with this name. Also 'Garnacha Tinta' was present in one of the plots. Also some foreign cultivars like 'Merlot' and 'Cabernet Sauvignon' were detected.

Three red cultivars with pigmented pulp ('Teinturier') were localized: 'Garnacha Tintorera', locally known as 'Alicante Tinta', 'Garnacho' that is the 'Morrastel Bouschet', and 'Negrón de Aldán' or 'Petit Bouschet' locally named 'Tinta Aragonesa'. All three are related: 'Petit Bouschet' is a cross of 'Aramon' and 'Teinturier'; 'Garnacha Tintorera' is a cross between 'Garnacha Tinta' and 'Petit Bouschet' and 'Garnacho' has 'Petit Bouschet' as one of its parents (http://www.vitis.org). All these three cultivars are used for improving the color of the red wines in the region. From the molecular viewpoint, 'Petit Bouschet' has at least one allele in common with any of the two other cultivars for any STMS loci (see Tab. 1), as expected by its genetic relationship.

Thirteen white cultivars were identified (Tab. 1). 'Godello' is grown in several places in 'Castilla y León'. 'Allarén' was mentioned by García de los Salmones (1914) as existing in León; only some isolated plants were detected. 'Malvasía Riojana', synonymie of 'Alarije' and 'Subirat Parent' (Chomé *et al.* 2003) is grown in several regions of Spain. 'Moscatel de Grano Menudo' or 'Muscat á petit grains' is locally known as 'Moscatel Rubio'. 'Temprano blanco' is a cultivar that has also been found in many localities of the region, in some of them under the synonymy of 'Pixa de Galo'. 'Palomino' is an Andalusian cultivar that was also planted in several places of Galicia along the last decades. Several plants of each of the following cultivars were also localized in the region: 'Cagarrizo', 'De Rey', 'Rio Abaixo' and 'Mando' or 'Prieto Picudo Blanco'. They had not previously described and are almost extinct, being of marked interest for preservation of the existing variability. Some plants of 'Picapoll' were also collected, as well as 'Chasselas Cioutat', already mentioned.

Only two cultivars with pink or red berries were identified: 'Temprano Colorado' or 'Chasselas Rose' and 'Alsacia', that has the same microsatellite genotype than 'Picapoll'. The ampelographical differences are reduced to the color of the berry, pink in 'Alsacia' and white in 'Picapoll', hence this variety could also be designed as 'Picapoll Rosa'. The seven cultivars, 'Aramon', 'Cagarrizo', 'Mandón', 'Negreda', 'Pan y Carne', 'Río Abaixo', and 'Picapoll Rosa' or 'Alsacia', not previously characterized, can be considered as endangered, as well as 'Allarén', 'De Rey', and 'Prieto Picudo Blanco', the last three being present in the grapevine germplasm collection of 'El Encín' (Spain) (Cabello 1995). Only isolated plants of them are present in commercial plantations. Two of these cultivars, 'Río Abaixo' and 'Negreda' are unique, not previously mentioned and non existing in maintenance collections. For the six first cultivars, the microsatellite profiles have been here published here for the first time.

As a conclusion of the present work, a marked molecular biodiversity was detected in the collected grapevine material at the 'Comarca del Bierzo'. Maintenance of minor endangered cultivars in germplasm collections is highly recommended.

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