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The Current Status of Wild Grapevine Populations (*Vitis vinifera* ssp *sylvestris*) in the Mediterranean Basin

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1. Introduction

The Eurasian grapevine (*Vitis vinifera* L) is the most widely cultivated and economically important fruit crop in the world (Mattia *et al.* 2008). *Vitis vinifera* L includes the cultivated form *V. vinifera* ssp *vinifera* and the wild form *V. vinifera* ssp *sylvestris*, considered as two subspecies based on morphological differences. However, it can be argued that those differences are the result of the domestication process (This *et al.* 2006). The wild form, considered the putative ancestor of the cultivated form, represents the only endemic taxon of the *Vitaceae* in Europe and the Maghreb (Heywood and Zohary 1991). Grapevine domestication has been linked to the discovery of wine (McGovern 2004). Although wild grapevines were spread over Southern Europe and Western and Central Asia during the Neolithic period, archaeological and historical evidence suggest that primo domestication events would had occurred in the Near-East (McGovern *et al.* 1996). In addition, several studies have shown evidence supporting the existence of secondary domestication events along the Mediterranean basin (Aradhya *et al.* 2003; Grassi *et al.* 2003, Arroyo-García *et al.* 2006; Lopes *et al.* 2009; Andres *et al.*, 2012). Recent genetic analyses using a large SNP platform provided genetic evidence supporting the Eastern origin of most cultivated germplasm as well as the existence of introgression from wild germplasm in Western regions, likely as the consequence of those predicted secondary domestication events (Myles *et al.* 2010). Distinction between wild and cultivated forms of *Vitis vinifera* L is mainly based on morphological traits. The most conspicuous differential trait is plant sex: wild grapevines are dioecious (male and female plants), while cultivated forms are mostly hermaphrodite plants, with self fertile hermaphrodite flowers (This *et al.* 2006).

Wild grapevines can still be found in Eastern and Western Europe (Arnold *et al.* 1998). The South Caucasus (Azerbaijan, Armenia and Georgia), together with eastern Anatolia, has

been considered for a longtime as the birth place for viticulture with the earliest examples of wine-making (This *et al.* 2006, McGovern 2003, Zohary 1995, Olmo 1995, Levadoux 1956, Negru 1938). A 1998 census (Arnold *et al.* 1998) showed that wild grapevine were present in Spain, Italy, Switzerland, Romania, Bulgaria, Hungary, Austria, and in the countries of former Yugoslavia (Figure 1). Apparently, Spain and Italy harbor the highest number of recorded populations and they were proposed to work as shelters for *V. vinifera* during the last glaciation (about 12,000 years ago) as well as putative sources of postglacial colonization and diversification (Levadoux 1956). Wild vines were abundant in their indigenous range in Europe until the middle of the 19th century, when the arrival of North American pests (Phylloxera) and pathogens (downy and powdery mildews) and the destruction of their habitats drove European wild vines close to extinction (IUCN 1997). The solution to generate resistance to Phylloxera was the use of American species and hybrids as rootstocks and many varieties of rootstocks were developed by breeders (Arraigo and Arnold, 2007).

Currently, vines found in natural habitats are considered to be a mixture of wild forms, naturalized cultivated forms and rootstocks escaped from vineyards as well as hybrids derived from spontaneous hybridizations among those species and forms (Laguna 2003, Lacombe *et al.* 2003, This *et al.* 2006). Recently, Arrigo and Arnold (2007) compared ecological features and genetic diversity among populations of naturalized rootstocks and native wild grapevines and did not detect the existence of genetic flux between them. The genetic analysis of wild grapevine populations from France and Spain (Di Vecchi *et al.* 2009; Andres *et al.* 2012) detected the existence of gene flow between cultivated and wild grapevine, estimating up to 3% of pollen migration between the cultivated fields and closely located wild grape. These pollen fluxes may have a significant effect on the evolution of those populations. Currently, wild grapevine is endangered throughout all its distribution range, (Di Vecchi *et al.* 2009) and conservation efforts are required to maintain the genetic integrity and survival of the remnant populations. Within this context, information on the amount and distribution of wild grapevine genetic diversity is crucial for the development of conservation strategies.



Figure 1. Localization of wild grapevine population in the Mediterranean basin. (Heywood and Zohary, 1991).

The principal key ideas of this chapter is a better understanding of the exact status of the remaining wild grape populations and their relationships with existing varieties using the molecular markers and genetic analysis approaches that it has been published about some wild grapevine populations around the Mediterranean basin.

2. Chlorotype variation and distribution in *V. vinifera* ssp. *sylvestris* around de Mediterranean basin

The chlorotype variation is based on specific features of the chloroplast genome as well as its conserved gene order and coding sequences in different species and its general lack of heteroplasmy and recombination. Furthermore, chloroplasts are uniparentally transmitted in most species (usually maternal in angiosperms and paternal in gymnosperms). The low mutations rates observed in the chloroplast genome represent a drawback to their wide application in the study of population history and dynamics within a given species. However, this problem has been overcome by the identification of variable intergenic regions and introns flanked by conserved sequences in many species as well as by the identification of chloroplast microsatellites which consist of mononucleotide repeats. Chloroplast microsatellites have been found in all plant species analyzed and they frequently are highly polymorphic (Provan *et al.* 2001). One problem associated with chloroplast microsatellites is their high homoplasmy due to the recurrent generation of alleles of the same length that creates alleles which being identical by state are not identical by descent. High levels of homoplasmy can confound estimates of population differentiation and the recurrent generation of alleles could mimic gene flow (Goldstein and Pollock 1997). The risk is however reduced in intra-specific analysis (Arnold *et al.* 2002).

As in other angiosperms, grapevine chloroplasts are maternally inherited (Arroyo-García *et al.* 2002) and therefore transmitted through seeds and cuttings. The chloroplast genome of grape is 160,928 bp in length and its gene content and gene order are identical to many other unarranged angiosperm chloroplast genomes (Jansen *et al.* 2006). Genetic diversity at the grape chloroplast has so far only been analyzed at the level of chloroplast microsatellite loci. Polymorphisms were searched by Arroyo-García *et al.* (2006) with 54 chloroplast microsatellite markers corresponding to 34 different loci in sample sets of four *Vitis* species (*Vitis berlandieri* Planchon, *V. riparia* Mich., *V. rupestris* Scheele and *V. vinifera* L.), using primer pairs developed for tobacco (Bryan *et al.* 1999; Weising and Gardner 1999; Chung and Staub, 2003) and *Arabidopsis* (Provan 2000). Nine loci were initially found polymorphic due to differences in the number of mononucleotide repeats in poly T/A stretches (Arroyo-García *et al.* 2006), which after comparison with the complete chloroplast genome sequence (Jansen *et al.* 2006) corresponded to five different loci: cpSSR3 (equivalent to NTCP-8), cpSSR5 (equivalent to NTCP-12 and ccSSR5), cpSSR10 (equivalent to ccSSR14), ccSSR9 and ccSSR23. These loci were genotyped in a sample of more than 1,200 genotypes of *V. vinifera* which uncovered the presence of two to three alleles per polymorphic locus and a total of eight chlorotypes. Among them, only four (A, B, C and D) had global frequencies greater than 5%. Chlorotype diversity is moderate in grapevine with diversity values (H) reaching 0.44 in the

most diverse populations or cultivars groups that contrast with average H values of 0.55 reported in *Arabidopsis* (Picó *et al.* 2008) or H values higher than 0.95 observed in *Pinus sylvestris* (Provan *et al.*, 1998).

Very small and isolated populations of *V. vinifera* ssp. *sylvestris* can still be found in European temperate regions along deep river banks. Among them, Arroyo-Garcia *et al.* (2006) have performed an exhaustive screening of Iberian and Anatolian populations in the two ends of the Mediterranean basin and have included additional populations representative of other regions; they considered that all the natural populations were grouped in eight population groups following a geographic criterion. No clear-cut geographic structure was found among the seven *sylvestris* population groups considered. However, the most frequent chlorotypes displayed a different geographic distribution. As seen in Fig. 2, chlorotype A is very prevalent in West European *sylvestris* populations (IBP, CEU), but was not found in the Near East (NEA, MEA). In contrast, chlorotypes C, D and G are frequent in Near Eastern populations (NEA, MEA), but were not found farther west (e.g. IBP and CEU).

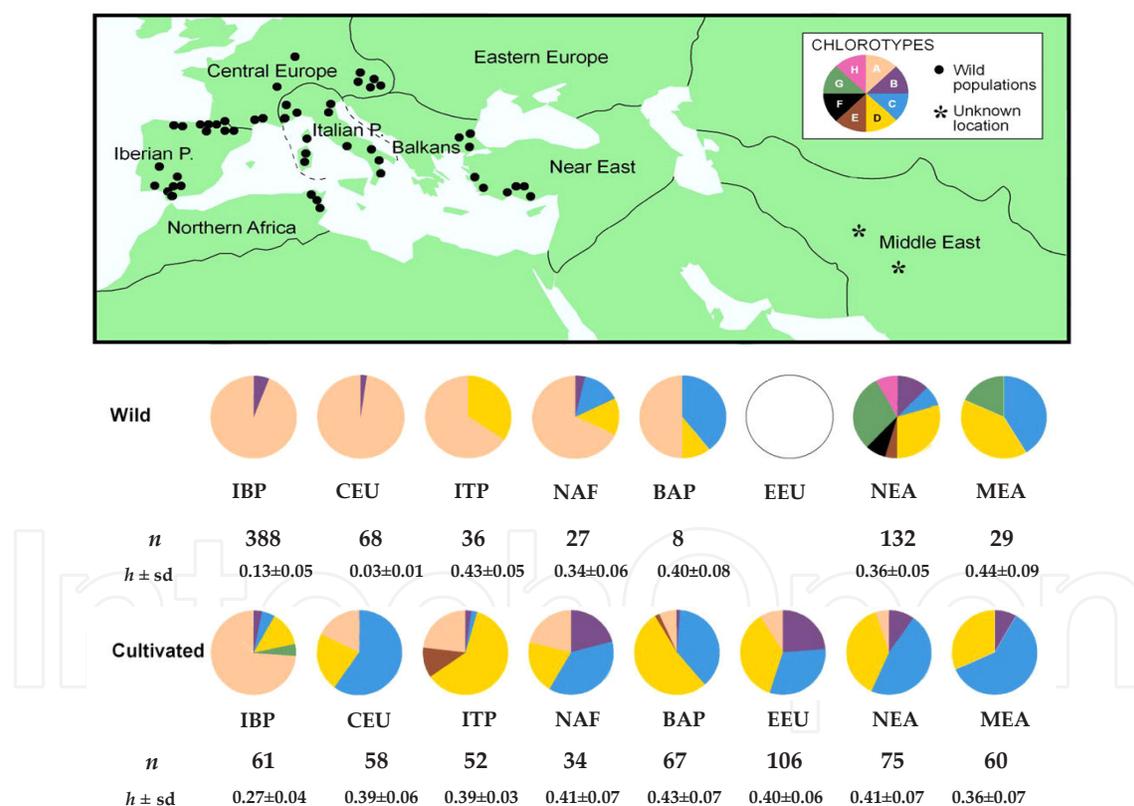


Figure 2. Chlorotype distribution in *sylvestris* and *sativa* population groups. Geographic areas considered are separated by lines when needed. Black periods do not mark specific *sylvestris* populations but river valleys where wild genotypes were collected at several locations. Asterisks indicate that specific locations of collection in the area are unknown. *Sativa* and *sylvestris* genotypes are grouped in eight population groups. From west to east: Iberian Peninsula (IBP), Central Europe (CEU), Northern Africa (NAF), Italian Peninsula (ITP), Balkan Peninsula (BAP), Eastern Europe (EEU), Near East (NEA) and Middle East (MEA). The figure also shows the values of unbiased chlorotype diversity and the number of genotypes considered within each population group. Chlorotype colour codes are as in Figure.

3. Multiple origins for cultivated grapevine

The chlorotype distributions observed among *sylvestris* populations allow for testing the two basic hypotheses on the origin of cultivated grapevine, proposed above, since they lead to different predictions regarding the amount and distribution of chloroplast genetic variation (Arroyo-García *et al.* 2006). The restricted origin hypothesis predicts that the chlorotype diversity of cultivated Eurasian grape should be limited to a few founder chlorotypes. In contrast, a multiple-origin hypothesis would predict greater diversity in cultivated grapevine groups than in *sylvestris* population groups. As shown in Fig. 2, unbiased chlorotype diversity is very similar in all the cultivated groups (from 0.36 to 0.43 with the exception of a lower value for IBP) and in most cases cultivated diversity values are higher than diversity values observed in *sylvestris* population groups. These results are also consistent with the existence of higher genetic differentiation (GST) among population groups of *sylvestris* (0.353 ± 0.10) than *sativa* (0.169 ± 0.07) grapevines. Interestingly, the geographic distribution observed for some chlorotypes in *sylvestris* groups can still be observed in cultivated groups (Fig. 2). In this way, cultivars with chlorotype A are highly abundant in Western Europe while they were not observed in Near and Middle East samples. Similarly, chlorotypes C and D, which are very common among NEA and MEA cultivars, are less frequent among IBP cultivars. To test further the origin hypotheses, they analyzed the genetic relationships among *sylvestris* and *sativa* population groups, since single- or multiple-origin hypotheses would predict different patterns of genetic relationships. All analyses grouped the cultivated population groups in two major clusters (Fig. 3). One cluster with high bootstrap values related the IBP cultivated group with the western, IBP, CEU, and Northern Africa, NAF *sylvestris*, population groups. The second main cluster showed that all the other cultivated groups considered are highly related to eastern *sylvestris* groups NEA and MEA. BAP and ITP *sylvestris* population groups appeared more related to the NEA/MEA cluster than to the western *sylvestris* cluster. These inferences were independent of the genetic model assumed, as the same partitioning was supported by all analyzed models. The statistical analysis was also robust for different clustering methods, including agglomerative and K-means, the latter indicating two as the optimum number of clusters. In summary, these results support the existence of a relevant genetic contribution of eastern and western *sylvestris* population groups to the genetic make-up of current grapevine cultivars and could suggest the existence of at least two origins of *sativa* cultivars: (i) an eastern origin related to NEA and MEA *sylvestris* population groups and characterized by chlorotypes C and D, and (ii) a western origin related to IBP, CEU and NAF *sylvestris* population groups and characterized by chlorotype A. Whether this second origin represents independent domestication events or developed as a consequence of the east to west transmission of the 'wine culture' will require further archaeological research. One palaeobotanical study (Hopf 1991) of grape pollen and seeds suggests that the Eurasian grapevine was exploited by Neolithic populations of the Iberian Peninsula before contact with Eastern cultures took place. This implies that grapevine could have been independently domesticated in Eastern and Western Europe. The putative existence of western and eastern domestication events is consistent with the morphotype classification of cultivated grapes proposed by Negrul (1938), who distinguish-

ed an *occidentalis* group, characterized by the small berry grapes of Western Europe, an *orientalis* group comprised of the large berry cultivars of Central Asia, and a *pontica* group including the intermediate types from the Black Sea basin and Eastern Europe. The results show by Arroyo-García *et al.* (2006) do not exclude the existence of additional genetic contributions of local *sylvestris* wild germplasm or even domestication events in other regions of the species distribution. However, sample size and the limited chloroplast genetic variation found in the Eurasian grape do not provide enough resolution to detect them. In fact, putative genetic relationships between cultivated varieties and local *sylvestris* populations have been proposed in other regions (Grassi *et al.* 2003; Di Vecchi *et al.*, 2009).

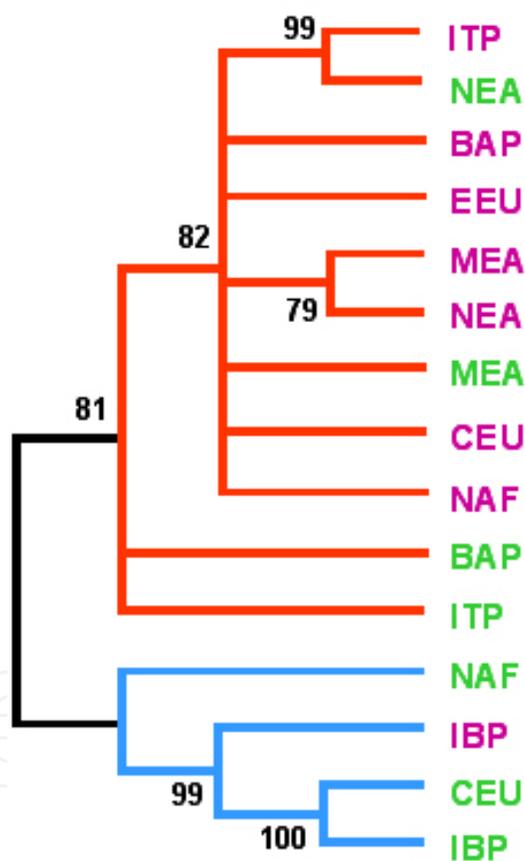


Figure 3. Genetic relationships among *sylvestris* and *sativa* grapevine population groups. The tree was constructed using the neighbor joining method on the Dmyu distance matrix calculated for all pairwise combinations of population groups. Bootstrap support values exceeding 70 are indicated. Branches with low bootstrap support were collapsed. Major clusters are depicted with red and blue colours. *Sylvestris* population groups are depicted in green and *sativa* population groups in magenta. Population codes are as Fig 2.

4. Nuclear diversity in cultivated and wild grapevine

The characterization of the genetic diversity and its distribution throughout the species range is important for our understanding about the adaptation and survival of wild species to ensure that genetic resources are available for use in research and breeding programs (This *et al.*, 2006). Microsatellite markers, being abundant, multi-allelic and polymorphic, provide a means of detecting genetic polymorphism. Due to their co-dominant structure this marker system enables studies on population genetic analysis, assessment of genetic structures and differentiation in germplasm collections and natural populations. The cultivated grapevine (*Vitis vinifera* L.) is very diverse, with 6,000–10,000 cultivars believed to exist in the world (Galet 2000), and many grape collections (http://www.vitaceae.org/index.php/Grape_Germplasm_Resources). This large diversity is mostly due to the long history of grapevine cultivation (McGovern 2003), and vegetative propagation, which has enabled the conservation of cultivars over centuries. There is also a large diversity of complex *Vitis* hybrids and rootstocks (Galet 2000).

Cipriani *et al.*, (2010) have analyzed a collection of 1005 grapevine accessions; they were genotyped at 34 microsatellite loci with the aim of analyzing genetic diversity and exploring parentages. This study constitutes the largest analysis of genetic diversity in cultivated grape and confirms previous analyses suggesting that grape is a very diverse species (Martinez *et al.* 2006; Ibanez *et al.* 2009). The genetic diversity on average is quite high for *V. vinifera* ssp *vinifera* (0.769) and even higher for rootstocks and hybrids. It is as diverse as poplar (Smulders *et al.* 2008), rose (Esselink *et al.* 2003), wild populations of rice (Gao *et al.* 2006), and much more diverse than tomato (Ranc *et al.* 2008). High genetic distance is a good indication that grape has been widely exchanged and crossed in order to increase its diversity level (This *et al.* 2006). The analysis of kinship uncovered 74 complete pedigrees, with both parents identified. Many of these parentages were not previously known and are of considerable historical interest. Grouping the accessions by profile resulted in a weak correlation with their geographical origin and current area of cultivation, revealing a large admixture of local varieties with those most widely cultivated, as a result of ancient commerce and population flow.

Several studies have described successfully used microsatellite markers to genotype *V. vinifera* ssp. *sylvestris* and *V. vinifera* ssp. *vinifera* (e.g., Aradhya *et al.* 2003; Dangl *et al.* 2001; Imazio *et al.* 2003; Lacombe *et al.* 2003; Regner *et al.* 2000; Lopes *et al.*, 2009; ; Laucou *et al.*, 2011; Andrés *et al.*, 2012). However, *V. vinifera* ssp *sylvestris* was found to be less diverse than Hybrids or Rootstocks, in accordance with previous observations (De Andres *et al.* 2007). In general, *V. vinifera* ssp *sylvestris* is less diverse than the domesticated forms, which could be due to the scarcity of the endangered wild form, small natural populations and the small number of samples available in the collections. The distribution of the wild grapevine has dramatically been reduced over the last 150 years, with the spread of pathogens from North America (phylloxera, oidium, mildew). Most of them died, except in floodplain forests as the root–host homoptera phylloxera was sensitive to flooding (Ocete *et al.*, 2004). Massive death also occurred in vineyards. In France, most vineyards were destroyed and replanted

afterwards using American rootstock. Phylloxera did not disappear and continued to infect populations of wild grapevines surviving in the floodplain forests in zones where the water table sank. Intensive river management, starting in the middle of the 19th century, enhanced this process. Two other human impacts also contributed to the destruction of populations of wild *Vitis*. Shortly after river management, most of the floodplain forests were fragmented and replaced by arable crops or meadows. In remnant forests, the intensification of forest management led to the removal of the vines, considered detrimental to tree growth. Fragmentation of wild grapevine habitats had an enormous impact on gene exchanges between populations, leading to a bottleneck, especially in gyno-dioicous plants. This also reduced the adaptability of the plant to habitat changes.

The total genetic diversity values found in wild grape individuals from Anatolia region are higher than of wild type accessions from other regions such as those described for the Mediterranean basin (Andrés *et al.*, 2012; Di Vecchi *et al.*, 2009; Lopes *et al.*, 2009; Zinelabidine *et al.* 2010). In general, these values are similar for outcrossing vegetative propagated perennial species (Bejaj *et al.* 2007). The observed heterozygosity (H_o) is not significantly different ($P \leq 0.01$) than expected heterozygosity (H_e) in the wild group, indicating a random mating population. However, reduction in observed heterozygosity has been observed in wild grapevine populations analyzed in Spain, Portugal, France or Italy (Andrés *et al.* 2012; Lopes *et al.* 2009; Di Vecchi *et al.* 2006; Grassi *et al.* 2003), most likely due to the reduction of these populations by human action. The comparison of the genetic diversity values with the autochthonous grape cultivars from Anatolia region indicated that diversity is greater in the wild grapes than in the cultivated ones. Similar results have been found in other studies (Lopes *et al.*, 2009; Riani *et al.*, 2010). The wild grapevine population from the both ends of the Mediterranean basin showed a higher genetic variability in Anatolian wild grape populations than in Spanish populations (Ergul *et al.*, 2011). This result is in agreement with the comparison of the number of alleles at the 15 shared SSR loci between Spanish and Anatolian populations. Of 229 total alleles detected at these loci, 189 were observed only in Spanish while 237 were observed only in Turkish populations. The number of unique alleles in Anatolian populations was also much higher than in Spanish populations. This result was expected as Anatolian populations are located at the primary center of diversity and thus are more diverse than in the peripheral populations. At the same time, the Iberian wild grape populations are small, showed lower genetic diversity values and suffered from inbreeding depression (Andrés *et al.*, 2012).

In conclusion, the present study suggests that there is no immediate reason for concern about any demographic bottlenecks facing the wild grape populations of the Anatolian region, and the presence of high number of rare alleles in populations investigated here is clear evidence for this finding. At the same time, the wild population from the western and central Europe pointed out that they are suffering inbreeding depression due to the low level of genetic diversity. For the future, in situ conservation of wild grapevine populations around the Mediterranean basin should be advanced by a dynamic approach to keep the level and composition of genetic diversity as high as possible for safeguarding these precious genetic resources for crop improvement.

5. Genetic relationship: Cultivated versus wild compartment of grape

The picture arising today is of a low but clear genetic differentiation of cultivars and wild grape based either on chloroplast markers (Arroyo-Garcia *et al.* 2006; Grassi *et al.* 2006), nuclear microsatellites (Snoussi *et al.* 2004; Grassi *et al.* 2003; Lopes *et al.*, 2009; Ergul *et al.*, 2011; Andres *et al.*, 2012) or both (Grassi *et al.* 2003; Sefc *et al.* 2003). The wild individuals also cluster according to their populations (Grassi *et al.* 2008). The positive Fis values observed in the wild grapevine accessions suggest a high level of genetic relationship among the individuals of the same wild populations. In fact, the detection of potential parent-progeny relationships within wild populations supports that possibility (Andres *et al.*, 2012). At the same time, the detection of gene flow between both compartments (Di Vecchi *et al.* 2009; Andres *et al.*, 2012) could have in the future strong consequences. Therefore, the histories of both compartments are also different and as a consequence linkage disequilibrium is more important in cultivated grape (Barnaud *et al.* 2006) than in wild individuals (Barnaud *et al.* 2010).

Until now a systematic genetic and morphological characterization of the individual accessions had been done with some wild grapevine population in order to confirm whether they could correspond to bona fide ssp. *sylvestris* individuals, naturalized grapevine cultivars, rootstocks, or spontaneous hybrids derived from wild and cultivated forms as previously described (Di Vecchi *et al.* 2009; Zecca *et al.*, 2011; Andres *et al.*, 2012). The results of the genotypic and phenotypic analyses of wild grapevine accessions from Spain allowed classifying approximately 19% of the samples as naturalized cultivated forms (Andres *et al.*, 2012). These samples could have “escaped” from old abandoned vineyards. As expected for an outcrossing dioecious subspecies they have observed the existence of spontaneous hybrids (4% of the collected samples) between wild and cultivated forms (Andres *et al.*, 2012). The existence of cross hybridization between wild and cultivated forms has been shown to be a widespread phenomenon in many species (Arnold 1998; Papa and Gepts, 2003; Di Vecchi *et al.* 2009). The detection of spontaneous hybrids in grapevine wild populations is in agreement with the previous detection of pollen flow between vineyards and wild plants reported by Di Vecchi *et al.* (2009). This level of gene flow between wild and cultivated forms taking place during many generations might have consequences, as introgression, pollution of the gene pool and genetic loss, on the evolution of these small wild populations (Grassi *et al.* 2006). In addition, these results showed no evidence of hybridization between rootstocks and wild individuals (Andres *et al.* 2012). This could be due to the existence of genetic barriers between both taxa such as the phenological mismatches suggested by Arrigo and Arnold (2007).

Different studies suggest genetic exchange between cultivated and wild grapevines (Cunha *et al.*, 2009; Di Vecchi *et al.*, 2009; Grassi *et al.*, 2003). The genetic relationship between cultivated varieties and wild grapevine populations from Spain suggests a genetic contribution of Southern wild populations in the autochthonous grapevine cultivars varieties (Andres *et al.*, 2012). Therefore, it seems that in opposition to the established dominant theory on the origin of the domestication of grapevine, many of the varieties of the Iberian Peninsula and from other European countries could have local origins.

The genetic analysis of wild grapevine from Spain and cultivars from European countries showed the partition in wild and cultivated forms from that region. The STRUCTURE analysis identifies two genetic groups (clusters C1 and C2) which included all the wild accessions from Spain and correspond to Northern and Southern populations and two other (C3 and C4) including the majority of the analyzed cultivars (Figure 4). The existence of two genetic groups within the wild accessions suggests some level of isolation among those genetic lineages. One possible scenario to generate such structure is that it derives from the isolation created by the last Pleistocene glaciations. As reviewed by Gomez and Lunt (2006), the fragmented nature of the Iberian Peninsula habitat favored the occurrence of multiple glacial refuges isolated from each other. Phylogeographic studies of different European species such as olive trees have shown the existence of strong genetic differentiation within the Iberian Peninsula (Belaj *et al.* 2007). Alternatively, these two genetic groups could represent different colonization events of the Iberian Peninsula by the species *Vitis vinifera* L. what could have taken place following Northern (the Pyrenees) and Southern pathways (Gibraltar). The common chlorotype A identified both in Western Europe and Northern Africa (Arroyo-Garcia *et al.* 2006) seems to suggest a single common origin for all the ancestral populations favoring the first hypothesis. Alternatively, we cannot discard that part of the moderate genetic differentiation observed between the two genetic groups could result from their different history of relationship with the cultivated forms. In fact, we have found a high number of wild genotypes from Southern group showing high ancestry values of clusters C3 and C4 that mainly group cultivated forms of grapevine. In the same direction, we found higher genetic differentiation ($F_{st} = 0.13$) between cluster 1 (Northern group) and the analyzed cultivars than between cluster 2 (Southern group; $F_{st} = 0.07$) and the analyzed cultivars. On the other hand, genetic differentiation between clusters 1 and 2 would be reduced by the existence of gene flow between both genetic groups, what seems to be suggested by the presence of some genotypes showing high ancestry values from both genetic clusters.

Two different genetic clusters could also be detected within the analyzed cultivars although showing very low genetic differentiation ($F_{st} = 0.0048$). This low genetic differentiation would result from the high level of gene flow between grapevine cultivars. Myles *et al.*, (2011) have proposed that the genetic structure of the *vinifera* cultivars represents a large complex pedigree resulting from a number of spontaneous and inter-generation crosses between cultivars that have been vegetatively propagated for centuries. Still within this complex pedigree structure, it could be possible to distinguish different groups of more strongly related cultivars that would vary depending on the set of cultivars analyzed. In this case, an analysis of cluster 3 and 4 identified mainly Iberian cultivars as having higher ancestry in genetic cluster 3 and central European cultivars and Northern Iberian cultivars as having higher ancestry in genetic cluster 4.

Interestingly, the analyses of the ancestry values showed by analyzed cultivars identify some of them with a high ancestry value of cluster 1 and cluster 2. These grapevine cultivars correspond to the Spanish cultivars; Allarén, Benedicto, Listan Negro, Malvasia de Lanzarote and Malvasia Blanca and the European cultivars Cabernet Franc, Petit Verdot, Pinot Meunier and Sangiovese. These cultivars have been described as more closely related to

wild accessions (This *et al.*, 2006) or are considered autochthonous cultivars. Therefore, these results support the existence of introgression from Western wild forms of *Vitis vinifera* in the pedigree of some of the current Western European cultivars. Finally, the genetic differentiation observed between wild and cultivated forms of grapevine in the Iberian Peninsula point out the interest to characterize and conserved that the existent Western populations as a source of novel alleles for the future understanding and improvement of the genetics of grapevine cultivated forms.

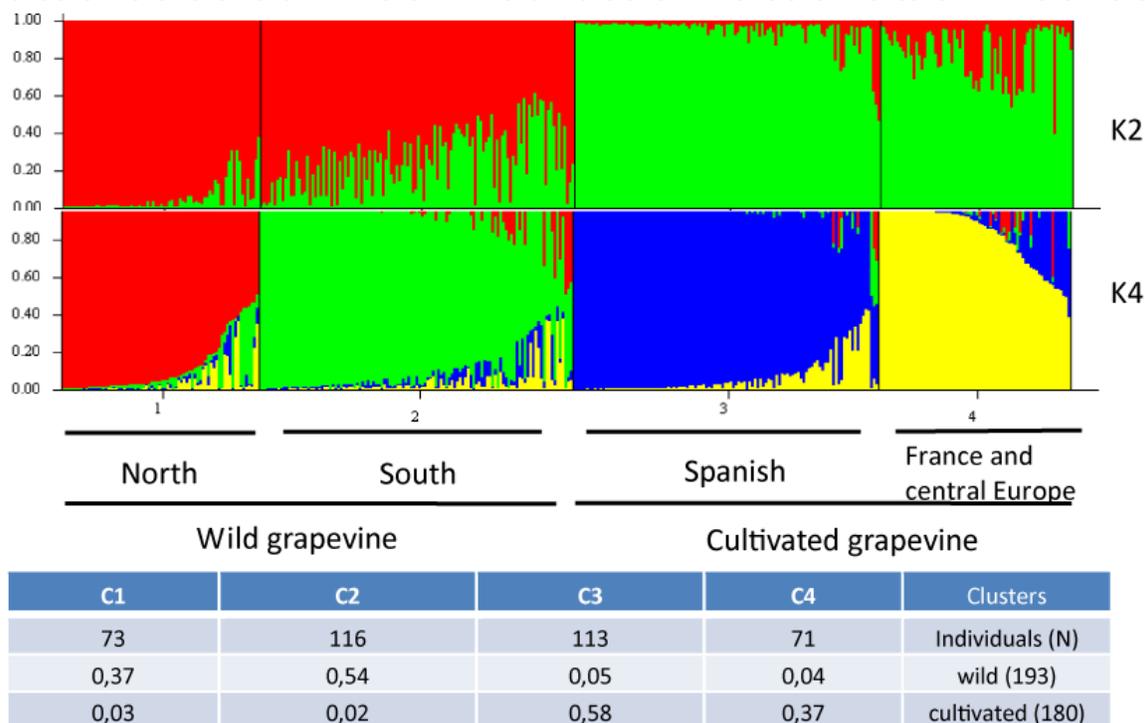


Figure 4. Graphical representation of ancestry membership coefficients of all individuals analyzed (Cultivated and wild grapevine from Spain). Each individual is shown as a vertical line divided into segments representing the estimated membership proportions in the two and four ancestral genetic clusters inferred with STRUCTURE. Individuals within each cluster are arranged according to estimated cluster membership proportions. (Bottom) Number of individuals and the mean membership fractions in the four genetic clusters.

In conclusion, molecular marker analysis have shown clear divergence between wild and cultivated grapes and low level of introgression (Grassi *et al.* 2003, Ergul *et al.* 2011, Andres *et al.* 2012), but they are still connected through gene-flow (Regner *et al.* 2000, Lopes *et al.* 2009). Some studies (Grassi *et al.* 2003, Arroyo-García *et al.* 2006, Lopes *et al.* 2009; Andres *et al.*, 2012) have reported the possibility of multiple domestication events in different geographic locations in the origin of cultivated grape. The several geographic sources of wild and cultivated grapes, supports at least two separate domestication events that gave raise to cultivated grape; one derived from the wild grape from Transcaucasia, and another from the wild grape of southern European and North African origin. Probably, with wider representation of wild grape, one may be able to demonstrate the multiple domestication events supporting diffused center of domestication of cultivated grape.

6. Wild grapes as phylogenetic resource

Genetic erosion was perceived as global scale problem in the middle of the twentieth century. It was found out that the introduction of new grapevine cultivars had rapidly displaced the varieties traditionally cultivated resulting in great uniformity of cultivated crops. Therefore, the genetic diversity of those species became alarmingly scarce. This situation led to the implementation of measures for the conservation of plant genetic resources. In the vine, as in other crops, genetic erosion or loss of variability is occurring. That is, it is reducing dangerously agrobiodiversity, the genetic base on which natural selection acts, increasing dramatically the vulnerability of different cultivars to new environmental changes or the appearance of new pests and diseases (Ocete *et al.*, 2007). It should be noted that the wild forms contain diversity for ongoing feedback to relatives (This *et al.*, 2006). These plant genetic resources are generally not a material that is exploitable in a direct way, but it can be used in plant breeding, because wild populations still conserves an overall important genetic diversity (Grassi *et al.*, 2003). This rich genetic pool can be used to avoid the loss of biodiversity affecting the current viticulture. Indeed, the number of allowed cultivars has been reduced to the detriment of several traditional minority varieties. Some international cultivars, like Cabernet Sauvignon, Merlot, Shyrah, Chardonnay, Sauvignon Blanc and so on are being planted in vineyards of all over the world. At the same time, only few numbers of clones from each cultivar are available (Ocete *et al.*, 2004). These facts contribute to a great extent to speed up the problem of genetic erosion in modern viticulture and mainly lead to increase a risk of rapid propagation of new devastating pests and diseases. Some interesting characteristics of wild plants can be transferred throughout the breeding to cultivars suitable of wine making, table grapes and also rootstocks.

Genetic resources in *V. vinifera* are likely limited to only several thousand genotypes in germplasm stock centers or in endangered wild populations. Inter-fertility between species of the genus *Vitis* opens the genetic variation available for breeding across the whole genus. Considering the relevance of genetic resources for the future of the crop and their current scarcity, major efforts should be dedicated to the collection and characterization of the existing resources in the species and the genus. Genomic tools and information can help to rapidly generate genotypic information; however, collection of phenotypic data requires more careful characterization at morphological, biochemical, physiological or pathological and environmental response levels. Open databases with these phenotypic and genotypic data are required as well as more efficient ways to store and exchange biological materials representing all the available genetic diversity.

Together with the genetic variation characterized in the population screened in European countries could be interesting to generate a collection of genotypes that can still represent part of the existent natural genetic variation of the species. This collection could be phenotype in different environments and these genetic tools could be the basis for further studies to establish the relationship between phenotypic variation and nucleotide diversity in grapevine. Understanding grapevine natural genetic variation will help the improvement and breeding of grapevine cultivars.

7. Phenotypic characterization of wild grapevine populations

The analysis of large sets of genetic resources at the morphological level has not been intensive. One of the reasons might be the complexity of the methods available so far or the fact that phenotyping grape is expensive, time consuming and requires a lot of space. Most of the work in the past years has been devoted to the development of methods for many traits from composition of berries to disease resistance and abiotic stresses tolerance but development of rapid methods and non-destructives ones should still be a priority in order to speed up the analysis of genetic resources.

7.1. Enological characterization of wild grapevine populations from Spain

The anthocyanin composition of female grape accessions, mostly Spanish, preserved at El Encin Germoplasm Bank (Madrid, Spain) was analysed during several years. After the extraction from grape skins, total anthocyanins were determined by spectrophotometry, and the anthocyanin fingerprint of grapes by HPLC, considering the relative amount of 15 anthocyanins (Revilla *et al.*, 2010). Some typical chromatograms are shown in Figure 5.

The anthocyanin fingerprint of grapes revealed the presence of three types of accessions (Revilla *et al.*, 2010; Revilla *et al.*, 2011). In the first group (23 accessions), grapes did not contain acylated anthocyanins (Revilla *et al.*, 2012). This character is unusual in cultivated grapevines, occurring primarily in Pinot noir and its mutants (Wenzel *et al.*, 1987, Mattivi *et al.*, 2007) and in some grey and rosé cultivars that may be mutants of red grapes (e.g., Pinot gris) or white grapes (e.g., Muscat Rouge de Madere). To our knowledge, this type of anthocyanin fingerprint has not been described in grape cultivars usually considered of Spanish origin (García-Beneytez *et al.*, 2002, Pomar *et al.*, 2005, Gómez-Alonso *et al.*, 2007). In the second group (17 accessions), grapes contained acylated anthocyanins and a high proportion of cyanidin-derived monoglucosides. This character is rare in cultivated grapevines, although it has been reported and was observed in 12 cultivars among the 64 studied (Mattivi *et al.*, 2007). Most were grey or rosé cultivars, or even mutants of white cultivars (e.g., Gewürztraminer). To our knowledge, this anthocyanin fingerprint is rare in grape cultivars usually considered of Spanish origin, with Brancellao as the most remarkable exception (Pomar *et al.*, 2005). In the third group (86 accessions), grapes contained acylated anthocyanins and a large proportion of delphinidin-derived monoglucosides, as do most grapevine cultivars (Wenzel *et al.*, 1987, García-Beneytez *et al.*, 2002, Pomar *et al.*, 2005, Mattivi *et al.*, 2007). In most of these accessions (53), *p*-coumarylated derivatives were more abundant than acetylated derivatives. This character is quite common in red cultivars usually considered as Spanish (e.g., Garnacha and Tempranillo), as described previously (García-Beneytez *et al.*, 2002). On the other hand, acetylated anthocyanins were more abundant than *p*-coumarylated derivatives in 33 accessions. This character is well documented in several French cultivars (e.g., Cabernet Sauvignon and Merlot), but is rare in Spanish cultivars. Among the Spanish cultivars commonly grown, only Mencía presents this type of fingerprint (García-Beneytez *et al.* 2002, Pomar *et al.* 2005).

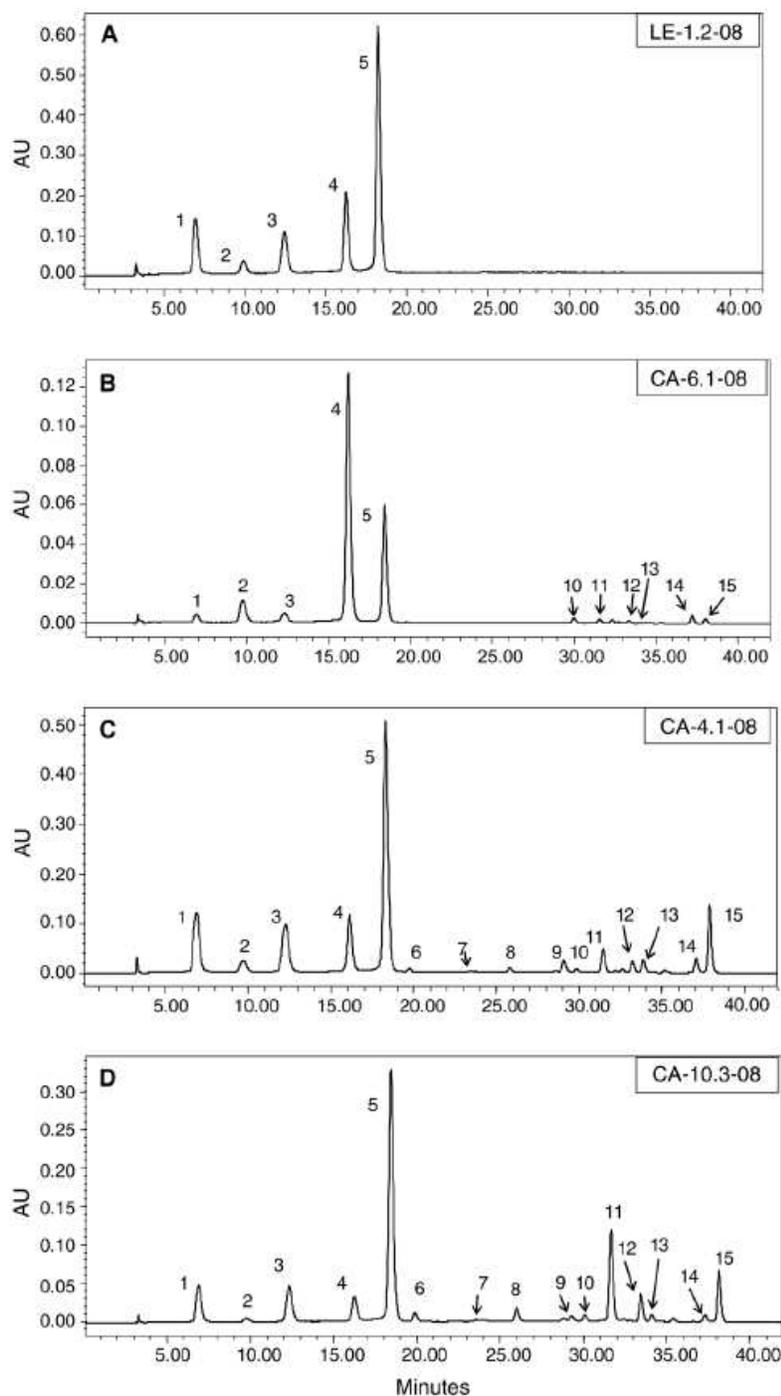


Figure 1 Chromatograms at 520 nm for grape skins extracts of four different wild grapevine accessions: LE-1.2.08, CA-6.1.08, CA-4.1.08, and CA-10.3.08. Peak 1, DpGI; 2, CyGI; 3, PtGI; 4, PnGI; 5, MvGI; 6, DpGIAc; 7, CyGIAc; 8, PtGIAc; 9, DpGICm; 10, PnGIAc; 11, MvGIAc; 12, MvGICf; 13, PtGICm; 14, PnGICm; 15, MvGICm.

Figure 5. Chromatograms at 520 nm for grape skins extracts of four different wild grapevine accessions: LE-1.2.08, CA-6.1.08, CA-4.1.08, and CA-10.3.08. Peak 1, DpGI; 2, CyGI; 3, PtGI; 4, PnGI; 5, MvGI; 6, DpGIAc; 7, CyGIAc; 8, PtGIAc; 9, DpGICm; 10, PnGIAc; 11, MvGIAc; 12, MvGICf; 13, PtGICm; 14, PnGICm; 15, MvGICm.

Nevertheless, the intensity of acylation is quite variable in this group of accessions, and in about 30% of them the proportion of acylated derivatives is <15%, revealing that the expression of genes involved in the acylation of anthocyanins is quite variable among the accessions.

Results obtained by two-factor ANOVA (accession and year) of the 15 variables used to describe the anthocyanin fingerprint of grapes, using a group of 21 accessions sampled during three consecutive years, suggest that variations in the anthocyanin profile among wild grape accessions were more important than differences among years for a given accession (Revilla *et al.*, 2010). Weather conditions affect to some extent the relative proportion of primitive anthocyanins (DpGl and CyGl) and of some acylated derivatives. Similar results were obtained previously in studies with cultivated varieties (Ryan and Revilla 2003, Revilla *et al.*, 2009). Variance component analysis confirmed that the factor *accession* contributed to variance more than the factor *year*, except for MvGlCf. Moreover, the factor *year* is relevant for primitive anthocyanins (DpGl and CyGl) and MvGl. The influence of year may be related to data on Tempranillo wines, which show different amounts of DpGl when grapes were grown in different environments, but collected at similar stages of ripening and made into wine with the same technology (Revilla *et al.*, 2005).

In conclusion, the maintenance of genetic variability and the phenotypic characterization within wild grape populations has become a priority primarily due to the concurrent risks of increased human impact on flood-plain areas and the spread of new pests. Fragmentation of species habitat will reduce both the number and size of the population, and decrease the genetic variation within populations. So the existence of different genetic pools within this population is remarkable and the conservation of this germplasm becoming more interesting. This population, as the rest situated in Spain, has not a specific preservation statute. It is necessary to take into account that Spain is the country with the largest area of vineyards all over the world, and it is affected by a heavy process of genetic erosion (Ocete *et al.* 2007). In consequence, there is an urgent need to bring this material that could be propagated to nurseries for use in the restoration of riparian forests and undertake breeding programs of cultivars and rootstocks. Particularly, the low incidence of pests and diseases is remarkable, the high acidity of the wines and their high intensity of color total, interesting characteristics can be transferred by crossing with cultivars from Mediterranean areas. On the other hand, the immersion tolerance, absence of rot root and symptoms caused by nematodes could be interesting for obtaining new rootstocks, hybridizing with traditional rootstocks, especially when many vineyards have fertirrigation or are planted on clayey soils under a rainy climate, as it was indicated by Ocete *et al.* (2010). These phenotypic data will be used to incorporate the wild populations found to the European *Vitis* Data Base, according to the postulates of the COST Action FA-1003 of Viticulture (EU).

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