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Genetic Structure and Molecular Variability of Grapevine Fanleaf Virus in Sicily

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Abstract: Grapevine fanleaf virus (GFLV) is one of the main causes of grapevine fanleaf degeneration disease (GFDD) and is present in almost all areas where grapevine is cultivated. In this work, we ascertained the presence and spread of GFLV in different commercial vineyards in four Sicilian provinces (Italy), and its genetic structure and molecular variability were studied. In detail, a total of 617 grapevine samples of 11 autochthonous grapevine cultivars were collected in 20 commercial vineyards. Preliminary screening by serological (DAS-ELISA) and molecular (RT-PCR) analyses for ArMV (arabis mosaic virus) and GFLV detection were conducted. Results obtained showed the absence of ArMV in all the samples analyzed, while 48 out of 617 samples gave positive results to GFLV, for a total of 9 out of 11 cultivars analyzed. Phylogenetic analyses carried out on the GFLV-CP gene of 18 Sicilian GFLV sequences selected in this study showed a certain degree of variability among the Sicilian isolates, suggesting a different origin, probably as a consequence of the continuous interchange of GFLV-infected propagating material with other Italian regions or viticultural areas located in other countries.

Keywords: GFLV; grapevine disease; molecular variability; DAS-ELISA; RT-PCR; phylogenetic analysis

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1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important and extensively cultivated crop worldwide; according to Faostat 2019 data [1], the global covered area reached about 7 million ha, with a total production of over 77 million tons. More than 3 million ha is located in the European continent, especially in the central and southern areas. In Italy, grapevine is one of the most economically important crops, with an area of 722,000 ha and a total production of over 810,000 tons [2]. Numerous grapevine cultivars are cultivated in Italy, and every region has its own autochthonous cultivars. In the last 20 years, Sicily has become one of the most important regions for the grapevine industry, due to the atmospheric conditions, which permit the production of very good wines obtained from autochthonous and imported cultivars, such as "Alicante", "Grillo", "Catarratto", "Carricante", "Inzolia", "Zibibbo", "Malvasia", "Nero d'Avola", "Nerello Cappuccio", "Perricone", "Nerello Mascalese", "Moscato", and "Grecanico".

In the last decade, in grapevine cultivation, important yields in terms of quantity and quality have been obtained; however, this crop is constantly threatened by the presence of several viral diseases which can lead to a progressive deterioration of the current

grapevine sanitary status of many autochthonous cultivars in the country used mainly for the production of "high-quality" wines.

Quality and quantity sustainability in grapevine production requires us to be aware of diseases that affect this crop. Similar to other crops that are vegetatively propagated, grapevine is exposed to many different pests and pathogens that play a major role, causing heavy losses, shortening the productive life of the crop, and endangering the survival of affected grapevines [3]. For this reason, through certification schemes application and taking into account the sanitary improvement of the crop, it is essential to produce propagation material (cuttings, rooted cuttings, and grafted plants) with improved sanitary traits that can reduce the inoculum potential. Furthermore, it is important to apply a massive and continuous phytosanitary monitoring of vineyards already present, applying appropriate strategies for restraining the production and distribution of infected stocks, in order to have a beneficial impact on the health conditions of the viticultural industry [4].

Viruses are the most dangerous grapevine pathogens, and, to date, 86 viral species belonging to 17 families and 34 genera have been identified infecting grapevine; some of them cause severe diseases, such as leafroll, infectious degeneration, and rugose wood [5]. Therefore, it is of crucial importance to know the genetic diversity and the spread of these pathogens, in order to develop new phytosanitary programs or new variety selection programs, especially in a perspective of ecologically sustainable environment [6].

As a first step, virus disease management relies on preventive measures, such as plant propagation material control and eradication programs' establishment, avoiding the pathogen introduction in a new area. However, when viruses are already present in an area, phytosanitary selection and sanitation programs through production of virus-free plant material and clonal selections of resistant or tolerant cultivars are required to control the disease.

The use of certified plant material remains one of the key strategies for a sustainable management of grapevine viral disease. The selection process involves screening for the presence of the main grapevine viruses, namely 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*), and Grapevine virus *A*–GVA (family, Betaflexiviridae; genus, *Vitivirus*). GLRaV-1, GLRaV-3, GFLV, ArMV, GVA, and Grapevine fleck virus–GFkV (family, Tymoviridae; genus, *Maculavirus*) (only for rootstocks) are considered harmful diseases by the European Commission directive (2005/43/EC), and their absence in nursery must be confirmed through official inspections [7].

Another dangerous grapevine virus is the Grapevine Pinot gris virus (GPGV), reported since 2003 in Northern Italy [8]; due to movement of infect propagation material in the grape-growing countries, it has spread rapidly [9]. The danger of GPGV lies in the fact that in some cases infected plants are asymptomatic, representing an increased risk for its spread; for this reason, GPGV should be included in the grapevine certification schemes for the production of virus-free plants [9]. Among the mentioned viruses, GFLV, which belongs to the *Nepovirus* genus of the Secoviridae family, is considered the most widespread and the main responsible of grapevine fanleaf degeneration disease (GFDD), which is one of the most severe grapevine virus diseases worldwide [10].

All nepoviruses involved in fanleaf degeneration/decline can cause similar symptoms expressed in the foliage [11], such as leaf distortion, yellow mosaic close to primary veins, bright yellow vein banding on leaves, widely open petiolar sinuses, double nodes, and short and malformed internodes [12].

In particular, GFLV is spread in almost all areas where grapevine is cultivated, including North and South America, Europe, Africa, Asia, and Oceania [13]. In the last years, GFLV has been observed in Spain [14], Croatia [15], Chile [16], Switzerland [17], Tunisia [18], Italy [19], and Canada [20]. The result of the extensive GFLV diffusion in these regions highlighted an urgent need for an efficient virus control strategy [21].

The GFLV virions are icosahedral with a diameter of 30 nm [22]. The genome consists of two single-stranded positive-sense RNAs, called RNA1 and RNA2, of ~7.3 and ~3.7 kb, respectively. These RNAs are polyadenylated at their 3′ ends and have a covalently attached small genome-linked viral proteins (VPg) at their 5′ ends. The RNA1-encoded polyprotein of 253 kDa (P1) is processed into six proteins, referred to as X1 (of unknown function), X2 (a putative protease cofactor), NTB (nucleotide triphosphate-binding protein), VPg, Pro (3C-like proteinase), and Pol (RNA-dependent RNA polymerase) [23]. The RNA2-encoded polyprotein of 122 kDa (P2) is cleaved by the RNA1-encoded protease into three functional fragments, namely the homing protein (2A), the movement protein (MP), and the coat protein (CP) [24,25].

GFLV can reduce grape yield, quality and, in some cases, it can also reduce the productive life of grapevine plants. Moreover, in GFLV-infected material, rooting of rootstocks and grafting are both significantly decreased [13]. Symptoms can vary considerably depending on virus isolate and grapevine cultivar or species, appearing as distorted, asymmetrical, and wrinkled leaves with irregular margins [26]; sometimes a greenish-yellow mosaic can be seen on the leaf surface (Figure 1B). The main veins are very close together, giving the leaf a partially open-fan shape. Symptoms on leaves persist throughout the growing season and subside in midsummer. The shoots have a zigzag pattern (Figure 1A), shortening of the internodes, double nodes, and banding, with abnormal bifurcations, while the bunches are reduced in number and size, with irregular ripening.

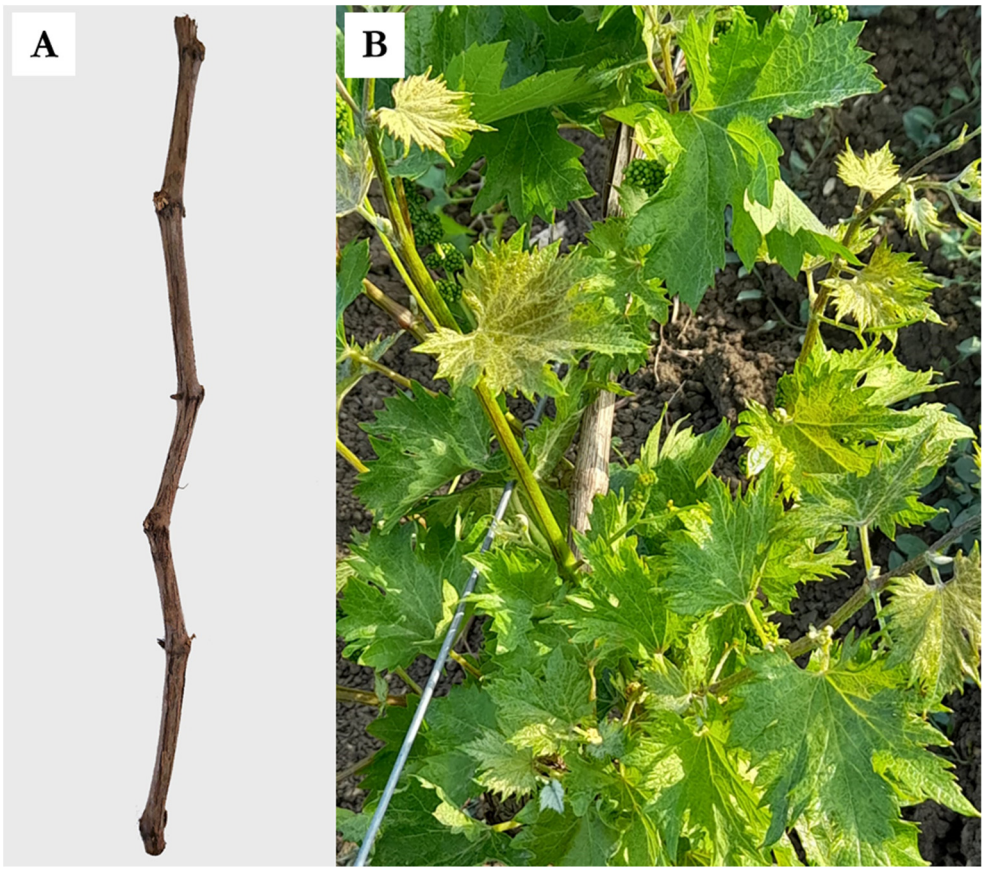

Figure 1. Typical zigzag pattern/shortening of the internodes (**A**) and greenish-yellow mosaic on the leaves (**B**) caused by Grapevine fanleaf virus in Nero d'Avola cultivar.

GFLV is transmitted plant-to-plant by the ectoparasitic nematode *Xiphinema index* [13,27–30].

The main objective of this study was to ascertain the presence and spread of GFLV in different cultivars collected in many commercial vineyards in Sicily, and to study its genetic structure and molecular variability.

2. Materials and Methods

2.1. Field Surveys and Samples Collection

In order to study the presence of GFLV in Sicily and evaluate his genetic structure, a total of 617 grapevine samples were collected in different surveys carried out during December 2020, January 2021, and February 2021. Field surveys were carried out in 20 commercial vineyards in Sicily located in the provinces of Trapani, Agrigento, Caltanissetta, and Ragusa. Grapevine materials were randomly collected according to the hierarchical sampling scheme developed by Gottwald and Hughes [31], with a minor correction for adapting the scheme to grapevine plants. All samples collected were geo-referenced by using the Planthology mobile application [32]. Sampling was carried out in 11 autochtonous CVSs: "Grillo", "Nerello Mascalese", "Zibibbo", "Nero d'Avola", "Carricante", "Nerello Cappuccio", "Grecanico", "Perricone", "Catarratto", "Alicante", and "Moscato" (Table 1). The sampling was made according to the major presence of the different cultivars in Sicily; in detail, Grillo, Zibibbo, Perricone, and Catarratto are among the most widespread cultivars. Moreover, in some cases, the area of some commercial vineyards was more extended than others, with a high number of plants of just one or two cultivars; in these cases, the samples number for each cultivar increased. Each sample, consisting of 4 dormant cuttings, was split into two subsamples to perform serological and molecular analyses.

Table 1. Number of samples for each cultivar analyzed by DAS-ELISA and resulted positive for ArMV/GFLV.

Cultivar	No. of Samples Analyzed	ArMV/GFLV Positive Samples
Grillo	114	0
Nerello mascalese	43	1
Zibibbo	106	7
Nero d'Avola	64	10
Carricante	30	4
Nerello Cappuccio	24	11
Grecanico	64	5
Perricone	74	1
Catarratto	66	8
Alicante	21	1
Moscato	11	0
Total	617	48

2.2. Preliminary Screening by Serological Analysis

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) [33] was performed using polyclonal antibodies to GFLV/ArMV (Agritest srl, Valenzano, Italy). The samples were also analyzed for ArMV in order to verify the presence of this virus in Sicilian commercial vineyards, as to date it has not been reported. Five-hundred mg of floematic tissue of each sample was mixed and homogenized with 5 mL extraction buffer (37.2 g TRIS-HCl, 32 g TRIS-base, 8 g NaCl, 20 g PVP MW 24000, 10 g PEG MW 6000, and 0.5 mL Tween 20 in 1 L of distilled water, pH 8.2), and a 1:10 dilution (*w*/*v*) of each sample was used for DAS-ELISA, following the manufacturer's instructions. Positive control was prepared from lyophilized plant tissue infected by GFLV or ArMV (Agritest srl, Valenzano, Italy), and negative control from healthy plant tissue (Agritest srl, Valenzano, Italy), was re-suspended in 2 mL of distilled water and used as positive and negative controls, respectively. Two hours after the addition of the p-nitro-phenylphosphate substrate, the optical densities (O.D.) at 405 nm, using a AMR-100 microplate reader (Hangzhou Allsheng Instruments, China), were measured. The sample was considered positive if its OD_{405} value was at least twice the negative control value, as reported in the protocol supplied by Agritest srl.

2.3. Total RNA Extraction

Positive samples by DAS-ELISA were used for the subsequent molecular analysis. Moreover, all the samples from "Grillo" and "Moscato" CVSs that resulted in being negative for the DAS-ELISA assay were also analyzed by molecular analyses. A total of 100 mg of floematic tissue of each sample was homogenized, and total RNA was extracted by using a GenUP Plant RNA kit (Biotechrabbit GmbH, Berlin, Germany), according to the manufacturer's instructions, and eluted in 50 µL RNase-free water. The total RNA concentration was measured twice with a UV–Vis Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and dilutions were adjusted to approximately 50 ng/µL and stored at −20 °C until subsequent analyses.

2.4. Molecular Analyses

Reverse-transcription polymerase chain reaction (RT-PCR) assays were carried out on samples that resulted in being positive for GFLV/ArMV by DAS-ELISA and "Grillo" and "Moscato" samples (Table 1). In detail, the GFLV coat protein (CP) and ArMV polyprotein genes were amplified by end point RT-PCR, using the EV00N1/EV00N3 [21] and ArMV 2B/ArMV 2BR [34] primer pairs, respectively (Table 2). The specific primers used for GFLV amplification designed by Vigne and co-workers [21] were modified in order to delete the sequence of the restriction enzyme *XbaI*.

Table 2. Primers list used for GFLV and ArMV specific detection by end point RT-PCR and sequencing.

Virus	Gene	Primer Name	Sequence (5′-3′)	Amplicon Size (bp)
GFLV	Coat protein	EV00N1 * EV00N3 *	GACTATCTAGACACATATATACACTTGGGTCTTTTAA ACTGTCTAGAGGATTRGCYGGYAGAGGAGT	1573
ArMV	Polyprotein	ArMV 2BF ArMV 2BR	AGGGTCGCTTCTAGTACAGC ATCCGAGGAAGAGCAACTCC	962

* The primers used for GFLV amplification [18] were modified by deleting the sequence of additional restriction enzyme *XbaI*.

One-step end-point RT-PCR for each virus was performed in a final volume of 25 µL containing 1 µL of total RNA extract, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM MgCl2, 0.4 mM dNTPs, 1 µM of each primer, 4U of RNaseOut, 20U of superscript II reverse transcriptase-RNaseH, 2U of *Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and RNase-free water to reach the final volume. RT-PCR was carried out in a MultiGene OptiMax thermal cycler (Labnet International Inc., Edison, NJ, USA). The cycling conditions were as follows: reverse transcription at 42 °C for 50 min, initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 54 °C (ArMV) [34]—55 °C (GFLV) [21] for 1 min and 72 °C for 90 s, and a final elongation step at 72 °C for 10 min. Total RNAs derived from grapevine infected by GFLV and ArMV (Agritest srl, Valenzano, Italy) were used as positive control, while molecular-grade water and total RNA extracted from healthy grapevine plant (Agritest srl, Valenzano, Italy) were used as negative controls.

The RT-PCR products were electrophoresed on 1.5% agarose gel, stained with Sybrsafe (Thermo Fisher Scientific, Waltham, MA, USA), and visualized by UV light.

2.5. Sequence Analyses

Since for each grapevine cultivar the positive samples were from the same field, and assuming a low genetic variability among the various isolates, a total of 18 out of 48 GFLV obtained amplicons, corresponding to ~40% of RT-PCR positive samples, were purified by using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. No amplification products were observed for ArMV by RT-PCR, except for the positive control. GFLV-purified amplicons were sequenced in both directions, using an ABI PRISM 3100 DNA sequence analyzer (Applied Biosystems, Foster

City, CA, USA). The sequences obtained were trimmed to remove the flanking regions, leaving only 1515 nt CP gene, and deposited in GenBank.

The nucleotide sequences obtained with other 24 sequences of CP-GFLV retrieved from GenBank from different countries (Italy, from DQ362921 to DQ362925, and from DQ362927 to DQ362935; Iran, FJ513385 and KJ913810; France, AY371023 and X16907; Brazil, EU038294 and EU258681; Chile, DQ526452; USA, X60775; Austria, U11768; and China, AJ318415) were used for phylogenetic analyses.

Multiple nucleotide sequence alignment was performed by using the CLUSTALW algorithm [35], and a mathematical model was applied to estimate the number of nucleotide substitutions, considering nucleotide frequencies and instantaneous rate change. The model that fitted best was the Tamura 3-parameter (T92) [36], modeled by using a discrete gamma distribution (+G) = 0.3838 with two rate categories. Phylogenetic relationships were inferred by the maximum-likelihood method (ML), with 1.000 bootstrap replicates to estimate the statistical significance of each node [37], performed with the MEGA X program [38]. 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 MEGA X program [38]. In order to investigate the presence of recombination events between the nucleotide sequences obtained from Sicilian isolates, analyses with RDP4 program (v.4.39) were carried out, including the algorithms GENECONV, BOOTSCAN, MAXCHI, SISCAN, 3SEQ, and RDP [39]; RDP4 parameters were set as default values. Only concordant results of in silico analysis between different algorithms were considered as a positive result. Nucleotide sequence diversity of GFLV CP gene was estimated within and between different countries, which were considered as geographic populations, using the Jukes–Cantor model [40]. The role of natural selection at the molecular level in the Sicilian isolates of GFLV was studied by evaluating the rate of synonymous substitutions per synonymous site (dS) and the rate of nonsynonymous substitutions per nonsynonymous site (dN), separately; these values were estimated by the Pamilo–Bianchi–Li method [41], using the MEGA X program.

Lastly, the pairwise percent identities of CP-GFLV isolates was calculated within the GFLV isolates from Sicily (Italy) and between the other reference isolates of the other countries, using the SDT v1.0 program [42].

3. Results

3.1. Incidence of GFLV and ArMV Infection in Sicilian Vineyards

A total of 617 samples of 11 CVSs from commercial vineyards located in different Sicilian provinces (Trapani, Agrigento, Caltanissetta, and Ragusa) were investigated for the presence of GFLV and ArMV by DAS-ELISA assay. Forty-eight out of 617 samples gave a positive result for at least one virus, representing 7.78% of the infection (Table 1). In addition, 9 out of the 11 cultivars tested were positive for at least one virus. Only the cultivars "Grillo" and "Moscato" tested negative for both viruses. In order to confirm the first screening results and ascertain the presence of ArMV and GFLV in single or mixed infections, all samples that resulted in being positive at the first screening by DAS-ELISA were subjected to molecular analyses. In addition, to avoid possible false-negative results, the "Grillo" and "Moscato" samples were also analyzed by RT-PCR end point.

3.2. Polymerase Chain Reaction and Sequencing

To distinguish the single or simultaneous presence of GFLV and ArMV, based on the data obtained from the DAS-ELISA assays, a total of 48 positive samples were analyzed by end-point RT-PCR, using specific primer pairs (Table 2). Moreover, "Grillo" and "Moscato" samples were analyzed. It is interesting to note that ArMV was not detected in any samples (except in the positive control), while 48 samples gave positive results for GFLV (Table 3), obtaining the expected amplicon size of 1573 nt.

Table 3. Number of samples analyzed by RT-PCR with specific primer pairs for Arabis mosaic virus and Grapevine fanleaf virus.

Cultivar	Number of Samples Analyzed	ArMV	GFLV
		No. Positive Samples	No. Positive Samples
Grillo	114	0	0
Nerello mascalese	1	0	1
Zibibbo	7	0	7
Nero d'Avola	10	0	10
Carricante	4	0	4
Nerello Cappuccio	11	0	11
Grecanico	5	0	5
Perricone	1	0	1
Catarratto	8	0	8
Alicante	1	0	1
Moscato	11	0	0
Total	173	0	48

In detail, the highest percentage of positive samples was recorded in the cultivars "Nerello Cappuccio", "Carricante", "Nero d'Avola", and "Catarratto", with an incidence of 45.83%, 13.33%, and 12.12%, respectively (Table 4). Meanwhile, the cultivars "Grecanico", "Zibibbo", "Alicante", "Nerello Mascalese", and "Perricone" had a lower incidence, with a percentage of 7.81%, 6.60%, 4.76%, 2.32%, and 1.35%, respectively (Table 4). The CP-GFLV sequences obtained were deposited in GenBank under the accession numbers from MZ027456 to MZ027473.

Table 4. Percentage of incidence for each cultivar tested by specific RT-PCR end point for Grapevine fanleaf virus.

Cultivar	No. of Samples Collected	% of GFLV Incidence
Grillo	114	-
Nerello mascalese	43	2.32
Zibibbo	106	6.60
Nero d'Avola	64	13.33
Carricante	30	13.33
Nerello Cappuccio	24	45.83
Grecanico	64	7.81
Perricone	74	1.35
Catarratto	66	12.12
Alicante	21	4.76
Moscato	11	-

3.3. Phylogenetic Analyses

Phylogenetic analyses were carried out on the CP gene of the 18 Sicilian GFLV sequences obtained in this study, as well as another 14 Italian isolates, 2 Iranian isolates, 2 French isolates, 2 Brazilian isolates, 1 isolate from Chile, 1 from USA, 1 from Austria, and 1 from China, retrieved from GenBank database. The phylogenetic tree reported in Figure 2 related to the evolutionary relationships among GFLV sequences used in the present work showed that GFLV isolates were separated into two statistically significant clusters: the first one including only two isolates from Iran, showing low variability (probably due to the limited number of sequences available in GenBank for this country); and the second one including isolates from different countries, suggesting a continuous exchange of genetic material between the countries of the second cluster.

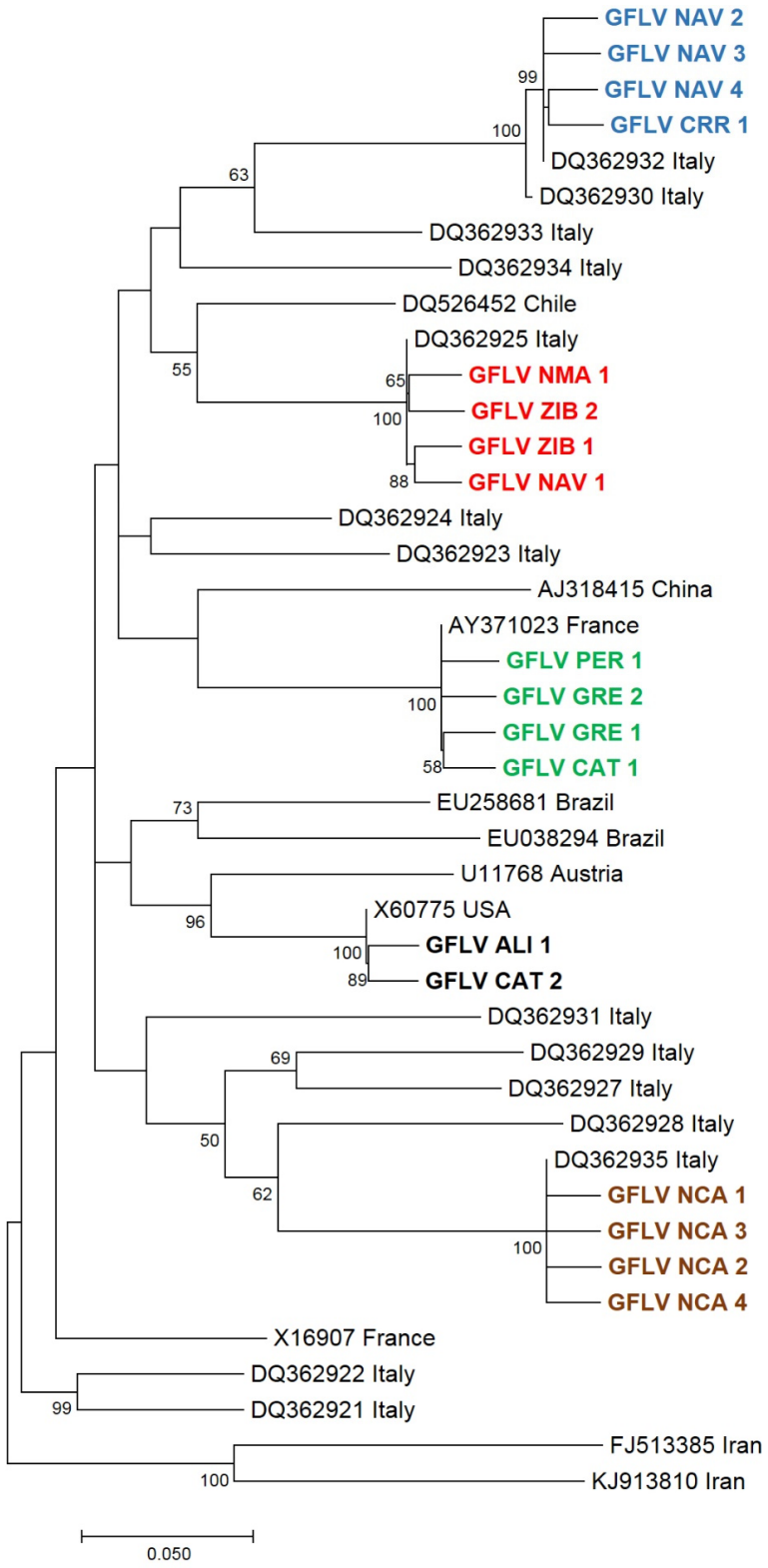

Figure 2. Phylogenetic relationships between coat protein genes of Italian Grapevine fanleaf virus (GFLV) isolates (18 Sicilian sequences obtained in the present work; 14 Italian isolates retrieved from GenBank) and isolates from other countries. The evolutionary history was inferred by using the maximum-likelihood method (ML) based on the Tamura 3-parameter model with bootstraps of 1000 replications, conducted with MEGA X program. Only bootstrap values $\geq$ 50% are indicated in the nodes. The sequences obtained in the present work are in bold.

Sicilian isolates were grouped into five sub-clades: GFLV-ALI1 and GFLV-CAT2 grouped with the USA isolate found in *Vitis rupestris* S. George maintained in grapevine collection in Davis (CA); GFLV-PER1, GFLV-GRE1-2, and GFLV-CAT1 grouped with the French isolate retrieved in Chardonnay vineyard; GFLV-NAV2-3-4 and GFLV-CRR1 grouped with Italian isolates found in *V. rupestris* S. George located in the Italian Piedmont region (DQ362932); GFLV-ZIB1-2, GFLV-NAV1, and GFLV-NMA1 grouped with an isolate found in a Sangiovese cultivar retrieved in Emilia Romagna region (Italy); and, finally, the isolates GFLV-NCA1-2-3-4 were closely related to an Italian isolate found in Piedmont region from *V. rupestris* S. George rootstock (DQ362935).

Recombination analyses showed that no candidate recombinant events were detected by GENECONV, BootScan, 3Seq, and RDP algorithms; however, SiScan and MaxChi algorithms identified putative recombination events. The SiScan algorithm detected one event in the GFLV-GRE-1 isolate, with a beginning breakpoint at position 924 (nt) and ending breakpoint at position 1161 (nt) (major parent GFLV-CAT-2 and minor parent GFLV-NMA-1, with a 87.5% and 93.7% of similarity, respectively) and an average *p*-value of 9.037×10^{-03}, while the MaxChi algorithm detected one recombination event in the GFLV-NMA-1 isolate, with a beginning breakpoint at position 1447 (nt) and ending breakpoint at position 55 (nt) (major parent GFLV-PER-1 and minor parent GFLV-NAV-2, with a 87.6 and 96% of similarity, respectively) and an average *p*-value of 4.066×10^{-01}.

In addition, the analysis of the nucleotide diversity showed a very low differentiation within Italian isolates (0.1258 ± 0.008) and between isolates from Italy and those from France, China, Chile, USA, Austria, and Brazil (0.1166 ± 0.0188, 0.1339 ± 0.0234, 0.1180 ± 0.0208, 0.1164 ± 0.0216, 0.1318 ± 0.0232, and 0.1354 ± 0.0208, respectively), suggesting a common origin, while a certain level of differentiation was observed between Italian and Iranian isolates (0.1726 ± 0.0263) (Table 5).

It was impossible to calculate the nucleotide diversity for China, Chile, USA, and Austria, because only one sequence for each group was available.

The CP gene of the Sicilian GFLV isolates showed dN and dS values of 0.073 and 0.293, respectively, with a dN/dS ratio of 0.249. These values confirm the hypothesis of negative selection. Finally, the pairwise percent identity of the Sicilian CP sequences ranged from 86 to 100% (nt) (Figure 3), while when the CP sequences included both the Sicilian and reference isolates, the range was lower, from 82 to 100% (nt) (Figure 4).

Table 5. Nucleotide diversity [a] of Grapevine fanleaf virus (GFLV) in different geographical populations.

	n [b]	France	Italy	China	Chile	USA	Austria	Brazil	Iran
France	2	**0.1062 ± 0.500**	0.1166 ± 0.0188	0.1227 ± 0.0614	0.1024 ± 0.0521	0.1062 ± 0.0535	0.1119 ± 0.0560	0.1153 ± 0.0579	0.1531 ± 0.0768
Italy	32		**0.1258 ± 0.008**	0.1339± 0.0234	0.1180 ± 0.0208	0.1164 ± 0.0216	0.1318± 0.0232	0.1354 ± 0.0208	0.1726± 0.0263
China	1			**n.c.**	0.1238 ± 0.0000	0.1246 ± 0.0000	0.1372 ± 0.0000	0.1360 ± 0.0681	0.1593 ± 0.0797
Chile	1				**n.c.**	0.0963 ± 0.0000	0.1107 ± 0.0000	0.1153 ± 0.0578	0.1565 ± 0.0782
USA	1					**n.c.**	0.0881 ± 0.0000	0.1123 ± 0.0562	0.1651 ± 0.0826
Austria	1						**n.c.**	0.1207 ± 0.0604	0.1561 ± 0.0782
Brazil	2							**0.1092 ± 0.500**	0.1639 ± 0.0822
Iran	2								**0.1476 ± 0.500**

Nucleotide diversity within a group is reported in bold in the diagonal, while nucleotide diversity between groups are reported above the diagonal. [a] Nucleotide diversity was measured by the Jukes–Cantor method. [b] Number of isolates for each population; n.c., not calculated.

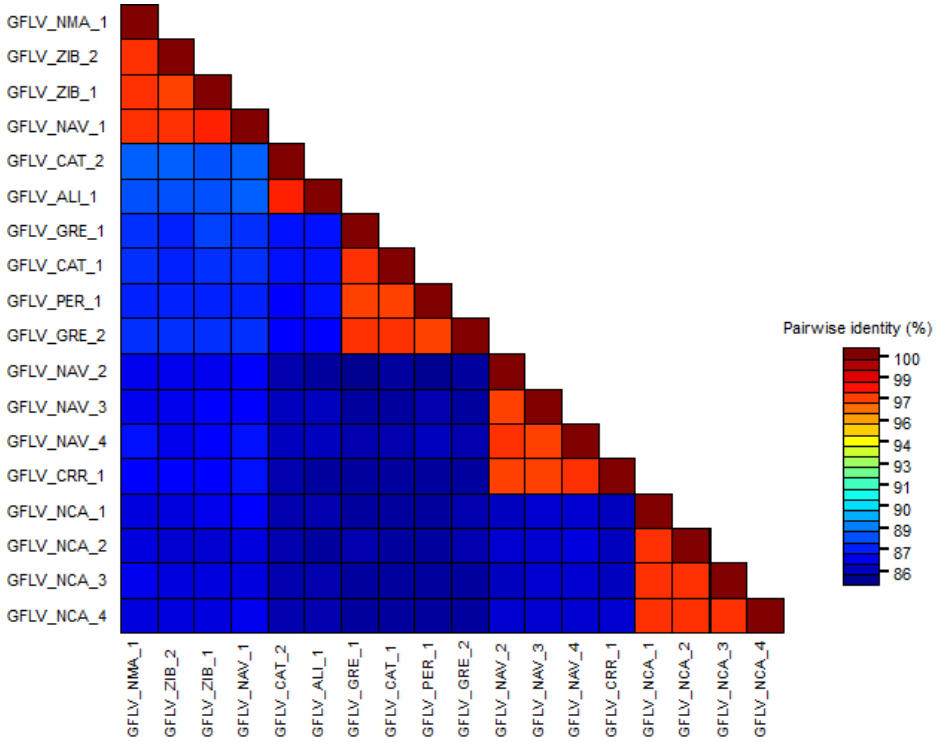

Figure 3. Graphical representations of pairwise percent identity of nucleotides of the sequenced coat protein gene between the GFLV isolates from Sicily (Italy), using SDTv1.0 program. Each colored key represents a percentage for the identity score between two sequences.

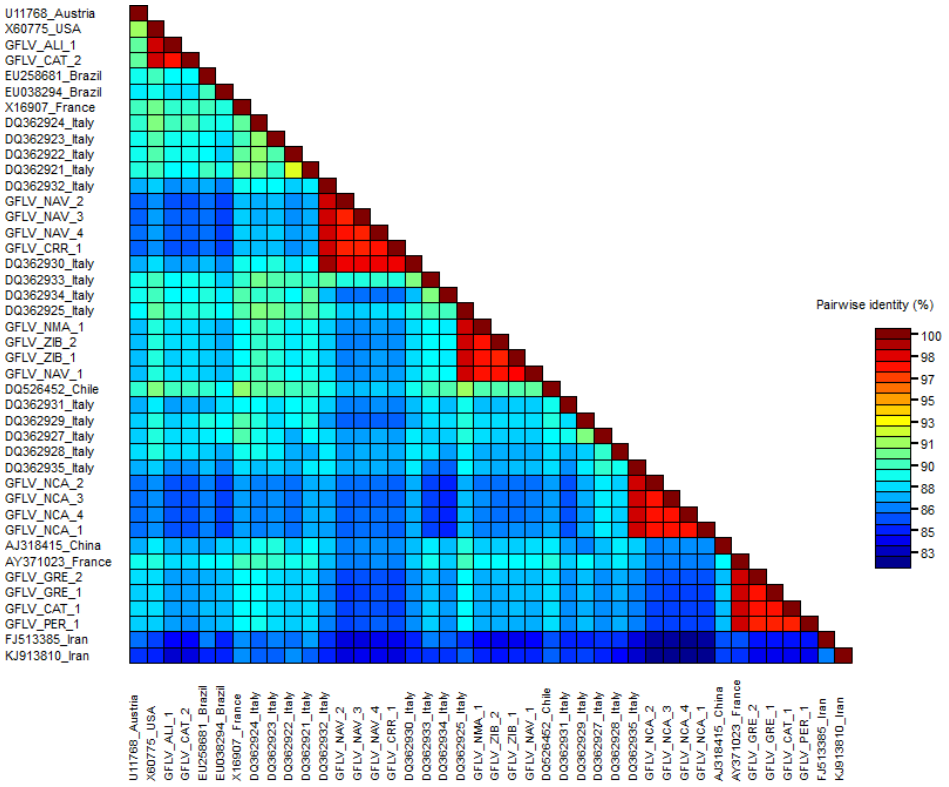

Figure 4. Graphical representations of pairwise percent identity of nucleotides of the sequenced coat protein gene between the GFLV isolates from Sicily (Italy) and reference isolates, using SDTv1.0 program. Each colored key represents a percentage for the identity score between two sequences.

4. Discussion

Italy is one of the Mediterranean Basin countries with the most important tree and vegetable crops in Europe, but, in the last decades, it has suffered heavy losses caused by the appearance and/or recrudescence of new viral pathogens [6,43–45].

GFLV is one of the greatest and most severe grapevine viruses worldwide, causing fanleaf degeneration disease (GFDD); it has a great genetic variation potential, and its replication process is error-prone, since it does not have a proofreading correction mechanism associated with RNA-dependent RNA polymerase; consequently, each viral isolate consists of a population of genetically related variants [46].

The survey conducted in this study in several vineyards located in four Sicilian provinces showed the absence of ArMV in all the samples analyzed, regardless of vineyard location and cultivar. Probably, the ArMV absence in Sicilian vineyards analyzed could be associated with the absence of the vector *Xiphinema diversicaudatum* [47] and/or the limited presence of the virus in propagation material.

The grapevine cultivars analyzed in this study, which represent the most widespread and important autochthonous species in Sicily, showed a different incidence of GFLV infection. In detail, a higher incidence of the disease was detected on "Nerello Cappuccio", "Carricante", "Nero d'Avola" and "Catarratto" cultivars, followed by "Grecanico", "Zibibbo" (Muscat of Alexandria), "Alicante", "Nerello Mascalese", and "Perricone"; meanwhile, in "Grillo" and "Moscato" (white Muscat of Noto) cultivars, no infection was found. Probably, the absence of the pathogen in these two varieties is due to the different cultivation practices used for plant growth. This result may be favored by the establishment of GFLV in the different vine-growing Sicilian areas due to the continuous exchange of GFLV-infected propagation plant material and the possible presence of its vector (*X. index*), as already reported in Italy [29]. In addition, it is possible to hypothesize that cultivars that showed a higher incidence of infection are more susceptible to GFLV infection than others, as was similarly found in autochthonous cultivars in another GFLV study [34].

The presence of GFLV-infections in the autochthonous Sicilian cultivars, or other grapevine viruses, could also be explained by the extensive use of *Vitis rupestris* Scheele in the last century as rootstock, a fundamental species imported from America, and together with it, probably, many viruses of the grapevine in order to contain phylloxera damages. The intensive use of *V. rupestris* Scheele, even if it allowed the eradication of phylloxera problems, favored the wide spread of several viruses in almost all areas of grapevine cultivation.

Phylogenetic analysis of Sicilian GFLV isolates obtained in this work highlighted a certain degree of variability within the sequences obtained, suggesting a different origin. In fact, this is probably due to the continuous interchange of plant propagation material with other Italian regions or European countries, especially the Piedmont region and France, respectively, suggesting that GFLV isolates from various regions, distant by thousands of kilometers, had similar or identical genetic structure with the Sicilian isolates.

Moreover, the isolates from the "Nerello Cappuccio" cultivar, which show a low variability, were grouped within the same clade with an isolate from Piedmont region, suggesting that these isolates originated from a single accession, probably introduced in Sicily by GFLV-infected propagation material.

The same situation is occurring for the other four clades: one includes three isolates from the "Nero d'Avola" cultivar and one from the "Carricante" cultivar, showing a high sequence homology with two isolates from Piedmont region, probably also in this case due to the introduction of infected rooted cuttings in Sicily; another clade groups viral variants isolated from "Nerello Mascalese", "Zibibbo", and "Nero d'Avola" cultivars with an Italian isolate from Emilia Romagna region. Another clade, grouping isolates obtained from the "Perricone", "Grecanico", and "Catarratto" cultivars with a French isolate, suggests the introduction of infected material from France; the same situation is present for the remaining clade composed by isolates from "Alicante" and "Catarratto" cultivars, showing a high sequence homology with an isolate coming from California. Moreover, in this case,

the results obtained by phylogenetic analysis suggest that this viral accession has probably arrived in Sicilian vineyards due to the introduction of GFLV-infected material—in this case, from the USA.

In summary, the relationship among geographical origin, sequence variability, and cultivar within the Sicilian isolates analyzed could be justified by the different introduction in Italy of sequence variants due to the exchange of GFLV-infected propagating material from distant viticultural areas of the world. The phylogenetic relationships obtained can be implemented when more CP-GFLV gene sequences from other countries will be available in the GenBank.

Regarding genome recombination events, which are common in many viruses attacking different plant species [44], the occurrence of recombination events, detected only with two algorithms, suggests that the possible slow movement of the vector does not favor mixed infections in the same cultivation area.

In some cases, the risk of the wide spread of viral diseases is increased by the fact that there are also many cases of latent infections without many symptoms; for this reason, it is very important to monitor the territory, paying attention to the sanitary status of plant propagation material. [48,49]. This will reduce the threat of grapevine viruses on the livelihood of the local grape and wine industry. Furthermore, growers are recommended to monitor and control the vectors and the symptomatic plants for known grapevine viruses [50], in order to avoid the establishment and spread of these pathogens. A further of grapevine virus control is the use of somatic embryogenesis, which can be a good alternative to obtain certified virus-free propagation material [51].

In addition, many authors have demonstrated that it is possible to contain the GFLV spread by selecting rootstocks resistant to *Xiphinema index*, using *Muscadinia rotundifolia* as a source of resistance [52,53].

In conclusion, the best strategy to control viral diseases must be preventive, using certified "virus-tested" and "virus-free" plants at the national level, during vineyard establishment. This can be implemented through the application of diagnostic techniques for early and reliable detection; in this context, serological tests (DAS-ELISA) and reverse-transcription polymerase chain reaction (RT-PCR) permit satisfactory and reproducible detection. Moreover, it is possible to adopt other diagnostic techniques that are more sensitive, such as quantitative RT-PCR (RT-qPCR) [54,55], possibly combined with rapid and affordable sample extraction methods in order to shorten the processing time, thus allowing simultaneous and multiple samples analysis and reducing the total cost for single analysis [56]. Last, but not least, RT-LAMP (Reverse-Transcription Loop-Mediated Isothermal Amplification) assay was developed for the rapid detection of GFLV [57]; this technique is extremely useful thanks to its higher sensitivity over ELISA and PCR techniques, especially in the case of low virus concentrations and the presence of inhibitors [58].

Future analyses of more clones from single GFLV isolates will be necessary to check for the presence of sequence variants and intra-isolate genetic diversity [21], in order to elucidate GFLV genetic population structure in Sicily and further improve the virus containment.

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