

Genetic Structure, Origins, and Relationships of Grapevine Cultivars from the Castilian Plateau of Spain

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Abstract: Four hundred twenty-one cultivated (*Vitis vinifera* ssp. *sativa*) and four alleged wild grapevine samples (putative *Vitis vinifera* ssp. *sylvestris*) from the Castilian Plateau in northern central Spain were genotyped at the six nuclear microsatellite loci (SSRs) proposed as a standard set for cultivar identification by the GENRES 081 project, yielding 121 different genotypes. The cultivated data set yielded 300 redundant samples, 13 homonyms, and 27 previously unreported genotypes, almost one-fourth of the nonredundant genotypes. Nonredundant genotypes were examined at another 16 nuclear and three chloroplast additional microsatellite loci for further analyses. Three differentiated genetic clusters were detected among them, separating (1) Muscat-type accessions and interspecific *Vitis* hybrids, (2) accessions from France and the western Castilian Plateau, and (3) accessions from the central Castilian Plateau together with local table grapes. The close relatedness of accessions from the western plateau among each other and to French varieties supported introduction of the latter along the pilgrimage route to Santiago de Compostela. White-berried cultivars from the central plateau were also closely related. Chlorotype data suggested that previously unpublished genotypes and autochthonous Castilian varieties had local origins or resulted from crosses between introduced and local varieties. Morphological features and allelic composition suggested that three of the four samples collected from wild habitats were closely related and might represent genuine *Vitis vinifera* ssp. *sylvestris* individuals.

Key words: Spanish grapevine cultivars, *Vitis vinifera* L., chlorotypes, genetic structure, parentage relationships

Cultivated grapevine (*Vitis vinifera* ssp. *sativa*) likely evolved from the domestication of wild grapevine (*V. vinifera* ssp. *sylvestris*), a dioecious climbing plant thriving in riverbank forests from Portugal to the Himalayas and from Germany to northern Africa. Archaeological evidence suggested early grapevine domestication in the Near East (Zagros Mountains, Iran) (Arroyo-García et al. 2006, and references therein). Genetic information agrees (Aradhya et al. 2003) and suggests at least one secondary domestica-

tion event in western Europe (Arroyo-García et al. 2006). Spontaneous hybridization among cultivated and between cultivated and wild populations and a combination of seed and vegetative propagation would have given rise to the current diversity of cultivars. Furthermore, the exchange of plant material among regions could have homogenized the pools of cultivated grapevines over time (This et al. 2006).

European wild grapevine, *Vitis vinifera* ssp. *sylvestris*, is faced with extinction because of habitat destruction and foreign pests such as phylloxera (Arrigo and Arnold 2007). It is confined to isolated populations subject to inbreeding depression (Di Vecchi-Staraz et al. 2009) and its endangered status was recognized by the International Union for Conservation of Nature in the 1980s. Current vines collected from natural habitats frequently correspond to a mixture of pure *V. vinifera* ssp. *sylvestris*, escapees from cultivation (*V. vinifera* ssp. *sativa* or pest-resistant rootstocks, which are hybrids between *V. vinifera* and northern American *Vitis* species), and spontaneous crosses between them (Arrigo and Arnold 2007). A number of surviving wild grapevine populations have recently been reported in the Iberian Peninsula (Arroyo-García et al. 2006).

The Castilian Plateau in northern central Spain is a large, mountainous region, crossed by the Duero River and its tributaries (Supplemental Figure 1). Viticulture likely predated Roman arrival and today represents the main and most productive agricultural activity of the area. Such environmental and historical factors make this region a likely candidate to contain a high number of genetically diverse cultivars. On the other hand, the cold continental winters on the Castilian Plateau restrict potential natural habitats

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for wild grapevines to a few, low frost-free valleys in the border sides of the region.

Despite their economic importance, the origins and genetic relationships of local cultivars from this area are poorly understood. Certain historical references provide clues, such as the possible introduction of French cultivars into the plateau via the pilgrimage route to Santiago de Compostela. Unfortunately, in most European vineyards, the incidence of pests like phylloxera, the abandonment of rural areas, and especially the globalization of wine companies and markets promote the progressive substitution of old local varieties by better-known cultivars and have led to a drastic decrease in cultivar diversity during the last century (This et al. 2006). As a result, most known, named grape varieties exist only in germplasm collections.

There are approximately 10,000 grapevine cultivars held in germplasm collections worldwide. However, based on DNA profiling, the number of grapevine varieties is estimated at ~5,000, many closely related (This et al. 2006). This proves the need for identifying synonyms and homonyms in collections to remove redundant accessions and improve their management. Moreover, this is the first step toward estimating the extent of genetic diversity in the species. Maintenance of local cultivars is critical for conserving grapevine diversity. Local genotypes, even those with poor quality traits, may hold underlying unique alleles or allele combinations that confer a high genetic potential as parents.

When considering cultivar identification of grapevines, nuclear microsatellite markers (SSRs) are the most used tools, as demonstrated by European Projects GENRES 081 (This et al. 2004) and GrapeGen06 (<http://www1.montpellier.inra.fr/grapegen06>). They have also been useful for parentage and pedigree studies (Vouillamoz and Grando 2006). Chloroplast DNA microsatellites (cpSSRs) have a conserved gene order, low mutation rate, and maternal inheritance and have therefore become widely used to investigate the origins, domestication events, and phylogeography of *Vitis vinifera* L. (Arroyo-García et al. 2006). The combination of nuclear and chloroplast SSRs is suitable to examine the origins and evolution of grapevines.

The central objective for this study was to clarify the composition and diversity of the grapevine gene pool in the Castilian Plateau in northern central Spain. More specifically, we investigated (1) genetic composition of grapevine cultivars present in the region, focusing on newly identified genotypes; (2) structure of the cultivated grapevine gene pool, especially with respect to possible hybridization between local and French cultivars; and (3) putative parentage relationships within this cultivated gene pool.

Materials and Methods

Four hundred twenty-one (421) samples of grapevines cultivated in the Castilian Plateau were analyzed. Forty-one were from the Grapevine Germplasm Bank of Castilla y León (BGVCyL). The rest were directly collected from vineyards. Several French varieties commonly cultivated in this area were included as references. Additionally, we collected

four samples from wild environments in the Arribes del Duero region that were surrounded by vineyards to determine their nature and relatedness to locally cultivated varieties. Three wild samples (labeled W1, W2, and W3) were from a population (Aldeadávila, 41°10'N; 6°30'W; 200 to 400 m asl) located on the Spanish bank of the Duero River, and a fourth (W4) sample was collected 5 km from **the former**.

DNA was extracted as previously described (Santana et al. 2008). We initially typed the six microsatellites proposed as a core set for grapevine cultivar identification by the European Research Project GENRES 081 (This et al. 2004): VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, and VrZAG79. Redundant genotypes were discarded and cultivar identification was performed by comparing the set of unique profiles with microsatellite data from the Vitis International Variety Catalogue (www.vivc.bafz.de) and various studies (Crespan et al. 1999, Crespan and Milani 2001, Lefort and Roubelakis-Angelakis 2000, Aradhya et al. 2003, Ibáñez et al. 2003, Martín et al. 2003, 2006, Hvarleva et al. 2004, Almadanim et al. 2007, Zinelabidine et al. 2010). Nonredundant genotypes were subsequently analyzed with an additional set of 16 SSRs previously used to study genetic relationships among grapevines (Di Vecchi-Staraz et al. 2009): VVMD21, VVMD24, VVMD25, VVMD28, VVMD32, VVIb01, VVIh54, VVIIn16, VVIIn73, VVIp31, VVIp60, VVIq52, VVIv37, VVIv67, VMC1b11, and VMC4f3. The 22 analyzed loci are distributed along the 19 linkage groups of the nuclear genome of *Vitis vinifera* L. All but VrZAG62 and VrZAG79 have proved powerful for paternity analysis in grapevine (Di Vecchi-Staraz et al. 2009). We also genotyped three chloroplast microsatellite loci: cpSSR3, cpSSR10, and ccSSR9 (Arroyo-García et al. 2006) to generate additional information on the putative geographical origin of the different accessions based on chlorotypes.

PCR amplifications for SSRs and cpSSRs were performed as described previously (Santana et al. 2008). Amplification products were mixed with 20 µL deionized formamide and 0.4 µL size standard GeneScan 500-ROX (Applied Biosystems, Foster City, CA), denatured at 95°C for 3 min and separated on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Fragment sizes were calculated with GeneScan 3.7. Each sample was independently amplified at least twice to prevent genotyping errors.

Genetic diversity of nonredundant genotypes at the six GENRES 081 SSRs, excluding wild individuals to allow comparison to similar studies, was evaluated using number of alleles, expected (H_e) (gene diversity) and observed (H_o) heterozygosity, and probability of identity (PI) using Identity software (v. 1.0; Centre for Applied Genetics, University of Agricultural Sciences, Vienna). Discrimination power (D) was calculated as

$$D = 1 - \sum P_i^2$$

(Tessier et al. 1999), where P_i is the frequency of the genotype i . Both PI and D describe the probability that two unrelated cultivars can be distinguished by a certain marker (Sefc et al. 2000). The data set of nonredundant 22-locus

genotypes also enabled an indirect appreciation of the power of SSRs to discriminate the cultivars under study, as opposed to *PI* and *D* which assume unrelated cultivars and therefore independence of loci and genotypes. Using Microsoft Excel, we compared each of the 117 unique cultivated genotypes with each other and counted the number of loci that were identical in each pairwise comparison of genotypes. Based on the resulting 117 x 117 matrix of identical loci, the average number of genotypes for six or more loci out of 22 was identified.

Genetic clustering was inferred with Structure v. 2.1. (Pritchard et al. 2000) based on data from 22 SSRs. Ten runs with a burn-in of 50,000 and a run length of 100,000 iterations were performed for an imposed number of clusters ranging from $K = 1$ to $K = 10$, using the admixture model, letting genotypes have ancestry in more than one cluster and allowing correlation of allele frequencies among clusters. The optimal number of genetic clusters, K , was chosen using the delta K method (Evanno et al. 2005), and individual proportions of ancestry in each cluster were summarized over runs using CLUMPP (Jakobsson and Rosenberg 2007). After assigning each individual cultivar to the Structure cluster in which it had >70% ancestry, genetic differentiation among pairs of clusters was computed as pairwise F_{ST} with FSTAT v. 2.9.3. (Goudet 1995). Significance of F_{ST} values was tested using exact tests in Genepop 4.0 (Raymond and Rousset 1995). The polymorphism of each cluster was characterized as gene diversity H_E , calculated with Identity, and as allelic richness (A_s), the number of alleles expected in a sample of a given size, calculated with FSTAT v. 2.9.3.

Genetic distances between the 22-locus genotypes were calculated as D_A and D_C , both considered appropriate to reflect a correct tree topology (Takezaki and Nei 1996), using the software Populations as described elsewhere (Santana et al. 2008). Four dendrograms were constructed with UPGMA and neighbor-joining algorithms. The dendrogram with the highest bootstrap support (1000 bootstrap samples) was used to check genetic relationships among accessions. Parentage relationships were proposed based on percentage of shared alleles relatedness estimator r (Queller and Goodnight 1989), and relationship category assignments (parent-offspring, full sibs, half sibs) were suggested from likelihood ratios obtained by Markov Chain Monte Carlo simulations performed with KinGroup software (Kononov et al. 2004).

Results

Varietal diversity and composition of the sample. Using the six GENRES 081 SSRs, 117 unique genotypes were discriminated among the cultivated and four among the wild samples out of 421 analyzed (Table 1). Three hundred (300) samples were identical to one of the discriminated genotypes, frequently under different names that correspond to synonyms. In addition, 13 homonyms were identified. Genetic diversity estimators for the nonredundant cultivated samples at the six nuclear GENRES loci are summarized

(Table 2). The mean discrimination power per locus was 94.0%, confirming that these are highly polymorphic microsatellites (Tessier et al. 1999). The cumulative probability of two unrelated cultivars sharing a genotype at the six loci was on the order of 10^{-7} , suggesting high power for identification of synonyms given the allele frequencies in this sample (Sefc et al. 2000). However, the pairwise comparison of 22-locus genotypes showed that each of the 117 unique cultivars shared on average the same genotype at six or more loci with 3.57 other cultivars, a fairly high figure, which suggested that some cultivars were related. Comparison to available online and published grape genotypes matched many samples considered local with Spanish, French, or other foreign-assigned genotypes, allowing their correct identification (Table 1). The complete 22-locus genotypes and corresponding chlorotypes are presented as supplementary information (Supplemental Table 1).

Twenty-seven genotypes (23.1%) collected from different viticulture areas (Supplemental Figure 1) did not match any genotype in the European *Vitis* database or in nine published SSR studies of grapevine germplasm. They corresponded to locally grown varieties for which no previous references or plant materials have been found in any Spanish grapevine collection or commercial nursery. Thirteen displayed chlorotype A, typical for Iberian and Central European wild grapevines (Arroyo-García et al. 2006), although there were also five accessions carrying chlorotype B, five with D, two with C, and one with G.

All samples collected from the wild presented unique alleles. Sample W4 showed outlier allele sizes for a number of loci in our sample (Supplemental Table 1) and was identified as the *Vitis* interspecific hybrid *Aramon* x *rupestris* Ganzin#1, typically used as rootstock (De Andrés et al. 2007). Six newly identified cultivated genotypes (TL16, TVZ7, ME5, ME6, ME8, and ME9) also showed extreme allele sizes for more than one locus. Five bore chlorotype B or C, the only two types found in the American *Vitis* species usually used for crosses (Arroyo-García et al. 2002). The remaining one, TVZ7, had chlorotype A and showed a high percentage of shared alleles with the demonstrated hybrid W4.

Genetic structure of Castilian Plateau grapevines. The large proportion (23.1%) of newly identified genotypes within the analyzed cultivated samples raised important questions about their origins. Different clustering approaches were used to establish their genetic relationships.

Neighbor-joining (NJ) and UPGMA dendrograms had very low bootstrap support of deep nodes, making the inference of genetic clusters impossible. The neighbor-joining dendrogram on D_A displayed the highest number of bootstrap values above 70% (10 relationships at terminal nodes; Figure 1) and divided the genotypes into three major groups, identified as Garnacha, France-Tempranillo, and Muscat (Moscatel) NJ clusters. The model-based clustering approach in Structure showed the highest probability of the data for $K = 3$ clusters ($\ln P(D) = -9247.5$, $\Delta K = 295.8$). Average proportions of ancestry over 10 Structure runs of each genotype in the proposed clusters are shown

Table 1 Accessions with unique genotypes and their varietal identification according to the European Vitis Database (www.vivc.bafz.de) and published research (Crespan et al. 1999, 2001, Lefort et al. 2000, Aradhya et al. 2003, Ibáñez et al. 2003, Martín et al. 2003, 2006, Hvarleva et al. 2004, Almadanim et al. 2007, and Zinelabidine et al. 2010).

Code	Accession name	Berry color ^a	Original collection zone	Varietal identification	Assignment to Structure cluster (>70% ancestry)
Heading need here					
Bi1	Tintorera	t	Bierzo	Negrón de Aldán	nd
Bi3	-	b	Bierzo	Rabo de Ovella/Sumoll	nd
Bi4	-	b	Bierzo	Negreda	3
Bi6	-	w	Bierzo	<i>Unknown</i>	nd
Bi7	-	b	Bierzo	Mandón/Morenillo II	nd
Bi8	-	t	Bierzo	Grand Noir/Morrastel-Bouschet	3
Bi9	-	-	Bierzo	<i>Unknown</i>	3
Bi10	-	w	Bierzo	Picapoll	3
Bi11	Doña Blanca	w	Bierzo	Doña Blanca	3
Bi12	Mencía	b	Bierzo	Mencía	nd
Bi13	Garnacha Tintorera	t	Bierzo	Garnacha Tintorera	2
Bi15	Palomino Macho	w	Bierzo	De Rey	2
Bi19	-	-	Bierzo	<i>Unknown</i>	1
Bi20	-	-	Bierzo	<i>Unknown</i>	1
Bi21	-	b	Bierzo	Brancellao	3
Bi22	-	w	Bierzo	Allarén	nd
Bi25	-	w	Bierzo	Perla de Csaba	1
Bi28	-	w	Bierzo	Cagarrizo	3
Bi29	Toledana	b	Bierzo	Aramon	2
Bi32	Godello	w	Bierzo	Godello	3
Bi36	-	b	Bierzo	<i>Unknown</i>	3
Bi37	-	b	Bierzo	Monastrell	3
Bi43	-	-	Bierzo	<i>Unknown</i>	1
Bi48	-	b	Bierzo	Petit Verdot	nd
Bi50	Tinta	b	Bierzo	Trepát	2
Bi54	-	w	Bierzo	Pedro Ximénez Canario	3
SF5	Verdejo Serrano	w	Sierra de Francia	<i>Unknown</i>	3
SF8	Moscatel Gordo Peludo	w	Sierra de Francia	Moscatel Gordo Peludo/Gordera Roja	1
SF12	-	b	Sierra de Francia	Moristell	2
SF13	Vigorosa	b	Sierra de Francia	Brujidera	2
SF14	Nudo Corto	b	Sierra de Francia	Mazuelo/Cariñena	2
SF15	Colgadera	b	Sierra de Francia	Gordera Negra	2
SF17	-	b	Sierra de Francia	Ariño	nd
SF18	Falso Rufete	b	Sierra de Francia	<i>Unknown</i>	3
SF27	-	w	Sierra de Francia	Legiruela	1
SF34	-	b	Sierra de Francia	Morate	2
SF46	Calabrés	b	Sierra de Francia	<i>Unknown</i>	3
SF50	Cojón de Gallo	r	Sierra de Francia	<i>Unknown</i>	2
SF60	-	w	Sierra de Francia	<i>Unknown</i>	2
SF61	Del Pipajo	w	Sierra de Francia	Zalema	2
AR4	Bastardillo Serrano	b	Arribes del Duero	Sinsó	nd
AR7	Tinta Jeromo	b	Arribes del Duero	Tinta Jeromo	nd
AR8	Puesta en Cruz	w	Arribes del Duero	Rabigato	1
AR13	-	w	Arribes del Duero	Mantúo	2
AR14	Juan García	b	Arribes del Duero	Juan García	3
AR17	-	-	Arribes del Duero	<i>Unknown</i>	3
AR35	Verdejo Colorado	r	Arribes del Duero	<i>Unknown</i>	2
AR38	Gajo Arroba	b	Arribes del Duero	Gajo Arroba	nd
PT5	Cornifesto	b	Arribes del Duero	<i>Unknown</i>	3
PT8	Trincadeira Preta	b	Arribes del Duero	Trincadeira	3
PT18	-	b	Arribes del Duero	<i>Unknown</i>	3
PT20	Poilta	b	Arribes del Duero	<i>Unknown</i>	nd
TL1	Albarín Blanco	w	Tierra de León	Albarín Blanco	3
TL3	Prieto Picudo	b	Tierra de León	Prieto Picudo	3
TL13	Francesa	w	Tierra de León	Centennial Seedless	1
TL16	-	b	Tierra de León	<i>Unknown</i>	1
BE13	-	-	Valles de Benavente	<i>Unknown</i>	3
BE26	Moscatel	w	Valles de Benavente	Italia	1
1BE1	Redondal	w	Valles de Benavente	Kamchiya	nd
1BE2	Teta de Cabra	w	Valles de Benavente	Bougseb	nd
1BE3	Moscatel Romano	w	Valles de Benavente	Muscat of Alexandria	1
1BE9	Santa Paula	r	Valles de Benavente	Molinera/Red Malaga	2

^ab: black; p: pink; r: red; t: teinturier; w: white.

continued

Table 1 (cont.) Accessions with unique genotypes and their varietal identification.

Code	Accession name	Berry color ^a	Original collection zone	Varietal identification	Assignment to Structure cluster (>70% ancestry)
1BE12	Moscatel Romano	b	Valles de Benavente	Muscat Hamburg	1
1BE14	Moscatel Blanco de Grano Menudo	w	Valles de Benavente	<i>Unknown</i>	1
1BE15	Moscatel Tinto de Grano Menudo	b	Valles de Benavente	Moscatel de Grano Menudo/Muscat aux Petit Grains/Moscato Bianco	1
2BE1	Morisco	w	Valles de Benavente	Jaén Blanco	2
2BE3	Tinta Fina	b	Valles de Benavente	Albarín Negro	3
4BE6	Molinera	b	Valles de Benavente	Corbeau	3
4BE13	Teta de Cabra Tinta	b	Valles de Benavente	Negra Dorada	nd
4BE14	Juan el Herrero	b	Valles de Benavente	Tarragoní	2
5BE1	-	w	Valles de Benavente	Pleita/Mandilari	2
TO1	Colgadera	w	Toro	Airén	2
TO4	-	p	Toro	Perlon	nd
TO21	Teta de Cabra	p	Toro	Teta de Vaca	2
TO37	Malvasía	w	Toro	<i>Unknown</i>	1
TO38	Merlot	b	Toro	Merlot	nd
TVZ2	Albillo Real	b	Zamora	Albillo	1
TVZ7	-	b	Zamora	<i>Unknown</i>	1
ME1	Blanca del País	w	Valle de Mena	De José Blanco	1
ME3	Hondarrabi	w	Valle de Mena	Petit Courbu	3
ME5	Tinta del País	b	Valle de Mena	<i>Unknown</i>	1
ME6	Tinta del País	b	Valle de Mena	<i>Unknown</i>	1
ME8	Tinta Redonda	b	Valle de Mena	<i>Unknown</i>	1
ME9	Blanca del País	w	Valle de Mena	<i>Unknown</i>	1
ME10	Tinta Gorda	b	Valle de Mena	Señá	nd
ME12	Hondarrabi	w	Valle de Mena	Matza Zuri	3
ME15	Tinta	b	Valle de Mena	Graciano	3
ME17	Blanca del País	w	Valle de Mena	Chardonnay	3
CoNV1	Blanca de Mesa	w	Valladolid	Beba/Calop Rojo	2
CoNV2	De Mesa Gorda	b	Valladolid	Alphonse Lavallé	1
Ci1	Albillo Mayor	w	Cigales	Albillo Mayor	2
Ci2	Temprana Media	w	Cigales	Pardillo	2
Ti1	Albillo Real Extremadur	w	Valle del Tiétar	Albillo Real	2
RU1	Palomino	w	Rueda	Palomino	2
RU2	Sauvignon blanc	w	Rueda	Sauvignon blanc	3
RU3	Verdejo	w	Rueda	Verdejo	3
RU4	Viura	w	Rueda	Viura/Macabeo	2
RD1	Cabernet Sauvignon	b	Ribera del Duero	Cabernet Sauvignon	3
RD2	Garnacha Tinta	b	Ribera del Duero	Garnacha	2
RD3	Malbec	b	Ribera del Duero	Malbec	nd
Accessions collected from the BGVCyL					
CyL-03	Alcazpepita	w	Rueda	Cañorrojo	2
CyL-04	Calagraña	w	Rueda	Calagraña/Katsano	2
CyL-06	Doradilla 2	w	Rueda	<i>Unknown</i>	2
CyL-09	Huerta del Rey	w	Cigales	Huerta del Rey/Marfal	2
CyL-10	Marta Nava	w	Rueda	Salvador	2
CyL-12	Pirulés Dorada	w	Ribera del Duero	Alarije	2
CyL-18	Temprana Agosteña	w	Cigales	Lairén	nd
CyL-22	Tempranillo de Nava	w	Rueda	Chasselas Doré	1
CyL-23	Tolociriana	w	Rueda	Tolociriana/Castellana Blanca	2
CyL-26	Juliana	r	Rueda	Juliana	2
CyL-27	Cenicienta	b	Rueda	Cenicienta	2
CyL-30	Pan y Carne 1	b	Bierzo	Merenzao	3
CyL-31	Pan y Carne 2	b	Bierzo	<i>Unknown</i>	3
CyL-34	Rufete	b	Sierra de Francia	Rufete	3
CyL-37	Tinta Madrid	b	Cigales	Bobal	2
CyL-39	Tinta del País	b	Ribera del Duero	Tempranillo	2
CyL-40	Verdejo Tinto	b	Sierra de Francia	Puesto Mayor	3
Samples collected from the wild					
W1	Wild individual 1	male	Arribes, wild habitat	<i>Unknown</i>	nd
W2	Wild individual 2	b	Arribes, wild habitat	<i>Unknown</i>	nd
W3	Wild individual 3	male	Arribes, wild habitat	<i>Unknown</i>	1
W4	Wild individual 4	male	Arribes, wild habitat	Aramon x <i>Rupestris</i> Ganzin#1 (hybrid)	1

^ab: black; p: pink; r: red; t: teinturier; w: white.

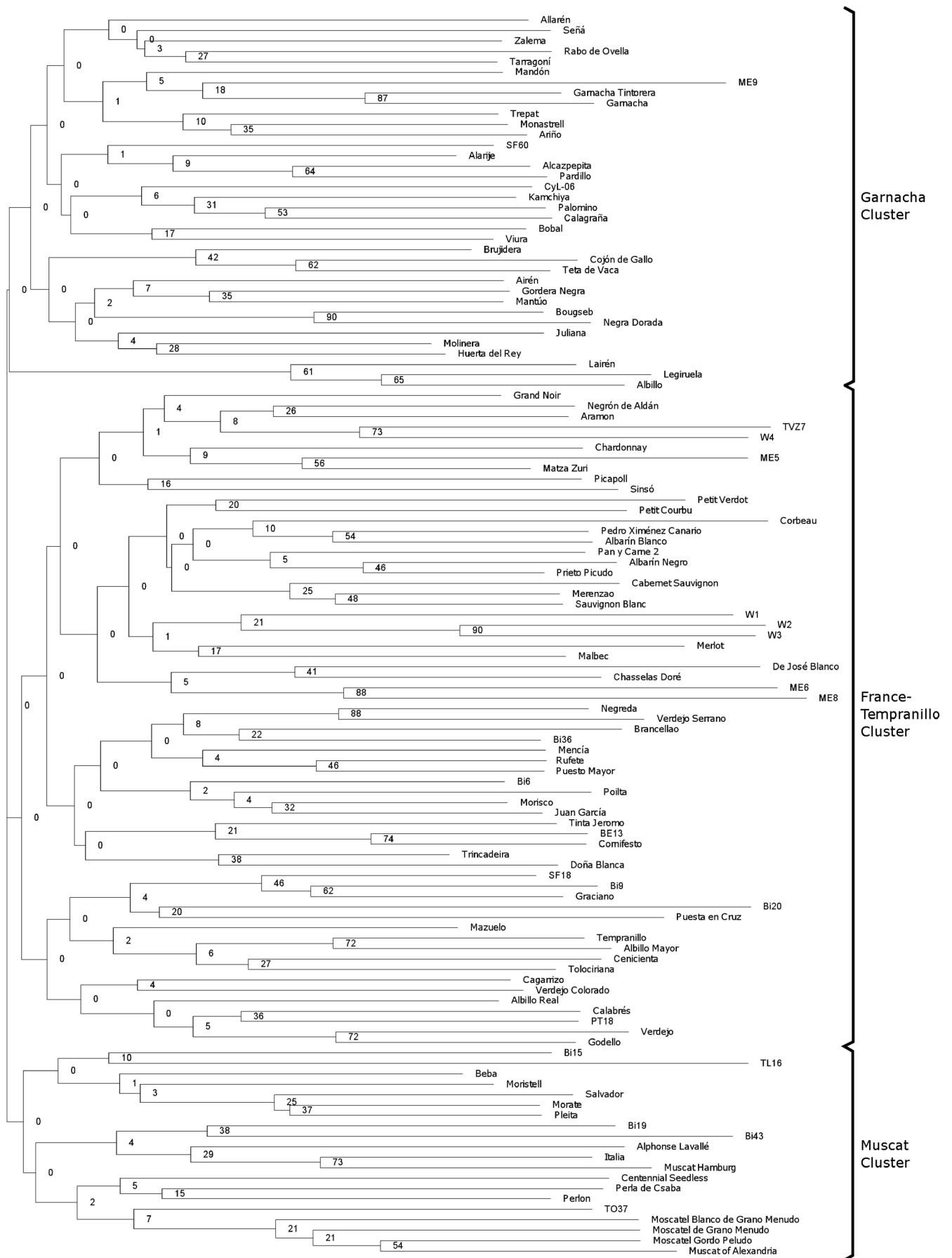


Figure 1 Neighbor-joining dendrogram constructed on D_A distances among 117 genotypes for 22 SSRs.

as supplementary data (Supplemental Figure 2). Assignment of individual genotypes to the cluster in which they had more than 70% ancestry (Table 1) left 22 genotypes (out of 121) unassigned. Cluster 1 contained all Muscats in the sample, several local accessions used as table grapes with names and morphology suggestive of the Muscat family, table grapes of foreign origin like Centennial Seedless or Perla de Csaba, and wild samples and hybrids (used as rootstocks in many vineyards), almost matching the Muscat cluster from NJ analysis. Cluster 2 contained the majority of local table grapes in the sample, most local white-berried accessions from the central plateau, and the well-known black-berried varieties Tempranillo and Garnacha, corresponding to the sum of the Garnacha cluster and Tempranillo subcluster from the NJ approach. All French wine accessions were in cluster 3, along with representative wine accessions from the western plateau, such as Juan García, Prieto Picudo, Rufete, Doña Blanca, and Godello, and some newly identified genotypes. This cluster closely matched the France subcluster from the NJ approach.

Genetic diversity and differentiation among clusters.

Allelic richness, gene diversity, and number of alleles per locus were highest for cluster 1 ($A_s = 9.86$, $H_e = 81.2\%$), with remaining clusters also fairly polymorphic ($A_s = 6.13$ and 6.22 , $H_e = 71.3\%$ and 71.8% for clusters 2 and 3 respectively) (Table 3). All differentiation tests between pairs of clusters were significant ($p < 0.001$, Table 4), with the greatest between clusters 2 and 3 ($F_{ST} = 0.077$), likely due to the heterogeneous composition of cluster 1.

Accessions from cluster 1 mostly exhibited chlorotypes B and D, typical of Muscat cultivars (Laiadi et al. 2009), with an important presence of chlorotype C. Chlorotype A, typical in wild populations from the Iberian Peninsula and in the majority of cultivated Iberian accessions (Arroyo-García et al. 2006), was predominant in clusters 2 and 3, with a limited presence of D and C, mostly in French accessions, and a complete absence of B.

Putative parentage relationships within the sample.

Many cultivars in each genetic cluster could be first-degree relatives, since many shared at least one allele at each of the 22 SSR loci, shared certain morphological traits, had

similar names, and were traditionally grown in the same areas. To test that possibility, putative parentage relationships were proposed based on the combination of several estimators: percentage of shared alleles identical by state (IBS), relatedness coefficient (r) and likelihood ratios (LRs) (Table 5), chlorotype information, pairwise bootstrap values in the dendrogram, and cultural and historical information. This strategy was chosen because the category of relationship based on LRs alone is not significant below a minimum of 57 microsatellite markers (Vouillamoz and Grando 2006). We first confirmed previously described parentage relationships, corroborating the suitability of LRs from KinGroup: the Sauvignon blanc/Cabernet Sauvignon parent-offspring (PO) relationship (Bowers and Meredith 1997), the Muscat Hamburg/Italia PO (www.vivc.bafz.de), the Muscat of Alexandria/Moscatel de Grano Menudo (syn: Muscat aux Petit Grains and Moscato Bianco) PO (Crespan and Milani 2001), and Garnacha Tintorera (syn: Alicante Bouschet) as an offspring of Garnacha x Petit Bouschet (Cabezas et al. 2003). Those confirmed relationships had relatedness coefficient values ranging from 0.470 to 0.602, so $r > 0.470$ was chosen as a threshold to investigate parentage relationships. This condition was fulfilled by 45 pairs of accessions, which displayed significant ($p < 0.001$) first-degree relationships when tested with KinGroup (Table 5).

The genotypes belonging to cluster 1 had seven interesting pairwise relationships. Moscatel Blanco de Grano Menudo, which had a previously unreported genotype, formed a PO pair with Moscatel de Grano Menudo (Ibáñez et al. 2003, Martín et al. 2003), which also corresponds to Muscat aux Petit Grains or Moscato Bianco (Crespan and Milani 2001), by LR analysis ($p < 0.001$ for PO over any other category of relationship, tested with KinGroup). These two genotypes were adjacent in the NJ dendrogram (NJ bootstrap = 54%), presented a high and significant relatedness ($r = 0.480$), carried similar names, and were white-berried and cultivated in the same vineyards. Moscatel Blanco de Grano Menudo had the Iberian chlorotype A, while the more widespread Moscatel de Grano Menudo had

Table 2 Genetic diversity of the 117 nonredundant cultivated genotypes at the GENRES 081 set of six nuclear loci. Average (\pm SD) number of alleles per locus (N_a), observed and expected heterozygosities (H_o and H_e), discrimination power (D), and probability of identity (PI).

	N_a	H_e	H_o	D	PI
VVS2	14	0.852	0.914	0.947	0.072
VVMD5	13	0.861	0.836	0.960	0.066
VVMD7	15	0.790	0.821	0.933	0.104
VVMD27	10	0.832	0.905	0.931	0.095
VrZAG62	12	0.792	0.846	0.923	0.117
VrZAG79	11	0.842	0.872	0.949	0.078
Averages per locus	12.5 ± 1.87	0.828 ± 0.012	0.866 ± 0.013	0.940	0.089
Cumulative	75				4.3×10^{-7}

Table 3 Genetic diversity within each cluster for 22-locus genotypes identified with Structure software (criterion of >70% cluster membership). Average (\pm SD) number of alleles per locus (N_a), observed and expected heterozygosities (H_e and H_o), and allelic richness (A_s). Data from 22 nuclear loci.

	Cluster 1	Cluster 2	Cluster 3
N_a	9.9 ± 3.0	6.4 ± 2.2	6.4 ± 1.77
H_o	0.762 ± 0.018	0.759 ± 0.015	0.799 ± 0.015
H_e	0.812 ± 0.016	0.713 ± 0.026	0.718 ± 0.027
A_s	9.86	6.13	6.22

Table 4 Differentiation among Structure clusters estimated by pairwise F_{ST} . All values significant, $p < 0.001$, tested with Genpop.

	Cluster 2	Cluster 3
Cluster 1	0.051	0.056
Cluster 2		0.077

Table 5 Parentage estimators. All *r* values were significant, *p* < 0.001, tested with KinGroup. Italic font indicate those accessions with corrected names after identifying their genotypes in available databases.

Pairs of genotypes	Chlorotype	Alleles IBS		<i>r</i>	LRs suggestion of category ^a
		Loci	%		
Cluster 1: Muscats					
Albillo Real (TVZ2)– <i>Legirueta</i> (SF27)	A – A	22	64	0.564	PO
<i>Lairón</i> (CyL-18)– <i>Legirueta</i> (SF27)	A – A	22	66	0.547	PO
Moscatel Gordo Peludo (SF8)– <i>Muscat of Alexandria</i> (1BE3)	A – B	21	66	0.552	FS ^b
Moscatel de Grano Menudo (1BE15)–Moscatel Blanco de Grano Menudo (1BE14)	D – A	22	59	0.480	PO
Moscatel de Grano Menudo(1BE15)– <i>Muscat of Alexandria</i> (1BE3)	D – B	22	61	0.562	PO
<i>Muscat Hamburg</i> (1BE12)– <i>Italia</i> (BE26)	D – C	22	61	0.470	PO
Wild 3 (W3)–Wild 2 (W2)	A – A	19	57	0.533	FS/HS
Cluster 2: Central Castilian Plateau					
Albillo Mayor (Ci1)–Doradilla2 (CyL-06)	A – C	21	61	0.482	FS ^b
Albillo Mayor (Ci1)– <i>Tempranillo</i> (CyL-39)	A – A	22	64	0.555	PO
Albillo Mayor (Ci1)–Tolociriana (CyL-23)	A – A	22	57	0.472	PO
Alcazpepita (CyL-03)–Calagraña (CyL-04)	A – D	21	61	0.481	FS ^b /HS
Alcazpepita (CyL-03)– <i>Pardillo</i> (Ci2)	A – A	20	70	0.565	FS
Alcazpepita (CyL-03)–Tolociriana (CyL-23)	A – A	22	57	0.485	PO
Cojón de Gallo (SF50)– <i>Brujidera</i> (SF13)	A – D	22	64	0.474	PO
Cojón de Gallo (SF50)– <i>Teta de Vaca</i> (TO21)	A – C	22	61	0.506	PO
Cojón de Gallo (SF50)–Verdejo Colorado (AR35)	A – A	21	66	0.563	PO
<i>Garnacha</i> (RD2)– <i>Garnacha Tintorera</i> (Bi13)	A – A	22	70	0.602	PO/FS
<i>Mantúo</i> (AR13)–Colgadera (SF15)	A – A	19	59	0.519	FS/HS
<i>Mantúo</i> (AR13)– <i>Teta de Cabra</i> (1BE2)	A – C	22	59	0.488	PO
Marta Nava (CyL-10)– <i>Morate</i> (SF34)	D – A	20	62	0.485	FS ^b
Marta Nava (CyL-10)– <i>Pleita</i> (5BE1)	D – D	22	59	0.503	PO
Morisco (2BE1)–Bi6	A – D	22	61	0.470	PO
<i>Pleita</i> (5BE1)– <i>Mazuelo</i> (SF14)	D – A	21	64	0.477	FS ^b
<i>Pleita</i> (5BE1)– <i>Morate</i> (SF34)	D – A	21	62	0.555	PO
Viura (RU4)– <i>Morate</i> (SF34)	A – A	20	60	0.478	HS
Cluster 3: Western Castilian Plateau					
Albarín Blanco (TL1)– <i>Pedro Ximénez Canario</i> (Bi54)	D – D	20	68	0.475	FS
Albarín Negro (2BE3)–Cornifesto (PT5)	A – A	22	59	0.490	PO
Albarín Negro (2BE3)–Prieto Picudo (TL3)	A – A	21	73	0.570	FS
<i>Ariño</i> (SF17)– <i>Monastrell</i> (Bi37)	A – A	20	66	0.505	FS
BE13–Cornifesto (PT5)	A – A	22	68	0.600	FS
Doña Blanca (Bi11)–Morisco (2BE1)	A – A	21	66	0.475	PO/FS ^b
Godello (Bi32)–Verdejo (RU3)	A – A	20	68	0.515	FS
<i>Graciano</i> (ME15)–Bi9	A – A	22	66	0.541	PO
<i>Graciano</i> (ME15)–Falso Rufete (SF18)	A – A	20	61	0.513	FS
<i>Graciano</i> (ME15)–Mandón (Bi7)	A – A	22	64	0.500	PO
<i>Graciano</i> (ME15)– <i>Monastrell</i> (Bi37)	A – A	21	66	0.486	FS
Juan García (AR14)–Morisco (2BE1)	A – A	22	66	0.505	PO
<i>Matza Zuri</i> (ME12)– <i>Negrón de Aldán</i> (Bi1)	A – A	20	64	0.480	FS
Pan y Carne 2 (CyL-31)– <i>Merenzao</i> (CyL-30)	D – A	21	66	0.495	FS ^b
<i>Puesto Mayor</i> (CyL-40)–Prieto Picudo (TL3)	A – A	22	75	0.539	FS
<i>Puesto Mayor</i> (CyL-40)–Rufete (CyL-34)	A – A	22	70	0.538	PO/FS
Sauvignon blanc (RU2)–Cabernet Sauvignon (RD1)	D – D	22	61	0.471	PO
Sauvignon blanc (RU2)– <i>Merenzao</i> (CyL-30)	D – A	21	73	0.558	FS ^b
Verdejo Serrano (SF5)– <i>Negreda</i> (Bi4)	A – A	22	66	0.526	PO/FS
Nonclustered					
<i>Teta de Cabra Tinta</i> (4BE13)– <i>Teta de Cabra</i> (1BE2)	C – C	22	66	0.523	PO

^aAbbreviations: LRs, likelihood ratios; PO: parent-offspring; FS: full siblings; HS: half siblings.

^bFull-siblings suggestions require two independent reciprocal crosses, since chlorotypes do not match. In all these pairs a single locus is in disagreement with a parent-offspring suggestion, with at least one member of each pair being homozygous for the nonconcordant locus. Hence, the incidence of not-displayed peaks remains a feasible explanation for the FS category suggestion.

chlorotype D, characteristic of Eastern vines (Arroyo-García et al. 2006) and Muscats (Laiadi et al. 2009). Similarly, Muscat of Alexandria (collected as Moscatel Romano in the region) with chlorotype B, present in the entire distribution area of the species at a low frequency (Arroyo-García et al. 2006) and also typical of Muscats (Laiadi et al. 2009). It was closely related ($r = 0.552$, NJ bootstrap = 54%) to Moscatel Gordo Peludo (Gordera Roja), a chlorotype-A accession collected in the western plateau.

Within cluster 2, 18 pairs of samples indicated likely first-degree relationships. Alcazpepita, a white-berried local accession (syn: Cañorrojo), had a possible FS (full sibling) relationship with Pardillo ($r = 0.565$ and NJ bootstrap = 64%); both have the typical Iberian chlorotype A. Alcazpepita was also closely related to Tolociriana (syn: Castellana Blanca, $r = 0.485$, chlorotype A) and Calagraña ($r = 0.481$ and chlorotype D), both also white-berried and collected from the same area in the geographical center of the plateau. The well-known variety Tempranillo was related to Albillo Mayor ($r = 0.555$, NJ bootstrap = 72%). The LRs suggested they may be PO ($p < 0.001$ when tested with KinGroup), as reported previously (Martín et al. 2006). Albillo Mayor was also closely related to other white-berried cultivars from the area, such as CyL-06 ($r = 0.482$) and Tolociriana ($r = 0.472$). Verdejo Colorado and Cojón de Gallo ($r = 0.563$), both newly identified genotypes with chlorotype A, were suggested PO ($p < 0.001$).

Accessions from cluster 3 showed 19 potentially closely related pairs. From their high relatedness ($r = 0.558$ and NJ bootstrap = 48%) and the suggestion from LRs, Sauvignon blanc (chlorotype D) may have a first-degree relationship with Merenzao (chlorotype A), a black-berried variety present in distinct areas of the western plateau. Albarín Negro (syn: Bruñal in the western plateau, syn: Alfrocheiro Preto in Portugal) and Prieto Picudo had a high relatedness ($r = 0.570$), shared chlorotype A, and were suggested to be FS by LRs ($p < 0.001$). Albarín Negro also took part in a likely first-degree relationship ($r = 0.490$) with Cornifesto, an accession with a previously unpublished genotype, which in turn was very closely related ($r = 0.600$ and NJ bootstrap = 74%) to the unknown BE13; both have the chlorotype A. Prieto Picudo also showed a high relatedness ($r = 0.539$) with Puesto Mayor, which is in turn related to Rufete ($r = 0.538$ and NJ bootstrap = 46%), all three representatives of western Castilian cultivars with typical Iberian chlorotype A. Finally, the A-chlorotyped Verdejo and Godello were suggested to be FS ($p < 0.001$), supported by high NJ bootstrap (72%) and a high relatedness coefficient ($r = 0.515$), confirming previous expectations (Santana et al. 2008).

Discussion

It is remarkable that the genotypic screening of cultivated grapevines from the Castilian Plateau (9.4 x 10⁶ ha) provided 117 genotypes using the GENRES 081 loci, among which 27 (23.1%) were newly identified in this study. Some others corresponded, unexpectedly, to international vari-

eties established in old vineyards of the region and perhaps involved in the origin of some local cultivars. These findings confirmed previous results (Santana et al. 2008) that the Castilian Plateau still has a significant number of poorly described local genotypes. Most analyzed materials were collected from old, traditional vineyards in which local cultivars were represented by few plants. No new plantations are being established from them and no material for propagation is available commercially. The inclusion of these newly characterized genotypes in germplasm banks both for conservation and breeding purposes is highly recommended, as is further screening of traditional vineyards.

Despite the high discrimination power (D) and PIs of the six GENRES 081 loci in our sample, the nonredundant cultivars (based on GENRES 081 loci) shared an identical genotype at six or more loci with more than three other cultivars, on average, when analyzed with an additional 16 loci. This result is most likely because the studied cultivars were related, as shown through potential parentage relationships. Alleles do not combine independently in the genotypes under study, as assumed for the computation of PIs and D , and the sharing of certain alleles becomes more likely than expected if mating occurred at random. The pairwise comparison of genotypes indicates that the definition of nonredundant genotypes based on the GENRES 081 loci may have left additional genotypes undetected in the study sample and suggests that the overall relatedness of cultivars may hamper their correct identification in some cases. Our observation reinforces the decision of the consortium of the European project GrapeGen06 (<http://www1.montpellier.inra.fr/grapegen06>)—the successor of the GENRES 081 project—to adopt three additional SSR loci to ensure proper identification of related cultivars.

Data from the six GENRES 081 loci, both number of alleles N_a and gene diversity H_e , indicated higher genetic diversity in Castilian cultivated grapevine than in previously analyzed samples from the Iberian Peninsula. Since corresponding studies had very different sample sizes and N_a increases with sample size, unlike H_e , we only compared gene diversities. We obtained a gene diversity of 82.8% \pm 1.2 based on 117 genotypes, compared with 75.8% on 49 genotypes (Lopes et al. 1999), 81.0% on 96 genotypes (Ibáñez et al. 2003), 80.6% on 163 genotypes (Martín et al. 2003), 78.1% on 37 genotypes (Lopes et al. 2006), and 78.4% on 73 genotypes (Fernández-González et al. 2007). These comparisons, together with the high observed heterozygosity (H_o) and allelic richness (As), confirm a high genetic diversity in the Castilian sample.

The genetic structure of Castilian grapevine samples corresponded to three genetic groups. Muscats (cluster 1) were differentiated from most accessions as documented in previous studies (Crespan and Milani 2001, Aradhya et al. 2003), which considered them an ancestral type of grapevines with characteristic flavor and morphological features. Many of the genotypes in cluster 2 are traditionally grown in the central plateau. They could be related among each other and to the important cultivars Garnacha

or Tempranillo, as they mostly bear chlorotype A, which indicates a linkage to the Iberian Peninsula. Cluster 3 grouped local varieties from western areas of the Castilian Plateau to French cultivars, in agreement with historical information (Santana et al. 2008). During the Middle Ages, grapevine cuttings were introduced to Spain from France through the east-west pilgrimage route to Santiago. Moreover, the southwestern zone (Sierra de Francia) of the plateau was repopulated with the French between the 11th and 12th centuries. A secondary north-south pilgrimage route was established in the 15th century, linking the route to Santiago with the Virgin of France's shrine, following the Silver Route and crossing the western side of the plateau. Along these routes, interbreeding between French and local varieties could have occurred.

Although all three clusters were fairly diverse, cluster 1, which contained hybrid and wild genotypes and Muscat and Muscat-like local genotypes, showed higher N_a , H_e , and A_s values, likely because of the unique alleles harbored by these accessions. Pairwise F_{ST} values among the three proposed clusters were inside the ranges obtained when differentiation among cultivars from seven European regions was investigated (Sefc et al. 2000). Clusters 2 and 3 were the most differentiated, representing local accessions from central and western areas of the plateau, respectively, suggesting at least two clearly separated origins for the typical cultivars grown in each area.

The suggested close genetic relationships inside each cluster are consistent with previous historical, viticultural, and genetic information. However, they should be considered with caution, since the number of analyzed loci was lower than recommended. The predominance of chlorotypes B, D, and C and the scarcity of chlorotype A in cluster 1 indicate that the majority of these accessions are foreign to the Castilian Plateau (Arroyo-García et al. 2006) and were therefore likely imported. The genotypes from the Muscat family presented close relationships among each other and with some local genotypes, suggesting that they may be ancestral to several Castilian cultivars like Moscatel Gordo Peludo (Gordera Roja) or the newly identified Moscatel Blanco de Grano Menudo. Surprisingly, the previously unknown genotype Bi20 showed chlorotype G, common in Near Eastern wild vines but absent from western Europe (Arroyo-García et al. 2006), and did not exhibit close relationships with any other in the sample, therefore supporting a likely introduction from the Near or Middle East.

Cluster 2 harbored a complex net of first-degree relationships among local accessions from the central plateau involving Tempranillo and several newly identified genotypes. Some clues indicate possible autochthonous origins for these unknown genotypes, such as the presence of chlorotype A in all but one (Doradilla 2, with chlorotype C) and the predominance of white berries, both conditions shared by typical local cultivars from central Castilian areas, such as Alcazpepita, Tolociriana, and Huerta del Rey.

Historical information, genetic structure, and previous analyses (Santana et al. 2008) suggest that French varieties

might be involved in the origin of some traditional cultivars grown in the western plateau (cluster 3). Although we identified only one putative first-degree relationship between a western Castilian accession and a French accession (Merenzao and Sauvignon blanc), further studies including more French genotypes might reveal new close relationships. French cultivars currently grown in the plateau, although probably related to the medieval ones, have been introduced more recently. As in cluster 2, cluster 3 harbored a complex net of close relationships involving local western Castilian cultivars and several newly identified genotypes, in which Albarín Negro and Prieto Picudo play key roles, and the abundance of chlorotype A suggests an Iberian origin.

The individuals collected in Castilian wild habitats were closely related, with the exception of the demonstrated rootstock W4. W2 and W3 were a possible FS/HS pair ($r = 0.533$, NJ bootstrap support = 90%). The samples W1, W2, and W3 belonged to the same dynamic population on the Spanish bank of the Duero River in the western plateau and exhibited typical *sylvestris* features such as unisexual flowers (two appeared to be male), small leaves and clusters, and small round black berries. They shared chlorotype A, characteristic of wild populations from Spain and not found in other *Vitis* species (Arroyo-García et al. 2002), and did not share either characteristic alleles or genetic relationships with the possible hybrids in the sample, which reduces the possibility of being rootstocks escaped from vineyards. Their genotypes did not show any genetic relationship to cultivated accessions in the sample. Although one example of a direct relationship between wild and cultivated individuals (Aradhya et al. 2003) and a few cases of gene flow from cultivated to wild populations (Di Vecchi-Staraz et al. 2009) have been reported, such relationships are scarce, since the flowering times of the two forms do not match, reducing the possibility of gene flow through pollen (This et al. 2006). Thus, the three samples included here may represent genuine *Vitis vinifera* ssp. *sylvestris* individuals.

Conclusions

Results show that the Castilian Plateau still harbors a high cultivar diversity of grapevines, of which a substantial fraction is insufficiently characterized to date. This diversity is mostly ignored, as only a small part of it is currently considered for use by DOCs in the region. This study may have underestimated the diversity in the region by using the six SSR recommended by GenRes081 for discrimination of genotypes. Recently, three additional SSR have been proposed to ensure proper identification of closely related cultivars by the GrapeGen06 project.

The analyses of genetic structure and relationships highlight three main groups of cultivars in the region, one of which supported crosses between French and Castilian cultivars, especially those cultivated in the western plateau along traditional pilgrimage routes. These results provide a view of how the cultivated grapevine genetic pool is structured in a viticultural region including germplasm of local and foreign origins and crosses between them. Further

research will be required to confirm the proposed genetic links among these cultivars.

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