Short Notes

Detection of viruses in olive cultivars in Greece, using a rapid and effective RNA extraction method, for certification of virus-tested propagation material

Matthaios M. MATHIOUDAKIS[1,*], Maria SAPONARI[2], Beata HASIÓW-JAROSZEWSKA[3], Toufic ELBEAINO[4], Georgios KOUBOURIS[5,*]

[1] *Institute of Olive Tree, Subtropical Crops and Viticulture, Plant Pathology Laboratory, Karamanlis Ave. 167, Gr-73134, Chania*
[2] *Consiglio Nazionale delle Ricerche-Istituto per la Protezione Sostenibile delle Piante (CNR-IPSP), Sede Secondaria di Bari, 70126, Bari, Italy*
[3] *Institute of Plant Protection-National Research Institute, Department of Virology and Bacteriology, ul. Wł. Węgorka 20, 60-318, Poznań, Poland*
[4] *Istituto Agronomico Mediterraneo di Bari, Via Ceglie 9, Valenzano, 70010, Bari, Italy*
[5] *Institute of Olive Tree, Subtropical Crops and Viticulture, Laboratory for Olive Cultivation, Karamanlis Ave. 167, Gr-73134, Chania*
*Corresponding authors: mathioudakis@nagref-cha.gr, koubouris@nagref-cha.gr

Summary. Although Greece is the world's third largest olive production country, information about the presence of olive viruses is limited. A survey for the presence of virus infections in the ten most important Greek cultivars was conducted in a germplasm collection olive grove located in Chania, Crete. Samples were RT-PCR assayed for the presence of *Arabis mosaic virus* (ArMV), *Cherry leafroll virus* (CLRV), *Strawberry latent ring spot virus* (SLRSV), and *Olive leaf yellowing-associated virus* (OLYaV), amplifying part of the capsid protein (ArMV), the 3′UTR (CLSRV, SLRSV) or the HSP70h (OLYaV) gene. Total RNAs were purified using the Trizol method, yielding good quality and purity, thereby confirming application of the method as a rapid economic extraction protocol for detection of olive viruses. SLRSV was the most predominant virus, with an infection rate of 55%, followed by CLRV and OLYaV in 5% of the tested samples. ArMV was detected only in one sample. Mixed virus infections were also commonly detected. The DNA amplicons of the obtained viruses from the infected samples were sequenced. The partial sequences of ArMV, CLRV and SLRSV from olives, which are reported for the first time, showed 74-100% nucleotide similarity with available homologous sequences from other crops, whereas OLYaV isolates showed high sequence variability of 25%. The phylogenetic analysis based on olive-OLYaV HSP70h partial-nucleotide sequences grouped the olive isolate sequences according to the geographical origins of the host germplasm collection. This is the first official report of the occurrence of olive viruses in Greece, emphasizing the need to implement a certification programme for production and distribution of high-quality (virus-free) olive propagation material, in Greece and more generally in the Mediterranean basin.

Keywords. Olive viruses, RT-PCR, olive RNA extraction protocol, virus-tested propagation material.

INTRODUCTION

Olive (*Olea europaea* L.) is one of the most important fruit tree crops in Greece, with total area of olive orchards of 830,000 ha, and total world annual production >250,000 t (IOC, 2018). There are over one hundred olive cultivars grown in Greece (Linos *et al.,* 2014), with 'Koroneiki' and 'Kalamon' being the most predominant (Metzidakis and Koubouris, 2006). Despite the richness of the olive autochthonous varieties cultivated in different parts of the country, which indicates their importance, it is estimated that over 90% of the new plantations employ only the ten most popular cultivars. In an attempt to create new plantations and increase their productivity of superior quality olive oil (monovarietal), the phytosanitary status of the most economically and commercially important olive cultivars was evaluated. This was to create high-quality olive germplasm, from which pre-basic certified propagation material could be produced. This is considered mandatory to enhance national and international olive industry.

Olive is susceptible to several pathogens, caused by fungi, bacteria, viruses and phytoplasmas, which are transmitted and disseminated in host propagation material. Fifteen viruses belonging to seven genera (*Nepovirus, Cucumovirus, Tobamovirus, Alphanecrovirus, Oleavirus, Necrovirus, Marafivirus*), and one unassigned member of the Secoviridae and one of the Closteroviridae families, have been recorded in olive (Martelli, 1999; Felix and Clara, 2002; Cardoso *et al.,* 2004; Al Abdullah *et al.,* 2010; Loconsole *et al.,* 2010). Many of these viruses cause latent infections, and are symptomless and restricted in olive trees; however, some other viruses have wide host ranges and can spread to numerous economically important crops which are virus reservoirs for serious diseases. Research conducted on the olive virus records in Italy, Spain, and Portugal was initiated in 1999 (Martelli, 1999). However, more attention has been recently drawn to the virus sanitary status of olive trees in several countries of the Mediterranean basin.

The recent outbreak of the olive quick decline syndrome, caused by *Xylella fastidiosa* in Italy (Saponari *et al.,* 2013), and the detection of this bacterium in other European countries (France, Spain, Germany), has prompted the necessity for annual surveys and inspections in the olive groves of Greece. This action was taken as a preventive measure to cope with the introduction of *X. fastidiosa* in Greece. However, there are no data regarding the presence of olive-infecting viruses in this country.

This paper reports results of a preliminary investigation into olive-infecting viruses, by testing a plot of an olive germplasm collection and using a rapid and effective RNA extraction protocol. This is the first survey report on the detection of olive viruses in Greece. This survey for the presence of *Arabis mosaic virus* (ArMV; *Nepovirus*), *Cherry leaf roll virus* (CLRV; *Nepovirus*), *Strawberry latent ring spot virus* (SLRSV; Secoviridae), and *Olive leaf yellowing associated virus* (OLYaV; Closteroviridae), according to the EU regulations PM 4/17(2) (EPPO, 2006) for the certification of olive propagation material, was conducted from the ten most widely-grown olive cultivars in Greece. Genetic diversity and phylogenetic relationships between virus isolates were also analyzed and are reported herein.

MATERIALS AND METHODS

Study area, plant material and virus sources

A survey was carried out during March 2018, and a total of 40 trees was collected from a germplasm collection plot located at the Institute of Olive Tree, Subtropical Crops and Viticulture (IOSV) in the region of Chania, Crete, Greece. This collection plot contained young olive trees (4–5 years old) from different accessions of Greek cultivars, which have been analyzed and genetically characterized (SSR) for their identification and authentication.

Table 1 lists the ten most important olive cultivars that were chosen, according to their economic importance throughout Greece. Samples were collected from four different trees of each cultivar. Each labelled sample consisted of four sub-samples of cuttings (each 25-35 cm in length) from 1- to 2-year-old twigs, from all four quadrants of the canopy of each plant (two shoots from each quadrant). No typical virus symptoms were observed in the collected samples, with the exception of light leaf yellowing in samples originating from two different cultivars.

Total RNA aliquots originally extracted from characterized olive material, infected with CLRV and OLYaV, or herbaceous hosts infected with ArMV and SLRSV, were used as positive controls to confirm the reliability of the method used for virus detection.

RNA isolation and evaluation of the method for olive tissues

Phloem and leaf tissues from at least eight shoots per surveyed tree were separately scraped and powdered in liquid nitrogen. Approximately 0.1 g of each sample was subjected to RNA isolation by the Trizol method, using

Table 1. Sampled Greek olive cultivars, and results of the molecular detection of ArMV, CLRV, OLYaV and SLRSV.

Cultivar	No. of tested trees	Detected virus and number of infected trees			
		ArMV	CLRV	OLYaV	SLRSV
Gaidourelia	4	0	1	0	0
Lianolia Kerkiras	4	0	0	0	1
Koroneiki	4	1	0	0	2
Throubolia	4	0	1	0	2
Megaritiki	4	0	0	0	4
Patrini	4	0	0	0	3
Kalamon	4	0	0	0	2
Amfissis	4	0	0	0	3
Chalkidikis	4	0	0	1	3
Mastoidis	4	0	0	1	2
Total	40	1	2	2	22

the TRIzol reagent (Invitrogen, ThermoFisher Scientific) as described in the manufacturer's instructions. Initially, the resulting RNAs from phloem and leaves were compared to each other for quality and quantity, and also with total RNAs extracted using the RNeasy Plant Mini kit (Qiagen) from the same ground tissues. The final RNAs were diluted to a final concentration of 150 ng μL^{-1}.

To evaluate the functionality and the sensitivity of the Trizol method in olive tissues, the following experiments were carried out: i) molecular assays for the partial amplification of the β-tubulin, an endogenous olive gene, and ii) molecular assays to assess the purity of the total RNA from genomic DNA (gDNA) during the extraction procedure.

Detection of olive viruses and olive-specific genes using One-step RT-PCR assays

A previously reported One-Step Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used for the detection of olive-infecting viruses, which used reliable sets of primers (Supplementary Table S1) that partially amplify the coat protein (CP) gene of ArMV, the 3′UTR of CLRV and SLRSV, and the Heat shock protein 70 homolog (HSP70h) gene of OLYaV (Loconsole *et al.,* 2010). This was applied under the following modifications. Briefly, 2 μL of total RNA was added in the reaction mixture (final volume 25 μL), along with 5× GoTaq Polymerase buffer, 1.5 mM MgCl$_2$, 5 mM DTT, 0.4 mM dNTPs, 0.2 μM of each virus-specific primer pair, 5 U AMV (Promega), 20 U RNase Out (New England Bio-

Labs) and 1.25 U GoTaq Polymerase (Promega). The cDNA synthesis was performed at 48°C for 45 min, followed by denaturation at 95°C for 5 min. The target amplification was carried out under the following cycling scheme: 35 cycles of 94°C for 30 s, 55°C for 45 s (for OLYaV 58°C was used), 72°C for 30 s, with a final extension step at 72°C for 7 min. The PCR conditions were the same for all viruses. Positive and water controls were used in all of the tests.

The same one-step RT-PCR protocol was applied for detection of the β-tubulin gene, using the following modifications: 0.5 mM dNTPs, annealing temperature of 59°C and 0.25 μM of the specific primers (Supplementary Table S1). To confirm the purity of RNA from gDNA, the same reaction was carried out with exclusion of the reverse transcription enzyme.

The amplified products were analyzed by electrophoresis in 1.5% TAE (1×) agarose gel stained with GelRed (Biotium Inc.).

Sequencing, recombination and phylogenetic analyses

The resulting DNA amplicons were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). The nucleotide sequences from all detected isolates were determined by direct sequencing using both of the virus-specific primers, with the Sanger method (Macrogen Inc.).

For the bioinformatics analyses, the partial olive virus sequences obtained here were aligned, after clipping the primer-binding region with all the known homologous deposited nucleotide viral sequences using Muscle software as implemented in MEGA X (Kumar *et al.,* 2018). Prior to phylogenetic analyses, the occurrence of potential recombination events within virus isolates was analyzed using the RDP, GENECONV, Chimaera, MaxChi, BootScan, SiScan, and 3Seq methods implemented in the RDP version 4 program (Martin *et al.,* 2015) with default settings. Recombination events were considered as significant if four or more of these methods had a $P < 0.05$, in addition to phylogenetic evidence of recombination. Sequence alignments were used for inferring phylogenetic evolutionary relationships and the construction of maximum-likelihood trees in MEGA X. The best nucleotide substitution model was calculated for each set of sequences. The Kimura 2-parameter model (Kimura, 1980) was applied for ArMV, CLRV and SLRSV, and the Tamura 3-parameter model (Tamura, 1992) for OLYaV. Confidence in branch points in the phylogenetic tree was assessed by the bootstrap method with 1,000 pseudorandom replicates (Felsenstein, 1985).

RESULTS AND DISCUSSION

The integrity and quality of the Trizol-total RNAs obtained from olive phloem and leaves was confirmed by electrophoresis, showing a similar pattern as those extracted using another commercial kit. The yield of total RNA was greater for phloem tissues than from the leaves (data not shown), varying from 0.2–0.3 µg µL^{-1} for the RNeasy Plant Mini kit (Qiagen) to 0.4–0.7 µg µL^{-1} for Trizol. The purity (ratio A$_{260}$/A$_{280}$) in both cases was in the optimal range. In previous studies, the use of the RNeasy kit has been commonly adopted for olive total RNA isolation, following various laboratory validations and a comparison with silica microchromatography preparations (Loconsole et al., 2010). In the present study, Trizol was successfully used for preparation of the olive total RNA templates, extracted from phloem, for detection of the target viruses. The β-tubulin gene was successfully detected (data not shown), thereby confirming the effectiveness and suitability of the Trizol-purified olive total RNAs for setting amplification reactions. No amplifications occurred when the total RNAs were directly subjected to PCR without a reverse transcription step (data not shown). This indicated that the Trizol-purified RNAs were free from gDNA. The Trizol method could be used as a simple and high-quality olive RNA extraction protocol (within 3 h duration), minimizing costs without the need of additional purification steps.

The one-step RT-PCR reactions generated the expected amplicons for all positive controls. All four viruses investigated were present in the tested Greek olive germplasm plants, with different infection rates. In Greece, occurrence of olive viruses has not been previously investigated. The only previous report of an olive-infecting virus in Greece was for *Olive mild mosaic virus* (Gratsia et al., 2012), which was detected in spinach.

The present study found at least one virus present in 25 out of 40 samples, with an average of 62.5% virus infection (Table 1). This infection level was comparable to those reported from other Mediterranean countries and Syria (51–86%) (Saponari et al., 2002; Al Abdullah et al., 2005; Faggioli et al., 2005; Youssef et al., 2010; Faggioli et al., 2017), but in contrast to Albania, where virus incidence was very low (2%) (Luigi et al., 2009). In Lebanon and Croatia, incidence of these viruses varied from 20 to 31% (Fadel et al., 2005; Luigi et al., 2011; Godena et al., 2016).

SLRSV was the most frequently detected virus, being present in 22 samples (55%), in almost all of the olive Greek cultivars except 'Gaidourelia' (Table 1). The high infection rates of SLRSV in Greece is in contrast to previous reports in other neighboring countries, where incidence of this virus varied from 0.5 to 29.2% (Saponari

et al., 2002; Al Abdullah et al., 2005; Fadel et al., 2005; Faggioli et al., 2005; Luigi et al., 2009; El Air et al., 2011; Godena et al., 2016). The high incidence of SLRSV (a nematode-borne virus) in the tested olive plants could indicate the presence of SLRSV in other crops or in olive itself, which have bordered the sampled grove as a reservoir of infection. The transmission of SLRSV by *Xiphinema diversicaudatum* in nature is well known, and this cannot be excluded; however, there is no evidence that this nematode is able to transmit SLRSV from olive to olive or from other crops to olive.

ArMV was detected only in one sample collected from a tree of 'Koroneiki' (designated as ArMV-Gr/Kr), whereas CLRV was detected in single infections on two olive cultivars, 'Throuboulia' and 'Gaidourelia' (designated, respectively, as CLRV-Gr/Tr and CLRV-Gr/Gd). The low infection percentages of ArMV (2.5%) and CLRV (5%), confirmed similar results reported in the Mediterranean basin. For ArMV, the absence of infection in Italy, Croatia, and Tunisia (Faggioli et al., 2005; El Air et al., 2011; Godena et al., 2016), and one report of sporadic infection (0.7%) from Egypt (Youssef et al., 2010), are noteworthy. The only reports available are from Lebanon and Syria that show similar infection rates (0.7–2%) (Al Abdullah et al., 2005; Fadel et al., 2005). The incidence of CLRV, as indicated in other countries, varied from very low (0.5%) in Spain (Bertolini et al., 2001) to higher infection rates such as 12% in Croatia (Godena et al., 2016), 13% in Tunisia (El Air et al., 2011), and 4.9–33.3% in Italy (Saponari et al., 2002; Faggioli et al., 2005).

OLYaV was also detected with low incidence (5%), in two olive cultivars, 'Mastoidis' and 'Chalkidikis' (these virus isolates designated, respectively, as OLYaV-Gr/Ms and OLYaV-Gr/Ck). This was similar to OLYaV incidence found in Albania (2%) (Luigi et al., 2009), but OLYaV infection-rates were greater in Tunisia (49%) (El Air et al., 2011), Italy (21–64%) (Saponari et al., 2002; Albanese et al., 2003; Faggioli et al., 2005), Lebanon (23.6%) (Fadel et al., 2005), Syria (14.5%) (Al Abdullah et al., 2005), and especially in California, where OLYaV is the most prevalent virus (93%) (Al Rwahnih et al., 2011). The low infection rate of OLYaV in Greece could be explained by the possible absence or limited presence of the vector of this virus in the country, which is not known but suspected to be the olive psyllid *Euphyllura olivinae* (Sabanadzovic et al., 1999). Another explanation could be the high genetic variability which is well known in Closteroviridae viruses. This could influence the specificity of the primers used in the detection assays. This is probably limited for OLYaV, as the primers were designed in a conserved HSP70h motif. There have been numerous divergent isolates of OLYaV detected to date, but it can-

not be excluded as a cause of particular reported greater variability compared to other closteroviruses (Essakhi *et al.,* 2006; Al Rwahnih *et al.,* 2011).

The proportions of mixed virus infections was of 8%, in 'Mastoidis' by SLRSV and OLYaV, and in 'Koroneiki' by ArMV and SLRSV. At least one host tree of each cultivar was free from the tested viruses, except for the cultivar 'Megaritiki' (Table 1).

Sequences of PCR-amplicons showed nucleotide homologies with the respective homologous viral sequences in the GenBank, confirming the specificity of the amplicons. No recombination events were detected within the analyzed sequences from each virus. The sequences obtained from ArMV, CLRV and SLRSV represent the first genetic information recovered from olive-infected material.

The comparative sequence analyses undertaken in this study revealed the presence of some unique nucleotide substitutions in the ArMV, OLYaV and SLRSV sequences from olives. The ArMV-Gr/Kr isolate showed two unique nucleotide substitutions, whereas the similarities varied from 74 to 94%. In the case of OLYaV, the sequence was only obtained from one of the RT-PCR amplicons from 'Mastoidis'. Molecular characterization of the Greek OLYaV-Gr/Ms isolate revealed the presence of a unique adenine substitution (position 66) and high molecular diversity with previously reported isolates, as indicated for European and American isolates (Essakhi *et al.,* 2006; Al Rwahnih *et al.,* 2011). The variability levels of the Greek isolate in comparison with isolates from California ranged from 3 to 25%, whereas the nucleotide similarities varied from 75 to 80% compared with Italian isolates. Within the sequence dataset used, there were also three isolates from Greek olive cultivars ('Tragolea', 'Conservolia', 'Gaidourelia') maintained in the germplasm repository of California sharing 75 to 93% nucleotide similarity with the Greek isolate reported here. This HSP70h sequence variability was significantly greater compared with other Closteroviridae viruses, suggesting the need of further studies to elucidate taxonomic relationships within the family.

Sequence analysis of the 22 SRLSV isolates revealed six diverse isolates from the olive cultivars 'Throuboulia', 'Kalamon', 'Amfissis' and 'Chalkidikis' (these virus isolates are designated, respectively, as SLRSV-Gr/Tr, SLRSV-Gr/Km1, SLRSV-Gr/Km2, SLRSV-Gr/Af and SLRSV-Gr/Ck), whereas all the others SRLSV isolates were identical with the sequence of SLRSV-Gr/Tr. A sequences alignment comparison with those available in the database (from mint, cherry, strawberry, black locust, Japanese rose and peach) showed different variability levels up to 24%. The most variable sequences

to the olive isolates were those from peach and mint. There was 100% similarity between the SLRSV from black locust (*Robinia pseudoacacia*) in Poland and the SLRSV-Gr/Ck isolate, demonstrating the significant role of possible reservoir hosts in the virus dissemination. Six unique nucleotide substitutions were present only in olive isolates, with the exception of the isolate from black locust. Between the Greek isolates, the divergency ranged from 1 to 17%, with the SLRSV-Gr/Af and SLRSV-Gr/Km1 isolates being the most variable showing, respectively, 26 and 14 unique nucleotide substitutions. The high sequence divergency values of olive isolates are in accordance with the results of Dullemans *et al.* (2019). In previous studies the primer pair used for the detection of SLRSV was reported to target part of the CP gene (Faggioli *et al.,* 2002) instead of the 3′UTR, probably due to the limited knowledge of the virus genome organization.

The CLRV Greek isolates (CLRV-Gr/Tr and CLRV/Gr/Gd) were 100% similar, and multiple nucleotide alignments with the available CRLV sequences from pome and stone fruits, woody and herbaceous hosts, revealed that the Greek isolates had two unique nucleotide substitutions. The olive isolates, except the nucleotide variations, exhibited a motif of eight nucleotide deletions (positions 127 to 134), which was also present in CLRV isolates from birch and walnut, but not from *Chenopodium quinoa* and stone fruit hosts. The variability at nucleotide levels ranged from 2 to 13%, depending on the host, with the lowest values detected with isolates from birch and walnut.

All of the sequences determined in this study were deposited in the EMBL-EBI database under the accession numbers LR593885, MK936232 to MK936236 and MN706529 to MN706532.

Phylogenetic analyses were also carried out to investigate evolutionary relationships among the virus isolates. In the case of ArMV and CLRV, there were no available sequences; consequently, these analyses only confirmed the clustering of olive isolates with those from different hosts. All the viruses grouped within the virus-specific tree with ArMV-Gr/Kr isolate being more closely related to those sequenced from narcissus and lily, whereas the most distantly-related isolate was from butterbur (Figure 1A). The CLRV olive isolates were grouped in a separate clade of a sub-cluster with closely related isolates from walnut and birch (Figure 1B). For SLRSV, all the six diverse olive isolates grouped together but in a separate cluster from the other isolates of different host origins, with the exception of an isolate from black locust which was grouped in a sub-cluster together with SLRSV-Gr/Tr and SLRSV-Gr/Ck isolates (Figure 1C). The olive SLRSV-
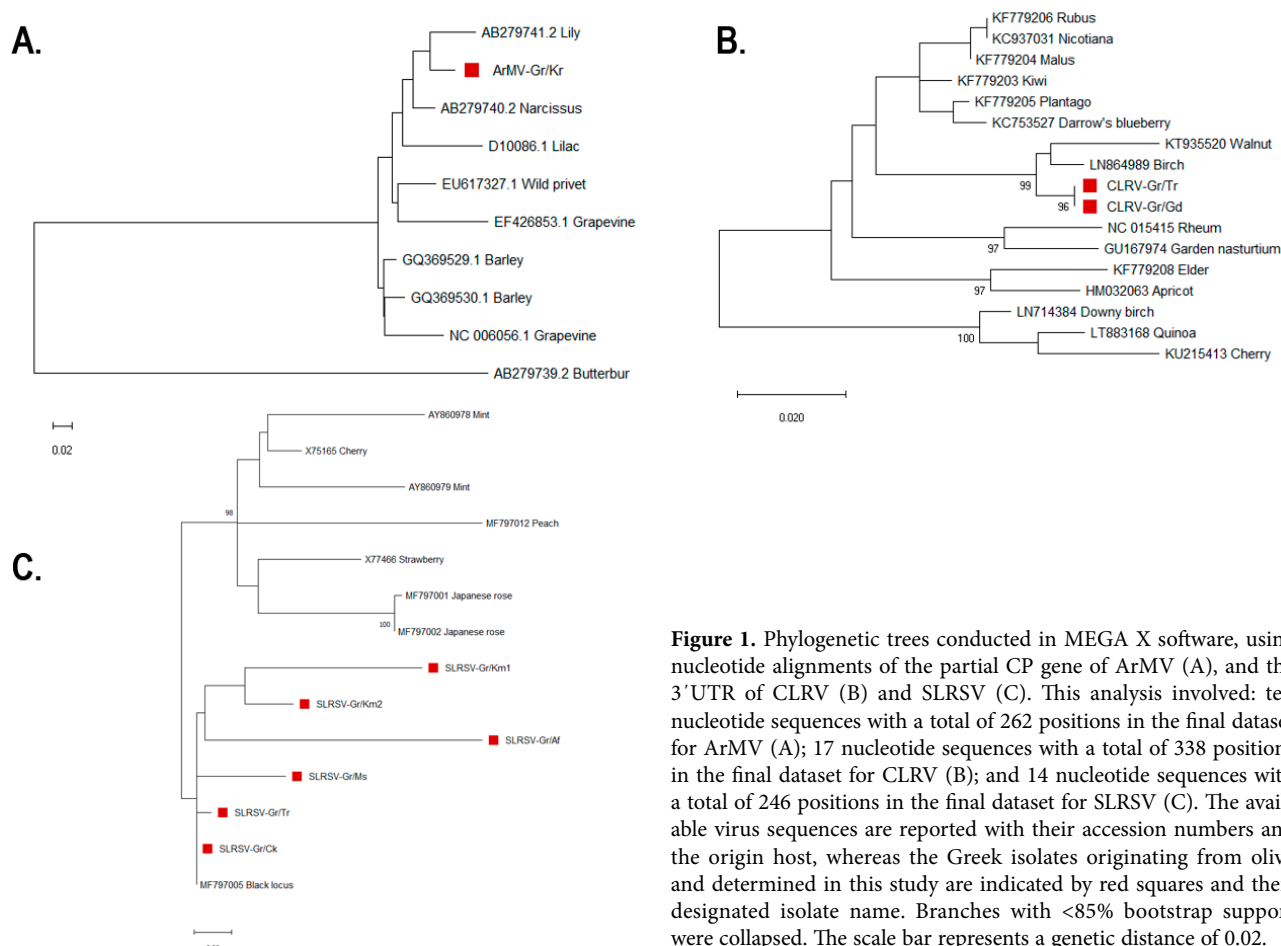
Figure 1. Phylogenetic trees conducted in MEGA X software, using nucleotide alignments of the partial CP gene of ArMV (A), and the 3′UTR of CLRV (B) and SLRSV (C). This analysis involved: ten nucleotide sequences with a total of 262 positions in the final dataset for ArMV (A); 17 nucleotide sequences with a total of 338 positions in the final dataset for CLRV (B); and 14 nucleotide sequences with a total of 246 positions in the final dataset for SLRSV (C). The available virus sequences are reported with their accession numbers and the origin host, whereas the Greek isolates originating from olive and determined in this study are indicated by red squares and their designated isolate name. Branches with <85% bootstrap support were collapsed. The scale bar represents a genetic distance of 0.02.

Gr/Af isolate, which was the most diverse, was alone in a separate clade, and the diverse peach isolate was grouped alone in a separate clade. These recently identified variable isolates from black locust, Japanese pear and peach were used for a new classification of the unassigned SLRSV by Dullemans *et al.* (2019). According to that study, a new taxonomic position is proposed for SLRSV as a member of the new genus *Stalarivirus*. Based on phylogenetic analyses of the CP full-genome, SLRSV-A, SLRSV-B and the *Lychnis mottle virus* are suggested as three distinct species of *Stalarivirus* (Dullemans *et al.*, 2019). In the present study, although the sequences used for inferring evolutionary relationships were from the 3′UTR of SLRSV, it is clear that the olive virus isolates are grouped with the SLRSV-A isolates, with an exception of the diverse peach isolate which is suggested as an isolate of SLRSV-B (Figure 1C).

Since the host range of OLYaV is limited to olive, the available homologous sequences in the database were from that host. In total, 56 OLYaV isolates from Italy and the United States of America were used to further characterize the evolutionary relationships of the Greek isolate with other OLYaV isolates. The results clearly showed two main different clusters with the first divided into two sub-clusters, A and B. The isolates clustering in groups A and C separated according to geographical origin of the germplasm collection. The Greek isolate clustered with the Californian isolates (Figure 2). The smaller sub-cluster group B contained two separate clades by sequences from the same geographical origin of the germplasm collection, with insertion of two isolates of different origin in each group. Previous phylogenetic studies on OLYaV comparable sequences were conducted using either isolates coming from Italian (Bari) or North American (California) olive tree collections. These studies demonstrated the existence of either three cluster groups of isolates from the Italian collection (Essakhi *et al.*, 2006) or six groups from isolates obtained from the Californian collection (Al Rwahnih *et al.*, 2011). Although each of these collections contained olive cultivars originating from different countries, the study of Essakhi et al. (2006) suggested a clustering of
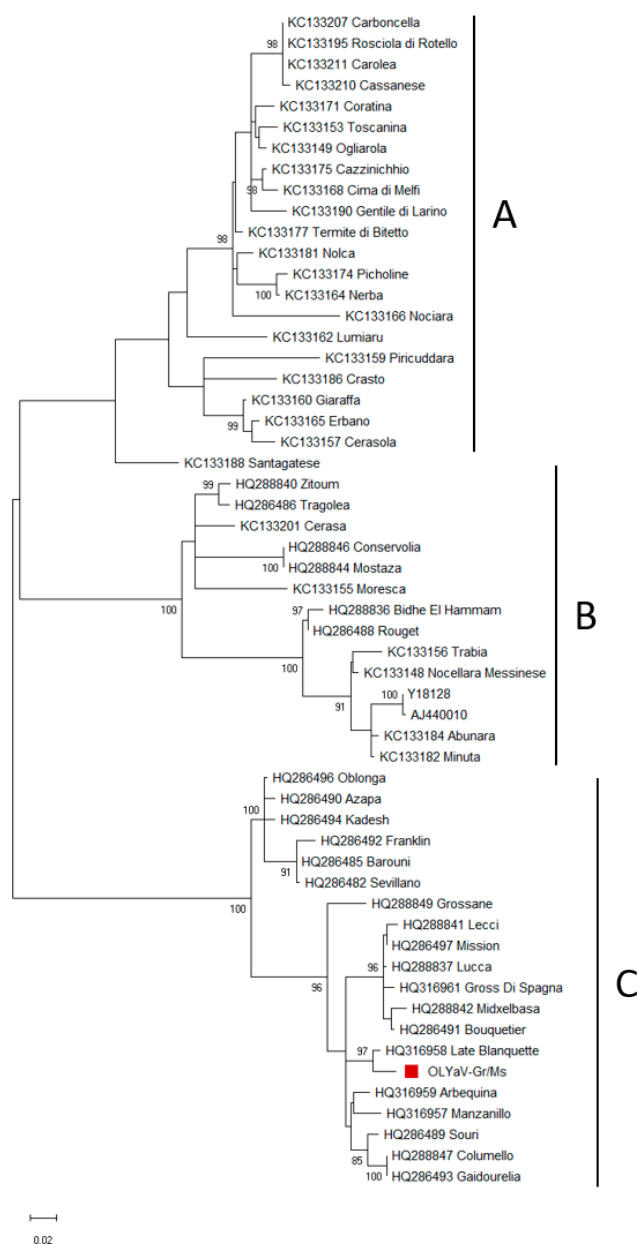
Figure 2. Evolutionary analyses conducted by MEGA X using nucleotide sequences of the OLYaV HSP70h gene. The analysis involved 56 nucleotide sequences. There was a total of 353 positions in the final dataset. The available OLYaV sequences originating from Italian and American germplasm collections (cultivars from different countries) are reported with their accession numbers and cultivar name, whereas the Greek isolate determined in this study is indicated by a red square and its designated name. Branches with <85% bootstrap support were collapsed. The scale bar represents a genetic distance of 0.02.

none of the clusters showed country of origin homogeneity (Al Rwahnih *et al.,* 2011). These studies cannot give clear interpretations of whether the relationships between isolates are geographically-dependent, as OLYaV isolates were not initially detected in their country of origin. Although the origin of the trees was from different countries, they were all maintained as part of an olive-tree germplasm collection, so the possibility of virus infection and dissemination after plantation establishment cannot be excluded for some viruses. In the present study we combined most of these previously reported sequences, and the results showed clear demarcation of the isolates based on the collections (Italian vs. American) into two clusters. Clusters A and C consisted of isolates from the Italian or North American collections, whereas the smaller sub-cluster B was mixed, but again the isolates from the different collections were grouped together in individual clades (Figure 2). The Greek OLYaV/Gr-Ms isolate was more closely related in terms of evolution with isolates from the North American collection, indicating a prominent role of exchange of infected host material in virus dissemination.

Although the present study should be subsequently upscaled to an extensive, country-wide sampling, to elucidate the virus infection status of olive trees in Greece, it has provided fundamental knowledge on the application of a rapid and economic RNA isolation protocol to facilitate virus detection techniques. The study has also made the first record of some olive viruses at unexpectedly high incidence proportions, in one of the leading olive producing countries. Olive virus sequences from olive hosts are also reported for first time. These results illustrate the urgent need to implement certification schemes for the production of virus-tested olive propagation material and prevention of virus dissemination. Phytosanitary controls are also of great importance for local cultivars in order to maintain their PDO recognition. A novel result from this study was the identification of sixteen virus-tested olive accessions from nine out of ten cultivars tested. Further research in large-scale surveys in this olive germplasm, in the National Germplasm Repository of Greece and in commercial olive groves will determine virus infection status for more Greek cultivars, which could represent potential candidates of a pre-basic mother plantation. This could supply increasing international demand for certified olive propagation material.

isolates based on the country of origin (except for the third group, which was geographically heterogeneous). This is in conflict with the second study, in which

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