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RESEARCH ARTICLE

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Characterization of the largest relic Eurasian wild grapevine reservoir in Southern Iberian Peninsula

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Abstract

Wild grapevine is becoming a threatened species in the Iberian Peninsula due to human impacts. The aim of this work was to carry out a holistic study for six years of the largest wild grapevine population found up to date in SW Iberian Peninsula. This population has 115 vines. Ampelographic and soil characteristics have been studied. Evaluation of its environment has also been studied by describing the main parasitic species and natural enemies of pests. The ability of this plant material for its micropropagation and storage in slow-growth conditions has been tested. Microvinification resulted in a wine with good acidity and medium color intensity, two interesting characteristics under a warm climatology. Finally, the identification of private alleles in this wild population, absent in other locations from the Northern and Southern Iberian territories, is a very valuable feature and confirms the importance of establishing conservation programs. The population here studied is genetically unique and potentially useful for commercial rootstocks and cultivars breeding that would improve viticulture and enology.

Additional key words: ecology; genetic analysis; *ex situ* conservation; sanitary status; *Vitis vinifera* subsp. *sylvestris*.

Abbreviations used: DC (dark condition); ELISA (enzyme linked immusorbent assay); F (fixation index); He (expected heterozygosity); Ho (observed heterozygosity); I (information index); K (number of populations); Na (number of alleles); NC (normal condition); Ne (effective number of alleles); SSR (simple sequence repeat).

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Introduction

Grapevine is currently considered the most important fruit crop all over the world (De Mattia *et al.*, 2008), with about 8 million hectares planted but it is seriously affected by the genetic erosion. For example, in Andalusia, only 5 cultivars (Palomino fino, Pedro Ximénez, Jaén, Muscat of Alexandria and Zalema), out of the 119 described in the region in 1807 by Clemente-y-Rubio are nowadays still cultured in commercial vineyards, full of imported international clonal cultivars (Ocete *et al.*, 2007).

Between the Iberian Peninsula and Afghanistan, there is only one autochthonous species of grapevine, *Vitis vinifera* L., within which two subspecies are included. One, wild and dioecious, *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi, is considered as the parental of the other, *Vitis vinifera* L. subsp. *sativa* (DC.) Hegi

(Arnold, 2002). This is a hermaphrodite subspecies developed along the domestication process, where most of the table grape, raisin or wine producing cultivars are included (This *et al.*, 2006).

The Eurasian wild grapevine, as the rest of *Vitis* species are lianae which take the bushes or trees of the surrounding vegetation as supporters. Probably the Iberian Peninsula was the westernmost refuge for wild grapevines during Quaternary glacial periods (Lehmann & Böhm, 2011). According to the literature (Laguna, 1566; Clemente-y-Rubio, 1807), wild grapevines were very common in Spain until middle 19th century. On the other hand, it is necessary to underline that these vines had their genetic contribution to the Iberian cultivars which were included in the *proles occidentalis* (Negrul, 1938). According to DNA microsatellite analysis, Iberian wild vines have provided the chlorotype 'A' to autochthonous cultivars (Arroyo-Garcia *et al.*, 2006), probably by hybridization from Phoenician age to present with imported hermaphrodite plants. A 4% of hybrid specimens between both subspecies were found in Spanish natural habitats (De Andrés *et al.*, 2012). Wild grapevine constitutes an interesting phytogenetic resource for cultivars and rootstocks breeding in order to cope the consequences of climatic change and the possible appearance of new pests and diseases (Mackey, 2009).

Canalization of rivers, dam and levee constructions, forestry and horticulture exploitations of floodplain forests, cleaning of riverbanks and the continuous extension of the road network resulted in direct eradications of wild grapevine populations throughout Europe (Arnold, 2002). Other indirect repercussions, derived from the importation of North American diseases and pests such as powdery and downy mildews and Phylloxera, led to the current status of the wild grapevine as a threatened taxon in the European territory (Thorsell & Sigaty, 1997), and hence in the Iberian Peninsula (Ocete *et al.*, 1999).

Preliminary studies on the localization and ecology of this Vitaceae were carried out in Ossa-Morena mountain range and surrounding areas of Portugal and Spain (Ocete *et al.*, 1999, 2002). The sources of Rivera de Huelva River are located on the Spanish side of these mountains, in the Extremadura region (Badajoz province). The banks of the river are poorly preserved due to aforementioned human impacts, highlighting the construction from 1940 to 1979 of four dams along its channel to supply drinking water to Seville metropolitan area.

The aim of the present paper was to study from a holistic point of view the largest wild grapevine population found up to date in the Mediterranean area of the Iberian Peninsula (Ocete *et al.*, 1999; Cunha *et al.*, 2010), including research on its genetic structure. Furthermore, to indicate those measures carried out for *in situ* conservation, considering the relevance of this wild genetic resource for the future of the crop and their current scarcity (Arroyo-García & Revilla, 2013).

Material and methods

Localization and in situ characterization

Wild grapevine population nuclei were mapped by a GPS receiver along Rivera de Huelva course. Accessions were assessed at flowering time (May), to determine the sex of the plants. Pollen samples from flowers were obtained by brushing the mature anthers from 10 male and 10 female vines. Grains were introduced in DPX (Fluka) and observed under optical microscope Olympus BX 61 in order to study the morphological structure of the grains.

The phenological development of the vines was followed twice per month from 2005 to 2011. The main ampelographic descriptors were evaluated following the OIV (2009) systematic list on a sample of 25 vines of each sex.

Analysis of soil

Soil samples were obtained at rooting depths (0-30 cm). The organic matter present in the soil was determined by the Walkley and Black´s (1934) method, organic carbon concentration by dichromate oxidation (Nelson & Sommers, 1982) and calcium carbonate by using a Bernard calcimeter (Álvarez-Iglesias *et al.*, 2003). The pH of the soils was determined in saturated paste extract (1:2.5 v/v) with a portable pH-meter and electrode system (Crison pH/mV p-506, Spain), calibrated in the field.

Samples were dried at 50 ºC for 48 h. Dried soil samples were ground and homogenized by sieving through nylon nets of 2 mm mesh in order to remove large stones and dead material and then ground again to <60 µm. Soil samples of 2 mm fraction were analyzed for size particle distribution by the hydrometer method (Gee & Bauder, 1986), Kjeldahl-N by the method described by Hesse (1971) and available P by the method of Olsen *et al.* (1954). The extraction was made according to Lindsay & Norvell (1978). Available metals (Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S and Zn) of all samples were measured by inductively coupled plasma (ICP) spectroscopy (Advantage de Thermo, Beverly, MA, USA) (Walinga *et al.*, 1995).

Sanitary status

Roots were unearthed up to 40-50 cm of depth to observe possible symptoms caused by subterranean phytophagous and pathogens. Samples of fine roots were observed under binocular to detect possible damages caused by Phylloxera (*Daktulosphaira vitifoliae* Fitch) (Homoptera, Phylloxeridae), root-knot nematodes and rot fungi.

The detection of possible symptoms caused by phytophagous arthropods, insects and mites, and diseases was carried out in spring and summer, every 15 days along the six years of this investigation, on shoots, leaves and bunches of 100 vines, situated up to 3 m height. In the case of mites 50 leaves/vine were observed to evaluate the intensity of infestation.

Special attention was paid to try to find mealybugs because some of them are vectors of virus, as *Planococcus citri* (Risso) (Hemiptera, Pseudococcidae) that transmits *Grapevine leafroll virus*. For this task, 25 vines exhibiting a perimeter higher than 20 cm were debarked from 2009 to 2012 to find the hemipteran.

The degree of infestation/infection caused by mites was evaluated according to the procedures of Ocete *et al.* (2007) and OIV (2009) in the case of both kinds of mildews. To detect the possible presence of *Grapevine fan leaf*, *Grapevine leafroll* and *Grapevine fleck viruses*, 10 samples from the oldest female and male vines were taken in June 2010. ELISA tests were performed using the Bioreba kit systems.

Together with phytophagous sampling, natural predatory species of Chrysopidae and Coccinelidae were identified. On the other hand, to investigate mite species found inside erinea, 25 available leaves with erinea were randomly chosen from ten individuals in July. The leaves were put in a chamber at 4 ºC for 20 min to slow down the activity of the predatory mites. Immediately after, erinea were studied under the binocular to pick up natural enemies of this mite in action. The Berlese-Tullgren funnel was also used to extract the mites from the erinea. Predatory specimens of *Colomerus vitis* (Pagenstecher) (Acari, Eriophyidae) were separated and introduced into tubes with ethanol (70%) with 2 drops of glycerin. Mites were cleared in lactic acid and mounted on Heinze-PVA medium. Observations were made using an interference contrast microscope. Generic nomenclature for the Phytoseiidae follows the criteria proposed by Chant & McMurtry (1994) for Typhlodrominae and those referred by De Moraes *et al.* (1986) for Amblyseiinae.

Microvinification

For microvinification berries from 3 available vines were harvested on 26th September 2012. In total, 10.55 kg of bunches were obtained. The use of the grape destemmer reduced the sample to 8.79 kg. After destalking, berries were pressed by hand, obtaining 1,788 mL of must. The alcoholic fermentation with maceration and daily removing was developed by wild yeasts, at 20 ºC, along 10 days and monitored by density measures. References of the official analytic methods used from OIV (2015) and data obtained are shown in Table 1.

Genetic analysis

Since wild grapevines are mostly dioecious while cultivated are generally hermaphrodites we have only used male plants for the genetic analysis as they cannot be escapes from cultivated fields nor the result of pollination between wild and cultivated plants. A sample of 63 male accessions were analyzed (Table 2) and compared with a published genotype data, corresponding to Spanish wild grapevine populations (De Andrés *et al.*, 2012).

Table 1. Analysis of the microvinification

Parameter	Value	Method
Ethanol content (% vol)	11.18	Near infrared (NIR)
Methanol (mg/L)	323	Gas chromatography
Malic acid (g/L)	4.13	OIV
Tartaric acid (g/L)	3.16	OIV
Citric acid (g/L)	3.10	Enzymatic
Total acidity (g/L)	6.80	OIV
Volatile acidity (g/L)	1.06	FCSA Autoanalyzer
pH	3.45	pHmeter
Dry extract (g/L)	28.0	Densimetric calculation
Color intensity	8.54	OIV
Absorbance 420 nm	2.65	Spectrophotometer
Absorbance 520 nm	4.99	Spectrophotometer
Absorbance 620 nm	0.89	Spectrophotometer
Total polyphenols index	39.10	Spectrophotometer (280 nm)

Table 2. Allelic patterns (means ± SD) and fixation index across wild grapevine populations from Spain and La Minilla population

	Population (No. of individuals)		
	La Minilla (63)	Northern (87)	Southern (131)
Na	6.375±0.596	8.25±0.796	9.125±0.757
Ne	4.125±0.227	3.875±0.350	4.733±0.397
I	1.379±0.079	1.318±0.096	1.707±0.085
No. of private alleles	0.875±0.479	0.750±0.313	1.500±0.267
He	0.691±0.027	0.635±0.019	0.757±0.022
Ho	0.687±0.028	0.608±0.020	0.668±0.021
F	0.012	0.076	0.121

Na: number of alleles; Ne: number of effective alleles; I: information index; He: expected heterozygosity; Ho: observed heterozygosity; F: fixation index

Total genomic DNA was extracted from young leaves, using the DNeasyTM Plant Mini Kit (Qiagen). Extracted DNA was quantified and used as a working DNA solution of 5 ng/µL. PCR included 11 microsatellites: VVS2 (Thomas & Scott, 1993), VVMD7, VVMD24, VVMD25 (Bowers *et al.*, 1999), VMC1B11 (Zyprian & Töpfer, 2005), VVMD5, VVMD21, VVMD27, VVMD28 (Bowers *et al.*, 1999), VVIV60 (Merdinoglu *et al.*, 2005) and VMC4F3.1 (Di Gaspero *et al.*, 2000). PCR amplifications were analyzed in an ABI 3130 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA) using GeneScan-LIZ 500 as internal marker (Applied Biosystems). Amplicon fragments were sized with GeneMapper 4.0 software.

Allele size and total number of alleles were determined for each SSR (Simple Sequence Repeat). Putative alleles were indicated by their estimated size in bp. Genetic diversity was estimated using the following statistics: number of alleles (Na); mean number of alleles per locus; effective number of alleles (Ne); observed heterozygosity (Ho); expected heterozygosity (He) (Nei, 1973); and fixation index (F), also called inbreeding coefficient. All those statistics were calculated using GenAlex software version 6.0 (Peakall & Smouse, 2006) and the Excel Microsatellite Toolkit (Park, 2001).

Bayesian clustering was applied on the SSR genotype data using the software package STRUCTURE, vers. 2.1 (Falush *et al.*, 2003). Analyses were performed with the total collection (63 unique genotypes). Only accessions with ancestry values superior to 0.7 were included in each population analyses. Admixture model and correlated allelic frequencies were used to analyze the dataset without prior population information. Ten simulations per K value were performed for each number of populations (K) (set from 1 to 10). Burn-in period and Monte Carlo Markov Chain length were set up to 100,000 and 300,000, respectively in each run. To assess the best K-value supported by the data we calculated the second order change of the likelihood function divided by the standard deviation of the likelihood (ΔK) (Evanno *et al.*, 2005).

Ex situ conservation

Micropropagation. From cuttings of the considered population, uninodal explants, 1 cm length were prepared. The explants were disinfected by the following steps: a) washing with water and detergent, gently rinsing with distilled water; b) immersion in 70% ethanol for 45 s; c) immersion in a solution of 45% sodium hypochlorite (3.5 % active chlorine) with some drops of Tween-20 (20 min at 30 ºC with stirring); d) gently rinsing with distilled, sterilized water.

After disinfection, the explants were placed individually in sterile test tubes (25 × 150 mm) with 8 mL of "VID" medium (Sarimento *et al.*, 1992) with 0.13 µmol/L indolebutyric acid (IBA) and 0.32 µmol/L benzylaminopurine (BA) with 0.6% agar, pH 5.7. Each tube was covered with a plastic cap, sealed with parafilm and placed in a growth chamber at 23±2ºC, 30 $\mu mol/m^2 \cdot s$ of light intensity and 16 h of photoperiod. Adaptation to outside conditions was carried out according to Cantos *et al.* (1998).

Conservation in germplasm banks. Sixty explants from the *in vitro* cultures were chosen and cultivated in the same conditions described above. After 15 days, 30 of these explants were cultured at the same conditions (NC) and the other 30 were placed in a fridge at 4 ºC and darkness to slow the growth (DC). After two and six months, 15 explants of each condition were taken and micropropagated again (1 cm and 1 bud) in De Wit tubes with 8 mL of the same medium and cultured at NC conditions of light and temperature. After 30 days the survival, stem length, bud (nodes), lateral shoots and root numbers, and rooting percentages were recorded in all situations considered.

Results

Localization and in situ characterization

Descending the river course, the best conserved portion of the bank forest is situated very close to La Minilla reservoir (37º40'2.8''N, 6º10'47.8''W - 37º40'27.5''N, 6º10'5.5''W) with 87 vines: 53 males and 34 females. Such kind of gallery forest constitutes an azonal formation with deciduous species crossing the Myrtocomunnis-Quercetorotundifoliae series of the climax vegetation of that geographical area (Valle, 2003). Very close to that main populational nucleus there are three more situated along occasional creeks: (37º40'5.7''N, 6º10'9.5''W), with 11 vines, 7 males and 4 females; (37º39'34.3''N, 6º9'25.3''W) with 7 vines, 6 males and 1 female, and (37º39'50.5''N, 6º9'47.2''W) with 10 vines, 7 males and 3 females. In total, the whole population comprises 115 vines.

The approximate phenological calendar was bud swelling: 21 March−3 April; flowering: 15 May−2 June; veraison (onset of ripening): 12 July−10 August; fruit ripening: 16 September−30 September; end of leaf fall: 20 November−7 December.

Main ampelographic descriptors are compiled in Table S1 [suppl.]. The main features found were as follows: A 63% of the vines are males, with a female/male ratio of 0.60. Inflorescences were more abundant

in male individuals than in female plants. Female flowers had reflexed stamens while all the male flowers found corresponded to type I (fully developed stamens, no gynoecium).

Young shoots had low intensity of anthocyanin coloration. Mature leaves were bigger in female plants and mainly wedge-shaped while in male plants leaves were more frequently pentagonal. The male grain of pollen is tricolporate, quite similar to that belonging to hermaphrodite cultivars, and the female one is a unaperturate ovoid sac.

Only female plants had berries. All the berries were small, rounded and reddish. The seed morphology is subspherical with a small beak. Wide/length ratio is higher than in the cultivated varieties.

Analysis of soil

The results of soil analysis are shown in Table 3. According to the percentages of fine sand, silt and clay the soil studied is classified as sandy loam (Haplic Fluvisol). The pH was 7.29, classified as neutral. Electrical conductivity was only 0.052 mS/cm, very low in relation to levels considered normal (< 4 mS/cm) (Báscones, 2004). Level of organic matter was normal and, in consequence, also C and N contents, with a C/N rate of 10.7 considered as medium level (Báscones, 2004). Soil was very poor in available phosphorous. Concentration of available potassium was also very low if compared to the 200 mg/kg of available potassium of normal agricultural soils (Pérez de Mora *et al.*, 2006). Because the percentage of $CaCO_3$ was very low, the available calcium levels were also low. On the contrary, available magnesium concentration, 274 mg/kg (Table 3), showed higher contents that those considered normal ranging from 180 mg/kg (González *et al.*, 2003), to 120 mg/kg (Báscones, 2004). Na level was within normal values and Fe concentration was slightly high.

Other microelements considered as toxic above certain thresholds as Ba, Cd, Cu, Mn, Pb, S and Zn presented normal values (Table 3).

Table 3. Character fertility and presence of heavy metals on soil of the wild grapevine population of Rivera de Huelva.

Parameter	Values
Coarse-grained sand (%)	77.7
Fine-grained sand (%)	4.2
Silt (%)	8.8
Clay (%)	9.2
pH	7.29
$CaCO_3$ (%)	0.9
CE (Ext 1:5) (mS/cm)	0.052
Organic matter (%)	1.39
Available K (mg/kg)	41.0
Available Ca (mg/kg)	1090
Available Mg (mg/kg)	274
Available P (mg/kg)	1.9
C (%)	0.806
N (%)	0.075
C-N rate	10.7
B (mg/kg)	4.72
Ba (mg/kg)	95.29
Cd (mg/kg)	0.01
Cu (mg/kg)	23.74
Fe (mg/kg)	34252.67
Mn (mg/kg)	491.67
Na (mg/kg)	233.76
Pb (mg/kg)	20.41
S (mg/kg)	135.95
Zn (mg/kg)	71.82

Sanitary status

On roots, no symptoms of infestation or infection attributable to Phylloxera, root-not nematodes or mycelium of rot fungi were detected.

In the vine leaves, damages caused by mites, concretely by the Erineum strain of *Colomerus vitis* were very frequent in all the vines progressively developing from March to the end of July. The percentage of leaves affected varied usually between 10 to 20%. Symptoms of the presence of *Calepitrimerus vitis* (Nalepa) (Acari, Eriophyidae) were scarcer, developing from March up to the beginning of June, and affecting from 3 to 18% of the leaves.

Small spots of infestation caused by larvae and adults of *Haltica ampelophaga* Guérin and Méneville (Coleoptera, Chrysomelidae) were very occasionally found on leaves. Several shoots and leaves showed bites caused by *Decticus albifrons* (Fabricius) (Ortopthera, Tettigonidae) and *Ephipiger ephipiger* Fiebig (Ortopthera, Tettigonidae). In June 2012, a very dry year, several specimens of *Doctiosaurus maroccanus* (Thunberg) (Ortopthera, Acrididae) were also detected in the external part of the gallery forest, but there was no evidence of feeding on vines. On the other hand, some small colonies of *Aphis gossypii* Glover (Hemiptera, Aphididae) were present only on 7 vines between middle of May to the end of June.

About the origin of the fungal diseases it is necessary to remark that in the surrounding area where this study was carried out there are not vineyards, only some cottages and ancient buildings with very isolated vines. The nearest wine producing area is about 40 km far from the studied zone. The presence of symptoms caused by *Erysiphe necator* (Schweinitz) Burrill was common in most of the vines, mainly on leaves and shoot axes, affecting between 10-21% of the leaves and around a 15%

of bunches. Oil spots on leaves together with other damages on shoot axes and bunches caused by downy mildew (*Plasmopara viticola* (Berkeley and Curtis) Berlease and de Toni), were also frequent mainly in those vines situated in the shadiest places of the gallery forest, where the level of infection on leaves reached 17% and around 9% on bunches. Symptoms caused by *Botrytis cinerea* Pearson were not found any year.

Regarding the possible virus infections, all the ELISA tests were negative for *Grapevine leafroll, Grapevine fan leaf* and *Grapevine fleck viruses*. No symptoms were detected in the wild vines.

Groups of pedunculated eggs laid by *Chrysoperla carnea* (Stephens) (Neuroptera, Chrysopidae) are frequent. About coleopterans, two species of ladybirds were identified: *Coccinella septempunctata* L. (Coleoptera, Coccinelidae), the most frequent species, and *Adalia decempunctata* (L.) (Coleoptera, Coccinelidae).

The maximum number of mobile forms of predatory mites per erineum of *C. vitis*, was three. The species found were: *Typhlodromus phialatus* Athias-Henriot (Acari, Phytoseiidae), *Neoseiulella litoralis* (Swirski and Amitai) (Acari, Phytoseiidae), *Euseius stipulatus* (Athias-Henriot) (Acari, Phytoseiidae) and *Phytoseiulus persimilis* Athias-Henriot (Acari, Phytoseiidae)

Microvinification

Analysis of the microvinification is shown in Table 1. The experimental wine resulted with medium concentration of alcohol, similar to that obtained with traditional cultivars from the region. The total acidity was relatively high, and consequently the pH value was much lower than that obtained with traditional Andalusian cultivars. It is a very important characteristic to highlight, because it would need no addition of tartaric acid to give stability. The volatile acidity was slightly high due to the absence of potassium metabisulphite addition. The color intensity is medium for a red wine due to the irregular degree of maturation of the berries, but it was not possible to delay the harvest due to heavy incidence of berry-consuming birds. The concentration of methanol is correct, and fulfills Spanish legislation, that allows up to 400 mg/L. The dry extract is normal for a red wine and the total polyphenols index measured at 280 nm is also normal.

Genetic analysis

Genetic diversity of the wild populations. The results of the genetic diversity analysis, according to DNA microsatellite genotyping are shown in Table 2. These genotypes showed a mean value of 6.3 alleles including an average of 0.875 private alleles (alleles found in a single population throughout the study region). Genetic comparisons between the wild grapevine genetic groups from the Iberian Peninsula named here as Northern and Southern Spain wild populations (De Andrés *et al.*, 2012) and La Minilla allowed to find out that the high number of private alleles presented in La Minilla population is similar to the number found in the Northern and Southern wild populations (Table 2). This result pointed out that some alleles in these populations are not present in the other Spanish populations. The mean number of alleles between the different populations ranged from 6.3 to 9.1. The number of effective alleles (Ne) ranged between 3.4 and 4.6. The information index (I) ranged from 1.3 to 1.7; Ho ranged between 0.608 and 0.687, whereas the values of He were slightly higher, ranging between 0.636 and 0.766. The fixation index (F) showed values ranging from 0.012 to 0.121, supporting the existence of inbreeding depression in some wild grape populations.

Population structure between wild grapevine populations from the Iberian Peninsula. The genetic structure analysis included Northern and Southern Spain wild grapevine populations (218 samples) and the wild accessions from La Minilla (63 samples). The choice of a fixed number of populations (K) is arbitrary, each of them characterized by a set of allele frequencies at each locus. Individuals in the sample are probabilistically assigned to genetic groups or jointly to one or more groups. Consequently, we performed an analysis where K varied from 1 to 10. This analysis showed a large increase of likelihood from K=1 to K=3 and smaller increases from K=3 to K=10. Using the methodology of Evanno *et al.* (2005), this result supports K=3 as the most likely number of genetic groups, which corresponds to wild individuals from Northern, Southern and La Minilla populations (Fig. 1). Then, it may be supposed that La Minilla genetic group comprise a unique population that could be the result of fragmentation due to the recent human alteration of the habitat.

Ex situ conservation

Micropropagation. The in vitro response of the explants obtained directly from the field individuals is indicated in Table 4. After 30 days of in vitro culture, the 64.9% of the explants formed stem and leaf with good growth (averages of 5.5 cm length stem, 9.2 nudes and 1.6 of lateral shoots), and 97.8% of rooting, with an average of 3.5 roots per plant with 7.3 cm of mean length with a very good aspect. In consequence, the acclimation to outside conditions was 80%.

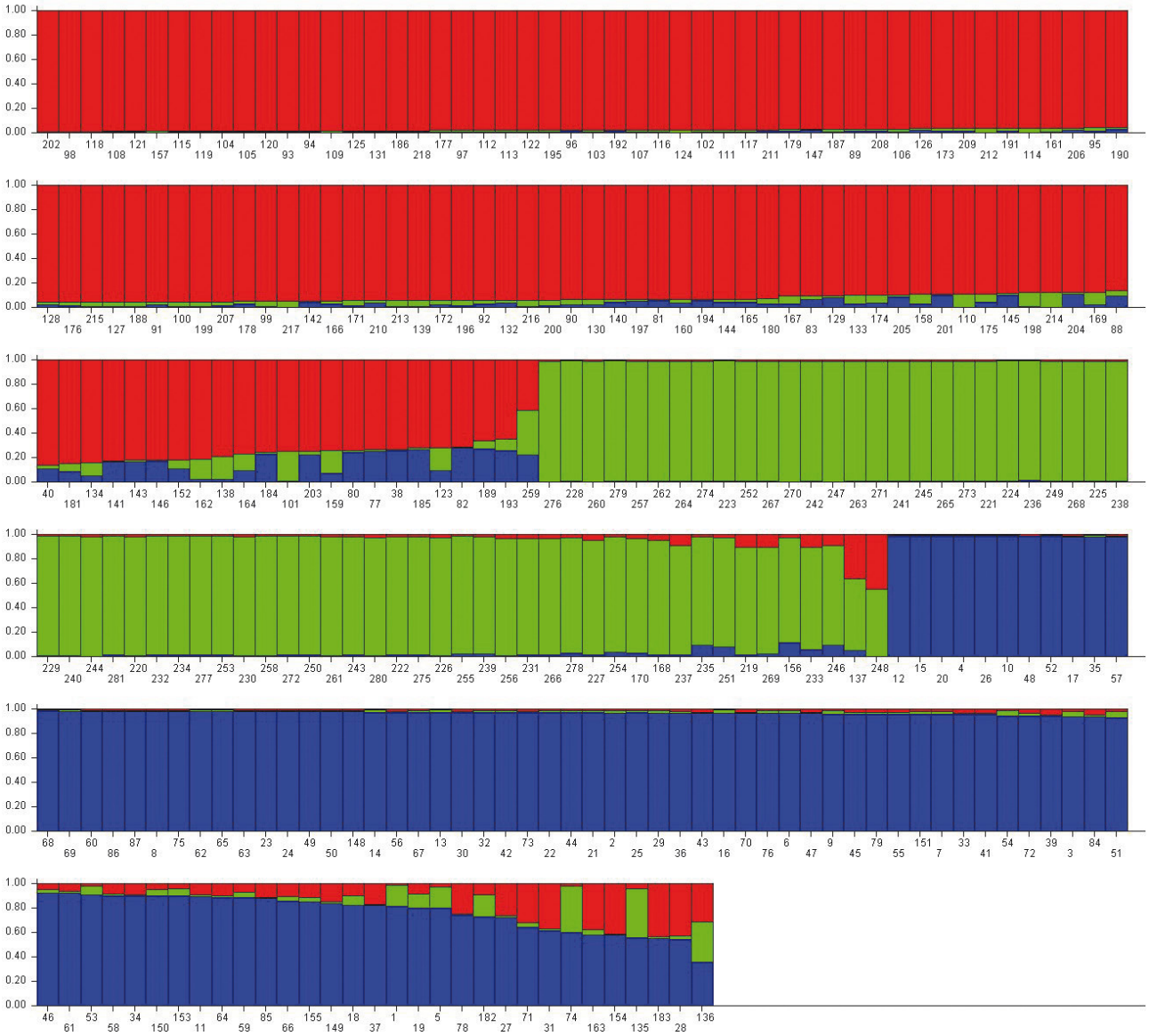

Figure 1. Graphical representation of ancestry membership coefficients of all 281 Spanish wild individuals. Each individual is shown as a vertical line divided into segments representing the estimated membership proportions in the three genetic clusters inferred with STRUCTURE. Red color corresponds to Southern wild individuals, green corresponds to La Minilla wild individuals and blue to Northern wild individuals.

Conservation in germplasm banks. Survival rate of explants was high after 2 months for both normal (NC) and dark cold room (DC) conditions. Although DC treatment showed more survival rate than NC cultures the difference was not significative, ranging from 80 to 100%. After 6 months of culture, survival rate fell to 40% both for NC and DC treatment (Table 5).

The DC treatments reduced the explants growth compared to NC cultures (Table 5). No great differences were observed between 2 or 6 months of culture in DC conditions. In standard conditions, 6 months of culture resulted in lower growth, both in terms of stem length and number of buds (Table 5). The mean number of lateral shoots per plants although higher in the NC culture, showed no significant differences between treatments (Table 5).

Rooting percentage was 100% for both culture conditions independently of the storage period. Mean number of roots was similar for all the conditions except for NC after 2 months that was higher (Table 5).

Discussion

According to the literature already revised in the Introduction section, this is the largest population found

Table 4. Micropropagation response of explants from Rivera de Huelva after 30 days of culture

Variable	Value
Explants number	99
Formed stem (%)	64.9
Stem length (cm)	5.5
Number of buds (nodes)	9.2
Number of lateral shoots	1.6
Rooting (%)	97.8
Number of roots	3.5
Root length (cm)	7.3

in the Mediterranean area of the Iberian Peninsula (Ocete *et al.*, 2002; Cunha *et al.*, 2004), where the distribution of this grapevine subspecies is highly fragmented. This natural riparian habitat has been intensely modified by man along the history. A proof of it is the presence of a Roman aqueduct ruins inside the location studied in the present paper.

The evaluation of the ampelographic characters is very similar to that described for other Andalusian wild grapevines (Ocete *et al.*, 2007). The sexual structure of female and male flowers is similar to the rest of the Spanish populations (Ocete *et al.*, 1999). the female pollen grain featured an unaperturate ovoid sac as described by Gallardo *et al.* (2009) in other Andalusian populations. Only female plants had berries, like in the case of the Spanish populations (Ocete *et al.*, 1999). Their seed typologies are close to the wild grapevine seeds described by Terral *et al.* (2010).

Wild grapevine is sensitive to Phylloxera under artificial laboratory conditions. The absence of symptoms on roots in the wild ecosystems from the Iberian Peninsula to the Caucasian region seems to be due to soil flooding several months each year. This edaphic condition could also determinate the absence of symptoms caused by species belonging to *Meloidogyne* and some root rot fungal species, as reported by Ocete *et al.* (2007).

The very scarce infestation caused by the coleopteran *H. ampelophaga* constitutes the first citation of this species in wild populations from Portugal to the Republic of Georgia. This coleopteran had an important incidence on Spanish vineyards from, at least, the 16th century (De Herrera, 1513) up to the sixties of the 20th century, until synthesis pesticides became routinely used (Hidalgo, 2002).

As in the rest of the Andalusian populations, *Colomerus vitis* (Pagenstecher) (Acari, Eryophidae) is present on all the vines sampled while infestations by *Calepitrimerus vitis* are less frequent (Ocete *et al.*, 2007). Probably, these monophagous species were imported from natural habitats to first vineyards during the domestication process started around 8,000 B.P in the Caucasian region (Ocete *et al.*, 2007).

On the contrary, damages caused by downy and powdery mildews were imported from American grapevine species along 19th and 20th centuries.

This population conserves a wide diversity of natural enemies of pests, as is frequent in other populations found in Andalusia region (Ferragut *et al.*, 2008). The neuropteran *Chrysoperla carnea* is very common in the Western area of the Iberian Peninsula, covered mainly by oak tree species, feeding on aphids, white flies and mites (Marín & Montserrat, 1987). This neuropteran has also been found feeding on the same arthropods that live on wild grapevine populations (Ferragut *et al.*, 2008). About coleopterans, the two species of ladybirds found are common in natural flora (Cardoso & Gomes, 1986) and crops (Ripollés, 1990; Down *et al.*, 2000) of the Iberian Peninsula.

In the case of Phytoseiidae species, its distribution varies with the location of the wild grapevine populations through different European regions, according to the very scarce literature existing on this subject (Gallardo, 2005). Therefore, along the Rhine´s flood plains in Germany and France, the most frequent predators were *Euseius finlandicus* (Oudemans), *Neuseiulella tiliarum* (Oudemans), *Typhlodromus tiliae* (Oudemans), *Phytoseius sp.* (Ocete *et al.*, 2000). *T. phialatus* can also be found in most of the Spanish Guarantees of Origin (Villaronga *et al.*, 1991) and on spontaneous flora situated around several French vineyards (Tixier *et al.*, 2000). *P. persimilis* is common in the Iberian Peninsula in natural habitats and different agroecosystems (García-Mari *et al.*, 1987) and also on wild grapevine species from USA (Karban *et al.*, 1995). It constitutes a trading species used in biological control of

Table 5. Survival and biometry of stem and root produced by Rivera de Huelva wild grapevine plantlets from normal (NC) and darkness and cold conditions (DC) after 2 and 6 months periods.

Months	Survival (%)		SL (cm)		NB		NLS		R (%)		NR	
	NC	DC	NC	DC	NC	DC	NC	DC	NC	DC	NC	DC
2	80 A	100 A	7.4 B	2.1 A	8.5 B	3.7 A	1.7 A	1.2 A	100 A	100 A	5.4 B	3.0 A
6	40 A	40 A	6.0 B	2.2 A	7.7 B	3.0 A	1.5 A	1.0 A	100 A	100 A	3.8 A	3.0 A

SL, stem length; NB, number of buds; NLS, number of lateral shoots; R, rooting; NR, number of roots. Different letter in rows mean differences ($p \geq 0.05$) between NC and DC at the same month.

Tetrannychiide, mainly to control *Tetranychus urticae* (Koch), as it occurs in Andalusian greenhouses (García-Mari, 1994). Despite *E. stipulatus* was found in a short number of erinea, this is probably the most representative Phytoseidae on traditional Mediterranean crops. This mite, together with other species of the same genus, such as *T. phialatus*, *T. rhenanoides* and *T. phialatus*, control *Panonychus ulmi* (Koch) (Acari, Tetranychidae) on pear and apple tree orchards from Northeastern Spain (Sarasúa *et al.*, 2000).

Only a few adults of *N. litoralis* were found in our samples. This species is relatively common on coastal crops, mainly in Middle East (De Moraes *et al.*, 2004).

The results obtained from *in vitro* propagation meant a very high *in vitro* precocity of this wild plant material, higher than the cultivated one, which normally needs at least 15 days more of culture to reach similar level of development (Gribaudo *et al.*, 1995; Troncoso *et al.*, 1999).

It is interesting to emphasize that the wild grapevine explants developed contemporarily shoots and roots in the culture medium, giving new plants in only one *in vitro* culture stage indicating a good balance of nutrients and growth regulators. Thus, the possibility of obtaining complete plants with only one *in vitro* stage saves time and costs in the propagation process. Lateral shoot formation increases multiplication factor and allows using a complete stem with much more possibilities of survival and development. It was possible to attain healthy self-rooted plants for an adequate adaptation to *ex vitro* conditions. Thus, following the indicated method for plant transplanting and hardening to outside conditions, an 80% of surviving plant was reached.

Survival is the most important factor to consider in conservation assays as it defines the efficiency of the process. Explant growth was reduced by dark and cold culture but a sufficient number of healthy plants could be obtained after 6 months. In NC conditions the growth, measured by stem length and number of buds formed, decreased slightly after 6 months of culture likely due to medium ageing and nutrient depletion. Nevertheless, the number of lateral shoots per explant did not seem to be affected by any of the storage conditions tested.

From the results of these experiments, a robust, quick and economical method, for the *in vitro* propagation and conservation of wild grapevine plants was set up.

Only about 20% of berry weight could be transformed into must due to the small berry size (0.8-1 cm of diameter), resulting in a low flesh/berry ratio, and the presence of big pips as in other Iberian regions such as Navarra (Ocete *et al.*, 2011b) and La Rioja (Ocete *et al.*, 2011a). The color intensity is lower than those obtained in microvinifications using wild grapevines from Cádiz province (Ocete *et al.*, 2007) and Sardinia (Lovicu *et al.*, 2009), where the values were 12 and 14 respectively (Table 1). According to the microvinification analysis (Table 1) it is a young wine, which according to absorbance data at different wavelengths, has as color components yellow (30.58%), red (58.56%) and violet (10.51%), being then very well balanced.

Precise detection and quantification of genetic variation is prerequisite for the successful conservation and exploitation of plant genetic resources. The results of the genetic analysis showed that La Minilla still harbors a number of wild grapevine accessions with low levels of heterozygosity (He). Similar result has been observed in wild grapevine populations analyzed in Morocco, Sardinia, Portugal, France or Italy (Di Vecchi-Staraz *et al.*, 2009; Cunha *et al.*, 2010; Zecca *et al.*, 2010; Zinelabidine *et al.*, 2010). However, we have detected that the observed heterozygosity (Ho) was not significantly lower ($p \leq 0.05$) than expected heterozygosity (He) in La Minilla populations, in parallel with the estimation of F values close to zero (Table 2). Since F values near 0 indicate random mating, the F values found for La Minilla wild populations are consistent with a random mating population status. At the same time, the relative lower genetic diversity in this population may be due to the sample size, because a positive correlation of number of alleles with sample size is expected (De Andrés *et al.*, 2012). However, the identification of private alleles in each wild population is valuable and confirms the importance to establish a conservation program of new wild populations from La Minilla. The analysis of the genetic structure reveals three main genetic groups corresponding to Northern, Southern Spain and La Minilla populations. These genetic groups showed very high average probability of assignment to their own cluster, in agreement with the hypothesis that they are genetically distinct. The specific alleles present in each wild population strongly support the importance of conservation of this wild germplasm.

There does not exist any legislation on the preservation of wild grapevines in Andalusia nor in Spain and in consequence this subspecies is not provided in the mandatory previous studies of environmental impact required for public works. Thus, in the case of this population 35 vines were completely destroyed during the cited period of re-building of the bridge. It is necessary to start an active program to stop the constant and alarming loss of wild grapevines in the region claimed by Ocete *et al.* (2007), including a legal figure of preservation and to start programs for *in situ* protection and restoration of

natural habitats as in the cases of France, Austria, Germany and Hungary. It is necessary to remember that wild grapevine is a species well represented in the Andalusian landscape along millennia. In this territory, the first location of *Vitis* pollen was found in El Padul bogs (Granada province) belonging to initial Upper Pleistocene phases (Florschütz *et al.*, 1971). Also, pollen remains are very abundant in the survey carried out in the Laguna de Las Madres (Huelva province), dating back to the 4,500 BP (Stevenson, 1985), in an area very close to the actual relic wild grapevine populations located in Doñana National Park, Biosphere Reserve and World Heritage Site (Ocete *et al.*, 2007).

The evolutionary process of plant domestication by humans led to morphological, physiological, behavioral and genetic differentiation of a wide range of species from their wild progenitors (Gerbault *et al.*, 2014).

As a conclusion, those wild resources and traditional varieties could be vital to ensure the future and sustainability of viticulture. They constitute a tremendous source of information and genetic material to be used in cultivar breeding, and to adapt them also to the new challenges of the sector and the market demands. In this work a relic population of wild grapevine has been described. According to genetic analysis results, this is a genetically unique population useful for cultivar improving.

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