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Original article

Detection and characterization of citrus tatter leaf virus (CTLV) and citrus yellow vein clearing virus (CYVCV) in citrus trees from Cyprus

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ABSTRACT

Citrus tatter leaf virus (CTLV) was firstly reported of in California. After that, it reported in Australia, Korea, Nigeria, Japan, South Africa, and China. The transmission of this virus from plant to plant is very easy with mechanically. Citrus yellow vein clearing virus (CYVCV) was reported on lemon trees in India, Pakistan, Turkey and China. Foliar distortion, necrotic spots, chlorosis and wrinkling symptoms were observed in young lemon orchards in newly established orchards with trees imported from abroad. Therefore, surveys of citrus trees in Cyprus were performed for CTLV and CYVCV from 2013 to 2016. A total of 64 leaf samples from symptomatic citrus trees (41 lemon, 10 orange, 10 mandarin and three grapefruit samples) were collected for total nucleic acid extraction and RT-PCR with CTLV primers to amplify a 309 bp and a 614 bp fragment, respectively, of the 5′ end (100%) and high nucleotide sequence identity (99%) with isolates BJNM-2 and QC4 from China and isolate BDZ-1 from Australia. To our knowledge, this is the first report of CTLV from Europe.
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1. Introduction

Citrus fruits are the most important fruits of the world because of their species have rich diversity; extended period of fruit maturity in the year and high vitamin C content for human nutrition are some important reasons which rapidly increased the demand for these fruits in the world markets. Four different citrus species are produced, which have economic importance about exportation and importation in the world. The first species which produces mostly are oranges and mandarins, lemons and grapefruits follow them. The citrus is most important fruit group for exportation in Cyprus. But; increasing of fertilizers, herbicides, pesticides, electric and water prices and labor costs are the most reasons to decrease the income for citrus production in last years. The drought, soil, and water salinity problems and some citrus diseases are some important problems for citrus production in Cyprus. It inspires to decrease fruit quality and yield so cause some problems for exporting.

The genus of citrus tatter leaf virus (CTLV) is *Capillovirus*. This genus includes two different viruses, 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 (*Citrus limon* × *Citrus sinensis*) orchards 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. CTLV is readily transmitted mechanically (Garnsey, 1964; Roistacher et al., 1980; Cowell et al., 2017). This virus was found in Korea and Nigeria (Fajinmi et al. 2011), Japan (Miyakawa and Matsui 1976), South Africa (da Graca, 1977) and Australia (Broadbent et al., 1994; Song et al. 2009).

Song et al. (2015) noticed CYVCV disease found in India, Pakistan, Turkey, and China on lemon trees. Sequence analysis of the CYVCV-RL isolate which obtained from Yunnan, China was same with CYVCV-Y1 (JX040635) isolate from Turkey.

The citrus producers in Cyprus imported young citrus trees from other countries without any care of quarantine regulations and they import young citrus transplants from Turkey. 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 years.

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This study was carried out on the detection of CTLV and CYVCV which were not studied molecularly in Cyprus. For this reason, molecular detection of CTLV and CYVCV in citrus plants in Cyprus was aimed in this study. In this paper, sequencing and analysis of CTLV isolates which obtained in Cyprus and their relationships with other CTLV isolates from different regions of the world were investigated.

2. Material and methods

2.1. Field survey and symptoms identification

The survey was 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 64 citrus samples (41 lemon samples, 10 orange samples, 10 mandarin samples and 3 grapefruit samples) chosen for analysis (Fig. 1).

2.2. Collection of samples

In this research, samples of severe symptoms from different citrus orchards were collected. We pay attention to collect the samples from leaves and young shoots which the symptoms of diseases were shown (Fig. 2). Samples were stored at +4 °C for 7–10 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 numbered.

2.3. Total nucleic acid extraction

The total nucleic acid extracts were obtained from leaves and young shoot tissues of citrus plants which the disease symptoms of CTLV and CYVCV 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). Then, extracts were centrifuged at 4 000 rpm for 3 min in 2 ml eppendorf. After the centrifugation, 50 µl Sodium Dodecyl Sulfate (20%) was added in eppendorf tube, and they were mixed in vortex. Subsequently, the extracts were kept at 65 °C for 15 min. After then, 250 µl potassium acetate (5 M) was added to tubes and they were transferred to ice for 20 min. The tubes were centrifuged again at 13 000 rpm for 15 min. at next stage. Then, nucleic acids were precipitated with ethanol. Finally, total nucleic acids were diluted with 50 µl RNAse free distilled water (Astruc et al., 1996).

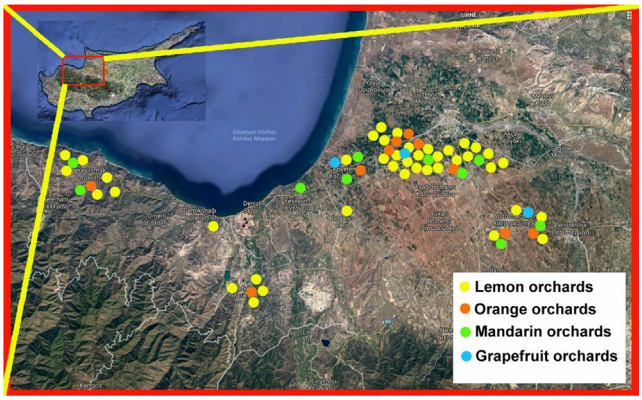


Fig. 1. Map of the sampling orchards.



Fig. 2. Leaf distortion, necrosis spots and chlorotic symptoms on citrus leaves which infected CTLV.

2.4. RT – polymerase chain reaction

RT – Polymerase Chain Reactions were conducted in thermal cycler using one pair of CTLV specific primer (TL1F: 5′-TGAAAACC TTTGCTGCCACCTCT-3′ and TL1R: 5′-TACTCTCCGAACCTGCCTC GAAA-3′) which yielded a 309 bp and one pair of specific CYVCV primer (614-F: 5′-TTTACCGCAGCTATCCATTTCCAC and 614-R: 5′-GCAGAAATCCCKAACCACTAG), which yielded 614 bp nucleotide fragment. cDNA synthesis was completed in a PCR tube containing 1 µl total RNA, 1 µl reverse and 1 µl forward primer, 2.5 µl 10X PCR buffer solution, 1.5 µl $MgCl_2$, 1 µldNTP, 0.2 µlTaq DNA polymerase and 16.8 µl of RNAse free sterile water. After that, PCR reactions were applied by the first denaturation of 2 min at 94 °C followed by 35 cycles of 30 sec at 94 °C, 30 sec at 59 °C and 2 min at 72 °C. Final extension was 10 min at 72 °C.

2.5. DNA Electrophoresis

Electrophoresis method was used to analyze the PCR products in 2% agarose gel at 60 V with TAE buffer. DNA was visualized by staining gel in ethidium bromide and photographed under UV light.

2.6. 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. 3), 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.

Analyses were carried using the Maximum Composite Likelihood model developed by Tamura et al. (2004) and evolutionary analyses were conducted in MEGA7 as suggested by Kumar et al. (2016).

3. Results and discussion

CTLV is a virus disease, which is transmitted mechanically. It is graft-transmissible and also it can be distributed from one plant to other with pruning application (Cowell et al., 2017). Moreover, CTLV is a non-vector transmissible pathogen (Zhou, 2018). Citrus producers have used pruning application and grafting methods in many orchards to change varieties in recent years in Cyprus. CTLV is common in different citrus species and more usual in lemon (Cowell et al., 2017). It causes chlorotic leaves on new grafted trees and new shoots (Lina et al., 2018). A lot of chlorotic leaves were

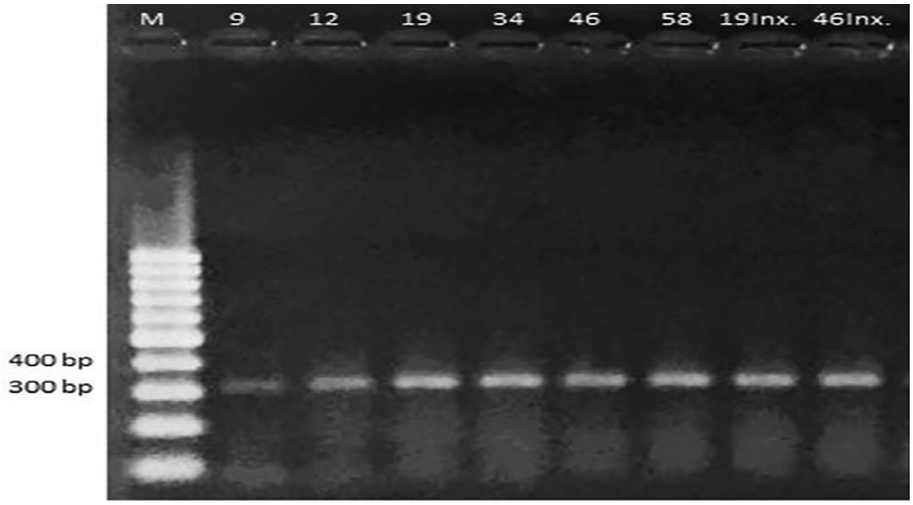


Fig. 3. Agarose gel electrophoresis of RT-PCR products amplified from citrus trees using Citrus tatter leaf virus TL1 primers. Arrow indicates the expected 309 bp PCR product. M: 1 kb plus DNA ladder. Lanes from 4 to 9: Samples positive of citrus in Cyprus.

determined in citrus orchards during the surveys of this research. These orchards were grafted with new variety's scions in Cyprus.

Electrophoresis analysis of the CTLV gene generated from leaf samples from symptomatic citrus plants which were collected from different citrus orchards in Cyprus was shown in Fig. 3. Lanes 4 (sample 19), 5 (sample 34), 6 (sample 46), 7 (sample 58), 8 (sample 19Inx) and 9 (sample 46Inx) were determined positive bands at 309 bp. Additionally, RT-PCR products of CYVCV were determined negative bands at 614 bp.

A fragment of the predicted size, 309 bp, was obtained by RT-PCR with TL1F and TL1R primers. RT – PCR results showed that 6 of 64 tested citrus plants were infected with CTLV, the presence of which has known in four different regions in Cyprus (Bostanci, Guneskoy, Aydinkoy, and Lefke). The six CTLV isolates (Sample 19: CTLV-KKTC-Bostanci, Sample 34: CTLV-KKTC-Guneskoy, Sample 46: CTLV-KKTC-Aydinkoy, Sample 58: CTLV-KKTC-Lefke, Sample 19Inx: CTLV-KKTC-Bostanci-Indexin, Sample 46Inx: CTLV-KKTC-Guneskoy-Indexin), which were found in Cyprus, have nucleotide sequence similarity with CTLV isolates from China and Australia. The highest nucleotide identity level (99%) was determined with two different isolates (CTLV-BJNM-2 and CTLV-QC4) from China and one other isolate (CTLV-BDZ-1) from Australia (Fig. 4). To our knowledge, this study is the first report of indexing for CTLV in Cyprus and Continental Europe.

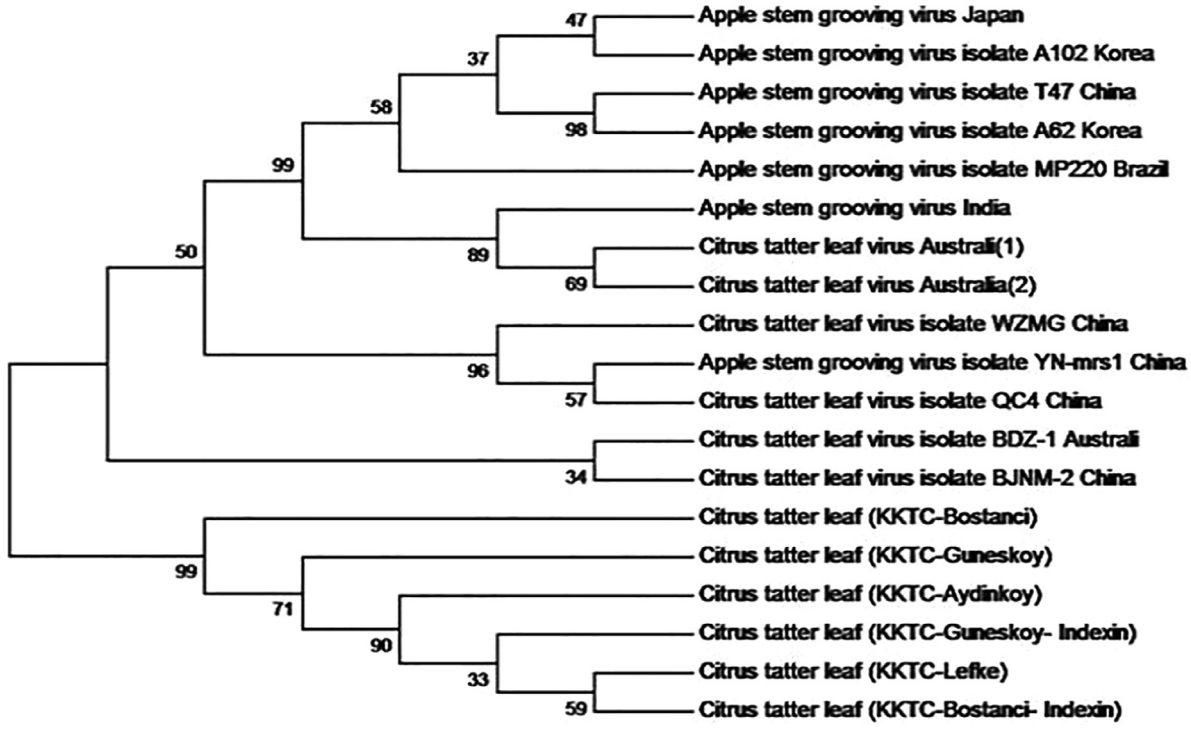


Fig. 4. Neighbor joining phylogenetic tree based on the complete nucleotide sequences of CTLV isolates (CTLV-KKTC-Bostanci, CTLV-KKTC-Guneskoy, CTLV-KKTC-Aydinkoy, CTLV-KKTC-Lefke, CTLV-KKTC-Guneskoy-Indexin, CTLV-KKTC-Bostanci-Indexin). Numbers at the branches indicate bootstrap percentages (1000 replications). The scale bar indicates nucleotide substitutions per site.

4. Conclusions

China is the origin of Meyer lemon, and it has been reported that the Meyer lemon trees are infected by CTLV. At the present time, CTLV is a widespread virus disease in China and Australia. 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. It might be concluded that, CTLV has carried to Cyprus from Australia with bud unions and scions. 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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