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Evidence of a secondary grapevine domestication centre detected by SSR analysis

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Abstract The origin of the grapevine was investigated with archaeobotanical, cultural and historical data. A primary domestication centre was located in the Near East region but there is no agreement on the existence or role of secondary domestication centres. In this work, PCR-based microsatellite analysis has been applied to study the origin of some Italian cultivated grapevines from in situ direct domestication of the wild autoctonous grapevine. Three different Italian locations in Grosseto, Cosenza and Nuoro were identified for this study, and domesticated grapevine as well as wild local accessions growing in these location, were analysed by SSR markers. Cluster analysis performed on Cosenza and Grosseto samples showed a high value of genetic distance between domesticated and wild accessions. On the contrary two cultivars (Bovale Murru and Bovale Muristellu) recovered in Nuoro (in the Sardinia island) were very close to some wild varieties. This suggests that the latter two cultivars may have originated from wild grapevines and consequently that in this location a secondary grapevine domestication event occurred. Six Lambrusco varieties were also included in this analysis as ancient putative ancestors of the cultivated grapevines. The molecular analysis excluded this hypothesis and suggest Lambrusco as an independent Vitis taxon.

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Introduction

The *Vitis vinifera* ssp. *sativa* grapevine is an Old World crop, which is thought to have been domesticated in the Near East region (Zohary and Spiegel-Roy 1975; Zohary and Hopf 1993) or in the Transcaucasian region (Olmo 1976), beginning from the second half of the 4th millennium B.C. Starting from the primary centre of domestication, the grapevine spread to the South-eastern Mediterranean regions, Palestine, Southern Lebanon and Jordan (Zohary and Spiegel-Roy 1975; Wasilykowa et al. 1991). Then, domesticated grapevines appeared during the first half of the 3rd millennium B.C. in Minor Asia, Southern Greece, Crete and Cyprus. At the beginning of the 2nd millennium B.C., domesticated grapevines were found in the Southern Balkans (Logothetis 1970; Kroll 1991), while they made their first appearance in Southern Italy in the second half of the 2nd millennium B.C. and in Northern Italy, Southern France, Spain and Portugal in the second part of the 1st millennium (Forni 1990; Hopf et al. 1991).

Characteristics of wild and domesticated grapevines are summarised in Table 1. The most relevant differentiating trait is the mating system: wild grapevine (*V. vinifera* ssp. *silvestris*) is dioecious with anemophilous pollination, while domesticated grapevine (*V. vinifera* ssp. *sativa*) is self-pollinating (hermaphrodite). Hermaphroditism was the crucial trait selected for by ancient farmers in order to warrant fruit production by each individual. In present days cultivated grapevines are hermaphrodite. A peculiar situation is the Lambrusco accessions, because they show morphological characteristics (Table 1) found in both domesticated and wild grapevine. Their position in the grapevine classification is under discussion (Levadoux 1956).

In this paper we focus on the possible existence of secondary centres of grapevine domestication, i.e., areas where wild varieties have been domesticated in subsequent times (Olmo 1995). No evidence for the existence of such centres has been yet produced, although their presence outside the Near East or Transcaucasian regions

Table 1 Comparative morphology of wild and domesticated grapevine based on Olmo (1976). Morphology of Lambrusco accessions is also shown. Data was obtained by Calo et al. 2001

Type	Wild grapevine	Domesticated grapevine	Lambrusco accessions
Mating system	Dioecious	Hermaphrodite	Hermaphrodite
Habitat	Humid soils	Dry habitats	Dry habitats
Berry shape	Small, round or oblated	Large and elongated	Small and round, in several case irregular; dimension is very variable
Trunk	Often branches, slender, bark separated in very long thin strips	Thick bark separates in wider and more-coherent strips	Similar to domesticated grapevine
Seeds	Small, rounded body, high width/length ratio (>0.70)	Large, pyriform body, lower width/length ratio (< 0.60)	Similar to domesticated grapevine
Fruit clusters	Small, globular to conical, irregular set, berry maturity variable in cluster	Large, elongated, compact to well-fitted, berry, uniform in maturity	Small, conical and irregular set
Leaves	Small, usually deeply three-lobed. Petioles short and slender, dull aspects	Large, many entire or with shallow sinuses, petiole thick, glabrous to drowny	Small, usually deeply and three-lobed

cannot be excluded. In support of this, archaeological remains indicate that ancient populations of these areas included wild grapes in their diet even before the 4th millennium B.C. (Núñez and Walker 1989).

Italy is a convenient place to carry out research on the origin of domesticated grapevines because this country is characterised by high biodiversity for both domesticated (*V. vinifera* ssp. *sativa*) and autochthonous wild grapevine (*V. vinifera* ssp. s*ilvestris*) relatives (Anzani et al. 1990; Calo et al. 2001).

On the basis of these considerations, we considered that the origin of Italian cultivars may be sought in two ways: (1) in situ direct domestication of wild autochthonous grapevines, and (2) introduction of domesticated varieties from different regions and at different times during the first phases of the establishment of viticulture and/or during the following historical periods.

To evaluate the contribution of these events, the genetic relationship between domesticated and wild grapevines growing in the same location, was investigated. In addition Lambrusco varieties were also analysed. Lambrusco accessions have already been proposed as ancient putative domesticated ancestors derived from wild grapevine (Levadoux 1956; Forni 1990). Actually the genetical position of Lambrusco varieties is unknown.

Historic knowledge on grapevine domestication is based on archaeo-botanical, cultural and historical evidence. This is considered informative, but insufficient to give conclusive evidence (Bisson 1999, Fossati et al. 2001). Recently, molecular markers have proven to be a more direct and effective tool to study crop origin. On the basis of genome-wide measures of genomic similarity, the origin of some domesticated cereals has been traced back to wild populations (Salamini et al. 2002). In the specific case of the grape, SSR (Simple Sequence Repeat) analysis has been mostly used to evaluate the genetic relationship among cultivars (Sefc et al. 2000; Labra et al. 2001a).

In this work, polymorphism at six SSR *loci* was investigated to define the genomic relationship among wild and domesticated Italian grapevines. Results give a substantial contribution to the rationalisation of knowledge on the grapevine domestication, as well as on genomic relationship among local germplasm accessions including traditional and modern-day cultivated grapevines.

Materials and methods

Plant material

Wild grapevines, distinguished from domesticated accessions because they are dioecious, were identified in three different Italian locations. In particular, 15 individuals from Nuoro – Sardinia (indicated as WN1 to 15), seven individuals were collected from Grosseto - Tuscany (indicated as WG1 to 7) and seven from Cosenza - Calabria (indicated as WC1 to 7). A total of 23 traditional domesticated grapevines growing in these regions were also selected and a complete list is given in Table 2. Domesticated accessions were collected from CI.VI.FRU.CE (the germplasm collection of Pavia, Italy). Finally, six Lambrusco accessions were also included in this study as old Italian varieties: Lambrusco Maestri, Lambrusco Cannet, Lambrusco Oliva, Lambrusco Foglia Frastagliata, Lambrusco Graspa Rossa and Lambrusco Montericco.

DNA extraction

DNA extraction was performed on young 1–2 cm long leaves that were harvested from rooted cuttings. These were frozen in liquid nitrogen and ground into a fine powder. Genomic DNA was extracted as described by Labra et al. (2001b).

Microsatellite analysis

In this study, six primer pairs of nuclear microsatellites, VVS4 (Thomas and Scott 1993), VVMD5, VVMD6, VVMD7 (Bowers et al. 1996) VVMD21 and VVMD32 (Bowers et al. 1999), were used. The PCR reaction was performed by adding 15 ng of genomic DNA to a 20-μl PCR mixture containing 10 ng of the DNA primer specified for each microsatellite *locus*, 200 μM of each of the four dNTPs, 0.5 U of Dynazyme (Celbio, Italy) and Dynazyme buffer as specified by the supplier. PCR amplification was performed with a Programmable Thermal Controller (PTC 100, MJ Research Inc., USA) using the following profile: 7 min at 94 °C; 35 cycles of denaturation (45 s at 94 °C), annealing (range from 48 °C to 52 °C) and extension (1 min at 72 °C); then a final step for 7 min at 72 °C. SSRs were analysed on 5% polyacrylamide gel using silver staining to detect the alleles, and sizes were defined by using the GEL DOC 2000 Biorad image analysis system.

Data analysis

The length of microsatellite alleles was assigned to each of 58 varieties analysed. Data obtained were used to calculate *gene diversity* values (Nei 1987) for each groups, and genetic distance (GD) was calculated by the GENETIX 4.02 computer package (Belkhir 1999).

In this paper the *gene diversity* (h) is simply a measure of genetic variability and was estimated as $h = n/n - 1(1 - \sum p^2_i)$ where n is the number of individuals sampled and p is the frequencies of the allele. The average gene diversity (H), over all loci, is estimated by sampling r *loci* from the genome,

$$H = \sum_{j=1}^{r} h_j / r.$$

Genetic distance between groups was estimated using the Nei unbiased genetic distance coefficient (Nei 1972). This parameter is defined as:

$$\mathrm{GD}_{\mathrm{ij}} = -\ln \sum p_a p_b \Big/ \left(\sum p_a^2 \sum p_b^2\right)^{\frac{1}{2}},$$

where p_a and p_b are the frequencies of a given allele in populations a and b. For multiple *loci* these values are calculated by summing over alleles at all *loci* studied. The resulting genetic distance matrix was used for a cluster analysis according to the unweighed pairgroup arithmetic average method in consideration of nuclear allele frequencies. The unrooted tree was computed using the UPGMA method by WebPHYLIP version 2.0 (Allison and Lonxin 1999) in conjunction with TREEVIEW 1.6.6. (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

In addition, samples (domesticated and wild grapevine) from the same location were compared. In this case, each microsatellite allele was scored as a binary character for its absence (0) or presence (1) for a total of 52 samples. The resulting data were analysed using software program NTSYSpc. Similarity matrices were computed using two different similarity coefficients: Dice and Jaccard indices (Sneath and Sokal 1973).

Result

A total of 58 plants was analysed by SSR markers. Of these 23 are hermaphrodite domesticated grapevines (*V. vinifera* ssp. *sativa*, 29 are wild grapevines (*V. vinifera* ssp. *silvestris*), and six are Lambrusco accessions (Table 2). To exclude the possibility that wild accessions collected were escaped from cultivation only dioecious plants were considered. Six microsatellite *loci* were investigated and a total of 71 alleles were detected. The allele number for each SSR *locus* ranged from 16 to 4 with an average of 9.

A high level of gene diversity was detected in wild as well as in domesticated grape populations (Fig. 1). In the case of wild grapevines, it can be postulated that the high level of gene diversity is correlated with the mating system of these dioecious and outbreeding plants; the domesticated grapevine plants, which were selected from wild ancestors, had their gene diversity preserved due to the practice of vegetative propagation.

Table 2 Accessions, abbreviations and cultivation area of the *V. vinifera* L. cultivars analysed in this study. D: domesticated grapevine; N: Nuoro (Island of Sardinia); G: Grosseto (Tuscany); C: Cosenza (Calabria)

Cultivation area	Accessions	Abbreviation
Nuoro (Sardinia)	Girò	DN1
	Bovale Murru	DN2
	Bovale Muristellu	DN3
	Bovale Grosso	DN4
	Bovale di Spagna	DN5
	Fiudedda	DN6
	Cannonau	DN7
	Gregu Nieddu Muristellu	DN8
Grosseto (Tuscany)	Canaiolo	DG1
	Canaiolo Bianco	DG2
	Malvasia	DG3
	Colorino	DG4
	Ciliegiolo	DG5
	Vernaccia di S. Gimignano	DG6
	Malvasia del Chianti	DG7
	Sangiovese Nielluccio	DG8
Cosenza (Calabria)	Montonico	DC1
	Greco Nero	DC2
	Guarnaccia	DC3
	Greco Bianco	DC4
	Pizzutello	DC5
	Arvino	DC6
	Magliocco	DC7

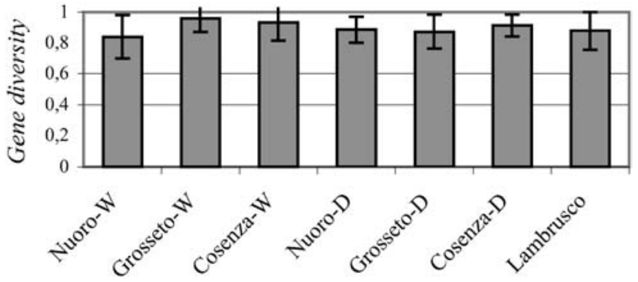

Fig. 1 Histogram and summary gene-diversity values calculated for each analysed grapevine grop following SSR analysis

Genetic distance among wild, domesticated and Lambrusco groups

The genetic distance index (GD) was calculated among each group. Table 3 summarised the GD value obtained by the pair-wise method. The average GD values among the three subspecies *silvestris* groups is 0.492, whereas 0.260 was the average GD detected among cultivated groups. Results obtained by comparing Lambrusco varieties with domesticated and wild grapevines showed an average value of 0.765 for plants of subspecies *sativa* and 0.804 for plants of subspecies *silvestris*, respectively. These data point out the existence of three major grape clusters grouping domesticated grapevines, wild grapevines and Lambrusco accessions. To define the genetic relationship among these cluster, an unrooted dendrogram was computed (Fig. 2). This shows a clear separation for the two *V. vinifera* subspecies.

Table 3 Genetic distance (Nei 1972) among three Italian populations of *V. vinifera* ssp. *silvestris*, three groups of *V. vinifera* ssp. *sativa* and Lambrusco accessions. Symbols are the results of significance tests (500 permutations) of genetic distances between pairs of groups. * = ($p < 0.001$), ** = ($p < 0.01$), *** = ($p < 0.05$), † = non-significant value. Levels of genetic distance among three different cluster were significant

G.D.	Nuoro-W	Grosseto-W	Cosenza-W	Nuoro-D	Grosseto-D	Cosenza-D	Lambrusco
Nuoro-W	–	*0.405****	*0.596**	0.567*	0.986*	0.869*	0.877*
Grosseto-W		–	*0.476****	0.553**	0.747*	0.642*	0.621*
Cosenza-W			–	0.717*	0.884*	0.714*	0.915*
Nuoro-D				–	0.344*	0.268†	0.871*
Grosseto-D					–	0.169**	0.652**
Cosenza-D						–	0.773*
Lambrusco							–

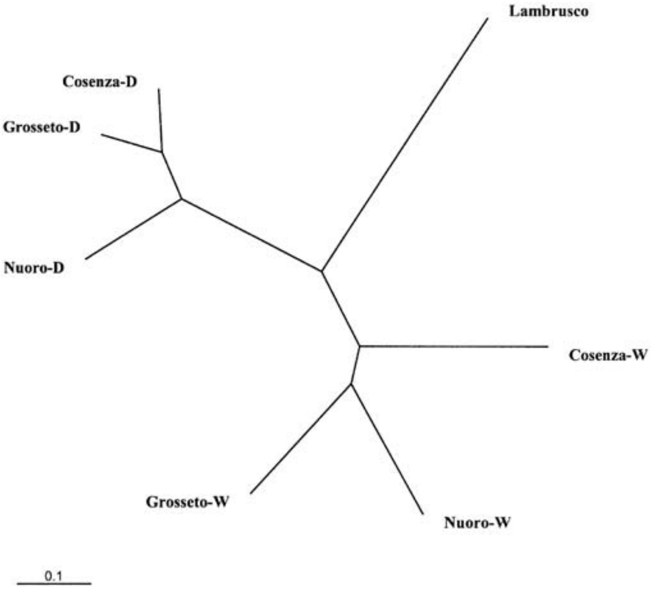

Fig. 2 Unrooted dendrogram (UPGMA) showing the genetic distance (GD – Nei 1972) among domesticated (D) and wild (W) grapevine varieties from the three analysed Italian regions. Lambrusco varieties were also analysed. Three different trees (UPGMA, WPGMA, Neighbor-joining; data not shown) were constructed and these showed the same topology

Morphological analysis suggest several common similarity between Lambrusco accessions and both *Vitis* subspecies, while molecular analysis showed a clear distinction of Lambrusco accessions from wild as well as domesticated grapevine. Based on these data, we can exclude Lambrusco as ancient putative domesticated ancestors of the domesticated grapevines sampled in Cosenza, Grosseto and Nuoro.

Genetic relationships among grapevine accession from three regions

To assess the relationship among accessions (domesticated and wild grapevine) from the same location, three independent similarity matrices were computed. UPGMA dendrograms based on genetic similarity were performed for each local germplasm. Initially, two different similarity coefficients (Jaccard and Dice) were considered; however, given that there was a high cophonetic correlation between them (ranging from 0.97 to 0.98, data not shown) it is probably appropriate to use only one or the other. We chose to consider the Dice coefficient.

The Dice similarity index detected in the three dendrograms (from 0.94, high genomic similarity, to 0.14, high genomic dissimilarity) demonstrated the high polymorphism of the accessions analysed.

The dendrograms constructed upon the SSR analysis of the Grosseto (Fig. 3A) and Cosenza (Fig. 3B) varieties show a clear separation between domesticated and wild accessions. A different situation was observed in the case of the Nuoro germplasm (Fig. 3C) where some accessions of subspecies *sativa* and *silvestris* were not clearly separate. Bovale Muristellu and Bovale Murru showed 50% of shared alleles with three *silvestris* accessions. We underline that all Nuoro domesticated accession are hermaphrodite while wild samples are dioecious. Based on mating system and morphological traits, Bovale Muristellu and Bovale Murru are classified as domesticated accessions, with exclusion for a few berry traits (small or medium globular berry) that link to wild accessions (Calo et al. 2001).

Discussion

Molecular markers were used to investigate the origin of domesticated plants. The progenitors of important crop species like einkorn (Heun 1997), olive (Lumaret and Ouazzani 2001) and barley (Badr et al. 2000) were identified by DNA molecular tools. The problem of the origin of today's grapevine cultivars is debated. The search for direct ancestors of local varieties (local wild vines or domesticated vines from other regions) is still an open issue, as is their genetic history. Vegetative propagation, seeds produced by self-pollination or breeding may have played intersecting roles in defining the present genomic constitution of each cultivar.

In this work, SSR markers have been used to investigate the origin of the Italian domesticated grapevine *V. vinifera* ssp. *sativa* from the wild subspecies *V. vinifera* ssp. *silvestris* and to verify the occurrence of a secondary grapevine domestication in Italy. The number of Italian

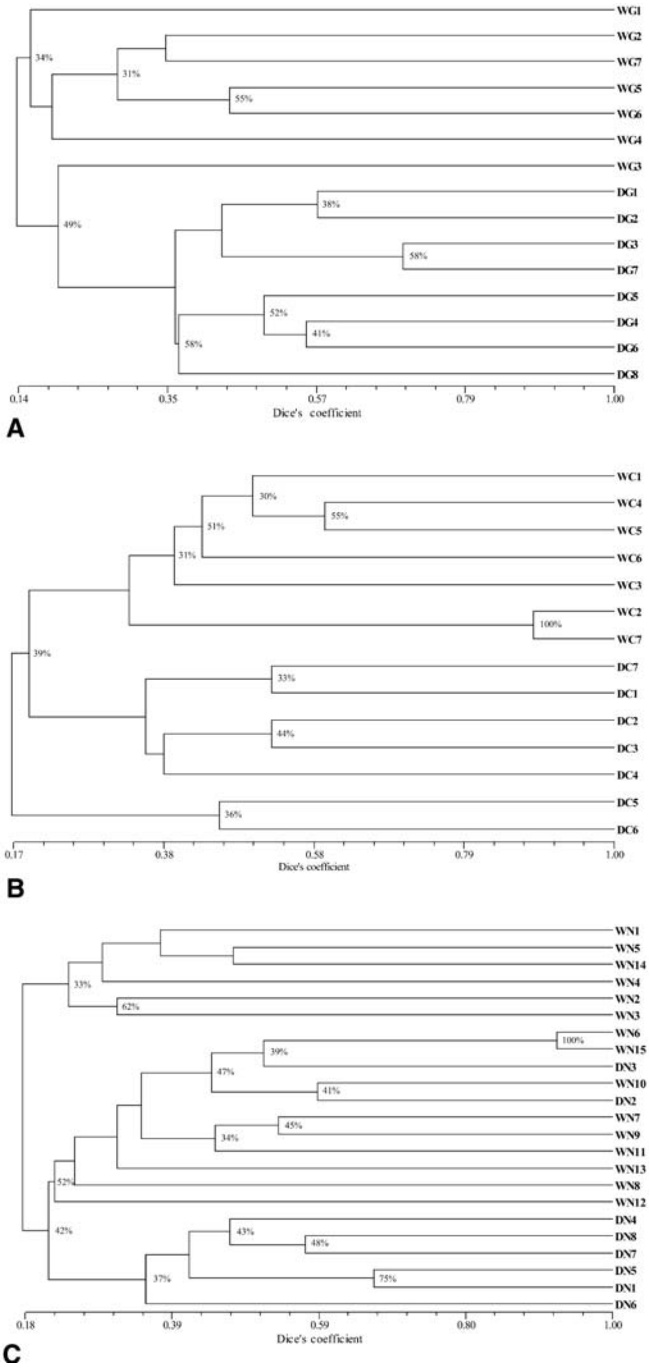

Fig. 3 UPGMA dendrograms obtained by SSR analysis performed on grapevine accessions from Grosseto (**A**), Cosenza (**B**) and Nuoro (**C**). We choose to find option of NTSYS software and obtained a tree for each analysis. Numbers on the branches are bootstrap values obtained from 300 replicate analyses (only values showing >30% support were reported). D = cultivated varieties. W = wild varieties

varieties is very large (Calo et al. 2001) and their ancestors remain largely unknown. The human selection develops huge number of varieties well adapted to the local conditions. Nevertheless, some varieties still present ancient traits characteristic of wild grapevine, i.e. small and round berries, and small leaves (Table 1). This is the case of the Lambrusco accessions, nowadays cultivated in Italy. On the basis of these considerations Lambrusco varieties were compared with *sativa* as well as *silvestris* accessions to verify their possible role as progenitors of some of the domesticated grapevines. Our results strongly suggest that Lambrusco varieties should be considered an independent taxon different from *sativa* and *silvestris*. The high genetic dissimilarity detected among *sativa* and *silvestris* accessions from Grosseto and Cosenza do not show a direct origin of the domesticated grapevine from the wild ones recovered in the same region.

We can suggest that the high genetic variability detected among the Italian domesticated grapevines is the result of multiple variety flows that link different viticultural European regions through different routes (Labra et al. 1999). This hypothesis is supported by the historical document, which demonstrates that the Italian viticulture was greatly influenced from human migrations through the Northern European regions (via dell'Ambra), between France and Spain (via di Eracle) or Greece and the middle-east region (via Egnazia) (Forni 1990; Scienza et al. 1998). This is also supported by recent molecular data produced when analysing regional grapevine germplasm with molecular tools, as is the case for grapevines in the Aosta Valley (Labra et al. 2002c) or in Southern Italian regions (Pellerone et al. 2001).

A different situation has now been observed in the Nuoro area, where two domesticated grapevines (cv Bovale Muristellu and cv Bovale Murru) were found to be very close to some wild varieties: this is the first evidence of the possible presence of a secondary grapevine domestication centre. Bovale Muristellu and Bovale Murru showed the complete morphological identity of domesticated grapevine. Historical information of Bovale cultivars suggest an unknown ancient origin, and based on the viticultural history of Sardinian varieties (Calo et al. 2001) the local origin was not excluded. The presence of a secondary domestication centre in Nuoro (Sardinia) was supported by the geographical position of the island and by the low cultural exchange that characterised the Sardinian human history (Cavalli-Sforza et al. 1994). It is conceived that the low agronomical varietal flow from outstanding regions induced the ancient Sardinian viticulturist to the domesticated wild grape.

These results complicate the viticulture history because it suggests that the Italian cultivars were derived both from cultivars introduced from other viticultural regions, where the primary centre of domestication was located, and from direct domestication from local wild vines.

This work provides a firm basis for the study of the origin of grapevines and of the existence of secondary domestication centres in isolated geographical regions. The latter may apply to several islands in the Mediterranean sea. In the future we will evaluate the contribution of primary and secondary domestication centres by analysing other populations of domesticated and wild grapes grown in several European regions and islands. The aim is to define the contribution of human population movement and of cultural exchange in the viticultural history.

Microsatellite (SSR) analysis which amplifies hypervariabile regions containing tandemly arranged di-, tri- or tetra-nucleotide repetitions, is expected to detect cryptic levels of DNA variation among closely related genomes (Bowers et al. 1996). In this work SSR analysis has been used to define genetic relationships between domesticated and wild accessions. We conclude that the use of this co-dominant marker is a good tool to estimate genetic relationships among domesticated as well as wild accessions, to solve cases of homonymies and synonymies, to fingerprint varieties, and to search or confirm parents of prominent grapevines varieties as also shown in previous papers (Bowers et al. 1999; Labra et al. 1999; Labra et al. 2001c; Anthony et al. 2002).

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