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An inventory of the relic Eurasian wild grapevine populational nuclei in Huelva province (Andalusia, Spain)

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Summary

This paper is focused on 21 relic wild grapevine populations within Huelva province (SW Europe, Spain) prospected between 2015-2017. Position of each population along river-bank forests, types of flower of this dioecious subspecies and morphology of the pollen grains were described. Analyses of five microvinifications were carried out, which indicate that the color intensity of the wines is between 14.6 and 17.6 and the pH between 3.26-3.27, which are suitable values for red wines under Mediterranean climatology. A list of the accompanying vegetation and the cultivated varieties in the "Condado de Huelva" Registered Appellation Origin Mark were also included. Moreover, the genetic diversity of 23 vines was characterized using 25 nuclear SSR loci, the results showed a slightly higher diversity than the one found in Iberian cultivars. However, the observed heterozygosity was significantly lower than the expected one for wild populations in the Huelva province. The inbreeding depression in these wild grape populations is suggested by the positive F values. Therefore, the conservation of this unique germplasm collection should be part of the process of maintaining the genetic diversity in this gene pool, especially, taking into account that no particular legal figure of preservation exists in Spain.

Key words: ampelography; ecology; genetic diversity; threatened phytogenetic resource; *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi.

Introduction

The Eurasian grapevine, *Vitis vinifera* L. is constituted by two subspecies: *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi and *Vitis vinifera* L. subsp. *sativa* (DC.) Hegi. The first one is the wild grapevine, the ancestor of the actual cultivars. The populations of these subspecies are spread from the Iberian Peninsula to Afghanistan (Arnold 2002) and some areas of the Maghreb (Ocete *et al.* 2007). The wild vines around Lake Tiberiades are genetically closely related to Israel's indigenous crop varieties. However, a large part of the current cultivars are proles orientalis, brought by the Arabs. (Drori *et al.* 2017). They are integrated by individuals of both sexes, male and female (Levadoux 1956, Olmo 1995). That dioecious character was observed by Dioscorides (I BC). He wrote: "One that never perfects its grapes, but always leaves them at flowering time and its fruit is called Enanthe (it means, flower of wine). And the other one that has just matured producing certain small black berries with an acid taste".

On the other hand, cultivated varieties are generally hermaphrodite, due to a long process of selection by human communities from the Neolithic age to present (Forni 2004). Nonetheless in Southern Caucasus, where the first cultivated pips were found with an about 8000 years age of (Chilashvili 2004), several female vines are still under cultivation (Maghradze *et al.* 2010). In the cultivated varieties that pressure exerted by man made it possible to concentrate characteristics useful for productivity (hermaphroditism, size of the clusters and berries) and for the quality of the product and facility of exploitation (accumulation of sugar, resistance to drought and degree of tolerance to parasitic species) (Ocete *et al.* 2007).

Already, Theophrastus (IV-III B.C.) differentiated both subspecies giving the name of *Agria ampelos* to wild exemplars and *Oenophoros ampelos* to cultivars (Laguna 1570). Theophrastus wrote literally on the wild grapevine "Liana with hard leaves, ashen color and calloused long reddish shoots". On the second one: "It is the name given to different cultivars". The geographic frame of the present article corresponds to the old Tartessian area situated at the most western limit of Andalusia (Iberian Peninsula). In

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this region, the presence of pollen grains of wild grapevine is confirmed from the Middle Pleistocene in El Padul bog (Granada province) (Florschütz *et al.* 1971). Another similar finding much later was registered in the Laguna de Las Madres. It is a lagoon near the Atlantic coast in Mazagón (Huelva province). Pollen is datable to 4,480 ± 150 BP (Stevenson 1985).

In Andalusia, the majority of the actual relic populations of wild grapevines are distributed along river-bank forests, as in the rest of the Mediterranean (Gallardo 2005). They harbor a high genetic diversity, suitable to be used for breeding projects inside the actual climate change period. In fact, biodiversity loss is a worldwide problem, and the grapevine is probably one of the most affected species. The number of cultivars has decreased in recent years, and the local varieties of each region have been displaced by other varieties that have expanded internationally due to globalization of markets (Marsal 2017). It is necessary to highlight that Iberian wild grapevines have provided the A chlorotype to autochthonous grapevine cultivars from this region and other Iberian areas (Arroyo-García *et al.* 2006).

Material and Methods

Prospection of wild grapevine populations: The prospection of wild grapevine individuals was carried out along river-banks of the main rivers: 'Guadalquivir mouth', 'Guadiana', 'Tinto' and 'Odiel', their tributaries and creeks at flowering time, in the first fortnight of may during 2015-2017. The position of each location was taken by GPS (Fig. 1).

The plant sampling strategy was the same for all populations, and was designed as to prevent errors as cultivars escaped from vineyards and rootstocks instead of wild plants. To reduce the risk, only dioecious individuals were selected, knowing that cultivated individuals are hermaphrodites, except around a 10 % and cultivars belonging to the wild subspecies are female.

The main ampelographical descriptors were evaluated at flowering time (second half of May) according to Organisation Internationale de la Vigne et du Vin (OIV) (2009) between 2014-2017. Pollen samples from flowers from each population were obtained brushing the mature anthers from male and female vines. Grains were included in a mixture of distyrene, plasticiser and xylene, (DPX) (Fluka) and observed under optical microscope Olympus BX 61.

Accompanying vegetation: The accompanying vegetation of the river-bank forest were identified using general botanical keys and the studies carried out by Valdés *et al.* (1987) on Western Andalusia.

Genetic evaluation: The genetic analysis has been analysed on 23 individuals belonging to 4 populations by using 25 nuclear microsatellites loci, the methodology has been described by De Andres *et al.* (2012). This genotype database was then compared with genotypes database of the 181 autochthonous cultivars from Spain.

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; 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 and Smouse 2006) and the Excel Microsatellite Toolkit (Park 2001).

Results and Discussion

Prospection of wild grapevine populations: The geographical position of the 21 wild grapevine populations found and the number and sex of the individuals is shown in Tab. 1 and Fig. 2. Eighteen populations are located in protected areas, four in Doñana National Park and fourteen within Picos de Aracena y Sierra de Aroche Natural Park.

Ampelographical descriptors of both types of sexes of wild grapevine confirm data from both Andalusian populations (Cantos *et al.* 2017) and the germplasm bank of El Encín, Alcalá de Henares (Benito 2015). The leaves

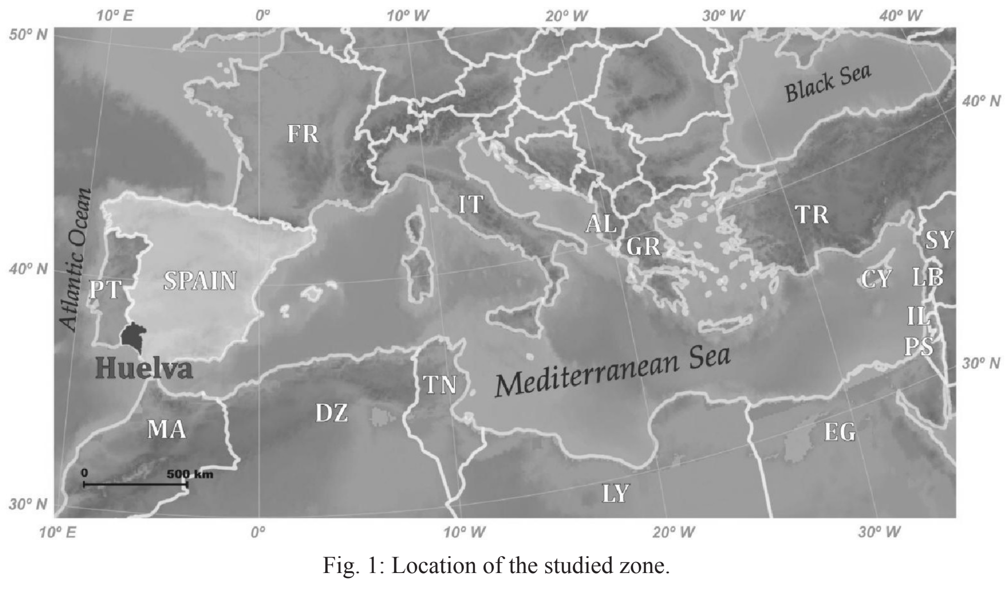

Fig. 1: Location of the studied zone.

Table 1

Coordinates and number of individuals of both sexes.

Location	Geographic coordinates (WGS84)		Number of specimens	
	Longitude	Latitude	Male	Female
Almonte/1	-6,39055	36,86055	5	2
Almonte/2	-6,38805	36,86416	4	1
Almonte/3	-6,38805	36,87472	3	2
Almonte/4	-6,50396	37,12048	17	6
Almonte/5	-6,54611	37,14222	32	20
Aracena/1	-6,52527	37,88194	3	1
Aracena/2	-6,50027	37,86694	4	2
Aroche/1	-7,04805	37,96666	11	4
Aroche/2	-7,03194	37,96805	9	3
Aroche/3	-7,00555	37,96861	14	5
Arroyo Candón/1	-6,75388	37,33861	17	9
Calañas/1	-6,91055	37,66666	5	3
Calañas/2	-6,90250	37,65944	3	1
Cortegana/1	-6,85944	37,93361	2	1
Cumbres de San Bartolomé/1	-6,78750	38,02944	4	2
Encinasola/1	-6,96138	38,13250	5	2
Fuenteheridos/1	-6,65861	37,90888	6	3
Higuera de la sierra/1	-6,46944	37,84416	1	0
Linares de la Sierra/1	-6,62722	37,87833	5	2
Linares de la Sierra/2	-6,60557	37,86581	5	3
Rosal de la Frontera/1	-7,13638	37,97666	8	4

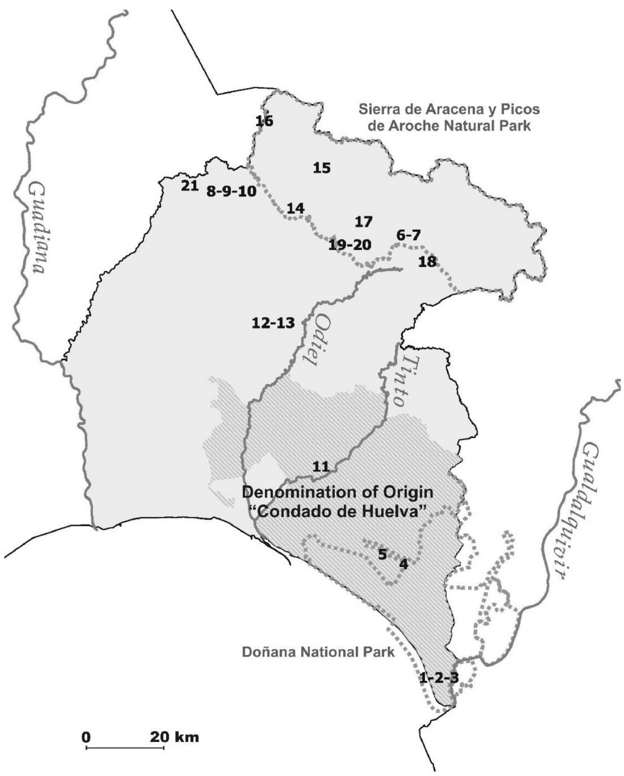

Fig. 2 Location of the wild grapevine populations

of female specimen are always bigger and less lobed than the male ones. The male flower is always *type I*, without gynoecium, and the female one with reflexed stamens. No male flowers type II, with primordium of gynoecium, or hermaphrodite were found, according to code number 151 OIV (2009). All the female individuals produced bunches with red berries, under 1 cm of diameter and one or two pips.

The morphology of male pollen grain is tricolporated, meanwhile the female one is acolporated, without holes for the exit of the pollen tube, according to the external morphology of the types described by Gallardo *et al.* (2009).

In Tab. 2 the main accompanying botanical species in wild grapevine habitats of Huelva province are listed.

It is necessary to remark the absence of legal protection for wild grapevine in Spain and consequently in Andalusia, so these relic populations are subject to continuous threads in those places outside natural reservations. In the case of the population Arroyo Candón, during preparation of the present paper, the 26 exemplars were selectively cut at soil level. The destruction of wild grapevine populations by anthropic actions was indicated the first time by Issler (1938). These progressive negative impacts have drastically reduced the numbers of wild grapevines in Europe. At present, this subspecies constitutes a threatened phytogenetic resource, so it is necessary to give a legal figure of preservation in the European Union (Ocete *et al.* 2015).

Genetic diversity analysis: A total of 23 wild samples analysed were collected around Rosal de la Frontera which corresponds to the Guadiana river bank. We have analyzed 181 autochthonous cultivars from Spain (De Andres *et al.* 2012) including the cultivars Zalema, Listan de Huelva, Garrido Fino and another from Condado de Huelva vineyards.

The results of the genetic diversity analysis, according to DNA microsatellite genotyping are shown in Tab. 3. In the Guadiana populations, genotypes showed a mean value of 7.15 alleles including one private allele (alleles found in

Table 2

List of the accompanying vegetation

Species	Species
Ailanthus altissima (Miller) Swingle	*Pinus pinaster* Aiton
Alnus glutinosa (L.) Gaertner	*Pinus pinea* L.
Arbutus unedo L.	*Pistacia lentiscus* L.
Arundo donax L.	*Populus alba* L.
Bryonia dioica Jacq.	*Populus nigra* L.
Celtis australis L.	*Pteridium aquilinum (*L.*)* Kuhn
Ceratonia siliqua L.	*Quercus coccifera* L.
Chamaerops humilis L.	*Quercus ilex* subsp. *ballota* (Desf.) Samp.
Cistus sp.	*Quercus suber L.*
Clematis vitalba L.	*Retama sphaerocarpa (*L.*)* Boiss.
Crataegus monogyna Jacq.	*Rhamnus alaternus* L.
Daphne gnidium L.	*Rosa sempervirens* L.
Eucalyptus globulus Labill.	*Rubus ulmifolius* Schott
Ficus carica L.	*Salix alba* L.
Foeniculum piperitum (Ucria)	*Salix atrocinerea* Brot.
Fraxinus angustifolia Vahl	*Salix fragilis* L.
Hedera helix L.	*Smilax aspera* L.
Nerium oleander L.	*Tamarix africana Poiret*
Olea europaea L.	*Ulmus minor* Miller
Phlomis purpurea L.	

Table 3

Genetic diversity of the wild populations.

Population	Sample size	Loci typed	total alleles	He Hz	Obs Hz	MNA	Private alleles	F
Spanish autochthonous cultivars	181	20	188	0.740 ± 0.0308	0.748 ± 0.0072	9.4 ± 2.84	28	-0.011
Wild samples	23	20	169	0.759 ± 0.0256	0.700 ± 0.0229	7.15 ± 2.35	1	0.079

Unbiased Hz: Nei's unbiased gene diversity; unbiased Hz SD: unbiased gene diversity. Obs Hz: observed heterozygosity; Obs Hz SD: observed heterozygosity; MNA: mean number of alleles; MNA SD: MNA standard deviation inter-locus standard deviation; fixation Index: F.

a single population throughout the study region) to mean values of 9.4 alleles with 28 private alleles in the cultivated grapevine. The genetic comparisons between the cultivated and wild grapevine groups from the Iberian Peninsula allowed to find out that the lower number of private alleles presented in Guadiana populations could be due to the different sample size analyzed. The mean number of alleles between the different populations ranged from 7.15 to 9.4. These differences could be due because a positive correlation of number of alleles with sample size is expected (De Andrés *et al.* 2012). The observed heterozygosity in Guadiana populations ranged between 0.700 and 0.748, whereas the values of expected He were slightly higher, ranging between 0.740 and 0.759. The results of the genetic analysis showed that Guadiana wild populations still harbor a number of wild grapevine accessions with high levels of heterozygosity (He). Similar results have been observed in wild grapevine populations analyzed in Morocco, Sardinia, Portugal, France or Italy (Arroyo-García and Revilla 2013). However, we have detected that the observed heterozygosity (Ho) was significantly lower ($p \leq 0.05$) than expected heterozygosity (He) in Guadiana populations (Tab. 3). The fixation index (F) showed values ranging from 0.079 to -0.011. Since F values near zero indicate random mating, the positive F values found for Guadiana wild populations are consistent with the existence of inbreeding depression in these wild grape populations. The wild accessions appeared severely endangered and the reason could be the existence of human activities, geographical barriers, leading to isolation of wild populations in their native habitat. On the other hand, the cultivated compartment showed negative F values indicating excess of heterozygotes (Tab. 3). Therefore, this deficiency of homozygotes in the majority of the cultivated groups suggests that they are made up of germplasm with divergent demographic (founder effects, bottlenecks, dispersal) and selection histories.

In order to know if Huelva province could be a secondary centre of domestication for Eurasian grapevine, we should take into account the analysis of chlorotype distribution between wild and cultivated accessions around the Mediterranean basin (Arroyo-García *et al.* 2006). The results suggest the existence of at least two important origins for the cultivated germplasm, one in the Near East and another in the western Mediterranean region, of which the latter gave rise to many of the current Western European cultivars. Indeed, over 70 % of the Iberian Peninsula cultivars display chlorotypes that are only compatible with them having derived from western *sylvestris* populations with A chlorotype. This result suggests a putative secondary center in the Iberian Peninsula.

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