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Genotypic and Sanitary Characterization of Minority Grapevine Varieties Prospected in Andalusia, Spain

Inmaculada Rodríguez-Torres [1,†], Ana Martín Carrillo [2,†], María del Pilar Ramírez [3], Francisco Jesus Gómez Gálvez [4], Leonardo Velasco Arjona [5], Carlos Padilla [6] and Enrico Cretazzo [2,*]

1 Agroindustry and Food Quality Area, IFAPA Rancho de la Merced Center, Ctra. Cañada de la Loba PK 3,1, 11471 Jerez de la Frontera, Spain; icrodrigueztorres@gmail.com
2 Biotechnology and Plant Breeding Area, IFAPA Rancho de la Merced Center, Ctra. Cañada de la Loba PK 3,1, 11471 Jerez de la Frontera, Spain; ana.martin.carrillo@juntadeandalucia.es
3 Agroalimentary Technologies and Engineering, IFAPA Cabra Center, Antigua Ctra. Cabra-Doña Mencía, Km. 2,5, 14940 Cabra, Spain; mariap.ramirez.perez@juntadeandalucia.es
4 Biotechnology and Plant Breeding Area, IFAPA Alameda del Obispo Center, Avda. Menéndez Pidal s/n, 14080 Cordoba, Spain; franciscoj.gomez.galvez@juntadeandalucia.es
5 Sustainable Plant Protection Area, IFAPA Málaga Center, Cortijo de la Cruz s/n, 29140 Malaga, Spain; leonardo.velasco@juntadeandalucia.es
6 Plant Protection Department, IMIDA, Calle Mayor s/n, 30150 Murcia, Spain; carlosv.padilla@carm.es
* Correspondence: enrico.cretazzo@juntadeandalucia.es
† These authors contributed equally to this work.

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 genotypic characterization of 98 specimens from six areas with a winemaking tradition in Andalusia was carried out between the years 2020 and 2022, by means of thirteen microsatellite markers, including the nine recommended by the OIV. A total of 33 different genotypes were obtained, 20 of which corresponded to profiles of already described varieties (11 of them are of 6 minority cultivars 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 12 profiles did not match with previously identified varieties. These profiles were registered in the database of the IFAPA "Rancho de la Merced" Germplasm Bank. The eco-geographical groups of the new identified genotypes were determined through an analysis of genetic diversity. The presence of grapevine fanleaf virus, grapevine fleck virus, and grapevine leafroll-associated viruses was also determined due to the requirement of healthy clones of the new varieties for their potential interest to be authorized for cultivation in Spain.

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

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1. Introduction

Andalusia has a surface area 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. Grapevine (*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 1000 m; in areas close to the sea, such as the hills of "Marco de Jerez" (Cádiz); and even in regions with high slopes, such as "La Axarquía" (Málaga). There are seven 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. Moreover, the region has 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 in this region [1,2]. Although this plague ended up destroying more than a million hectares with the consequent loss of many local varieties, it is estimated that about 300 out of the 600 varieties that were originally cultivated before the invasion in 1878 were conserved.

Currently, 56 grape varieties are authorized for cultivation in Andalusia in accordance with the List of Authorized Varieties for Andalusia (Real Decreto 111/2022, of 9 February, which modifies Real Decreto 1338/2018, of 29 October, which regulates the potential of viticultural production). Despite this number, only four white grapes account for 77% of the total cultivated area: 'Palomino Fino', 'Pedro Ximénez', 'Moscatel de Alejandría', and 'Zalema' (unpublished, according to "Consejería de Agricultura, Pesca, Agua y Desarrollo Rural, Junta de Andalucía, 2022). Furthermore, the sum of the 29 least cultivated varieties only represents 2% of the total surface. It can be understood from these data that many 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 authorized in Spain, their conservation is endangered. For all these reasons, it is crucial to investigate them in situ and to identify, recover, and evaluate the possibility of including some of these varieties in the Andalusian wine heritage. However, as has already been mentioned, in many cases, these varieties are not authorized for cultivation in Spain. Therefore, if there is genuine interest in growing some of these varieties, it would be necessary to carry out the official authorization process. The first step of this process consists of forwarding plants grafted onto '110R' rootstock to the National Reference Collection of Grape Varieties (maintained at IMIDA, Murcia, and funded by MAPA, Madrid). A regulated ampelographic assessment is then carried out when the vines are formed, in which 44 characters (including leaves, shoots, flowers, and fruits) according to CPVO and UPOV will be recorded for at least two years. In addition, to qualify the candidate plant materials, the MAPA requires the absence of grapevine fanleaf virus (GFLV), grapevine leafroll-associated viruses 1 and 3 (GLRaV-1 and -3) and grapevine fleck virus (GFkV) as tested by serology. These are the viruses contemplated by Orden APA/2474/2006, of 27 July, which modifies certain annexes of the Technical Regulation for the Control and Certification of Vine Nursery Plants approved by Real Decreto 208/2003, of 21 February. Therefore, to include a variety in the Register of Commercial Grapevine Varieties in Spain (RVCV) and then obtain authorization for its cultivation in a given Autonomous Community, it is required to identify or proceed through sanitation propagation (i.e., in vitro cultivation) with a clone that is certifiable from the phytosanitary point of view.

It is well known that a big concern in most viticultural areas around the world is the scarceness of varietal diversification [4]. Moreover, frequently, only a very few clones are used for a given variety, resulting in a substantial reduction in genetic variability [5]. In this study, we show a methodology for approaching the initial steps necessary to increase the pool of cultivated grape varieties and clones in a given area. Multiple goals could be pursued: (a) to add a commercial variety to the list of authorized ones in a given region; (b) to recognize new synonymies of already authorized varieties and have available new local clones that are sanitary guaranteed; (c) to recover old varieties, possibly described in the past, that are very well adapted in a given ecosystem; (d) to discover new autochthonous varieties that could provide the basis for climate change adaptation in a given area [6]. Here, we present the results of surveys of minority varieties carried out on 98 specimens in six wine-producing areas spanning Andalusia (Figure 1).

Figure 1. Map of the prospected areas.

2. Materials and Methods

2.1. Areas and Specimens Prospected

Expert staff from local entities such as Regional Agricultural Offices, Rural Development Groups, Regulatory Councils of Denomination of Origin, and autonomous viticulturists and oenologists have intensively collaborated with IFAPA, pointing out the old vineyards as the object of the study and selecting the vines included in the prospection because of their interesting productive and qualitative features. Next, more details are provided about the areas studied:

a. "Altiplano de Granada", a region in the northeast of the Granada province that includes the Huéscar and Baza municipalities, is characterized by its high average altitude (between 700 and 1200 m). Grapevine cultivation occupies an area of about 350 ha and it is widely distributed in more than 1000 small vineyards with an average surface of 0.3 ha, thus showing great fragmentation and being owned by many local winemakers for domestic consumption. This area is protected by the PDO "Granada" and the PGI "Altiplano de Sierra Nevada". Given that an exhaustive survey had been previously carried out in this area, only two plants have been studied in this work [7].

b. "Alpujarra de Granada", located in the southeastern part of the Granada province. It is characterized by its rugged relief, occupying most of the southern face of the Sierra Nevada. The 25 municipalities that make it up are located at an altitude between 1200 and 1500 m. Consisting of 2000 ha of vineyards with slate and stony soils and a slightly limestone subsoil. This region is protected by the PDO "Granada" and the PGI "Cumbres del Guadalfeo". Nine specimens were surveyed in this area from two plots whose vineyards are more than 60 years old, and their origin could be dated back to the pre-phylloxera period.

c. PDO "Montilla-Moriles", in the central area of Andalusia belonging to the Córdoba province, has the Guadalquivir River to the north, the Subbética Mountains to the south, the Genil River to the east, and the Guadajoz River to the west. This zone includes 17 municipalities. This PDO has nearly 6000 ha of vineyards that produce fortified wines (Fino, Amontillado, Oloroso, and Palo Cortado) and sweet wines with the 'Pedro Ximénez' variety. 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 specimens were surveyed from eight plots located in the municipalities of Montilla, Moriles, and Cabra, with the aim of clarifying the situation of a supposed different variety locally called "Montepila", given the strong interest of the winemaking sector that requires its inclusion in the registry of authorized varieties in Andalusia.

d. "Valle de los Pedroches", a region of 3162 km^2 also located in the Córdoba province, represents the northernmost territory of Andalusia. This area is characterized by its high 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 an average altitude of between 500 and 700 m. Valle de los Pedroches is protected by the PGI "Córdoba". The vineyards of this area reached their maximum splendor in the 17th and 18th centuries, when they exceeded 2000 ha. It has been estimated that about 1800 ha were destined for the cultivation of vines shortly before the arrival of phylloxera at the end of 1800. Grapevine cultivation started disappearing shortly after this plague, although isolated or semi-isolated vine plants remained in the area, mainly for the production of self-consumption wines. In recent years, there has been a growing interest in these family orchards in order to preserve the biodiversity and traditional landscapes of the area. This indicates 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/or international varieties have been avoided. Thirty-six vines were surveyed in this area, most of them isolated specimens located near the dividing boundaries between the plots.

e. "Pago Burujena", a territory located within El Marco de Jerez (PDO "Jerez-Xérès-Sherry" and "Manzanilla-Sanlúcar de Barrameda"), is the most emblematic and important area for the production of fortified and sweet Andalusian wines, having the same typologies mentioned for the PDO "Montilla-Moriles" but using instead the traditional 'Palomino Fino' variety. A greater varietal diversification has been recently pursued in the Jerez DO, and one of the objectives of producers is to rescue pre-phylloxera varieties for the production of both fortified and still wines. In the Jerez viticulture and its surroundings area, "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 Duchy of Medina Sidonia, and consists of an area of about 22 ha characterized by high quality limestone soils and the presence of different traditional varieties of the area. [8]. Seven specimens from a plot located in the Pago were surveyed.

f. "Moguer", whose municipality is protected by the PDO "Condado de Huelva", is a territory with a high vocation for growing vines, characterized by vineyards at an average altitude of 25 m, 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 currently cultivated, with a high degree of fragmentation of the plots (average surfaces between 2 and 3 ha), many of them older than 40 years. For these reasons, and because of its long winemaking tradition, it is an ideal area to survey for minority varieties linked to possible wine diversification. In the province of Huelva, this production is mostly based on the cultivation of 'Zalema'. Twelve vines over 50 years old belonging to this municipality and collected from a single plot were surveyed.

2.2. Plant Materials

For the genotypic study, shoots were collected from each of the 98 grapevine plants and stored at 4 °C during 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.

For virus tests, five adult leaves were collected from different shoots of each plant in the late spring of 2022, and quickly stored at 4 °C. From each of the 5 leaves, 1 cm of petiole and a leaf surface of about 2 cm^2 attached to the same petiole were kept apart, stored in the cold and used for the determination of virus infections.

2.3. Genotypic Analysis

The DNeasy 96 Plant Kit (Qiagen, Hilden, Germany) extraction kit was used to purify total DNA. DNA concentration was recorded with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, NC, USA).

Microsatellite markers (SSR) were chosen for varietal identification since they are one of the most widespread tools for identifying grapevine genetic resources [9]. Thirteen SSR were analyzed, nine of them (VVS2 [10], VVMD5, VVMD7 [11], VVMD25, VVMD27 [12], 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 [13]), have been proven to be quite polymorphic in previous studies. For the GrapeGen06 SSR set, data were coded to compare microsatellite profiles as reported by Maul et al. [14] in order to include them in the European Vitis database (http://www.eu-vitis.de/index.php (accessed on 1 May 2023)). 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. Reactive volumes and PCR conditions were set according to previous studies [13,15]. The amplicons were separated on the 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 into the IFAPA Rancho de la Merced database, which has around 1500 genotypes.

2.4. Cluster Analysis

A set of 258 genotypes with a clear ancestry inferred by Cretazzo et al. [15] in accordance with the eco-geographic origin of the cultivars [16], was used as a template composed of six groups representing different putative geographical origins. The twelve genotypes not previously described in this work were integrated into this dataset in order to investigate their ancestry. 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 [17], according to the default setting recommended by the software, with the only modification being to increase bootstrap replicates up to one thousand to obtain more accuracy.

2.5. Pedigree Analysis

The software CERVUS v.3.0.7 (Field Genetics, London, UK) [18] 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 profiles had been fully characterized [15]. The profiles of the 12 unknown varieties were included as candidates as well, with 541 cultivars, the set of possible parents used. This analysis relies on allele frequencies and is based on the difference in the log-likelihood ratio (LOD) between related and unrelated relationships to assign parentage, combined with a simulation of parentage analysis to determine the confidence of assignments. The parameters considered for the simulation were the following: number of offspring = 100,000; 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 equal to two. The parameters considered for the simulation and criteria were adopted according to the default settings recommended by the software as well as the bibliography [19–21].

2.6. DAS-ELISA Test

Serological tests for the determination of GFLV, GLRaV-1, GLRaV-2, GLRaV-3, and GFkV were performed using the Bioreba (Reinach, Switzerland) and Agritest (Valenzano, Italy) specific DAS-ELISA tests. Although GLRaV-2 analysis is not currently required in certification schemes in Spain, its determination is recommended by the ICVG [22] and its importance in Spain is well known [23]. For virus determination, plant tissue was homogenized in sterile bags with phosphate buffered saline at pH = 7.2–7.4, including 0.2% *w*/*v* diethyldithiocarbamic acid (DIECA) and 2% *w*/*v* polyvinylpyrrolidone average mol wt 10,000 (PVP). Extracts were then analyzed according to Sánchez-Vizcaino et al. [24]. Homogenates from three healthy vines were included as negative controls in all the DAS-ELISA plates. Samples were considered positive when OD405 readings were at least two times the average of controls [25].

2.7. Quantitative RT-PCR

Total RNA was obtained using 100 mg of leaf material that was crushed in liquid nitrogen and homogenized in the lysis buffer from the SpectrumPlant^{TM} Total RNA kit (Sigma-Aldrich Co., San Luis, AZ, USA), and extracted following the manufacturer's recommendations. For each biological replicate, genomic DNA was removed by the On-Column Dnase I Digestion Set (Sigma-Aldrich Co., San Luis, AZ, USA) during the extraction protocol. Total RNA yield and purity were determined using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, 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 of 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, Waltham, MA, USA) as follows: 6.1 µL of one-step RT-PCR Master Mix, 0.6 µL of primer/probe mix (400 nM primers and 80 nM probe), 0.3 µL of MuLV/RNA inhibitor, and 3 µL of total RNA template in a 12 µL reaction. Reactions were carried out in a Biorad I-cycler (Biorad, Hercules, 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 °C for 35 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The data were analyzed quantitatively by measuring the threshold cycles (CT) in the Microsoft Excel program and graphically by an amplification plot. For the GLRaVs, GFkV, and GFLV, the primers and probes used were previously described by Osman et al. [26,27] and Cepin et al. [28], respectively.

3. Results

According to the genetic analyses, 71 of the 98 vines analyzed in this work corresponded to varieties already described in the literature, discerning 21 different genotypes (Table 1). The remaining 27 individuals showed genotypes that had not been previously described; in particular, 12 unknown varieties were identified (Table 2).

Most of the unidentified genotypes fit into the eco-geographical group of varieties of the Mediterranean Iberian Peninsula (Figure 2), according to the clustering deduced by Cretazzo et al. [15]. Remarkably, one of the eight unidentified varieties found in "Valle de los Pedroches" fit into the group of varieties that represents *Prole orientalis*, whose geographic origin extends from the Middle East to East Asia [16,29]. 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.

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
	Jarrosuelto	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 Prieta	Beba	1
	Moguer	Airén	1
	Moguer	Listán Prieto	2
	Mantúo de Sanlúcar	Listán del Condado	3
	Mesa Plaza Tinta	Alphonse Lavallée	1

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

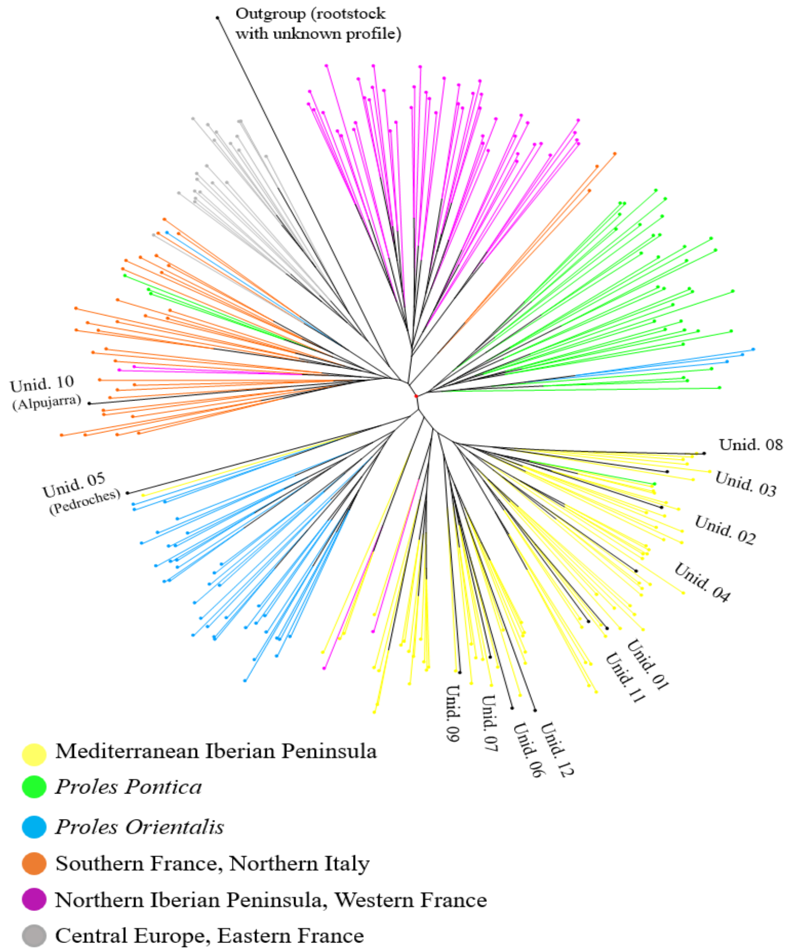

Figure 2. Phylogenetic tree obtained from the 13 SSR profiles of 271 varieties. The 12 unidentified genotypes, indicated with the abbreviation "unid.", detected in this study have been integrated into the set of varieties distributed by eco-geographic groups, according to Cretazzo et al. [15].

A third of the unidentified varieties ('Unidentified 01', 'Unidentified 04', 'Unidentified 06' and 'Unidentified 08') were assigned to a parent-pair with a trio LOD score above the 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'. Furthermore, 'Montúa' and 'Jarrosuelto' would be the parents of 'Unidentified 06' For 'Unidentified 08' the best trio was formed with 'Roal' and 'Gabriela', showing the highest LOD score. 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 zero 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. Summary statistics obtained from the allele frequency analysis conducted in CERVUS 3.0 are provided in Appendix A, Table A1.

The virus incidence was very different among the surveyed areas (Appendix A, Table A2). Nevertheless, it was always possible to identify virus-free specimens in each area that could be useful as starting material in the process of registering the unregistered varieties in Spain. Additionally, it is also possible to recover potential certified local clones for the already registered varieties in case they are authorized in Andalusia under their main name or under some synonymy in the future.

4. Discussion

Most of the confirmed varieties identified in this study clustered in the Mediterranean-Iberian Peninsula group (Table 1), according to the origin proposed by VIVC and a previous phylogenetic study [15]. Furthermore, varieties 'Rojal Tinta', 'Listán del Condado', 'Alarije', and 'Cayetana Blanca' were identified in more than one of the areas prospected (Table 1). Likewise, ten out of the twelve unidentified varieties fit into the Mediterranean-Iberian Peninsula group (Table 2). Within the "Valle de los Pedroches", it is worth noting that the unidentified genotypes 06 and 07 share a common putative parent ('Montúa'), as well as 289 genotypes 01 (Amparo Tinta) and 07 showed a first-grade relationship (Figure 3). Likewise, we propose another direct relationship between specimens from two different areas (as in 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 natural hybridization, breeding, selection, human-mediated movements of seeds and cuttings, and other factors inside the Andalusian territory.

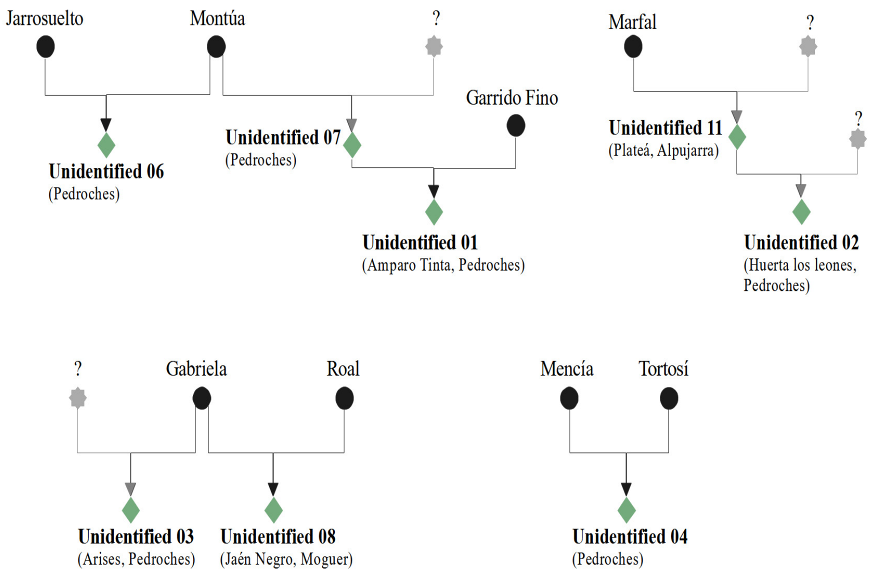

Figure 3. First-order kinship relationships were obtained among the unidentified varieties found in this study. Trios with a LOD score above the strict critical threshold and a minimum pair loci mismatching and parents with zero pair loci mismatching are represented. The character "?" means undetermined parent.

Next, we highlight the most relevant aspects of the results in each area studied:

a. "Altiplano de Granada". One specimen was shown to correspond to 'Rojal Tinta', a suitable variety for producing rosé wines that is not currently authorized in Andalusia but authorized in Castilla-La Mancha, an adjoining Community. Interestingly, two other prospected vines, each in both locations of La Alpujarra, corresponded to 'Rojal Tinta', suggesting the intentional cultivation of this variety in Eastern Andalusia. The microsatellite profile of 'Rojal Tinta' matched the profile described for the 'Rojal' variety in Castilla-La Mancha by Fernández-Gonzalez et al. [30]. The other specimen, white grape, showed an unidentified genotypic profile; thus, additional studies would be necessary to further characterize this variety. It is remarkable the low rate or absence of virus infections in the "Altiplano de Granada" area [7]. Possible explanations rely on the one hand, on the absence of massive introductions of propagation material from other origins due to the marginal viticulture in this area and, on the other hand, on the high altitude that prevents the presence of insect vectors of grapevine leafroll-associated viruses, mainly *Planococcus ficus* and *P. citri*. The results of the ELISA and RT-RT-qPCR tests of the two varieties surveyed in this study have confirmed the absence of virus infections. The presence of healthy phytosanitary material enables the possibility of certifying a local 'Rojal Tinta' clone in Andalusia.

b. "La Alpujarra de Granada". The presence of 'Rojal Tinta' in this area has already been highlighted. Regarding the other genotypes present in this area, there are two local varieties ('Rome' and 'Mantúo de Pilas') for which there is a strong interest among the winemakers to include them in the Register of Commercial Varieties in Spain, followed by authorization in Andalusia. 'Rome' has been already described in other areas of Andalusia, such as "La Axarquía" in Málaga province [31]. In addition, we identified one specimen of 'Airén', the most cultivated white variety in Spain; two other specimens corresponded to 'Jacquez', a direct producer hybrid with red berries (skin and pulp, therefore it is a dyera) and characterized by a high acidity in wines, 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. The red grape vine was shown to be virus-free. "La Alpujarra" is also characterized by vineyards at high altitude. However, unlike Altiplano, viticulture in La Alpujarra has historically been more intensive, and the exchange of plant materials has been frequent. This might explain why four of the vines showed simple or multiple virus infections (Appendix A, Table A2).

c. PDO "Montilla-Moriles" (Córdoba). A total of 31of the 32 vines prospected from eight different locations are named "Montepila" by local viticulturists. The literature regarding the term 'Montepila' is unclear, with several conjectures about the origin of this name (unpublished). Microsatellite analysis allowed us to clarify that it corresponded to 'Zalema', the main white variety grown in the Huelva province, usually known in the Córdoba province as "Torrontés". Moreover, 27 of the prospected specimens showed the "Zalema" genotype, three additional vines matched to 'Cayetana Blanca' (a common variety in several regions of Spain, known in the Córdoba province as "Baladí-Verdejo") and another vine matched to 'Pedro Ximénez'. Therefore, 'Montepila' is not an unidentified minority variety, although it is necessary to further determine by ampelography that it is not a somatic mutant. In a preliminary study by studying 11 basic grouping characters, we found no differences compared with 'Zalema'. 'Zalema' is authorized in Andalusia; however, the synonymy 'Montepila' is not yet considered; consequently, its authorization could be undertaken through a request to the MAPA, supported by its corresponding technical report. The last specimen studied, displaying black berries, corresponded to 'Negra Rayada', a Spanish variety not authorized in the RVCV. This variety has also been found in the Andalusian province of Almería [32]. In one of the eight locations, two vines showing the 'Zalema' genotype resulted virus-free, and, therefore, they could be plausibly proposed as certified clones once the synonymy 'Montepila' is authorized.

d. "Valle de los Pedroches" (Córdoba). Eight previously unidentified genotypes were identified in this area. Two of these genotypes were detected 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, our survey suggests that there was some intentional cultivation of these two varieties, presumably in the pre-phylloxera period. They consist of red and white varieties that are currently experimentally vinified at IFAPA. According to the classification established by Muñoz-Organero et al. [29], 'Tinta Amparo' and 'Arises' would be novel autochthonous minority varieties. Two additional novel genotypes were found twice;

in both cases, they were collected in the same location, similarly to the other four unidentified genotypes that were found only once. Among the other varieties found in the area, there 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 wine grape cultivar described since the 16th century [33]. It was traditionally grown in Andalusia [2], but currently it is residual in the provinces of Córdoba, Granada, Badajoz, Guadalajara, Toledo, and Cádiz [34,35]. There has been no interference through the introduction of foreign propagation material to the area in more than a century since the cultivation of these vineyards was almost abandoned. On the other hand, isolation and altitude might explain the absence of virus infections in the area. Hence, we identified healthy candidates for each variety, suitable as starting material to eventually proceed with the registration process and possible authorization in Andalusia, a strictly necessary condition for its permitted cultivation, even in plots for self-consumption.

e. “Pago Burujena” (Cádiz). There has been a growing interest in recent years in the recovery of varieties grown in the past and suitable for the production of fortified and still wines by the regulatory council of the PDOs “Jerez-Xérès-Sherry” and “Manzanilla-Sanlúcar de Barrameda” [1,36]. In Pago Burujena, one of the most traditional areas in the Jerez DO, clones of ‘Mantúo Castellano’ and ‘Mantúo de Pilas’ were apparently maintained in a vineyard for which three and two vines known by these denominations were respectively surveyed. In addition, two vines known as ‘Barcelonés’ were also collected. ‘Mantúo Castellano’ was shown to be a synonym of ‘Listán del Condado’, a variety commonly cultivated in the province of Huelva, while ‘Mantúo de Pilas’ and ‘Barcelonés’ was shown to correspond to ‘Alarije’. For the former, it can be assumed that it was a naming error, while for the latter, it was a synonymy. In the case of ‘Mantúo Castellano’, it would be interesting to request the recognition of synonymy since this is the name by which it has traditionally been known in Jerez. In addition, one of the three plants was virus-free, so a certified clone could be derived from it.

f. “Moguer” (Huelva). In this municipality, we identified an specimen of the most cultivated variety in Spain, ‘Airén’; two specimens of ‘Beba’, recently authorized in Andalusia, of significant interest for its cultivation in the Jerez area; a specimen of ‘Mollar Cano’; three specimens of ‘Listán del Condado’; a specimen of the table variety ‘Alphonse Lavallée’; two genetically identical specimens 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 specimens of ‘Listán Prieto’. The last one is possibly the most significant result. In his book from 1513, Agricultura General Herrera [33] described the ‘Uvas Prietas’ variety cultivated in the center of the Iberian Peninsula, which could possibly correspond to ‘Listán Prieto’. This variety was also explicitly mentioned in Andalusia in the year 1807 [1]. As a result of the America and the Canary Islands conquests in the 15th century, colonists from the Iberian Peninsula (Galician, Castillan, Andalusian, Extremaduran or even Portuguese) settled down in these lands, bringing grapevines together with their customs and crops [37]. Around 1550, a Jesuit introduced the ‘Listán Prieto’ variety in Peru [38], and cultivars were introduced in Mexico between the years 1520 and 1540 [39]. In California, it is known as Mission’ [40] as an allusion to the vines carried by the friars in their evangelizing work. In the 19th century, phylloxera obliterated almost half of the peninsular vineyards [39], but this insect did not reach the Canary Islands. The use of this variety is widespread in the Canary Islands and in America [41,42]. There is no record of its cultivation on the Iberian Peninsula since the last century [40]. Therefore, the location of several specimens of this variety, some over 50 years old, in the township of Moguer has 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. We identified three virus-free vines in this zone, one belonging to 'Beba', another to 'Listán Prieto' and another to the unidentified "Jaén Negro". They could represent the starting point for obtaining locally certified clones of these varieties.

In this work, we have considered a vine as a certifiable clone only when both ELISA and RT-qPCR analyses tested negative for all viruses considered in the official certification schemes in addition to GLRaV-2. In the case of the unidentified varieties for which a registration process might be requested by viticulturists and winemakers, these same virus-free vines could be forwarded to the National Reference Collection of Grape Varieties for their evaluation. As mentioned above, MAPA requires these plants to be virus-free only through serological assays. In view of these results, we recommended requiring both ELISA and RT-qPCR (and additionally including GLRaV-2) in the analyses, as RT-qPCR is more sensitive than ELISA [43]. We have frequently observed along this study that some samples resulting in negative results in the serological assays tested positive in RT-qPCR (see Appendix A, Table A2, sections La Alpujarra de Granada, PDO Montilla-Moriles, Pago Burujena, and Moguer). Nonetheless, the intrinsic genetic variability of viruses prevents, in some cases, the efficient use of amplification techniques, which could result in false-negatives [44,45]. We provide here two examples for which a negative RT-qPCR corresponded to a positive ELISA (Mantúo de Sanlucar/Listán del Condado for GLRaV-3 and Marenas/Zalema for GLRaV-1) (see Appendix A, Table A2). Therefore, we suggest testing the vines before sending the material for registration in a first stage by serology, which is less expensive and more accessible, and in case of negative results, performing an RT-qPCR test. In conclusion, our results show that it is necessary to pursue research on minority grapevine varieties through prospecting, recovery, and conservation, as well as study their agronomic and oenological characteristics, since they represent a viticultural heritage at both regional and national levels. Finally, this work can be representative of similar studies carried out in other regions, mainly European, but not limited to those where viticulturists and winemakers are interested in recovering and diversifying their local varieties and wines.

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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
ICVG	International Council for the Study of Virus and Virus-Like Diseases of the Grapevine

Appendix A

Table A1. Summary statistics obtained from the allele frequency analysis conducted in CERVUS 3.0.

Locus	k	N	HObs	HExp	PIC	NE-1P	NE-2P	NE-PP	NE-I	NE-SI	HW	F (Null)
md7	14	541	0.797	0.803	0.779	0.551	0.375	0.189	0.063	0.364	NS	0.0046
md32	11	540	0.863	0.820	0.797	0.527	0.353	0.173	0.055	0.354	NS	−0.0285
zag62	10	541	0.824	0.807	0.782	0.548	0.371	0.187	0.061	0.362	*	−0.0138
zag79	13	539	0.818	0.854	0.838	0.448	0.287	0.118	0.037	0.332	NS	0.0208
eva2	19	540	0.854	0.865	0.851	0.420	0.264	0.100	0.031	0.326	NS	0.0060
isv2	23	541	0.884	0.851	0.833	0.464	0.300	0.132	0.040	0.335	NS	−0.0212
vvs2	15	541	0.850	0.842	0.824	0.477	0.311	0.137	0.043	0.340	**	−0.0042
md5	11	541	0.854	0.859	0.842	0.449	0.286	0.122	0.036	0.330	NS	0.0022
md27	9	541	0.848	0.829	0.804	0.520	0.347	0.173	0.053	0.349	NS	−0.0122
md25	14	540	0.785	0.767	0.728	0.632	0.454	0.273	0.093	0.390	NS	−0.0118
md28	19	540	0.852	0.870	0.856	0.417	0.262	0.103	0.031	0.323	NS	0.0107
isv4	11	539	0.844	0.814	0.788	0.544	0.368	0.189	0.060	0.358	*	−0.0210
isv3	10	540	0.830	0.673	0.613	0.750	0.590	0.418	0.167	0.456	***	−0.1167

Significance of Hardy-Weinberg equilibrium test (HW): *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; NS = $p > 0.05$

Table A2. 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

Table A2. *Cont.*

Zone	Input Name	Variety	ELISA	PCR
PDO Montilla-Moriles	Peñalista	Negra Rayada	m.d.	m.d.
	Cp1 Marenas fila 3 cp5	Zalema	GFkV	GFkV
	Cp2 Marenas fila 4 cp18	Zalema	GFkV, GLRaV-3	GFkV, GLRaV-3
	Marenas fila 7 cp3 cp44	Zalema	GFkV, GLRaV-3	GFkV, GLRaV-2, GLRaV-3
	Cp 4Marenas fila 8 cp16	Zalema	GFkV, GLRaV-1	GFkV
	Cp5 Marenas fila 9 cp20	Zalema	GFkV	GFkV
	Cp9 La Plata fila 7	Zalema	m.d.	m.d.
	La Plata fila 13 p7	Pedro Ximénez	-	GFkV, GLRaV-2
	Cp6 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 Naranjos 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.
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 1° Amparo Tinta	Unidentified 01	-	-
	Recio 2° Amparo TInta	Unidentified 01	-	-
	Recio 3° Amparo TInta	Unidentified 01	-	-
	Recio 4° Amparo TInta	Unidentified 01	-	-
	Recio 5° Amparo TInta	Unidentified 01	-	-
	Tinta Amparo malla Rafael	Unidentified 01	-	-

Table A2. *Cont.*

Zone	Input Name	Variety	ELISA	PCR
	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	GFkV, GLRaV-2, GLRaV-3
	Mantúo Castellano	Listán del Condado	GFkV, GFLV, GLRaV-3	GFkV, GFLV, GLRaV-2, GLRaV-3
	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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