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A NEWLY REPORTED CITRUS VIRUS DISEASE IN CYPRUS: CITRUS TATTER LEAF VIRUS (CTLV)

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ABSTRACT

This study has been performed on the detection and determination of *Citrus Tatter Leaf Virus* (CTLV), which has not been studied at the molecular level in Cyprus. Therefore, this study focused on the molecular characterization of CTLV in citrus plants in Cyprus. This paper reports the sequencing and analysis of a CTLV isolate obtained in Cyprus and examines its relationship to other CTLV isolates from different parts of the world. Surveys were conducted in various regions of Cyprus between 2013 and 2016. As a result, 84 different citrus orchards, which showed CTLV symptoms, like chlorosis, leaf wrinkled, asymmetrical leaves, yellow lesions and mosaic shaped spotswere selected for sampling. ReverseTranscriptase – Polymerase Chain Reactions were performed in the thermal cycler using one pair of CTLV specific primer (TL1F: 5′-TGAAAACCTTTGCTGCCACCTCT-3′ and TL1R: 5′-TACTCTCCGAACCTGCCTCGAAA-3′), which yielded a 309 nucleotide fragment overlapping the N – terminal part of the coat protein (CP). Polymerase Chain Reaction products were analyzed by electrophoresis in a 2 % agarose at 60 volts, for one hour with TAE buffer. DNA was visualized by staining gel in ethidium bromide (0.5 µg/mL) and photographed under UV light. Six samples were found positive for CTLV. After that, Reverse Transcriptase – Polymerase Chain Reaction products were sequenced and the nucleotide sequencing analysis showed that all CTLV isolates which were obtained from Cyprusare same and they were similar to the three different CTLV isolates from China (CTLV-BJNM-2 and CTLV-QC4) and Australia (CTLV-BDZ-1). The results revealed that, this is the first report of a complete nucleotide sequence of a CTLV isolate for Cyprus and Europe.

***Keywords*:**Citrus, RT - PCR, Molecular analysis, Virus, Cyprus

1. INTRODUCTION

Citrus is among the most important fruits in the world because of their species have rich diversity; extended period of fruit maturity throughout the year and high vitamin C content which is helpful for human health. These are the most important reasons which caused a rapid increase in the demand for citrus fruits in the world markets. Four different citrus species are produced, which have economic importance in international markets. Among these four species, orange is the most produced one in the world and is followed by mandarins, lemons and grapefruits. Citrus is among the most important export crops in Cyprus. However, farmer's income from citrus production is decreasing due to the: increasing prices of fertilizers, herbicides, pesticides, electricity, water and labor, and these are the reasons of the decrease in the production too. The drought, soil, and water salinity problems and some citrus diseases are some other important problems for citrus production in Cyprus. It inspires to decrease fruit quality and yield so cause some problems for exporting.

Citrus tatter leaf virus (CTLV) is a member of *Capillovirus* genus. It includes two different species, *Apple stem grooving virus* (ASGV) and *Cherry virus A* (CVA) definitely (King et al. 2011).

CTLV disease was firstly reported in California in Meyer lemon (*C. limon* X *C. sinensis*) orchards, which was originated when infected Meyer lemon was introduced into the United States from China in 1908 (Wallace and Drake, 1962; Zhang et al., 1988). China is the origin of Meyer lemon, and it has been reported that the Meyer lemon trees are infected by CTLV. At present, CTLV is widespread in China (Song et al. 2009), and it is readily transmitted mechanically (Garnsey, 1964; Roistacher et al., 1980). After that, this virus was reported in Korea and Nigeria (Fajinmi et al. 2011; Song et al., 2016), Japan (Miyakawa and Matsui 1976), South Africa (da Graca, 1977) and Australia (Broadbent et al., 1994; Song et al., 2016).

The citrus producers in Cyprus have been importing young citrus trees from other countries without any care of quarantine regulations. After the

establishment of young citrus orchards, some leaf distortion, necrotic spots, chlorotic and wrinkled leaf symptoms have seen in young lemon orchards in Cyprus in last decade.

This study has been performed with the aim of detection and determination of CTLV, which has not been studied at the molecular level in Cyprus. According to authors' knowledge, no studies have been performed before about *Citrus Tatter Leaf Virus* in Cyprus and on the other hand, the leaf symptoms in citrus orchards were very similar with CTLV. Although there is not any control measures against viruses, it is very important to clearly identify virus type to be able to prepare a management plan. Therefore, this study focused on the molecular characterization of CTLV in citrus plants in Cyprus. This paper reports the sequencing and analysis of CTLV isolates which obtained in Cyprus and examines their relationships to other CTLV isolates from different parts of the world.

2. MATERIAL AND METHODS

2.1. SURVEY OF FIELDS AND SYMPTOMS IDENTIFICATION

The surveys were conducted in various regions of Cyprus in an area of 5.000 da citrus orchard during between 2013 and 2016. The citrus trees which showed diseases symptoms like chlorosis, leaf wrinkled, asymmetrical leaves, yellow lesions and mosaic shaped spots were determined and 84 citrus orchards chosen for analysis.

2.2. COLLECTION OF SAMPLES

In this research, samples of severe symptoms from different citrus orchards were collected. Authors paid attention to collect the samples from leaves and young shoots where ch the symptoms of diseases were shown (Fig. 1). Leaf samples were stored at +4 °C for 7 to 10 days and -20 °C for a longer period until when they were extracted. The samples were determined for each citrus species and varieties, and then they were coded.

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Fig. 1. Leaf distortion, necrosis spots and chlorotic symptoms on citrus leaves which infected by CTLV.

2.3. EXTRACTION OF TOTAL NUCLEIC ACIDS

The total nucleic acid extracts (Astruc et al., 1996) were obtained from leaves and young shoot tissues of citrus plants which the symptoms of citrus tatter leaf virus were seen. Tissues were triturated in extraction buffer (100 mMTris-HCl pH.8.0, 50 mM EDTA b-pH. 7.0, 500 NaCl and % 0,1 10 mM 2. Mercapto-ethanol). After that, they were centrifuged at 4 000 rpm for 3 minutes in 2 ml eppendorf tube. After the centrifugation, 50 µl Sodium Dodecyl Sulfate (%20) was added in Eppendorf tube, and they were mixed in vortex. Then, the extracts were kept at 65 °C for 15 minutes. After then, 250 µL potassium acetate (5M) was added, and tubes were transferred to ice for 20 minutes. The tubes were centrifuged again at 13 000 rpm for 15 minutes at next stage. Then, nucleic acids were precipitated with 500 µL ethanol. Finally, total nucleic acids were diluted with 50 µlRNAse free distilled water.

2.4. REVERSE TRANSCRIPTASE – POLYMERASE CHAIN REACTION

Reverse Transcriptase – Polymerase Chain Reactions (RT – PCR) were performed in thermal cycler using one pair of CTLV specific primer (TL1F: 5′-TGAAAACCTTTGCTGCCACCTCT-3′ and TL1R: 5′-TACTCTCCGAACCTGCCTCGAAA-3′), which yielded a 309 nucleotide fragment overlapping the N – terminal part of the coat protein (CP) (Roy et al., 2005).The cDNA synthesis was done in a PCR tube containing 1 µl total RNA, 1 µl reverse primer and 1 µl forward primer,2.5 µl 10X PCRbuffer solution (100mMTris- HC1 (pH 8.8), 500 mM KC1, % 0.8 Nonidet P40), 1.5 µl MgCl2, 1 µldNTP (2 mM), 0.2 µlTaq DNA polymerase and 16.8µl of RNAse free sterile water. After that, PCR reactions were applied by the first denaturation of 2 minutes at 94 °C followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 59 °C and 2 minutes at 72 °C. Final extension was 10 minutes at 72 °C.

2.5. NUCLEIC ACID SEQUENCING AND SEQUENCE ANALYSIS

PCR products, which were obtained by RT-PCR method and gave positive bands of the desired size in agarose gel electrophoresis step (Fig. 2), were directly sequenced. Nucleic acid sequencing was done for reverse and forward primers. The obtained results were evaluated in sequence program and the degree of relationship with the differences between them and the other isolates which discovered in the other countries.

The number of base substitutions per site from between sequences is shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). The analysis involved 19 nucleotide sequences. All positions containing missing data and gaps were eliminated. There were a total of 339 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2015).

2.6. DNA ELECTROPHORESIS

Ten µl of PCR product was analyzed by electrophoresis in a 2 % agarose at 60 volts, for one hour with TAE buffer. DNA was visualized by staining gel in ethidium bromide (0.5 µg/mL) and photographed under UV light (Sambrook et al., 1989).

3. RESULTS AND DISCUSSION

3.1. REVERSE TRANSCRIPTASE – POLYMERASE CHAIN REACTION AND ELECTROPHORESIS

Electrophoresis analysis of the RT – PCR products of CTLV gene generated from leaf samples from symptomatic citrus plants which were collected from different citrus orchards in Cyprus was shown in Figure 2. Lanes 3, 4, 5, 6, 7 and 8 were determined positive bands at 309 bp.

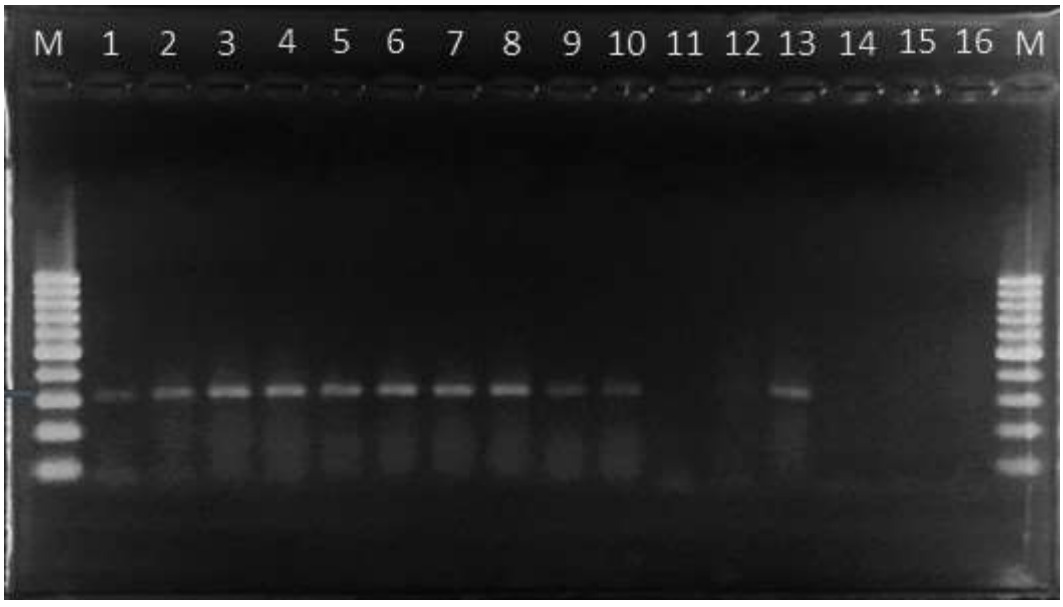


Fig. 2. Agarose gel electrophoresis of RT-PCR products amplified from citrus trees using Citrus tatter leaf virus TL1 primers. Arrow indicates the expected 309 bp PCR product. M: 1 kb plus DNA ladder. Lanes from 3 to 8: Samples positive of citrus in Cyprus.

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3.2. NUCLEIC ACID SEQUENCE AND PHYLOGENETIC ANALYSIS

A fragment of the predicted size, 309 bp, was obtained by RT-PCR with *Citrus Tatter Leaf Virus* specific primers (TL1F and TL1R). Sequence comparisons showed that the six *Citrus Tatter Leaf Virus* isolates (CTLV-KKTC-Bostanci, CTLV-KKTC-Guneskoy, CTLV-KKTC-Aydinkoy, CTLV-KKTC-Lefke, CTLV-KKTC-Guneskoy-Indexin, CTLV-KKTC-Bostanci-Indexin), which were found in Cyprus, have nucleotide sequence identity with CTLV isolates from different parts of the world. The highest level of nucleotide identity (99 %) was detected with two different isolates (CTLV-BJNM-2 and CTLV-QC4) from China and one other isolate (CTLV-BDZ-1) from Australia (Fig 3). According to author's knowledge, this is the first report of a complete nucleotide sequence of a CTLV isolate that has found in Europe.

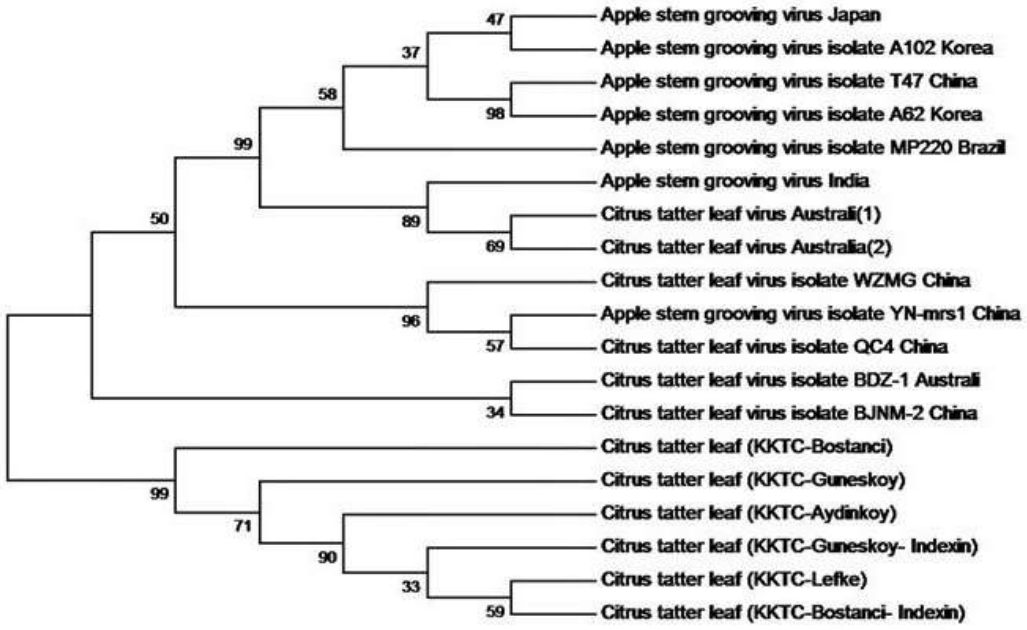


Fig. 3. Neighbor joining phylogenetic tree based on the complete nucleotide sequences of CTLV isolates (CTLV-KKTC-Bostanci, CTLV-KKTC-Guneskoy, CTLV-KKTC-Aydinkoy, CTLV-KKTC-Lefke, CTLV-KKTC-Guneskoy-Indexin, CTLV-KKTC-Bostanci-Indexin). Numbers at the branches indicate bootstrap percentages (1000 replications). The scale bar indicates nucleotide substitutions per site.

4. CONCLUSIONS

RT – PCR results showed that 6 out of 84 tested citrus plants were infected with CTLV, the presence of which has known in four different regions in Cyprus (Bostanci, Guneskoy, Aydinkoy, and Lefke). This study is the first report of indexing for *Citrus Tatter Leaf Virus* in Cyprus and Continental Europe. Reverse Transcriptase – Polymerase Chain Reaction products were sequenced, and the nucleotide sequencing analysis showed that all CTLV isolates which were obtained from Cyprus (CTLV-KKTC-Bostancı, CTLV-KKTC-Guneskoy, CTLV-KKTC-Aydınkoy, CTLV-KKTC-Lefke, CTLV-KKTC-Guneskoy-Indexin, CTLV-KKTC-Bostancı-Indexin) are same and they were similar to three different CTLV isolates from China (CTLV-BJNM-2 and CTLV-QC4) and Australia (CTLV-BDZ-1). China is the origin of Meyer lemon, and it has been reported that the Meyer lemon trees are infected by CTLV. At present, CTLV is widespread in China (Song et al., 2009) and Australia (Broadbent et al., 1994; Song et al., 2016). Citrus producers in Cyprus imported young citrus trees from other countries without any care of quarantine regulations, also some Cypriot people live in Australia, and they visit the Cyprus for holiday. When they visit Cyprus, they bring some plant tissues, like bud unions or scions for grafting. CTLV is transmitted with mechanically rapidly (Garnsey, 1964; Roistacher et al., 1980), graft-transmissible, and is able to infect many citrus species and hybrids (Cowell et al., 2017). It might be concluded that, CTLV has been carried to Cyprus from Australia with bud unions and scions. Moreover, Cypriot farmers import the used (second hand) agricultural machines and equipments from China for some agricultural applications. It might be the reason of CTLV transmission from China to Cyprus. In future, scientists should investigate the effect of this virus on yield and plant development. Furthermore, the period of the virus's maximum concentration in plants should be determined in Cyprus conditions to be able to molecularly analyze the positivity of virus.

Cyprus, being an island offering bio-isolation and quarantine opportunities, deserves to be one of the most suitable places for citrus research.

Scientific research pertaining to the selection, breeding and/or adaptation of citrus varieties, in response to the demands of the prospective consumers, in both variety and supply dating would secure Cyprus as a good place for citrus production. Moreover, this natural isolation provides a good condition for prevention of virus infections, but it needs to be supported by legal quarantine measures.

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