Article

Genotypic and sanitary characterization of minority grapevine varieties prospected in Andalusia, Spain

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Abstract: Andalusia is a Spanish region that is home to numerous minority varieties due to its diversity and territorial extension, offering the local viticulture the possibility of diversifying its wine production. The molecular identification of 98 specimens from six areas with a winemaking tradition in Andalusia was carried out between the years 2020 and 2022. Thirteen microsatellite markers were used in this study, including the nine recommended by the OIV. 31 different genotypes were obtained, 20 of which corresponded to profiles of already described varieties (11 of them are of minority cultivation in Andalusia: 'Rojal Tinto', 'Beba', 'Zurieles', 'Rome', 'Hebén', 'Mollar Cano', 'Listán Prieto', 'Listán del Condado', 'Jarrosuelto', 'Negra Dorada' and 'Mantúo de Pilas'), while the other 11 profiles did not match with previously identified varieties. This profiles were integrated into the database of the IFAPA "Rancho de la Merced" Germplasm Bank. The eco-geographical groups of the new identified genotypes were determined through analysis of genetic diversity. The presence of the Grapevine Fanleaf Virus, the Grapevine Fleck Virus and the Grapevine Leafroll associated Viruses was also analyzed using an ELISA test due to the necessity of having certifiable clones of the new varieties for their potential interest in being authorized for its cultivation in Spain.

Keywords: genetic resources; molecular markers; local cultivar; certification

1. Introduction

Andalusia has a surface of about 87,000 km^2, which represents 17.3% of the Spanish territory. This wide extension entails a great diversity of landscapes and ecosystems. The vine (*Vitis vinifera* L.) is one of the most rustic crops and one of the best adapted to the multiple edaphoclimatic conditions of Andalusia. Vineyards can be found in high areas, such as "La Alpujarra" (Granada), located at more than 1,000 m; in areas close to the sea, such as the hills of "Marco de Jerez" (Cádiz); and even in regions with a high slope, such as "La Axarquía" (Málaga). There are 7 Protected Denominations of Origin (PDO) for still wines in Andalusia: "Jerez-Xérès-Sherry", "Manzanilla de Sanlúcar de Barrameda", "Granada", "Condado de Huelva", "Montilla-Moriles", "Málaga" and "Sierras de Málaga"; there are also 16 Protected Geographical Indications (PGIs) and a PDO of flavored wines. Andalusia has an enormous wine-growing potential due to its plant genetic resources. Numerous bibliographical references show the great heterogeneity of vine varieties that were cultivated before the phylloxera arrived to this region [1,2] . Although phylloxera ended up destroying more than a million hectares, with the consequent loss of many indigenous varieties, it is estimated that about 300 of the 600 varieties that were cultivated before the invasion in 1878 were saved.

There are 56 vine varieties allowed for cultivation in Andalusia in accordance with the List of Authorized Varieties for Andalusia (Real Decreto 111/2022, of February 9, which

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modifies Real Decreto 1338/2018, of October 29, which regulates the potential of viticultural production).

Despite this number, 77% of the cultivated area is occupied by only four white grape varieties: 'Palomino Fino', 'Pedro Ximénez', 'Moscatel de Alejandría' and 'Zalema' (data not published, supplied by "Consejería de Agricultura, Pesca, Agua y Desarrollo Rural, Junta de Andalucía, 2022); furthermore, the sum of the least cultivated varieties only represents 2% of the total surface. It can be deduced from this data that a large number of varieties are barely cultivated and, therefore, are not well known by viticulturists [3]. In addition, not all the minority cultivated varieties are included in the previously mentioned list and, since their cultivation is not allowed in Spain, the protection for their conservation is even worse.

For all these reasons, it is necessary to track down in the field, identify, recover and evaluate the possibility to incorporate these varieties into the Andalusian wine patrimony. However, as it has been mentioned, in several cases they could be varieties that are not allowed in Spain. In this case, if there would be a specific interest in cultivating these varieties, it would be needed to undertake the official authorization process. The first step of this process consists in sending about 10 plants grafted onto '110R' rootstock to the National Reference Collection of Grape Varieties (maintained at IMIDA, Murcia and funded by MAPA, Madrid). An official analysis by ampelography would be carried out when the vines are formed, in which 44 characters (of leaf, shoot, flower and fruit) established by CPVO (Community Plant Variety Office) and UPOV (International Union for the Protection of New Varieties of Plants) would be observed for at least two years. To accept these materials, MAPA requires the absence of Grapevine Fanleaf Virus (GFLV), Grapevine Leafroll associated Viruses (GLRaV-1 and -3) and Grapevine Fleck Virus (GFkV) verified by at least ELISA test. These are the viruses contemplated by Orden APA/2474/2006, of July 27, which modifies certain annexes of the Technical Regulation for the Control and Certification of vine nursery plants approved by Real Decreto 208/2003, of February 21st. Therefore, to register a variety in the Register of Commercial Grapevine Varieties in Spain (RVCV), and then to be able to authorize its cultivation in a certain autonomous community, it is necessary to find or to obtain through sanitation propagation material that is certifiable from a phytosanitary point of view.

This work shows the results of the varietal identification of 98 specimens located in different areas of Andalusia. It made be possible by means of the collaboration of local entities such as Regional Agricultural Offices, Rural Development Groups, Regulatory Councils of Denomination of Origin, and viticulturists and oenologists. In particular, surveys were carried out in the following wine-producing areas (Figure 1):

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1. Altiplano de Granada
2. Alpujarra de Granada
3. PDO Montilla-Moriles
4. Valle de los Pedroches
5. Pago Burujena
6. Moguer

Figure 1. Map of the prospected areas.

a) "Altiplano de Granada", a region in the northeast of the Granada province made up of the Huéscar and Baza regions and characterized by its high average altitude (between 700 and 1,200 m). The vineyard occupies an area of about 350 ha and it is widely distributed in more than 1,000 small plots, with an average size of 0.3 ha, which shows a great fragmentation, as well as the abundance of traditional winemakers for domestic consumption. This area is protected by the PDO "Granada" and by the PGI "Altiplano de Sierra Nevada". An exhaustive prospecting study had been carried out before in this area, so only two strains were studied [4].

b) "La Alpujarra de Granada", a region in the southeastern part of the Granada province, characterized by its rugged relief, occupying most of the southern face of Sierra Nevada. The 25 municipalities that make it up are located at an altitude between 1,200 and 1,500 m. It has about 2,000 hectares of vineyards with slate soils and stony and slightly limestone subsoil. This region is protected by the PDO "Granada" and by the PGI "Cumbres del Guadalfeo". Nine vines were surveyed in this area from two farms whose vineyards are more than 60 years old and their origin could date back to pre-phylloxera times.

c) PDO "Montilla-Moriles", a territory located in the heart of Andalusia, in the Córdoba province, with the Guadalquivir to the north, the Subbética mountains to the south, the Genil river to the east and the Guadajoz river to the west, made up of a total of 17 municipalities. This PDO has nearly 6,000 hectares of vineyards that produce fortified wines (Fino, Amontillado, Oloroso and Palo Cortado) and sweet wines with 'Pedro Ximénez'. There has been a commitment to the parallel production of other types of wine in the recent years, possibly accompanied by a greater varietal diversification. In this area, thirty-two vines were surveyed from eight farms located in the municipalities of Montilla, Moriles and Cabra, with the aim of clarifying the situation of a supposed local variety called "Montepila", due to the strong interest of the sector that wants its inclusion in the list of authorized varieties in Andalusia.

d) "Valle de los Pedroches", a region of 3,162 km^2 located in the Córdoba province that represents the northernmost territory of Andalusia. This area is characterized by its

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diversity of landscapes (it goes from the holm oak dehesa to the peneplain until reaching Sierra Morena) and soils (with a predominance of slate in agricultural areas), and with an average altitude of between 500 and 700 m. Valle de los Pedroches is protected by the PGI "Córdoba". The vineyard of this region knew its maximum splendor in the 17th and 18th centuries, exceeding 2,000 hectares of surface. It was estimated that 1,800 hectares were destined for the cultivation of vines shortly before the arrival of the phylloxera at the end of 1800. The wine culture started disappearing after the phylloxera, although isolated or semi-isolated vine plants have been preserved in some parts of the region, mainly for the production of self-consumption wines. In recent years, projects have been promoted for the management of these family “vineyards” in order to preserve the biodiversity and traditional landscapes of the area. This suggests that there has been no influence from modern viticulture in this area, making it particularly attractive in terms of prospecting for possibly pre-phylloxera varieties, since the introduction and spread of major national and international varieties has been unlikely. Thirty-six vines were prospected in this region, most of them isolated and located near dividing lines between properties.

e) "Pago Burujena", a territory located within El Marco de Jerez (PDO "Jerez-Xérès-Sherry" and "Manzanilla-Sanlúcar de Barrameda"), the most emblematic and important area for the production of fortified and sweet Andalusian wines, being the same typologies mentioned for the PDO "Montilla-Moriles", but using the traditional ’Palomino Fino’ variety in this case. Greater varietal diversification has been committed in Jerez in recent years, and one of the objectives is to try to rescue pre-phylloxera varieties for the production of both fortified and still wines. In the viticulture of Jerez and its surroundings, the "Pagos" (rural places) represent cultivation areas geographically delimited by orographic elements and characterized by uniformity in terms of soil, microclimate, variety and even the human qualities of the viticulturists. In this context, the definition of Pago Burujena dates back to the 16th century, under the dukedom of Medina Sidonia, and it is an area of about 22 ha characterized by limestone soils of superior quality and the presence of different traditional varieties of the area [5]. Seven vines from a farm located in said Pago were prospected.

f) "Moguer", whose township is protected by the PDO "Condado de Huelva", is a territory with a high vocation for growing vines, characterized by an average altitude of 25 m for the vineyards, loamy or sandy soils with a certain content of lime (which gives them a slightly basic pH) and a Mediterranean climate with Atlantic influences. Around 550 ha of vineyards are cultivated, with a high degree of fragmentation of the farms (which have average surfaces of between 2 and 3 ha), many of them older than 40 years. For this reason, and because of its long winemaking tradition, it is an ideal area to search for minority varieties linked to a possible wine diversification. In the province of Huelva, this production is mostly based on the cultivation of ’Zalema’. Twelve vines with more than 50 years old maintained on a farm were prospected in this township.

2. Materials and Methods

2.1. Vegetal Material

Vine shoots were collected from each of the 98 vine plants and kept in a cold room at 4 ºC in the winter of 2021. The shoots were placed in buckets with water to force their sprouting later in the spring of 2022. Once sprouted, samples of about 50 mg were taken from the apical meristem of each of them for DNA extraction.

Five adult leaves were collected from several shoots of each plant analyzed by SSR and stored at 4 ºC in the late spring of 2022. From each of the 5 leaves, 1 cm of petiole and a leaf area of about 2 cm^2 attached to the same petiole were successively cut and, as a whole, the crude extract was obtained for virus detection.

2.2. Genotypic analysis

The DNeasy 96 Plant Kit (Qiagen) extraction kit was used to obtain DNA. DNA concentration was measured with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

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Microsatellites markers (SSR) were chosen for varietal identification since they are one of the best tool for identification of grapevine genetic resources [6]. Thirteen SSR were analyzed, nine of them (VVS2 [7], VVMD5, VVMD7 [8], VVMD25, VVMD27 [9], VVMD28, VVMD32, VrZag62 and VrZag79) are those recommended by the European project GrapeGen06 and the OIV (International Office of Vine and Wine), and the other four (ISV3, ISV4, VVS2 and VMCNG4b9), used by CREA-UTV (Turi, Italy, [10]), have been proven to be quite polymorphic in previous studies. For the GrapeGen06 SSR set, data was coded to compare microsatellite profiles as reported by Maul et al. [11], in order to include them in the European Vitis database (http://www.eu-vitis.de/index.php). Four multiplex PCRs were carried out depending on the annealing temperatures of the different primers, which were purchased from Biomers (Ulm, Germany), using the 6-FAM, Hex and Atto 550 fluorophores. The amplificates were separated in an ABI3130 sequencer. Subsequently, allelic sizes were determined using the Gene mapper program. The International Variety Catalog (VIVC) was chosen as a reference to compare the profiles obtained; other databases were used for this as well. The varieties 'Garnacha', 'Tempranillo', 'Merlot' and 'Syrah' were used to harmonize the size of the alleles and to be able to compare the genotypes. Results were integrated in the IFAPA Rancho de la Merced database, which has around 1,500 genotypes.

2.3. Cluster Analysis

A set of 258 genotypes with a clear ancestry inferred by Cretazzo et al. [12] in accordance with the eco-geographic origin of the cultivars [13], was combined with twelve genotypes not previously described found in this work, in order to clarify their putative geographical origin. An unweighted neighbor-joining (NJ) tree was constructed based on the Simple Matching dissimilarity index (SM) between the unique genetic profiles using Darwin software package v6.0 [14]. One thousand bootstrap replicates were performed.

2.4. Pedigree Analysis

The software CERVUS v.3.0.7 (Field Genetics, London, UK) [15] was used to identify first-order kinship relationships, mother-father-offspring trios, among the unknown grapevine cultivars found in this study and a set of 529 cultivars of *Vitis vinifera* from the Germplasm Bank of the IFAPA Rancho de la Merced whose 13 SSR profile had been fully characterized [12]. The profiles of the 12 unknown varieties were included as candidates as well, being 541 cultivars the set of possible parents used.

This analysis relies on allele frequencies and is based on the difference in the log-likehood ratio (LOD) between related and unrelated relationships to assign parentage combined with simulation of parentage analysis to determine the confidence of assignments. The parameters considered for the simulation were the following: number of offspring = 100000; number of candidate parents = 100; proportion of candidate parents sampled = 0.3; prop. loci typed = 0.8; and prop. loci mistyped = 0.01. Three criterions were considered to establish strict parentage relationships: i) 10 minimum type loci, ii) a confidence level of the LOD score higher than 95% (strict) or 80% (relaxed) and iii) a maximum number of tolerated trio loci mismatches equals to two [16–18].

2.5. ELISA Test

Serological tests for the detection of GFLV, GLRaV-1, GLRaV-2, GLRaV-3 and Grapevine fleck virus (GFkV) were performed using BIOREBA (Reinach, Switzerland) or AGRITEST (Valenzano, Italy). The technique used was DAS-ELISA (Standard Double-Antibody-Sandwich Assay).

GLRaV-2 analysis is not required by certification protocols, but it is recommended by the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICGV; [19] and its importance in Spain is well-known [20])

Plant tissue was homogenised with phosphate buffered saline (8 g L-1 NaCl, 0.2 g L-1 KCl, 0.2 g L-1 KH2PO4, 1.14 g L-1 Na2HPO4, 0.2% w/v diethyldithiocarbamic acid

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(DIECA), 2% w/v polyvinylpyrrolidone average mol wt 10,000 (PVP), pH = 7.2 - 7.4) by triturating in sterile bags. Samples were screened following the protocols described by Sánchez-Vizcaino [21]

Crude extracts from three healthy vines were used as controls. The substrate used was p-nitro-phenyl phosphate at 1 mg mL-1 in 10% v/v diethanolamine solution (with 0.2% w/v of sodium azide). Readings were performed at A405nm. Samples were considered positive when readings were more than double of the average of controls (above 0.16 and 0.6, after 2 and 24 h, respectively).

2.6. Quantitative RT-PCR

Total RNA was extracted starting from 100 mg of leaf material using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich Co., MI, USA), following the manufacturer's instruction. For each biological replicate, DNA was removed by On-Column DNase I Digestion Set (Sigma-Aldrich Co., MI, USA) during the extraction protocol. Total RNA yield and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) for A260/A280 ratio verification, expected to range from 1.85 to 2.05. The primer/probe mixes for the one-step TaqMan®RT-PCR protocols were as follows: 20 μl each of the 100 pmol/μl forward and reverse primers and 4 μl of the 100 pmol/μl TaqMan®probe were added to 196 μl water to bring the final volume to 240 μl. Single-tube TaqMan®RT-PCR reactions (12 μl) were set up in 96-well reaction plates using a TaqMan®core reagent kit (Thermo Fisher Scientific, Massachusetts, USA) as follows: 6.1 μl one-step RT-PCR Master Mix, 0.6 μl primer/probe mix (400 nM primers and 80 nM probe), 0.3 μl MuLV/RNA inhibitor and 3 μl of total RNA template in 12 μl reaction. Reactions were carried out in a Biorad I-cycler (Hercules, California, USA) in a one-step reaction as recommended by Thermo Fisher Scientific (RT-PCR Master Mix procedure). Reverse transcription and amplification conditions were as follows: 45 ºC for 35 min, 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min (28). The data were analyzed quantitatively by measuring the threshold cycles (CT) in a Microsoft Excel program and graphically by an amplification plot. The threshold cycle (CT) is the cycle at which a significant increase in fluorescence occurs; hence a CT value below 40 indicates a positive result in this setup. For GLRaVs, GFkV and GFLV, primers and probes were described by Osman et al. [22,23] and Cepin et al. [24], respectively.

3. Results

71 of the 98 vines analyzed corresponded to varieties already described in the bibliography; 21 different genotypes were detected (Table 1).

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Table 1. Known varieties found in this study.

Zone	Input name	Confirmed variety	Number of individuals
Altiplano de Granada	Rosada Hornico	Rojal Tinta	1
La Alpujarra de Granada	Rome	Rome	1
	Mollar Cano	Rojal Tinta	1
	Tinta	Rojal Tinta	1
	Tinta Cortijo La Paz	Jacquez	1
	Llaqui	Jacquez	1
	Ricardera	Mantúo de Pilas	1
	Desconocida blanca	Airén	1
PDO Montilla-Moriles	Peñalista	Negra Rayada	1
	Montepila	Zalema	27
	Montepila	Cayetana Blanca	3
	Montepila	Pedro Ximénez	1
Valle de los Pedroches	Risquez	Ahmeur bou Ahmeur	2
	Risquez	Cayetana Blanca	1
	Merino	Cayetana Blanca	2
	Vieja Primera	Cayetana Blanca	2
	Blanca Lagareyes	Alarije	1
	Villaharta Llanos Suelo	Alarije	1
	Tío Kiko Camino	Negra Dorada	1
	Hebén	Hebén	1
	Jarosuelto	Jarrosuelto	1
	Schiava Grossa	Schiava Grossa	1
	Entreárboles	Zurieles	1
Pago Burujena	Mantúo Castellano	Listán del Condado	3
	Mantúo de Pilas	Alarije	2
	Barcelonés	Alarije	2
Moguer	Mollar Cano	Mollar Cano	1
	Beba	Beba	1
	Listán Prieto	Beba	1
	Moguer	Airén	1
	Moguer	Listán Prieto	2
	Mantúo de Sanlúcar	Listán del Condado	3
	Mesa Plaza Tinta	Alphonse Lavallée	1

The remaining 27 individuals showed genotypes that had not been previously described; in particular, 12 unknown varieties were identified (Table 2).

Table 2. Unidentified varieties found in this study.

Zone	Input name	Confirmed variety	Number of individuals
Altiplano de Granada	Blanca Hornico	Unidentified 09	1
La Alpujarra de Granada	Tinta Piedras Blancas	Unidentified 10	1
	Plateá	Unidentified 11	1
Valle de los Pedroches	Tinta Amparo	Unidentified 01	10
	Huerta de los Leones	Unidentified 02	2
	Arises	Unidentified 03	4
	Falda de la Sierra	Unidentified 04	1
	Arroyo Lorito	Unidentified 05	1
	Autóctona Miguel	Unidentified 06	1
	Risquez	Unidentified 07	1
	Lagarreyes	Unidentified 12	2
Moguer	Jaén Negro	Unidentified 08	2

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Most of the unidentified genotypes fitted into the eco-geographical group of varieties of the Mediterranean Iberian Peninsula (Figure 2), according to the clustering deduced by Cretazzo et al. [12]. It is noteworthy that one of the eight unidentified varieties found in "Valle de los Pedroches" fitted into the group of varieties that represents the *Prole orientalis*, whose geographic origin extends from Middle East to East Asia [13,25]. On the other hand, one of the two unidentified varieties found in "La Alpujarra de Granada" fitted into the group of Northern Italian and Southern French varieties.

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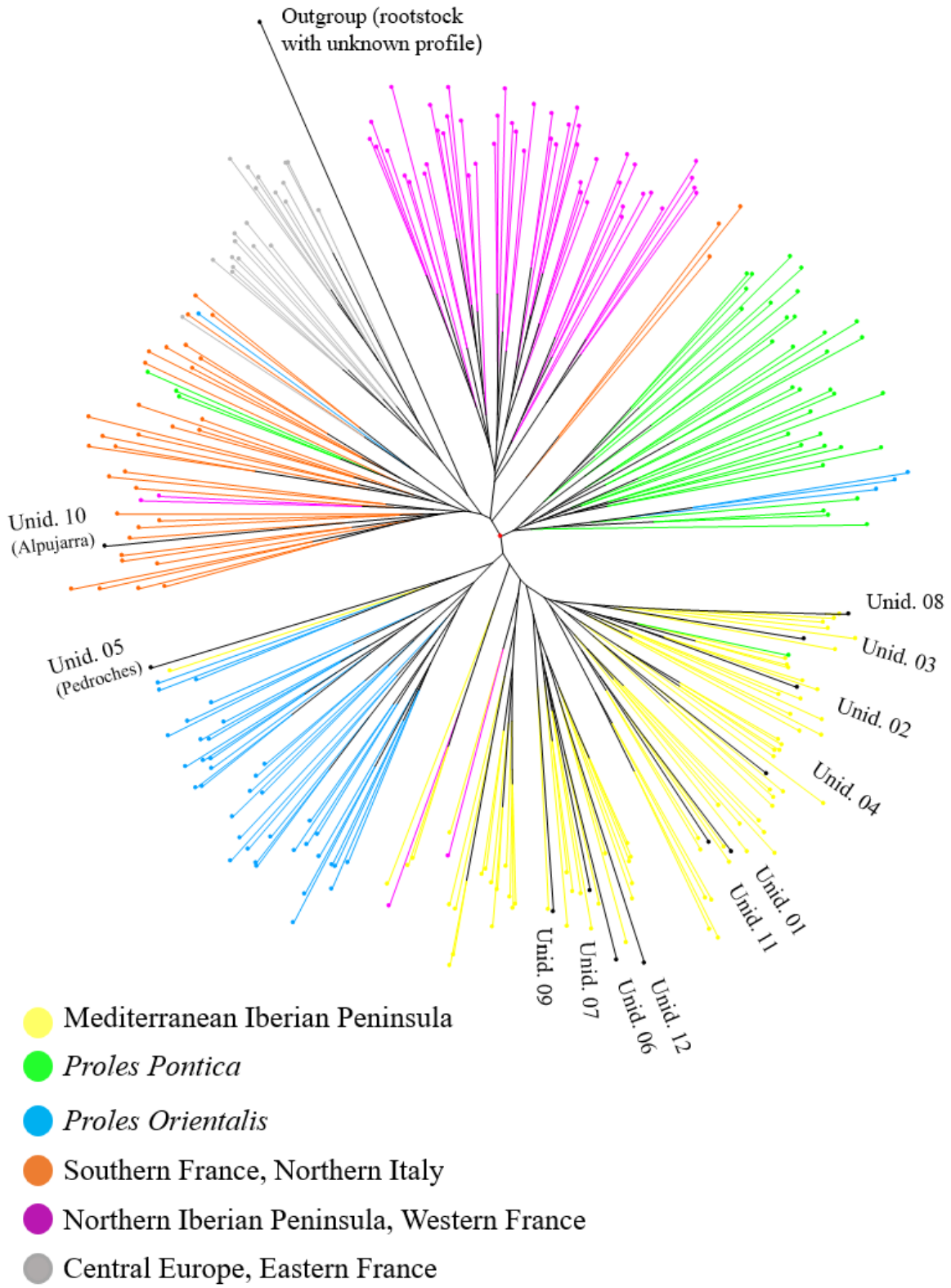

Figure 2. Phylogenetic tree obtained from the 13 SSR profiles of 271 varieties. The 12 unidentified genotypes, indicated with abreviation "unid.", detected in this study have been integrated into the set of varieties distributed by eco-geographic groups, according to Cretazzo et al. [12]

A third of the unidentified varieties ('Unidentified 01', 'Unidentified 04', 'Unidentified 06' and 'Unidentified 08') were assigned to a parent-pair with trio LOD score above the

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strict critical threshold (14.04). Three unidentified cultivars ('Unidentified 02', 'Unidentified 03' and 'Unidentified 09') were assigned to a trio with a LOD between the critic and the relaxed threshold (10.19). The remaining unidentified cultivars were assigned to trios under the relaxed critical LOD ('Unidentified 07', 'Unidentified 10' and 'Unidentified 11') or showed no trios inferred from the parent-pair analysis ('Unidentified 05' and 'Unidentified 12').

For the trios with a LOD score above the strict critical threshold, the pedigree analysis showed that 'Garrido Fino' and 'Unidentified 07' were inferred as putative parents of 'Unidentified 01'. Futhermore, 'Montúa' and 'Jarrosuelto' would be the parents of 'Unidentified 06'. For 'Unidentified 08' the best trio was conformed with 'Roal' and 'Gabriela', showing the highest LOD score. And lastly, 'Mencía' and 'Tortosí' would be the parents of 'Unidentified 04'.

For the trios with a LOD score between the critic and the relaxed threshold, we only considered the parent with 0 pair loci mismatching. This analysis showed 'Montúa' as one of the parents of 'Unidentified 07', 'Gabriela' as one of the parent candidates of 'Unidentified 03', and 'Marfal' as one of the parents of 'Unidentified 11', which would be one of the parents of 'Unidentified 02' as well.

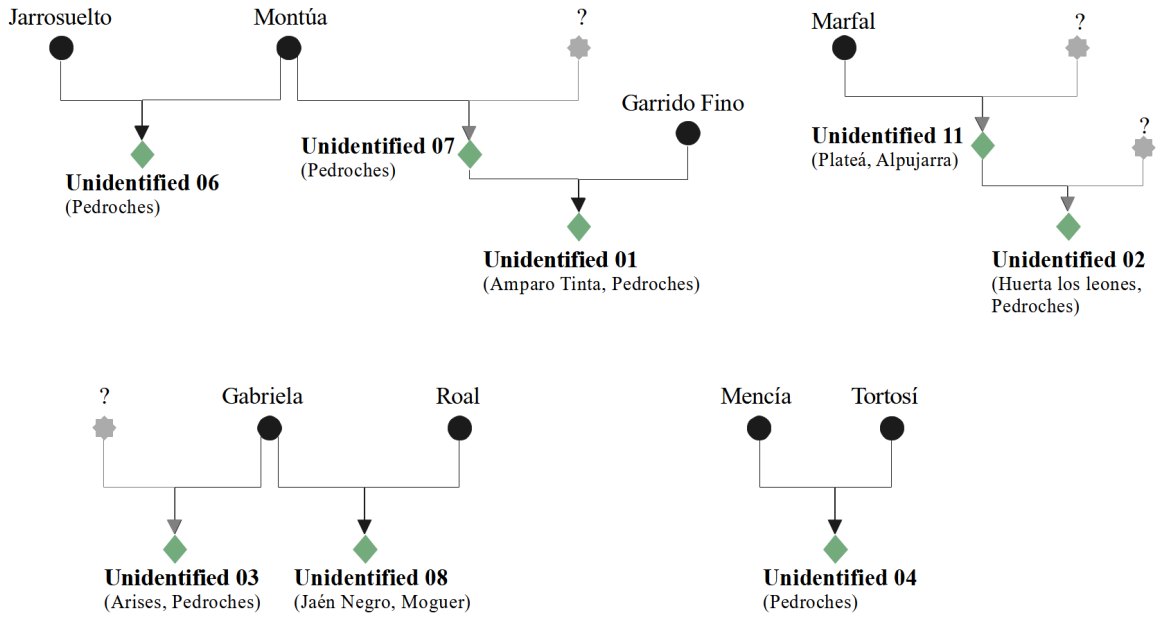

Figure 3. First-order kinship relationships obtained among the unidentified varieties found in this study. Trios with a LOD score above the stric critical threshold and a minimum pair loci mismatching and parents with 0 pair loci mismathing are represented.

Virus incidences among the surveyed areas showed by the ELISA and qPCR tests was very different (Table A1). It was possible to detect some healthy material in each area that could serve to start the process of registering the unregistered varieties in Spain or to certified local clones for the already registered varieties just in case they would be authorized in Andalusia under their main name or under some synonymy in the future.

4. Discussion

Most of the confirmed varieties found in this study (Table 1) showed belonging to the Mediterranean Iberian Peninsula group, according to the origin proposed by VIVC and the previous phylogenetic study [12]. Furthermore, varieties 'Rojal Tinta', 'Listán del Condado', 'Alarije' and 'Cayetana Blanca' were identified in more than one area prospected (Table 1). Likewise, ten out of the twelve unidentified varieties fitted in the Mediterranean Iberian Peninsula group (Table 2). Within the "Valle de los Pedroches", it is worth noting that unidentified genotypes 06 and 07 share a common putative parent ('Montúa'), as

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well as genotypes 01 (Amparo Tinta) and 07 showed a first-grade relationship (Figure 3). But also, another direct relationship is proposed between individuals from two different areas (as the case of Unidentified 11 from “La Alpujarra de Granada” and Unidentified 02 from “Valle de los Pedroches”), as well as a common putative parent (’Gabriela’) that is shared by Unidentified 03 (Arises, “Valle de los Pedroches”) and Unidentified 08 (Jaén Negro, “Moguer”). All that suggests a long history of combination of natural hybridization, breeding, selection, human-mediated movements of seeds and cuttings and other factors inside the Andalusian territory.

Next, the most relevant aspects of the results in each area studied are described:

a) "Altiplano de Granada". One strain turned out to be ’Rojal Tinta’, a suitable variety for producing rosé wines that is not authorized in Andalusia, but it is authorized in Castilla-La Mancha, an adjoining region. It should be noted that two other prospected vines, each in both locations of La Alpujarra, turned out to be ’Rojal Tinta’, which suggest an intentional cultivation of this variety in Eastern Andalusia. The microsatellite profile of ’Rojal Tinta’ matched with the profile described for the ’Rojal’ variety in Castilla-La Mancha by Fernández-Gonzalez et al. [26]. The other strain, white grape, turned out to have an unidentified genotypic profile, so further study would be necessary. It was already possible to ascertain the absence of infection by viruses listed in the current grapevine nursery plant legislation in the entire area [4]. This is probably due to, on one hand, the absence of massive introduction of propagation material from other origins due to the marginal viticulture in this area; and, on the other hand, to the accentuated altitude that prevents the presence of insect vectors of the Grapevine Leafroll-associated Virus, mainly *Planococcus ficus* and *P. citri*. The results of the ELISA and qPCR tests of the two strains prospected in this study have confirmed the absence of virus infection. The presence of healthy phytosanitary material implies the possibility of certifying a local Andalusian clone in case ’Rojal Tinta’ were to be authorized in Andalusia in the future.

b) "La Alpujarra de Granada". The interesting presence of ’Rojal Tinta’ has already been mentioned. Among the other genotypes found in this area, there are two local varieties (’Rome’ and ’Mantúo de Pilas’) for which there is a strong interest to have them included in the Register of Commercial Varieties in Spain and then authorized in Andalusia. ’Rome’ has been described in others Andalusia areas, such as "La Axarquía", in Málaga [27]. Futhermore, one individual of ’Airén’, the most cultivated white variety in Spain; two individuals who turned out to be ’Jacquez’, a direct producer hybrid with red berries (skin and pulp, therefore it is a dyera) and characterized by high acidity, a highly appreciated and sought-after quality in the indigenous red varieties of Andalusia, which usually lacks it; and two not previously identified genotypes, corresponding to a white grape vine and a red grape one, were found. The red grape vine turned out to be virus-free. This area is also characterized by the remarkable altitude at which the vineyards are cultivated; however, unlike Altiplano, viticulture in La Alpujarra has been historically more intense. It could explain that four vines showed simple or multiple virus infections (Appendix A1).

c) PDO "Montilla-Moriles" (Córdoba). 31 vines of the 32 prospected from eight different locations were named "Montepila" by the viticulturists. The literature that can be found regarding the term ’Montepila’ is very confusing, with several theories about the origin of this name (not published data). Microsatellite analysis clarified that it was actually ’Zalema’, the main white variety in the Huelva province, usually known in the Córdoba province as “Torrontés”. In fact, 27 of the prospected individuals showed "Zalema" genotype; three vines turned out to be ’Cayetana Blanca’ (a common variety in several regions of Spain, known in the Córdoba province as “Baladí-Verdejo"); and one vine turned out to be ’Pedro Ximénez’. Therefore, it is not an unidentified minority variety, although it is necessary to ampelographically verify that it is not a somatic mutant (a first approximation has already been carried out by studying 11 basic grouping characters that have shown no differences). ’Zalema’ is authorized as a variety in Andalusia; however, the synonymy ’Montepila’ is not yet contemplated, and its authorization could be undertaken through a request to the MAPA, supported by its corresponding technical report. The last strain studied, black

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berry, turned out to be 'Negra Rayada', a Spanish variety not authorized in the RVCV. This variety has also been located in the Andalusian province of Almería [28]. In one of the eight locations, the two vines with the 'Zalema' genotype were virus-free and, therefore, it could be interesting to use them to obtain a certified clone once the synonymy 'Montepila' is authorized.

d) "Valle de los Pedroches" (Córdoba). Eight not previously described genotypes were found in this area. Two of them were found 10 times (in eight different locations) and four times (in four different locations), respectively. Although viticulture has practically disappeared from the Valle de los Pedroches, these data indicate that there was some intentional cultivation of these two varieties, presumably in the pre-phylloxera period. In particular, these are a red and a white variety that are already being experimentally vinified by IFAPA technician. According to the classification established by Muñoz-Organero et al. [25], 'Tinta Amparo' and 'Arises' would be new autochthonous minority varieties. Two other genotypes were found twice; however, in both cases it took place in the same location and, therefore, they are unidentified varieties, just as the other four unidentified genotypes that were found once. Among the others varieties found were two Spanish commercial varieties ('Alarije' and 'Cayetana Blanca'), a table variety ('Ahmeur bou Ahmeur'), a foreign variety ('Schiava Grossa') and four known minority varieties ('Hebén', 'Jarrosuelto', 'Zurieles' and 'Negra Dorada'). 'Hebén' is a winegrape cultivar, already described in the 16th century [29]. It was grown in Andalusia [2], but currently it is hardly cultivated in the provinces of Córdoba, Granada, Badajoz, Guadalajara, Toledo and Cádiz [30,31]. There has been no interference through the introduction of foreign propagation material to the area in more than a century due to the fact that the cultivation of the vineyard is almost abandoned; for this reason, and taking into account the altitude of the valley, the ELISA ans qPCR tests showed the absence of viruses. These results were extremely interesting because a pool of new varieties was found. Healthy material for each variety to undertake the processes of registration to the RVCV and authorization in Andalusia, a strictly necessary condition for its legal cultivation, even for plots for self-consumption, was found.

e) "Pago Burujena" (Cádiz). There has been a growing interest in the recent years in the recovery of varieties used in the past and suitable for the production of fortified and still wines on the part of the regulatory council of the PDOs "Jerez-Xérès-Sherry" and "Manzanilla-Sanlúcar de Barrameda" [1,32]. In Pago Burujena, one of the most traditional estates in the Jerez area, copies of 'Mantúo Castellano' and 'Mantúo de Pilas' were supposedly preserved on a farm, for which three and two vines known by these denominations were respectively prospected. In addition, two vines known as 'Barcelonés' were also prospected. 'Mantúo Castellano' turned out to be a synonym of 'Listán del Condado', a variety cultivated in the province of Huelva, while 'Mantúo de Pilas' and 'Barcelonés' turned out to be 'Alarije'; for the first one it can be affirmed that there was a denomination error, while for the second one it was a synonymy. In the case of 'Mantúo Castellano', it would be interesting to request recognition of synonymy, since this is the name by which it has always been known in Jerez. In addition, one of the three plants turned out to be free of viruses, so a certified clone could be obtained from it.

f) "Moguer" (Huelva). In this township area were found an individual of the most cultivated variety in Spain, 'Airén'; two individuals of 'Beba', recently authorized in Andalusia, with a special interest for its cultivation in the Jerez area; an individual of 'Mollar Cano'; three individuals of 'Listán del Condado'; an individual of the table variety 'Alphonse Lavallée'; two genetically identical individuals of an unidentified variety locally called "Jaén Negro" (it would be a homonym, since 'Jaén Negro' also refers to a synonym of the 'Jaén Tinto' variety); and two individuals of 'Listán Prieto'. The last one is possibly the most important result. In the book from 1513, Agricultura General, Herrera [29], described the 'Uvas Prietas' variety cultivated in the center of the Iberian Peninsula, which could possibly be 'Listán Prieto'. This variety is also expressly mentioned in Andalusia in the year 1807 [1]. As a result of the America and the Canary Islands conquest in the 15th century, people from the Iberian Peninsula (Galician, Castillan, Andalusian, Extremaduran or even

Portuguese) settled down in these lands, bringing among their customs and crops the vine [33]. Around 1550 a Jesuit implanted the 'Listán Prieto' variety in Peru [34], and cultivars were introduced in Mexico between the years 1520 and 1540 [35]. In California it is known as 'Mission' [36] as an allusion to the vines carried by the friars in their evangelizing work. In the 19th century, the phylloxera obliterated almost half of the peninsular vineyards [35], but this insect did not reach the Canary Islands. The use of this variety is widespread in the Canary Islands and in America [37,38]. There was no record of its cultivation in the Iberian Peninsula since the last century [36]. Therefore, the location of several specimens of this variety, with over 50 years old, in the township of Moguer has a great historical relevance. 'Listán Prieto' usually produces wines with a high color intensity and good acidity, characteristics highly desired in Andalusian red wines, so its detection in these surveys can support the intention of authorizing it in Andalusia. Three virus-free vines were found in this zone, one belonging to 'Beba', another one to 'Listán Prieto' and another to the unidentified "Jaén Negro". They could represent the starting point for obtaining local certified clones of these varieties.

In this paper we have considered a vine as a certifiable clone only when both ELISA and qPCR analyses displayed the absence of all viruses required by certification rules with the addition of GLRaV-2. In the case of the unidentified varieties for which a registration process wanted to be undertaken, these virus-free vines should also be used for obtaining propagation material to send to the National Reference Collection of Grape Varieties for evaluations needed. As mentioned before, MAPA requires these material to be virus-free at only ELISA assays. However, in order to really maintain this collection free of viruses, we strongly advised MAPA to require both virus analysis, ELISA and qPCR (and possibly include also GLRaV-2). In fact, on one hand, it is well known that qPCR is much more sensitive than ELISA, allowing to detect viruses at very low concentration where ELISA would display false-negative results [39]. In this work there are several examples in which negative ELISA correspond to positive qPCR (see Appendix A1., sections La Alpujarra de Granada, PDO Montilla-Moriles, Pago Burujena and Moguer). However, on the other hand, viruses undergo frequent mutation events that can modify the virus sequence in the hybridation regions for both primers and probe, which could result in false-negatives with a major rate for qPCR than ELISA [40,41]. In the Appendix A1 are two cases in which negative qPCR correspond to positive ELISA (Mantúo de Sanlucar/Listán del Condado for GLRaV-3 and Marenas/Zalema for GLRaV-1). In our opinion, a correct strategy to test vines before sending material for its registration in the RVCV, or deciding to start a sanitation protocol, should be testing by ELISA (less expansive) before and then performing qPCR only for negative test.

The results obtained showed that it is necessary to continue the research of minority varieties, prospecting, recovering and conserving them, as well as studying their agronomic and enological characteristics, as they represent a wine patrimony both at regional and national levels.

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Abbreviations

The following abbreviations are used in this manuscript:

OIV	International Organisation of Vine and Wine
ELISA	Enzyme-Linked ImmunoSorbent Assay
PDO	Protected Designation of Origin
PGI	Protected Geographical Indications
IMIDA	Instituto Murciano de Investigación y Desarrollo Agrario y Medioambiental
MAPA	Ministerio de Agricultura, Pesca y Alimentación
CPVO	Community Plant Variety Office
UPOV	International Union for the Protection of New Varieties of Plants
GFLV	Grapevine Fanleaf Virus
GLRaV	Grapevine Leafroll associated Virus
GFkV	Grapevine Fleck Virus
DNA	Deoxyribonucleic Acid
SSR	Short Sequence Repeat
CREA-UTV	Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria
PCR	Polymerase Chain Reaction
VIVC	Vitis International Variety Catalog
IFAPA	Instituto Andaluz de Investigación y Formación Agraria, Pesquera y Alimentaria
RVCV	Registro de Variedades Comerciales de Vid

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Appendix A

Table A1. Viruses detected in each studied area.

Zone	Input name	Variety	ELISA	PCR
Altiplano de Granada	Rosada Hornico	Rojal Tinta	-	-
	Blanca Hornico	Unidentified 09	-	-
La Alpujarra de Granada	Rome	Rome	-	-
	Mollar Cano	Rojal Tinta	-	GFkV
	Tinta	Rojal Tinta	-	-
	Tinta Cortijo La Paz	Jacquez	-	-
	Llaqui	Jacquez	-	-
	Ricardera	Mantúo de Pilas	-	GFkV, GLRaV-3
	Desconocida Blanca	Airén	GFLV, GLRaV-2	GFLV, GFkV, GLRaV-2
	Tinta Piedras Blancas	Unidentified 10	-	-
	Plateá	Unidentified 11	GFLV	GFLV, GFkV
PDO Montilla-Moriles	Peñalista	Negra Rayada	m.d.	m.d.
	cp1 Marenas fila 3 cp5	Zalema	GFkV	GFkV
	cp2 Marenas fila 4 cep18	Zalema	GFkV, GLRaV-3	GFkV, GLRaV-3
	Marenas fila 7 cp3 cp44	Zalema	GFkV, GLRaV-3	GFkV, GLRaV-2, GLRaV-3
	cp4 Marenas fila 8 cp16	Zalema	GFkV, GLRaV-1	GFkV
	cp5 Marenas fila 9 cp20	Zalema	GFkV	GFkV
	cp9 La Plata fila 9	Zalema	m.d.	m.d.
	La Plata fila 13 p7	Pedro Ximénez	-	GFkV, GLRaV-2
	cp 6 La Plata fila 17	Zalema	GFkV	GFkV, GLRaV-5
	cp3 La Plata fila 19	Zalema	-	GLRaV-3, GLRaV-5
	Los Rosales 1	Zalema	GFkV	GFkV
	Los Rosales 2	Zalema	-	GFkV, GLRaV-2
	Los Rosales 3	Zalema	-	GFkV, GLRaV-3
	Los Rosales 4	Zalema	GFkV	GFkV, GLRaV-3
	Colección Montepila 1	Zalema	-	GFkV
	Colección Montepila 2	Zalema	-	GFkV
	Montepila 1 recinto 16	Zalema	GFkV	GFkV
	Montepila 2 (recinto 16)	Zalema	-	GFkV
	Montepila 3 recinto 16	Zalema	GFkV, GLRaV-3	GFkV, GLRaV-2, GLRaV-3
	Montepila 4 recinto 16	Zalema	GLRaV-3	GFkV, GLRaV-3
	Montepila los Naranjos 1	Zalema	m.d.	m.d.
	Montepila (2 linde) los Naranjos	Zalema	m.d.	m.d.
	Montepila los Naranjo 3	Zalema	m.d.	m.d.
	Montepila 4	Zalema	m.d.	m.d.
	Montepila los Naranjos 5	Cayetana Blanca	m.d.	m.d.
	La Primilla 1	Zalema	-	-
	La Primilla 2	Cayetana Blanca	m.d.	m.d.
	La Primilla 3	Zalema	-	-
	La Primilla 4	Cayetana Blanca	m.d.	m.d.
	Cuesta Blanca 1	Zalema	m.d.	m.d.
	Cuesta Blanca 2	Zalema	m.d.	m.d.
	Cuesta Blanca 3	Zalema	m.d.	m.d.

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Zone	Input name	Variety	ELISA	PCR
Valle de los Pedroches	Risquez 1	Ahmeur bou Ahmeur	-	
	Risquez 2	Ahmeur bou Ahmeur	-	-
	Risquez 3	Unidentified 07	-	-
	Risquez 4	Cayetana Blanca	-	-
	Vieja Primera	Cayetana Blanca	-	-
	Vieja Primera	Cayetana Blanca	-	-
	Merino 1	Cayetana Blanca	-	-
	Merino 2	Cayetana Blanca	-	-
	Lindero C1	Unidentified 01	-	-
	La Torre Amparo Tinta	Unidentified 01	-	-
	Camino falda de la sierra	Unidentified 01	-	-
	Recio 1º Amparo Tinta	Unidentified 01	-	-
	Recio 2º Amparo Tinta	Unidentified 01	-	-
	Recio 3º Amparo Tinta	Unidentified 01	-	-
	Recio 4º Amparo Tinta	Unidentified 01	-	-
	Recio 5º Amparo Tinta	Unidentified 01	-	-
	Tinta Amparo malla Rafael	Unidentified 01	-	-
	Tinta Amparo huerta Rafael	Unidentified 01	-	-
	Huerta Los Leones C5	Unidentified 02	-	-
	Huerta Los Leones C2	Unidentified 02	-	-
	Isleta (Arises)	Unidentified 03	-	-
	Garrido (Arises)	Unidentified 03	-	-
	Arises cuadra Rafael	Unidentified 03	-	-
	Arises huerta Rafael	Unidentified 03	-	-
	Camino falda de la sierra (Portillo)	Unidentified 04	-	-
	Arroyo Lorito	Unidentified 05	-	-
	Autóctona Miguel	Unidentified 06	-	-
	Lagareyes	Unidentified 12	-	-
	Lagareyes	Unidentified 12	-	-
	Blanca Lagareyes	Alarije	-	-
	Villaharta llanos suelo	Alarije	-	-
	Tío Kiko Camino	Negra Dorada	-	-
	Hebén	Hebén	-	-
	Jarrosuelto	Jarrosuelto	-	-
	Schiava Grossa	Schiava Grossa	-	-
	Entreárboles	Zurieles	-	-
Pago Burujena	Mantúo Castellano	Listán del Condado	GFkV, GLRaV-3	GLRaV-2, GLRaV-3, GFkV
	Mantúo Castellano	Listán del Condado	GFkV, GFLV, GLRaV-3	GLRaV-2, GLRaV-3, GFkV, GFLV
	Mantúo Castellano	Listán del Condado	-	-
	Mantúo de Pilas	Alarije	GFkV	GFkV
	Mantúo de Pilas	Alarije	GFkV	GFkV
	Barcelonés	Alarije	GFkV	GLRaV-2, GLRaV-3, GFkV
	Barcelonés	Alarije	GFkV	GLRaV-2, GLRaV-3, GFkV
Moguer	Mollar Cano	Mollar Cano	GLRaV-3	GLRaV-3
	Listán Prieto	Beba	-	GFkV, GLRaV-3
	Beba	Beba	-	-
	Moguer	Airén	-	-
	Moguer	Listán Prieto	-	-
	Moguer	Listán Prieto	-	GLRaV-3
	Mantúo de Sanlúcar	Listán del Condado	-	GLRaV-3
	Mantúo de Sanlúcar	Listán del Condado	GLRaV-3	-
	Mantúo de Sanlúcar	Listán del Condado	-	GLRaV-3
	Mesa Plaza Tinta	Alphonse Lavallée	-	GLRaV-3
	Jaén Negro	Unidentified 08	GFkV, GFLV	GFkV, GFLV
	Jaén Negro	Unidentified 08	-	-

*Virus-free vines are indicated in red

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