Increased Diversity of Citrus Tristeza Virus in Europe

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Abstract

This study investigated the genetic diversity of citrus tristeza virus (CTV) isolates from Montenegro and Croatia, European countries with the northernmost citrus growing regions situated on the Eastern Adriatic coast. Fifteen complete or nearly complete CTV genomes were reconstructed from high-throughput sequencing of samples collected in distinct municipalities in Montenegro and Opuzen municipality in Croatia. Phylogenetic analyses assigned some of the sequences to VT and T30 strains, previously recorded in Europe, while remarkably other isolates were placed in S1 and RB groups, which have not been reported in Europe so far. In addition, a new phylogenetic lineage including only isolates from Montenegro was delineated and tentatively proposed as the MNE cluster. Recombination analysis revealed evidence of 11 recombination events in the sequences obtained in this study, between isolates of related strains,

within isolates of the same strain, and between distant strains. These findings show that CTV diversity in Europe is higher than reported before and calls for the re-evaluation of management strategies.

Keywords: complete genomes, CTV, HTS, genotyping, non-EU strains

Introduction

Citrus tristeza virus (CTV) is one of the most damaging pathogens affecting citrus plants worldwide. Over the past century, CTV epidemics have drastically reduced citrus production and caused the death of more than 100 million trees propagated on sour orange (*Citrus aurantium* L.) rootstock (Jones 2021). The most destructive epidemics began in Argentina in 1930 and thereafter spread throughout South America, North America and the Mediterranean region, whereas South Africa and Australia remained unaffected due to the limited use of the sour orange rootstock (Dawson et al. 2015; Moreno et al. 2008). Further losses in citriculture caused by the decline of citrus trees in many regions were prevented by eradicating diseased trees and introducing the tristeza-tolerant rootstock *Poncirus trifoliata* (L.) Raf. (Moreno et al. 2008).

CTV belongs to the *Closteroviridae* family (genus *Closterovirus*) and possesses the largest nonsegmented genome (19.3 kb) among plant viruses (Folimonova and Sun 2022; Karasev et al. 1995). The virus has a positive single-stranded RNA genome with two untranslated regions (UTRs) at the 5’- and 3’-termini and 12 open reading frames (ORFs) encoding at least 19 proteins (Karasev 2000). The 5’-terminal ORF1a contains methyltransferase (MET), helicase (HEL) and two protease (PRO) domains, while ORF1b encodes the RNA-dependent RNA-polymerase (RdRp) (Karasev et al. 1995). The 3’-terminal portion of the genome includes ten ORFs that encode major (p25) and minor (p27) coat proteins (CPs), three proteins (p61, p65 and p6) required for virion assembly and movement (Satyanarayana et al. 2000), three proteins (p33, p18 and p13) as determinants of the host range of the virus, and three RNA silencing suppressors (p25, p20 and p23) (Lu et al. 2004).

CTV is transmitted over long distances by the movement of infected propagation material and over short distances by aphids such as *Toxoptera citricidus* Kirkaldy and *Aphis gossypii* Glover, which transmit the virus in a semi-persistent manner (Bar-Joseph et al. 1989). The severity and type of symptoms associated with CTV depend on several factors, among which are the virus strain, or a mixture thereof, the host species, the combination of scion and rootstock, and environmental factors (Moreno et al. 2008). Mild CTV strains elicit only very weak symptoms or latent infections in citrus plants, while severe strains lead to three major syndromes in susceptible hosts: quick decline (QD), also called tristeza, stem pitting (SP) and seedling yellows (SY). QD, induced by severe CTV strains in citrus grafted on sour orange or lemons, leads to

phloem necrosis and dieback of the infected tree within a few weeks or may progress as ‘slow decline’ with symptoms developing over months or years (EFSA 2014). SP-inducing strains cause deep elongated pits in the wood of the trunk and branches of grapefruit or sweet orange regardless of the rootstock, whereas SY strains affect seedlings and cause chlorosis and stunting of grapefruit, sour orange and lemon (Moreno et al. 2008). The QD and SP are the biggest problem in citrus production.

The classification of CTV strains is not related to a specific phenotype of the diseased tree, but to the genotype of the virus (Dawson et al. 2015) and eight groups (T30, T36, T3, T68, VT, RB, HA16-5 and S1) are currently recognized (Harper 2013; Yokomi et al. 2018). Although CTV strains have traditionally been characterized as ‘mild’ or ‘severe’, subsequent research suggests that such classification may be simplistic, as isolates from groups T-36 and VT can cause both mild and severe (QD, SP and SY) symptoms (EPPO 2023; Varveri et al. 2015). The isolates belonging to groups T-30, S1 and RB cause latent infection or mild symptoms (EPPO 2023; Yokomi et al. 2018), while the members of groups T-68 and T3 belong to the severe genotypes (Albiach-Marti 2013). The group VT includes two subgroups, “Western” VT-like isolates from the USA and Israel and “Asian” VT-like isolates from Asia and Spain (Harper 2013). Coinfection of citrus trees with multiple genetic variants and strains of CTV is a common occurrence and has been associated with the wide range of phenotypes in citrus species and cultivars (Scott et al. 2013). However, there is no direct relationship between a specific genetic strain of CTV and an array of symptoms (Harper 2013).

Various methods have been used in the past to distinguish mild and severe strains of CTV: biological indexing on different indicator hosts (Ballester-Olmos et al. 1993), serological analysis using monoclonal antibodies MCA13 (Permar 1990), single-strand conformation polymorphism (SSCP) analysis (Rubio et al. 1996), restriction fragment length polymorphism (RFLP) analysis of the CP gene (Jiang et al. 2008; Roy et al. 2003), RT-PCR and phylogenetic analysis based on partial sequences of the 5’-half of the genome (Ayllón et al. 2001; Cerni et al. 2009). The advent of high-throughput sequencing (HTS) has opened a new era in the study of CTV genetic diversity (Bester et al. 2021; Pais da Cunha et al. 2021), allowing the rapid reconstruction of full-length genomes, which is indispensable for the accurate assignment of an isolate to a strain group (Harper 2013).

Although CTV isolates are widely distributed in Europe, they are mainly variants that cause mild disease. CTV isolates associated with severe diseases such as QD, SP and SY in citrus have been reported on few occasions in Spain (Ruiz-Ruiz et al. 2009), Italy (Ferretti et al. 2014; Scuderi et al. 2016) and Croatia (Cerni et al. 2009), while resistance-breaking (RB) isolates are not known to exist in Europe (EFSA 2019).

Montenegro is a small country located in the middle of the northern Mediterranean basin (Supplementary Fig. 1). Citrus fruits represent the most significant crop on the Montenegrin Adriatic coast (294 km). The geographical position of the coastal region (from 41^{o} 51^{l} to 42^{o} 43^{l} north latitude) favours cold-tolerant citrus varieties (Radulovic and Plamenac 1988). Thus,

Satsuma mandarins (*Citrus unshiu* Marc.), whose seedlings were first brought to Montenegro from Japan in 1930, accounts for about 85% of citrus production, with the major varieties Kawano Wase, Zorica Rana and Chahara also widely cultivated in Croatia (Škorić et al. 2002). The remaining 15% of Montenegrin citrus production is distributed among clementines, sweet oranges, lemons and kumquats (Bitz et al. 2020; Radulovic et al. 2020). The most favourable rootstock for citrus cultivation in Montenegro is *P. trifoliata.* Despite the economic importance of citrus production, there are few data on the occurrence of CTV in Montenegro and molecular analyses of CTV genotypes have been extremely limited. The occurrence of CTV was first reported in 2005 (Papic et al. 2005), and since then only two isolates have been partially characterised based only on the nucleotide sequence of the CP gene (Cerni et al. 2009). Given its quarantine status and economic importance, CTV has been sporadically monitored in nurseries over the past decade, however, a citrus certification programme has not yet been developed.

In this context, the aim of this study was i) to monitor the occurrence of CTV in main citrus growing regions in Montenegro, including a sample from the neighbouring Croatia; ii) to reconstruct complete genomes from different plant sources using HTS technology, and iii) to study the genetic diversity and strain-group assignment of Montenegrin and Croatian strains, also in relation to CTV sequences previously reported. With the aim to better understand the evolution of CTV, we investigated putative recombination events as a driving force contributing to the emergence of new virus strains in the past.

Materials and methods

Field surveys and sample collection

To assess the presence of CTV in citrus orchards, nurseries and mother-plant blocks, field surveys were conducted from May to October (avoiding the hottest period in July and August when the virus titre usually declines) 2016 to 2022 in the main citrus growing regions of Montenegro (Supplementary Fig. 1). The surveys were conducted at different locations in 8 municipal districts (Ulcinj, Bar, Budva, Tivat, Kotor, Herceg-Novi, Podgorica and Danilovgrad) of Montenegro and in different citrus species and varieties. A total of 419 samples were taken randomly including 193 plants from nurseries, 153 from commercial orchards and 73 from mother trees. The majority of samples (183) were collected from *Citrus unshiu* (Satsuma mandarin), the dominant citrus species in the country, while 79 samples belonged to *C. sinensis* (sweet orange), 73 to *C. limon* (lemon), 46 to *Fortunella* spp., 12 to *C. madurensis* (calamondin), 10 to *C. paradisi* (grapefruit), 7 to *C. aurantiifolia* (lime), 6 to *C. medica* (citron) and 3 to *Poncirus trifoliata*. One sample of sweet orange (Corrugated Navelina) from the Neretva River valley, the main cultivation region of the neighbouring Croatia situated even more to the north than the Montenegrin citrus production region, was also considered (Supplementary Fig. 1). This orange was preferentially sampled due to the symptoms of severe yield loss and included in the study. The two countries share citrus

cultivation practices, the historic origin of many varieties and trade propagation material. To reduce the effect of uneven virus distribution in the plant, each sample consisted of 20-30 leaves from different parts of the tree. Collected samples were transported to the laboratory in hand-held cooler and stored at -20°C until the analyses were performed. Petioles and midribs of citrus leaves were used as a plant tissue (phloem) source for further analysis.

Serological and molecular detection of CTV

All collected samples were assayed by Double Antibody Sandwich - Enzyme Linked Immunosorbent Assay (DAS-ELISA) using polyclonal antibodies against recombinant coat protein (Bioreba, Switzerland) of CTV which enables the detection of mild and severe QD, SP and SY isolates from different regions of the world. The colour reaction was examined visually and spectrophotometrically (Multiscan EX, Thermo LabSystems) by measuring absorbance at 405 nm. A sample with the absorbance value at least three times greater than the absorbance of the negative control was considered positive.

ELISA positive samples were subjected to conventional RT-PCR to confirm the obtained results. Total RNAs were extracted from the petioles and midribs of citrus leaves. Briefly, 100 mg of plant material was homogenised in 1 ml of 2% CTAB buffer (2% CTAB, 2% PVP-40, 100 mM TRIS-HCl pH 8, 1.4 M NaCl, 20 mM EDTA), incubated at 65°C for 15 min and centrifuged 5 min at 10000 x g. The supernatant (650 µl) was mixed with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged 10 min at 15000 x g. The supernatant was collected and 350 µl of isopropanol was added. The mixture was centrifuged 10 min at 15000 x g and resulting pellet was washed with 1 ml 70% ethanol and centrifuged 10 min at 15000 x g. The pellet was dried in Speed Vacuum concentrator (Uniequip, Germany), dissolved in 100 µl of RNase-free water and stored at -80°C. The concentration and purity of RNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). RT-PCR detection of CTV was performed using the one-step RT-PCR protocol with primers PIN1/PIN2 (Olmos et al. 1999) which amplify a 131 bp fragment within the conserved 3'-UTR. One-step RT-PCR reaction mix contained 1x Qiagen One-step RT-PCR buffer, 1x Q-Solution, 10 µM of each dNTP, 0.6 µM of each primer and 1.25 U One-step RT-PCR Enzyme mix (Qiagen, USA). The RT-PCR conditions in T3 Thermocycle (Biometra, Germany) were 30 min at 50°C for reverse transcription, 15 min at 95°C for hot-start Taq activation, followed by 40 cycles of amplification for 20 s at 94°C, 20 s at 60°C and 45 s at 72°C, and final extension for 10 min at 72°C. The RT-PCR amplicons were visualised on a 1.5% agarose gel stained with ethidium-bromide.

High-throughput sequencing and bioinformatics

A total of nine CTV samples from Montenegro from different hosts (4 from Satsuma mandarin, 4 from sweet orange, and one from lemon), three municipal districts (Bar, Herceg-Novi and

Podgorica) and three different years of sampling (2018, 2020 and 2022), were selected for further analyses by HTS. A Croatian isolate from a sweet orange tree from Opuzen municipality, collected in 2022, was also included in the analysis.

Total RNA extracts were treated with DNase I (Ambion, USA) and used as input for cDNA synthesis (Maxima H Minus Reverse Transcriptase, Thermo Scientific), and second strand synthesis using random octamer primers (NEBNext Ultra II Non-Directional RNA Second Strand Module). The libraries were prepared according to a Nextera XT DNA Library Preparation Kit or an Illumina DNA Prep kit (Illumina, USA) and sequenced on a MiSeq or NextSeq2000 instrument (Illumina, USA) as paired-end reads at the Leibniz Institute-DSMZ (Braunschweig, Germany). In the bioinformatic analyses, total reads were paired, trimmed, aligned against host sequences (chromosomes and predicted transcripts acc. PRJNA225998, chloroplast acc. NC_008334 and mitochondrion acc. NC_037463) and *de novo* assembled using GeneiousPrime software v. 2023.1.1 (Biomatters, Auckland, New Zealand). The obtained contigs were screened by BLASTn and BLASTp against a virus reference database for virus discovery and reconstruction of viral genomes.

Fifteen complete or nearly complete genome sequences from nine Montenegrin and one Croatian CTV samples were obtained and deposited into GenBank via web-based BankIt-NCBI-NIH platform (http://www.ncbi.nlm.nih.gov/WebSub) and assigned to sequence accession numbers (Supplementary Table S1).

Sequence Demarcation Tool (SDT)

Sequence Demarcation Tool (SDT) enables computing of pairwise sequence identities based on multiple independent pairwise alignments and visualizing results in colour-coded pairwise-identity plots and matrices. The Muscle software (Edgar 2004) in SDT v1.2 (Muhire et al. 2014) was used to calculate and visualize the pairwise identity between complete genome sequences from 15 isolates from this study and 45 from different countries around the world retrieved from NCBI GenBank.

Sequence Alignment and Phylogenetic analysis

To assess the divergence level, a multiple alignment of complete genome sequences of 14 Montenegrin, one Croatian and 45 known CTV isolates from NCBI GenBank (Supplementary Table S1) was performed using Muscle software in MEGA 11 (Tamura et al. 2021). Phylogenetic trees were reconstructed using two methods, maximum parsimony (MP) and neighbour network (NN). MP with the subtree-Pruning-Regrafting algorithm and average pathway method to calculate branch length was performed in MEGA 11. NN was conducted using Splits Tree 4.19.1 (Huson and Bryant 2006) with LogDet distance correction, exclusion of gaps and parsimony-uninformative sites. The reliability of the phylogenetic trees was assessed by computing bootstrap values from

1000 replicates. The mean genetic distance within and between phylogenetic groups were calculated using p-distance model in MEGA 11.

Recombination analysis

To identify recombination patterns and potential recombination breakpoints within the 15 CTV complete genomes from this study, we used the Recombination Detection Program version 4.101 (RDP4, Martin et al. 2015). In addition to the CTV isolates from this study, the dataset included 45 available CTV complete genome sequences from previously described CTV genotypes from the NCBI GenBank database. The analysis was performed with seven algorithms included in RDP: RDP, Geneconv, Bootscan, MaxChi, Chimaera, SiScan and 3Seq using default parameters values for different methods (Martin et al. 2015). Whole genome sequences in the dataset were aligned with Clustal Omega Tool available at https://www.ebi.ac.uk/Tools/msa/clustalo/ (Madeira et al. 2022). Recombination events predicted by four or more methods at a probability value threshold of 0.01 were considered as a proof of a putative recombination event. Potential recombination events where parental sequence could not be identified were discarded.

Confirmation of CTV genotypes by RT-PCR and design of a novel assay for specific detection of the MNE group

The assignment of CTV from Montenegrin and Croatian samples to specific strain-groups was confirmed by one step RT-PCR (Qiagen, Germany), following the manufacturer's procedure, and using primers specific for T30 and VT (Roy et al. 2010), RB (Cook et al. 2016) and S1 genotype (Bester et al. 2021). Based on alignment of complete genome sequences of CTV isolates from this study and NCBI, Decipher v2.0 package (Wright 2016) was used to design oligonucleotides for detection of the T30 and newly proposed MNE group (Table 1) using default parameters. The oligonucleotides were synthetized by Macrogen Europe (the Netherlands). The RT-PCR products were generated under the following thermal cycling conditions: 30 min at 50°C for reverse transcription, 15 min at 95°C for hot-start Taq activation, followed by 35 cycles of amplification for 30 s at 94°C, 30 s at 59-63°C and 1 min at 72°C, and final extension for 10 min at 72°C. The RT-PCR products, together with positive and negative controls were visualized on a 1.5% agarose gel stained with ethidium bromide and photographed with gel documentation system (Bio-Rad, USA).

Results

CTV incidence and distribution

A survey on the occurrence and spread of tristeza disease in Montenegro was conducted during a seven-year period (2016-2022). In total, 419 leaf samples from citrus orchards, nurseries and

mother trees from 8 municipalities were analysed by DAS-ELISA, of which 106 (25.3%) reacted positively with antisera against CTV. Furthermore, virus infection in each ELISA-positive sample was confirmed by RT-PCR amplifying an expected 131 bp DNA product. The results showed that the incidence of CTV in orchards was higher (45.1%) than the rates in nurseries (16.1%) and mother trees (8.2%) (Table 2). Various citrus species including mandarins (49.2% infection rate), calamodin (16.7%), orange (12.7%), grapefruit (10.0%) and lemon (4.1%) were found to be infected, while lime, citron, *Fortunella* spp. and *Poncirus trifoliata* proved to be CTV-free (Table 3). Virus incidence level varied significantly according to the municipal districts. The highest incidence of CTV was recorded in Ulcinj (39.1%), the municipality with most intensive citrus-fruit cultivation, followed by Herceg-Novi (25.9%), while Danilovgrad and Bar (25.0%), Kotor (20.0%), Podgorica (16.0%) and Budva (11.1%) showed lower virus incidence rates. No infections of CTV were detected in samples from Tivat municipality (Table 4).

HTS analyses and reconstruction of complete genome sequences

For further HTS analysis, nine Montenegrin samples from different citrus hosts, municipal districts and year of sampling, and one sample of sweet orange from Croatia collected in 2022 were chosen.

The high-throughput sequencing generated ~4 to 38 millions of raw reads per library (Supplementary Table S2). In the bioinformatic analyses, after quality trimming and subtraction of host sequences, the remaining high-quality reads were *de novo* assembled (read statistics at different steps of bioinformatic analysis are given in Supplementary Table S2). The following BLASTn/BLASTp analysis against a reference plant virus database assigned contigs to CTV, which were further assembled to reconstruct (nearly) complete virus genomes. The analyses revealed single as well mixed infections with multiple CTV isolates, overall resulting in the reconstruction of 15 complete or nearly complete genomes (missing a number of nucleotides at the extreme 5'- or/and 3'-UTR's) from the 10 samples/libraries. In the Montenegrin mandarin sample 151/22 from HajNehaj three genome sequences (named 151/22-1, 151/22-2 and 151/22-3) were assembled, while in the mandarin sample 186/18 from Tološi, and in the orange (125/22) and mandarin (168/22) from Tabija Polje, two CTV sequences each were reconstructed, and named 186/18-1 and 186/18-2, 125/22-1 and 125/22-2, and 168/22-1 and 168/22-2, respectively. In all other isolates from this study one consensus sequence was generated covering the complete or nearly complete genome sequence of CTV. The relevant data related to virus isolates, GenBank accession number of the genomic sequences, coverage and percentage identities are reported in Supplementary Tables S1, -S2 and -S3, respectively.

Sequence Demarcation Tool, Phylogenetic and Recombination analyses

Pairwise comparison of the nucleotide sequences from this study and those from NCBI was performed using the SDT program (Supplementary Fig. 2). The results of SDT analysis revealed

that Montenegrin and Croatian isolates share 81.4-100% identity between each other. The lowest nucleotide identity (81.4%) among isolates from this study was between a mandarin isolate (151/22-3) from Haj-Nehaj and isolates from orange (125/22-2) from Tabija Polje locality, mandarin (186/18-2) from Tološi and a lemon isolate (60/20) from Pečurice. In terms of the highest nucleotide identity, Montenegrin isolates from orange (125/22-1) and mandarin (168/22-2) located at Tabija Polje, 125/22-2 and 168/22-1, and two orange isolates (127/22 and 170/22) from Novakovići locality showed 100% identity between each other.

The phylogenetic analysis based on genome sequences was conducted to study the phylogenetic relationship of CTV isolates from this study and isolates from all over the world (Fig. 1 and 2). The analysis revealed four different CTV genotypes among Montenegrin and Croatian isolates clustering them into VT, RB, T-30 and S1 phylogenetic groups. Out of the 15 CTV genomes obtained in this study, one Montenegrin isolate 151/22-1 (Fig. 1) clustered in the phylogenetic analyses within T30 group, while another Montenegrin 186/18-1 and the Croatian isolate 9A were members of the VT group, more precisely, the “Asian” subgroup (Harper 2013). Two Montenegrin isolates 151/22-3 and 166/22 were classified in the S1 group and five in the RB (186/18-2, 60/20, 125/22-1, 151/22-2 and 168/22-2) (subgroup I). Remarkably, five Montenegrin CTV isolates (167/18, 125/22-2, 127/22, 168/22-1, 170/22) clustered in a new, distinct phylogenetic group, which is here proposed as the MNE group and may correspond to a novel virus genotype (Fig. 1 and 2). Additionally, amino acid sequence comparison revealed that all Montenegrin RB isolates possess an invariable motif “RVENV” in the p23 protein sequence which distinguishes the RB group from other CTV genotypes (Ghosh et al. 2022). Overall, out of the eight CTV phylogenetic groups known so far (Harper 2013; Yokomi et al. 2018), these isolates were distributed in four lineages, plus in a new lineage where five Montenegrin isolates were consistently clustered in both types of phylogenetic analyses (Fig. 1 and 2), with 92.2% (Table 5) of nucleotide (nt) identity to the closest T30 group over the whole genome. Nucleotide identity level within the new MNE cluster was 98.1%. The lowest nucleotide sequence identity (80%) among different genotypes was between T68 and T36 group members. In the nucleotide sequence comparisons of the 5’-proximal ORF1a in the CTV genome the sequence identity calculated by using MEGA 11 showed that the closest genotype group to the putatively new MNE phylogroup was T30 with nucleotide identity of 91.1% and amino acid identity of 92.7% (Table 6).

BLASTn comparisons of complete genome sequences from our study, including the ones in the MNE cluster, with corresponding CTV sequences in the NCBI GenBank (accessed July 18, 2023) were performed to reveal nucleotide identities with 96 currently available CTV genomes having a complete coding sequence (CDS). Within the MNE cluster isolates 127/22, 167/18 and 170/22 had the highest identities with the French isolate C7B1 (MZ648331) from the Corsican mandarin hybrid belonging to the T30 genotype with nucleotide identities 92.7%. On the other hand, isolates 168/22-1 and 125/22-2 were the most similar to the USA mandarin isolate FS701-T30 (KC517489) with respective identity of 92.2%.

Different consensus sequences reconstructed from the same plants considered in this study reflect complex CTV population structure with genotypes belonging to different phylogenetic groups (Fig. 1). In the sample 152/22, the first sequence (152/22-1) belongs to T30, the second to RB, and the third to S1 genotypes. Two genotypes are present in the sample 186/18 (-1 is in the VT and -2 in the RB genotype). Likewise, 125/22-1 belongs to RB and 125/22-2 to MNE, whilst 168/22-1 is assigned to MNE and 168/22-2 to the RB group.

RDP4 analyses of 15 complete CTV genomes obtained in this study with 45 complete CTV genomes from GenBank revealed putative recombination events in almost all of 60 isolates included in the dataset. However, the support for majority of these events was weak, i.e., they were detected by fewer than four algorithms (with $p<0.01$) or the parental sequences could not be identified. Considering only sequences obtained in this study, 11 potential strong recombination events were detected (Table 7) where the recombination event was supported with four or more algorithms (with $p<0.01$) and parental sequences were identified. Detailed results for each recombination event, including the determined p-value, the start and end points of the recombination events, and the potential parent sequences, are shown in Table 7. Potentially novel CTV strain (MNE) from Montenegro showed evidence of recombination events in the part of the genome responsible for virion assembly and cell-to-cell movement, where minor donors were isolates of the T36 strain, while major parents were isolates of strain T3 or Maxi isolate of the VT strain. In the part of the genome responsible for replication, MNE isolates are also potential recombinants between isolates of the T30 strain and H16-5. Inter-strain recombination was also detected in isolates of the MNE strain with the isolates of the T30 strain as minor parent. Montenegrin isolates classified as RB strains showed evidence of recombination between isolates of T30 and T36 strains. The only Montenegrin isolate belonging to the T30 strain showed evidence of recombination between RB and VT and between T36 and T3 isolates. Montenegrin isolates of the S1 strain also showed evidence of recombination between and within strains. According to this analysis, the isolate VT (186/18-1) from Montenegro is an intragroup recombinant of VT isolate from Greece and VT isolates from Italy.

Confirmation of CTV genotypes by RT-PCR and development of a new assay specific for MNE isolates

The phylogenetic findings were firstly verified by RT-PCR using genotype specific primers previously described for clades S1, RB subgroup 1 and VT (Bester et al. 2021; Cook et al. 2016; Roy et al. 2010) producing amplicons of 715, 628 and 302 base pairs (bp), respectively (not shown). Also, T30 specific primers producing an amplicon of 206 bp were used to confirm the finding of the member of T30 genotype (151/22-1). However, three isolates from MNE lineage (167/18, 127/22, 170/22) could also be amplified with T30 genotype specific primers (not shown). The other two isolates from the same lineage (151/22-1 and 125/22-2) failed to amplify. Sequence inspections of the multiple alignments revealed substantial nucleotide differences in

the primer annealing regions, prompting the design of new pair of primers per each genotype group, able to distinguish T30 and MNE isolates (Table 1). The MNE specific primers, resulting in a 546 bp-long amplicon, consistently amplified all five MNE isolates, and both pairs of T30 specific primers designed in this work (resulting in 267 bp and 720 bp products) amplified only T30 Montenegrin isolate (not shown). No RT-PCR cross reactivity was detected between T30 and MNE isolates. (Bester et al. 2021; Cook et al. 2016; Roy et al. 2010)

Discussion

CTV is present in all citrus growing regions of the world, including the Mediterranean (EPPO 2023; Moreno et al. 2008). In Europe, most of CTV isolates have been associated with mild disease, while severe isolates have only been recorded in a few cases (Cerni et al. 2009; Ferretti et al. 2014; Ruiz-Ruiz et al. 2009; Scuderi et al. 2016). The virus is listed as a quarantine pest in many citrus-growing countries including Montenegro (Official Gazette of Montenegro, No 32/23). In the European Union non-EU isolates are listed in Annex II, Part A of Commission Implementing Regulation (EU) 2021/2285.

The survey conducted in the present study revealed that the incidence of CTV in mother trees was the lowest (8.2%, Table 2) although the phytosanitary status of mother trees and propagation material has not been regularly checked in Montenegro as in countries where a citrus certification program is fully implemented. More importantly, infected mother trees continued to be used for propagation of plant material due to the inadequate enforcement of phytosanitary measures. Although the number of *P. trifoliata* samples as the main rootstock in Montenegro was low, all of them were from eight-year-old mother trees (Table 3) and they were CTV-free. This is expected as trifoliate rootstock plants are produced from seeds, which are not the route of CTV transmission, and not manipulated before grafting. It also reinforces previously proposed epidemiological scenario that in this part of Europe, in the absence of the most efficient insect vector *Toxoptera citricidus*, CTV still spreads via infected scions, and possibly whole plants, produced locally or introduced (Černi et al. 2005; Papic et al. 2005). As a result of uncontrolled multiplication of infected plant material from mother trees, the level of CTV infection in nurseries was almost twice as high (16.1%). In Montenegrin citrus orchards almost half of tested plants were CTV infected (45.1%, Table 2). CTV infection rate in the neighbouring Croatia was reported previously (Černi et al. 2005; Cerni et al. 2009) and was about 40% in the production orchards reflecting similar horticultural practices in the two countries. The most propagated Satsuma mandarin had the highest infection rate in Croatia (about 60%) (Cerni et al. 2009) which was higher than in our studies (49.2%, Table 3). However, in this study more hosts were investigated, and new citrus species including grapefruit and ornamental calamondin were found infected in this restricted agroecological niche of the East Adriatic (Montenegro, Croatia and Albania) (Cerni et al. 2009; Stamo et al. 2000). Expectedly, the highest CTV incidence in the plants of all production stages (mother trees, nurseries and production orchards) was recorded in Ulcinj municipality (39.1%), the main citrus cultivation region in Montenegro (Table 4).

The complete or nearly complete CTV genome sequences obtained in this study from Montenegrin and Croatian samples could finally be assigned to one of eight previously reported genotypes defined as single phylogenetic lineages or strains (EPPO 2023; Harper 2013; Yokomi et al. 2018). Previous reports from Montenegro and Croatia included partial molecular characterization of a few CTV isolates (Černi et al. 2005; Cerni et al. 2009; Papic et al. 2005) and only on the basis of the CP gene, which is not informative enough for correct genotyping (Harper 2013). Similarly, in other European countries previous attempts of CTV genotyping included only conserved CP and/or p20 sequences (Chatzivassiliou and Nolasco 2014; Davino et al. 2013; Ferretti et al. 2014). Thus, this study significantly contributes novel insights into the diversity of CTV strains in Europe.

Members of genotypes T30, VT and T36 (Catara et al. 2021; EFSA 2019; Scuderi et al. 2019) are known to occur in Europe. One of the Montenegrin isolates belongs to genotype T30 (151/22-1, Satsuma mandarin 'Kawano Wase', Fig. 1 and 2, Supplementary Table S1), which is known to harbour phenotypically mild isolates (EPPO 2023), and it was found in mixed infection with two other CTV genotypes, with 151/22-2 belonging to the RB cluster, which may include strains with a more severe phenotype (e.g., slow decline of citrus was reported for the Greek strain from the locality of Koufos in western Crete, GenBank acc. no. KF908013, EPPO 2023; Owen et al. 2014), and 151/22-3 belonging to the S1 cluster with mild phenotypes (Fig. 1 and 2). Unfortunately, the assignment of a particular phenotype to a particular phylogenetic lineage in CTV is still complicated and remains a problem without clear guidelines (Harper 2013). Mixed infections of sequence variants from different genotypes, which are common in citrus (Bar-Joseph et al. 1989; Harper 2013; Moreno et al. 2008; Scott et al. 2013), further complicate the situation. The occurrence of multiple isolates in the same sample has been previously reported in the area (Cerni et al. 2009) and, besides sample 151/22, it has been found in this study in samples 186/18, and 168/22 from mandarins, as well as in sample 125/22 from orange (Fig. 1 and 2, Supplementary Table S1)

The only Croatian isolate in this study (9A, 'Corrugated Navelina' orange) and another Montenegrin isolate (186/18-1, Satsuma mandarin 'Kawano Wase', Supplementary Table S1) were clustered with VT, the second CTV genotype already recorded in Europe including mild and severe isolates (EPPO 2023; Varveri et al. 2015). In the isolate 9A, severe symptoms were detected in the field (Supplementary Fig. 3). Montenegrin mandarin 186/18 is also one of the samples with multiple CTV sequence variants belonging to different genotypes. In this case, 186/18-1 belongs to VT and 186/18-2 to RB genotype (Fig. 1 and 2). In addition to the presence of non-European RB genotypes detected for the first time in Montenegro (Fig. 1 and 2), two isolates (the 'Kawano Wase' mandarin isolate 151/22-3 and the orange isolate 166/22, Supplementary Table S1) were assigned to the S1 genotype, a non-European cluster with mild phenotype members (EPPO 2023). This requires a reassessment of the epidemiological situation in Europe (EFSA 2017; EFSA 2019) and further investigations due to the proximity of Croatian (EU

country) and Montenegrin (non-EU) citrus growing regions, intensive trade in citrus propagating material, and the possible common origin of some plant accessions.

Surprisingly, phylogenetic analyses clearly showed the grouping of five Montenegrin sequences (Fig. 1 and 2) in a statistically well-supported separate cluster. We tentatively named it the MNE cluster and examined identities of sequences within this cluster with other sequences representing previously defined genotypes according to the given criteria (Harper 2013; Yokomi et al. 2018). The nucleotide identity level across the entire genome within the MNE cluster was 98.1% (Fig. 1, Table 5). This new cluster was closest to T30 genotype and had a nucleotide identity of 92.2%. As Harper (2013) set the threshold for genotype delineation at 7.5% nt differences (92.5% identity) at the time fewer genomes were available in the databases, we performed BLASTn analyses for all five MNE isolates against 96 currently available CTV genomes in the NCBI GenBank database to examine if MNE cluster could be considered a new genotype. The nt identity level ranged from 92.2 to 92.7% in these calculations. Additional criterion for delineating a new genotype to be examined (Harper 2013) was >8% nt difference of ORF1a gene or encoded protein. The analyses have shown that these differences were 8.9% at the nucleotide level for MNE cluster members and 7.3% at the amino acid level with members of the genotype T30 (Table 6). Taken together, these results suggest that MNE cluster could be accommodated in a new genotype. (Catara et al. 2021; EFSA 2017). The current criteria for delineating CTV genotypes (Harper 2013) should perhaps be revisited taking into account the larger number of CTV genome sequences available in the databases. Likewise, the number of genotypes currently recognized on the base of the last revision in the Mediterranean area including 13 whole-genome sequences (Catara et al. 2021) and in Europe (EFSA 2017) should be reconsidered.

Recombination events can be an important source of genetic variation in plant viruses (García-Arenal et al. 2003) and are important evolutionary force leading to the emergence of new genotypes (Harper 2013; Martín et al. 2009). Recombination analyses performed with the new genome sequences obtained in this study (Table 7) showed the existence of recombination signals in almost all isolates. In CTV populations recombination between closely related strains are more common than between more diverse strains, with few exceptions (Harper 2013; Yokomi et al. 2018). This study also demonstrated the presence of recombination between similar strains and within isolates of the same strain (intra-strain recombination). However, some of the recombination recorded in this study occurred between distant strains (recombination of Montenegrin RB in which isolates from T30 and T36 were detected as parent strains, and recombination in the isolates belonging to the tentative new MNE group, in which parent isolates were from the following strain groups: T36, T3, VT, H16-5). Similar results were presented by Yokomi et al. (2018), where the S1 strain isolates underwent diverse recombination events.

In conclusion, the new findings presented here on the occurrence of distinct CTV genotypes on the eastern Adriatic coast (principally in Montenegro), including genotypes assigned to non-European lineages S1 and RB, and the delineation of the potentially new genotype MNE,

significantly contribute to a better understanding of the CTV diversity and underline the need for further research and continuous CTV surveillance. This is particularly important considering that *P. trifoliata* is the most used rootstock in the area where sequences of genotype RB have been newly discovered, as this could influence management schemes and cultivation practices in this specific agroecological niche.

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Table 1. Primers used for detection of Citrus tristeza virus MNE and T30 group of isolates.

Primer	Sequence 5'-3'	Nucleotide position	Annealing temperature	Size of amplicon (bp)
CTV-MNE F6	GACGTATCTCGGTGTGTTGT	6011-6030	63°C	797
CTV-MNE R6	GTCAGAAGAACCGTTACCTAAC	6786-6807		
CTV-T30 F1	AAGTTATCAGGAGGATTATAGGCGA	10030-10054	59°C	720
CTV-T30 R1	TCTCGAACGATAAATAATTGGACCGT	10724-10749		
CTV-T30 F2	AACGGTGACGATTTTGCTGTC	1995-2015	59°C	267
CTV-T30 R2	CGGTCGGTCTGAACTAACCTG	2256-2277		

Table 2. Incidence of CTV infection in orchards, nurseries and mother trees during 2016-2022

Sample origin	Number of positive/number of tested samples							Total
	2016	2017	2018	2019	2020	2021	2022	
Orchards	11/22	23/30	-	17/36	-	-	18/65	**69/153 (45.1%)**
Nurseries	11/38	0/42	3/29	14/43	3/41	-	-	**31/193 (16.1%)**
Mother trees	-	1/11	3/22	0/10	0/10	0/10	2/10	**6/73 (8.2%)**
Total	**22/60 (36.7%)**	**24/83 (28.9%)**	**6/51 (11.8%)**	**31/89 (38.7)**	**3/51 (5.9%)**	**0/10 (0%)**	**20/75 (26.7%)**	**106/419 (25.3%)**

Table 3. Incidence of CTV on different citrus species in Montenegro during 2016-2022

Host species	Number of positive/ number of tested samples							Total
	2016	2017	2018	2019	2020	2021	2022	
Mandarin	19/33	24/52	6/11	28/50	1/5	-	12/32	**90/183 (49.2%)**
Orange	2/14	0/3	0/9	2/12	0/7	0/2	6/32	**10/79 (12.7%)**
Lemon	0/8	0/21	0/11	1/16	1/12	0/1	1/4	**3/73 (4.1%)**
Fortunella sp.	-	0/7	0/11	0/6	0/15	0/2	0/5	**0/46 (0%)**
Calamodin	-	-	0/5	0/2	1/3	0/1	1/1	**2/12 (16.7%)**
Grapefruit	1/5	-	0/1	0/1	0/2	0/1	-	**1/10 (10%)**
Sweet lime	-	-	0/2	0/1	0/3	0/1	-	**0/7 (0%)**
Citron	-	-	0/1	0/1	0/3	-	0/1	**0/6 (0%)**
Poncirus sp.	-	-	-	-	0/1	0/2	-	**0/3 (0%)**
Total	**22/60 (36.7%)**	**24/83 (28.9%)**	**6/51 (11.8%)**	**31/89 (38.7%)**	**3/51 (5.9%)**	**0/10 (0%)**	**20/75 (26.7%)**	**106/419 (25.3%)**

Table 4. Incidence of virus infection in different municipalities in Montenegro during 2016-2022

Municipal district	Number of positive/ number of tested samples							Total
	2016	2017	2018	2019	2020	2021	2022	
Bar	11/30	9/43	0/11	3/15	2/14	0/2	9/21	**34/136 (25%)**
Ulcinj	-	15/34	-	11/20	0/6	-	8/27	**34/87 (39.1%)**
Budva	-	-	-	1/9	-	-	-	**1/9 (11.1%)**
Tivat	-	-	-	-	-	-	0/3	**0/3 (0%)**
Kotor	-	-	0/1	2/9	-	-	-	**2/10 (20%)**
Herceg-Novi	11/30	0/1	1/4	1/5	-	-	1/14	**14/54 (25.9%)**
Podgorica	-	0/5	3/31	11/28	1/24	0/8	1/4	**16/100 (16%)**
Danilovgrad	-	-	2/4	2/3	0/7	-	1/6	**5/20 (25%)**
Total	**22/60 (36.7%)**	**24/83 (28.9%)**	**6/51 (11.8%)**	**31/89 (34.8%)**	**3/51 (5.9%)**	**0/10 (0%)**	**20/75 (26.7%)**	**106/419 (25.3%)**

Table 5. Average nucleotide sequence identities of the complete genome within and between different genotypes (data for the MNE cluster are in bold).

Genotype	RB	MNE	T30	S1	VT	T36	T3	T68	HA16-5
RB	96.2								
MNE	**81.9**	**98.1**							
T30	82.0	**92.2**	98.9						
S1	81.3	**86.6**	85.4	95.7					
VT	81.4	**90.4**	90.4	83.7	96.7				
T36	90.8	**82.7**	81.4	81.8	80.6	98.3			
T3	81.1	**89.9**	90.0	83.5	92.4	80.3	95.9		
T68	80.7	**84.7**	84.5	87.6	87.8	80.0	86.5	94.5	
HA16-5	81.3	**83.9**	83.5	88.2	84.1	80.3	84.4	87.5	94.5

Table 6. Average nucleotide and amino acid sequence identities of the ORF1a within and between different genotypes (data for the MNE cluster are in bold; data for amino acid sequence identity are italic).

Genotype	RB	MNE	T30	S1	VT	T36	T3	T68	HA16-5
RB		***74.7***	*74.4*	*73.2*	*75.0*	*85.2*	*74.5*	*74.0*	*73.7*
MNE	**73.1**		***92.7***	***79.9***	***92.2***	***68.7***	***91.9***	***83.1***	***80.4***
T30	72.7	**91.1**		*80.0*	*92.1*	*68.6*	*91.5*	*82.8*	*79.9*
S1	71.9	**77.8**	77.7		*79.8*	*67.1*	*79.9*	*86.4*	*87.6*
VT	73.1	**90.8**	90.7	77.8		*69.2*	*91.4*	*83.1*	*79.7*
T36	86.2	**69.0**	68.8	67.8	69.0		*68.5*	*68.2*	*67.7*
T3	72.9	**90.4**	90.3	77.9	90.6	68.8		*82.5*	*79.9*
T68	72.3	**80.1**	80.0	85.6	80.5	68.1	79.8		*86.5*
HA16-5	72.1	**78.2**	77.8	86.7	77.9	68.1	78.1	84.9	

Table 7. Genome location and origin of potential recombination events predicted in the isolates considered in this study

Recombinant Sequence(s)	Breakpoints		Minor Parent	Major Parent	RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan	3Seq
	Begin	End									
MNE (all isolates)	10750	18366	T36s	T3s, Maxi_VT	5.13E-129	NS*	2.35E-139	8.73E-39	6.54E-41	3.46E-42	4.91E-12
MNE (all isolates)	1247	10750	T30s	H16-5	NS	NS	NS	2.77E-05	1.37E-04	1.84E-80	7.36E-12
167/18, 127/22, 170/22	101	1212	T30s	MNE (125/22-2, 168/22-1)	5.26E-61	1.63E-55	1.60E-61	4.56E-17	8.86E-18	3.52E-22	7.36E-12
125/22-2, 168/22-1	18615	19147	T30s	MNE (127/22, 170/22, 167/18)	4.07E-09	1.04E-10	7.03E-12	NS	NS	NS	6.95E-06
RB (all isolates)	10510	14076	T30	T36	3.95E-129	6.23E-41	1.52E-121	9.02E-32	1.84E-22	3.43E-34	1.72E-11
RB (all isolates)	17791	18775	T30s	T36s	4.29E-27	NS	1.19E-28	8.52E-09	1.14E-12	1.61E-07	1.72E-11
151/22-1	14021	15161	RBs	VTs	9.75E-40	1.45E-04	3.44E-42	1.03E-11	8.06E-13	2.41E-02	9.81E-12
151/22-1	12837	17191	T36	T3s	4.95E-15	NS	6.00E-15	6.68E-08	7.93E-03	2.12E-136	1.72E-11
166/22, 151/22-3	9869	10612	MNEs, VTs	HA16-5s	2.59E-14	NS	2.27E-13	5.28E-07	7.75E-09	8.22E-05	9.81E-12
166/22	1176	1949	T68	S1	2.79E-03	NS	1.55E-03	NS	1.77E-04	NS	2.67E-05
186/18-1	9743	13852	VT (Greece)	VTs (Italy group)	1.32E-08	NS	2.87E-10	1.49E-09	5.21E-09	1.70E-06	6.11E-08

*NS - No significant P-value was recorded for this recombination event using this method.

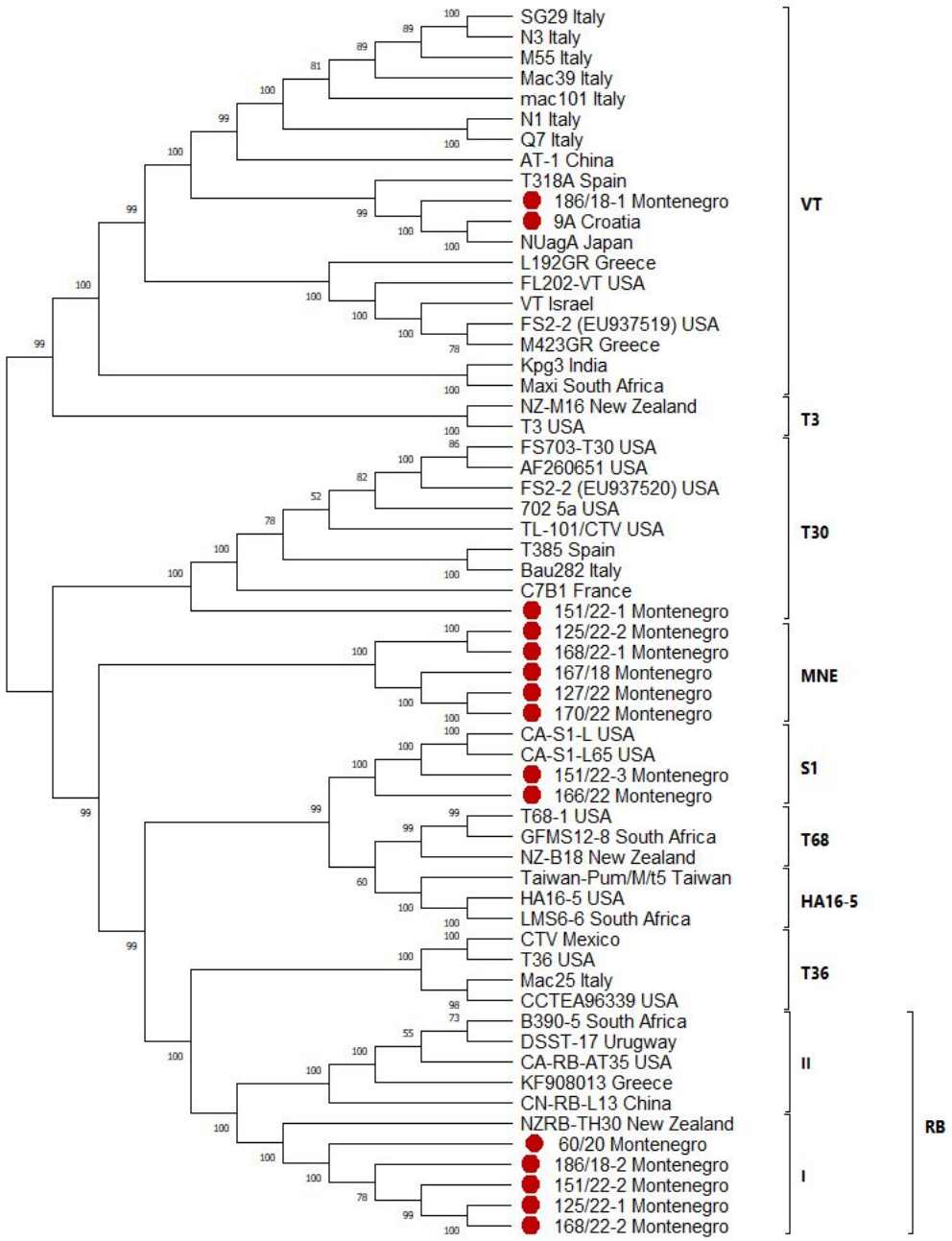


Figure 1.
Phylogenetic tree constructed using the Maximum Parsimony (MP) method with the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates) in MEGA 11 program. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. This analysis involved 60 complete nucleotide sequences of CTV including 15 from this study and 45 from the NCBI database. For each isolate, name of the isolate and country of origin are indicated. CTV complete genome sequences from this study are marked with a red circle.

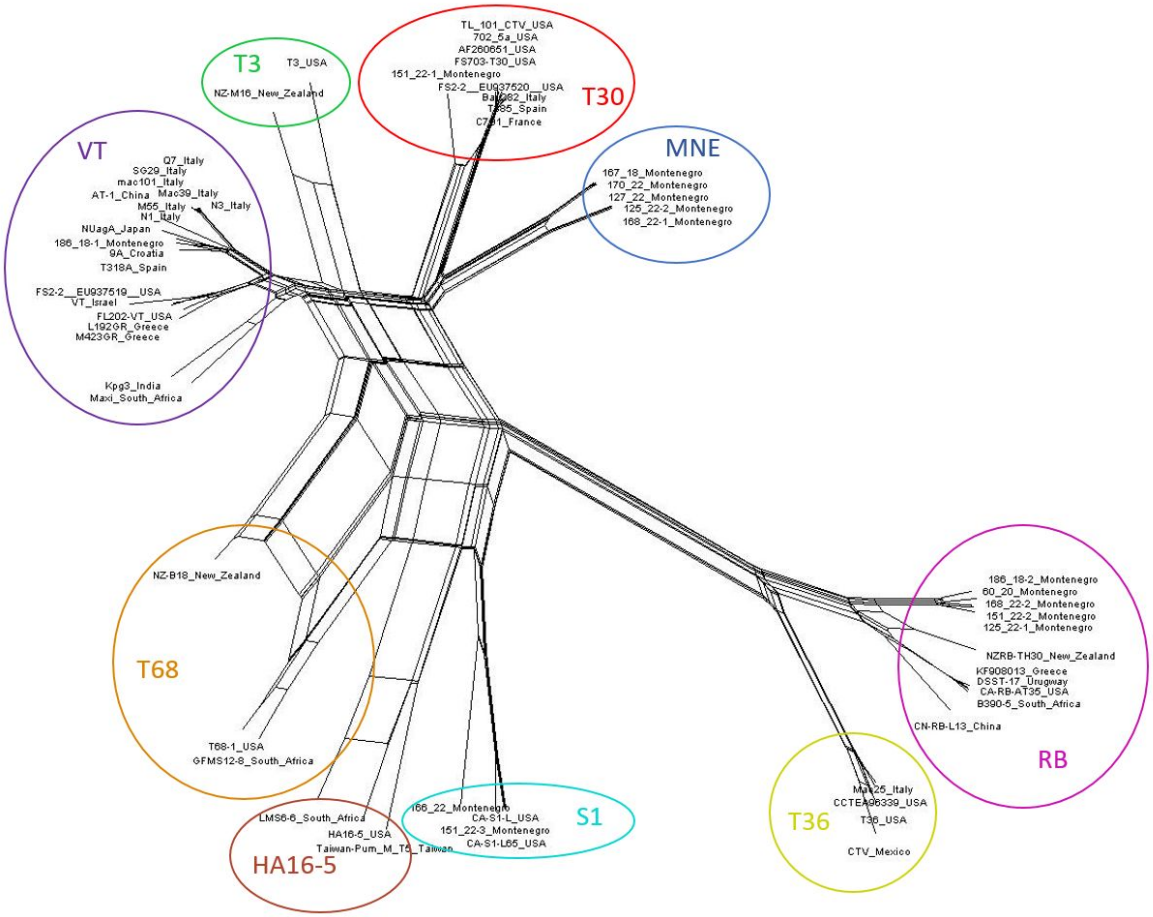


Figure 2. Neighbour network reconstruction of Citrus tristeza virus complete genomes sequences from this study. Major genotypes are indicated with coloured circles.

Supplementary Table S1. CTV isolates used in phylogenetic study and their NCBI's Database accession numbers

Isolate	Host species and cultivar	Country/District/Locality	Year	Accession Number
167/18	Satsuma mandarin cv Kawano Wase	Montenegro/Herceg Novi	2018	OR122725
186/18-1	Satsuma mandarin cv Kawano Wase	Montenegro/Podgorica/Tolosi	2018	OP006457
186/18-2	Satsuma mandarin cv Kawano Wase	Montenegro/Podgorica/Tolosi	2018	OP006458
60/20	lemon cv unknown	Montenegro/Bar/Pecurice	2020	OP006456
125/22-1	orange*cv unknown	Montenegro/Bar/Tabija Polje	2022	OR147841
125/22-2	orange cv unknown	Montenegro/Bar/Tabija Polje	2022	OR147842
127/22	orange cv unknown	Montenegro/Bar/Novakovici	2022	OR122726
151/22-1	Satsuma mandarin cv Kawano Wase	Montenegro/Bar/Haj-Nehaj	2022	OR122727
151/22-2	Satsuma mandarin cv Kawano Wase	Montenegro/Bar/Haj-Nehaj	2022	OR122728
151/22-3	Satsuma mandarin cv Kawano Wase	Montenegro/Bar/Haj-Nehaj	2022	OR122729
166/22	orange cv unknown	Montenegro/Bar/Celuga	2022	OR122730
168/22-1	Satsuma mandarin cv Kawano Wase	Montenegro/Bar/Tabija Polje	2022	OR147838
168/22-2	Satsuma mandarin cv Kawano Wase	Montenegro/Bar/Tabija Polje	2022	OR147839
170/22	orange cv unknown	Montenegro/Bar/Novakovici	2022	OR147840
9A	orange cv Corrugated Navelina	Croatia/Opuzen	2022	OR184846
FS2-2	orange cv Hamlin	USA/Florida	2004	EU937519
AT-1	orange cv unknown	China	2011	JQ061137
NUagA	*Citrus* sp.	Japan	-	AB046398
T318A	unknown	Spain	-	DQ151548
Kpg3	mandarin** cv unknown	India	2002	HM573451
T36	*Citrus* sp.	USA/Florida	-	U16304
CCTEA96339	orange *cv* Madam Vinous	USA/California	2014	MH279617
CTV	*Citrus* sp.	Mexico	-	DQ272579
Taiwan-Pum/M/T5	pomelo cv unknown	Taiwan	-	JX266713
-	*Citrus* sp.	USA/Florida	-	AF260651
T385	*Citrus* sp.	Spain	-	Y18420
702 5a	Mexican lime	USA/California	-	MH279618
FL278-T30	grapefruit	USA	2012	KC517490
-	orange cv unknown	China	2005	MK779711
B165	mandarin cv Ellendale	India	-	EU076703
FL202-VT	orange cv unknown	USA	2012	KC517493
Mac39	alemow	Italy	2012	KJ790175
Maxi	lime	South Africa	2011	KU883266
NZ-M16	lime	New Zeland	2006	EU857538
T3	alemow	USA	2005	KC525952
VT	unknown	Israel	-	U56902
T68-1	alemow	USA	-	JQ965169
GFMS12-8	lime	South Africa	1996	MK033511

NZ-B18	orange cv unknown	New Zealand	2005	FJ525436
NZRB-TH30	*Poncirus trifoliata*	New Zealand	2005	FJ525434
B390-5	lime	South Africa	2000	KU883265
DSST-17	orange cv unknown	Uruguay	2014	MH186146
CN-RB-L13	pomelo	China	2017	MH558666
HA16-5	Persian lime	USA	2001	GQ454870
LMS6-6	lime	South Africa	1996	KU883267
CA-RB-AT35	mandarin cv Shiranui	USA/California	2010	KU358530
L192GR	lemon	Greece	-	KC262793
SG29	orange cv Sanquinello	Italy	2007	KC748392
Bau282	orange cv TDV	Italy	2007	KC748391
N3	orange cv Tarocco	Italy	-	OP345181
Mac25	alemow	Italy	2014	KR263170
mac101	alemow	Italy	-	MW689620
Q7	orange cv Tarocco	Italy	-	OM803129
M55	alemow	Italy	-	OP345183
N1	orange cv Tarocco	Italy	-	OP345182
FS2-2	orange cv Hamlin	USA	2004	EU937520
FS703-T30	mandarin	USA	2012	KC517491
CA-S1-L	citron S1	USA	2010	KU589212
CA-S1-L65	orange cv Navel	USA	2001	KU589213
TL-101/CTV	orange cv Madam Vinous	USA	-	MZ330116
C7B1	mandarin	France	2020	MZ648331
1825	orange	Greece	2010	KF908013
M423GR	mandarin x orange ortanique	Greece	-	MF595989

*Orange stands for sweet orange (Citrus sinensis). **Mandarin denotes mandarin species that are not Satsuma mandarins.

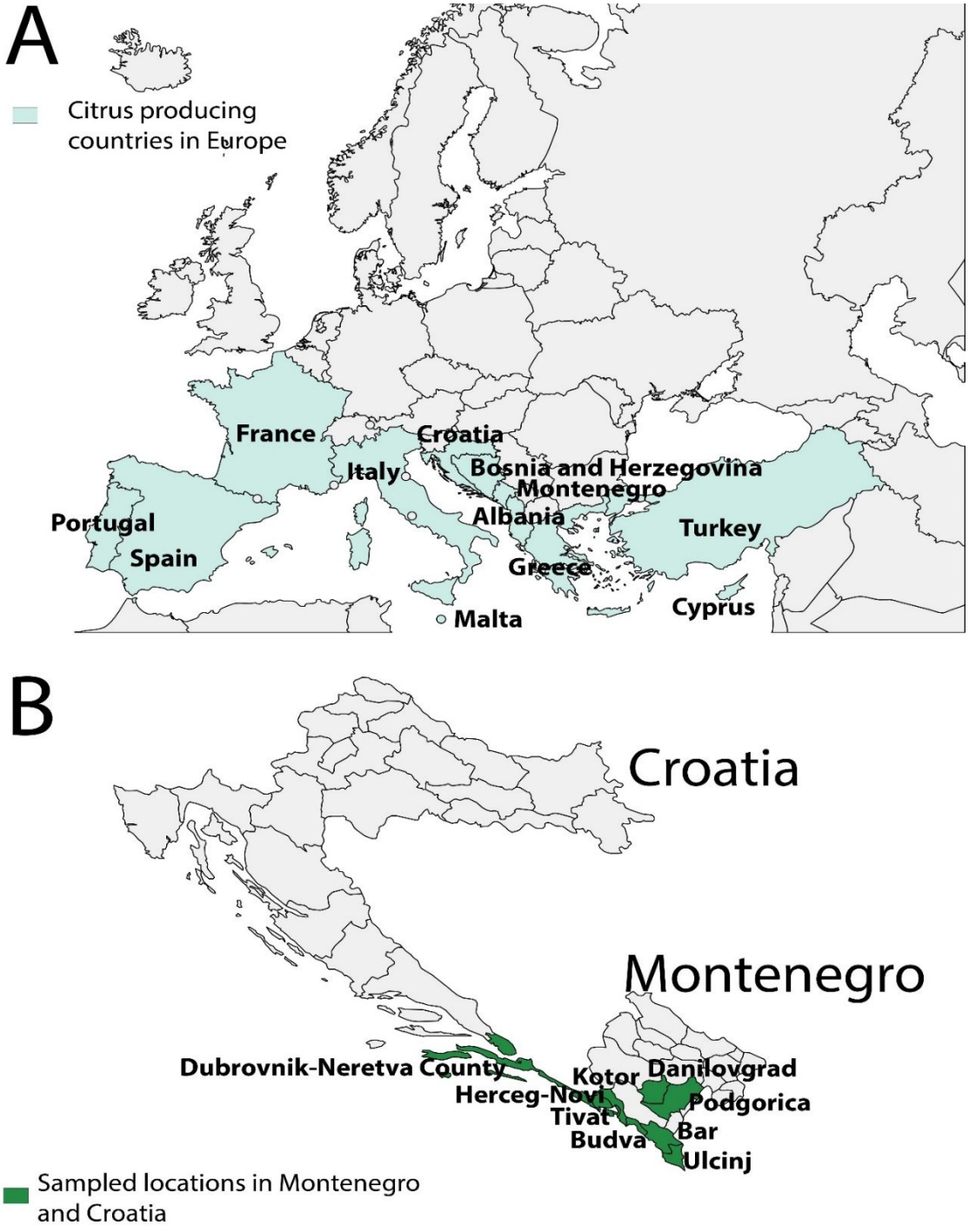
Supplementary Table S2. Number of trimmed reads mapped to the assembled (nearly) complete CTV genomes. Mapping was performed in GeneiousPrime, allowing 10% maximum mismatches per read.

Accession	CTV Isolate	Raw reads of library	Trimmed reads	Normalized reads	Reads after subtraction of host sequences	Trimmed reads mapped to virus genome	% Mapped reads
OR184846	9A	27.864.860	26,712,644	18.568.122	2.330.208	29,036	0.11
OR147842	125/22-2	14.322.448	12,367,202	2.548.942	202.460	15,680	0.13
OR147841	125/22-1	14.322.448	12,367,202	2.548.942	202.460	21,112	0.17
OR147840	170/22	15.933.776	13,513,476	1.243.092	117.931	37,854	0.28
OR147839	168/22-2	24.079.430	20,504,876	3.762.408	479.856	70,965	0.35
OR147838	168/22-1	24.079.430	20,504,876	3.762.408	479.856	61,561	0.30
OR122730	166/22	38.596.788	35,682,538	2.630.798	320.172	255,532	0.72
OR122729	151/22-3	33.655.986	31,664,788	18.175.638	2.558.784	7,040	0.02
OR122728	151/22-2	33.655.986	31,664,788	18.175.638	2.558.784	27,586	0.09
OR122727	151/22-1	33.655.986	31,664,788	18.175.638	2.558.784	10,906	0.03
OR122726	127/22	30.318.524	28,344,426	9.342.404	1.095.236	104,269	0.37
OR122725	167/18	7.646.702	6,309,600	317.036	91.672	1,860,525	29.49
OP006458	186/18-2	5.325.326	4,550,844	910.250	147.147	203,284	4.47
OP006457	186/18-1	5.325.326	4,550,844	910.250	147.147	370,470	8.14
OP006456	60/20	4.076.074	3,560,646	299,162	47.326	176,750	4.96

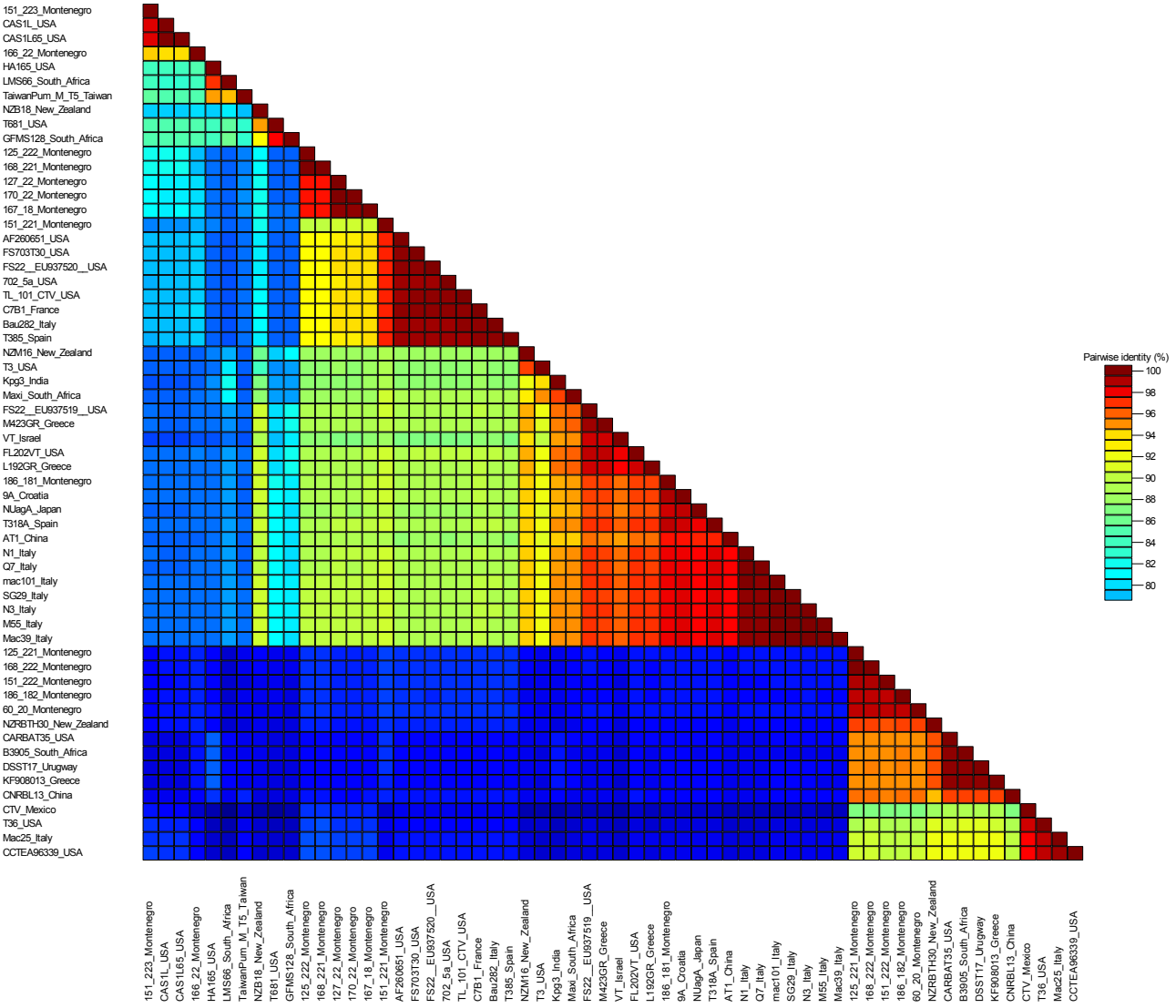
Supplementary Table S3. Percentage identity between the CTV genome sequences assembled in this study. Values were calculated with the Clustal Omega algorithm implemented in GeneiousPrime 2023.2.1

	OP006458	OP006456	OR147841	OR147839	OR122728	OR122730	OR122729	OR184846	OP006457	OR122727	OR147842	OR147838	OR122725	OR147840
OP006458														
OP006456	98.7													
OR147841	98.8	98.8												
OR147839	98.8	98.8	100.0											
OR122728	98.6	98.6	98.9	98.9										
OR122730	81.8	81.9	82.0	81.9	81.8									
OR122729	81.3	81.2	81.3	81.3	81.2	93.0								
OR184846	81.5	81.6	81.6	81.6	81.5	83.7	83.7							
OP006457	81.5	81.7	81.6	81.6	81.5	83.7	83.7	98.9						
OR122727	82.7	82.7	82.7	82.7	82.6	85.0	84.1	90.9	91.0					
OR147842	82.4	82.5	82.5	82.5	82.4	85.5	87.2	90.5	90.5	90.8				
OR147838	82.4	82.5	82.5	82.5	82.4	85.5	87.2	90.5	90.5	90.8	100.0			
OR122725	82.0	82.1	82.2	82.1	82.1	86.2	86.7	90.5	90.6	91.2	97.0	97.0		
OR147840	82.0	82.1	82.1	82.1	82.0	86.2	86.7	90.5	90.5	91.1	97.0	96.9	99.7	
OR122726	82.0	82.1	82.1	82.1	82.0	86.1	86.7	90.5	90.5	91.1	96.9	96.9	99.6	100.0

Supplementary Fig. 1. Map of the most important European citrus growing countries (A) and map of Montenegro and Croatia showing the sampling locations of the material analyzed in this study (B). Maps were created with MapChart.net.



Supplementary Fig. 2. Sequence Demarcation Tool analyses displays colour-coded pairwise identity matix generated from 60 CTV genome sequences (15 from this study and 45 from NCBI)



Supplementary Fig. 3. Symptoms in the sweet orange (*Citrus sinensis*) cv Corrugated Navelina grafted on *Poncirus trifoliata* (sample 9A) from Croatia: A – leaf dropping, weaker turgor, shortened internodes and leaf mottling, B – honey-pitting of the bark, C - inverse stem pitting of the trunk above the bud union, necrosis of the bud union with *P. trifoliata* rootstock (photos by Luka Mustapić).