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Authors
Bester, Rachelle
Karaan, Maryam
Cook, Glynnis
et al.

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Journal of Citrus Pathology

Brief Report

First report of citrus virus A in citrus in South Africa

R Bester[1], M Karaan[1], G Cook[2] and HJ Maree[1,2*]

[1]Department of Genetics, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa; [2]Citrus Research International, P.O. Box 28, Nelspruit, 1200, South Africa

*Correspondence to: hjmaree@sun.ac.za

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Abstract

High-throughput sequencing (HTS) of citrus indicator hosts, originally inoculated from field samples and showing transient chlorotic flecking or oak leaf patterns, revealed the presence of the first South African variant of citrus virus A (CiVA). This virus was first identified in citrus in Italy and was classified as a member of the second species (*Coguvirus eburi*) of the genus *Coguvirus* within the order *Bunyavirales*. The South African sequence variants of CiVA share 95.26-95.55% (RNA1) and 94.82-97.85% (RNA2) nucleotide sequence identity with the CiVA sequences from the Italian isolate. The discovery of CiVA in South African citrus orchards indicates a wide distribution of CiVA and further investigations are required to determine the association of CiVA with citrus disease symptoms.

Keywords: CiVA, impietratura, coguvirus, chlorotic flecking, oak leaf pattern

Introduction

The movement of citrus germplasm between citrus growing countries carries the risk of disseminating graft-transmissible diseases worldwide. Most citrus producing countries established certification programs and quarantine regulations to prevent the spread of diseases through budwood interchange and supply. Processes for the detection and elimination of known pathogens are well established. However, for diseases of unknown etiology it is not possible to perform specific tests and long-term biological indexing is required. The identification and association of pathogens with diseases is therefore required to develop rapid and specific diagnostics.

The application of high-throughput sequencing (HTS) for virus discovery using a metagenomic approach has been highly successful in identifying new viral and viroid sequences (Al Rwahnih et al. 2009; Kreuze et al. 2009; Al Rwahnih et al. 2012; Espach et al. 2012; Villamor et al. 2016; Navarro et al. 2018a; Navarro et al. 2018b; Rott et al. 2018; Diaz-Lara et al. 2019; Bester et al. 2020a; Bester et al. 2020b; Chiapello et al. 2020). This technology allows for the study of virus population structure, ecology, evolution or to differentiate between virus variants that may influence disease symptomology.

Recently the genus *Coguvirus* included in the family *Phenuiviridae* in the order *Bunyavirales* (Kuhn et al. 2020), was established to include citrus concave gum-associated virus (CCGaV) that was identified using HTS (Navarro et al. 2018a; Abudurexiti et al. 2019). This virus was shown to be associated with a severe and ancient citrus disease called concave gum (CG) (Navarro et al. 2018a). Even though CCGaV is phylogenetically related to other species in the order *Bunyavirales*, the virus can be distinguished based on a bipartite RNA genome, not coding for any glycoproteins and virions lacking an external envelope (Navarro et al. 2018a). The second species of the new genus *Coguvirus*, called *Coguvirus eburi* (Kuhn et al. 2020), was created to include citrus virus A (CiVA), a new phenui-like virus identified first in citrus (Navarro et al. 2018b) and later in pear (Svanella-Dumas et al. 2019). The coguviruses have a negative-stranded RNA1, encoding the viral RNA-dependent RNA polymerase (RdRp), and an ambisense RNA2, coding for the putative movement protein and nucleocapsid protein.

Concave gum, impietratura and cristacortis are citrus diseases that occur worldwide, however the causal agents have remained elusive (Da Graça 1978). Inoculated to sweet or sour orange indicator hosts, these diseases are associated with transient chlorotic flecking or oak leaf patterns in subsequent young flush, similar to that obtained for psorosis (Bar-Joseph and Loebenstein 1970; Vogel and Bovè 1974). The distribution of affected plants and the symptoms observed after grafting symptomatic plant material would suggest the presence of virus-like causal agents (Scaramuzzi et al. 1968; Servazzi et al. 1968; Ieki and Ito 1996). Impietratura has been observed in South Africa for many years and reported on grapefruit and sweet

orange (Da Graça 1978). Symptoms include abnormal fruit drop, circular, green patterns on fruit, occasionally observed as sunken lesions and pockets of gumming found in the albedo of affected fruit. Concave gum and cristacortis have not been reported from South Africa. The identification of the genomes of CCGaV and CiVA enables investigation into their relationship with diseases of previously unknown aetiology (Navarro et al. 2018a; Navarro et al. 2018b).

In this study HTS data was used to identify potential viral pathogens in citrus repository plants that displayed transient chlorotic flecking or oak leaf patterns and led to the discovery of the first South African CiVA infection.

Materials and Methods

Budwood of citrus field trees, displaying various disease symptoms, were collected over years and inoculated to 'Madam vinous' sweet orange (*Citrus sinensis*) and maintained as disease sources in a glasshouse repository. Unfortunately, a complete symptom description of the original sources was never recorded and the maintained sources only described as ‘psorosis-like’. These indicator trees were maintained in a temperature-controlled greenhouse (24-28°C) with natural light in summer, but with additional lighting provided in winter months to supply a total of 16 hours light per day. Two indicator plants, sources 1.5 and 1.8, displayed transient chlorotic flecking or oak leaf patterns on young flush. The trees were tested for citrus psorosis virus (CPsV) with standard RT-PCR analysis (Citrus Research International (CRI), Nelspruit, South Africa).

Total RNA was extracted from the phloem layer, petioles and midribs from each sample using a modified cetyltrimethylammonium bromide (CTAB) method (Ruiz-García et al. 2019). Ribo-depleted RNA libraries were constructed with the Illumina TruSeq Stranded Total RNA Sample Preparation kit with Plant Ribo-Zero at Macrogen (South Korea). Paired-end sequencing (2x100 bp) was performed on an Illumina NextSeq instrument (Macrogen, South Korea). Adapter sequences were removed, and data trimmed for quality using Trimmomatic (Bolger et al. 2014) (SLIDINGWINDOW of 3 nts with Q20, MINLEN of 20 nts). Trimmed data were subjected to a de novo assembly using CLC Genomics Workbench 10.1.1 (Qiagen) (default parameters). The de novo assembled contigs were identified using BLAST+ standalone against a local copy of the NCBI GenBank nucleotide database (BLASTn cut-off e-value of 0.001).

The contigs identified with the highest nucleotide sequence identity to CiVA RNA1 and RNA2 (MG764565; MG764566) were selected as the draft genomes of the South African variants of CiVA. The contigs were used to design eight primer sets for RNA1 (Table 1) and five primer sets for RNA2 (Table 1) to produce overlapping amplicons, allowing for the amplification of the whole genome by RT-PCR (Table 1). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using random hexamers (Promega) and Maxima Reverse Transcriptase (Thermo Scientific) according to manufacturers’ instructions. The PCRs were performed using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) according to the manufacturers’ instructions.

The 5’ ends of the CiVA genetic variant of sample 1.8 were determined using two micrograms of total RNA in the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen). Three genome specific reverse primers for RNA1 and RNA2 (Table 1) were designed and used according to the manufacturer’s instructions. Poly(A) tailing of total RNA with E. coli Poly (A) Polymerase (New England Biolabs) was used to determine the 3’ ends of the genomes. Complementary DNA (cDNA) was synthesized from 5 µg of total RNA using an oligo(dT) primer (Table 1) and Maxima Reverse Transcriptase (Thermo Scientific) according to the manufacturer’s instructions. A genome-specific forward primer for RNA1 and RNA2 (Table 1) was designed and used in combination with the oligo(dT) primer in PCR amplifications with Phusion® High-Fidelity DNA Polymerase (New England Biolabs). Amplicon DNA was sent for bi-directional Sanger sequencing at the Central Analytical Facility (CAF) at Stellenbosch University, South Africa.

Open reading frames (ORFs) were predicted using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) and SmartBlast (NCBI). Pairwise alignments between CiVA sequence variants were performed using CLC Genomics Workbench 11.0.1 (Qiagen). Phylogenetic relationships between CiVA and representative members of the family *Phenuiviridae* and the newly identified genus *Coguvirus* were assessed. A multiple sequence alignment of the RdRp sequences were performed with MAFFT version 7 (Katoh et al. 2019), and a phylogenetic tree was constructed with 1,000 bootstrap replicates, using the maximum-likelihood method and the Jukes-Cantor model in MEGA X (Jukes and Cantor 1969; Felsenstein 1985; Kumar et al. 2018).

Results

'Madam vinous' sweet orange (*Citrus sinensis*) inoculated with two different field sources, plants 1.5 and 1.8, presented with the oak leaf pattern on young leaves (Table 2). These trees tested negative for CPsV by RT-PCR.

The raw RNAseq HTS data contained an average of 18 million paired-end reads per sample. This was reduced to an average of 16 million paired-end reads per sample after trimming and filtering. On average, the de novo assemblies generated 62,441 contigs with an average N50 of 845.

The BLASTn analysis identified citrus tristeza virus (CTV) and viroids in samples 1.5 and 1.8 based on the contig assemblies (Table 2). Additionally, contigs with 95% nucleotide identity to the CiVA RNAs (MG764565; MG764566) were assembled from both these samples.

The contigs identified as CiVA in sample 1.5 and 1.8 were 98.49% (RNA1) and 97.40% (RNA2) and 99.78% (RNA1) and 99.52% (RNA2) identical on nucleotide (nt) level to the sequences generated using Sanger sequencing,

respectively. The de novo assembled contigs represented 99.79% (sample 1.5) and 99.84% (sample 1.8) of the complete sequence for RNA1 and 99.70% (sample 1.5) and 99.52% (sample 1.8) of full-size RNA2.

The South African CiVA genome is composed of two RNAs (RNA1 and RNA2) of 6690 and 2731 nt, respectively. The two sequence variants identified (1.5 and 1.8) share 98.79% (RNA1) and 95.15% (RNA2) nt identities. The Sanger-validated sequence variants 1.5 and 1.8 were deposited in the NCBI GenBank database (MT720883; MT720884; MT720885; MT720886).

The South African CiVA RNA1 and RNA2 share identical nucleotide sequences at their 5′ and 3′ ends as reported previously for coguviruses (Navarro et al. 2018a; Navarro et al. 2018b).

Table 1
Primer properties for citrus virus A (CiVA) genome amplification

Primer name	Sequence (5'-3')	Application	Amplicon size (bp)	Primer concentration (µM)	Annealing temperature (℃)
CiVA_1_1F	TTTATAAGTTTTTTTATCAAGA	Genome amplification	321	0.3	50
CiVA_1_321R	AATTATATCCCAGAGAGTTA				
CiVA_1_87F	TTCCTTCAGTCTTGTAACCA	Genome amplification	1232	0.3	58
CiV_1_1318R	AATTTTGAGTGAGGGCTTCA				
CiVA_1_1107F	CTAACACCCACCCAGACTTT	Genome amplification	1301	0.3	60
CiVA_1_2407R	CACCCATGCGGAAGAGTATA				
CiVA_1_2273F	ACATCATCTAATGACCGCTC	Genome amplification	1224	0.3	60
CiVA_1_3496R	CTCTTTCACACACACTCTCA				
CiVA_1_3340F	GGTCTTATTATGGGATGGCA	Genome amplification	1131	0.3	60
CiVA_1_4470R	ACAGTCACTTCTACCTTCAG				
CiVA_1_4250F	GAGTCATCATAGGATCTGGA	Genome amplification	1197	0.3	58
CiVA_1_5446R	ACTAACAGACACAGAGGATA				
CiVA_1_5269F	GCAGGCAATAGTGAACTGTA	Genome amplification	1195	0.3	58
CiVA_1_6463R	GAGAGAGTTAGATGTGTCCT				
CiVA_1_6291F	CTACTTCTAACACTGATTTAAA	3' end amplification with GVA-dT(17)	381	0.5	53
CiVA_1_6661R	CCCAAACTTTAAAATAAAGT	Genome amplification CiVA_1_6291F	371	0.3	50
CiVA_2_1F	ATAACTTTTTTGTTAAAAAGC	Genome amplification	285	0.3	50
CiVA_2_285R	AATCTTGTTCCTTCACTAT				
CiVA_2_76F	CACTCCAAAACCTGTTCTAC	Genome amplification	1159	0.3	55
CiVA_2_1234R	ACACTAGACTCCAAACTCAT				
CiVA_2_1076F	GAATCTGAAGAGCCTGAAGA	Genome amplification	1116	0.3	60
CiVA_2_2191R	ACAAAGTTTCTGCTGCTGAC				
CiVA_2_2028F	ACCACTGCCTCTTTTGATTC	Genome amplification	614	0.3	55
CiVA_2_2641R	CACTCCACCAAAATCCCAAG				
CiVA_2_2454F	CTGCTCGAAACTCATAAGAA	3' end amplification with GVA-dT (17)	266	0.5	53
CiVA_2_2718R	ACACATAGAACCCATAACTTT	Genome amplification with CiVA_2_2454F	265	0.3	55
CiVA_1_307R	AGTTATAAACTCTTGAATAA	5' RACE	307	0.5	55
CiVA_1_265R	AGAGCTGAGCTGGATCAACA	5' RACE	265	0.5	55
CiVA_1_179R	TGGTAAGCCCTATCTAACAACA	5' RACE	179	0.5	55
CiVA_2_375R	TTATTCATTCTTGGTGT	5' RACE	375	0.5	55
CiVA_2_357R	AATGTGACAAGCCTTCTTACCC	5' RACE	357	0.5	55
CiVA_2_279R	CCTTCACTATACTCCTTTGCCA	5' RACE	279	0.5	55
GVA-dT (17)	TACGATGGCTGCAGTTTTTTTTT TTTTTTTT	3' end amplification (with CiVA_1_6291F or CiVA_2_2454F)	381 and 266	0.5	53

Table 2. BLASTn results of the de novo assembled high-throughput sequencing (HTS) data for samples showing transient foliar symptoms.

Sample	Symptom description	BLASTn-identified citrus viruses and viroids
1.5	Oak leaf symptoms observed on 'Madam vinous' sweet orange indicator.	citrus tristeza virus (CTV), **citrus virus A (CiVA),** citrus exocortis viroid (CEVd)
1.8	Oak leaf symptoms observed on 'Madam vinous' sweet orange indicator.	citrus tristeza virus (CTV), **citrus virus A (CiVA),** citrus exocortis viroid (CEVd), citrus dwarfing viroid (CDVd), hop stunt viroid (HSVd)

Both South African CiVA sequence variants shared more nucleotide sequence identities to sequence variant TX-NO (MK689372, MK689373) identified in the United States of America (RNA1: 95.92% and 96.13%; RNA2: 98.39% and 95.33%) compared to the Italian sequence variant W4 (MG764565, MG764566) (RNA1: 95.26% and 95.55%; RNA2: 97.85% and 94.82%). Sequence variants 1.5 and 1.8 were on average 96% (RNA1) and 77% (RNA2) identical to the pear sequence variant identified in France (MK273077; MK273078).

RNA1 of CiVA, in both sequence variants, possesses one ORF (ORF1) which is 6555 nt in length and encodes a putative protein of 2,184 amino acids (aa). SMARTBLAST analyses using the predicted aa sequences (1.5 and 1.8) of ORF1 show a 97% identity to the RNA-dependent RNA polymerase (RdRp) of CiVA sequence variant TX-NO (QEU56214). RNA2 contains two ORFs. ORF2a is 1188 nt long and encodes a putative protein of 395 aa. SMARTBLAST analyses using the derived aa sequences of ORF2a showed a 96.71% identity to the putative movement protein (MP) of CiVA sequence variant TX-NO (QEU56215) for sequence variant 1.5 and 97.22% identity to sequence variant W4 (AYN78569) for sequence variant 1.8. ORF2b is 1113 nt long in sequence variant 1.5 and 1119 nt long in sequence variant 1.8 and encodes a protein of 370 aa and 372 aa in sequence variants 1.5 and 1.8, respectively. SmartBLAST analyses using the derived aa sequences of ORF2b revealed a 98.38% (1.5) and 96.49% (1.8) identity to the nucleocapsid protein of CiVA sequence variant W4 (AYN78570).

A phylogenetic tree based on the maximum likelihood method and Jukes-Cantor model, in which CiVA sequence variants 1.5 and 1.8 group with the current members of the genus *Coguvirus*, is shown in Figure 1.

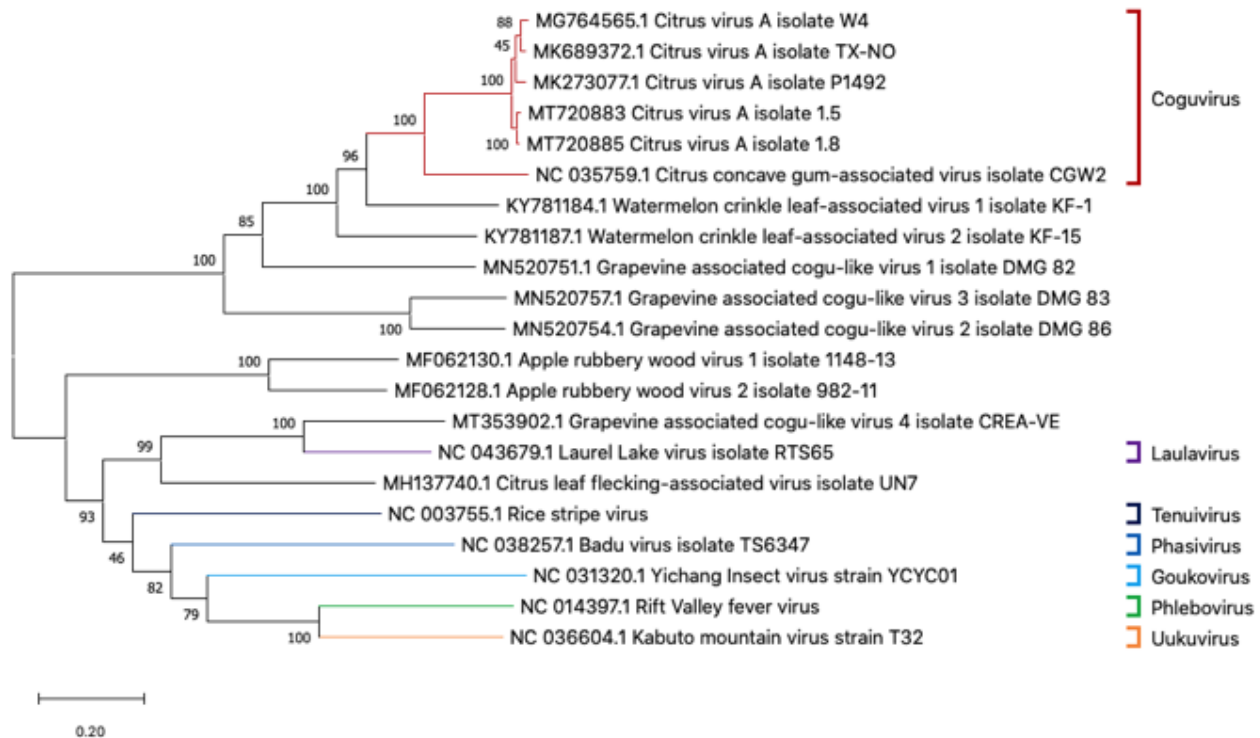

Fig. 1. Evolutionary relationships of the RdRp sequences of citrus virus A (CiVA) (sequence variants 1.5 and 1.8), representative members of the family *Phenuiviridae*, the newly identified genus *Coguvirus* and unclassified cogu-like viruses. The phylogenetic tree was constructed with 1,000 bootstrap replicates using the maximum likelihood method and Jukes-Cantor model in Mega X (Jukes and Cantor 1969; Felsenstein 1985; Kumar et al. 2018). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Discussion

This study is the first report of CiVA in citrus in South Africa. The complete genomes of two sequence variants were sequenced using both HTS and Sanger sequencing. The South African sequence variants share all the features of the previously identified negative-stranded RNA viruses of the newly confirmed genus *Coguvirus* (Navarro et al. 2018a; Navarro et al. 2018b). Phylogenetically, the South African sequence variants cluster in the genus *Coguvirus* clade with previously identified CiVA sequence variants, CCGaV and watermelon crinkle leaf-associated virus 1 and 2 (Xin et al. 2017). Even though the recently identified grapevine associated cogu-like virus 2 and 3 (Chiapello et al. 2020) clusters with the coguviruses and not with representative members of the family *Phenuiviridae*, it is possible that they represent a different genus. The phylogenetic analysis also revealed that apple rubbery wood virus 1 and 2 (Rott et al. 2018), cluster separately from the other plant-infecting bunyaviruses. This may indicate that there is greater diversity within this novel group of plant-infecting viruses of the order *Bunyavirales* and that more representative members and phylogenetic analyses are needed to resolve the diversity within this order.

The identification of CiVA in South African expands its distribution. Both prevalence of CiVA and its potential association with symptoms or disease require further investigation. A previous report found no association between CiVA and concave gum disease (Navarro et al. 2018b). In this study, two indicator plants that were inoculated with field-collected sources, developed oak leaf or flecking patterns in young leaves and were positive for CiVA. This study demonstrates the ability of HTS to identify viruses that can assist in resolving disease etiology.

In conclusion, this study reports the identification and complete genome sequence of CiVA from South Africa. Further research can focus on the incidence and host range of CiVA and on the possible association with citrus diseases.

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