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Short Notes

Detection and phylogeny of viruses in native Albanian olive varieties

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Summary. Forty samples representing 14 native Albanian and two foreign olive varieties were collected from an olive varietal collection plot in the Valias region (Tirana, Albania). The samples were assayed by RT-PCR for presence of olive-infecting viruses, including arabis mosaic virus (ArMV), cherry leaf roll virus (CLRV), cucumber mosaic virus (CMV), olive latent ringspot virus (OLRSV), olive latent virus 1 (OLV-1), olive leaf yellowing-associated virus (OLYaV), strawberry latent ringspot virus (SLRSV) and by PCR for the bacterium *Xylella fastidiosa* (Xf). Ninety-eight percent of the samples were infected with at least one virus. OLYaV was the most prevalent (85% of samples), followed by OLV-1 (50%), OLRSV (48%), CMV (28%), SLRSV (3%) and CLRV (5%), whereas ArMV and Xf were absent. Fifty-five percent of the samples were infected with one virus, 13% with two viruses, 20% with three, and 5% with four. Analyses of the nucleotide sequences of the Albanian virus isolates generally showed low genetic variability, and that most were phylogenetically related to Mediterranean isolates, in particular to those from Greece and Italy. Five olive trees, representing three native cultivars ('Managiel', 'Kalinjot' and 'Kushan-Preze') and one foreign ('Leccino'), were found to be plants of the *Conformitas Agraria Communitatis* ("*CAC*") category i.e. free of ArMV, CLRV, SLRSV and OLYaV. Only one tree of the native cultivar 'Ulliri i kuq' was free of all tested viruses, so this is plant material of the "Virus-tested" category. Olives derived from both categories could be used for propagation of standard quality plant materiel in a future certification programme for olive in Albania. This is the first report of CLRV, OLRSV, CMV and OLV-1 in Albania. The study also reveals the precarious health status of native olive varieties in the Valias varietal collection plot. However, the discovery of six plants representing two certifiable categories is a first step in a future olive tree certification program in the country.

Keywords. RT-PCR, sequence and phylogenetic analyses, certification programme.

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INTRODUCTION

Olive (*Olea europaea*) is one of the oldest and most important fruit tree crops in Albania (Belaj *et al*., 2003). Currently, there are 54,000 ha of olive groves containing approx. 10 million trees in Albania, of which thousands are secular (Ismaili, 2009). The olive growing area in this country extends

from the northern border of Shkoder to Konispol in the south, penetrating the mainland towards the east, through river valleys, creating a continuum of olive groves (Velo and Topi, 2017). Until 2009, the olive groves were distributed as follows: 10% on flat land, 83% on hillsides and 7% in steep areas (MAFCP, 2009); however, this situation has changed in favour of flat land and hilly areas making olive cultivation a dominant feature of the landscapes (Kolaj *et al*., 2017).

Most olive production in Albania is concentrated in the coastal and mountainous areas (Fier, Berat, Elbasan and Vlora regions) with Mediterranean climatic conditions. In 2018, almost 100,000 tons of olives were produced, an increase of 3.2% compared to previous years, and the olive oil industry produced 6,000 tons of product (Lazemetaj, 2018).

The genetic resources of Albanian olive groves are varied, with many ancient native varieties (approx. 50 varieties), but only six ('Kalinjot', 'Krypsi Beratit', 'Bardhi Tiranë', 'Krypsi Elbasanit', 'Mixan' and 'Himara') make up 85% of the national production (MoA, 2017). Olive tree propagation is mainly by grafted or self-rooted cuttings, which undergo a *Conformitas Agraria Communitatis* ("*CAC*") certification. This is based on visual assessment of the health status of the olive tree and is managed by the "National Seed and Plant Institute" of the Ministry of Agriculture (MoA). To preserve Albanian olive germplasm, the MoA with the collaboration of the Agricultural University of Tirana has established a varietal collection plot in the Valias region of Albania. This plot contains the most important native varieties (Genetic Bank of the University of Tirana, Albania). Initially, these "mother" plants of different varieties were selected based on visual inspections, regardless of their phytosanitary status.

Although this approach can be valid for excluding some diseases, it is not appropriate for virus infections which can be masked in plants in latent forms and\or at low concentrations, thus leading to the selection of false virus-free olive trees. As with other crops, propagation of olive by vegetative means favours virus spread. Olive is affected by 15 viruses (Martelli, 2013), some of which are agents of identified diseases, while most cause latent infections, the effects of which have yet to be determined.

Three surveys of virus diseases (Saponari *et al*., 2002; Çakalli *et al*., 2006; Luigi *et al*., 2009), carried out on, respectively, 38, 37 and 50 trees, have summarized knowledge on olive viruses present in Albania. Only strawberry latent ringspot virus (SLRSV) and olive leaf yellowing-associated virus (OLYaV) were identified. Following the growing interest of farmers in the use of high-quality nursery plants, identification of healthy plants that can be used as a sources of propagation material for growers has become a necessity to improve the national olive industry. Albania has therefore started a certification programme for the multiplication of olive plant material. As part of this programme, a survey was conducted in the varietal collection plot of "mother" plants (in the Valias region) to assess the virus phytosanitary status of native varieties that could be used in the future. Furthermore, the recent outbreak of the olive quick decline syndrome, caused by *Xylella fastidiosa* (Xf) in Italy (Saponari *et al*., 2013), and its identification in other European countries (France, Spain, Germany) made it necessary to extend this investigation to survey for this bacterium, for which the results of both studies are reported in the present paper.

MATERIALS AND METHODS

Field survey and source of plant material

In March 2018, a survey was conducted in a plot for the varietal collection of olive (Valias, Tirana, Albania) which was established by the Albanian Ministry of Agriculture to include the most important olive varieties in the country. The olive germplasm in this plot has been periodically subjected to phenotypic and pomological evaluations in order to identify trees that could be used as propagation material in the Albanian certification programme.

Forty olive trees with no apparent symptoms were each sampled. Leaves and cuttings (approx. 25-30 cm long) aged between 1 and 2 years, were taken from each tree canopy. In total, 16 cultivars were sampled, of which 14 were native and two were foreign (Table 1).

Three clones of each cultivar were sampled, and the samples were labelled, stored in plastic bags at 4°C and brought to the laboratory for analysis. Cuttings from olive seedlings kept in screenhouses at the Mediterranean Agronomic Institute of Bari, which were healthy as indicated by RT-PCR assays were used as negative control samples, and different plants infected (also indicated by RT-PCR) with arabis mosaic virus (ArMV), cherry leaf roll virus (CLRV), cucumber mosaic virus (CMV), olive latent ringspot virus (OLRSV), olive latent virus 1 (OLV-1), OLYaV and SLRSV, were used as positive control samples.

Extraction of total nucleic acids

For virus detection, total nucleic acids (TNAs) were extracted from 0.2 g of phloem tissues of each sample

Table 1. Olive samples collected from the varietal collection plot of Valias region (Tirana, Albania), assayed by RT-PCR for the presence of seven olive-infecting viruses. * indicates *CAC* category plants; ** indicates *Virus-tested* category plant.

Cv. N°	Cultivar	Sample N°.	ArMV	SLRSV	CLRV	OLYaV	OLRSV	CMV	OLV-1
1	Boç	1	-	-	-	+	+	-	-
		2	-	-	-	+	+	-	-
		3	-	-	-	+	+	-	-
2	Managjel	1*	-	-	-	-	+	-	+
		2	-	-	-	+	+	-	+
3	Frangu	1	-	-	-	+	-	-	+
		2	-	-	-	+	-	-	+
		3	-	-	-	+	-	-	+
4	Kushan	1	-	-	-	+	-	-	+
		2	-	-	-	+	+	-	+
		3	-	-	-	+	-	-	+
5	I bardhi i Tiranes	1	-	-	-	+	+	-	+
		2	-	-	-	+	+	-	+
		3	-	+	-	+	+	-	+
6	Kalinjot	1*	-	-	-	-	+	+	-
		2*	-	-	-	-	-	+	-
		3	-	-	-	+	-	+	-
7	Krypsi i Krujes	1	-	-	-	+	+	+	-
		2	-	-	+	+	+	+	-
		3	-	-	-	+	+	-	-
8	Kokermadhi i Beratit	1	-	-	+	+	-	+	-
		2	-	-	-	+	-	+	+
		3	-	-	-	+	-	+	+
9	I holli i Himares	1	-	-	-	+	+	-	-
		2	-	-	-	+	+	-	-
		3	-	-	-	+	+	-	-
10	Mixan	1	-	-	-	+	-	+	-
		2	-	-	-	+	-	-	+
		3	-	-	-	+	-	+	-
11	Lecino Valias	1	-	-	-	+	+	-	-
		2	-	-	-	+	-	-	-
		3*	-	-	-	-	-	-	+
12	Frantoio-Valias	1	-	-	-	+	+	-	-
		2	-	-	-	+	+	+	+
13	Kushan-Preze	1*	-	-	-	-	-	-	+
		2	-	-	-	+	-	-	+
14	I bardhi i Tiranes-Preze	1	-	-	-	+	-	-	-
		2	-	-	-	+	-	-	+
15	Mixan-Preze	1	-	-	-	+	-	-	+
16	Ulliri i kuq-Preze	1**	-	-	-	-	-	-	-
	Number of infected trees		**0**	**1**	**2**	**34**	**19**	**11**	**20**
	% infected trees		**0**	**2.5**	**5**	**85**	**47.5**	**27.5**	**50**

(cortical scrapings). Each sample was homogenized in 1 mL of grinding buffer (0.4 M guanidine thiocyanate, 0.2 M NaOAc (pH 5.2), 25 mM EDTA, 1.0 M KOAc (pH 5.0), and 2.5% w/v PVP-40). Nucleic acids were then purified with silica particles (Foissac *et al*., 2001).

For Xf detection, DNA was extracted following the CTAB protocol (2% hexadecyl trimethyl-ammonium bromide, 0.1 M Tris-HCl (pH 8), 20 mM EDTA, and 1.4 M NaCl) (Hendson *et al*., 2001). For each sample, 0.3 g of fresh leaf midrib and petiole was homogenized with 2 mL of CTAB buffer, using an automated hammer. Extracted sap was incubated at 65°C and then chloroform treated. TNA was precipitated with 0.6 volume of cold 2-isopropanol and resuspended in 120 µL of sterile water for PCR assays.

Reverse transcription (RT) and polymerase chain reaction (PCR)

Samples were assayed by RT-PCR for the presence of seven olive viruses, including ArMV, CLRV, CMV, OLRSV, OLV-1, OLYaV and SLRSV. Virus RNAs were reverse transcribed using 200 U of *Moloney Murine Leukaemia virus* reverse transcriptase enzyme (Invitrogen Corporation), 4 µL of 5XFS M-MLV buffer, 2 µL of DTT (0.1 M), and 0.5 µL of dNTPs (10 mM). The mixture was incubated at 39°C for 1 h and then at 70°C for 10 min. PCR was performed using 2.5 µL of cDNA, together with 2.5 µL of 10× Taq polymerase buffer (Promega Corporation), 1 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 0.5 µL of 10 µM of sense and antisense primers (Table 2) and 1 unit of Taq DNA polymerase (5U μL^{-1}) in a final volume of 25 µL. Amplifications were carried out in a thermocycler (Biometra) after a preliminary denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 35 s, annealing at 55°C for 35 s (58°C for OLYaV) and 72°C for 35 s, and a final extension step at 72°C for 7 min. Amplified products were electrophoresed in 5% TBE polyacrylamide gel (PAGE) and visualized by silver nitrate staining.

DNA was extracted from olive samples following the CTAB protocol and assayed by PCR using primers RST31/33, which are widely used in the detection of different Xf subspecies (Minsavage *et al.,* 1994). Reactions consisted of a 1× amplification buffer in a final volume of 25 µL, containing 2.5 µL of TNA, 1 µL of dNTPs (10 mM), 0.5 µL of each sense and antisense primers (10 µM), and 1.25 U of Taq DNA polymerase. PCR cycles consisted of 94°C for 1 min followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final step of 72°C for 5 min. PCR reactions were electrophoresed in 1.2% TAE agarose gels.

Cloning, sequencing and bioinformatic analyses

Based on the occurrence of each virus in the tested samples, amplified RT-PCR products yielded from different infected olive trees were transformed in StrataClone^{TM} PCR Cloning vector pSC-A (Stratagene), subcloned into *Escherichia coli* DH5α cells, and three DNA clones from each sample were custom sequenced bidirectionally (Eurofins Genomics). Nucleotide sequences were analyzed with the DNA Strider 1.1 program (Marck, 1988). Multiple alignments of nucleotide sequences were performed using the default options of CLUSTALX 1.8 (Thompson *et al*., 1997). The BLASTn program was used to search for nucleotide homology in GenBank (Altschul *et al*., 1990). Tentative phylogenetic trees were constructed with the MEGA 6 version software, using the Neighbor-joining method, with 1000 bootstrap replicates (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

Detection of olive viruses and Xylella fastidiosa

RT-PCR detection of olive viruses showed that almost all the native Albanian olive cultivars tested were infected with a least with one virus (97.5% of infection). Only one tree was virus-free. OLYaV was the most prevalent virus (in 85% of samples), followed by OLV-1 in 50% of samples (Table 1). The high incidence of OLYaV in the sampled Albanian olive trees was similar to that reported in other countries, including Lebanon (24%), Syria (15%), Tunisia (49%), Italy (42%), and the United States of America (93%) (Saponari *et al*., 2002; Albanese *et al*., 2003; Al Abdullah *et al*., 2005; Fadel *et al*., 2005; Faggioli *et al*., 2005; Al Rwahnih *et al*., 2011; El Air *et al*., 2011). The transmission of this virus occurs through the exchange of infected plant propagation material; however, it is strongly suspected that the olive psyllid (*Euphyllura olivinae*), which has been repeatedly found to host OLYaV, is the vector of this virus in nature, although this has not been demonstrated with experimental transmission tests (Sabanadzovic *et al*., 1999). For this reason, it is difficult to establish which path OLYaV has taken in the varietal collection plot to reach such high incidence.

OLV-1 is a polyphagous soilborne virus capable of infecting crops without being transmitted by vectors. These two characteristics make this virus very transmissible. OLV-1 has also been shown to be present in blossoms (Lobão *et al*., 2002), pollen (Saponari *et al*., 2002), and fruit pulp (Félix, unpublished) of infected trees, as well as in high proportions (> 80%) of seedlings originating from infected olive trees (Saponari *et al*., 2002)

Table 2. List of primers used in RT-PCR assays for detecting the olive-infecting viruses. CP, Coat protein; RdRP, RNA-dependent RNA Polymerase; HSP70-like protein, Heat shock protein 70 kDa-like protein.

Virus	Genus	Amplified region	Primer sequence (5' to 3')	Amplicon length (bp)	Reference
ArMV	Nepovirus	CP gene	TTGGTTAGTGAATGGAACGG TCAACTCACCCTCCAAATCCC	504	Grieco *et al*., 2000
CLRV	Nepovirus	CP gene	TTGGCGACCGTGTAACGGCA GTCGGAAAGATTACGTAAAAGG	416	Faggioli *et al*., 2005
OLRSV	Nepovirus	3'terminal	TTGCAAAACTAGTGCCAGAGG TGCATAAGGCTCACAGGAG	480	Grieco *et al*., 2000
CMV	Cucumovirus	RdRP gene	TAACCTCCCAGTTCTCACCGT CCATCACCTTAGCTTCCATGT	513	Grieco *et al*., 2000
OLV-1	Alphanecrovirus	3'terminal	ACACAGAAATCATAAGTGCC CCATAGCACCATCATACC	299	Faggioli *et al*., 2005
OLYaV	Closterovirus	HSP70 gene	CGAAGAGAGCGGCTGAAGGCTC GGGACGGTTACGGTCGAGAGG	383	Sabanadzovic *et al*., 1999
SLRSV	Sadwavirus	CP gene	CCCTTGGTTACTTTTACCTCCTCATTGTCC AGGCTCAAGAAAACACAC	293	Faggioli *et al*., 2005

indication transmission through seeds. By means of ovule fertilization with infected pollen and\or by grafting of cultivar plants that are 'recalcitrant' to rooting onto seedlings originated from infected seeds, all these approaches make again OLV-1 a very infectious virus. Factors favouring the high OLV-1 incidence in the olive trees of Valias field merits future investigation.

The OLRSV incidence particularly high (48%) compared with olive orchards in other Mediterranean countries, i.e., Tunisia (17%), Lebanon (14%), and Syria 12% (El Air *et al*., 2011; Al Abdullah *et al*., 2005; Fadel *et al*., 2005). No vector for OLRSV is known; however, as a nepovirus, its natural transmission could occur through nematodes. These pests have not been reported from the Valias collection plot, suggesting that the transmission of OLRSV may have occurred through infected self-rooted olive tree cuttings.

Among the viruses tested, CMV was the third most commonly found (28% of the samples). This incidence is similar to reports from other countries, i.e., Syria (23%) and Tunisia (26%) (Al Abdullah *et al*., 2005; El Air *et al*., 2011). Although CMV is transmitted through many different aphid vectors (*Myzus* spp.), the presence in Albanian olive cultivars may not be attributed to these, because their association with olive has not been demonstrated.

SLRSV and CLRV were found at low incidence, i.e., 1% for SLRV and 3% for CLRV, and ArMV was not detected. The rare presence\absence of these three polyphagous neopoviruses in Albanian olives is satisfactory.

Excluding the OLYaV infections, whose investigation on olive plants is regulated on the basis of a simple visual inspection for the presence of leaf yellowing symptoms (EU Directives n°. 2016/97 for "*CAC*" [*Conformitas Agraria Communitatis*] category), and not on laboratory tests as conducted in the present study, the phytosanitary status of the 14 native Albanian cultivars tested here can be considered as acceptable.

A high proportion (93%) of the tested olive plants material was free of ArMV, CLRV, SLRSV, and symptoms of yellowing of the leaves. It is therefore legitimate to classify the plants as "*CAC*" category. The presence of OLYaV found in the samples based on laboratory tests is worrying, because the latent period of this virus in the infected plant material is unpredictable. For phytosanitary safety, therefore, this material should be excluded from the "*CAC*" category in the future Albanian certification programme. Consequently, five trees of three 3 native varieties and one foreign cultivar ('Leccino') were eligible for the "*CAC*" category. Only one plant of the native cultivar 'Ulliri i kuq' was found free of all the viruses tested, so this plant can be designated in the "*Virus-tested*" plant material category. For the remaining olive cultivars, for which no virus-free plants were detected, sanitation measures (thermotherapy and\or *in vitro* shoot tip culture), or sanitary selections for a greater number of olive plants, are recommended for the future certification programme in Albania. It is notable that attempts to correlate the presence of any single virus or group of viruses with specific host symptoms in Valias plot were unsuccessful. This agrees with other reports on the symptomatology of these viruses (Martelli, 2013).

PCR results showed that Xf was not present in any of the tested samples, confirming previous reports of absence of this pathogen in Albania (Cara *et al*., 2016).

Sequence variability

All of the virus sequences obtained in this study were deposited in the GenBank under different accession numbers (acc.n°), and are shown in the phylogenetic trees (Figure 1). The sequence of SLRSV found only in one Albanian olive tree of cultivar 'I Bardhi i Tiranes' (isolate *I Bardhi3*) had 80.5% to 93.5% similarity with those in GenBank; whereas two isolates *Gr\Ts* and *Gr\Ms* (acc.n°, respectively, MK936233 and 706532), were recently identified in olive trees in Greece (Manthoudiakis *et al*., 2020), and these were the most related isolates (93.5% similarity). The SLRSV isolates present in the Genbank database showed 22% of variability, in the coat protein homologue region investigated here.

The sequence analyses of CLRV isolates found in two Albanian olive cultivars, i.e., 'Krypsi' and 'Kokermadhi', showed, respectively, 86% and 87% to 98% similarity with homologue isolates in the database. Both SLRSV isolates (*Krypsi 2* and *Kokermadhi 4*) shared 98.2% similarity with the *Adilcevaz* isolate from Turkey (acc.n° FJ785323), and *Gr\Gd* (acc.n° MK936236) and *Gr\Tr* (acc. n° MK936235) from Greece, whereas the sequence similarity between the two SLRSV isolates was 96%.

The incidence of OLV-1 was greater than that of SLRSV and CLRV; thus, 11 isolates, i.e., one from each OLV1-infected cultivar (Table 1), were chosen for sequencing. The comparison of their sequences showed three different representative sequence-types of all 11 OLV-1 isolates that were present as single infections in cultivars 'Kokermadhi', 'Kushan' and 'Mixan', and in mixed virus infections. Albanian OLV-1 isolates shared 88% to 98.3% similarity with those of the GenBank. Whereas isolate *Kushan3* had similarity of 98.3% with isolate *V10* from Portugal (acc.n° KF804063), isolate *Kokermadhi3* had 96% similarity with *P33* (acc.n° MN586597, from Tunisia), and *Mixan2* had 97% similarity with *G1A* (acc.n° KF804056, from Portugal).The Albanian OLV-1 isolates showed 93.3% and 98% similarity.

In the case of OLYaV, one isolate from each of the 15 OLYaV-infected cultivars was sequenced. After the sequence analysis, seven different sequence-types were

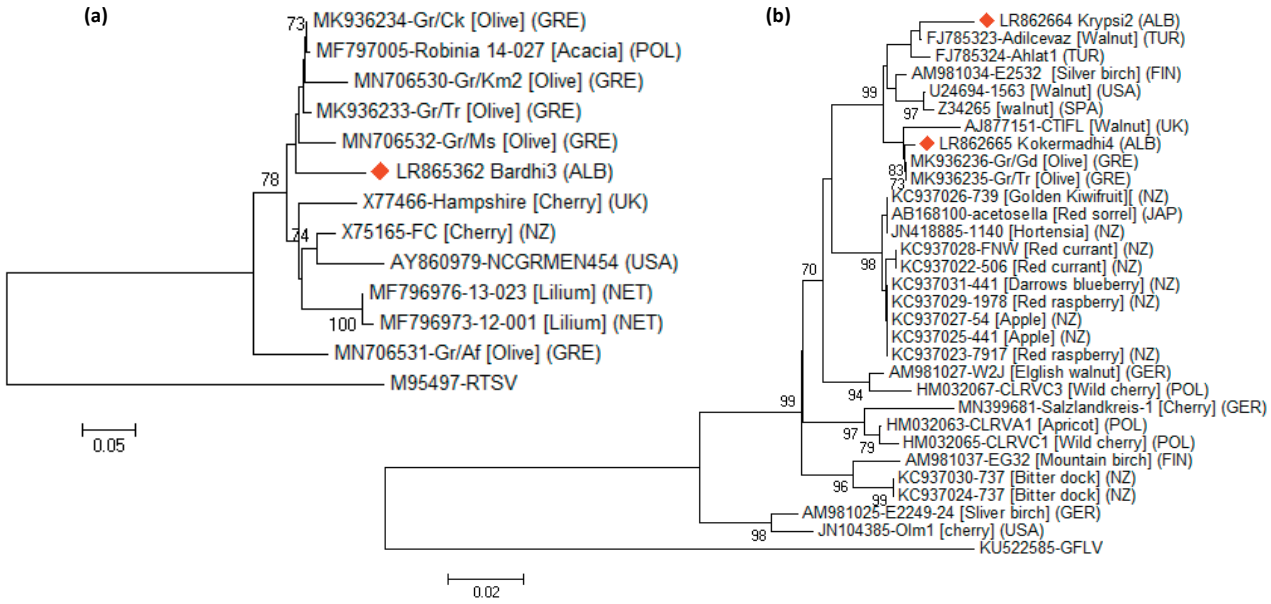

Figure 1. Phylogenetic trees based on nucleotide sequences of different partial genes\domains of SLRSV (a), CLRV (b), OLV-1 (c), CMV (d) and OLYaV (e). Alignments were obtained using Clustal X 1.8, and analyzed by the Neighbor-Joining method with 1000 bootstrap replicates. The percentage of replicate trees (when >70%) in which the virus isolates clustered together is shown next to each branch. GenBank accession number, name, isolation sources and countries of origin of each corresponding virus isolate used in the analysis are reported in the phylogenetic tree. *Rice tungro spherical virus* (RTSV) of the genus *Waikavirus*, *Grapevine fanleaf virus* (GFLV) of the genus *Nepovirus*, *Olive mild mosaic virus* (OMMV) of the genus *Alphanecrovirus*, *Fig mosaic virus* (FMV) of the genus *Emaravirus* and *Daucus carota* HSP70 gene, were used as outgroup species. The abbreviations used for countries of origin of the isolates are: Albania (ALB); Australia (AUS); Chile (CHI); Croatia (CROA); Egypt (EGY); Finland (FIN); France (FRA); Germany (GER); Greece (GRE); India (IND); Israel (ISR); Italy (ITA); Japan (JAP); Malaysia (MAL); Netherlands (NET); New Zealand (NZ); Palestine (PAL); Poland (POL), Portugal (PORT); South Korea (S-KOR); Spain (SPA); Tunisia (TUN); Turkey (TK); Uganda (UGA); United Kingdom (UK); and United States of America (USA). Albanian virus isolates are indicated in red.

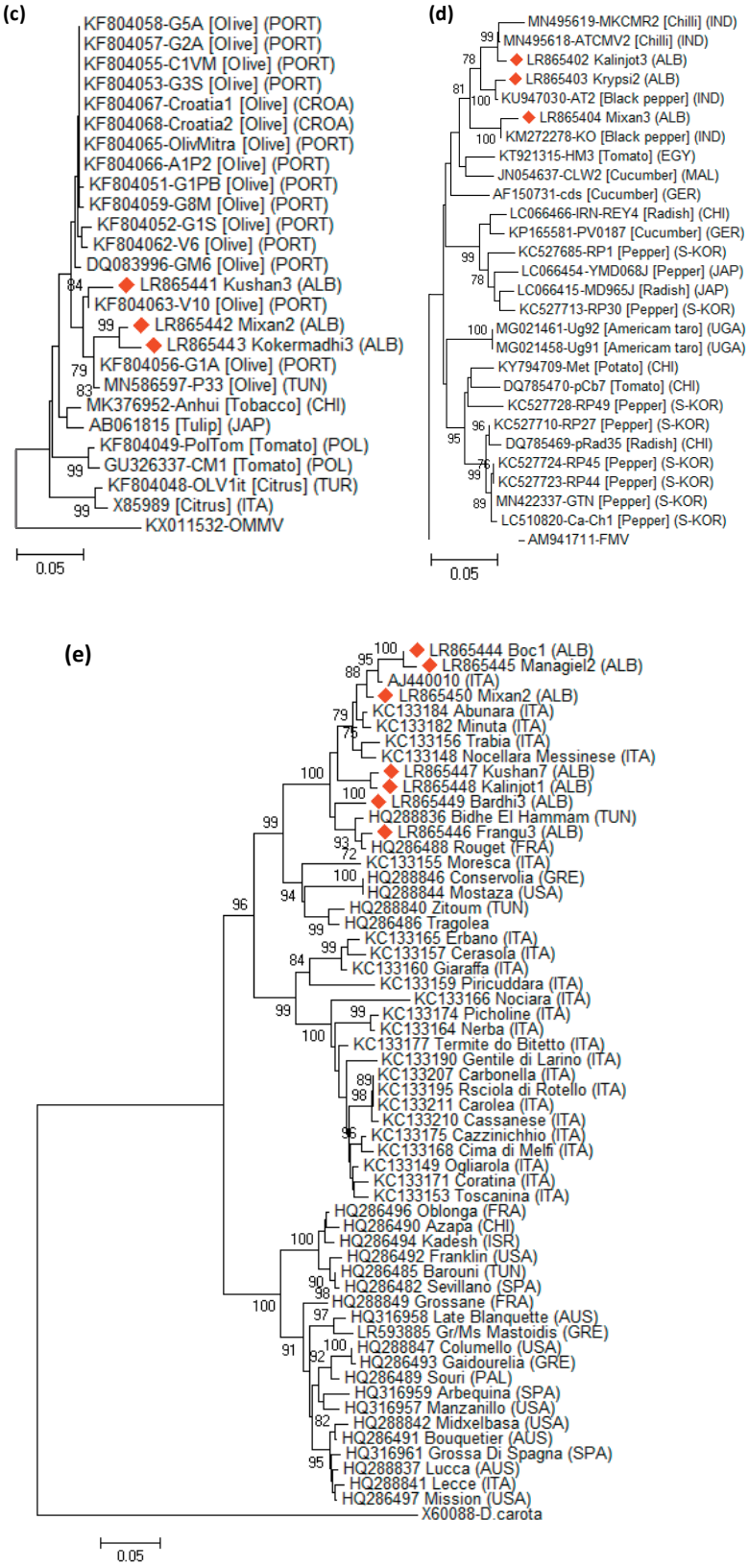

Figure 1. Continued.

identified, sharing 88.2% to 98.6% similarity. BLASTn sequence analysis showed that isolate *Mangiel2* was the most variable among the Albanian isolates and with those of the GenBank, with sequence similarity of 69.9%. Opposite to this, greatest similarity was found with *Frangu3* isolate that showed 98.9% similarity with GenBank isolates. In general, the similarity margin, comparing the sequences of the seven Albanian isolates with those in Genbank, ranged from 90.3% (*Bardhi3*) to 98.9% (*Frangu3*). In particular, isolates *Boç1* and *Mangiel2* had the greatest similarity (respectively, 97.1% and 96%) with the Italian isolate *AJ* (acc.n° AJ440010). *Mixan2* shared 97.9% similarity with isolate *Abunara* from Tunisia (acc.n° KC133184). Of the three OLYaV isolates, *Kushan7* had 93.7% similarity with isolate *Rouget* (acc.n° HQ286488, from France), *Kalinjot1* had 92.4% similarity with *Bidhel Hammam* (acc.n° HQ288836, from Tunisia), and *Frangu3* had 98.9% similarity with *Nocellara messinese* (acc.n° KC133148, from Italy).

Nine Albanian olive cultivars were shown to be infected with OLRSV, so their RT-PCR amplicons were sequenced. BLASTn analyses of obtained sequences (acc.n° LR865438, isolate *Kalinjot3*; LR865403, isolate *Krypsi2*; and LR865404, *Mixan3*) showed that these isolates shared 99.1% to 99.3% similarity with the unique OLRSV isolate in the GenBank (acc.n° NC_038863); whereas the intraspecies variability ranged from 0.9% to 1.5%.

Five Albanian cultivars were infected with CMV (Table 1), so their RT-PCR amplicons were sequenced. Nucleotides sequence comparison for these isolates showed three different sequence types, found as single infections in the olive cultivars 'Kalinjot', 'Krypsi' and 'Mixan'. The sequence from 'Kalinjot' shared 89.8% to 99.4% similarity with the homologue from the GenBank, that from 'Krypsi' was 89.0% to 99.2% similar, and the sequence from 'Mixan' was 87.8% to 98.8%. The Albanian isolates were 93.3% to 96.1% similar to each other. The CMV sequences of the other two infected cultivars ('Kokermadhi' and 'Frantoio Valias') were identical to that of the isolate from 'Kalinjot'.

Phylogenetic analyses

The phylogenetic analyses conducted in this study aimed to determine relationships between viruses found in the Albanian olive cultivars and their homologues of international origins. In the case of SLRSV, the analysis was conducted on the few sequences of isolates from olive reported in the GenBank, together with those from other crops. In the phylogenetic tree, the Albanian isolate of SLRSV (*Bardhi3*) clustered in one clade together with Greek isolates from olive (*Gr\Tr*, *Gr\Ms* and *Gr\km2*) (Figure 1a). A similar clustering was found for CLRV isolates from Albania, i.e., *Kokermadhi4,* that grouped together with olive isolates from Greece (*Gr\Gd*, *Gr\Tr*). In contrast, *Krypsi2* clustered closely to *Adilcevaz* and *Ahlat1* isolates, both reported from walnuts in Turkey (Figure 1b).

The OLV-1 phylogenetic tree had four differentiated clusters based on infected hosts rather than geographic origins. The largest cluster was composed of isolates from olive from Portugal, for which the Albanian isolates *Kokermadhi2*, *Kushan3* and *Mixan2* were part of in a distinct clade (Figure 1c).

Analogous to SLRSV, the lack of CMV sequences from olive in GenBank conditioned the phylogenetic analysis that was based on isolates from different crops rather than on those from olive. The phylogenetic tree for CMV isolates did not show any distribution, based on host species and\or geographic origin. However, the Albanian isolates from olive formed one clade with those from chili and black pepper from India (Figure 1d). It is likely that this indistinct distribution of CMV isolates in the phylogenetic tree was conditioned by the polyphagous nature of this virus, exposing it to sequence recombination that has generated a quasi-species which is difficult to be differentiated by phylogenetic analysis.

The presence of a single for OLRSV sequence in GenBank has limited the design of a phylogenetic scenario for this virus. The present analysis was therefore limited, only reporting the genetic variability found among the three isolates that were sequenced.

The phylogenetic tree of OLYaV showed two clusters, one which was diversified with isolates from different origins, i.e., the United States of America, Australia, Chile, France, Greece, Italy, Israel, Palestine, Spain, and Tunisia, while the second was composed of Mediterranean isolates, i.e., from France, Greece, Italy, and Tunisia. The exception was isolate from the United States of America, which has a Spanish denomination (cultivar 'Morteza'). The Albanian isolates all grouped together in a clade close to Italian isolates.

CONCLUSIONS

This study was carried out to identify a "*Virus-free*", or at least "*Virus-tested*", plant material that could be part of primary resources for a future certification programme for olive in Albania. This research is not unique, because two decades ago, two similar investigations were conducted in this country to identify possible virus infections in native olive trees, using double-

stranded RNA analyses (Çakalli *et al*., 2006; Saponari *et al*., 2002). Both investigations reported a virus incidence of 22 to 24%; but did not specify the identity of the viruses in the Albanian olive cultivars. Eight years later, a third investigation was undertaken on 50 olive trees, and this reported the presence of only two viruses i.e., SLRSV and OLYaV in a few olive cultivars (Luigi *et al*., 2009). Excluding these three investigations, little is known of the occurrence and distribution of other viruses that may be present in Albanian olive cultivars.

The present study reports for the first time the presence of four viruses (CLRV, OLRSV, OLV-1 and CMV) in the most important Albanian olive cultivars. Although the number of samples tested was limited and does not allow a comprehensive definition of the incidence of these viruses as only the Valias collection plot was assayed, discovery of these viruses in most of the 14 cultivars is of great concern. This is particularly because these native cultivars are pomologically and phenotypically important and are likely to be part of the future olive certification programme in Albania.

This study identified five olive trees of three native cultivars ('Managiel', 'Kalinjot' and 'Kushan-Preze') and one foreign cultivar ('Leccino') that were found free of ArMV, SLRSV, CLRV and OLYaV, and one olive tree of cultivar 'Uliri i Kuq-Preze' that was free of all the tested viruses. Based on EU directives (EU nº. 2016/97 for the category "*CAC*" [*Conformitas Agraria Communitatis*]), the plant material of these Albanian cultivars, found in the phytosanitary categories "*CAC*" and "*Virus-tested*", are valuable candidate clones that can be used in the propagation of high quality material in the future olive certification programme. At the genome level, all the viruses found in the Albanian cultivars presented low genetic variability, compared to that reported from other Mediterranean countries (Mathioudakis *et al*., 2020; Al Rwahnih *et al*., 2011; El Air *et al*., 2011; Essakhi *et al*., 2006; Al Abdullah *et al*., 2005). The limited exchange of olive plant material in the Balkan area, in particular of Albanian native olives, has preserved the phytosanitary status of this material from the introduction of international viruses isolates. Further laboratory analyses should be carried out on more olive clones, and on cultivars not tested in this study, to identify healthy candidates that could be part of a future olive certification programme in Albania.

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