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Research Article

Genetic Diversity and Structure in a Spanish Grape Germplasm Collection Assessed by SSR Markers

Hiba Ghrissi,[1] María Teresa De Andrés,[2] Luis Javier Andreu,[3] and Yolanda Gogorcena[1]

[1]*Group of Genomics of Fruit Trees and Grapevine, Dpto. de Pomología, Estación Experimental de Aula Dei-Consejo Superior de Investigaciones Científicas (EEAD-CSIC), Avenida de Montañana 1005, Zaragoza 50059, Spain*
[2]*Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA), Finca El Encín. Carretera A-2, PK 38,200, Alcalá de Henares 28805, Spain*
[3]*Centro de Transferencia Agroalimentaria Del Gobierno de Aragón, Avenida. de Montañana 930, Zaragoza 50059, Spain*

Correspondence should be addressed to Yolanda Gogorcena; aoiz@eead.csic.es

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Background and Aims. *Vitis vinifera* L. is a major global horticulture crop which holds historical connections contributing to the development of human culture. The main objective of the study was to explore the genetic diversity and structure of grapes curated at the germplasm bank of Aragón and link them to cultivar utilization, putative geographic origin, and historical events. *Methods and Results*. The genetic diversity of the 411 accessions of the Grapevine Germplasm Bank was assessed using 26 SSR markers. SSR markers VVIP31, VMC4F3-1, VVIV67, and VVS2 distinguished the 156 nonredundant genotypes found in the collection. The profiles were compared to the El Encin database, and 105 were identified as known cultivars, of which 93 were Spanish, 12 were from other European origins, and 51 others were not known. The 51 profiles, as they were all collected in Spain, were designated as Spanish unknown for further analysis. To establish a comparative study for principal coordinate analysis (PCoA) and structure, we kept 113 identified genotypes from this collection but added 61 representative genotypes with diverse European and oriental origins. Bayesian analysis and PCoA showed four distinct groups of grape cultivars: (1) traditional Spanish wine grape from Aragón or cultivated along the Ebro River, (2) Spanish wine grape, (3) Spanish and oriental table grape, and (4) Italian, Portuguese, French, and German-Slovenian wine grape varieties. *Conclusions*. The 51 unknown Spanish genotypes were not registered yet in any European commercial list and are of special interest. These genotypes could be ancient cultivars adapted to local climatic and environmental conditions and probably resilient to face the new conditions derived from climate change.

1. Introduction

Grapevine, being considered as one of the oldest crops, has been used to produce such as table fruits, dry fruits, juice, and wine [1]. Archaeological records suggest that the cultivation of domesticated grapes began approximately 6000–8000 years ago in the near East [2–4]. It is considered one of the most important fruit species in the modern world and is profoundly connected with the development of human culture [5]. It was estimated that in 2020, the world vineyard area was 7.3 million hectares (Mha); five countries represent 50% of the world's vineyard area: 13% in Spain, 11% in China, 10% in France, 9% in Italy, 7% in Turkey, and 50% in the rest of the world [6].

Vitis vinifera L. is a species that is a widely cultivated and economically important fruit crop in the world [7] that has motivated efforts in genomics to accelerate the exploitation of *Vitis* germplasm [8]. According to Negrul [9], cultivated groups can be subdivided into three ecotypes: the occidentalis, the orientals, and the pontica.

Grapevine cultivars derived from planned breeding activities appeared when Phylloxera and fungal diseases began to destroy the European vineyards in the middle of

the 19th century. These diseases diminished the abundance of wild vines in their indigenous habitat in Europe along with the destruction of their habitats which drove the European wild vines close to extinction [10]. Indeed, the diversity of the grapevine started to be preserved in exotic germplasm collections: autochthonous, cultivars, and minorities. In Spain, there are 18 varietal collections of grapevines situated in the main wine-producing regions [11]. The main ones are El Encin Germplasm Bank (BGVCAM in Madrid, Spain) and Rancho de la Merced in Jerez (Cádiz, Spain), which are the Spanish collections of reference since the beginning of the 19th century [12]. The Grapevine Germplasm Bank of Aragón (BGVA) (Zaragoza, Spain), was created in 1990 to preserve the varietal diversity of the region [13]. These endangered materials were collected in fluvial valleys of the northern areas all over Spain, from the Pyrenees to the Balearic and Canary Islands [11]. Nowadays, the collection is still enlarging with material on the edge of extinction prospected in old vineyards.

Traditionally, the identification and classification of grapevines have been based on morphological characteristics mainly known as ampelography. Morphological descriptions are useful to prevent possible mistakes in plantations but later need verification by molecular methods. The combination of ampelographic descriptions and molecular markers indeed establishes varietal identity and leads to reliable and objective results [14, 15]. Since 1993, different types of DNA-based markers have been illustrated for grape molecular characterization such as RFLPs [16, 17]; and [18], RAPDs [19–21]; and [22], AFLPs [23, 24], SSRs [13, 25–28], and SNPs [29–33]. Yet, the great revolution in grape molecular genetics did not take place until the advent of SSR markers and later SNPs markers that allowed the discrimination between wild and cultivated populations and the study of its genetic relationships [34]. These markers have also been used to establish a structure of grapevine from European and orientals origins [30, 32, 33, 35].

A previous study conducted by Buhner-Zaharieva et al. [13] identified 200 accessions curated at the Movera grapevine collection by using six SSR markers. The present study aimed to conduct the molecular characterization of other 411 accessions of the same Grapevine Germplasm Bank collection (BGVA) by using 26 SSR markers and linking them to the utilization of cultivars, putative geographic origin, and historical events. We have analyzed the genetic diversity of the 156 *Vitis vinifera* L. nonredundant genotypes that have been collected in old vineyards in abandoned rural areas of the Aragón region, and later, structure analysis was performed to investigate the genetic relationship and origin of the studied germplasm. The interest of this study is to provide the molecular identification of ancient grapevine accessions prospected in old vineyards useful for future research. The characterization of the collection is the previous step to start vineyard assays to evaluate wine quality to challenges faced by winemakers in an unpredictable climate to reach the desired quality of the final product.

2. Material and Method

2.1. Plant Material. A set of 411 accessions from *Vitis vinifera* L. preserved at the BGVA (41°11′ 36.4920″' N and 1°46′ 56.3700″ W) were selected for molecular characterization. Fresh young leaves were collected, frozen in liquid nitrogen, grounded to a fine powder by using the mixer mill MM400 (RETSH, Haan Germany), and stored at −20°C until analysis.

2.2. DNA Extraction and Microsatellite Analysis. DNA was extracted by using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Extracted DNA was quantified by using the NanoDrop 2000 spectrophotometer (NanoDrop, Delaware, USA).

A panel of 26 microsatellites [36] was chosen because they presented clear profiles and minimal problems in allele conversions: VVIP60, VVIB01, VRZAG83, VRZAG79, VVMD7, VRZAG62, VMC1B11, VVIQ52, VVMD25, VVS2, VVIH54, VRZAG112, VVMD24, VVIN73, VVIP31, VRZAG29, VVMD28, VVMD32, VVMD27, VVMD21, VRZAG67, VVIV37, VMC4F3-1, VVIV67, VVMD5 and VVIN16 (Table 1). For each one, a sequence forward marked with fluorescence PET, VIC, NED, and FAM and reverse were used to perform a multiplex loading strategy in the sequencer based on the allele ranges in pairs of *loci* and fluorochrome.

Two multiplex PCR were performed by combining more than one pair of primers in the same tube's reaction (Supplementary-Table 1). All of the samples were amplified with two sets of SSRs; set A: VVIP60, VVIB01, VVIQ52, VVIH54, VVIN73, VVIP31 [37], VRZAG83, VRZAG79, VRZAG62, VRZAG112 [38], VVMD7, VVMD25, VVMD24 [39, 40], VMC1B11 [41], and VVS2 [42]; set B: VRZAG29, VRZAG67 [38], VVMD28, VVMD32, VVMD27, VVMD21, VVMD5 [39, 40], VVIV37, VVIV67, VVIN16 [37], and VMC4F3-1 [41].

The Qiagen multiplex PCR Kit was used for multiplex PCR amplification. Polymerase chain reactions for both sets were performed in 21 μl adding 5 ng of DNA template to a mix containing 1X PCR Master Mix (Qiagen, Hilden, Germany) with an equimolar amount of each primer ranging from 0.1 μM to 0.22 μM for PCR-A and from 0.08 μM to 0.15 μM for PCR-B (Supplementary-Table 1) [36].

PCR amplifications were carried out on a Gene Amp 2700 thermocycler (Applied Biosystems, Foster City, CA, USA) with the following temperature profile. For multiplex PCR A: a starting denaturation step at 94°C for 15 min, followed by eight cycles at 94°C for 1 min, 60°C for 1.5 min, and at 72°C for 2 min; two touchdown programs were coded for PCR A with ten touchdown cycles at 94°C for 1 min, 60°C-0.3°C/cycle for 1.5 min, and at 72°C for 2 min followed by twenty touchdown cycles at 94°C for 1 min, 57°C-0.2°C/cycle for 1.5 min, and at 72°C for 2 min and a final step at 72°C for 90 min. For multiplex PCR B: an initial denaturation step at 94°C for 15 min, followed by 3 cycles at 94°C for 1 min, 60°C for 1.5 min, and at 72°C for 2 min, also, two-touchdown

Table 1: Number of alleles (Na), the effective number of alleles (Ne), Shannon's information index (*I*), observed and expected heterozygosity (Ho and He), Wright's fixation index (*F*), polymorphism information content (PIC), number of genotypes, and discrimination power (PD) with the 26 SSR markers in 156 *Vitis vinifera* L. unique genotypes.

Locus	Na	Ne	*I*	Ho	He	*F*	PIC	#Genotypes	PD	Size range
VMC4F3-1	15	7.9	2.23	0.84	0.88	0.04	0.86	49	0.97	165–208
VVIN16	6	3.0	1.29	0.60	0.67	0.10	0.61	15	0.81	146–159
VVIV37	12	6.7	2.10	0.83	0.85	0.03	0.83	39	0.96	150–181
VVIV67	15	7.2	2.15	0.91	0.86	−0.06	0.85	47	0.96	339–399
VVMD21	9	3.3	1.45	0.69	0.70	0.004	0.65	19	0.87	226–265
VVMD27	10	5.8	1.82	0.85	0.83	−0.03	0.80	25	0.94	175–206
VVMD28	15	6.3	2.13	0.83	0.84	0.01	0.82	46	0.66	220–270
VVMD32	10	5.6	1.85	0.83	0.82	−0.01	0.80	28	0.94	238–272
VVMD5	10	6.9	2.02	0.89	0.86	−0.04	0.84	36	0.95	219–248
ZAG29	4	1.3	0.45	0.20	0.21	0.06	0.20	6	0.36	111–121
ZAG67	10	5.9	1.91	0.84	0.83	−0.01	0.81	32	0.94	124–164
VMC1B11	11	4.7	1.79	0.78	0.79	0.01	0.76	32	0.92	167–194
VVIB01	5	2.3	1.06	0.60	0.57	−0.06	0.51	9	0.75	288–307
VVIH54	9	2.2	1.09	0.58	0.55	−0.05	0.51	15	0.84	147–181
VVIN73	6	1.3	0.54	0.24	0.25	0.03	0.23	8	0.41	254–267
VVIP31	15	7.8	2.25	0.86	0.87	0.01	0.86	50	0.97	166–200
VVIP60	13	3.7	1.64	0.72	0.73	0.02	0.69	30	0.86	306–333
VVIQ52	4	2.8	1.11	0.65	0.65	−0.02	0.57	9	0.79	82–88
VVMD24	7	3.2	1.48	0.75	0.69	−0.09	0.66	19	0.87	206–218
VVMD25	10	3.8	1.57	0.78	0.74	−0.06	0.70	21	0.88	237–269
VVMD7	12	3.9	1.70	0.78	0.75	−0.05	0.71	30	0.89	231–261
VVS2	15	7.3	2.18	0.89	0.87	−0.03	0.85	47	0.96	123–159
ZAG112	5	3.4	1.31	0.77	0.71	−0.09	0.66	11	0.84	187–201
ZAG83	10	4.2	1.67	0.80	0.76	−0.05	0.73	25	0.89	228–260
ZAG62	9	4.0	1.64	0.78	0.75	−0.04	0.72	26	0.90	181–204
ZAG79	14	5.8	2.02	0.82	0.83	0.01	0.81	38	0.95	235–265
Total	**261**	**120.4**						**715**	**=1**	
Average	**10.03**	**4.63**	**1.63**	**0.74**	**0.73**	**−0.014**	**0.69**	**27.5**	**0.85**	

programs were coded with 10 touchdown cycles at 94°C for 1 min, 60°C-0.3°C/cycle for 1.5 min, and at 72°C for 2 min, followed by twenty touchdown cycles at 94°C for 1 min, 57°C-0.3°C/cycle for 1.5 min, and at 72°C for 2 min, followed by 5 cycles at 94°C for 1 min, 50°C for 1.5 min, and at 72°C for 2 min and a final step at 72°C for 90 min [36].

PCR products were first checked in 2% agarose gel and later were separated by capillary electrophoresis in an ABI3130 sequencer (Applied Biosystems). The loading mixture that contained 1 μL of diluted PCR product, 0.1 μL of GeneScan-500LIZ (Applied Biosystems), and 14 μL of Hi-Di Formamide (Applied Biosystems) were denatured at 95°C for 5 min before being loaded into the sequencer. Raw data were transformed into allelic sizes by using the GeneMapper v4.1 software (Applied Biosystems).

2.3. Data Analysis

2.3.1. Genetic Diversity. The diversity analysis was conducted on the 156 Vitis vinifera L. unique genotypes from the BGVA (Supplementary-Table 2) from the set of 411 that contains duplicates. Genetic diversity was estimated using the following statistics: number of observed alleles per locus (Na), effective number of alleles per locus [43] (Ne), observed heterozygosity (Ho), expected heterozygosity (He), Wright's fixation index (F), Shannon's information index (I) and discrimination power (PD) are calculated by using GenAlEx 6.5 software [44]. The polymorphism information content (PIC) was calculated employing Cervus 3.0 software [45]. To estimate the minimum number of microsatellites to distinguish all the genotypes, each microsatellite was tested for redundancy by using the Microsatellite tool kit (MSTools) [46]. Redundancy was determined as the proportion of distinguishable genotypes [47]. The identification of cultivars was carried out, comparing the SSR profiles with those in the database of the Spanish grapevine reference collection of El Encin. We assigned the cultivar name and genotype as referred to in this database.

2.3.2. Analysis of Population Structure. To establish a comparative study, STRUCTURE analysis was performed on a total dataset with 174 *Vitis vinifera* L. genotypes (supplementary Table 5) from different European and oriental origins. From the 156 *Vitis* unique profiles of the BGVA collection, we chose 113 representative genotypes (47 Spanish known, 51 Spanish unknown, and 15 European and orientals all presented in bold in supplementary Table 5) curated at the germplasm collection. We added 61 profiles of genotypes from different origins according to our database and literature (7 German-Slovenian, 10 French, 7 Greek, 9 Italian, 8 Portuguese, 11 Spanish known, and 9 orientals) provided by the Spanish reference collection BGVCAM to balance the groups.

The program structure (version 2.3.4) implements a model-based clustering criterion for inferring population structure by using genotypic data from unlinked markers [48]. A preliminary test was performed based on an admixture model where the allelic frequencies were correlated, and a burn-in period of 5.000 and 50.000 MCMC iterations was for data collection. The analysis was run for K values ranging from one to ten inferred clusters with ten independent runs each. To assess the best K value supported by the data, the ΔK method was described by [49] through Structure Harvester v 0.6.94 application [50] to examine the rate of change in successive posterior probabilities over the range of K values. Once defined the most probable K-value, a final single run was performed using a burn-in period of 100.000 and 1.000.000 MCMC iterations, with K values ranging from one to ten inferred clusters with twenty independent runs each. The model assignment was used to determine the final placement of a genotype in a group. The results were displayed graphically in a barplot. A minimum membership (qI) of 0.7 was assigned for each accession to belong to a subpopulation.

Later, the principal coordinates analysis (PCoA), which is based on standardized covariance of genetic distances calculated for codominant markers, was performed to detect the genetic distance between populations in the first place and genetic distance among varieties in the second place by using the GenAlEx 6.5 software [44].

3. Results

3.1. Amplification Tests and Molecular Identification. Figure 1 shows an example of the electropherogram obtained from the capillary electrophoresis of the "Tempranillo" genotype. Alleles were clearly differentiated using this PCR conditions, Figure 1(a) shows the peaks obtained from the set A: VVIP60, VVIB01, VRZAG83, VRZAG79, VVMD7, VRZAG62, VMC1B11, VVIQ52, VVMD25, VVS2, VVIH54, VRZAG112, VVMD24, VVIN73, and VVIP31 (except for VVIQ52, homozygous with 84 bp, not included in this window that shows markers over 130 bp). Figure 1(b) shows alleles obtained from set B: VRZAG29, VVMD28, VVMD32, VVMD27, VVMD21, VRZAG67, VVIV37, VMC4F3-1, VVIV67, VVMD5, and VVIN16.

We checked the peaks obtained for all the 411 studied accessions in order to discriminate each peak from the other to eliminate any possibility of "pull-up" peaks. The collection contains duplicate accessions, and only 156 unique profiles or genotypes were found different.

In the collection, we identified 105 grape genotypes, of which 93 were Spanish and twelve from different European and oriental origins. We did not find identity (not matched with an exact profile in the El Encin database) for 51 profiles that were named as Spanish unknown genotypes since these accessions were all collected in Spain.

3.2. Genetic Diversity Measures for SSR Markers. Parameters of the genetic diversity calculated for the 26 SSR markers used to characterize the 156 cultivars of *Vitis vinifera* L. of the BGVA are shown in Table 1.

All *loci* analyzed in this study were multiallelic and polymorphic. The number of alleles detected for each *locus* ranged from 4 to 15, with a total number of 261 alleles for all *loci* and an average of 10.04 alleles per *locus* (Table 1). The alleles obtained for each *locus* and their respective frequencies are shown in supplementary Table 3. The most frequent alleles in this study were detected for the *loci* ZAG29 at 111 bp and for the *loci* VVIN73 at 263 bp, which showed respectively frequencies of 88.6% and 86.2%. Nine alleles (3.4%) showed frequencies between 40% and 60%, and 14 alleles showed frequencies between 20% and 40%. While 32 alleles (12.6%) showed low allele frequencies (AF = 0.003), (supplementary Table 3). On the other hand, from 26 *loci* analyzed, each *locus*, except for VVIV37, VVMD32, ZAG29, VVIB01, VVIH54, VVIN73, VVIQ52, and VVMD24, showed at least one to three rare or unique alleles with AF ≤ 0.003. Samples in which only a single allele per *locus* was detected were homozygous genotypes instead of heterozygous with a null allele that did not amplify for computing genetic diversity parameters. The observed genotypes found with the 26 microsatellites ranged from six to 50 (supplementary Table 4), with a total of 715 different monolocus genotypes. In absolute terms, the *locus* ZAG29 showed the most frequent genotype, 111/111, with a frequency of 78.6%, followed by the VVIN73 *locus,* with the genotype 263/263, with a frequency of 74.8%. Most of the SSR loci, except for VVMD28 and VVIN73, presented genotypes with very low frequencies (0.006; in bold in supplementary Table 4). These rare genotypes ranged from one in the *loci* ZAG29, VVIB01, and ZAG112 to 21 for the *locus* VVIV67. Shannon's information index (I) ranged from 0.45 to 2.25, with an average value of 1.63. The observed heterozygosity (Ho) varied between 0.20 to around 0.90, with an average value of 0.74. The expected heterozygosity (He) ranged from 0.21 to around 0.90, with an average value of 0.73 (Table 1). Wright's fixation index (F) compares He with Ho, estimating the degree of allelic fixation. The F average obtained in this study (−0.014) indicates the absence of inbreeding mating since the F values are close to zero under random mating. The PIC values are equal to or slightly lower than the expected heterozygosity and are correlated with the corresponding Ne (effective number of alleles) values (Table 1). Both PIC and Ne values are very useful for the evaluation of adequate SSR markers to distinguish unambiguously related *Vitis vinifera* L. cultivars. The most informative *loci* of this study were VMC4F3-1 and VVIP31, with PIC values of 0.86 (Ne = 7.9) and 0.86 (Ne = 7.8), respectively. The less informative *loci* were ZAG29 and VVIN73, with a PIC of 0.2 and 0.23, respectively (Ne = 1.3) (Table 1). The highest discrimination power (PD) was observed in VMC4F3-1 and VVIP31 with a PD of 0.97, whereas ZAG29 showed the lowest one with (PD = 0.36).

The selection of the four most polymorphic *loci* which revealed the highest number of different genotypes, VVIP31 (50); VMC4F3-1 (49); VVIV67 (47); and VVS2 (47), allowed us to distinguish unambiguously all the 156 *Vitis vinifera* L. nonredundant genotypes curated at the Aragón germplasm bank.

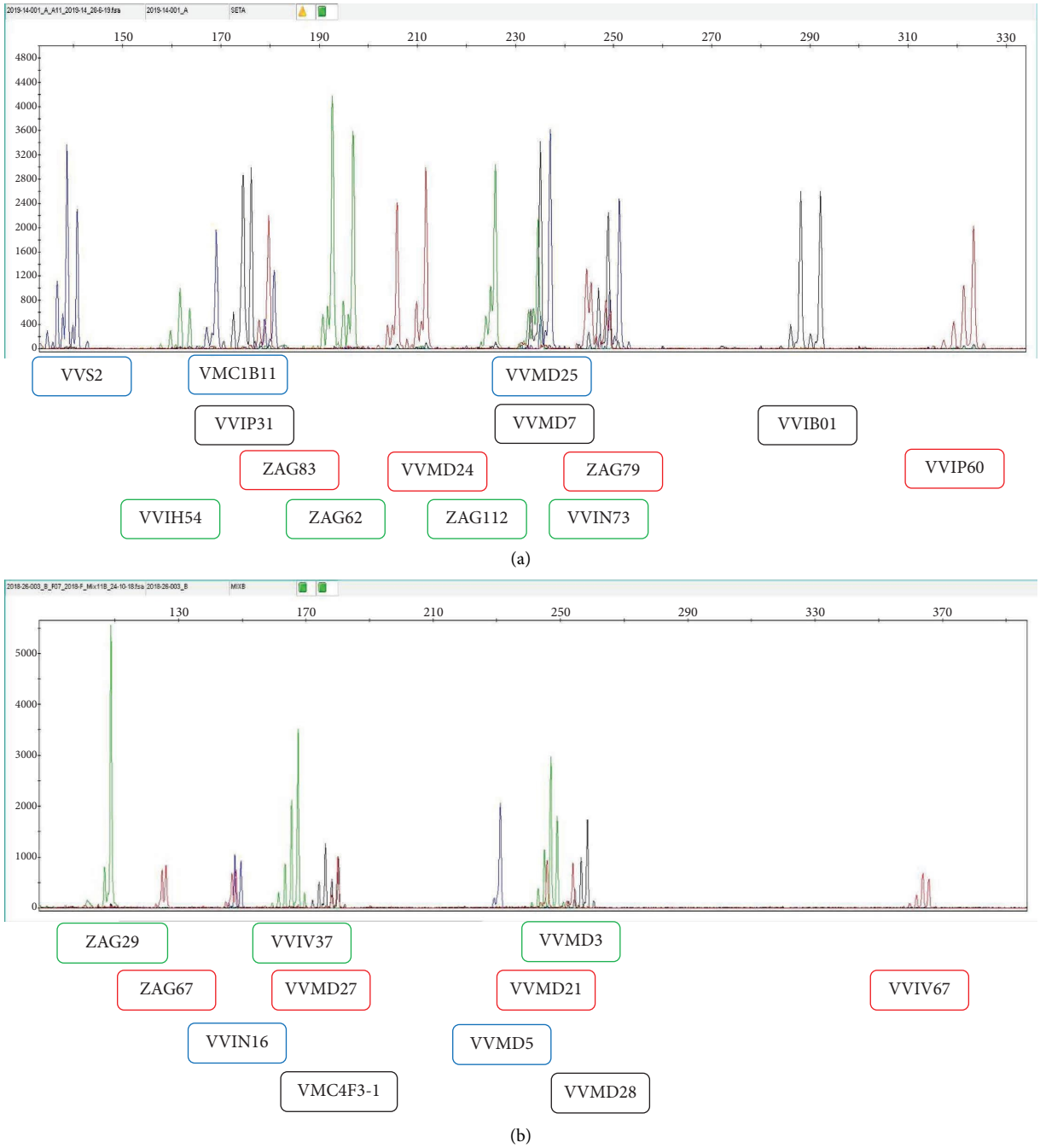

Figure 1: SSR profile obtained from the genotype "Tempranillo" for PCR A (a) and PCR B (b). SSR were labeled with the dye color.

3.3. Genetic Relationship and Structure. The genetic diversity and population structure of the 174 different genotypes were analyzed by using PCoA and structure (supplementary Table 5). First, based on the genetic distance, the data set was used to visualize the level of similarity among populations (Figure 2). The principal coordinates analysis (PCoA) explained 86.18% of the total variance and separated four groups, Spanish, French/Portuguese, Italian/Greek/orientals, and German/Slovenian. The first axes explained 50.13% of the overall variation in the sample of genotypes and separated populations 6, 7 Spanish known and unknown genotypes in the negative axis plot, from populations 1, 2, 4, and 5, mostly enriched in German-Slovenian, French, Italian, and Portuguese genotypes. The second axis explained 25.97% of the population variation and separated populations 3, and 8 (Greek, and Oriental origins, respectively) from the former groups.

The genetic structure of the eight populations defined according to the potential geographical origin of the cultivar (German-Slovenian, French, Greek, Italian, Portuguese, Spanish: known and unknown, and orientals) was analyzed by using structure. The 174 unique genotypes (supplementary Table S1) were analyzed independently. The ln $[Pr(X/K)]$ reached a maximum value at $K = 2$ ($\Delta K = 578.02$,

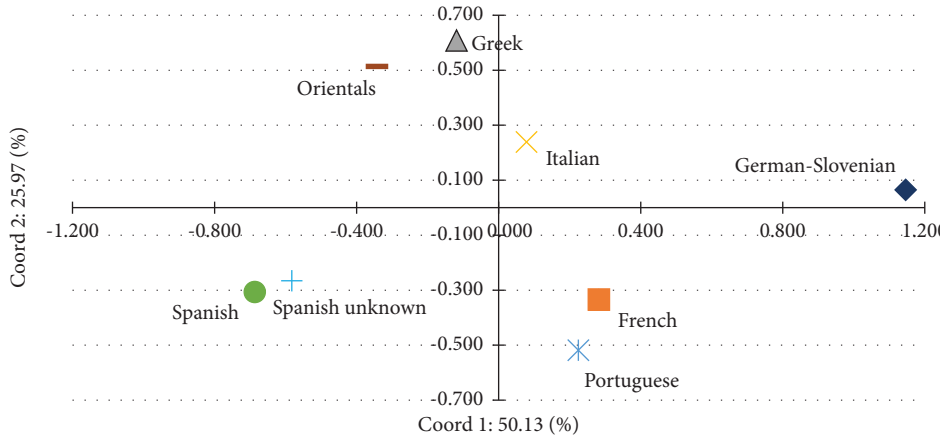

FIGURE 2: Principal coordinates analysis (PCoA) based on genotypic data obtained from 26 SSR *loci* in the eight populations including 174 different genotypes (supplementary Table 5).

Figure 3), which corresponded to a strong differentiation of two main groups of genotypes. Another peak was found at $K = 4$ ($\Delta K = 65.19$, Figure 3). Based on the Bayesian clustering analysis, each vertical line represents a genotype, and overlapping colored segments indicate membership fraction. In both markers systems, the estimated Ln Prob showed that an increase in K (population number) resulted in higher likelihood values. Accordingly, the Evanno et al. [49] method strongly confirmed $K = 2$ and $K = 4$ as the optimal numbers of clusters (Figure 4).

For $K = 2$, with a membership of $qI \geq 0.7$, the first $Q1$ retained 76 genotypes including 73 Spanish cultivars, and the second $Q2$ clustered 74 genotypes (43%), including all foreign cultivars mixed with 6 Spanish genotypes. In addition, 24 genotypes with $qI < 0.7$ were considered as admixed between both groups (Figure 4) (supplementary Table 6). These two groups at $K = 2$ were divided into 2 groups, each at $K = 4$ (Figure 4, supplementary Table 7).

For $K = 4$, considering $qI \geq 0.7$, 100 genotypes were divided into four groups (17 $Q1$ + 26 $Q2$ + 27 $Q3$ + 30 $Q4$) (supplementary Table 7). The first $Q1$ contained 17 genotypes in which, most of them are Spanish originated in Aragón, and were traditionally cultivated in regions along the Ebro River. The $Q2$ contained 26 genotypes, all Spanish cultivars that mostly represent wine grapes cultivars. The $Q3$ contained 27 genotypes from different origins: Spanish, orientals, and some Greek cultivars, all or most of them are considered table grapes. Finally, $Q4$ grouped 30 genotypes that include varieties of three European groups (German-Slovenian, French and Portuguese) mixed with two Spanish genotypes (Hondarrabi and Albariño). The rest, 74 genotypes with $q < 0.7$, were considered as admixed in all subgroups studied (German-Slovenian, French, Greek, Spanish, and orientals).

The PCoA analysis was conducted with the genetic distance of all 174 genotypes (Figure 5). The PCoA was labeled based on the Q values of the STRUCTURE results to show a spatial differentiation among the four groups in which $Q1$, $Q2$, $Q3$, and $Q4$ presented a $qI \geq 0.7$ (full symbol). Genotypes that presented a $qI < 0.7$ were pointed out as mixed: $Q1$ MIX, $Q2$ MIX, $Q3$ MIX, and $Q4$ MIX (Figure 5, open symbols). The PCoA analysis reflects well the four structure groups, the admixed cultivars being located between the 4 groups $Q1$, $Q2$, $Q3$ and $Q4$ (Figure 5 and details in Supplementary-Table 7).

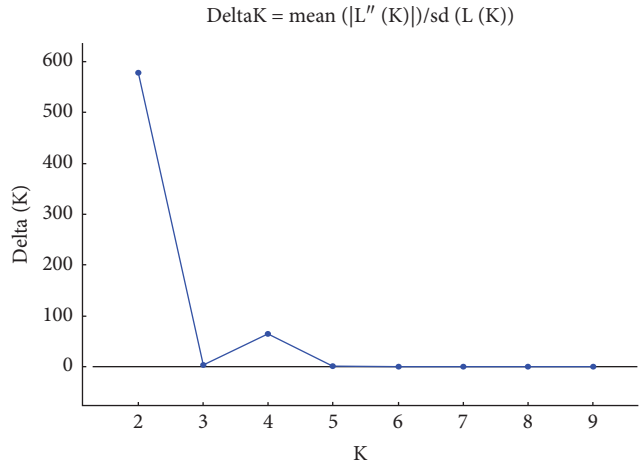

FIGURE 3: Exploration of K value for structure analysis of 174 *Vitis vinifera* (L) genotypes by estimates the rate of change of the slop log-likelihood curve (ΔK) calculated according to Evanno et al. [49] plotted against (K). Plot of delta K per K for the analysis on the 174 genotypes giving a robust ΔK maximum at $K = 2$ and at $K = 4$.

All known varieties from the $Q2$ originated from Spain, citing, for example, Hebén, Jarrosuelto, Moristel, Rojal Tinta, Cayetana Blanca, and interestingly includes nine of the Spanish unknown assigned to pop 7 named as ARAG (ARAG_27_05, ARAG_20_03, ARAG_22_02, ARAG_22_05, ARAG_25_08, ARAG_28_08, ARAG_33_04, ARAG_34_14, and ARAG_03_14) that are preserved at the Aragón germplasm bank. As from the $Q2$ MIX, we may cite other Spanish known varieties such as Garnacha, Fumat, Trepat, Santa Fé, Miguel de Arco, Boton de Gato, also grouping nine Spanish unknown genotypes (ARAG_18_08, ARAG_01_16, ARAG_18_11, ARAG_20_01, ARAG_30_13, ARAG_33_06, ARAG_33_08, ARAG_34_17, and ARAG_65_15). A second group on the negative side includes the distribution of the $Q1$ belonging to Spanish genotypes with known origin from Aragón and cultivated in adjacent

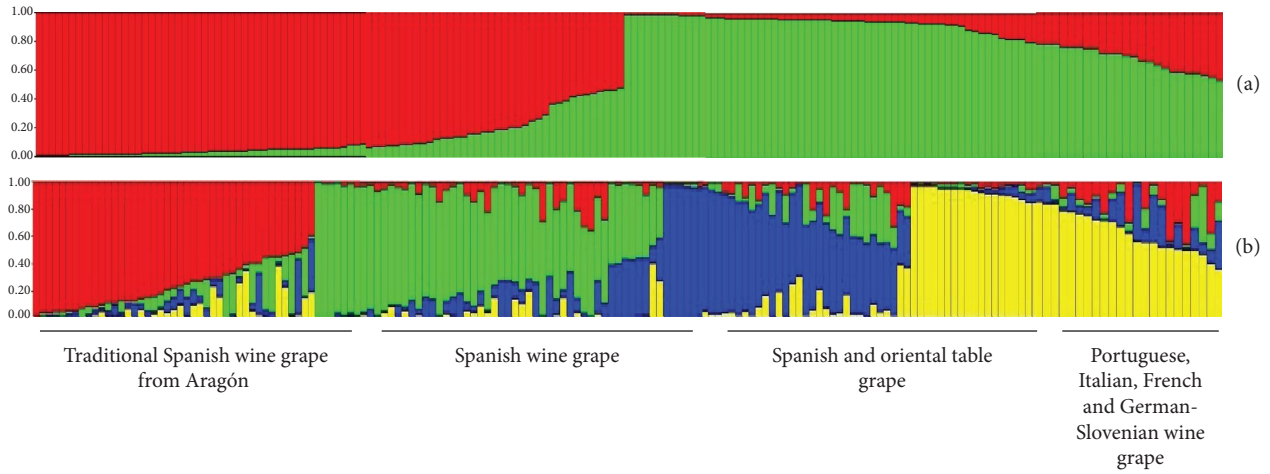

FIGURE 4: Bayesian clustering analysis. (a) ($K = 2$) and (b) ($K = 4$) of 174 genotypes that include: Spanish, other European, and oriental cultivars. Each single vertical line represents a genotype that is partitioned into colored segments in proportion to the estimated membership in two or four subpopulations (genotypes are listed in supplementary Table 6 and supplementary Table 7).

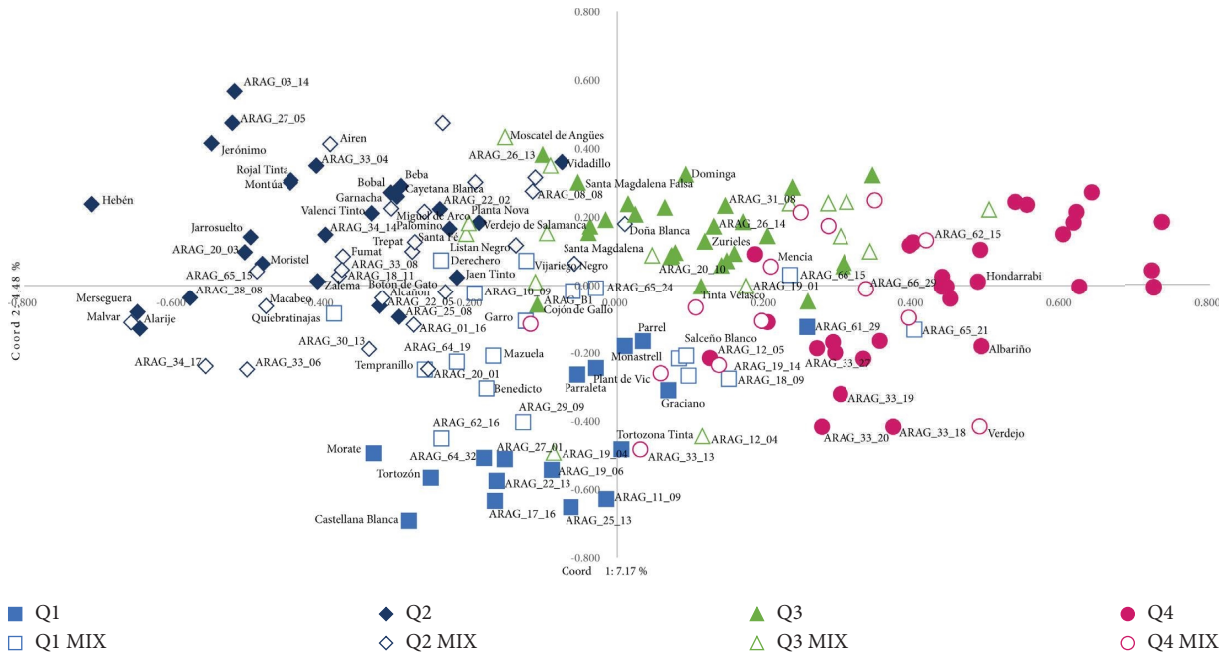

FIGURE 5: Principal component analysis PCoA based on genotypic data from 26 SSR *loci* in 174 genotypes. Clusters *Q*1, *Q*2, *Q*3, and *Q*4 are inferred by structure represented by different symbols and colors. Only the Spanish genotypes of pop 6 and pop 7 were labeled by their coordinates in supplementary Table 7.

regions along the Ebro River as we cite some of the most recognized: Castellana Blanca, Tortozón, Plant de Vic 98 N4, Parraleta, Parrel, Monastrell, Morate, and Graciano, plus eight Spanish unknown genotypes (ARAG_17_16, ARAG_19_06, ARAG_22_13, ARAG_25_13, ARAG_27_01, ARAG_11_09, ARAG_61_29, and ARAG_64_32). As for the Q1 MIX, we mention as an example: Benedicto, Tempranillo, Mazuela, Derechero, Garro, Salceño Blanco, and nine Spanish unknown (ARAG_18_09, ARAG_29_09, ARAG_10_09, ARAG_62_16, ARAG_64_19, ARAG_65_21, ARAG_65_24, ARAG_66_15, and ARAG_B1). Moreover, some of the Spanish table grape cultivars were mixed with Greek and oriental cultivars in the *Q*3: Dominga, Zurieles, Santa Magdalena Falsa, Cojón de Gallo, and two Spanish unknown ARAG_31_08, and ARAG_26_13; and *Q*3 MIX: Moscatel de Angües, Planta Nova and four Spanish unknown accessions (ARAG_19_01, ARAG_20_10, ARAG_12_04, and ARAG_19_04). Finally, on the right side, clear disposal of the *Q*4 that grouped the foreign genotypes mixed with two of the Spanish known genotypes: Albariño and Hondarrabi, and five Spanish unknown (ARAG_33_18, ARAG_33_19, ARAG_33_20, ARAG_33_27, and ARAG_12_05). The *Q*4 MIX included: Mencia, Verdejo, and

four of the unknown Spanish cultivars (ARAG_19_14, ARAG_33_13, *ARAG_62_15*, and ARAG_66_29). The PCoA distribution confirmed the results obtained by the structure.

4. Discussion

4.1. Genetic diversity on the Aragón germplasm bank grapevine collection measures for SSR markers. The main objective of this study was to explore the genetic diversity and structure of cultivated grapes curated at the BGVA and link them to cultivar utilization, putative geographic origin, and historical events. Most of the accessions have been collected in old vineyards in abandoned rural areas of the Aragón region, and some of them have already become extinct.

The genetic diversity values observed in this collection ranged from 4 to 15 alleles with a total number of 261 alleles for all *loci*, showed quite similar results with other collections (Na ranged from 5 to 18 alleles with a total number of 202; from 3 to 17 alleles with a total of 225; and from 4 to 13 alleles with a total number of 88; Oualkadi et al. [51]; De Andrés et al. [10]; and Sefc et al. [52], respectively). On the other hand, Laucou et al. [53] detected a total of 524 alleles with a mean of 26.20 alleles per locus with a larger collection of 4,370 accessions by using 20 SSR markers without exploring the ZAG series.

The results for the observed genotypes ranged from 6 to 50, with a total of 715 different monolocus genotypes in 411 accessions. Results obtained by Oualkadi et al. [51] showed a lower number of different genotypes (429) with a smaller collection (94) without exploring the ZAG series. Whereas, Laucou et al. [53] obtained a total number of 2,630 genotypes without exploring the ZAG series but with a larger and more diverse collection. The Shannon's information index (*I*) ranged from 0.45 in ZAG29 to 2.25 in VVIB3 *locus* (Table 1) as found by De Andrés et al. [10] in which the (*I*) varied between 0.72 in ZAG29 and 2.45 in VVMD28 in 192 wild accessions of *Vitis*. The high value of Shannon's information index for microsatellite *loci* indicates their effectiveness in establishing genetic relationships and diversity among accessions of grapevine in a collection [54].

The Ho varied between 0.2 and around 0.9 with an average value of 0.74, similar to those obtained by De Andrés et al. [10], which ranged between 0.3 and 0.8. Oualkadi et al. [51] showed that Ho was a little bit higher (0.32 in VVIN73 to 0.93 in VVIP31) with an average overall *locus* of 0.76, exploring 20 SSR markers without exploring the ZAG series. This can be explained by the use of the set of the highest polymorphic microsatellites. The He ranged from 0.21 to around 0.9 with an average of 0.73, and the values shared with other authors were similar to our results (He ranged between 0.4 and 0.9; 0.3 to 0.9; De Andrés et al. [10]; Oualkadi et al. [51], respectively). The expected heterozygosity is a better measurement to compare with other works since the number of alleles per *locus* is sensitive to the number of cultivars analyzed [51, 55]. The high allele number observed in this study (average = 10.03) and the high expected heterozygosity reflect the ability of these SSR markers to provide unique molecular profiles for individual plant genotypes. Furthermore, the *F* average obtained in this study (−0.014), as stated by Peakall and Smouse [44], *F* values close to zero are those expected under random mating, as found in other studies [32]. Negative values indicate an excess of heterozygosity due to negative assortative mating or selection for heterozygotes. On the contrary, the fixation index ($F = 0.54$), as found by De Andrés et al. [10], indicated evidence of inbreeding in the Spanish wild grapevine population studied and suggested a high level of genetic relationship among these individuals.

One major application of microsatellite markers in viticulture is the identification of cultivars and the distinction among them [39, 56, 57]. Therefore, the potential of the markers to yield different genotypes for as many cultivars as possible is of great interest. Indeed, in our collection, all *loci* distinguished up to 715 different monolocus genotypes. Also, it is important the selection of the most informative marker to reduce the number of *loci* to be investigated for reliable cultivar distinction. The SSR with the highest number of genotypes and discrimination power were VVIP31 (50, 0.97), VMC4F3-1 (49, 0.97), VVIV67 (47, 0.96), and VVS2 (47, 0.96). The PD variation among *loci* was due to the number of alleles per *locus*, as well as to the allele's frequencies.

The discrimination power (PD) of the SSR *loci* used in another study [51] varied from 0.31 in VVIN73 to 0.91 in VMC4F3-1 in which locus VVMD5, VVIV37, VVMD27, VVMD32, and VVS2 were also relevant for discriminating as in this study. Other authors observed the highest number of genotypes (41) in VVS2 out of 199 [26], and others described that *loci* VMC4F3-1 and VVIN73 to be respectively the most and least informative SSR markers [58, 59] since higher discrimination power (PD) implicates a lower probability for the confusion of cultivar identification [52].

4.2. Genetic Structure in the Studied Grapevine Collection. The principal coordinate analysis showed the eight assigned populations clearly separated (Figure 2). However, the PCoA that included symbols for all studied genotypes revealed that only the Spanish-labeled genotypes included in our collection were extended along the diagram and mixed with the other European groups (Figure 5). The structure analysis implies the existence of a high genetic overlap among the Spanish and other representative genotypes of several European and oriental origins (Figure 4(b), supplementary Table 7). The moderate level of mixture between the Spanish and the rest of the European and oriental groups of varieties may be due to an extensive exchange of genetic material coming from Europe [35]. This indicates that genotypes from diverse geographic regions are genetically mixed because of the migration process (probably as cutting exchanges). Interestingly, Spanish cultivars traditionally grown along the Camino de Santiago from Spanish Pyrenees to Galicia such as Albariño, Mencia, Verdejo, and Hondarrabi, all were *Q*4 and *Q*4 MIX mixed with other European genotypes but closer to the French and Portuguese genotypes in agreement with its geographic proximity.

In this study, the method for inferring population structure and genetic relationships revealed similar results,

Table 2: Putative crosses tested with 26 SSR markers which involve unknown accessions curated at the germplasm bank of Aragón.

Genotype code in El Encin Bank	Passport code-offspring	Genotype code in El Encin Bank-parent 1	Genotype code in El Encin Bank-parent 2
MEXT_1849	**ARAG_22_02**	GEN_0092-Hebén	GEN_0091-Vidadillo
MEXT_1889	**ARAG_33_06**	GEN_0092-Hebén	GEN_0150-Prieto Picudo Blanco I
MEXT_1900	**ARAG_34_14**	GEN_0092-Hebén	MEXT_0201-**ARAG_18_08**
MEXT_1954	**ARAG_65_15**	GEN_0092-Hebén	MEXT_0003-ARAG_B_1

as shown in Figure 5. The distance-based method analyses of the genetic relationships (PCoA analyses) identified four main genetic groups corresponding, the first to Spanish wine grapes varieties ($Q1$) most originated from Aragón or travelled along the Ebro River, the second and biggest one included Spanish wine grapes varieties ($Q2$), the third included table grape varieties ($Q3$) mixed with Greek and oriental origins, and the last with wine grape varieties from different European regions ($Q4$). The four groups showed high probabilities of assignment to their own cluster (supplementary Table 7), in agreement with the four groups being genetically distinct. In addition, the level of genetic differentiation detected between the Spanish varieties and other European varieties indicates the existence of a restrained genetic exchange between them. According to the study of Aradhya et al. [55], the two-dimensional projection revealed four clusters in which the French wine types representing the group occidentalis formed a separate cluster (4) from those wine-type cultivars belonging to the group pontica that constituted two clusters (2 and 3), and finally the groups predominant in table type belonging to orientalis and some to pontica formed cluster 1. The two-dimensional projection results agree with the PCoA based on the binary data matrix (244 accessions with 94 SSR alleles). According to Emanuelli et al. [30], the ΔK criterion suggested by Evanno et al. [49] gave the highest value in the two groups, although peaks of ΔK were also found at $K = 3$ and $K = 6$. Since different K values were detected with different methods, the inferred population structure of the studied *Vitis* collection was shown for K ranging from 2 to 6. The following runs of structure revealed four groups, the first group represented Italian/Balkan wine grapes, the second group presented Mediterranean table/wine grapes, the third group showed Muscats (wine/table grapes), and finally, the fourth group represented Central Europe wine grapes.

Concerning genetic structure, although not exactly similar, our results agree substantially with the clustering obtained in a wide French grapevine collection by Bacilieri et al. [35], who reported three clusters: wine-West and Central Europe, Wine-Balkans and East Europe, and Table-East. Indeed, while our populations $Q1$, $Q2$, $Q3$, and $Q4$ correspond respectively to Spanish cultivars (denominated as Wine-West), Spanish table + Greek + oriental (denominated as Table-East), and French + German-Slovenian (denominated as Central Europe), revealed by these authors at $K = 3$ [35]. Similarly, results obtained by Zarouri et al. [36] with 207 nonredundant grapevine genotypes from El Encin in which found three main groups: one group included almost wine-West Europe (most cultivars from France and Spain), the second group represented table grapes (most from Spain and oriental countries) but mixed with grapes cultivars from the Iberian Peninsula such as Albillo Mayor, Bobal, Hebén, and Tempranillo and the third group included table wine cultivars from the Balkan and Eastern Europe. Another study showed that structure analysis and the ΔK criterion suggested $K = 2$ for the set of 131 nonredundant genetic profiles [60] separated genotypes sampled as cultivated (81) or wild (44). However, additional stratification of the profiles for the structure analysis allowed the identification of three genetic groups: the first group contained the ancient Turkish cultivar Razaklija, the second group included genotypes native to the Western Balkans, and lastly, the third group formed the renowned internationally cultivars (Cabernet Franc, Merlot, and Savagnin). These studies revealed similarities with our study but also differenced, all based on the genetic background of each collection.

4.3. Pedigree Analysis. A preliminary analysis of parent-offspring compatibility shows that several genotypes share one allele per locus with Hebén, such as Cadrete or Trepat, but also new genotypes such as ARAG_22_02, ARAG_33_06, ARAG_34_14, and ARAG_65_15 (MEXT_1849, MEXT_1889, MEXT_1900, and MEXT_1954) (Table 2).

Zinelabidine et al. [61] proposed more than 23 parent-offspring relationships involving "Hebén" as a female parent in the Iberian genetic network and stated its predominant role in the spread of the most frequent chlorotype in Western Europe. Other authors also found possible first-degree relationships among grapevine varieties involving "Hebén" as one of the parents and putative parent-offspring relationships with several genotypes [62]. Further analysis should be conducted in order to establish the founders of these Spanish unknown genotypes that will help to better understand the origin and dissemination along Europe of the studied materials.

5. Conclusion

In this study, we provided the molecular characterization of the *Vitis vinifera* L. accessions of the germplasm bank of Aragón, also evaluating the genetic diversity in the collection. The 26 *loci* assayed in the 411 accessions were polymorphic and multiallelic. Most of the studied *loci* showed at least one rare allele in the collection; the results showed the absence of inbreeding mating since the F values are close to zero. The four most informative *loci* which showed the highest number of different genotypes and were able to distinguish the 156 different

genotypes studied were: VVIP31, VMC4F3-1, VVIV67, and VVS2. The PCoA distribution showed the existence of a high genetic overlap among 174 grapevines from different European and oriental origins. Structure results showed the differentiation of four distinct groups: the first population represented traditional Spanish wine grapevine genotypes from Aragón or cultivated around the Ebro River, the second population consisted of the Spanish wine grape cultivars, the third grouped the Spanish and oriental origins table grape, and finally, the fourth population grouped wine grape cultivars of Portuguese, Italian, French, and German-Slovenian origins. The preliminary pedigree analysis showed that Hebén is a compatible parent of several genotypes. This study provides the identification of several Spanish unknown accessions, which are the foundational basis for studying the behavior and adaptability of these grapes in the context of the environment and climate change. These genotypes could be ancient cultivars that are probably resilient to face the new conditions derived from climate change. These cultivars, grown traditionally for years in local conditions, and often submitted to extreme environments, are an alternative to modern varieties. Thus, these genetic resources are available to take the necessary adaptation measures, where appropriate, such as the relocation of vineyard cultivation to northern areas or higher altitude areas with lower average temperatures to reach the desired quality of the final product.

Data Availability

All datasets generated for this study are included in the article in the supplementary materials. SSR data will be available at https://www.comunidad.madrid/coleccion-variedades-vid and the complete database with 26 SSR will be available at the institutional repository of the Spanish National Research Council DIGITAL CSIC. DIGITAL.CSIC, https://digital.csic.es/?locale=en.

Disclosure

HG analyzed data and wrote the draft manuscript. MTDA run SSR samples and provided all SSR profiles. LJA selected and collected the accessions to be analyzed from the Aragón germplasm bank collection. YG conceived the idea, provided funds, supervised all activities, and with help from MTDA revised the final version of the manuscript. All authors discussed the results and commented on the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Supplementary Table 1. Name, dye, sequences, and the final concentration of the primers used in the PCR A and PCR B set. Supplementary-Table 2. List of the 156 *Vitis vinifera* L. studied cultivars curated in the Aragón germplasm bank collection. Vitis International Variety Catalogue (VIVC). Supplementary Table 3: Allele size (AS) in base pairs and allele frequency (AF) observed for the 156 *Vitis vinifera* L. accessions studied with 26 SSR markers. Supplementary Table 4: Observed genotype (OG) and genotype frequencies (GF) in 156 *Vitis vinifera* L. accessions analyzed with 26 microsatellites. Supplementary-Table 5. List of the 174 *Vitis vinifera* L. genotypes studied. In bold presented genotypes from Aragón germplasm bank collection (BGVA). Vitis International Variety Catalogue (VIVC). Supplementary Table 6. The values of qI for $K = 2$ ($Q1$ on the left and $Q2$ on the right side). Supplementary-Table 7. The values of qI for $K = 4$, Label and PCoA coordinates used for Figure 5. (*Supplementary Materials*)

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