IN SITU AND GENETIC CHARACTERIZATION OF WILD GRAPEVINE POPULATIONS IN THE CASTILIAN AND LEON REGION (SPAIN)

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Abstract

Aim : Assessing levels and patterns of population genetic variation in combination with morphological characterization represents an important step for evaluating rare or endangered species and determining appropriate conservation strategies. This is particularly important for ensuring the preservation of valuable genetic variation in wild relatives of crops, which could provide beneficial alleles for plant breeding and improvement.

Methods and results : A survey of the relict wild grapevine population was carried out in the province of Burgos, in the Castilian and Leon region (Spain). Genetic diversity, inbreeding extent and possible hybridization with cultivated grapevine were investigated using molecular markers. Results showed that overall, genetic diversity was low but inbreeding was not present. Private alleles were found in the Burgos wild genotypes, suggesting the potential value of these accessions. Comparisons of morphological traits and molecular features among the wild population showed a close genetic relationship among them and no genetic relationship to Castilian grapevine cultivars.

Conclusion : The genetic differentiation observed between wild and domesticated forms of grapevine points out the interest to characterize and conserve the existing populations as a source of novel alleles for future grapevine breeding programs. At the same time, the observed enological differences between wild and domesticated grapevine populations could be interesting for the wine industry. Finally, given that these populations are at severe risk of extinction, we recommend that this population be prioritized for *ex situ* and possibly on-farm conservation as well as *in situ* protection.

Significance and impact of the study : This work showed the existence of novel alleles and enological characters in natural wild grapevine populations, which represents a first step in the potential contribution of these natural populations to viticulture, suggesting a possible role in future breeding programs.

Key words : *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi, genetic diversity, genetic relationship, morphological characterization, pathogen infection, conservation programs

Résumé

Objectif : Le fait d'évaluer les niveaux et les types de variations génétiques des populations en relation avec les caractéristiques morphologiques représente un pas important dans l'évaluation des espèces rares et en danger et la détermination des stratégies de conservation appropriées. Ceci est particulièrement valable pour assurer la préservation d'une variation génétique importante dans les espèces sauvages, ce qui peut fournir des allèles bénéfiques pour la culture de la plante et son développement.

Méthodes et résultats : Une étude a été menée sur un reliquat de vigne sauvage dans la province de Burgos, dans la région de Castille-et-León (Espagne). La diversité génétique, l'extension de la consanguinité et une possible hybridation avec des vignes cultivées ont fait l'objet d'une investigation au moyen de marqueurs moléculaires. Les résultats ont démontré que, dans l'ensemble, la diversité génétique est faible mais que la consanguinité n'existe pas. Des allèles particuliers trouvés dans les génotypes sauvages de la province de Burgos soulignent la valeur de ces acquis. Des comparaisons de caractères morphologiques et de caractéristiques moléculaires parmi les populations sauvages ont montré une étroite relation génétique entre elles et aucune relation génétique avec les vignes cultivées de Castille.

Conclusion : La différenciation génétique observée entre les vignes sauvages et les vignes cultivées souligne l'intérêt d'évaluer et de conserver les populations existantes en tant que source de nouveaux allèles pour les futurs programmes de sélection de la vigne. En même temps, les différences œnologiques observées entre les vignes sauvages et les vignes cultivées pourront se montrer intéressantes pour la production du vin. Enfin, ces populations courant un grand risque d'extinction, nous recommandons leur conservation aussi bien "ex situ" que dans des centres spécialisés ("à la ferme") ainsi que leur protection "in situ".

Signification et impact de l'étude : Cette étude montre l'existence de nouveaux allèles et de caractères œnologiques dans les populations de vignes sauvages, ce qui représente un premier pas dans la contribution potentielle de ces populations à la viticulture, suggérant ainsi un rôle possible dans les programmes de sélection.

Mots clés : *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi, diversité génétique, relation génétique, infection pathogène, programmes de conservation

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INTRODUCTION

It has been argued that germplasm collections not only play an important role in the protection of native biodiversity (Primack, 2002), but can also serve as valuable sources of alleles and traits for ongoing plant breeding and crop improvement efforts (Hajjar and Hodgkin, 2007). Indeed, the gene pools of crop relatives often harbor beneficial alleles (Nevo and Chen, 2010), and in the case of rare and endangered species, such alleles are at risk of extinction. The availability of genetic diversity among wild relatives opens up opportunities for exploring trait and stress tolerance mechanisms that domestication and modern agriculture have left behind and also provides the scope for novel allele discovery.

The ancestor of the cultivated grapevine is *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi, a woody heliophilous dioecious liana growing up to the top of the canopy of the associated arboreal vegetation (Vigier 1718). Currently, its relict populations spread out from Portugal to the Hindu Kush mountain-range (Arnold, 2002), approximately limited by parallels 50 North (Rhine Valley, Germany) and 30 North (Ourika Valley, Morocco) (Ocete *et al.*, 2007). This taxon constituted a plant resource strongly linked to the development of several ancient civilizations along its distribution range. In fact, from the Neolithic Age up to some decades ago, berries were harvested for direct consumption, vinification or vinegar making (Rivera and Walker, 1989; Gorny, 1996; Ocete, 2011a). In Spain, seeds of this plant are relatively frequent in archaeological sites belonging to the Argar culture (Bronze Age), meanwhile the oldest cultivated ones appear later in the Phoenician colonies from the 7th century BC (Ocete *et al.*, 2007). Recently, new evidences of Spanish viticulture dating back to the 1st millennium BC have been described by Martínez and Maronda (2011) and Vera and Echevarría (2011).

Powdery and downy mildew fungal diseases arrived in Europe during the 19th century and caused a heavy impact on vineyards and wild populations. Phylloxera (*Daktulosphaira vitifoliae* Fitch) probably caused little damage, because the kind of locations, soil characteristics and climate of wild grapevine habitats are not suitable for this North American homopteran (Muñoz del Castillo 1878; Ocete *et al.*, 2006). The negative effect caused by these pests and diseases on wild grapevines was increased by human activities, mainly forest exploitations, road construction and river management. Furthermore, the introduction of American native species and hybrid cultivars, for use as Phylloxera-resistant rootstocks for European grapevine cultivars, represented a threat for the European wild subspecies because these invasive plants are able to displace from natural habitats to autochthonous vines (Terpó, 1969 and 1974; Laguna, 2003; Arrigo and Arnold, 2007). However, gene flow does not seem to be frequent between naturalized rootstocks and wild grapevines due to different ecological behaviors (De Andrés *et al.*, 2012). Nowadays, grapevine is considered an endangered taxon in Europe (Arnold *et al.*, 1998; Walter and Gillett, 1998), mainly in those countries, like Spain, Portugal and Italy, where there is a lack of legal protection for this plant, whereas in France, Switzerland, Germany, Austria and Hungary, it has been included in the list of endangered plant species. Its future represents a major stake in biodiversity conservation (Terral *et al.*, 2010) because most populations have a small number of individuals and possibly there could be a gene flow from cultivars, which could modify their original genetic structure (De Andrés *et al.*, 2012).

The main objective of our work was the morphological characterization and population genetic analysis of available, wild-collected individuals of *V. vinifera* subsp. *sylvestris* from the Castilian and Leon region in Spain to conserve and evaluate the valuable sources of alleles and traits for ongoing plant breeding and crop improvement efforts. Firstly, a complete *in situ* morphological characterization, including main plant pathogen/disease symptom identification and grape enological characterization, was concluded. Secondly, we estimated the level of genetic diversity and the extent of population structure in this rare species, also comparing the results to other wild populations, in order to define the proper strategy for *in situ* and *ex situ* programs of germplasm conservation. At the same time, the possible genetic relationships between wild and cultivated genotypes were analyzed.

MATERIALS AND METHODS

1. *In situ* characterization

The survey planning was to explore the riverbank forests of the main rivers and their tributaries in the province of Burgos. Each population was mapped by a GPS receiver in 2005. In this way, we considered populations in 18 different locations where wild grapevine accessions were analyzed by *in situ* morphological characterization and collected 38 wild grapevine leaf samples from 7 locations for genetic characterization (Table 1). Accessions were assessed at flowering time (June-July) to determine the sex of the plants. Pollen samples from flowers were obtained by brushing the mature anthers from 10 male and 10 female vines. Grains were maintained in DPX (Fluka)

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and observed under optical microscope Olympus BX 61 to study the morphological structure of the grains.

Other observations were carried out twice a month between 2005-2011 to establish an approximate phenological calendar of the vines from bud break to leaf fall. The main ampelographical descriptors were evaluated following the list provided by the International Organisation of Vine and Wine (OIV, 2009) on 57 females and 100 males over 2009-2010. The main botanical supporters were identified using general botanical keys and the study of Aizpuru *et al.* (2003) in the first and second year of the study.

The detection of possible symptoms caused by pests and diseases was carried out in spring and summer times on the aerial organs of the plants (shoots, leaves and bunches; 3 m above ground) over the seven years of the investigation. The roots were excavated down to a depth of 40-50 cm to observe possible symptoms caused by subterranean phytophagous and pathogens. Samples of fine roots were observed under binocular to detect possible Phylloxera damage, root-knot galls caused by nematodes, as in the case of *Meloidogyne* species, and rot fungi. The degree of damages caused by parasitic species was evaluated according to the grade systems proposed by Ocete *et al.* (2007) for mites and OIV (2009) for mildews. Plant materials taken at random from two plants (one male and one female) of each population, except in the case of Paradores and Entrambasaguas, where there were no female vines, were used to detect the presence of Grapevine Fan Leaf, Grapevine Leafroll and Grapevine Fleck viruses using ELISA tests (Martelli, 2000).

2. Microvinification

On October 26th, 2011, 6.3 kg of bunches were harvested in the Angulo Valley from 6 different grapevines. After destalking, berries were pressed by hand and 1200 ml of must were obtained. Fermentation with maceration occurred spontaneously from wild yeasts and lasted 7 days, then some basic enological analyses were performed following reference methods (OIV, 2009). Moreover, a sensory analysis of the wine was performed by a panel of experts (winemakers, enologists and consumers) following the free-choice profiling descriptive method (Murray *et al.*, 2001).

3. Genetic diversity analysis

The samples collected from the Burgos province were analyzed at the morphological and genetic levels. In order to avoid considering feral plants, we used the sex of the flower to discriminate *V. vinifera* subsp. *sylvestris* versus *V. vinifera* subsp. *sativa* and morphological observation to discriminate other *Vitis* ssp. Furthermore, given the known genetic determinism of sex in *V. vinifera* L. (Negi and Olmo, 1971), wild male plants cannot escape from cultivated fields of hermaphrodite or female cultivars and cannot result from pollination between wild females and cultivated hermaphrodite or female plants, whereas wild female plants could be. Because of the possible differences in the origin of male and female *sylvestris* plants, we initially analyzed wild male plants. A sample of 38 wild male accessions from the Burgos province was analyzed (Table 1) and compared with published genotype data, corresponding to 192 Spanish wild grapevine genotypes (De Andrés *et al.*, 2012), 210 autochthonous grapevine cultivars from the Castilian and Leon region (Santana *et al.*, 2010) and 36 European grapevine cultivars (De Andrés *et al.*, 2012).

Total genomic DNA was extracted from young leaves using the DNeasy™ Plant Mini Kit (Qiagen). Extracted DNA was quantified and used as a 5ng/µl stock solution. A set of 20 simple sequence repeat (SSR) microsatellite loci was analyzed to study genetic diversity. The genotypes were obtained using two independent multiplex PCRs, labeled as A and B, as previously described by Ibáñez *et al.* (2009). PCR A included 11 microsatellite markers: VVS2 (Thomas and Scott, 1993), VVMD7, VVMD24, VVMD25 (Bowers *et al.*, 1996 and, 1999), VVIB01, VVIH54, VVIN73, VVIP31, VVIP60, VVIQ52 (Merdinoglu *et al.*, 2005) and VMC1B11 (Zyprian and Topfer, 2005); PCR B included 9 microsatellite markers: VVMD5, VVMD21, VVMD27, VVMD28, VVMD32 (Bowers *et al.*, 1996 and 1999), VVIN16, VVIV37, VVIV67 (Merdinoglu *et al.*, 2005) and VMC4F3.1 (Di Gaspero *et al.*, 2000). PCR amplifications were analyzed in an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using GeneScan-LIZ 500 as internal marker (Applied Biosystems). Amplified fragments were sized with GeneMapper 4.0 software.

Allele size and total number of alleles were determined for each SSR. Putative alleles were indicated by their estimated size in bp. Private alleles are considered alleles that are found only in a single population among a broader collection of populations. Genetic diversity was estimated using the following statistics: number of alleles (Na); mean number of alleles per locus (MNA); effective number of alleles (Ne); observed heterozygosity (Ho); expected heterozygosity (He) (Nei, 1973); number of private alleles; and fixation index (F), also called inbreeding coefficient. These statistics were calculated using GenAlex software version 6.0 (Peakall and Smouse, 2006) and the Excel Microsatellite Toolkit (Park, 2001). Redundant genotypes were excluded from all analyses.

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4. Population structure and genetic differentiation analysis

Bayesian clustering was applied on the SSR genotype data using the STRUCTURE software package (Pritchard *et al.*, 2000) revised version 2.1 (Falush *et al.*, 2003). Analyses were performed with the total collection (460 unique genotypes) and with each population obtained from this first analysis. Only accessions with ancestry values higher than 0.7 were included in each population analysis. Admixture model and correlated allelic frequencies were used to analyze the dataset without prior population information, as suggested by Falush *et al.* (2003). Ten simulations per K value were performed for each population (K) (set from 1 to 10). Burn-in period and Monte Carlo Markov Chain (MCMC) length were set up at 100,000 and 300,000 in each run, respectively. To assess the best K value supported by the data, we calculated the second order change of the likelihood function divided by the standard deviation of the likelihood (ΔK) (Evanno *et al.*, 2005). However, natural populations are expected to show more complex relationships, and this may affect the manner in which STRUCTURE assigns individual to clusters. For that reason, in order to know the most likely K value after the first run of the STRUCTURE program, we have split the dataset on the base of optimal K to repeat the procedure in each genetic group.

Analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) was performed to characterize the partition of the observed genetic variation among and within populations and genetic groups using GenAlex software. Pairwise Fst values (Slatkin, 1995) among the populations and subpopulations inferred by STRUCTURE were estimated with Arlequin 3.5 (Excoffier *et al.*, 2005).

RESULTS

1. *In situ* characterization

During this survey, 18 wild grapevine populations totaling 157 accessions were analyzed (Table 1). In a previous study, only two populations in the Castilian and Leon region were revealed (Ocete *et al.*, 1999). Wild populations were only found in the Mena and Angulo Valleys, located in Northern Burgos close to the borders with the Cantabria and Basque regions (Figure 1). The list of sites along gallery forests and colluvial positions appears in Table S1a. The accompanying flora corresponds to Riparian-colluvial Atlantic vegetation, with cool thermal regime and annual precipitation of about 1200 mm (Table S1b). The approximate phenological calendar was : bud swelling : April 21-May 3 ; flowering : June 16-27 ; veraison (onset of ripening) : July 28-August 22 ; fruit ripening : November 4-16 ; and end of leaf fall : November 11-27. The main morphological descriptors of female and male vines (young and mature shoot, young and mature leaf, woody cane, flower, bunch, berry, seed and phenology) demonstrated very low polymorphism (Table S2). Pollen grains were tricolporated in male and acolporated in female flowers. No symptoms of infestation or infection attributable to Phylloxera (*Daktulosphaira vitifoliae*

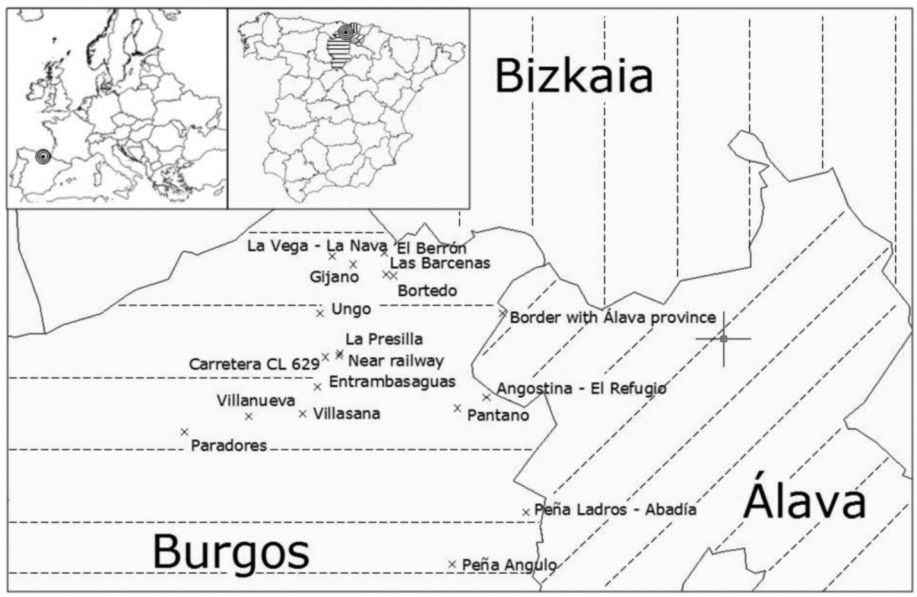


Figure 1. Distribution of the 18 wild grapevine populations in the Burgos province (Spain).

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Fitch), root-knot nematodes or mycelium of rot fungi were detected. Leaf damages caused by mites, namely the Erineum strain of *Colomerus vitis* (Pagenstecher) and *Calepitrimerus vitis* (Nalepa) (Acari, Eriophyidae), were very frequent in all populations. Symptoms of the *Erysiphe necator* (Schwein.) Burriel fungus were common in the majority of the vines, mainly on leaves and shoot axes. Oil spots on leaves together with other damages on shoot axes and bunches caused by downy mildew, *Plasmopara viticola* (Berkeley and Curtis) (Berlese and de Toni), were also frequent. The intensity of infestation or infection varied among vines of the same population (Table 1) but was not considerably different from the domesticated grapevines. Regarding viral infections, all the Elisa tests were negative for the three pathogens tested.

2. Microvinification

The experimental wine showed lower alcohol content in comparison to wines produced with cultivated varieties in the area. Total acidity was high and pH was low, given the medium/high malic acid content; color intensity and anthocyanin concentration were very high; and tannin levels were medium (Table S3). According to the sensory evaluation, the wine revealed noticeable fruit aromas of wild berry and acidity.

3. Genetic diversity of the wild populations

The genetic diversity present in wild grapevine populations from the Burgos province was analyzed using microsatellite genotyping performed in the initially selected 38 wild male grapevine samples (Table S4). 22 unique genotypes were identified, the remaining 16 samples corresponded to redundant genotypes. These unique genotypes showed a mean number of 5.5 alleles, including an average of 0.5 private alleles (Table 2). The genetic comparisons between the wild grapevine genetic groups from the Iberian Peninsula, which are here called Northern and Southern Spain wild populations (De Andrés *et al.*, 2012), and the Burgos province populations led to the identification of private alleles in the Burgos wild populations (Table S5). They are not present in other Spanish wild populations and some have a frequency higher than 10 %. This result pointed out the selection

Table 1. Number and sex of the vines and incidence of pests and diseases (number of individuals affected and intensity of infestation/infection) according to Ocete *et al.* (2007) and OIV (2009).

Location	N. of vines	Male vines	Female vines	*Colomerus vitis*	*Calepitrimerus vitis*	*Erysiphe necator*	*Plasmopara viticola*
El Berrón	5	3	2	5 (1-3)	3 (1-3)	2 (1)	3 (1-3)
Bortedo	9	7	2	9 (1-3)	4 (1)	3 (1)	6 (1-3)
Las Bárcenas	23	13	10	20 (1-3)	11 (1)	6 (1-3)	11 (1-3)
Gijano	11	7	4	9 (1-3)	7 (1)	4 (1)	2 (1-3)
La Vega-La Nava	17	11	6	16 (1-3)	12 (1)	3 (1)	6(1-3)
Ungo	13	8	5	13 (1-3)	8 (1)	2 (1)	4 (1-3)
La Presilla	7	4	3	7 (1-3)	5 (1)	5 (1-3)	1 (1-3)
Carretera CL 629	9	6	3	9 (1-3)	6 (1-3)	4 (1-3)	4 (1-3)
Near railway	6	4	2	6 (1-3)	3 (1)	3(1)	3 (1-3)
Entrambasaguas	3	3	0	3 (1-3)	0	1 (1-3)	1 (1-3)
Villasana	11	7	4	11 (1-3)	4 (1)	2 (1)	6 (1-3)
Villanueva	4	3	1	4 (1-3)	0	0	2 (1-3)
Paradores	1	1	0	1 (1-3)	0	0	0
Pantano	7	5	2	7 (1-3)	3 (1-3)	2 (1-3)	2 (1-3)
Angostina-El Refugio	11	7	4	11 (1-3)	4 (1)	5 (1)	6 (1-3)
Peña Angulo	5	3	2	4 (1)	0	2 (1-3)	2 (1-3)
Peña Ladros- Abadía	7	4	3	7 (1-3)	2 (1)	4 (1-3)	3 (1-3)
Border with the Alava province	8	4	4	8 (1-3)	3 (1)	3 (1)	4 (1-3)
TOTAL	157	100	57	150	75	49	66
Percentage (%)	100	63.7	36.3	95.5	47.8	31.2	42.0

In bold, locations where the male vines were genetically analyzed

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of alleles that are not present in the other studied Spanish populations. Mean number of alleles in the different populations ranged from 5.5 to 9.1; effective number of alleles ranged from 2.4 to 4.7, with an average of 2.4 in the Burgos wild populations; Information Index ranged from 1 to 1.7; observed heterozygosity ranged from 0.5 to 0.6, whereas expected heterozygosity was slightly higher, ranging from 0.5 to 0.7; and Fixation Index ranged from 0.012 to 0.121, pointing to the putative existence of inbreeding depression in some wild grape populations (Table 2). In general, the wild populations from the province of Burgos showed lower values in all the allelic patterns studied, probably due to the small population size.

4. Population structure between wild grapevine and cultivated genotypes

The genetic structure analysis included Northern and Southern Spain wild grapevine populations (192 samples), wild accessions from the Burgos province (22 samples), cultivated grapevines from Spain including the Castilian and Leon region (210 samples), and European cultivars (36 samples), especially from France. The analysis was achieved using the STRUCTURE program; the choice of a fixed K is not arbitrary, each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are probabilistically assigned to genetic groups or jointly to one or more groups. Consequently, we performed an analysis where K could vary from 1 to 10. This analysis showed a large increase of likelihood from K = 1 to 2 and smaller increases from K = 2 to 10. Using the methodology of Evanno *et al.* (2005), this result supported K = 2 (Figure S1) as the most likely number of genetic groups, which corresponds to wild and cultivated grapevine genotypes (Figure 2). Taking into consideration the genetic structure analysis done by De Andrés *et al.* (2012) reporting the genetic structure in the Spanish wild populations, it is possible to suppose that these genetic groups comprise more than one subpopulation. For that reason, in order to know the most likely K value after the first run of the STRUCTURE program, we have split the dataset on the base of optimal K to repeat the procedure in each genetic group. Analyses were performed with 221 genotypes from the cultivated group and 148 genotypes from the wild group; 91 genotypes were discarded because only accessions with ancestry values higher than 0.7 were included in each population analysis. This analysis showed that the most likely number of genetic groups was K = 5. The first cluster (wild grapevine) was divided in two subclusters (SW1 and SW2), which correspond to southern and northern wild grapevine accessions including the wild genotypes from the Burgos province; the second cluster (cultivated grapevine) was divided in three subclusters (SC1, SC2 and SC3) (Table S6). SC1 contained the majority of northern Spanish cultivars and some autochthonous cultivars from the Castilian region; SC2 included the majority of autochthonous cultivars from the Castilian region and one French accessions; and SC3 included autochthonous Spanish grapevine cultivars and some European cultivars. However, the consistency of the subpopulations described by STRUCTURE was very weak. In fact, the STRUCTURE program was run several times with different input data, resulting in different subpopulations for the cultivated grapevine. For this reason, we concluded that K = 2 was the most likely number of genetic groups, corresponding to wild and cultivated grapevine genotypes (Figure 2).

The output of the STRUCTURE analysis showed the ancestry value, which is an estimation of the proportion of the genome of an individual that originated from a given population. The ancestry value varies from 0 to 1. An ancestry value close to 0 or 1 in one group suggests no evidence of introgression for the individual studied. Intermediate values suggest introgression. Using these data, we can conclude that the wild

Table 2. Allelic patterns (means ± standard deviation) and Fixation Index across wild grapevine populations from Spain.

	Population (number of individuals)					
	Burgos province (22)		**Northern** (61)		**Southern** (131)	
Na - number of alleles	5 500	±0.366	6 950	±0.461	9 100	±0.757
Ne -effective number of alleles	2 462	±0.154	3 050	±0.177	4 733	±0.397
I - Information Index	1 098	±0.074	1 315	±0.059	1 707	±0.085
Number of private alleles	0.500	±0.198	0.250	±0.123	2 050	±0.473
He - expected heterozygosity	0.554	±0.037	0.651	±0.019	0.757	±0.022
Ho - observed heterozygosity	0.564	±0.019	0.608	±0.020	0.668	±0.021
F - Fixation Index	0.012		0.076		0.121	

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accessions from the Burgos province showed the same ancestry values as the wild accessions from Northern Spain (Table S6). In contrast, the autochthonous Castilian cultivars showed high ancestry values with the French cultivars (Table S6). The rest of the Spanish cultivars showed different levels of introgression with the European cultivars (Table S6).

5. Genetic differentiation among genetic groups

AMOVA analysis using the genetic groups showed that most of the existing genetic diversity was distributed within (85 %) rather than among (15 %) populations. The Fst values among the five genetic groups are shown in Table 3. The highest and most significant Fst values were observed in pairwise comparisons between the wild genetic group and the cultivated genetic group (Fst = 0.22). Fst values were found to be significantly different from zero (P <0.01) between populations. In addition, we observed a moderate genetic differentiation between subclusters SW1 and SW2 and between subclusters SC1, SC2 and SC3. On the other hands, the cultivated subclusters SC1, SC2 and SC3 showed high genetic differentiation with the wild subclusters SW1 and SW2 (Table 3). All values were significantly different from zero in all pairwise comparisons (P <0.05). These results confirm the low genetic relationship between wild grapevine from Burgos and the cultivated grapevine.

DISCUSSION

In the province of Burgos, which belongs to the Castilian region in north-central Spain, relict populations of wild grapevines are found only in the north under Atlantic climate. The environmental factors, like the cold continental climate of the Castilian region, restrict potential natural habitats for wild grapevines to a few low "frost-free" valleys on the border of the region.

Table 3. Pairwise estimates of Fst values based on data from 20 SSR loci among the model-based clusters inferred by STRUCTURE.
All values were significantly different from zero in all pairwise comparisons (P <0.05).

	SW1	SW2	SC1	SC2	SC3
SW1	0.000				
SW2	0.107	0.000			
SC1	0.168	0.109	0.000		
SC2	0.206	0.135	0.047	0.000	
SC3	0.158	0.129	0.042	0.063	0.000

SW1 and SW2, wild grapevine subclusters; SC1, SC2 and SC3, cultivated grapevine subclusters



Figure 2. Graphical representation of ancestry membership coefficients of all 460 individuals.
Each individual is shown as a vertical line divided into segments representing the estimated membership proportions in the two genetic clusters (wild and cultivated grapevine genotypes) inferred with STRUCTURE.

In these conditions, these populations are unique in the Iberian Peninsula and have probably developed ecological adaptive traits that have not been developed in other areas. Here, we compared the morphological and genetic diversity previously described by De Andrés *et al.* (2012) in wild grapevine populations from Spain with the wild grapevine populations from the Burgos province in the Castilian region, in order to conserve and evaluate the valuable sources of alleles and traits, which can be useful for the domesticated grapevine.

The morphological characters resulted very similar to those shown by wild grapevine populations from the neighboring Basque country, mainly in the case of the locations along the Cadagua River (Bizkaia province) and the Navarre region, which were described by Ocete *et al.* (2004 and 2011b). It is important to remark that the Iberian Peninsula constituted a refuge area for grapevine during the Ice Age in the Quaternary (Antunes and Böhm 2011). About 64 % of the vines were males. This corresponds to a female/male ratio of 0.57, confirming the data obtained by Anzani *et al.* (1990) and Ocete *et al.* (1999). All the observed male flowers corresponded to morphological type I (fully developed stamens and no gynoecium) (OIV 2009), as it occurs in the rest of the Spanish populations (Ocete *et al.*, 1999). No male plants with flower type II (fully developed stamens and reduced gynoecium) were found, confirming that this latter type of flower, which could be interpreted as a step towards hermaphroditism (Orrú, 2012), is very scarce in Spain. Several male individuals had a secondary flowering time in the middle of July. There was also pollen grain dimorphism: the male one was tricolporated, similar to that belonging to hermaphrodite cultivars, and the female one was an aperturate ovoid sac, as described in Andalusian populations by Gallardo *et al.* (2009). All the female plants had red berries, like in almost all the Spanish populations (Ocete *et al.*, 1999). This intense color of berries probably is an adaptive strategy to successfully attract birds and favor seed dispersal

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(Hidalgo, 2003). However, berry ripening was not uniform and complete within each bunch. Besides dioecism, seed morphology was a phenotypic trait characteristic of this wild taxon in comparison to cultivars of *V. vinifera* L. subsp. *sativa* (DC.) Hegi (Stummer, 1911). Seeds from the wild population of the Burgos province were subspherical with a small beak, close to those of *V. vinifera* subsp. *sylvestris* studied by Anzani *et al.* (1990) in Italy, Ocete *et al.* (2007) in Spain, Terral *et al.* (2010) in France and Orrú (2012) in Sardinia. Considering the morphological characteristics, vines from the populations involved in this survey were also comparable to the ones found in Northern Spain, namely in Navarra and La Rioja (Ocete,, 2011a ; Ocete *et al.*, 2011b). Regarding pathogen infestation, we previously found that wild grapevine populations are sensitive to Phylloxera under artificial laboratory conditions (Ocete *et al.*, 2011b). The absence of symptoms on roots in the wild ecosystems seems to be due to the edaphic conditions of their habitats, where there is several months of flooding each year. Similarly, this would explain the absence of symptoms caused by root-knot nematodes, such as galls and secondary rootlets, similar to those caused by *Meloidogyne,* as cited by Palm and Walter (1991). The mite *Colomerus vitis* was always present on all the grapevine populations, as in the rest of the Iberian sites cited by Ocete *et al.* (1999) and also in the Caucasian region (Ocete *et al.*, 2012). It is interesting to note that in some specimens with a low level of infestation, erinea was occasionally found on the upper leaf surface. Symptoms caused by another eriophid, *Calepitrimerus vitis*, were less frequent. Its presence was cited, also, in populations on the coast of Guipúzcoa and the Ebro Valley (Ocete *et al.*, 2004) and some riverbank forests from Andalusia (Ocete *et al.*, 2007). The constant presence of *Colomerus vitis* and the frequent one of *Calepitrimerus vitis* on the sites sampled would suggest that both species of obligatory and monophagous parasites have cohabited with vines since remote times, without causing any serious damage. On the contrary, damages caused by North American downy mildew are occasionally serious depending on the seasonal weather circumstances and the specific microclimatic conditions of the particular vine (Ocete, 2011a). In summary, we did not find any individual significant resistance to pathogen infestation that could be useful to the domesticated grapevine.

On the other hand, it should be underlined that the analytical characteristics of the wine obtained by the microvinification of wild grapes demonstrated interesting traits for the wine industry like high color intensity and total acidity (Ayala *et al.*, 2011). The experimental sample corresponded to a full-scale red wine, similar to those obtained from cultivars, such as Alicante Henri Bouschet or Syrah, or made with wild grapes from other Spanish neighboring regions, such as Navarra and La Rioja (Ocete, 2011a ; Ocete *et al.*, 2011b). Furthermore, it was comparable to wines made with wild grapes from Mediterranean regions as Andalusia (Ocete *et al.*, 2007) and Sardinia (Lovicu *et al.*, 2009). It revealed noticeable fruity aromas and a good ageing potential as a consequence of its tannin levels. Characteristics such as an intense color and a good total acidity could be very interesting in the Mediterranean climate. A blend of musts from wild and domestic grapes could produce red wines enhanced in color and not requiring acidity correction by tartaric acid addition. The cultivation of wild grapevines in commercial vineyards with the objective to improve the characteristics of the wine made from conventional cultivars could represent a sustainable approach for the on-farm conservation of wild grapevines. Moreover, the qualitative traits of wild grape fruits could be evaluated by breeding programs to develop new cultivars with improved polyphenol and organic acid accumulation. Finally, this survey provided a view of the potential contribution of these natural populations to viticulture, given the occurrence of such a genetically unique population ; our recommendations include population surveys of the Castilian region in the existing germplasm collections.

Precise detection and quantification of genetic variation is a prerequisite for the successful conservation and exploitation of plant genetic resources. Firstly, we found 16 redundant genotypes in the 38 wild grapevine accessions. This result can be explained because, as mentioned before, the wild ancestor is a woody liana growing up to the top of the canopy of the associated arboreal vegetation and in the sampling it is easy to get the same sample. In addition, we found higher genetic relationships between accessions from the same population because the number of grapevine plants found per population is low (5-8 on average) and that the possibility of hybridization and seed dispersion is higher for plants from the same population. At the same time, this result indicates that in the future the sampling method could be one or two accessions per population.

The results of the genetic analysis with the unique genotypes showed that the Castilian region (Burgos) still harbors a number of wild grapevine accessions with low levels of heterozygosity. A similar results has been observed in wild grapevine populations in Morocco (Zinelabidine *et al.*, 2010), Sardinia (Zecca *et al.*, 2010), Portugal (Cunha *et al.*, 2007 ; Lopes *et al.*, 2009), France (Di Vecchi-Staraz *et al.*, 2009) and Italy (Grassi *et al.*, 2003). However, observed heterozygosity was not significantly lower ($P \leq 0.05$) than expected heterozygosity in wild Burgos populations, in parallel

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with the estimation of F values close to zero (Table 2). Since F values are expected to be close to zero under random mating, and that F values close to zero have been observed in Burgos wild populations, a random mating population status could be supposed. This result is different from the analyses done by De Andrés *et al.* (2012), which highlighted a deficiency of heterozygotes (inbreeding) in the Spanish wild population. We can explain our result as a consequence of a random genetic drift of allele frequencies in the Burgos province populations due to their small size or to some sampling bias (sample composed only of male accessions). At the same time, the relative lower genetic diversity in this population may be due to the sample size because a positive correlation of number of alleles with sample size was expected (De Andrés *et al.* 2012). However, the identification of private alleles in Burgos wild individuals is valuable and confirms the importance to establish a conservation program for the Burgos wild populations. Conservation is very urgent because the Cadagua riverbank management in Villasana, the destruction of vegetation following roads and path ditches, as well as the gallery forest management of the rivers and creeks of Mena and Angulo have destroyed a lot of vines during the last decades. In addition, there is also a general systematic coppicing of the surviving plants included in the marginal hedges during the maintenance of the borders of the pasture plots. Fragmentation of wild grapevine habitats has a high influence on effective gene exchanges between populations, leading to the isolation of the populations (Arnold *et al.* 2005 ; Grassi *et al.* 2006). Therefore, according to Fullenwarth (1997), effective practices for conserving the natural biodiversity may be to collect seeds from wild grapevines, to produce plants in nurseries, and to afforest the riverbanks with wild grapevines. It is necessary to remark that the Burgos province spreads out 14.300 square kilometers where a total of about 100 vines were found distributed among several populations, so this grapevine subspecies constitutes a rare plant. In our opinion, there is an urgent need to develop a strategy for its *in situ* and *ex situ* protection. For this purpose, a program of *ex situ* conservation and propagation is in progress thanks to collaboration between the Laboratory of Applied Entomology of the University of Sevilla and the nursery of the Fondo Forestal Ibérico.

The genetic relationship between the wild genotypes from Burgos and the wild genotypes previously described showed high genetic similarity with the Northern Spain populations, supporting the genetic structure between northern and southern wild grapevine populations from Spain (De Andrés *et al.* 2012). The genotype comparison between wild Burgos individuals and grapevine cultivars did not show any correspondence, which excludes gene flow events of seeds or pollen dispersion from domestic grapevines towards the wild conditions. At the same time, wild grapevine samples did not show any genetic relationship with domesticated forms from the same region, as observed in the STRUCTURE analyses. Although a few cases of hybrid from cultivated to wild populations have been reported (Di Vecchi-Staraz *et al.* 2009), our findings are supported by the picture obtained thanks to several scientific reports based on nuclear SSR which demonstrate a clear differentiation between cultivars and wild grapevines (Snoussi *et al.* 2004 ; Grassi *et al.* 2008 ; De Andrés *et al.* 2012). However, we cannot exclude the putative genetic relationship between the wild genotype and other domesticated grapevines that have not been included in the survey.

The analyses of the genetic structure highlight two main genetic groups corresponding to domesticated and wild germplasm. Both groups showed very high average probability of assignment to their own cluster, in agreement with the hypothesis that they are genetically distinct. Actually, the genetic comparison between domesticated and wild forms of grapevine is still limited, and the picture arising today is a clear differentiation of domesticated and wild grapes based on nuclear SSR (Snoussi *et al.* 2004 ; Grassi *et al.* 2008 ; De Andrés *et al.* 2012). In addition to the major partition in wild and domesticated forms, the genetic structure analysis identified five genetic groups : clusters SW1 and SW2, which include all the wild accessions, and three other clusters, SC1, SC2 and SC3, which include the majority of the analyzed cultivars. The existence of two genetic groups within the wild accessions could suggest some level of isolation among those genetic lineages (Fst = 0.10) as it has been suggested by De Andrés *et al.* (2012). The genetic structure of the domesticated grapevine samples is very weak because the *vinifera* cultivars represent a large complex pedigree resulting from a number of spontaneous and inter-generation crosses between cultivars that have been vegetatively propagated for centuries (Myles *et al.* 2011 ; Bacilieri *et al.* 2013 ; Lacombe *et al.* 2013). In fact, the three different genetic clusters detected within the analyzed cultivars showed very low genetic differentiation (Table 3). This low genetic differentiation would result from the high level of hybridization between grapevine cultivars. These results suggested that close genetic relationships within each cluster are consistent with previous historical, viticultural and genetic information. The close relatedness of cultivars from Castilian and French varieties also supports the putative introduction of these cultivars along the pilgrimage route to Santiago de

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Compostela. Putatively, Castilian varieties could have local origins or result from crosses between introduced and local varieties (Santana *et al.* 2010). These results provide a view of how the domesticated grapevine genetic pool is structured in a viticultural region, including germplasm of local and foreign origins and crosses between them.

CONCLUSIONS

The genetic differentiation observed between wild and domesticated forms of grapevine in Spain points out the interest to characterize and conserve the existing western populations as a source of novel alleles for the future understanding and improvement of the genetics of grapevine domesticated forms. At the same time, with the recent loss of suitable habitats resulting from direct and indirect human impact, *V. vinifera* L. subsp. *sylvestris* is now endangered through its distribution range. As a consequence, populations are generally small and dispersed and this means a significant risk of extinctions and potential inbreeding depression of wild grapevine. Finally, given our findings as well as the observed genetic and enological differences between wild and domesticated populations, we recommend that this population be prioritized for *ex situ* and possibly on-farm conservation as well as *in situ* protection.

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