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Multiple origins of cultivated grapevine (*Vitis vinifera* L. ssp. *sativa*) based on chloroplast DNA polymorphisms

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Abstract

The domestication of the Eurasian grape (*Vitis vinifera* ssp. *sativa*) from its wild ancestor (*Vitis vinifera* ssp. *sylvestris*) has long been claimed to have occurred in Transcaucasia where its greatest genetic diversity is found and where very early archaeological evidence, including grape pips and artefacts of a 'wine culture', have been excavated. Whether from Transcaucasia or the nearby Taurus or Zagros Mountains, it is hypothesized that this wine culture spread southwards and eventually westwards around the Mediterranean basin, together with the transplantation of cultivated grape cuttings. However, the existence of morphological differentiation between cultivars from eastern and western ends of the modern distribution of the Eurasian grape suggests the existence of different genetic contribution from local *sylvestris* populations or multilocal selection and domestication of *sylvestris* genotypes. To tackle this issue, we analysed chlorotype variation and distribution in 1201 samples of *sylvestris* and *sativa* genotypes from the whole area of the species' distribution and studied their genetic relationships. The results suggest the existence of at least two important origins for the cultivated germplasm, one in the Near East and another in the western Mediterranean region, the latter of which gave rise to many of the current Western European cultivars. Indeed, over 70% of the Iberian Peninsula cultivars display chlorotypes that are only compatible with their having derived from western *sylvestris* populations.

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Introduction

The Eurasian grape (*Vitis vinifera* L.) is the most widely cultivated and economically important fruit crop in the world (Vivier & Pretorius 2002). Cultivated grapevines (*Vitis vinifera* spp. *sativa*) are thought to have been domesticated from wild populations of *Vitis vinifera* spp. *sylvestris* (Levadoux 1956). These wild vines are dioecious plants still occurring in small isolated populations along riverbank forests from the Atlantic coast of Europe to Tajikistan and the western Himalayas (Zohary & Hopf 2000). The exploitation of wild grape fruit as a food source by Palaeolithic hunter-gatherer populations is well documented at many prehistoric sites across Europe (Zohary 1996). However, the domestication of the grapevine has been linked to the production of wine, which required more fruit to be viable as well as storage containers made of pottery to preserve the beverage, developments that did not occur until the Neolithic period (*c*. 8500–4000 BC) (McGovern *et al*. 1986). The domestication process involved the selection of hermaphrodite genotypes producing larger and sweeter berries of attractive colours and the development of techniques for their vegetative propagation (Zohary & Hopf 2000).

Major questions regarding grapevine domestication concern the number of domestication events and the geographic locations where they took place. Two basic divergent hypotheses can be formulated: (i) a restricted origin hypothesis in which domestication took place from a limited wild stock in a single location, with those cultivars subsequently being transplanted to other regions (Olmo 1976); and (ii) a multiple-origin hypothesis in which domestication could have involved a large number of founders recruited during an extended time period and along the entire distribution range of the wild progenitor species (Mullins *et al*. 1992). In agreement with the first possibility, archaeological research has traced the earliest evidence for large-scale winemaking, presumably exploiting a domesticated plant, to the Neolithic period in the northern mountainous regions of the Near East, encompassing the northern Zagros, eastern Taurus and Caucasus Mountains (McGovern & Rudolph 1996; Zohary & Hopf 2000; McGovern 2003). From there, wine and grape cultivars were transplanted southwards to the Jordan Valley (*c*. 4000 BC) and Egypt (*c*. 3000 BC) on the western side of the Fertile Crescent, and to the central and southern Zagros Mountains, bordering Mesopotamia on the east, by *c*. 3000 BC. Western expansion of the wine culture is later documented in Crete, *c*. 2200 BC, and on the coasts of the Italian and Iberian Peninsulas (*c*. 800 BC) (McGovern 2003). On the other hand, the existence of morphological differentiation among cultivars from distinct geographical areas in the Near East and in the western Mediterranean region supports the second possibility in which wild local *sylvestris* germplasm significantly contributed to the generation of grape cultivars, possibly through multiple domestication events (Negrul 1938; Levadoux 1956; Mullins *et al*. 1992). This possibility is compatible with an eastern ancestral origin for the wine culture and viticulture practices and its spread from east to west. Resolving this issue has important implications for understanding the origin of current grape cultivars and provides information on the processes involved in the domestication of woody plant species.

The analysis of the amount and distribution of genetic variation in cultivated (*V. vinifera* ssp. *sativa*) and wild (*V. vinifera* ssp. *sylvestris*) populations can help in understanding the process of grapevine domestication. *V. vinifera* plants are highly heterozygous and the vegetative propagation of cultivars has maintained their high heterozygosity levels. When cultivars from the same geographic regions are grouped, nuclear DNA microsatellite markers provide weak discrimination between different geographic groups, with the greatest variation existing within the cultivar groups themselves (Aradhya *et al*. 2003). Additionally, European grape cultivars have a complex history of movement over growing regions, which hampers the recognition of clear geographic trends in their distribution (Sefc *et al*. 2000). To try overcoming these problems we have used variation in the chloroplast genome to analyse the relationships between *sylvestris* and *sativa* grapevine groups. The chloroplast genome has a lower evolutionary rate than the nuclear genome (Provan *et al*. 1999), and being maternally inherited in grapevine (Arroyo-García *et al*. 2002) can only be disseminated by seeds or cuttings. Furthermore, preliminary genotyping of grapevine cultivars from different locations for a few chloroplast microsatellite loci have shown the existence of a reduced number of chlorotypes, which could show specific geographic distributions (Arroyo-García *et al*. 2002; Imazio *et al*. 2006).

We have analysed chloroplast DNA variation at nine polymorphic microsatellite loci of 1201 *V. vinifera* genotypes belonging to both *sativa* and *sylvestris* subspecies, grouped into eight large geographical groups. The analysis of their genetic relationships supports the existence of an important contribution of wild *sylvestris* germplasm from the Near East and Western Europe to the origin of current Eurasian

cultivars. These results suggest the existence of at least two origins for grapevine cultivars, one in the Near East and a second one in the western Mediterranean region that gave rise to many of the Western European cultivars.

Materials and methods

Plant materials

The lists of *sylvestris* and *sativa* genotypes analysed and their region of origin and location are given in Tables S1 and S2 of the Supplementary material. Eight large population groups were delineated in the total sample according to physical geographic barriers: 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), with *sylvestris* and *sativa* samples being considered separately (Tables S1 and S2). In assigning cultivated genotypes to specific geographic regions, we only considered nonredundant genotypes traditionally cultivated in a specific geographic area, as determined by either regional viticulturists or as described by Galet (2000). When considering *sylvestris* samples, we relied on current relict populations with very few individuals per population and, in most cases, sampled all the present individuals. The current location of those populations is not close to actual vineyards. However, the geographic distribution of *sativa* and *sylvestris* groups has changed as a consequence of human population expansion. We reduced the possibility that they could be escapes of cultivated genotypes or had originated from cross-hybridization between cultivated *sativa* and wild *sylvestris* plants by using morphological criteria. Apart from other morphological features, *sylvestris* genotypes are mostly male and female plants while cultivated ones are generally hermaphrodites. Furthermore, given the known genetic determinism of sex in *Vitis vinifera* (Negi & Olmo 1971), wild male plants cannot be escapes 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 analysed the existence of genetic differentiation between wild male and female subpopulations within each *sylvestris* population group, using the Fisher exact test (Raymond & Rousset 1995) on chlorotype frequencies. The lack of significant subpopulation differentiation in any of the population groups supported the consideration of all the genotypes as being part of the same *sylvestris* population groups. To discard redundant genotypes, the genetic identity of each *sylvestris* and *sativa* genotype was confirmed in each sample using a set of six nuclear microsatellite loci (data not shown).

DNA extraction and analysis

Total genomic DNA was isolated from young frozen leaves using slightly different procedures depending on the laboratory (Doyle & Doyle 1990; Lodhi *et al*. 1994; QIAGEN DNeasy Plant Mini kit). To identify grapevine polymorphic chloroplast microsatellite loci, we tested 54 primer pairs developed for tobacco (Bryan *et al*. 1999; Weising & Gardner 1999; Chung & Staub 2003) and *Arabidopsis* (Provan 2000) (Table S3, Supplementary material) in samples of three *Vitis* species (*Vitis berlandieri* Planchon, *Vitis riparia* Mich., and *Vitis rupestris* Scheele) and 20 *V. vinifera* cultivars representative of previously identified chlorotypes (Arroyo-García *et al*. 2002). Nine polymorphic loci were found displaying two or three alleles per locus in *V. vinifera* (Table S4, Supplementary material). The PCR products from 22 primer-template combinations corresponding to the different alleles observed for the nine polymorphic chloroplast microsatellite loci were directly sequenced on both DNA strands by the dideoxy-nucleotide chain termination method using an Applied Biosystems 373 A. In all cases, size variation resulted from differences in the number of mononucleotide repeats in poly T/A stretches (Fig. S1, Supplementary material). Microsatellite polymorphisms were detected radioactively based on allele size following the procedure described by Arroyo-García *et al*. (2002). Every sample was analysed at least twice to ensure genotype reproducibility.

Data analysis

Chlorotype frequencies were directly estimated for each of the 15 population groups considered. An unbiased estimate of chlorotype diversity (h) and its standard deviation were calculated according to Nei (1987). A chlorotype median network was constructed using NETWORK (Bandelt *et al*. 1999). Genetic differentiation among *sylvestris* and among *sativa* groups were estimated from G_{ST} values calculated with PERMUT (Pons & Petit 1996). Distances among populations were calculated following the methods of Nei (Nei 1972), Reynolds (Reynolds *et al*. 1983), linearized F_{ST} (Slatkin 1995) and Dmyu (delta mu square) (Goldstein *et al*. 1995). These analyses were implemented using the programs ARLEQUIN (Schneider *et al*. 2000) and POPTREE (N. Takezaki; ftp://ftp.nig.ac.jp/pub/Bio/njbafd). Based on Dmyu values, a neighbour-joining tree (Saitou & Nei 1986) was constructed using PHILIP, version 3.57c, and visualized by TREEVIEW (Page 1996). Euclidean distances between pairs of populations were calculated in the space defined by chlorotype frequencies, methods used for clustering are described in Everitt (1993), and were carried out using MATLAB.

Results and discussion

Vitis vinifera *chlorotypes*

To study the genetic relationships between *Vitis vinifera* ssp. *sylvestris* and cultivated grapevine, we analysed a total of 1201 individual grapevine genotypes — 513 *sativa* cultivars and 688 *sylvestris* plants from more than 130 locations — from the Eurasian region of the species distribution (Tables S1 and S2). Genotypic analyses for nine polymorphic chloroplast microsatellite loci (Table S3 and Fig. S1) identified eight different chlorotypes (Table 1 and Table S4). Among them, only four (A, B, C and D) had global frequencies greater than 5%. The three most common chlorotypes were A, B and D in *sylvestris* and A, C and D in *sativa* samples. Among *sativa* cultivars, chlorotype A was twice as frequent in wine grape cultivars as in table grape ones, while chlorotype C had double frequency among table grapes. Chlorotypes B and D showed similar frequencies in table and wine grape cultivars.

Chlorotype relationships were analysed under a network model (Everitt 1993), given the existence of a significant ($P = 0.04$) phylogenetic incompatibility among sites when tested by the method of Jakobsen & Easteal (1996). The results showed that three of the most frequent chlorotypes (A, C, and D) corresponded to three major chlorotype lineages, with chlorotype B occupying a central position (Fig. 1). Chlorotypes G and H were closely associated to chlorotype A, chlorotype E was associated with D, and chlorotype F was closer to B. The intermediate relationship of chlorotype B to all other chlorotypes suggests that it could be an ancestral *V. vinifera* chlorotype (Fig. 1).

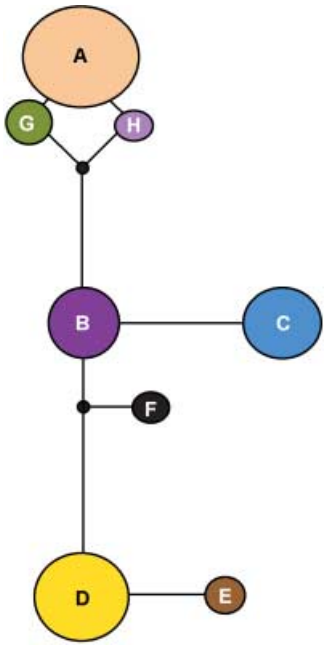

Fig. 1 Chlorotype median network representing all chlorotypes identified in grapevine. Circle areas are proportional to chlorotype frequencies in the global sample.

Chlorotype variation and distribution in V. vinifera *ssp.* sylvestris

Very small and isolated populations of *V. vinifera* ssp. *sylvestris* can still be found in European temperate regions along deep river banks. Among them, we 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. As explained in the Materials and Methods, all the natural populations were grouped in seven 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 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). In

Table 1 Chlorotype frequencies in global samples. Cultivar usage was based on Galet descriptions. Cultivars with both wine and table aptitude were considered as table grape cultivars for simplicity

	Frequency				
Chlorotype	Wine grapes ($n = 328$)	Table grapes ($n = 185$)	*sativa* ($n = 513$)	*sylvestris* ($n = 688$)	Total ($n = 1201$)
A	0.253	0.101	0.193	0.619	0.433
B	0.071	0.087	0.082	0.122	0.113
C	0.256	0.471	0.335	0.033	0.161
D	0.390	0.341	0.370	0.156	0.241
E	0.027		0.017	0.004	0.012
F				0.005	0.003
G	0.003		0.003	0.057	0.034
H				0.004	0.003

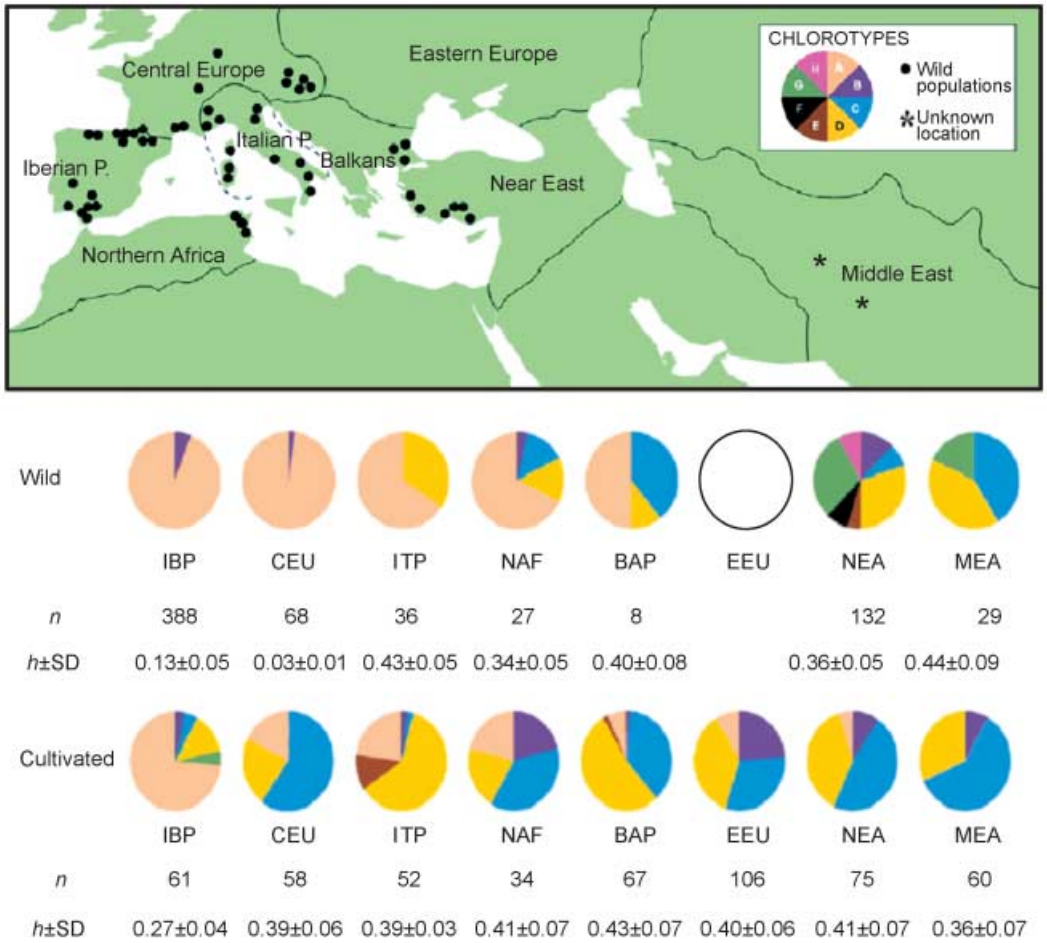

Fig. 2 Chlorotype distribution in *sylvestris* and *sativa* population groups. Geographic areas considered are separated by lines when needed. Black periods do not mark specific *sylvestris* populations but river valleys where wild genotypes were collected at several locations. Asterisks indicate that specific locations of collection in the area are unknown. See Table S1 (Supplementary material), for the specific locations. *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 Fig. 1.

particular, chlorotype G was only found in NEA and MEA *sylvestris* samples, while chlorotypes C and D coexist with chlorotype A in Mediterranean populations BAP and NAF and A and D coexist in ITP. Chlorotype B did not show a clear eastern or western pattern, occurring across the whole distribution area at low frequency, in agreement with its proposed ancestral position. Finally, infrequent chlorotypes, such as E, F or H, were only found in NEA *sylvestris* populations.

The large number of *sylvestris* samples analysed for IBP and NEA geographic groups allows concluding that chlorotype A is infrequent in NEA, while chlorotypes C, D and G are infrequent in IBP or CEU, which represent the western periphery of *V. vinifera* ssp. *sylvestris* area of distribution. Even if those chlorotypes were eventually found elsewhere, their frequencies should be lower than 1%. Analysis of unbiased chlorotype diversity in *sylvestris* populations showed central Mediterranean and eastern populations had higher diversity values than western population groups such as IBP and CEU (Fig. 2). These results are in agreement with Negrul's theory (1938), proposing the Anatolian Peninsula and Transcaucasian regions as the 'diversity centre' of *V. vinifera*, based on phenotypic variation. By contrast, IBP and CEU would represent peripheral populations.

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. 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 (G_{ST}) 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, we analysed 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 analysed models and when phenotypic distances were calculated from microsatellite morphs frequencies. 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

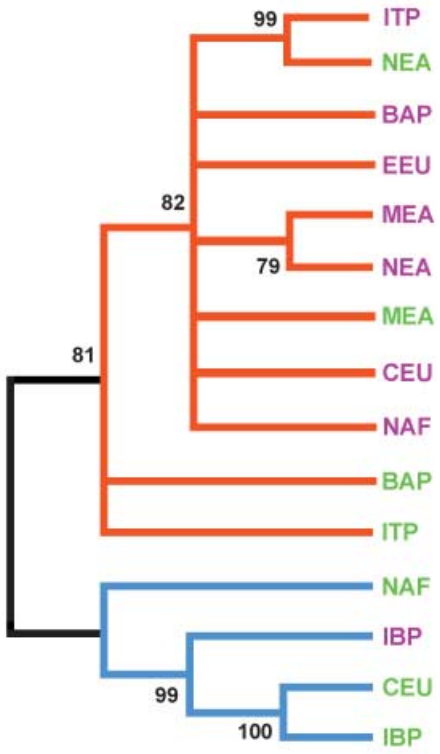

Fig. 3 Genetic relationships among *sylvestris* and *sativa* grapevine population groups. The tree was constructed using the neighbour-joining method on the Dmyu distance matrix calculated for all pairwise combinations of population groups. Bootstrap support values exceeding 70 are indicated. Branches 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 in Fig. 2.

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 distinguished 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. Our results 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).

In conclusion, Eurasian grapevine domestication could be described as a long-term process of selection of suitable genotypes in different locations followed by their vegetative propagation. Genetic variation would have increased during this process as a result of somatic variation and the occasional generation and propagation of spontaneous hybrids derived from crosses between cultivated plants, as previously documented (Bowers *et al*. 1999) or between cultivated and *sylvestris* plants. Since many of the current grape varieties can be traced back hundreds and even thousand years based on historical records (Bowers *et al*. 1999; Galet 2000), they are probably separated from their wild relatives by a low number of sexual generations. This generation number is not higher than 80, when assuming a generation time > 100 and an initial domestication event in the mountainous Near East 8000 years ago.

Interestingly, chlorotype analyses of cultivated olive trees and wild oleaster populations along the range of the *Olea europaea* species distribution yield a parallel picture to that of grapevine. The existence of western olive cultivars carrying chlorotypes that are only found in western oleaster populations also led to propose a multilocal origin of olive cultivars (Besnard *et al*. 2001). Multiple origins, together with limited manipulation restricted to the incorporation of somatic variants and a few spontaneous hybrid generations, might be common features of the domestication process of many woody species (Armelagos & Harper 2005). Future genomic analyses in grape and other woody species, including the analysis of well-dated ancient DNA, should unravel the details behind domestication of these species and the origin of specific cultivars.

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Supplementary material

Fig. S1 Nucleotide sequence of allelic variants identified at polymorphic chloroplast microsatellite loci.

Table S1 Wild *Vitis vinifera* ssp. *sylvestris* genotypes analysed in this study

Table S2 Grapevine cultivars analysed in this study

Table S3 Consensus chloroplast microsatellite primers tested in *Vitis vinifera*. Primer pairs were initially designed for *Nicotiana tabacum* L. chloroplast genome, ccSSR (1), cpSSR (2) and NTCP (3) and *Arabidopsis thaliana* chloroplast genome, ATCP (4)

Table S4 Chlorotype genotypes at polymorphic microsatellite loci

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Authors are interested in the characterization of grapevine genetic diversity and understanding the genetic relationships between cultivated and wild genotypes.