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Epidemiological Survey of Grapevine Leafroll-Associated Virus 1 and 3 in Sicily (Italy): Genetic Structure and Molecular Variability

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Abstract: Background: the most widely distributed and virulent Grapevine leafroll-associated viruses (GLRaV) that affect grapevine are GLRaV-1 and GLRaV-3, transmitted semi-persistently by different mealybugs and soft scales, mainly causing downward rolling of the leaf margins and interveinal reddening. Methods: the main objectives of this study were to investigate the genetic structure and molecular diversity of GLRaV-1 and GLRaV-3 in 617 samples from 11 autochthonous Sicilian grapevine cultivars, ascertaining their presence and spread. The detection was implemented by serological and molecular analyses and subsequently phylogenetic analyses on selected Sicilian isolates were conducted. Results: in total, 33 and 138 samples resulted positive to GLRaV-1 and GLRaV-3, with an incidence of 5.34% and 22.36%, respectively; 9 out of the 11 cultivars resulted positive, while the presence of both viruses was not found in 'Grillo' and 'Moscato' cultivars. Conclusions: phylogenetic analyses of the coat protein (CP) gene of 12 GLRaV-1 selected sequences showed a close relationship with European isolates; the discrete nucleotide differentiation and positive selection could demonstrate a current increase in population fitness. The phylogenetic analyses of the CP gene of 31 GLRaV-3 Sicilian CP sequences demonstrates a close relationship between Sicilian and different countries isolates; a certain stability of GLRaV-3 in the different cultivars analyzed is suggested by the discrete differentiation nucleotide and negative selection of the Sicilian isolates.

Keywords: grapevine disease; GLRaV-1; GLRaV-3; *Closteroviridae*; *Ampelovirus*; DAS-ELISA; RT-PCR; phylogenetic analyses

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Citation: Caruso, A.G.; Bertacca, S.; Ragona, A.; Matić, S.; Davino, S.; Panno, S. Epidemiological Survey of Grapevine Leafroll-Associated Virus 1 and 3 in Sicily (Italy): Genetic Structure and Molecular Variability. *Agriculture* **2022**, *12*, 647. https://doi.org/10.3390/agriculture12050647

Academic Editor: Florence Fontaine

Received: 9 February 2022
Accepted: 28 April 2022
Published: 29 April 2022

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important and extensively grown crops worldwide, with about 7 million ha of global covered area and ~78 million tons of total production, according to the latest data available [1]. In the European continent, more than 3 million ha are cultivated, particularly in the southern and central regions. In Italy, the grapevine has been cultivated for several millennia, with 0.7 million ha and 8.1 million tons; to date, it is one of the most economically important crops [2].

Italy has the highest number of grapevine cultivars (cvs) with different autochthonous cvs grown in each region [3]. In particular, Sicily has become one of the leading Italian regions for the wine powered industry in the last two decades. Thanks to the pedo-climatic conditions, such as average high temperatures, particular exposure to sunlight and characteristic physical–chemical properties of soils, distinctive wines are produced

from autochthonous cultivars, such as 'Catarratto', 'Grillo', 'Nero d'Avola' and 'Zibibbo', followed by 'Alicante', 'Carricante', 'Inzolia', 'Malvasia', 'Nero Cappuccio', 'Perricone', 'Nerello Mascalese', 'Moscato', and 'Grecanico' [3].

To obtain products with excellent quality and a good yield in terms of productions, it is not possible to overlook the diseases that can affect this crop. As most of the vegetative propagated crops, vineyards are threatened by different pests and pathogens that shorten the productive life of the plantations, endanger the survival itself of affected grapevines, and cause heavy yield losses [4]. In recent years, several diseases have been identified on grapevine caused by fungi, such as *Diplodia seriata*, *Lasiodiplodia* sp., *Neofusicoccum parvum*, and *N. vitifusiforme* [5], and systemic pathogens, as bacteria, such as *Agrobacterium tumefaciens* [6] and *Xylophilus ampelinus* [7], and viruses. Among these pathogens, viruses are considered the most dangerous and damaging to viticulture worldwide; exactly, 86 species of grapevine viruses have been identified, belonging to 17 families and 34 genera [8]. Therefore, it is very important to investigate the genetic diversity, the dispersion, and possible favorable recombination events of these pathogens [9–12], that may allow the overcoming of possible populations bottlenecks and the establishment of new recombinant isolates with more pronounced virulence characteristics, particularly in the perspective of ecologically sustainable management.

Preventive measures are fundamental for the management of viral diseases and reduction in their presence in propagation material, which is possible through clonal selection activities and phytosanitary actions in order to propagate virus-free plant material [13,14].

Certainly, the use of certified material remains the most effective way to control grapevine viruses; for this reason, the propagating material must conform to specific conditions and requirements to be qualified as C.A.C. (*Conformitas Agraria Communitatis*) [15]. The selection process involves monitoring for the presence of the six prevalent grapevine viruses which are considered harmful pathogens by the European Commission directive (2005/43/EC). These viruses are: Grapevine leafroll-associated virus 1 (GLRaV-1; family *Closteroviridae*; genus *Ampelovirus*), Grapevine leafroll-associated virus 3 (GLRaV-3; family *Closteroviridae*; genus *Ampelovirus*), Grapevine fanleaf virus (GFLV; family *Secoviridae*; genus *Nepovirus*), Arabis mosaic virus (ArMV; family *Secoviridae*; genus *Nepovirus*), Grapevine virus A (GVA; family *Betaflexiviridae*; genus *Vitivirus*), and Grapevine fleck virus (GFkV; family *Tymoviridae*; genus *Maculavirus*) only for rootstocks. Their absence must be confirmed in nurseries through official inspections [16].

Grapevine leafroll disease (GLD) is one of the most important viral diseases affecting grapevine, causing up to 40% yield losses [17]. This disease can be caused by several viruses of the *Closteroviridae* family, including Grapevine leafroll-associated viruses (GLRaV) 1–9 and a group of recently described GLRaV viruses (GLRaV-De, GLRaV-Pr, and GLRaV-Car) [18].

GLD is widely distributed throughout the world, affecting almost all areas where grapevine is cultivated (Europe, North and South America, Asia, Africa, and Oceania) [19]. In the last decade, it has been reported also in Washington, Texas, and Mississippi (USA) [20–22], Nigeria [23], Pakistan [24], and Russia [25]. To date, the ones causing the most virulent symptoms are GLRaV-1 and GLRaV-3, the most widely distributed across grapevine-growing regions [26,27]. GLRaV-1 and GLRaV-3 have both a single-stranded positive-sense RNA (ssRNA+) molecule of ~18,946 and ~17,919 nucleotides (nt), respectively. GLRaV-1 genome sequence reveals nine open reading frames (ORFs). The ORF 1 encodes two replication-associated proteins constituting the "replication gene block" (RGB). The remaining eight ORFs are positioned downstream of the RGB and the first five assigned as p7, heat-shock protein 70 homolog (HSP70h), p55, coat protein (CP), and the first divergent copy of the CP (CPd1) encompasses the quintuple gene block (QGB). The last three ORFs designated sequentially as CPd2, p21, and p24 are unique to GLRaV-1 [18,28]. GLRaV-3 presents a genome containing 12 ORFs; the 34 kDa major CP covers the entire virion length with the exception of 5′ extremity (ca. 100 nm). The 5′ end of the genome might be encapsidated by the virion tail structure, which contains proteins encoded by ORF4 (HSP70h),

ORF5 (p55), and ORF7 (CPm), and involved in cell-to-cell and systemic transport [28]. So far, no research about the composition of a 5′ virion tail/head structure is available; the proteins associated with such a structure are deduced from homologous proteins for other viruses (e.g., Beet yellows virus (BYV) and Citrus tristeza virus (CTV)) [18].

The symptom manifestation and severity of the disease is highly variable among the different *V. vinifera* cultivars, by scion–rootstock combinations and environment. Generally, GLD symptoms initiate on mature leaves around or soon after veraison, becoming more visible through the season: in red-berried cultivars infected with GLRaV-1, symptoms mainly consist of interveinal reddening of the leaves, while in white-berried cultivars, in single or mixed GLRaVs infections, leaves appear feeble/mild chlorotic that often may not be recognizable in vineyards [29]. Regarding GLRaV-3 in red-berried infected cultivars, the specific leaf symptoms consist of interveinal red/reddish-purple coloration, while in white-berried cvs show only a slight yellowing or chlorotic mottling. The characteristic downward rolling of leaf margins on symptomatic leaves appears toward the end of the season. The symptoms described may remain confined to the basal and central part of the shoots or extend upward along the shoots [29].

In addition, many reports indicate that mealybugs and soft scales are vectors of GLRaV-1 and GLRaV-3 [18,30,31], transmitting these viruses in a semi-persistent manner [32,33]. Both viruses are transmitted by the mealybugs *Heliococcus bohemicus*, *Phenacoccus aceris* (*Pseudococcidae*) and the soft scale *Parthenolecanium corni* (*Coccidae*), with a transmission efficiency of 14%, 23%, and 29%, respectively [34]; GLRaV-1 is also transmitted by the scale insect *Neopulvinaria innumerabilis* [35]. GLRaV-3 is vectored by the mealybugs *Planococcus ficus*, *P. citri*, *Pseudococcus longispinus*, *Ps. calceolariae*, *Ps. viburni*, *Ps. maritimis*, and *Ps. comstocki* [30,34,36–38], and by the scale insect *Pulvinaria vitis* [39]. GLRaV-1 and GLRaV-3 are also frequently observed in association with Grapevine virus A (GVA) [40].

In the present work, a total of 11 of the most important cultivars in terms of diffusion and production, collected in 20 Sicilian commercial vineyards, were investigated to evaluate the GLRaV-1 and GLRaV-3 presence and dispersion through serological and sensitive and reliable RT-PCR-based detection methods. In addition, their genetic structure and molecular diversity were evaluated, in order to detect possible different introduction of infected propagative material in Sicily, new recombination events, the nucleotide diversity, and selection pressure of both virus populations.

2. Materials and Methods

2.1. Field Surveys and Sample Collection

During December 2020, January and February 2021, a total of 617 grapevine samples were collected, in order to study the GLRaV-1 and GLRaV-3 presence in Sicily and investigate their genetic structure and molecular variability. The sampling was performed in 20 Sicilian vineyards located in Trapani, Ragusa, Agrigento, and Caltanissetta provinces. All vineyard ages range from 10 to 20 years, and all samples were collected from grapevine plants grafted on 140 RU (140 Ruggeri) rootstock. Random sampling was carried out following the hierarchical sampling scheme [41]; all collected samples were geo-referenced with the Planthology mobile application [42]. Specifically, for each province a total of 150 samples were collected, except for Trapani province, where a total of 167 samples were collected. Subsequently, each sample, consisting of four dormant cuttings, was divided into two subsamples for subsequent serological and molecular analyses. A total of 11 autochthonous cultivars were sampled (Table 1). The sampling was carried out with respect to the major presence of the different Sicilian cultivars; 'Grillo', 'Zibibbo', 'Perricone', and 'Catarratto' are among the most widespread cultivars. The higher number of 'Grillo' and 'Zibibbo' samples is due to the major extension area of some commercial vineyards compared to others.

Table 1. List of grapevine cultivars analyzed by DAS-ELISA for GLRaV-1/GLRaV-3.

Cultivar	Acronym ID	No. of Samples Analyzed	No. of Vineyards Analyzed
Grillo	GLL	114	3
Zibibbo	ZIB	106	3
Perricone	PER	74	2
Catarratto	CAT	66	2
Nero d'Avola	NAV	64	2
Grecanico	GRE	64	2
Nerello Mascalese	NMA	43	1
Carricante	CRR	30	2
Nerello Cappuccio	NCA	24	1
Alicante	ALI	21	1
Moscato	MOS	11	1
Total		**617**	**20**

2.2. Preliminary Screening by Serological Analysis

The preliminary screening was performed using GLRaV-1/GLRaV-3 polyclonal antibodies (Agritest srl, Valenzano, Italy) by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) [43]. The antibodies used do not allow to discriminate between the two viruses. Phloem tissue was obtained from each sample by scraping each dormant cutting with a sterile scalpel. For each sample (Table 1), 500 mg of phloem tissue was homogenized in an extraction plastic bag (BIOREBA AG, Reinach, Switzerland) containing 5 mL of extraction buffer (0.5 M Tris-HCl, 0.14 M NaCl, 2% PVP MW 24000, 1% PEG MW 6000, and 0.05% Tween 20 in 1 L of distilled water, pH 8.2) using HOMEX 6 homogenizer (BIOREBA AG, Reinach, Switzerland). The 1:10 diluted samples (*w*/*v*) were used for DAS-ELISA according to the manufacturer's instructions. Lyophilized plant tissue infected by GLRaV-1 or GLRaV-3 and healthy plant tissue provided by Agritest srl was used as positive and negative controls, respectively. The optical densities at 405 nm (OD_{405}) were measured two hours after the addition of the p-nitro-phenylphosphate substrate, using an AMR-100 microplate reader (Hangzhou Allsheng Instruments, Hangzhou, China). The sample was considered positive if the mean OD_{405} value was at least two-fold higher than that of the negative control value, as reported by Agritest srl. This preliminary test allowed us to detect the presence of 1-/3-associates and subsequently subject the positive samples to molecular analysis.

2.3. Total RNA Extraction

Total RNA was extracted from 100 mg of phloem tissue of each sample homogenized as described above and then a GenUP Plant RNA kit (Biotechrabbit GmbH, Berlin, Germany) was used, following the manufacturer's instructions. Two yield/quality measurements with a UV–Vis NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) were performed; samples were adjusted to a concentration of 50 ng/µL and stored at −80 °C.

2.4. Molecular Analyses

Samples that were positive by DAS-ELISA were subsequently tested by molecular analysis, in order to discriminate between GLRaV-1 and GLRaV-3 infections and for subsequent phylogenetic analyses. In addition, samples that were negative for the DAS-ELISA assay from 'Grillo' and 'Moscato' cultivars, were also analyzed by molecular analyses. Two-step end-point reverse-transcription polymerase chain reaction (RT-PCR) assays were performed for amplification of the GLRaV-1 and GLRaV-3 CP genes by using the CPF/CPR [44] and LR3_8504V/LR3_9445C [45] primer pairs, respectively (Table 2).

Table 2. List of primers for GLRaV-1 and GLRaV-3 specific detection by end point RT-PCR and sequencing.

Virus	Gene	Primer Name	Sequence (5′-3′)	Position on Virus Genome	Product Size (bp)	Reference
GLRaV-1	CP	CPF	CGCGCTTGCAGAGTTTAAGTGGTT	6957–6980	734	[44]
		CPR	TCCGTGCTGCATTGCAACTTTCTC	7667–7690		
GLRaV-3	CP	LR3_8504V	ATGGCATTTGAACTGAAATT	13,269-13,288	942	[45]
		LR3_9445C	CTACTTCTTTTGCAATAGTT	14,191-14,210		

The reverse transcription (RT) was performed in a final reaction volume of 20 µL, containing 3 µL of total RNA extract, 0.4 mM dNTPs, 4 µL of 5X First Strand Buffer (50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$) (Thermo Fisher Scientific, Waltham, MA, USA), 1 µM of reverse primer (CPR and LR3_9445C for GLRaV-1 and GLRaV-3, respectively), 20U of M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), and RNase-free water to reach the final volume. The cycling conditions were as follows: initial incubation at 65 °C for 10 min, reverse transcription at 42 °C for 45 min, and enzyme inactivation at 95 °C for 10 min. The cDNA obtained was used for the subsequent PCR assay, carried out in a 25 µL reaction volume, containing 2 µL of cDNA, 1X DreamTaq Buffer with $MgCl_2$, 0.4 mM dNTPs, 1 µM of each primer, and 2U of DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR was performed with initial denaturation at 95 °C for 5 min, following 40 cycles of 95 °C for 1 min, 50 °C (GLRaV-1) [44] to 48 °C (GLRaV-3) [45] for 1 min and 72 °C for 1 min, and a final elongation at 72 °C for 10 min, in a MultiGene OptiMax thermal cycler (Labnet International Inc., Edison, NJ, USA). GLRaV-1 and GLRaV-3 RNAs derived from lyophilized infected tissues were used as positive controls. Molecular-grade water and total RNA extracted from healthy lyophilized grapevine tissue were used as negative controls. The RT-PCR products were electrophoresed on 1.5% (*w*/*v*) agarose gel at 100V, stained previously with SYBR Safe (Thermo Fisher Scientific, Waltham, MA, USA), and visualized under UV light.

2.5. Sequence Analyses

A total of 12 GLRaV-1 and 31 GLRaV-3 RT-PCR amplicons, corresponding to ~36% and ~22% of positive samples, respectively, were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. These products were subsequently sequenced in both directions, using an ABI PRISM 3100 DNA sequence analyzer (Applied Biosystems, Foster City, CA, USA). The sequences of GLRaV-1 (734 nt) and GLRaV-3 (942 nt) were deposited in GenBank (NCBI).

The nucleotide sequences obtained were used to perform the phylogenetic analyses, together with 31 GLRaV-1 and 73 GLRaV-3 CP sequences from different countries retrieved from the NCBI database. In addition, the GLRaV-1 GenBank sequences were trimmed to remove the external fragment of the CP gene, leaving only the 734 nt CP gene, as the sequences obtained in this work.

Multiple nucleotide sequence alignment was carried out using the CLUSTALW algorithm [46]. A mathematical model was applied in order to estimate the nucleotide substitution number, taking into consideration the nucleotide frequencies and instantaneous rate change. The best model both for GLRaV-1 and GLRaV-3 was the Kimura 2-parameter (K2) [47], using a discrete gamma distribution (+G) = 0.7487 with two rate categories for GLRaV-1, and a discrete gamma distribution (+G) = 1.8087 with two rate categories and invariant sites (+I) = 0.2336 for GLRaV-3. Maximum-likelihood method (ML) for phylogenetic inference, with 1,000 bootstrap replicates to estimate the statistical significance of each node [48], was used with the MEGA X program [49]. Initial trees for the heuristic search were obtained automatically by applying neighbor-joining and BIONJ algorithms to a matrix of pairwise distances estimated by using the maximum composite

likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. All analyses were performed by using the MEGA X program [49].

The presence of recombination events between the sequences from Sicilian GLRaV-1 and GLRaV-3 isolates was evaluated by using the RDP4 program (v.4.39), applying 3Seq, Bootscan, GENECONV, MaxChi, RDP, and SiScan algorithms [50]. RDP4 parameters were set as default values. Only concordant results of in silico analysis, between different algorithms, were considered as a positive result.

GLRaV-1 and GLRaV-3 CP nucleotide diversity was estimated using the Jukes–Cantor model implemented in the MEGA X program, within and between different countries. Each country was considered as a geographic population [51]. The role of natural selection at the molecular level in the Sicilian isolates was evaluated studying separately the rates of synonymous substitutions per synonymous site (dS) and nonsynonymous substitutions per nonsynonymous site (dN). dS and dN values were predicted using the Pamilo–Bianchi–Li method [52], implemented in the MEGA X program.

Lastly, the pairwise percent identities of GLRaV-1 and GLRaV-3 CP isolates was calculated within the GLRaV-1 and GLRaV-3 isolates from Sicily, respectively, and between the other reference isolates from the other countries, using the SDT v1.2 program [53].

3. Results

3.1. GLRaV-1 and GLRaV-3 Incidence in Sicilian Vineyards

In total, 157 out of 617 samples, collected from different commercial vineyards in Trapani, Agrigento, Caltanissetta, and Ragusa provinces (Sicily), gave a positive result for at least one virus, representing an infection rate (calculated on the total number of samples collected) of 25.4% (Table 3). Out of the 11 cultivars, 9 resulted positive for at least one virus, while 'Grillo' and 'Moscato' cvs tested negative for both viruses. The serological screening results were further confirmed by molecular analyses, in order to ascertain the presence of GLRaV-1 and GLRaV-3 in single or mixed infections. Molecular analyses were performed on all positive samples and on 'Grillo' and 'Moscato' samples, to avoid possible false-negative results and reconfirm the absence of GLRaV-1/3 infection.

Table 3. Number of samples analyzed for GLRaV-1 and GLRaV-3 by DAS-ELISA.

Cultivar	No. Samples Analyzed	GLRaV-1/3 Positive Samples	Percentage (%) of GLRaV-1/3 Incidence
Nerello Mascalese	43	23	53.5
Nero d'Avola	64	33	51.5
Carricante	30	15	50.0
Alicante	21	9	42.8
Catarratto	66	28	42.4
Grecanico	64	22	34.4
Nerello Cappuccio	24	6	25.0
Perricone	74	12	16.2
Zibibbo	106	9	8.5
Grillo	114	0	0
Moscato	11	0	0
Total	**617**	**157**	**25.4**

3.2. Polymerase Chain Reaction and Sequencing

In total, 157 GLRaV-1/GLRaV-3 positive samples were analyzed by end-point RT-PCR to differentiate the presence of single or mixed infections by using specific primer pairs. A total of 33 and 138 samples were positive for GLRaV-1 and GLRaV-3 (Table 4), obtaining the expected amplicon size of 734 and 942 nucleotides, respectively.

Table 4. Number of samples collected and incidence percentage of Grapevine leafroll-associated virus 1 and Grapevine leafroll-associated virus 3 obtained by specific end point RT-PCR.

Cultivar	No. of Samples Analyzed	No. of GLRaV-1 Positive Samples by End Point RT-PCR	Percentage (%) of GLRaV-1 Incidence	No. of GLRaV-3 Positive Samples by End Point RT-PCR	Percentage (%) of GLRaV-3 Incidence	No. of Samples with Mixed Infection	Percentage (%) Incidence of Mixed Infection
Grillo	114	0	0	0	0	0	0
Zibibbo	106	2	1.9	7	6.6	0	0
Perricone	74	2	2.7	10	13.5	0	0
Catarratto	66	7	10.6	26	39.4	5	7.6
Nero d'Avola	64	7	10.9	29	45.3	3	4.7
Grecanico	64	5	7.8	21	32.8	4	6.2
Nerello Mascalese	43	5	11.6	19	44.2	1	2.3
Carricante	30	4	13.3	13	43.3	2	6.7
Nerello Cappuccio	24	0	0	6	25.0	0	0
Alicante	21	2	9.5	7	33.3	0	0
Moscato	11	0	0	0	0	0	0
Total	**617**	**33**	**5.3**	**138**	**22.3**	**15**	**2.4**

Regarding the infection percentage of each cv analyzed, the results obtained showed that the highest percentage of GLRaV-1 infection was observed in 'Carricante', 'Nerello Mascalese', 'Nero d'Avola', and 'Catarratto' cvs, with 13.3%, 11.6%, 10.9%, and 10.6% incidences, respectively, whereas a lower incidence was detected in 'Alicante', 'Grecanico', and 'Perricone' cvs, ranging from of 9.5% to 2.7%. The lowest incidence was detected in 'Zibibbo' cultivar, with a percentage of 1.9% in a total of 106 samples analyzed, and no positive samples were detected in 'Nerello Cappuccio' cultivar (Table 4). Regarding GLRaV-3, the highest percentage of positive samples was observed in 'Nero d'Avola', 'Nerello Mascalese', 'Carricante', and 'Catarratto' cvs, with 45.3%, 44.2%, 43.3%, and 39.4% incidences, respectively. A lower incidence was detected in 'Alicante', 'Grecanico', 'Nerello Cappuccio', and 'Perricone' cvs, ranging from 33.3% to 13.5%. The lowest incidence was detected in 'Zibibbo' cultivar, with a percentage of 6.6% (Table 4).

Moreover, the incidence of mixed infections detected by end point RT-PCR was higher in 'Catarratto' (7.6%), 'Carricante' (6.7%), and 'Grecanico' (6.2%), cvs, followed by 'Nero d'Avola' (4.7%) and 'Nerello Mascalese' (2.3%) cvs; no mixed infections were detected in 'Zibibbo', 'Perricone', 'Nerello Cappuccio', and 'Alicante' cvs (Table 4).

Regarding the GLRaV-1 and GLRaV-3 distribution in the four Sicilian provinces where the samples were collected, the highest GLRaV-1 incidence was observed in Ragusa province (8.0%), followed by Trapani, Caltanissetta, and Agrigento provinces (4.8%, 4.7%, and 4.0%, respectively); regarding the GLRaV-3 incidence, the highest value was recorded in Trapani province (27.5%), followed by Ragusa, Agrigento, and Caltanissetta provinces (24.7%, 23.3%, and 13.3%, respectively) (Table 5).

3.3. Phylogenetic Analyses

The CP phylogenetic analyses were performed on the 12 Sicilian GLRaV-1 sequences obtained in this study. GLRaV-1 sequences from Portugal, Canada, and China (6 sequences from each country), USA (4), Poland (3), France (2), and Australia, Brazil, South Korea, and Czech Republic (1 sequence from each country), retrieved from the GenBank database were included for the analyses.

GLRaV-1 isolates were separated into two statistically significant clusters (Figure 1). Cluster A (black) shows a greater differentiation than cluster B (red). (Figure 1). The Sicilian isolates are present in both clusters. Within cluster A, 7 Sicilian isolates were grouped with isolates from Portugal, USA, France, Canada Poland Brazil, South Korea, China, and Australia, and were closely related in the same sub-clade with 3 Portugal isolates found in *Vitis vinifera* cvs Sousao Vinhos Verdes and Vinhao Douro (KC567968, KC567972, and KC567977), showing a low variability. In the cluster B (red), the remaining 5 Sicilian isolates

were grouped and were closely related in the same sub-clade with 1 French isolate found in *Vitis vinifera* cv Pinot noir (MG925331). Regarding GLRaV-3, 31 Sicilian sequences were subjected to phylogenetic analyses, together with other GLRaV-3 sequences retrieved from GenBank database from USA, China, and Brazil (11 sequences from each country), Bosnia and Herzegovina (10), South Africa (6), Portugal (5), Chile (4), Canada (4), Pakistan (3), New Zealand (2), Poland (2), and from Israel, Croatia, Slovakia, and Italy (1 sequence from each country) (Figure 2). In this case, the isolates were grouped into two statistically significant clusters and in both clusters the Sicilian isolates are present. In cluster A (black), 2 sub-clades can be identified, which group 7 Sicilian isolates with 1 Bosnian isolate found in *Vitis vinifera* cv. Zilavka (MT432372) and 8 Sicilian isolates with 1 Portugal isolate (HQ401018) *Vitis vinifera* cv. Carrega-Tinto, respectively. In cluster B (red), a total of 16 Sicilian isolates were grouped and were closely related into the same sub-clade with one Italian isolate found in *Vitis vinifera* cv. Nero d'Avola (KY707826).

Table 5. Number of samples collected and incidence percentage of Grapevine leafroll-associated virus 1 and Grapevine leafroll-associated virus 3 for each vineyard.

Province	Vineyard ID	No. of Samples Collected	No. of GLRaV-1 Positive Samples	Percentage (%) of GLRaV-1 Incidence	No. of GLRaV-3 Positive Samples	Percentage (%) of GLRaV-3 Incidence
Trapani	1T	30	1	3.3	13	43.3
	2T	30	0	0	9	30.0
	3T	30	4	13.3	0	0
	4T	30	2	6.7	23	76.7
	5T	47	1	2.1	1	2.1
TOTAL		**167**	**8**	**4.8**	**46**	**27.5**
Agrigento	6A	30	0	0	8	26.7
	7A	30	0	0	11	36.7
	8A	30	0	0	6	20.0
	9A	30	4	13.3	1	3.3
	10A	30	2	6.7	9	30.0
TOTAL		**150**	**6**	**4**	**35**	**23.3**
Ragusa	11R	30	4	13.3	0	0
	12R	30	1	3.3	15	50.0
	13R	30	0	0	12	40.0
	14R	30	2	6.7	10	33.3
	15R	30	5	16.7	0	0
TOTAL		**150**	**12**	**8**	**37**	**24.7**
Caltanissetta	16C	30	0	0	3	10.0
	17C	30	4	13.3	4	13.3
	18C	30	0	0	0	0
	19C	30	0	0	7	23.3
	20C	30	3	10.0	6	20.0
TOTAL		**150**	**7**	**4.7**	**20**	**13.3**

The CP GLRaV-1 and GLRaV-3 sequences were submitted to the GenBank database under the accession numbers from OM286753 to OM286764 and from OM286765 to OM286795, respectively.

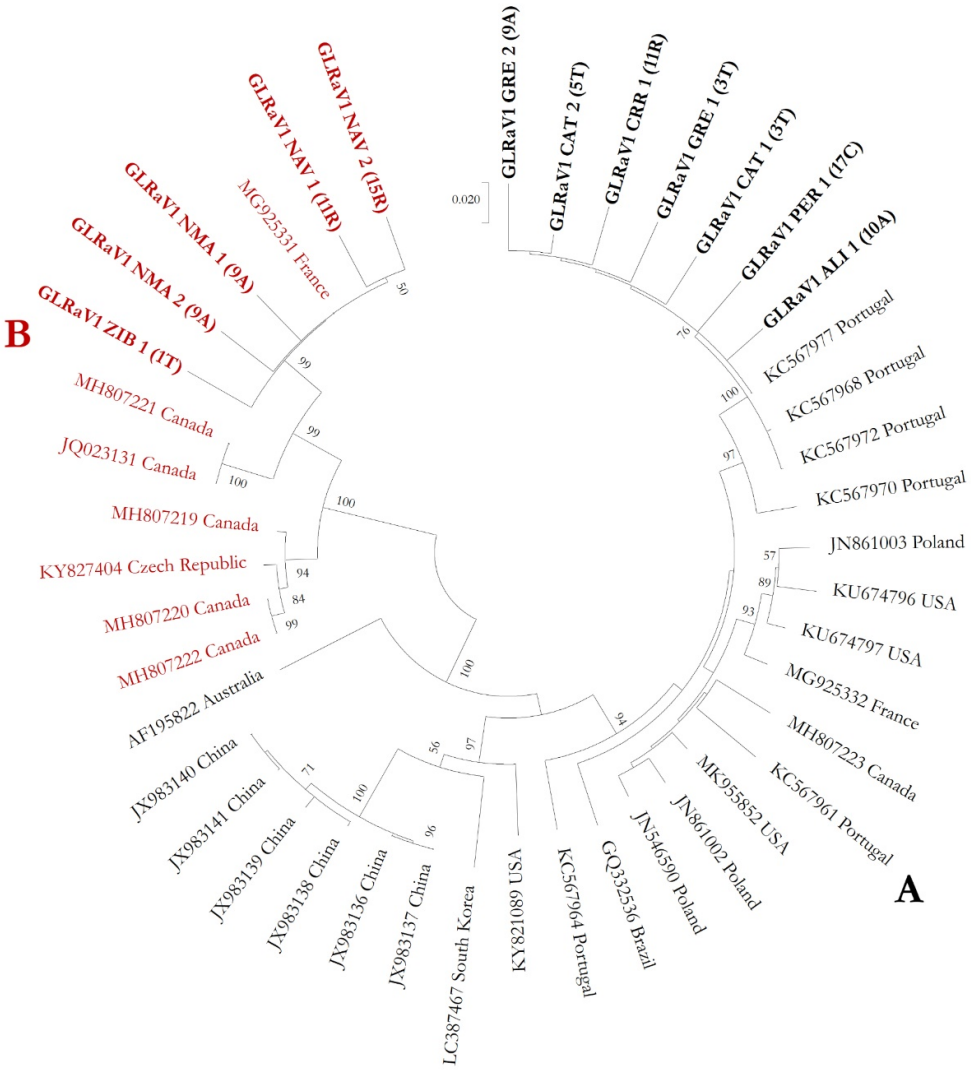

Figure 1. Phylogenetic relatedness between 12 Sicilian GLRaV-1 isolates and reference isolates retrieved from NCBI, based on coat protein gene. The evolutionary history was inferred by the maximum-likelihood method (ML) using the Kimura 2-parameter model (K2) (1000 replication bootstraps), conducted with the MEGA X program. Bootstrap values ≥50% are shown in the nodes. The sequences obtained in the present work are in bold. In brackets, the vineyard ID of origin (reported in Table 5). Black: cluster (**A**); red: cluster (**B**).

3.4. Recombination Analyses

The recombination analyses conducted using the RDP4 program detected different recombination events within Sicilian isolates and between isolates from other countries.

In detail, concordant results of in silico analysis showed that3Seq, Bootscan, GENECONV, MaxChi, RDP, and SiScan algorithms detected two candidate recombinant events in the Sicilian GLRaV-1 isolates (Table 6). The Bootscan and MaxChi algorithms detected the same event in the GLRaV-1_ZIB-1 isolate, with the 252 nt beginning breakpoint and the 560 nt ending breakpoint (major parent GLRaV-1_NMA-1 and minor parent MG925331 from France, showing a 91.5% and 100% of similarity, respectively) and a mean *p*-value of 1.13×10^{-2} and 4.11×10^{-8}, respectively. Moreover, the 3Seq algorithm detected another recombination event in the same Sicilian isolate (GLRaV-1_ZIB-1) with an identical position of beginning and ending breakpoints detected above, but with reversed parents (major parent MG925331 and minor parent GLRaV-1_NMA-1, with a 100% and 91.6% of similarity, respectively) and a mean *p*-value of 1.03×10^{-8}. Two putative recombination events among the Sicilian isolates were identified by SiScan and MaxChi algorithms; specifically, the

SiScan algorithm detected one event in the GLRaV-1_CRR-1 isolate, with 320 nt beginning breakpoint and 444 nt ending breakpoint (major parent GLRaV-1_CAT-2 and minor parent GLRaV-1_PER-1, with a 96.7% and 92.8% of similarity, respectively) and a mean *p*-value of 2.99×10^{-2}; whereas, with the MaxChi algorithm one putative recombination event was detected in the GLRaV-1_NMA-1 isolate, with the 198 nt beginning breakpoint and 524 nt ending breakpoint (major parent GLRaV-1_NAV-2 with a 95.4% of similarity and minor parent unknown), and a mean *p*-value of 1.31×10^{-2}. In both cases, major parents belonged to the Sicilian GLRaV-1 isolates.

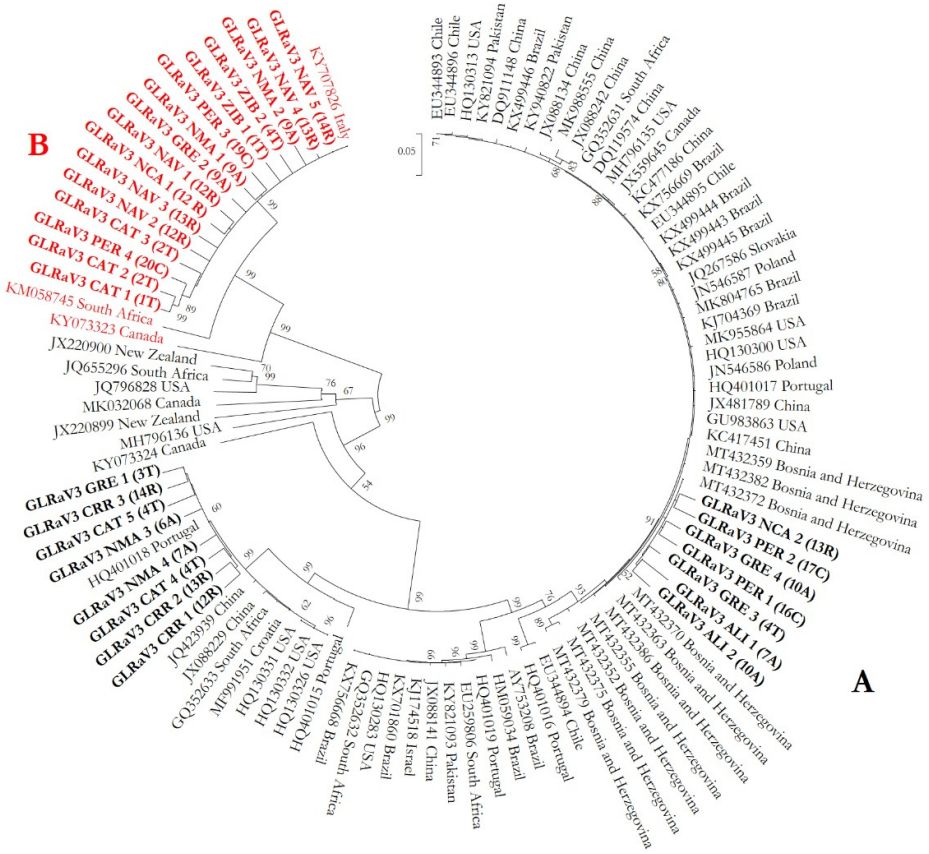

Figure 2. Phylogenetic relatedness between 31 Sicilian GLRaV-3 isolates and reference isolates retrieved from NCBI, based on coat protein gene. The evolutionary history was inferred by the maximum-likelihood method (ML) using the Kimura 2-parameter model (K2) (1000 replication bootstraps), conducted with the MEGA X program. Bootstrap values $\geq$ 50% are shown in the nodes. The sequences obtained in the present work are in bold. In brackets, the vineyard ID of origin (reported in Table 5). Black: cluster (**A**); red: cluster (**B**).

Regarding the Sicilian GLRaV-3 isolates, three candidate recombinant events involving the same Sicilian isolate (GLRaV-3_ALI-1) were detected as follows: the 3Seq algorithm detected one event with the 18 nt beginning breakpoint and 838 nt ending breakpoint (major parent GLRaV-3_CAT-5 and minor parent JX088134, with a 100% and 96.6% of similarity, respectively), and a mean *p*-value of 2.30×10^{-7}; Bootscan and 3Seq detected one event with the 838 nt beginning breakpoint and 927 nt ending breakpoint (major parent MT432372 and minor parent GLRaV-3_CAT-5, with a 97.3% and 100% of similarity, respectively), and a mean *p*-value of 3.46×10^{-9}; lastly, SiScan detected one event with the 152 nt beginning breakpoint and 838 nt ending breakpoint (major parent unknown and minor parent MK988555 from China, with 96.34%), and a mean *p*-value of 1.11×10^{-7} (Table 6). In addition, three putative recombination events among the Sicilian isolates analyzed were detected in the GLRaV-3_NCA-1 isolate; 3Seq, GENECONV, and RDP algorithms detected one event with the 603 nt beginning breakpoint and 633 nt ending breakpoint (major parent GLRaV-

3_NAV-1 with a 96.2% of similarity and minor parent unknown), and a mean *p*-value of 2.71×10^{-6}, 7.84×10^{-9} and 7.27×10^{-10}, respectively. The second one was detected using the Bootscan algorithm, with the same beginning and ending breakpoint position (major parent GLRaV-3_CAT-3 with a 95.4% of similarity and minor parent unknown), and a mean *p*-value of 1.39×10^{-9}. The third one by MaxChi algorithm with the 596 nt beginning breakpoint and 661 nt ending breakpoint (major parent GLRaV-3_ZIB-2 with a 95.7% of similarity and minor parent unknown), and a mean *p*-value of 1.01×10^{-3} (Table 6).

Table 6. Candidate and putative recombinant events detected on Sicilian GLRaV-1 and GLRaV-3 isolates.

Isolate	Algorithm	Major Parent	Minor Parent	*p*-Value
GLRaV-1_ZIB-1	Bootscan	GLRaV-1_NMA-1	MG925331	1.13×10^{-2}
	MaxChi	GLRaV-1_NMA-1	MG925331	4.11×10^{-8}
	3Seq	MG925331	GLRaV-1_NMA-1	1.03×10^{-8}
GLRaV-1_CRR-1	SiScan	GLRaV-1_CAT-2	GLRaV-1_PER-1	2.99×10^{-2}
GLRaV-1_NMA-1	MaxChi	GLRaV-1_NAV-2	Unknown	1.31×10^{-2}
GLRaV-3_ALI-1	3Seq	GLRaV-3_CAT-5	JX088134	2.30×10^{-7}
	Bootscan	MT432372	GLRaV-3_CAT-5	3.46×10^{-9}
	3Seq			
	SiScan	Unknown	MK988555	1.11×10^{-7}
GLRaV-3_NCA-1	3Seq	GLRaV-3_NAV-1	Unknown	2.71×10^{-6}
	GENECONV	GLRaV-3_NAV-1	Unknown	7.84×10^{-9}
	RDP	GLRaV-3_NAV-1	Unknown	7.27×10^{-10}
	Bootscan	GLRaV-3_CAT-3	Unknown	1.39×10^{-9}
	MaxChi	GLRaV-3_ZIB-2	Unknown	1.01×10^{-3}

3.5. Nucleotide Diversity and Selection Pressure Analyses

The analyses of nucleotide diversity indicated a discrete differentiation within GLRaV-1 Italian isolates (0.1392 ± 0.0340), as well as isolates from France (0.1487 ± 0.500), while between isolates from Portugal, Poland, USA, Canada, and China (0.0504 ± 0.0960, 0.0382 ± 0.2720, 0.0691 ± 0.1770, 0.0821 ± 0.1290 and 0.0073 ± 0.1220, respectively), a very low differentiation was observed. The nucleotide diversity for Brazil, South Korea, Australia, and Czech Republic was not calculated, due to the availability of only one sequence for each group in GenBank (Table S1).

Regarding the GLRaV-3 nucleotide diversity, the analysis identified a certain level of differentiation within Italian isolates (0.2043 ± 0.1921), as well as Canada, New Zealand, and South Africa isolates (0.2600 ± 0.2403, 0.2148 ± 0.1868, and 0.1969 ± 0.1864, respectively); unlike isolates from Poland, Portugal, China, Brazil, Bosnia and Herzegovina, Chile, and Pakistan (ranging from 0.0031 ± 0.0031 to 0.0811 ± 0.0805). In this case, it was impossible to calculate the nucleotide diversity for Slovakia, Israel, and Croatia, due to the availability of one sequence for each group in GenBank (Table S2).

The CP gene of the Sicilian GLRaV-1 isolates showed dN and dS values of 0.1662 and 0.0528, respectively, with a dN/dS ratio of 3.147, confirming the hypothesis of adaptive evolution or positive selection. While regarding GLRaV-3 isolates, dN and dS values of 0.0984 and 0.3906 were obtained, respectively, with a dN/dS ratio of 0.2519, confirming the hypothesis of negative selection.

Lastly, the pairwise percent identity of nucleotides for the Sicilian GLRaV-1 and GLRaV-3 CP sequences ranged from 80 to 100% and from 71% to 100%, respectively (Figures S1 and S2); the same ranges were obtained including both the Sicilian and reference isolates (Figures S3 and S4).

4. Discussion

In recent decades, emerging and re-emerging viral pathogens have caused substantial production and quality losses in different tree and horticultural crops (including grapevine) in the Italian territory [12,54–58].

Among the most common cultivated tree crops, the global cultivation of grapevine has an important economic value in producing wine, table fruit, juice, and other products [58], but it is continuously threatened by these pathogens, that cause major decreases in fruit yield and wine quality.

Information on viruses affecting grapevines in Sicily has been limited in recent years. In the present work, a different incidence of GLRaV-1 and GLRaV-3 was detected in the main autochthonous Sicilian cultivars. The absence of GLRaV-1 in 'Grillo', 'Nerello Cappuccio', and 'Moscato' cultivars could be due to a low prevalence of infected propagation material in Sicily, different cultivation practices and, moreover, a low presence of potential GLRaV-1 vectors. Future surveys with updated data on GLRaV-1 vectors distribution could be useful to clarify the infection risk and distribution in commercial grapevines cultivation.

The GLRaV-1 phylogenetic analyses show a close relationship of 7 sequences isolated from 'Grecanico', 'Catarratto', 'Carricante', 'Perricone', and 'Alicante' cvs with isolates from Portugal, and the remaining 5 sequences were closely related with 1 isolate from France; this might confirm a movement of infected propagation material within the European territory. This is partially supported by the detected recombination events among the Sicilian isolates that clustered with the isolate from France. Moreover, the discrete nucleotide differentiation and positive selection of the Sicilian isolates analyzed, could demonstrate a current increase in population fitness.

GLRaV-3 was found to be more prevalent in Sicilian vineyards; a higher incidence for each cultivar was detected than GLRaV-1. The data obtained show that GLRaV-3 is clearly more widespread in Sicily.

The higher number of sequences allowed to better discriminate the isolates analyzed in the phylogenetic analyses; our results indicate a close relationship of 16 Sicilian isolates from 'Nerello Mascalese', 'Nero d'Avola', 'Catarratto', 'Grecanico', 'Nerello Cappuccio', 'Perricone', and 'Zibibbo' cvs with 1 isolate from Italy. Moreover, it is important to note that all the three sequences retrieved from 'Carricante' cultivar are closely clustered with one isolate from Portugal [59], while the two sequences retrieved from 'Alicante' cultivar are closely clustered with one isolate from Bosnia and Herzegovina [15], both together with other Sicilian isolates. This shows that there was likely an exchange of propagating material between these countries, partially confirmed by the recombination events detected. Despite this, the higher nucleotide differentiation and the negative selection of the Sicilian isolates suggest a certain stability of GLRaV-3 in the different cultivars analyzed.

The significant genetic diversity among the new genetic variants that are regularly found, as in the case of GLRaV-3, means that up-to-date diagnostic tests need to be constantly developed for a great disease surveillance.

Moreover, the intensive use of an important species (*V. rupestris* Scheele) imported from America in the last century as rootstock, in order to reduce phylloxera damage, facilitated the diffusion of different viruses in major areas of grapevine cultivation; the presence of GLRaV-1 and GLRaV-3 in the autochthonous Sicilian cvs could also be caused by the massive use of *V. rupestris* in the last century and, subsequently, through the use of infected propagation material over the years. To date, the Eurasian grapevine (*V. vinifera* L.) cultivars have no known natural resistance to GLD; thus, it is impractical to obtain resistant lines through conventional selection or intraspecific crossbreeding programs, imposing severe limitations in disease control [18].

In this scenario, an essential component for grapevine viral disease management is the prevention. Preventive measures to be taken mainly consist of identification and production of virus-tested vines [13]; the establishment, production, and maintenance of virus-tested propagation material are extremely important for the obtainment of healthy vineyards [8]. Indeed, this can be achieved through the certification programs, that control the production

and distribution of clean propagation material; they are critical and meaningful for the production and delivery to growers of healthy and high-quality planting material [60], with great benefits in terms of economic and environmental importance.

Similarly, early diagnosis is another important element for good prevention. Satisfactory and reproducible results can be achieved both by serological and molecular assays to control the diffusion and implement a reliable technical support for the screening of GLRaV-1/3-free grapevine stock materials. Moreover, in recent years, different quantitative RT-PCR (RT-qPCR) have been developed as more sensitive diagnostic techniques for different viruses [61] and also for early GLRaV-1 and GLRaV-3 detection [62–65]. RT-qPCR techniques might also benefit from an important reduction in total cost for a single analysis, redefining the sample extraction with rapid, highly effective, and low-cost methods that reduce the processing time of multiple samples [66,67]. The rapid and reliable detection of GLD can be also achieved by RT-LAMP (Reverse-Transcription Loop-Mediated Isothermal Amplification) assay [68], that shows a higher sensitivity than serological and conventional molecular techniques, thanks to the robustness of the technique in the presence of inhibitors and low viral titer [69], allowing the detection in two hours by using a crude nucleic acid extraction method from grapevine petioles. These diagnostic methods can also be very useful for the monitoring of propagation material to be used for new vineyards, avoiding the introduction of new GLD variants from foreign countries, and the consequent deteriorating sanitary status [70] of Italian vineyards.

The introduction of new sensitive and economic diagnostic techniques mentioned above may contribute to further minimizing sanitary risks in the production of both local grape and wine industry, and could be applied for territory monitoring, disease spread [71], and to evaluate the genetic diversity and molecular variability of these pathogens [72].

5. Conclusions

To our best knowledge, this represents the first extensive survey on GLRaV-1 and GLRaV-3 genetic structure and molecular variability in Sicily. This study provides useful data on the sanitary status of most important red- and white-berried Sicilian cvs. It will be interesting to carry out further analyses of more clones from single isolates, clarifying the genetic population structures in Sicily region, improving the containment of epidemics and providing valuable information for the implementation of good management practices, in order to verify the presence of new sequence variants and the intra-isolate genetic diversity GLRaV-1 and GLRaV-3 in Sicilian wine-growing locations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12050647/s1, Table S1. Nucleotide diversity[a] of Grapevine leafroll-associated virus 1 (GLRaV-1) in various geographical populations; Table S2. Nucleotide diversity[a] of Grapevine leafroll-associated virus 3 (GLRaV-3) in various geographical populations; Figure S1. Nucleotide pairwise percent identity of the sequenced coat protein gene between the Sicilian GLRaV-1 isolates using SDTv1.2 program. The percentage to the identity score between two sequences is indicated by each colored key; Figure S2. Nucleotide pairwise percent identity of the sequenced coat protein gene between the Sicilian GLRaV-3 isolates using the SDTv1.2 program. The percentage to the identity score between two sequences is indicated by each colored key; Figure S3. Nucleotide pairwise percent identity of the sequenced coat protein gene between the Sicilian GLRaV-1 isolates and reference isolates using the SDTv1.2 program. The percentage to the identity score between two sequences is indicated by each colored key; Figure S4. Nucleotide pairwise percent identity of the sequenced coat protein gene between the Sicilian GLRaV-3 isolates and reference isolates using the SDTv1.2 program. The percentage to the identity score between two sequences is indicated by each colored key.

Author Contributions: Conceptualization, S.D. and S.P.; methodology, A.G.C., S.B., S.D. and S.P.; software, A.G.C., S.B. and S.D.; validation, A.G.C., S.B., S.M., S.D. and S.P.; formal analysis, A.G.C., S.B. and A.R.; investigation, A.G.C., S.B., A.R., S.D. and S.P.; resources, S.P. and S.D.; data curation, A.G.C., S.D. and S.P.; writing—original draft preparation, A.G.C., S.D. and S.P.; writing—review and editing, A.G.C., S.B., A.R., S.M., S.D. and S.P.; visualization, A.G.C., S.B., A.R., S.M., S.D. and S.P.; supervision, S.D. and S.P.; project administration, S.D. and S.P.; funding acquisition, S.D. and S.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank the 'CON-0339-Analisi certificazione virus vite' project, for the technical support.

Conflicts of Interest: The authors declare no conflict of interest.

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