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Population biology/Biologie des populations

Current distribution and characterization of the wild grapevine populations in Andalusia (Spain)

Distribution et caractérisation actuelles des populations sauvages de vigne en Andalousie (Espagne)

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ABSTRACT

For decades, human activities have gradually destroyed the natural habitats of wild grapevine, *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi, and nowadays this species is endangered in southern Europe. In this paper, 94 populations of this species have been localized and characterized in the Andalusian region in the Iberian Peninsula between 1989 and 2013. Location, ecological aspects, and sanitary characteristics are described. Must properties and *in vitro* tolerance to calcareous conditions were also checked. The paper also contains a global description of female and male individuals. Two hundred individuals from six river basin populations have been sampled, and their genetic structure analyzed by using 25 nuclear microsatellites loci to investigate the gene diversity of wild grape populations in Andalusia at two levels: total individuals and at river basin populations. Also, the genetic relationship of wild and cultivated accessions has been tested. Wild grapevine is considered the ancestor of the cultivated varieties and should be preserved as this material could be used to start breeding programs of cultivated varieties and also to restore riverbank forests, which constitute one of the worst preserved ecosystems in the area.

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RÉSUMÉ

Pendant des décennies, les activités humaines ont progressivement détruit les habitats naturels de la vigne sauvage, *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi et, de nos jours, cette espèce est menacée dans le Sud de l'Europe. Dans cet article, 94 populations de cette espèce ont été localisées et caractérisées en Andalousie, dans la péninsule Ibérique, entre 1989 et 2013. L'emplacement, les aspects écologiques et les caractéristiques sanitaires sont décrits. Les propriétés du moût et la tolérance *in vitro* aux conditions calcaires ont été également vérifiées. L'étude contient pareillement une description globale des individus féminins et masculins. Deux cents individus de six populations de bassins fluviaux ont été échantillonnés et leurs structures génétiques analysées en utilisant 25 microsatellites nucléaires pour étudier la diversité génétique des populations de raisins sauvages en Andalousie à deux niveaux : les individus totaux et les populations de bassins fluviaux. De plus, la relation génétique des accessions sauvages et cultivées a été testée. La vigne sauvage est considérée comme l'ancêtre des variétés cultivées et elle doit être préservée, car elle pourrait être utilisée pour lancer des programmes de sélection de variétés cultivées et pour restaurer les forêts riveraines, qui constituent l'un des écosystèmes les plus préservés de la région.

1. Introduction

Andalusia is a Spanish region situated in the South of the Iberian Peninsula, spreading out 87,268 km^2 with a coastline around 800 km long. Due to its location the region is under a Mediterranean climatology. Grapevine has been present in this territory from ancient times as proves the pollen grains conserved in El Padul bog (Granada province) attributed to the Middle Pleistocene [1] or in the Laguna de Las Madres in Mazagón (Huelva province) datable back to 4500 BP [2].

Several archaeological findings demonstrate that grapevine cultivation existed in the region throughout the first millennium BC [3], mainly linked to Phoenician colonies situated along the Atlantic and Mediterranean coasts of Andalusia [4,5].

More recently, before the arriving in the 19th century of the North American parasitic species, powdery and downy mildews and phylloxera, 119 cultivars could be found in the region [6]. At present, Zalema, Palomino fino, Pedro Xímenez, Muscat of Alexandria, and other minor varieties, such as Tintilla de Rota, Rome and Vigiriega among others, are practically the only traditional cultivars still in use in the area [7]. Meanwhile, Tempranillo and several international varieties, mainly Cabernet Sauvignon, Merlot, Petit Verdot, and Shyrah, have recently spread in the area in order to improve red wine quality.

Wild grapevine (*Vitis vinifera* L. ssp. *sylvestris* (Gmelin) Hegi) is a dioecious subspecies considered the parental of the cultured grapevine (*Vitis vinifera* L. ssp. *sativa* (DC) Hegi). In fact, microsatellite DNA analysis has shown that Iberian wild vines have provided the A chlorotype to autochthonous grapevine cultivars from Andalusia and other Iberian regions [8].

Wild grapevine was linked to different human activities in the Iberian Peninsula, and concretely in Andalusia it has been used during millennia to produce must, wine, vinegar, ropes, fishing traps and also as rootstock [9,10]. Bunches have been found in burials from the Argar culture as a part of funerary rituals [11]. The seeds found have morphological characteristics similar to those of the wild ones described by [12]. Berries were collected to produce homemade vinegar in the provinces of Cádiz and Jaén, and stems were used to produce fishing traps for lobster in the province of Cádiz up to about 25 years ago [13]. As stated above, the number of cultured grape varieties has been drastically reduced, leading to a huge loss of biodiversity in the Andalusian vineyard. This fact constitutes a serious drawback in the case of the appearance of new pests and diseases as well as to face the forthcoming climate change. Genetic diversity is crucial for food production, for the environment, and for sustainable development [14]. In this context, it should be very important to prospect and conserve the wild parentals of the current crops as wild grapevine is.

Moreover, some wild grapevine populations show a higher tolerance to pests and diseases [15] or to soil lime [16,17] and, possibly, to saline soils [18]. Also, their musts provide high intensity of color and good level of acidity, interesting characteristics for the production of quality red wines in Mediterranean areas [19]. These traits could be of high interest considering that global climate change will probably affect viticulture in next future and convert wild grapevine into a genetic pool useful for breeding [20].

On the other hand, the ability of some wild grapevines to store high concentrations of copper in roots from contaminated soils opens up a new research field on the phytoremediation capabilities of wild grapevine [21,22].

Considering the relevance of genetic resources for the future of the crop and their current and increasing scarcity, major efforts should be dedicated to the collection and characterization of this subspecies [23], cited in the white book on the Andalusian phytogenetic resources to counterbalance the risk of genetic erosion [24].

Due to diverse anthropic impacts on natural habitats of wild grapevine, including the presence of invasive vines, such as American rootstocks and direct producer hybrids, their populations are disappearing in an alarming way,

[25–31], leading wild grapevine to be considered as an endangered subspecies in Europe [32]. Consequently, the International Organization of Vine and Wine (OIV) has exhaustively recommended through Resolution OVI-VITI 424/2010 and COST Action FA1003 the *in situ* and *ex situ* conservation of this dioecious subspecies of grapevine. Within this context, information on the distribution, ampelographic characterization, sanitary status and genetic diversity is crucial for the development of conservation strategies in whichever territory [23].

Until now, no global inventory of wild grape individuals is available in Andalusia. For this reason, it is difficult to know the relationship between cultivated and wild grapevine individuals. In the present study, we have established an inventory of wild grape in Andalusia and analyzed the genetic diversity of these populations.

Accordingly, the aim of the present paper is to know the current distribution and main habitats of the relic fragmented populations of wild grapevine in the region, to establish a global ampelographic description of male and female vines along their phenological development, to know its sanitary status, and to investigate its main genetic characteristics, to evaluate its lime tolerance in soil in order to compare with traditional cultivars of the region.

2. Material and methods

2.1. Prospection of wild grapevine populations

Prospection for vines was accomplished between 1989 and 2013 in gallery forests situated along main water courses, their tributaries and nearby creeks of the Andalusia river basins. Identification of dioecious wild grapevine was carried out in flowering time from 15 May to 10 June. The coordinates of each population were registered using a GPS.

The plant sampling strategy was the same for all populations, and was designed to prevent collecting individuals from cultivated subspecies (*Vitis vinifera* L. subsp *sativa*) and rootstocks instead of wild plants. To further reduce this risk, only dioecious individuals were collected, since only cultivated individuals are hermaphrodites.

2.2. Description of the populations

The main ampelographic descriptors were evaluated according to OIV [33] systematic list between 2010 and 2013. Pollen samples from flowers were obtained by brushing mature anthers from male and female vines of each location. Grains were included in DPX (Fluka) and observed under an optical microscope Olympus BX 61 to study the morphological structure of the grains.

Observations on the phenological development of the vines were carried out twice per month to establish an approximate calendar from sprouting to leaf fall.

Main botanical supporters and the rest of the accompanying vegetation were identified using general botanical keys and the studies carried out on Western and Eastern Andalusia by [34] and [35], respectively. The nomenclature followed was unified according to criteria of Flora Ibérica updates [36].

2.3. Sanitary status

The detection of possible symptoms caused by pests and diseases was performed from 2010 to 2013, in spring and autumn, on shoots, leaves and bunches of plants situated up to 3 m of height. The intensity of damages caused by parasitic species was evaluated on leaves according to the following grade system: 1–3 means symptoms affecting up to a 20% of the leaves, 5–7 between 20–40%, and 9 more than 40 %.

To observe the possible symptoms caused by subterranean phytophagous and pathogens, the roots were unearthed up to 40–50 cm in depth. The samples of root hairs were observed under a binocular microscope to detect possible damages caused by phylloxera, root-knot nematodes, and rot fungi.

To determine the level of Grapevine Fan Leaf Virus (GFLV) infection, one adult leaf per plant of each population was sampled at the end of spring. Every leaf was washed gently first with tap water and then with distilled water. After that, the mesophyll of each leaf was cut into small pieces and analyzed by the ELISA test (Bioreba) according to [37].

2.4. In vitro tolerance to calcareous soils

Upon prospection was completed, soil lime tolerance was checked in two wild grapevine populations: 14/Montoro/4 (number 33, Table 1) and 14/Rute/1 (number 43, Table 1). Both populations are vigorous and well developed, but the first one is growing in a lime deprived soil (Fluvisol Humic), while the second one lives on an hypercalcic soil with a lime content ranging from 66.7 in the first 40 cm of depth to 62.2% at deeper levels (40–80 cm) [17]. Axillary buds were taken from individuals of both populations and washed with water and household detergent before gently rinsing with distilled water. The buds were then sterilized by immersion in absolute ethanol (1 min) and thereafter in a solution of sodium hypochlorite 20% (5% of active chloride) with some drops of Tween-20, for 20 min and finally rinsed three times with sterilized water (5 min each time). They were then placed individually into sterile test tubes (21×150 mm) with 8 ml of the nutritive medium reported by [38], modified to include 0.32 μM of benzylaminopurine (BAP) and 0.13 μM of naphthalene acetic acid (NAA) as growth regulators. The tubes were covered with polypropylene caps, sealed with parafilm and placed in a culture chamber at 24 °C, 30 $\mu E \cdot m^{-2} \cdot s^{-1}$ of light intensity and a photoperiod of 16 hours of light. In addition, plants of the hybrid rootstock "41B" (*Vitis vinifera* L. cv. Chasselas × *Vitis berlandieri* Planch) were used for comparison with the two wild grapevine populations. This rootstock is considered as lime-tolerant [39,40] and widely used in current viticulture on calcareous soil. The material of this rootstock was taken from the *in vitro* germplasm bank of the Institute of Natural Resources and Agrobiology of Seville (IRNAS) (CSIC). Buds from the three accessions were subcultured

Table 1
Geographical situation of the populations found.

Population number	Population name	Province	Longitude	Latitude	Height
1	11/Alcalá de los Gazules/1	Cádiz	−5.64611 W	36.36444 N	0/50
2	11/Alcalá de los Gazules/2	Cádiz	−5.64194 W	36.36778 N	0/50
3	11/Alcalá de los Gazules/3	Cádiz	−5.70139 W	36.42889 N	50/100
4	11/Alcalá de los Gazules/4	Cádiz	−5.59389 W	36.40361 N	250/300
5	11/Alcalá de los Gazules/5	Cádiz	−5.5925 W	36.41222 N	200/250
6	11/Alcalá de los Gazules/6	Cádiz	−5.62444 W	36.34583 N	50/100
7	11/Arcos de la Frontera/1	Cádiz	−5.56306 W	36.71194 N	200/250
8	11/Benaocaz/1	Cádiz	−5.49278 W	36.72361 N	250/300
9	11/El Bosque/1	Cádiz	−5.50361 W	36.74389 N	300/350
10	11/El Bosque/2	Cádiz	−5.49611 W	36.72444 N	250/300
11	11/Grazalema/1	Cádiz	−5.49639 W	36.76972 N	250/300
12	11/Jerez de la Frontera/1	Cádiz	−5.55417 W	36.56167 N	500/550
13	11/Jerez de la Frontera/2	Cádiz	−5.59111 W	36.5575 N	400/450
14	11/Los Barrios/1	Cádiz	−5.54306 W	36.20917 N	0/50
15	11/Los Barrios/2	Cádiz	−5.56722 W	36.18944 N	150/200
16	11/Prado del Rey/1	Cádiz	−5.5475 W	36.76333 N	250/300
17	11/Sanlúcar de Barrameda/1	Cádiz	−6.32056 W	36.86806 N	0/50
18	11/Ubrique/1	Cádiz	−5.47333 W	36.6525 N	250/300
19	11/Ubrique/2	Cádiz	−5.45139 W	36.65778 N	250/300
20	11/Ubrique/3	Cádiz	−5.44167 W	36.64389 N	300/350
21	11/Ubrique/4	Cádiz	−5.44667 W	36.6375 N	400/450
22	11/Vejer de la Frontera/1	Cádiz	−5.98583 W	36.28389 N	100/150
23	11/Villamartín/1	Cádiz	−5.57361 W	36.87917 N	150/200
24	11/Zahara de la sierra/1	Cádiz	−5.49861 W	36.82306 N	300/350
25	14/Adamuz/1	Córdoba	−4.52611 W	38.07639 N	350/400
26	14/Córdoba/1	Córdoba	−4.65722 W	37.94667 N	100/150
27	14/Córdoba/2	Córdoba	−4.65611 W	37.94056 N	100/150
28	14/Córdoba/3	Córdoba	−4.6375 W	37.94444 N	100/150
29	14/Hornachuelos/1	Córdoba	−5.20861 W	37.77333 N	50/100
30	14/Montoro/1	Córdoba	−4.29028 W	38.05722 N	200/250
31	14/Montoro/2	Córdoba	−4.28417 W	38.07556 N	300/350
32	14/Montoro/3	Córdoba	−4.31 W	38.11194 N	350/400
33	14/Montoro/4	Córdoba	−4.2725 W	38.13167 N	500/550
34	14/Montoro/5	Córdoba	−4.35722 W	38.00861 N	150/200
35	14/Posadas/1	Córdoba	−5.10972 W	37.85111 N	100/150
36	14/Posadas/2	Córdoba	−5.09472 W	37.85806 N	200/250
37	14/Posadas/3	Córdoba	−5.11639 W	37.89056 N	300/350
38	14/Posadas/4	Córdoba	−5.16611 W	37.86944 N	200/250
39	14/Posadas/5	Córdoba	−5.12139 W	37.82472 N	100/150
40	14/Posadas/6	Córdoba	−5.17917 W	37.78639 N	50/100
41	14/Posadas/7	Córdoba	−5.18111 W	37.78417 N	50/100
42	14/Posadas/8	Córdoba	−5.17861 W	37.79 N	50/100
43	14/Rute/1	Córdoba	−4.35472 W	37.38139 N	500/550
44	14/Villaviciosa/1	Córdoba	−4.995 W	38.10472 N	450/500
45	14/Villaviciosa/2	Córdoba	−5.12139 W	38.04444 N	500/550
46	14/Villaviciosa/3	Córdoba	−5.1175 W	38.06083 N	600/650
47	14/Villaviciosa/4	Córdoba	−5.03444 W	38.03417 N	500/550
48	14/Villaviciosa/5	Córdoba	−5.06833 W	38.0075 N	450/500
49	18/Loja/1	Granada	−4.09393 W	37.20836 N	550/600
50	21/Almonte/1	Huelva	−6.39056 W	36.86056 N	0/50
51	21/Almonte/2	Huelva	−6.38806 W	36.86417 N	0/50
52	21/Almonte/3	Huelva	−6.38806 W	36.87472 N	0/50
53	21/Almonte/4	Huelva	−6.50397 W	37.12049 N	0/50
54	21/Almonte/5	Huelva	−6.54611 W	37.14222 N	0/50
55	21/Aroche/1	Huelva	−7.04806 W	37.96667 N	250/300
56	21/Aroche/2	Huelva	−7.03194 W	37.96806 N	250/300
57	21/Aroche/3	Huelva	−7.00556 W	37.96861 N	250/300
58	21/Calañas/1	Huelva	−6.91056 W	37.66667 N	150/200
59	21/Calañas/2	Huelva	−6.9025 W	37.65944 N	200/250
60	21/Cortegana/1	Huelva	−6.85944 W	37.93361 N	550/600
61	21/Cumbres de San Bartolomé/1	Huelva	−6.7875 W	38.02944 N	250/300
62	21/Encinasola/1	Huelva	−6.96139 W	38.1325 N	250/300
63	21/Fuenteheridos/1	Huelva	−6.65861 W	37.90889 N	650/700
64	21/Higuera de la sierra/1	Huelva	−6.46944 W	37.84417 N	550/600
65	21/Rosal de la Frontera/1	Huelva	−7.13639 W	37.97667 N	200/250
66	23/Guarromán/1	Jaén	−3.83556 W	38.08917 N	250/300
67	23/Guarromán/2	Jaén	−3.84361 W	38.08278 N	200/250
68	23/La Iruela/1	Jaén	−2.83406 W	37.99439 N	800/850
69	23/La Iruela/2	Jaén	−2.91803 W	37.95617 N	800/850
70	23/Pozo Alcón/1	Jaén	−2.93229 W	37.71497 N	900/950

Table 1 (*Continued*)

Population number	Population name	Province	Longitude	Latitude	Height
71	23/Santa Elena/1	Jaén	–3.50417 W	38.39722 N	600/650
72	23/Santiago-Pontones/1	Jaén	–2.87361 W	38.01053 N	650/700
73	23/Santo Tomé/1	Jaén	–2.86231 W	38.01486 N	700/750
74	23/Santo Tomé/2	Jaén	–2.89778 W	37.99527 N	700/750
75	29/Antequera/1	Málaga	–4.44639 W	36.92861 N	550/600
76	29/El Burgo/1	Málaga	–4.96528 W	36.78417 N	600/650
77	29/Ronda/1	Málaga	–5.23833 W	36.78222 N	650/700
78	41/Castilblanco de los Arroyos/1	Sevilla	–5.89361 W	37.715 N	150/200
79	41/Cazalla de la Sierra/1	Sevilla	–5.70472 W	37.93194 N	400/450
80	41/Constantina/1	Sevilla	–5.60558 W	37.86581 N	500/550
81	41/El Castillo de las Guardas/1	Sevilla	–6.31139 W	37.71333 N	250/300
82	41/El Castillo de las Guardas/2	Sevilla	–6.32222 W	37.72 N	250/300
83	41/El Ronquillo/1	Sevilla	–6.1775 W	37.70333 N	300/350
84	41/Guillena/1	Sevilla	–6.15694 W	37.65944 N	200/250
85	41/Guillena/2	Sevilla	–6.12083 W	37.58917 N	150/200
86	Peña de los Enamorados	Málaga	–4.48292 W	37.06473 N	450/500
87	Coripe	Sevilla	–5.43244 W	36.88719 N	400/450
88	Algar	Cádiz	–5.65284 W	36.6342 N	150/200
89	Límite Málaga	Málaga	–4.11341 W	36.9183 N	350/400
90	Arroyo Candón	Huelva	–6.75469 W	37.33673 N	0/50
91	Rio del Valle	Cádiz	–5.69956 W	36.10044 N	0/50
92	Playa Punta Paloma	Cádiz	–5.72595 W	36.06683 N	0/50
93	Mesón Sancho	Cádiz	–5.52817 W	36.06963 N	250/300
94	Sendero La Miel	Cádiz	–5.45967 W	36.10738 N	0/50

every 45 days in the same medium to obtain a very homogeneous group of plants. Four different $CaCO_3$ treatments (0, 20, 40 and 60%) with 20 replicates per treatment were tested. The $CaCO_3$ doses were obtained by mixing fine siliceous sand (Quality Chemicals, Ref. 7631-86-9) with finely divided $CaCO_3$ (particle size $< 5\ \mu m$ in diameter) (Panreac Ref. 141212.0416) to the appropriate proportion plus 4 ml per tube of the same medium used for obtaining material by micropropagation. After capping the tubes, they were sterilized by autoclaving. The fine, clay-sized, fraction of $CaCO_3$, or active lime [16,41], maintains high levels of HCO_3 in the soil solution [42], and is therefore a reliable indicator for predicting the development of lime-induced chlorosis [43]. Twenty explants 0.5–1 cm in height, with 1 bud per accession and treatment, were transferred individually to test tubes with the different contents of $CaCO_3$. The explants were subcultured in the culture chamber in the same conditions as those indicated above during 60 days and in the end of experiment, survival, stem length, bud number and rooting percentage were measured.

2.5. Microvinification

Harvest was undertaken in one population of each province on the second week of October 2011. The berries were de-stemmed by hand and the microvinification carried out with the ripest fruits because maturation is not uniform along the same bunch. Fermentation was performed with indigenous yeasts along 10-day maceration with two daily stirrings at 20 °C, without the addition of potassium metabisulfite. Microvinification was characterized by different analytical parameters determined according to the procedures described by OIV [44]: near-infrared for the determination of ethanol concentration; automatic potentiometry for pH and total acidity; FCSA autoanalyser for the volatile acidity, and the OIV method for the determination of the color intensity.

2.6. Genetic evaluation

The genetic structure has been analyzed by using 25 nuclear microsatellites loci. This genotype database was then compared with genotypes database of the 168 autochthonous cultivars from Spain.

2.7. DNA extraction and PCR amplification

Total genomic DNA was extracted from young leaves, using the DNeasy™ Plant Mini Kit (Qiagen). The extracted DNA was quantified and used as a 10-ng/μl working DNA solution. A set of 25 microsatellite loci well scatted on the genome was analyzed: VVMD5; VVMD7, VVMD21, VVMD24, VVMD25, VVMD27, VVMD28, VVMD32 [45,46]; VVIN16, VVIV67, VVIV37, VVIQ52, VVIP60, VVIH54, VVIB01, VVIN73, VVIP31 [47]; VVS2 [48] ZAG29, ZAG62, ZAG67, ZAG83, ZAG112 [49] and VMC1B11, VMC4F3.1 (Vitis Microsatellite Consortium). Six of these markers belong to the core set chosen by the international grape community [50] to allow the comparison of our data with most other germplasm.

Amplification reactions were performed in a total volume of 20 μl with 30 ng of DNA template, 0.25 to 0.5 μM of forward primer labelled either with 6: FAM, HEX, NED or PET fluorophore, 0.5 μM of non-labelled reverse primer, 150 μM of each dNTP (Boehringer, Manheim, Germany), 2.5 mM $MgCl_2$, 1X buffer ampliTaq and 0.8 units AmpliTaq polymerase (PE/Applied Biosystems, Foster City, CA). The PCR was carried out using a GeneAmp PCR system 9700 thermocycler (PE/Applied Biosystems). The cycling program consisted of the following steps: 10 min 94 °C followed by 35 cycles of 45 s at 92 °C, 1 min at (52–57 °C)

according to the literature and 1 min 30 s at 72 °C and a final extension step of 5 min at 72 °C.

The labelled amplification products were resolved onto an automated 310 ABI PRISM DNA sequencer (PE/Applied Biosystems), using a HD400-ROX as an internal size standard. Allelic data were cored using GENEMAPPER 3.0 software and the genotype of each sample was determined.

2.8. Genetic diversity

To carry out the genetic analysis 200 wild accessions were collected from six river basins: Guadiana, Guadalquivir, Guadalhorce, Guadalete, Palmones and Doñana National Park, and the surrounding areas (Table 2).

Allele size and the total number of alleles were determined for each SSR (Simple Sequence Repeat). Putative alleles were indicated by the estimated size in base pairs. Genetic diversity was estimated using the following statistics: number of alleles (N_a); effective number of alleles (N_e); allelic richness (R_s); observed heterozygosity (H_o) calculated as the number of heterozygous genotypes over the total genotypes analyzed for each locus; expected heterozygosity (H_e) [51]; and fixation index (F), also called inbreeding coefficient. All the calculations were performed using GenAlex software version 6.0 [52].

The Wright's inbreeding coefficient (F_{IS}) was estimated according to Weir and Cockerham [53], and its significance ($F_{IS} \neq 0$) tested after 1000 permutations. A positive value of F_{IS} indicates a deficit in heterozygotes in comparison with the Hardy–Weinberg equilibrium expectations. A negative value of F_{IS} indicated an excess of heterozygous individuals. All calculations and tests were performed using FSTAT program [54].

2.9. Genetic differentiation

Analysis of molecular variance (AMOVA) [55] was performed to partition the observed genetic variability among and within populations using GENEALEX program. F_{ST} was estimated over all populations and between each pair of populations using the method of Weir and Cockerham [53]. Since some of the microsatellite markers have imperfect or compound loci and therefore did not follow the stepwise mutation model (SMM), we chose F_{ST} instead of R_{ST}. The calculations were tested using FSTAT program [54]. Principal component analysis (PCA) was used to display genetic divergence among samples in a multidimensional space using GENEALEX program version 6.0 [52].

Table 2
River basin populations analyzed.

River Basin	Sample size
Guadiana	45
Guadalquivir	64
Marismas	16
Guadalete	62
Palmones	7
Guadalhorce	6

2.10. Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics V.22. Data were analyzed using analyses of variance (F-test). Tukey tests were applied to significant test results for the identification of important contrasts.

3. Results

Ninety-four populations of wild grapevine have been identified during prospecting (Table 1; Fig S1). No population was found in the province of Almería, the easternmost of Andalusia. The size of the populations varies between 1 and 162 vines situated between 0 and 900 m in height. The total number of exemplars registered was 952, 372 females, and 580 males with a sex ratio of 0.64. No hermaphrodite wild vine was observed. Rootstocks, direct producer hybrids and some European cultivars were also found as feral vines in the most anthropized areas.

A global ampelographic description of the vines is displayed in Table S1.

As the number of populations is rather high, a global description is very difficult. However, the main general characteristics are as follows: young shoots with fully open tip aperture, low density of prostrate hairs and low anthocyanin coloration. One or two very short consecutive tendrils were present. Young leaves with copper-reddish color. The mature leaf size is small-medium with medium-green color and anthocyanin coloration absent both in upper and lower sides. These leaves do not show undulation of blade between main and lateral veins, they have short teeth in their border with absence of lateral sinus. The woody shoot is brownish without erect hairs and with elliptic cross section. The female flowers have reflexed stamens and fully developed gynoecium and the male ones fully developed stamens and no gynoecium.

In all samples, the male pollen grain is tricolporated, while the female one is acolporated, without holes for the exit of the pollen tube, according to the external morphology of the types described by [56].

The number of inflorescences per shoot is 1.1 to 2 in female flowers and 2.1 to 3 in male. Bunches are small, with low density and cylindroconical shape. Berries are short and narrow with not uniform distribution and blue-black color, thick skin and visible hilum. Seeds are present in all cases.

An approximated phenological calendar with a cycle from mid-March to mid-December is summarized in Table 3.

Table 3
Phenological Calendar.

Phenological phase	Period
Sprouting time	18 March–5 April
Flowering time	18 May–30 May
Veraison	16 July–15 August
Ripening time	27 September–16 October
Leave falling	9 November–17 December

Table 4
Microvinification parameters (musts and wines).

	Population number (see Table 1)						
	9	30	49	54	71	89	84
Sugar (g/l)	120.8	219.5	191.2	120.8	198.8	224.1	233.7
Ethanol (%)	13.3	12.4	10.8	13.4	11.3	12.7	12.5
pH	3.52	3.36	3.25	3.61	3.43	3.54	3.41
Total acidity	8.71	9.10	9.36	8.59	8.73	8.77	8.91
Volatile acidity	0.68	0.65	0.76	0.81	0.79	0.82	0.76
Intensity of color	12.1	11.3	10.6	11.5	11.9	10.9	12.4

Results of microvinification are shown in Table 4. The ethanol content ranged between 10.80 in the only population found in Granada province (number 49) and 13.40 obtained in a population (number 54) of Doñana National Park in Huelva province, very close to the Atlantic coast. The highest initial concentration of sugar in musts, measured by refractometry, was 233.70 g/l. The color of the wines obtained was very dark, with intensities ranging between 10.60 and 12.40 and pH values between 3.25 and 3.61.

A list of the main accompanying vegetation with 74 species, including botanical supporters, is shown in Table 5, where the total number of wild grapevine populations where the species was found is also indicated.

The species most commonly found as accompanying flora are blackberry (*Rubus ulmifolius*), sarsaparilla (*Smilax aspera*), oleander (*Nerium oleander*) and ash (*Fraxinus angustifolia*) with 63, 43, 37 and 34 occurrences, respectively.

3.1. Parasitic organisms

The most frequent symptoms of infestation are those caused on leaves by the erineum strain of *Colomerus vitis*

Table 5
List of the accompanying vegetation.

Species	*n*	Species	*n*
Ailanthus altissima (Miller) Swingle	3	*Pinguicula vallisneriifolia* Webb	1
Alnus glutinosa (L.) Gaertner	7	*Pinus nigra* subsp. *salzmannii* (Dunal) Franco	3
Arbutus unedo L.	4	*Pinus pinaster* Aiton	2
Aristolochia baetica L.	2	*Pinus pinea* L.	3
Arundo donax L.	19	*Pistacia lentiscus* L.	30
Berberis vulgaris L. subsp. *australis* (Boiss.) Heywood	1	*Pistacia terebinthus* L.	3
Bituminaria bituminosa (L.) C. H. Stirt.	1	*Populus alba* L.	8
Bryonia dioica Jacq.	9	*Populus nigra* L.	24
Buxus sempervirens L.	2	*Pteridium aquilinum* (L.) Kuhn	6
Calicotome villosa (Poiret) Link	1	*Quercus canariensis* Willd.	13
Celtis australis L.	6	*Quercus coccifera* L.	2
Ceratonia siliqua L.	4	*Quercus faginea* Lam.	3
Chamaerops humilis L.	5	*Quercus faginea* subsp. *broteroi* (Coutinho) A. Camus	1
Cistus sp.	1	*Quercus ilex* subsp. *ballota* (Desf.) Samp.	17
Clematis vitalba L.	17	*Quercus suber L.*	10
Cornus sanguinea L. subsp. *sanguinea*	2	*Retama sphaerocarpa* (L.) Boiss.	9
Crataegus monogyna Jacq.	20	*Rhamnus alaternus* L.	1
Cyperus longus L.	1	*Ricinus communis* L.	1
Daphne gnidium L.	7	*Rosa canina* L.	13
Erica arborea L.	3	*Rosa sempervirens* L.	1
Erica erigena R. Ross	2	*Rosmarinus officinalis* L.	1
Eucalyptus globulus Labill.	6	*Rubus ulmifolius* Schott	63
Ficus carica L.	25	*Ruscus aculeatus* L.	8
Flueggea tinctoria (L.) G. L. Webster	1	*Salix alba* L.	8
Foeniculum piperitum (Ucria)	8	*Salix atrocinerea* Brot.	8
Fraxinus angustifolia Vahl	34	*Salix fragilis* L.	6
Halimium halimifolium (L.) Willk.	1	*Salix pedicellata* Desf.	1
Hedera helix L.	1	*Salix purpurea* L.	1
Hypericum perforatum L.	1	*Salix* sp.	10
Laurus nobilis L.	2	*Scirpoides holoschoenus* (L.) Soják	2
Ligustrum vulgare L.	1	*Silybum marianum* (L.) Gaertner	1
Mentha pulegium L.	1	*Smilax aspera* L.	43
Mentha suaveolens Ehrh.	1	*Tamarix africana Poiret*	5
Myrtus communis L.	6	*Tamarix gallica* L.	1
Nerium oleander L.	37	*Tamarix* sp.	8
Olea europaea L.	25	*Teucrium fruticans* L.	1
Phillyrea angustifolia L.	3	*Ulmus minor* Miller	15
Phlomis purpurea L.	8	*Viburnum tinus* L.	5

(Pagenstecher) (Acari, Eriophyidae). Its presence was observed in all the locations, affecting 87.4% of the total number of vines studied. The presence of *Calepitrimerus vitis* (Nalepa) (Acari, Eriophyidae) is scarce, affecting only 11 vines, a 0.01%.

Occasionally, some vines situated in areas of the Ossa-Morena mountain range showed small infestations caused by *Bemisia tabaci* (Hemiptera, Aleyrodide) and *Jacobiasca lybica* (Bergenin and Zanon) (Hemiptera, Cicadellidae) [57]. The presence of *Planococcus* citri (Risso) (Homoptera, Pseudococcidae), a vector of the grapevine leafroll virus was detected only in one population located in Zahara de la Sierra (Cádiz province) (number 24, Table 1).

The presence of symptoms caused by powdery mildew (*Erysiphe necator* (Schwein) Burriel) on leaves were present in all the populations, affecting 76.3% of the vines.

Oil spots on leaves together with other damages on shoot axes and bunches caused by downy mildew (*Plasmopara viticola* (Berlease and de Toni)) were observed also in all the populations, but affecting a lesser percentage of the vines than powdery mildew (59.4%).

It is necessary to remark that the levels of infestation or infection of the different parasitic species varied from one liana to another within the same location.

No symptoms of infestation or infection attributable to phylloxera (*Daktulosphaira vitifoliae* Fitch), root-not nematodes or mycelium of rot fungi, were detected on roots.

No symptoms of GFLV were found on shoots in the field. Also, all the ELISA tests were negative for this virus.

3.2. Soil lime tolerance

The plant responses to increasing lime contents under *in vitro* conditions are shown in Table 6.

In the absence of lime, survival was higher in the wild grape plants compared to rootstock 41B. On the other hand, the stem length average was higher in 41B plants than in wild accessions. The bud number per plant and the rooting percentage were similar in all cases, with average values of 5.3 and 67.8 respectively. Increasing the calcium carbonate content to 20% decreased significantly the survival and rooting of 41B plants (54.2% and 27.1%, respectively). The stem length and bud number at this lime level were lower in 41B rootstock plants, although without statistical significance. At 40% of lime content, 90% of 43 plants survived, a percentage significantly higher than that of 41B and statistically similar to that of 33 plants. A similar behavior was found in rooting, as all plants with aerial development had also roots. The best stem development was reached also by the 43 plants, with an average of 1.93 cm. At the highest level of lime (60%), there were no significant differences in stem length or bud number per plant among the three accessions. However, survival and rooting were higher in 43 plants, although no statistical differences were recorded.

3.3. Identification of identical genotypes

To estimate the total genetic diversity of the populations found, all the individuals were genotyped with 25 SSR and the identical genotypes eliminated. The samples were considered identical when they shared exactly the same alleles across all 25 loci showing to be the same individual. Identical genotypes corresponded to samples collected in close proximity in the same site, thus considered a mistake in the sampling. As accessions identified as duplicates were excluded in the subsequent analysis for population genetic diversity analysis, the final number of distinct genotypes was 160.

3.4. Total genetic diversity in Andalusia

All 25 microsatellites loci examined were polymorphic when considered over all populations with 160 unique genotypes. The largest number of alleles was detected for the VVIP31 locus (17 alleles) and the lowest for the ZAG29 (3 alleles) with an average of 8.84 alleles per locus (Table 7). The allele size range was generally a good prediction of the number of alleles present for locus and vice versa. Overall the number of alleles was correlated with the size range. Allele frequencies ranged from 0.002 to 0.631. No fixed alleles (allele frequency > 0.9) were found (Table 7). Out of 221 alleles detected, 45 were rare alleles (alleles with frequency lower than 1%; data not shown). Rare alleles were detected in all the loci, except ZAG29, VVIQ52 and VVIN16 and VVIV67; the former ranged from 1 to 6 rare alleles, with an average of 1.8 rare alleles. Most of the markers were highly polymorphic, except marker ZAG29.

Table 6
Biometrical parameters of plants of two wildgrape populations and rootstock 41B cultured *in vitro* under increasing lime contents for 60 days.

Lime content (%)	Population	Survival (%)	Stem length (cm)	Bud number	Rooting (%)
0	33	90.0 B	3.68 A	5.42 A	70.0 A
	43	100.0 B	3.19 A	4.31 A	66.7 A
	41B	70.8 A	7.53 B	6.18 A	66.7 A
20	33	91.9 B	3.64 A	4.97 A	67.6 B
	43	82.5 B	4.06 A	4.33 A	75.8 B
	41B	54.2 A	2.41 A	3.27 A	27.1 A
40	33	70.0 AB	1.04 A	2.07 A	70.0 AB
	43	90.0 B	1.93 B	1.97 A	90.0 B
	41B	54.2 A	0.92 A	1.81 A	54.2 A
60	33	57.5 A	1.03 A	2.10 A	57.5 A
	43	87.5 B	1.29 A	1.68 A	87.5 B
	41B	60.4 AB	0.70 A	1.44 A	60.4 AB

Means in columns with the same capital letter do not differ at $p \leq 0.05$ among populations for each lime concentrations.

Table 7
Diversity indices[a] calculated for 160 distinct genotypes of wild grapes determined from 25 nuclear microsatellite data.

Locus	N_a	N_e	I	H_o	H_e	F
ZAG29	3.000	1.905	0.772	0.444	0.475	0.066
ZAG62	7.000	4.047	1.576	0.650	0.753	0.137
ZAG67	13.000	4.456	1.884	0.669	0.776	0.138
ZAG83	5.000	2.024	0.981	0.481	0.506	0.049
ZAG112	7.000	2.657	1.270	0.594	0.624	0.048
VVIB01	4.000	2.437	1.035	0.488	0.590	0.173
VMC1B11	11.000	5.822	1.942	0.688	0.828	0.170
VVIH54	11.000	4.487	1.749	0.681	0.777	0.123
VVMD7	11.000	3.802	1.727	0.644	0.737	0.126
VVMD24	7.000	4.374	1.597	0.719	0.771	0.068
VVMD25	9.000	5.063	1.804	0.719	0.803	0.104
VVIN73	7.000	3.200	1.342	0.631	0.688	0.082
VVIP31	17.000	8.523	2.365	0.856	0.883	0.030
VVIP60	8.000	5.733	1.859	0.706	0.826	0.145
VVIQ52	4.000	2.801	1.143	0.500	0.643	0.222
VVS2	13.000	6.141	2.053	0.788	0.837	0.059
VVMD5	9.000	4.053	1.752	0.675	0.753	0.104
VVIN16	5.000	2.581	1.185	0.600	0.613	0.020
VVMD32	10.000	5.507	1.869	0.744	0.818	0.091
VVIV37	9.000	5.169	1.845	0.675	0.807	0.163
VVMD28	14.000	9.158	2.413	0.794	0.891	0.109
VMC4F3.1	12.000	6.172	2.126	0.744	0.838	0.112
VVMD21	6.000	2.219	1.158	0.388	0.549	0.295
VVMD27	9.000	6.311	1.957	0.756	0.842	0.101
VVIV67	10.000	6.287	1.998	0.731	0.841	0.130

N_a: number of alleles; N_e: number of effective alleles; I: information index; H_o: observed heterozygosity; H_e: expected heterozygosity; and F: fixation index.
[a] Indices calculated for all the samples analysed.

The number of effective alleles (N_e) values ranged from 1.9 (ZAG29) to 9.1 (VVMD28). The most informative markers are VMC4F3.1; VVMD28; VVS2 and VVIP31 (Table 7).

The observed heterozygosity ranged from 0.444 in ZAG79 to 0.794 in VVMD28 (mean of 0.654). The expected heterozygosity ranged from 0.475 in ZAG79 to 0.891 in VMD28 (mean of 0.741). Comparison between the two parameters was carried out based on the Wright's fixation index (F). This parameter was positive for the 25 loci, meaning a deficit of heterozygosity (Table 7).

3.5. Genetic diversity in river basins

In order to study the sample size, we have compared the diversity indices in the river basin samples with the total individuals. The effect of the sample size has been corrected by calculating allelic richness (R_s). The average of allelic richness in all the individuals (8.858) was significantly higher than for the river basins (3.371) (Table 8). The mean number of alleles in river basin populations range between 2 and 5 alleles with an average of 2.523 alleles. The number of alleles of the total sample is 26.7% higher than in the river basin samples (Table 8).

The mean genetic diversity values (H_e) of river basin populations (0.668) was significantly lower than the total genetic diversity (0.722) (Table 8). In the river basin populations, the observed heterozygosity ($H_o = 0.635$) was lower than the expected values ($H_e = 0.668$) according to the Hardy–Weinberg equilibrium (HWE), suggesting a deficiency of heterozygous mainly due to the tight genetic relationship among individuals. However, the F_{IS} values obtained in all the river basin populations are not significantly different from zero ($P < 0.05$).

Therefore, this result points out that in the river basin populations the mean of diversity indices is significantly lower than in the total genetic diversity. The population size is strongly correlated with the genetic diversity indices. Smaller populations are expected to have a reduced level of genetic diversity because they have effects on the genetic drift, inbreeding, and reduced migration.

3.6. Genetic differentiation among wild populations and cultivated grapevine

The pairwise genetic differentiation values (F_{ST}) among the six river basins are shown in Table 9. The highest differences were observed between vines from Guadalhorce and Palmones basins. F_{ST} values were found to be significantly different from zero ($P < 0.01$) in almost all the

Table 8
Diversity indices[a] calculated for mean values of different populations.

Population	N_a	N_e	R_s	H_o	H_e	F_{IS}
General (160 indiv)	8.86 ± 0.827	4.17 ± 0.393	8.858	0.658 ± 0.012	0.722 ± 0.018	0.148
6 River basins	2.523 ± 0.341	3.17 ± 0.076	3.371	0.635 ± 0.020	0.668 ± 0.030	0.011

N_a: number of alleles; N_e: number of effective alleles; R_s: allelic richness; H_o: observed heterozygosity; H_e: expected heterozygosity; F_{IS}: inbreeding coefficient.
[a] Indices calculated for all the samples analyzed, river basins populations.

Table 9
Pairwise F_{ST} values between 6 river basins. In bold are indicated pairwise values significant at the 0.1% nominal level.

River Basins	Guadiana	Guadalhorce	Guadalete	Guadalquivir	Palmones
Guadalhorce	0.1370				
Guadalete	0.0517	0.1257			
Guadalquivir	0.0376	0.1211	0.0464		
Palmones	0.0959	**0.2085**	0.0494	0.1007	
Marismas	0.1234	0.1937	0.1145	0.1027	**0.1703**

cases. However, AMOVA analysis between populations showed that most of the genetic diversity (86%) was attributable to differences within groups rather than inter-groups (14%).

To investigate the genetic differentiation between *V. vinifera* subsp. *sylvestris* and *V. vinifera* subsp. *sativa*, 20 SSR data obtained in this study were compared with the data from 145 autochthonous Spanish and 36 European grapevine cultivars contained in the database of the *Vitis* Germplasm Bank of El Encín (IMIDRA, Spain) [58]. F_{ST} analysis revealed a small genetic differentiation between southern wild grape and traditional cultivated varieties from the Iberian Peninsula (F_{ST} pairwise value = 0.086) and the rest of Western Europe (F_{ST} = 0.080), respectively.

4. Discussion

The census of wild grapevine populations as well as the number of vines found (952) in Andalusia is higher than in other European regions according to the information collected by [25]. In spite of this fact, Andalusian locations are relic and isolated in comparison with those existing around the Skadar Lake (Montenegro) as our research team observed in 2013 (unpublished data). The latter constitutes a good example of ecosystems conservation due to the low intensity of anthropic impacts, exactly the opposite of the Andalusian situation.

The presence of American vines in natural areas originally occupied by wild grapevine indicates an invasive character of these rootstocks, according to the considerations of Crawley [59]. These exotic vitaceae try to regain their original ecological American niches, mainly alluvial positions, quite similar to the Eurasian wild grapevine's [60]. However, these naturalized populations show a higher genetic diversity than the reduced and often isolated wild grapevine populations. Able to reproduce sexually, such kind of interconnected populations tends to create new active swarms of hybrid rootstocks. The spread of naturalized rootstocks in the environment, the acceleration of the decline of the European wild grapevine, and the propagation of genes of viticultural interest in natural populations are potential consequences that should be kept in mind when undertaking appropriate management measures [61] suitable to be implemented by environmental authorities of the autonomous territory of Andalusia.

The ampelographic characters are similar to those of different European and Asian wild populations [62,63]. In all the Andalusian populations, the female leaf is always bigger than the male one, while in the North of Spain this is not a constant [64]. It is also remarkable that in the population #10 situated along the Majaceite river, near El Bosque village (Cadiz province), three female vines exhibited bunches with white berries, while in the rest of the cases were red.

The tricolporated grain of pollen from male plants is the cause of the dioecious character of this subspecies because the female pollen is unfertile. Its morphological characteristics are quite similar to those from hermaphrodite cultivars. According to Dr. Lovicu (personal communication) in Sardinia there is at least one plant with male and hermaphrodite flowers on different branches.

The harvest is a fairly complicated process, given the diffuse bunch distribution and its high heights on the botanical supporters. The amount of must extracted per berry is very low, around 16–17% of fresh weight.

Taking into account the value of the theoretical probable ethanol concentration, yeast is able to consume practically all the sugar. The wine color is deep red as in other microvinifications carried out in the Iberian Peninsula [31,65,66] and Sardinia [19]. The color intensities showed in this paper are very far from the value of 26.57 obtained from the Ega River bank forest in Northeastern Spain [31]. As a reference, in the Rioja Designation of Origin of Great Quality, a wine with color intensity 3.5 is considered red. It is remarkable that wines have a good acidity, considering the fact that the reference value for Spanish quality wines is 3.40 [67]. In the case of Andalusia, the market demands new red wines. Musts from traditional cultivars have two problems under Mediterranean climatology: low acidity and color intensity, due to the disruption of the anthocyanin/sugar ratio in berries with consequences for color/alcohol balance in red wines [68]. Hence, the Shyrah variety is being planted in several wine producing areas of the region. Probably some new hybrids between wild and red autochthonous cultivars could reduce that dependence of foreign varieties as the cited ones.

The ecosystems where wild grapevine prospers are mainly gallery forests in alluvial position, river-bank formations. Only those populations situated in the mouth of the Guadalquivir River around Doñana National Park (Cádiz and Huelva provinces) are growing on arenosols developed on deep sandy Quaternary sediments. Due to climatology, there are no populations on floodplains and those situated in colluvial sites are very scarce. It is a difference with populations from Northern Spain [64] and Central European countries [25] under a most rainy climatology. In the closest areas of the watercourses always appear arboreal species of willow (*Salix alba*, *S. fragilis*) or shrub ones (*Salix atrocinerea*, *S. pedicellata* and *S. purpurea*) and alder (*Alnus glutinosa*). At some distance,

poplars are often found (*Populus nigra*, *P. alba*), sometimes also elms (*Ulmus minor*), hackberries (*Celtis australis*) and ashes (*Fraxinus angustifolia*) in large banks. On high-salinity soils, the presence of *Tamarix africana* or *T. gallica* is relatively frequent.

Blackberry bushes (*Rubus ulmifolius*) are always present, which are the most cited species in this botanical study, associated with wild roses (*Rosa canina*, *R. sempervirens*) and oleanders (*Nerium oleander*). As climbing species were observed ivy (*Hedera helix*), sarsaparilla (*Smilax aspera*), clematis (*Clematis vitalba*) and *Bryonia dioica*, besides wild grapevine.

In the creeks, components of the climax vegetation appear at short distance from the water course. That is the case of arboreal plants: holm oak (*Quercus ilex* ssp. *ballota*), cork oak (*Quercus suber*) and another oaks (*Quercus faginea*, *Q. canariensis*) or pines (*Pinus pinea*, *P. nigra*). Also some shrub species can be found, such as myrtle (*Myrtus communis*), daphne (*Daphne gnidium*), rosemary (*Rosma*-Rosmarinus officinalis), butcher's broom (*Ruscus aculeatus*), *Phillyrea angustifolia*, *Cornus sanguinea*, *Chamaerops humilis, Buxus sempervirens*, *Viburnum tinus*, *Ligustrum vulgare* and *Erica* and *Cistus* species, among others.

Foeniculum piperitum is considered a nitrophilous plant and can be found mostly in roadsides, fallow, and cultivated fields. Although sometimes forms shrubs, its real ecological requirements are not known in undisturbed natural formations as in the cited gallery forests.

Wild olive and fig trees or carobs were observed relatively frequently.

On the other hand, relatively recent introduced exotic species, such as Eucalyptus (*Eucalyptus globulus*) and Chinaberry (*Ailanthus altissima*) are also cited.

However, according to the list of species and their occurrence, the following considerations can be made: the wild grapevine in Andalusia lives often associated with brambles (*R. ulmifolius*) and is also common with other lianae as sarsaparilla (*S. aspera*). In half of the 94 locations, the presence of ashes (*F. angustifolia*) was indicated, mainly in potential ash tree formations. About a quarter of those locations include the presence of *Arundo donax* canes. Only one-twentieth of them are associated with the presence of *Tamarix* species on soils of a higher salinity.

On leaves the presence of erinea caused by *Colomerus vitis* constitute a constant symptom, as in the rest of the European and Trancaucasian populations [63,66], where the presence of the other mite, *Calepitrimerus vitis* is also more frequent than in Andalusian ones, probably due to climatic conditions, such as a lower pluviosity, except in those populations situated in Cazorla, Segura y Las Villas mountain range (Jaén province) and Los Alcornocales Natural Park (Cádiz province).

The symptoms caused by powdery mildew are also another constant disease on leaves. Dark spots are also frequently visible on branches. These perithecas constitute the sexual phase of the fungus, a form of resistance to develop again the disease in the next spring. The presence of downy mildew, with a minor incidence, was also detected on bunches situated in the areas with higher humidity in river-bank forests situated in the Sierra Morena mountain range. Both fungal diseases are present in the totality of the Eurasian wild grapevine [25,63].

It should be highlighted that the intensities of infestation or infection of the different parasitic species varied from one liana to another within the same location.

No symptoms of infestation or infection attributable to phylloxera (*Daktulosphaira vitifoliae* Fitch), root-not nematodes or mycelium of rot fungi, were detected on roots. It could be due to the edaphic conditions, mainly flood profiles of the soils near the water course during several months per year and the sandy texture of these soils around the Guadalquivir River's mouth, as it was indicated in several European countries by [66]. This article remarks that all the populations tested, from the Iberian Peninsula to Hungary, under artificial infestation in plots, showed symptoms of phylloxera on roots. In the 19th century, when phylloxera started to destroy the French vineyard, some viticulturists thought that wild grapevines could become good rootstocks, but outside of their natural habitats developed infestation [69].

The absence of GFLV and symptoms caused by nematodes can be due to the situation of the population on alluvial and very damp soils, where the nematodes survival, main vector of this virus, is very difficult.

The better *in vitro* behavior of the plants of population #43 (14/Rute/1) in high levels of lime could be linked to a natural tolerance acquired through growing in a habitat with carbonate-enriched soils. Cambrollé [16] has already showed that the plants from this population were very tolerant to calcareous soils in greenhouse conditions. As a consequence, the better plant development in these conditions should be considered as a varietal character. These results strengthen the possibility of considering wild grapevine as a phytogenetic resource for improving the current cultivated grapevines.

4.1. Genetic diversity *in* Vitis vinifera *subsp.* sylvestris

Genetic analyses identified a total of 160 distinct genotypes as estimated from neutral SSR loci.

Surveying wild grape populations from Andalusia for a set of 25 microsatellites loci distributed throughout the genome revealed a high genetic variability in these Spanish populations considering total genetic diversity ($H_e = 0.722$). This value is common among outcrossing and vegetatively propagated perennial species [70]. The mean values of genetic diversity between the river basin populations were significant lower ($H_e = 0.668$) and slightly lower than the values reported in the literature for cultivated grapevine that range between $H_e = 0.758$ [71] and $H_e = 0.816$ [70]. This could be due to the sample size because smaller populations are expected to have reduced levels of genetic diversity [72].

4.2. Genetic differentiation of wild grape populations

Partitioning of the genetic variability by means of gene diversity statistics [73] indicated that most of the SSR diversity was distributed within the populations than between populations. This is consistent with the findings from other studies done for woody plants that

considerable genetic diversity is partitioned within, rather than between [74,75]. The low divergence scored between the groups and the large variability detected among accessions could be explained by the occurrence of gene flow in the natural close populations and the reproductive mode. A source of pollen and the lack of inter wild populations flow may be the reasons of the low heterozygosity of wild populations [32].

The present results support the hypothesis that wild grape has a low genetic diversity and the diversity has a spatial structure in the native habitat of wild grapevine in Andalusia. The distribution patterns of intraspecific genetic variation can provide data concerning the temporal and special dynamics of this economically important crop. According to [76], Eurasian wild grapevine is an opportunistic liana with reproductive patterns characterized both by vegetative propagation and sexual reproduction. Vegetative propagation would ensure rapid vine regeneration and land colonization, while sexual reproduction would assure genetic recombination and chromosome re-assortment crucial for evolution and survival of the vines.

The results of the analyses showed a genetic differentiation between the cultivated and the wild populations. Moderated differences between southern wild populations and cultivated grapevine have been found, and also some autochthonous cultivars that shared alleles with high frequency with southern wild accessions. These results suggest that grapevine cultivars from Spain could have had a local genetic contribution from Southern wild populations which provided the A chlorotype to some autochthonous cultivars [8,77]. From the first domestication event in the Transcaucasian area, different civilizations have spread viticulture throughout the Mediterranean Basin [78,79]. There exist archeological evidences that Phoenicians introduced viticulture in the South of Spain [3,80]. Also, it is possible that gene flow could have contributed to the observed distribution of genetic diversity. Hybridization with cultivars, and the long history of exchange of cultivated genetic resources through the Mediterranean basin [81] could have contributed to moderate the differentiation between these distant populations.

In recent years, the maintenance of genetic variability 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 habitats will reduce both the number and size of the populations, and decrease the genetic variation within them as described previously [61,82]. As a consequence, it is necessary to establish a program for the conservation of this germplasm.

The recent loss of suitable habitats due to direct and indirect human impact, *V. vinifera sylvestris* is now endangered through its range. As a consequence, populations are generally small and dispersed [61]. It could contribute to a significant risk of extinctions and potential inbreeding depression of wild grapevine. The results from the present paper on specific alleles in the wild populations, not present in cultivated grapevine, make this germplasm very interesting for conservation programs.

The Andalusian wild grapevine populations found and characterized are relic resources of a threatened subspecies and, as a consequence, must be conserved both for its ecological value as well as to ensure the future and sustainability of viticulture because they constitute a tremendous source of genetic material to be used in cultivar breeding and to adapt them also to the new challenges of the sector as the market demands and climatic change.

Disclosure of interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.crvi.2017.01.004.

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