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Research Papers

Detection of citrus leaf blotch virus in sweet orange in Croatia, using metagenomics and extraction-free RT-PCR

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Summary. In late 2022, uncharacteristically small fruits of Washington Navel sweet orange (*Citrus sinensis* L. 'Washington Navel') with fruit creasing symptoms were sampled in a small orchard on the coast of Croatia. The virome in the fruits was investigated using metagenomic analysis, which showed presence of citrus dwarfing viroid (CDVd) and citrus leaf blotch virus (CLBV). The record of CLBV is the first for Croatia. Nucleotide sequence analysis of the RdRp region indicated that the Croatian CLBV isolate shared closest sequence similarity to two isolates from kumquat Nagami from Sicily. Full-genome phylogeny of CLBV accessions indicate that isolates from citrus cluster together and are mostly distinct from isolates from non-citrus hosts. The use of Fast Technology for Analysis (FTA) cards was evaluated, as an alternate for CLBV sampling, and as a transport-safe method for obtaining viral RNA templates for RT-PCR. FTA cards in combination with an extraction-free protocol were shown to be usable when columella tissue was used to imprint, but leaf prints were unreliable.

Keywords. CDVd, *Citrus sinensis* 'Washington Navel', CLBV, FTA cards.

INTRODUCTION

Citrus leaf blotch virus (CLBV) was originally described from Corsica, in plants of the kumquat cultivar 'Nagami' (*Fortunella margarita* (Lour.) Swingle. The infected plants were grafted for propagation on Troyer citrange (*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf), a trifoliate hybrid rootstock, and had bud union creasing symptoms (Navarro *et al*., 1984; Galipienso *et al*., 2000, 2001). The virus was also associated with vein clearing and chlorotic blotching of 'Dweet' tangor leaves or stem pitting in Etrog citron, the so called Dweet mottle disease (Galipienso *et al*., 2000). Unlike the initially reported causal relationship of CLBV with Dweet mottle disease (Vives *et al*., 2005; 2008), the link with bud union creasing was not experimentally corroborated (Vives *et al*., 2002a).

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CLBV has been transmitted to experimental herbaceous hosts, mainly *Nicotiana* (Vives *et al*., 2008; Guardo *et al*., 2009; Chavan *et al*., 2013), where systemic infections were usually asymptomatic. The number of recorded natural CLBV host plants has increased as shown by high-throughput sequencing (HTS) studies, and will probably continue to grow. CLBV hosts of interest for European countries are primarily fruit trees, including sweet cherry (Wang *et al*., 2016), mulberry (Xuan et *al*., 2021), kiwifruit (Chavan *et al*., 2013; Zhao *et al*., 2019), apple (Feng *et al*., 2021), the woody ornamentals *Forsythia viridissima* (Huang *et al*., 2024), *Nandina domestica* (Kamitani *et al*., 2021), and *Viburnum lentago* (Kim *et al*., 2023), or *Paeonia lactiflora* (Gress *et al*., 2017). Geographical distribution of the virus includes Mediterranean (Spain, Italy, France, Morocco), North American (Cuba, United States of America), Asian (Turkey, China, Japan, South Korea), and Australasian (Australia, New Zealand) countries. In most EPPO countries, including Croatia, CLBV is categorized as a regulated non-quarantine pest (EPPO, 2024). As such, CLBV infections in planting material should be prevented, especially in citrus. CLBV is transmitted by grafting and, in kumquat, citrange and sour orange, also through seed (Guerri *et al*., 2004), causing international implications in certification and quarantine programmes.

CLBV belongs to *Citrivirus citri*, within subfamily *Trivirinae*, and *Betaflexiviridae* (ICTV, 2024). CLBV has typical *Trivirinae* genomic features (Silva *et al*., 2022), with a single-stranded, positive-sense RNA genome of 8747 nucleotides (nt), excluding the poly-A tail, organized in three open reading frames (ORFs) (Vives *et al*., 2001; Hajeri *et al*., 2010). The ORF1 codes for a large polyprotein (approx. 227 kDa) possessing the molecular motifs methyltransferase, AlkB-like, Otu-like peptidase, papain-like protease, helicase, and RNA-dependent RNA polymerase (RdRp), consistent with its replicase activity (Vives *et al*., 2002a). The downstream ORF2 is expressed through subgenomic RNA translated into a 40 kDa-movement protein (MP) that also serves as a virus silencing suppressor (Vives *et al*., 2002a; Renovell *et al*., 2012). The viral 41 kDa coat protein (CP), encapsidating the CLBV genome in a flexuous particle (960 × 14 nm), is encoded by the ORF3 (Galipienso *et al*., 2001; Vives *et al*., 2001), and is expressed through a second subgenomic RNA (Vives *et al*., 2002c; Renovell *et al*., 2010, 2012).

Routine plant RNA virus molecular detection and characterization is primarily achieved using reverse transcription and polymerase chain reaction (RT-PCR), followed by Sanger sequencing to confirm amplicon identity. However, unbiased high-throughput sequencing (HTS) approaches have become increasingly important for virus discovery (Boonham *et al*., 2014), and citrus viruses are no exception (Bester and Maree, 2024; Chen *et al*., 2025). In either approach, sensitivity, repeatability and reliability of detection is influenced by the quality and quantity of the nucleic acids, which can be degraded by plant or environmental RNases or damaged by chemical or physical factors. Detection can also be hampered by inhibitory plant-derived compounds in nucleic acid extracts. Hence, diagnostic protocols emphasize sample preservation at low temperatures, especially for unstable viruses, and for polyphenol-rich tissues commonly in woody host plants. To streamline workflows, extraction free methods have been explored. Microliter-scale crude sap obtained by pricking plant tissues (Kimura *et al*., 2023), or printing freshly cut tissue pieces to paper or nitrocellulose membranes, enable bonding and preservation of nucleic acids (Chang *et al*., 2011; Olmos *et al*., 1996), and have proven effective. Cellulose based Fast Technology for Analysis (FTA) cards have also been used for extraction-free detection of DNA and RNA viruses (Ndunguru *et al*., 2005; Cardona-Ospina *et al*., 2019).

In late 2022, in a family-owned orchard in Trogir (Croatia), uncharacteristically small fruits of a Washington Navel sweet orange (*Citrus sinensis* L. 'Washington Navel') with severe creasing symptoms were observed and sampled for metagenomic analyses. CLBV and citrus dwarfing viroid (CDVd) were identified, and confirmed with specific RT-PCR assays (Vives *et al*., 2002b; Wang *et al*., 2013a) and Sanger sequencing. In the attempt to conduct a CLBV survey in the orchard with virus detection in a laboratory hundreds of kilometres away, a transport-safe method for obtaining viral RNA templates for RT-PCR was evaluated, together with an extraction-free protocol using FTA cards, which was further optimized in the present study for practical initial CLBV screening from field-grown citrus trees.

MATERIALS AND METHODS

Plant samples

Two fruits of sweet orange (*C. sinensis* L. 'Washington Navel') with severe creasing symptoms had been initially collected from a tree originating from a nursery in Opuzen, and located in a small family orchard in Trogir (43°31'23"N, 16°15'20"E), in the late autumn of 2022. The fruits were refrigerated and individually sampled at the beginning of 2023. Tissues (500 mg) taken from individual fruits (flavedo, albedo, columella) were used for CTAB-based total nucleic acids (TNA) extractions

(Šeruga Musić *et al*., 2003), and were assessed using HTS and RT-PCR.

Creased fruits were collected again from the same tree in September 2023. Twigs (stems with leaves) were also collected from all four quadrants of the symptomatic tree, and from two asymptomatic neighbouring Washington Navel orange trees of the same age and origin. Two samples for CTAB extractions were prepared from each asymptomatic tree: leaves from all four quadrants were used for one type of samples, and fruit columella plus stem phloem scrapings for the second type. For the symptomatic tree, eight samples were made in total in the second growing season. This was done by separating leaf tissues from each quadrant into four samples, and doing the same with the fruit tissue (mostly from columella).

Eight fresh tissue samples equivalent to the ones from the symptomatic tree used for CTAB-extraction in 2023 were prepared for tissue printing on FTA™ Classic Micro cards, also known as QIAcard™ Non-Indicating FTA™ Cards (Qiagen). Two FTA™ cards were printed per quadrant of the symptomatic tree, one from leaf and the other from fruit tissues. Two or three symptomatic leaves with petioles from each quadrant were rolled, cut perpendicularly to the midvein with a sterile blade, and then printed several times on an individual FTA™ card applying pressure by gloved fingers. When the leaf sap trace was no longer visible, the cut was repeated several millimetres above the first one, and the whole procedure was repeated until the surface of the card dedicated to analysis was filled. One creased fruit from each quadrant was selected for printing, and was cut longitudinally, and pieces of columella were taken out and printed on the card, first longitudinally and then as transversal cuttings. Out of eight printed cards from tissues known to contain CLBV, based on positive CTAB-extract RT-PCR results, two were selected for RT-PCR optimization of the extraction-free FTA™ card procedure, one from leaves and the other from columellae.

Total nucleic acid extractions

The extraction protocol described by Šeruga *et al.* (2003) was used for each sample, from 500 mg of refrigerated or frozen plant tissue. The protocol was modified by omitting 2-mercaptoethanol from the 2%-CTAB buffer and adding 2% PVP (10 kDa). Air-dried TNA pellets were each resuspended in 50-100 μL of sterile deionized water, depending on the pellet size. Quantities and quality of nucleic acids was assessed by NanoDrop 2000c (ThermoFisher).

HTS sample preparation and sequencing

An aliquot of CTAB-extract from two creased orange fruits collected at the end of 2022 was subjected to shotgun metagenomic sequencing at Leibniz Institute-DSMZ. After DNase I treatment (Ambion) and cDNA synthesis (Maxima H Minus Reverse Transcriptase, Thermo Scientific) including a ribosomal-RNA depletion treatment (QIAseq FastSelect, Qiagen), the second cDNA strand was synthesized using random octamer primers (NEBNext Ultra II Non-Directional RNA Second Strand Module, New England Biolabs). DNA libraries were prepared with an Illumina DNA Prep kit (Illumina), and were sequenced on a NextSeq2000 instrument (Illumina) as paired-end reads (2× 150 bp).

HTS data analyses

The bioinformatic analysis was carried out following the *in-house* discovery pipeline at DSMZ. Briefly, raw reads were paired, trimmed, aligned to host-related sequences (*Citrus sinensis* chloroplast sequence, GenBank accession number NC_008334; mitochondrion sequence, acc. no. NC_037463; chromosome and transcript sequences, bioproject acc. no. PRJNA225998), and the unmapped reads were then *de novo* assembled using Geneious Prime software v. 2023.1.1 (Dotmatics). The resulting contigs were screened by BLAST (Altschul *et al*., 1990) against a custom virus plus viroid reference database.

Preparation of viral RNA for RT-PCR from FTA cards

Millimetre paper and a sterile blade were used to cut 3 × 3 mm pieces from each printed FTA™ card. The procedure for preparing card pieces for RT-PCR was as described for nitrocellulose membranes (Chang *et al*., 2011), wherein printed pieces were incubated together in one Eppendorf tube (1.5 mL capacity) containing 200 μL of 5% (v/v) Triton X-100 detergent in sterile deionized water. The tubes were then briefly centrifuged at 1000 rpm (Centrifuge 5424, Eppendorf) followed by incubation at room temperature for 5 min. The detergent was then removed by pipetting, and this step repeated twice. Card pieces were then twice washed with 200 μL of low EDTA TE buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0), were air dried on a sterile filter-paper placed in a sterile Petri dish, and were used in 20 μL of RT-PCR reaction mixture for CLBV detection (see below).

The initial FTA™ card detection procedure was attempted 39 d post card printing. Optimization of the extraction protocol commenced 2 months post-printing, when three 3 × 3 mm pieces per card were cut out, processed together twice with 300 μL of detergent, rinsed twice with 300 μL of TE buffer, and then dried and individually tested in different volumes of RT-PCR reaction mix (20, 30, or 40 μL). The optimization of the extraction protocol experiment was repeated 7 months after the card printing.

RT-PCR for CLBV and CDVd detection, amplicon sequencing and phylogenetic analyses

For the verification of CLBV metagenomic identification and optimization of extraction-free protocols, RT-PCR reactions were carried out using the CLBV-specific primers KU-27 and KU-15, allowing amplification of a 456 bp product containing the conserved RdRP domain within ORF1 (Vives *et al*., 2002b). The OneStep RT-PCR Kit (Qiagen) was used, with reaction mix as recommended by the manufacturer but scaled down to 10 μL (0.5 μL of TNA + 9.5 μL of reaction mix, including 0.4 μL of each primer 1 μM working solution). The programs in the ProFlex PCR System cycler (ThermoFisher) consisted of a reverse transcription step (30 min at 50°C), then 15 min at 95°C followed by 40 PCR cycles each with denaturation for 20 sec at 94°C, primer annealing for 20 sec at 50°C and elongation of 30 sec at 72°C, then a final elongation step for 10 min at 72°C.

For CDVd HTS detection verification, specific primers CDVd Rev 92-70 and CDVd For 87–107 (Wang *et al*., 2013a) were used with the same OneStep RT-PCR Kit (Qiagen) and similar reaction conditions, but with 37 PCR cycles and the annealing step at 59°C for 15 sec.

Amplicons were electrophoretically analysed (10V cm^{-1}) at room temperature in 1.5% or 1.8% agarose-TBE gels prepared with StainIN™ GREEN (highQu) fluorescent dye, according to the manufacturer's instructions. Results were visualized under UV-light and documented in Amersham ImageQuant 800 (Cytiva).

Selected amplicons were sent for Sanger sequencing (Macrogen Europe), followed by sequence analysis using GeneiousPrime software v. 2023.1.1 (Dotmatics). An online BLAST search was then carried out to determine closest sequence identity and identify datasets for alignment for CLBV phylogenetic analysis. A heat map of the pairwise nucleotide identity was constructed based on the 414 nt portion of the RdRP CLBV gene, using the Sequence Demarcation Tool v. 1.2. A phylogenetic tree was constructed based on the full genome CLBV isolates using the neighbour-joining method and T3+G substitution model, with bootstrapping of 1000 repetitions (Tamura and Nei 1993; Saitou and Nei, 1987) in Mega11 (Tamura *et al*., 2021). Branches with less than 60% bootstrap support were collapsed.

RESULTS

Sweet orange samples from two vegetative seasons

The fruit creasing symptom observed in the Washington Navel orange from Trogir in 2022 was likewise present in 2023, and fruits on the symptomatic tree were again reduced in size, were lumpy (Figure 1 A), asymmetric in the longitudinal sections, and with unequal rind thickness resulting from flavedo changes spreading into albedo (Figure 1B). At the beginning of 2024, chlorotic leaf symptoms developed on the symptomatic tree, but only in one of its quadrants. Leaves from sample in 2023 (Figure 1A) were used for virus testing along with fruits. Neighbouring orange trees remained symptomless, both on fruits and leaves, during the monitoring period.

CLBV and CDVd RT-PCR identification

Six out of eight CTAB extracts used for detection of CLBV by RT-PCR using primers KU-27 and KU-15 (Vives *et al*., 2002b) yielded amplicons of expected size (456 bp). Six positive samples originated from vegetative (mostly leaves) and generative (fruit) tissues from three of four quadrants of a symptomatic sweet orange tree. No CLBV amplicons were obtained from the fourth quadrant single samples or from two asymptomatic orange trees.

RT-PCR testing for CDVd showed presence of viroid RNA in the fruits and leaves of the sweet orange tree with fruit creasing symptoms. Samples from two neighbouring asymptomatic orange trees tested positive for CDVd in a separate experiment, indicating presence of CDVd in the symptomatic and asymptomatic selected orange trees in the orchard.

Sanger sequencing of CLBV PCR amplicons obtained from creased fruit samples separately extracted in two vegetative seasons, and the subsequent BLAST analyses, confirmed CLBV detection. As the sequences were identical, one 414 nt-long sequence representing a portion of CLBV RdRp was deposited to NCBI GenBank under the accession number OR729894.

Sanger sequencing was carried out for CDVd PCR amplicons from two leaf samples from the orange tree with creased fruits. After trimming the primers, obtained partial (259 nt) sequences were analysed by BLAST, revealing high similarity to other CDVd Gen-

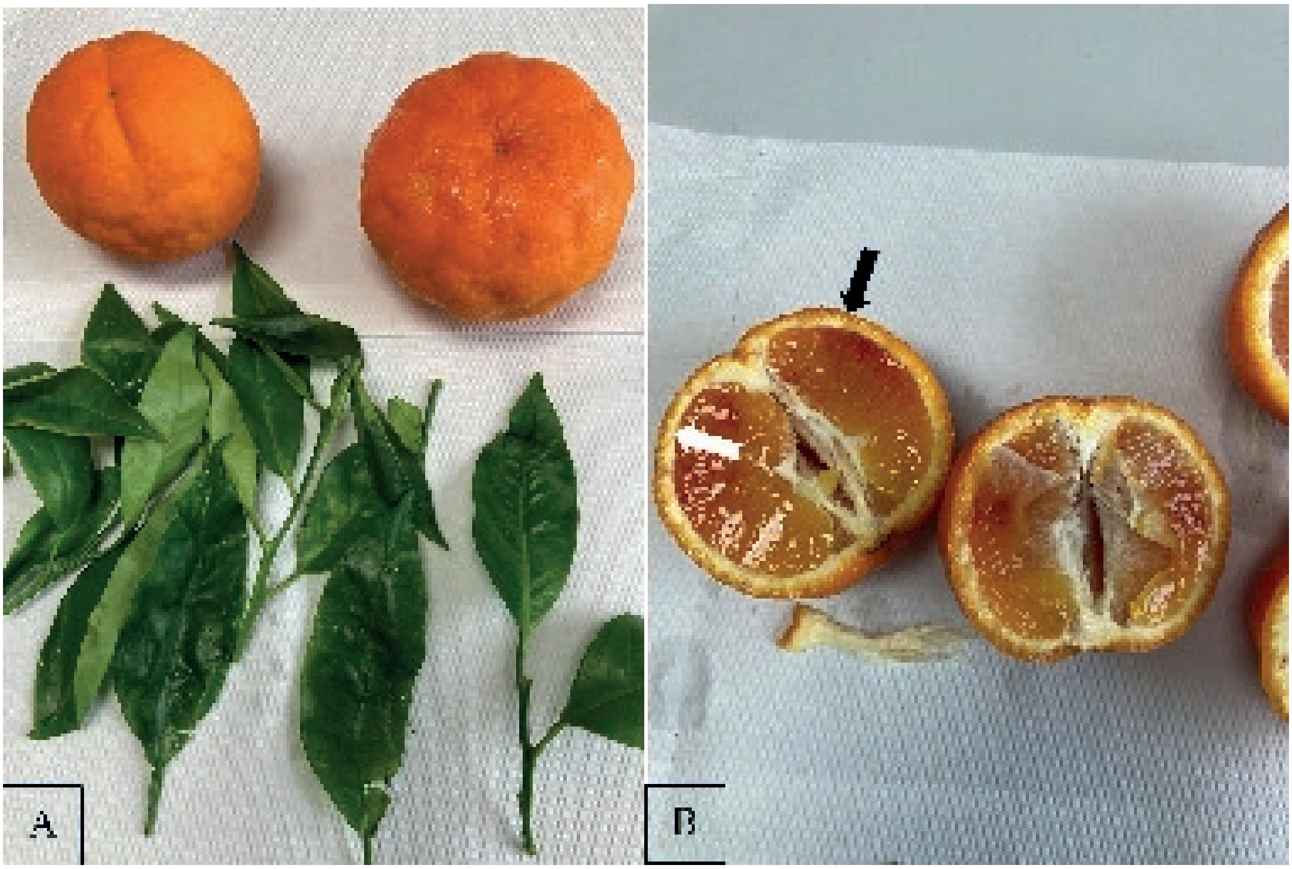

Figure 1. A) Sweet orange (*Citrus sinensis* 'Washington Navel') from Trogir (Croatia) used in this study. Small creased fruits (A, top) and leaves from the same tree (A, bottom). B) Longitudinally cut creased fruits with visible tissue changes including discolouration spreading from the flavedo (black arrow) into albedo (white arrow), and extracted columella prepared for tissue printing (B, beneath the section fruit.).

Bank sequences. The two generated sequences were identical, and a single representative CDVd sequence was deposited in GenBank under the acc. no. PP405019.

HTS results and CLBV phylogeny

From the creased fruits of the Croatian Washington Navel tree, 35,078,352 raw reads were obtained by Illumina sequencing. By performing the above-described pipeline, a complete CLBV genome of 8,737 nt, covered by 218,971 reads, was reconstructed. The sequence was deposited in GenBank under the acc. no. OR838782. This genome has the greatest (96.09%) nucleotide identity to a mandarin (*C. unshiu*) isolate (acc. no. LC758584) from South Korea. Similarly, CDVd 297 nt-long full genomes were reconstructed from 32 and 18 reads representing, respectively, one major (acc. no. OR838783) and one minor (acc. no. OR838784) viroid sequence variant, differing by one nt. There was 100% identity between the HTS sequence from the major viroid variant and the Sanger sequence. No other contigs assigned to citrus viruses or viroids were obtained from the metagenomic analysis.

A pairwise percentage sequence identity analysis (distance matrix) of the RdRp fragment showed that the Croatian CLBV isolate shared closest sequence identity to isolates from Italy (Guardo *et al*. 2007, Guardo *et al*. 2015), both originating from kumquat Nagami grown in the Sicilian localities of Messina (acc. no. EF203229) and Catania (acc. no. FJ449704) (Figure 2). Only these two isolates exceed 98% nucleotide identity in this conserved genome portion, while the nucleotide identity percentages versus other closely related isolates from France, China, South Korea, New Zealand or the United States of America were within the 95-98% range.

A selection of available full CLBV genomes obtained from GenBank was used to compile a phylogenetic tree (Figure 3). The sequences clustered into two primary

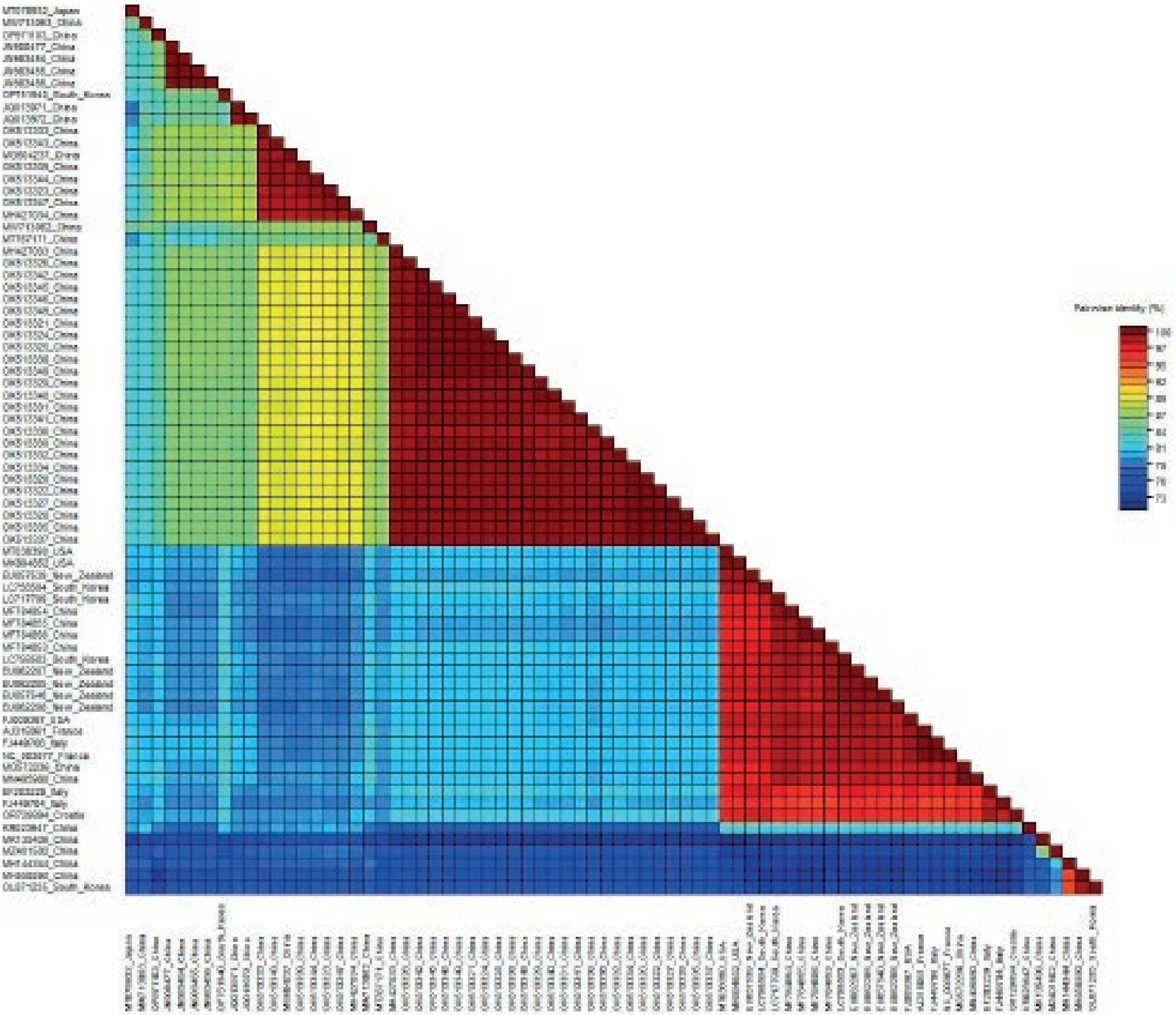

Figure 2. Heat map of the pairwise nucleotide identity matrix calculated for the 414-nt RdRp gene portion of CLBV isolates indicated by their GenBank accession numbers and the countries of origin.

clades. The first cluster was further divided into two sub-clusters (referred in Figure 3 as A and B). Citrus isolates, including CLBV from Croatia, were in the sub-cluster B. This sub-cluster was formed by accessions from Australasia and Asia (New Zealand, South Korea, China), United States of America, and two Mediterranean countries (France and now Croatia), consistent with grouping genomes unrelated with geographical origins. Many different cultivated citrus trees, including sweet oranges grafted on *P. trifoliata* from China and other countries, are included in this cluster. CLBV isolates from kiwifruit and other hosts instead clustered in separate branches.

Extraction-free CLBV detection optimization

After confirming the results of the HTS and proving presence of CLBV in the symptomatic tissue by RT-PCR with the templates obtained by CTAB-extraction, an investigation was conducted on two positive sample prints (Figure 4), from leaves and fruit (columella) of the symptomatic sweet orange tree. This was to verify the success of RT-PCR detection from the FTA™ cards.

The CLBV RdRp amplicon (Vives *et al.*, 2002b) was detected only from columella, as a faint band on the gel by using two 3 × 3 mm printed card pieces per RT-PCR tube (Figure 5, A). This initial positive result was obtained in 20 μL of reaction mix 39 d after tis-

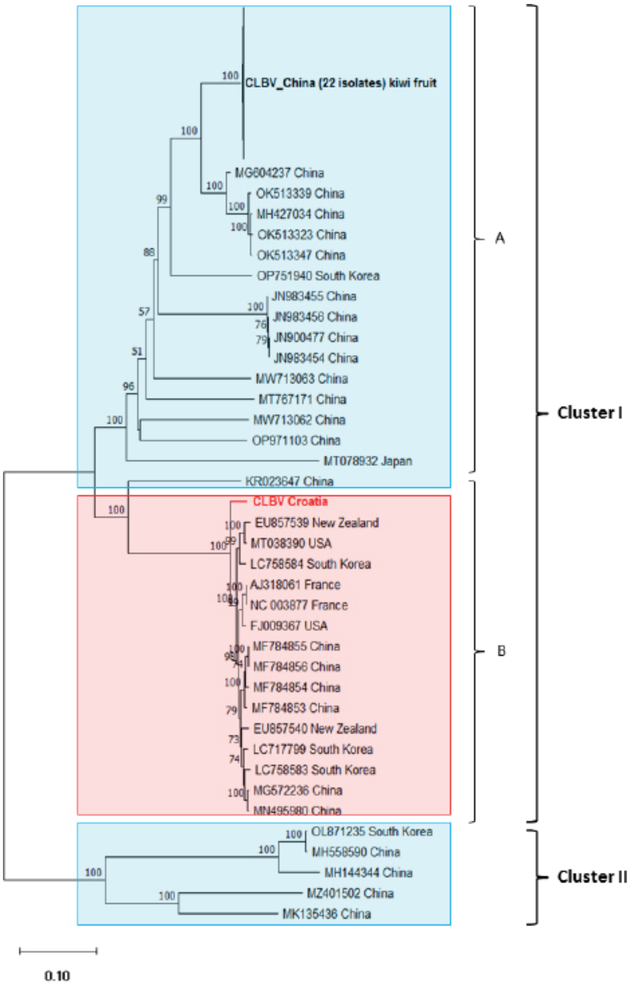

Figure 3. Neighbour-joining phylogenetic tree constructed from complete CLBV genome sequences with 1000 bootstrap repetitions. The isolate from Croatian Washington Navel sweet orange is shown in red font (GenBank acc. no. OR838782). For other virus isolates the countries of origin are shown next to the accession numbers. The red block includes CLBV isolates from *Citrus* hosts, while the blue blocks indicate CLBV isolates from non-*Citrus* hosts. Branches with bootstrap support value less than 60% were collapsed. A and B refer to sub-clusters of cluster I.

Figure 4. FTA^{TM} Classic Micro cards (Qiagen) printed with leaf (left) and fruit (right) tissues of a sweet orange tree infected with CLBV than had fruit creasing and leaf blotching symptoms. Cut-out pieces were used for optimization of the protocol for detection of CLBV by extraction-free RT-PCR. The photo was taken two months post printing.

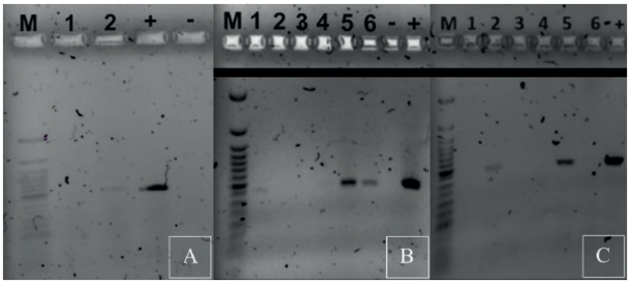

Figure 5. Gel electrophoresis of CLBV amplicons (456 bp) in three different detection attempts from FTATM Classic Micro cards (Qiagen). A) RT-PCR 39 d post card printing (pcp); B) optimized attempt 2 months pcp. C) optimized attempt 7 months pcp. Lanes M, 50 bp marker (New England Biolabs), and + and – indicate the positions of positive and water controls. Lanes in A: 1 = leaf vein and petiole prints, 2 = fruit columella prints. Lanes in B and C: 1 to 3 = leaf vein and petiole (reaction mix volumes, respectively, of 20, 30 or 40 μL), 4 to 6 = fruit columella prints (volumes, respectively, of 20, 30 or 40 μL).

sue printing and storage of the cards at room temperature.

For procedure optimization, three card pieces were simultaneously prepared in one tube with 1.5× increased volumes (300 μL) of detergent and TE buffer. This procedure saved time for the preparation, and reduced the chemicals used in this part of the process. Subsequently, only one card piece was used in the RT-PCR reaction with different volumes (20, 30 or 40 μL) of reaction mixture. This experiment took place 2 months after card printing, and confirmed that columella prints served as better material for virus detection, resulting in clearly visible amplicons in the gels (Figure 5 B). Columella prints of the same size also produced the most intense band in 30 μL volume of the reaction mix, which was clearly visible in 40 μL volume, and as a faint band in 20 μL reaction volume (Figure 5 B). At 7 months after card printing, columella card pieces prepared as described above (in triplicate) and tested singly in different volumes of RT-PCR reaction mixture were still adequate for CLBV detection in the optimum volume (30 µL) of the mixture, and overperformed the leaf prints (Figure 5 C).

DISCUSSION

Citrus trees are grown commercially in Dalmatia, in the Croatian mid and south regions of the East Adriatic coast. These are mostly producing Satsuma mandarins (*C. unshiu*) because of their cold tolerance, especially when grafted on cold hardy trifoliate (*P. trifoliata* syn. *C. trifoliata*) rootstocks. Lemons and sweet oranges are commercially less important, but are widespread as backyard and small family orchard trees. Sweet oranges are the third citrus group in terms of planting material production in Croatia. Between 1999 and 2017, approx. 188,000 orange trees were produced in Croatia, which is less than 13% of the total number of citrus plants produced in that period (Biško *et al.*, 2021). The most common cultivars are Washington Navel, Skaggs Bonanza Navel and Tarocco (Černi *et al.*, 2020). Viruses that could negatively affect the phytosanitary status of the trees and fruit yields are usually tested for, either in the framework of EU legislation (European Union, 2016) or in scientific research (Černi *et al.*, 2020). Targeted analyses, including immunochemical (ELISA) and/or molecular (RT-PCR), were usually performed in those studies. There have been increased records of new citrus viruses with use of unbiased metagenomic analyses (Černi *et al.*, 2022). CLBV has not been amongst these records, although many types of citrus planting material in Croatia have originated from the Mediterranean countries where this virus is known to occur (Afechtal *et al.*, 2021; Navarro *et al.*, 1984; Guardo *et al.*, 2007; Vives *et al.*, 2002c; Wang *et al.*, 2013a).

In the present study, sweet orange Washington Navel fruits from Trogir showing fruit creasing symptoms (Li and Chen, 2017) were analysed using HTS and targeted RT-PCR (Vives *et al.*, 2002a) in the 2022/23 growing season. This analysis gave the first record of CLBV in Croatia. CDVd (acc. no. OR838784) was also detected in this sample, as well as in surrounding asymptomatic orange trees. CDVd has been known to be present in Croatian citrus groves (Škorić, 2000) and was not further analysed in detail in the present study.

In the CLBV positive sweet orange tree, the fruit creasing observed in 2022/23 reappeared in 2023/24, accompanied by leaf blotching. The leaf blotching symptom has been described in CLBV infected trees, but in citrus indicators including pineapple sweet orange and 'Dweet' tangor (Galipienso *et al.*, 2000; Vives *et al.*, 2008). Leaf blotching and fruit creasing were not observed in two Washington Navel orange trees adjacent to, and of the same age as the CLBV positive sweet orange tree, as determined by RT-PCR. It is possible that the leaf blotching observed could have been associated with other biotic and abiotic factors (Roistacher, 1991). Although the link between sweet orange fruit creasing and CLBV could indicate presence of the virus in the symptomatic tree and its absence in the asymptomatic ones, this observation is limited to a few trees. Fruit creasing has not been associated with viral pathogens so far (Huai *et al.*, 2022; Li and Chen, 2017).

The complete genome sequence of CLBV from Croatia was reconstructed (acc. no. OR838782) from HTS data, and detection of the virus was confirmed by complementary methods including RT-PCR (Vives *et al.*, 2002a) and Sanger sequencing of the viral RdRp gene portion amplicon (acc. no. OR729894). Sequence analysis of the RdRp fragment indicated that the viral isolate from Trogir orange was a CLBV variant most closely related to Italian isolates from kumquat 'Nagami' (Guardo *et al.*, 2015) from Sicilian locations in Messina (acc. no. EF203229) and Catania (acc. no. FJ449704), with 98% sequence identity in the RdRp gene portion, and 95-98% identity with other isolates. At whole genome sequence level, the clustering of the CLBV isolate from Croatia with other isolates from cultivated citrus was consistent with low diversity among CLBV isolates (Vives *et al.*, 2002c). As there were disproportionately more sequences from south-east Asia (e.g. China) than other citrus growing regions available in the databases, this may have introduced bias. Nevertheless, the possibility should be assessed that south-east Asia is the centre of CLBV diversity, which is consistent with the geographical origin of many cultivated citrus species and hybrids that serve as hosts of this virus (Wu *et al.*, 2018).

Due to the remoteness of the orange field trees from the diagnostic laboratory, and temperature conditions that can result in degradation of nucleic acids, a further objective of the present study was to find a rapid, simple, sensitive, and practical way to detect CLBV that included RT-PCR (Vives *et al.*, 2002b). This also needed to allow safe transport of virus RNA templates for successive reactions in the laboratory, and storage at room temperature, without the need to transfer tissues in a cooler. In addition to the standard laboratory method for RT-PCR template preparation by CTAB buffer (Šeruga *et al.*, 2003), methods were considered that do not include extractions, such as tissue pricking (Kimura *et al.*, 2023) and FTA^{TM} card printing. Reduction of chemicals and costs, and the environmentally-friendly approach, prompted devising and testing of a procedure that used existing laboratory chemicals rather than requiring new ones (e.g., chemicals from an FTA™ card extraction kit).

Although the method described by Chang *et al.* (2011) was evaluated for printing and detection of poty- and cucumo-viruses from nitrocellulose membranes, its simplicity motivated adaption for FTA™ cards. The optimized preparation of card pieces effectively reduced the volume of chemicals per sample, and enabled rapid sample turnaround. The punchers recommended for cutting out the card pieces in the FTA™ card manufacturer's (Qiagen) protocols were not used, because of the need for equipment cleaning and accurate disinfection steps between the samples. The small cylindrical cutting devices (several mm in diameter) introduce risk of cross-contamination, especially during processing of large numbers of samples and the usual low numbers of punchers available in a laboratory. Instead, use of disposable sterile blades is recommended. Also, card printing should include tissue from all four quadrants of a fruit tree, and from different types of tissue, whenever possible. Uneven distribution of viruses in fruit trees is a factor even in metagenomic detection (Malgioka *et al.*, 2018). Although CLBV was not detected in one of the four quadrants of the positive tree, the inclusion of different tree parts and tissue types enabled detection using all approaches tested in the present study. Fruit tissue, mainly columella, was better for card printing and RT-PCR extraction-free CLBV detection than leaves with petioles, possibly due to more sap present and/or larger columella area available than in leaves. Green fruits were also successfully used (not shown). This is because CLBV is present in host meristem tissues (Agüero *et al.*, 2013) and is not restricted to phloem.

The one-step RT-PCR reaction mixture (Qiagen) optimized to 30 μL per piece of FTA™ card proved reliable for CLBV detection several weeks or months after tissue printing on the card, and this could be performed in the field. Successful CLBV detection 7 months after card printing and storage at room temperature indicates that this procedure is an effective method for storing templates. Unlike practical and fast loop-mediated isothermal CLBV detection (Peng *et al.*, 2021), RT-PCR detection from cards enables downstream analyses (cloning, sequencing) suited for virus characterization and other research. While the sensitivity of FTA™ card-based detection was lower than that of the CTAB-purified nucleic acids, this result underscores the potential for its routine use, pending the optimization of sample selection and protocol refinement to further improve performance. The printed cards are small and light, and their transport is easy within a country or internationally. Card transport is likely to be easily facilitated, as unlike plant tissues or infectious viruses, nucleic acids exchange is not restricted by international or national phytosanitary regulations.

In Croatia, the FTA™ card RT-PCR protocol will be used for future CLBV surveys from citrus, and possibly other hosts, to provide increased information on occurrence and distribution of the virus in field trees and planting material, and to determine its possible impacts on fruit production.

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