Survey of Citrus tristeza virus (CTV) strains in *Citrus x limon* (L) Burm f. (Lemon) in Tucumán Province, Argentina

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Abstract

Citrus tristeza virus (CTV) causes various syndromes of citrus and consists of diverse strains which may cause symptoms of differing severity. Lemon is the most important citrus crop produced in Tucumán province, Argentina, but the diversity of CTV strains within this region has been poorly studied. In this study we identified strains of CTV in lemons in 29 trees of five commonly planted lemon cultivars from this area using direct Sanger and next generation sequencing (NGS) of amplicons derived from the CTV p33 gene. The Kpg3/SP/T3 genotype was dominant in 28 of the 29 samples analysed, with one sample being dominant for a genotype of RB. This was confirmed with NGS in all but one instance. In addition, all thirteen samples tested by NGS were infected with RB, Kpg3/SP/T3 and HA 16-5 genotypes. One sample also had a minor VT component, while a further two samples also had a minor AT-1 component.

Keywords: Citrus tristeza virus, CTV, lemon, *Citrus x limon,* genotype, Argentina

Introduction

Citrus tristeza virus (CTV) is recognized as the cause of various serious syndromes on citrus (Bar-Joseph *et al.* 1989). Symptoms that may develop include Tristeza disease (quick decline), stem pitting, seedling yellows, reduced yield and fruit sizes (Moreno *et al.* 2008). At least six distinct CTV sequence lineages (strains) have been identified and are described as: T3, T30, T36, T68, VT and the potentially novel HA 16-5 strain (Harper 2013), which may elicit variable symptoms on different hosts and environmental conditions (Broadbent *et al.* 1996; Garnsey *et al.* 1991; Sambade *et al.* 2003; Van Vuuren 2002). Even different isolates of a single strain, often with only small differences in nucleotide sequence, may cause different severities of symptoms (Harper 2013). Symptoms are further complicated by the fact that the strains often exist as mixtures within a single citrus sample (Scott *et al.* 2013) and under these conditions cannot be ascribed to individual strains.

Citrus tristeza disease was reported in Northeast Argentina in 1930 (Zeman 1931) and in the Northwest in 1947 (Foguet 1961), with millions of citrus trees on sour orange dying from quick decline in both citrus regions. More recently, orange, grapefruit and tangerines are only grafted on CTV tolerant rootstocks. Tucumán province in the Northwest Argentina, has subsequently become one of the world's leading lemon producing areas and several lemon cultivars are very well adapted to the region. The most common cultivars planted are Frost Eureka, Frost Lisbon, Limoneira 8 A Lisbon and Feminello Santa Teresa, introduced from California, as well as Genova EEAT, a local cultivar developed by the EEAOC (Foguet, *et al*. 1987). The most efficient vector of CTV, *Toxoptera citricida* (Kirkaldy), along with other aphid species, are present in Tucumán province and consequently, the disease is endemic in this area.

CTV infection of lemon has previously been reported (Mc Clean 1950; Cordas 1975) but generally the plants are symptomless and yield and growth are not affected. However, when lemon is grafted on macrophylla rootstock (*Citrus macrophylla)*, the rootstock becomes susceptible to severe stem pitting and the tree becomes stunted (Broadbent et al 1980; Calavan and Burns 1968; Herrera et al. 1995; Piquer et al. 2005; Foguet, J.L., personal communication).

Figueroa *et al.* (2012), confirmed the natural spread of CTV in lemons in Tucumán province, where it took 5,5 years after planting for all trees of each cultivar tested to become infected (Figueroa, J. personal communication). Figueroa *et al.* (2009) performed biological characterization of a number of CTV isolates from the main lemon cultivars in Tucuman province and observed variability in symptom expression among isolates.

CTV diversity is generally poorly studied in South America (Benitez-Galeano *et al.* 2015), especially on lemon. In the late 1960s CTV was reported to be widespread on lemons in Chile (Weathers and Sanchez 1970), but at that time CTV strains could not be differentiated. Multiple molecular markers were used to characterize 100 CTV isolates from Chile, six of which were from lemon (Besoain *et al.* 2015). Two of these were amplified with T30 specific primers, while the remaining four could only be amplified with universal primers, which cannot differentiate between CTV strains (T36CP) (Hilf et al. 2005). Palacios *et al.* (2013), determined the CTV strain composition of various citrus cultivars from Northwest Argentina using a strain-specific RT-PCR protocol (Roy *et al.* 2010). Fifteen samples were lemon cultivars, with VT shown to be present in all of these samples. T3 was shown to be present in thirteen of the samples and T36 in only two (which were later shown to amplify with RB-

specific primers (Palacios *et al.*, *unpublished*)). Benítez-Galeano *et al.* (2015), analysed the nucleotide sequence of the p20, p23 and p25 genes for 13 CTV sources from Uruguay, seven of which were originally obtained from lemon cultivars and maintained on rough lemon hosts for many years. In four of the seven isolates, only the VT strain occurred, while one contained only the T3 strain. The remaining two isolates were shown to be mixed population of VT, T3 and T36-like strains in the one instance, and, as well as, in the second instance, a recombinant strain named NC.

During recent studies, Read and Pietersen (2016a; 2016b), demonstrated the usefulness of Illumina amplicon sequencing for the analysis of the strain composition of CTV populations using primers that amplify the p33 gene. This gene was demonstrated to be important in superinfection exclusion of CTV strains (Folimonova *et al.* 2010; Folimonova 2012) and a determination of the variability in p33 gene sequences within citrus production areas is an important first step in understanding the disease aetiology, epidemiology and ultimately its control by cross protection. This gene was also necessary for systemic infection of lemon trees and it may be involved in interactions with host proteins of lemon for successful long-distance transport of CTV (Dawson *et al.* 2013).

The aim of this study was to determine the presence of CTV strains in lemons in Tucumán province, Argentina, within a survey of 34 samples of five commonly planted lemon cultivars, collected at three separate sites using direct Sanger- and Illumina sequencing of the p33 gene amplicons.

Materials and Methods

Collection of material

Thirty-four lemon (*Citrus limon* (L) Burm f.) samples were collected from three sites within Tucuman province, Argentina in April, 2015. Leaf material was collected from various parts of each tree. Each sample was assigned a unique accession number.

Biological indexing on indicator plants.

Four plants of each of Mexican lime, Sour orange, Duncan grapefruit and Pineapple sweet orange indicators (Garnsey *et al.* 1987) were graft-inoculated using three bark patches from each source plant separately. Indicator plants were cut back after inoculation to force new growth, and then grown as a single stem. Greenhouse temperatures during the study were maintained between 18 to 27 ºC. Symptoms (vein clearing, leaf cupping, stunting, stem pitting and others) were evaluated five times during a seven-month period (one, two, three, four and seven months after inoculation) on each of the indicator plants. Stem pitting was determined at the end of the test by peeling the bark. Symptoms were rated as: 0 = no reaction; 0.5 = very mild reaction; 1 = mild reaction; 2 = moderate reaction; and 3 =severe reaction.

RNA isolation, reverse transcription and PCR amplification

RNA extractions were carried out in Argentina with a GeneJET plant RNA isolation kit (Thermo, Vilnius, Lithuania) using 100mg of leaf midrib macerated in liquid nitrogen. The amplification of the p33 gene from each sample was carried out using a two-step RT-PCR as described by Read and Pietersen (2015). Amplicons were then shipped in 200 µl 70% ethanol to South Africa where re-amplification of the purified product was done using the same PCR protocol.

Direct sequencing of p33 gene amplicons

To remove single stranded DNA from PCR products, 0.5µl of 10 U exonuclease I (Thermo, Vilnius, Lithuania) and 2µl of 2U FastAP® (Thermo, Vilnius, Lithuania) was added to 19µl amplification products and reaction was carried out as per manufacturer's instructions. Sanger sequencing reactions were carried out by adding 1µl BigDye® Terminator mix v3.1 (Applied Biosystems, Foster City, CA, USA), 2.25µl 5x BigDye® v3.1 sequencing buffer, 0.75µl 2µM Univ-p33-F primer and molecular grade water to a total volume of 10µl, to 2µl of the purified PCR products and using 1 cycle of 94°C for 1 min, 30 cycles of 94°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Sequencing products were purified using ethanol precipitation, according to Sambrook (2001). The purified sequencing products were submitted to the African Centre for Gene Technologies (ACGT), Automated Sequencing Facility, Department of Genetics, University of Pretoria, South Africa and sequenced using an ABI Prism® 3500xl Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Sequences not conforming to a quality criterion of a minimum PHRED score of 30 were discarded from further analysis or sequencing re-done.

Phylogenetic analysis of the direct sequence data

Chromas Lite 2.1 (Technelysium, Brisbane, Australia) was used to edit and correct errors in chromatograms. Alignments of sequences were carried out using the CLUSTAL W alignment software (EBI, Cambridgeshire, England) within the BioEdit Sequence alignment editor 7.1.3 (Hall 1999). The cognate p33 gene region was trimmed from 60 full-genome reference sequences accessed from GenBank (www.ncbi.nlm.nih.gov/genbank) and include the following accession numbers (strain names in brackets): AB0463981 (NUagA); AF001623 (SY568); AF260651 (T30); AY170468 (T36); AY340974 (Qaha); KC333868 (CT-ZA3); DQ151548 (T318A); DQ272579 (Mexico); EU076703 (B165); EU857538 (SP); EU937519

(VT); EU937521 (T36); FJ525431 (NZRB-M12); FJ525432 (NZRB-G90); FJ525433 (NZRB-TH28); FJ525434 (NZRB-TH30); FJ525435 (NZRB-M17); FJ525436 (NZ-B18); GQ454869 (HA18-9); GQ454870 (HA16-5); HM573451 (Kpg 3); JF957196 (B301); JQ061137 (AT-1); JQ798289 (A18); JQ911663 (CT14A); JQ911664 (CT11A); JQ965169 (T68); JX266713 (Taiwan-Pum/M/T5); JX266712 (Taiwan-Pum/SP/T1); KC262793 (L192GR); KC333868 (12-8); KC333869 (12-9); KC517485 (FS674-T36); KC517486 (FS701-T36); KC517487 (FS703-T36); KC517488 (FS577); KC517489 (FS701-T30); KC517490 (FL278-T30); KC517491 (FS703-T30); KC517492 (FS703-VT); KC517493 (FL202-VT); KC517494 (FS701-VT); KC525952 (T3); KF908013 (Crete 1825); KJ790175 (Mac39); KR263170 (Mac25); KU356770 (CA-RB-AT25); KU358530 (CA-RB-AT35); KU361339 (CA-VT-AT39); KU361340 (CA-RB-115); KU578007 (CA-T30-AT4); KU589212 (CA-S1-L); KU589213 (CA-S1-L65); KU883265 (B390-5); KU883266 (Maxi); KU883267 (LMS6-6); NC_001661 (T36); U16304 (T36); U56902 (VT); Y18420 (T385).

Neighbour-joining phylogenetic trees were constructed for each alignment, using MEGA 6 (Tamura *et al.* 2013) and the Maximum Composite Likelihood substitution model with a 1000 bootstrap replicates.

Illumina MiSeq sequencing

The p33 amplicons from a subset of samples were subjected to Illumina MiSeq sequencing (Illumina, San Diego, CA, United States). Paired-end DNA libraries were prepared using the Nextera V2 sample kit (Epicentre, Madison, WI, United States). The samples were sequenced at the Agricultural Research Council (ARC), Biotechnology Platform, Pretoria, South Africa.

Illumina MiSeq data analysis

All trimming and analyses of the Illumina MiSeq datasets was carried out using CLC Genomics workbench 5.5.1 (CLC, Aarhus, Denmark). Data were imported as paired-end reads with a distance range of 180-300. Adapter and quality trimming was performed using the default program settings with Nextera V2 transposase adapter sequences (Transposase1: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG; Transposase2: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG). Quality control was carried out using the Fast QC function. Datasets were mapped to the cognate p33 region of a set of reference sequences and the following reference assembly parameters: Length fraction: 0.9; similarity fraction: 0.9; global alignment: off; non-specific match handling: map randomly (Read and Pietersen 2015).

Cloning and sequencing of amplified products

p33 gene amplicons of sample 15-4058 were cloned using the pGEM-T Easy vector (Promega, Madison, WI, United States) according to the manufacturer's specifications and used to transform competent *E. coli* JM109 cells. Putative recombinants were selected using blue/white selection and plasmid extractions were performed using alkaline lysis. Plasmid inserts were amplified using the T7 (5'- TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'- ATT TAG GTG ACA CTA TAG AA –3') vector specific primers and PCR conditions described earlier. 48 amplicons displaying the correct amplicon size were purified using FastAP and ExoI enzymes as described earlier, followed by Sanger sequencing using the same conditions as for the p33 gene amplicons, except for the use of T7 as the sequencing primer.

Results

The location, year planted and host cultivar of lemon samples collected, along with the results of sequence comparisons using the nucleotide sequence of the p33 gene (Figure 1) is presented in Table 1. In the majority of cases, this method identifies the dominant CTV genotype present within any CTV source.

Thirteen samples, representative of lemon cultivar, geographic area or dominant CTV strain were selected for further analysis of the composition of the CTV strain in the population. The number of reads mapping to various references strains are presented in Table 2. Argentinian strains had a sequence identity between 0.869 and 0.998 amongst each other within the p33 gene sequenced area.

A discrepancy between direct sequencing and Illumina sequencing with regards to the strain identified as dominant was observed in only one sample (15-4058). To resolve this discrepancy, the p33 gene amplicon was cloned and multiple clones sequenced and the sequence aligned with cognate regions of reference CTV strains (Figure 3). This confirmed that both a Kpg3/SP/T3 (the sequence within this region of the genome does not differ amongst these three strains) strain and an RB strain (most closely related to the Taiwan-Pum/SP/T1 isolate) were present at high levels.

When inoculating the five CTV isolates which were selected to represent the diversity of sequences observed (listed in Figure 1, *Table 3*), they all induced similar symptoms on the indicator hosts. All isolates were associated with severe leaf symptoms (vein clearing or leaf cupping), mild to moderate stem pitting on all replicates of Mexican Lime as well as little or no stunting (depending on replicate) on this host. None of them induced seedling yellows on Duncan grapefruit or Sour orange. They also did not cause stem pitting in any replicate of sweet orange. Isolates 15-4049 and 15-4057, both from Eureka lemon, induced mild stem-pitting on some replicates of Duncan grapefruit.

Discussion and conclusions

This study involved the collection of 34 leaf samples from various lemon cultivars in the province of Tucumán, Argentina. Lemon cultivars were selected as the focus of this study, since Tucumán province is one of the leading lemon producing regions worldwide, as well as the fact that populations of CTV on lemon have been poorly characterized. The primary objective was to characterise the diversity of the CTV populations, associated with the 34 samples collected, through the amplification of the p33 gene (Read and Pietersen 2015), followed by direct Sanger and Illumina sequencing on selected samples. In addition, five populations, each representing a specific collection site, were selected and inoculated onto various indicator hosts to index symptom severity.

All 34 of the collected samples were sequenced with Sanger direct sequencing, however the identities of five of these could not be determined, probably due to population heterogeneity. The populations of 28 of the remaining 29 samples were dominated by a CTV strain with a p33 gene sequence of the Kpg3/SP/T3 genotype, while only one population contained a dominant RB-like p33 gene sequence. A subset of thirteen samples was selected for Illumina MiSeq amplicon sequencing. Analysis of the Illumina sequencing reads suggests that all thirteen samples contained RB, Kpg3/SP/T3 and HA 16-5-like sequences. Kpg3/SP/T3-like sequences were dominant in eleven of the thirteen datasets and present at levels between 29% and 72% of total mapped reads. The remaining two populations were dominant for RB-like sequences at 46% and 66% of total mapped reads. HA16-5-like sequences were always present as minor components at levels between 5% - 25% of total mapped reads. Additionally, VT and AT-1-like sequences were detected at very low levels (less than 1% of total mapped reads) in three of the Illumina MiSeq datasets. Agreement was found between the dominant sequence-type among the Illumina MiSeq datasets and the strain identified with direct Sanger sequencing in all but a single instance (15-4058). Sequencing of multiple clones

derived from the 15-4058 population, confirmed the presence of both RB (33% of sequenced clones) and Kpg3/SP/T3 (66% of sequenced clones). The relative ratio of these genotypes contradicts the results of the Illumina MiSeq data. However, since the Illumina Miseq dataset contains more information in orders of magnitude, coupled with the fact that the cloning experiment failed to detect HA 16-5-like sequences, more confidence should be placed in these results.

Specific strains could not be attributed to symptoms observed in the empirical indicator host experiment. There was also a large degree of variability between the symptoms observed among biological replicates. There was also no correlation between the symptoms observed and the specific indicator hosts, suggesting that the symptom expression is closely associated with cultivars.

The relative uniformity of CTV population structure among lemons was surprising when compared with grapefruit populations from north-western Argentina that were analysed with the same protocol (Read *et al.* unpublished results). When considering the phylogeny of CTV whole genome sequences, Kpg3 clusters as a divergent member of the VT strain and therefore our data supports the study of Palacios *et al.* (2013), who showed the primary presence of VT and T3 along with a further strain, shown later to be RB on lemons. In this study we report the occurrence of an RB strain in Argentina for the first time. Additionally, HA 16-5-like reads were detected in all the CTV populations in this study. The presence of this strain on lemon was previously reported in neighbouring Uruguay, where it was referred to as “NC” (Benitez-Galeano *et al.* 2015). The T30 genotype was previously reported on lemons in neighbouring Chile (Besoain *et al.* 2015), however it was not found to be present among any of the populations in this study. We did not observe the divergent CTV strain from lemons in Greece described by Varveri *et al.* (2015) (Figure 1 KC262793-L192GR). The results from this study cannot be directly compared to the specific CTV genotypes found

by Harper and Pearson (2015) on lemon (13 samples) from New Zealand and the South Pacific as different regions of the genome were utilized for phylogenetic analysis. However, Harper and Pearson (2015) showed that lemon cultivars may harbour populations of CTV composed of all currently classified strains. Their results showed that T3 is the most common strain found on lemon in New Zealand, followed by RB and VT. HA16-5 was not considered as a defined strain by Harper and Pearson (2015). The incidence of T3 and RB in New Zealand lemon is comparable to the results of this study where either Kpg3/SP/T3 or RB genotypes were dominant. The virtual absence of the other strains defined by Harper (2013), namely B165, VT, T30, T36 and T68 could suggest their absence from the overall CTV population in Tucumán.

While lemon cultivars are generally tolerant to CTV infections, it is essential to identify which strains persist in a particular region (Varveri *et al.* 2015), in order to determine the risks posed to citriculture by CTV. This is especially important to a country such as Argentina, which does not make use of CTV cross-protection as a control measure. Therefore, caution should be taken when introducing CTV sensitive cultivars like grapefruit (van Vuuren and Manicom, 2005) to an area such as Tucumán, as the spread of endemic CTV strains to these cultivars could lead to a reduction in productivity. Empirical biological data from this study already shows that lime and grapefruit cultivars could be at risk of stunting and decline symptoms. More work should be carried out to isolate individual components of the CTV populations from Tucumán, which will allow for the determination of which strains are responsible for eliciting severe disease responses (Read and Pietersen 2016b). In addition to this, CTV populations on different cultivars in their respective production areas should be characterized, in order to gain a more comprehensive representation the CTV strains present in Argentina.

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Table 1: CTV genotype identified using the direct, Sanger-determined sequence of the p33 gene amplicon for lemon samples collected in Tucumán, Argentina

Acc no. (SA)	*Citrus limon* (Lemon) cultivar	Rootstock	Collection site in Tucumán, AR	Year planted	Genotype by direct sequencing
15-4017	Femminello Santa Teresa	Sour orange (*Citrus aurantium*)	EEAOC	1974	n/d
15-4018	Femminello Santa Teresa	Sour orange (*Citrus aurantium*)		1974	Kpg3/SP/T3
15-4023	Limoneira 8A Lisbon	Sour orange (*Citrus aurantium*)		1977	Kpg3/SP/T3
15-4024	Limoneira 8A Lisbon	Sour orange (*Citrus aurantium*)		1977	Kpg3/SP/T3
15-4025	Limoneira 8A Lisbon	Sour orange (*Citrus aurantium*)		1977	Kpg3/SP/T3
15-4034	Frost Eureka	Benton citrange (*Poncirus trifoliata x Citrus sinensis*)		1979	Kpg3/SP/T3
15-4035	Frost Eureka	Benton citrange (*Poncirus trifoliata x Citrus sinensis*)		1979	Kpg3/SP/T3
15-4036	Frost Eureka	Benton citrange (*Poncirus trifoliata x Citrus sinensis*)		1979	Kpg3/SP/T3
15-4037	Frost Eureka	Cleopatra mandarin (*Citrus reshni*)	Finca Santa Isabel	1980	Kpg3/SP/T3
15-4038	Frost Eureka	Cleopatra mandarin (*Citrus reshni*)		1980	Kpg3/SP/T3
15-4039	Frost Eureka	Cleopatra mandarin (*Citrus reshni)*		1980	Kpg3/SP/T3
15-4040	Genova EEAT	Swingle citrumelo *(Poncirus trifoliata* x *Citrus paradisi*)		1985	Kpg3/SP/T3
15-4041	Genova EEAT	Swingle citrumelo *(Poncirus trifoliata* x *Citrus paradisi*)		1985	Kpg3/SP/T3
15-4042	Genova EEAT	Swingle citrumelo *(Poncirus trifoliata* x *Citrus paradisi*)		1985	n/d
15-4043	Genova EEAT	Swingle citrumelo *(Poncirus trifoliata* x *Citrus paradisi*)		1985	Kpg3/SP/T3
15-4044	Genova EEAT	Swingle citrumelo *(Poncirus trifoliata* x *Citrus paradisi*)		1985	Kpg3/SP/T3
15-4045	Limoneira 8A Lisbon	Swingle citrumelo *(Poncirus trifoliata* x *Citrus paradisi*)		1987	Kpg3/SP/T3
15-4046	Limoneira 8A Lisbon	Swingle citrumelo *(Poncirus trifoliata* x *Citrus paradisi*)		1987	Kpg3/SP/T3
15-4047	Frost Eureka	Volkamer lemon (*C. volkameriana*)	Finca El Naranjo	1979	n/d
15-4048	Frost Eureka	Volkamer lemon (*C. volkameriana*		1979	Kpg3/SP/T3
15-4049	Frost Eureka	Volkamer lemon (*C. volkameriana*		1979	Kpg3/SP/T3
15-4050	Frost Lisbon	Rubidoux trifoliate (*Poncirus trifoliata*)	Finca Monte Grande	1992	n/d
15-4051	Frost Lisbon	Rich trifoliate (*Poncirus trifoliata*)		1995	Kpg3/SP/T3
15-4052	Limoneira 8A Lisbon	Troyer citrange (*Poncirus trifoliata* x *Citrus sinensis)*		1987	n/d
15-4053	Limoneira 8A Lisbon	Troyer citrange (*Poncirus trifoliata* x *Citrus sinensis)*		1984	Kpg3/SP/T3
15-4054	Limoneira 8A Lisbon	Flying Dragon trifoliate (*Poncirus trifoliata* var monstrosa)		1998	Kpg3/SP/T3
15-4055	Limoneira 8A Lisbon	Flying Dragon trifoliate (*Poncirus trifoliata* var monstrosa)		1997	Kpg3/SP/T3
15-4056	Frost Eureka	Rangpur lime (*Citrus limonia*)		1976	Kpg3/SP/T3
15-4057	Frost Eureka	Rangpur lime (*Citrus limonia*)		1991	RB
15-4058	Frost Lisbon	Flying Dragon trifoliate (*Poncirus trifoliata* var monstrosa)		1997	Kpg3/SP/T3
15-4059	Frost Lisbon	Flying Dragon trifoliate (*Poncirus trifoliata* var monstrosa)		1996	Kpg3/SP/T3
15-4060	Genova EEAT	Troyer citrange (*Poncirus trifoliata* x *Citrus sinensis)*		1993	Kpg3/SP/T3
15-4061	Genova EEAT	Troyer citrange (*Poncirus trifoliata* x *Citrus sinensis)*		1992	Kpg3/SP/T3
15-4062	Frost Lisbon	Rich trifoliate (*Poncirus trifoliata*)		1993	Kpg3/SP/T3

Table 2: Results of Illumina MiSeq read mapping to various CTV strains for representative samples from lemon trees collected in Tucumán, Argentina. Samples grouped by collection site. † = sequence type found with the direct sequencing.

				Percentage of total reads mapping to reference sequence types					
Sample number	***C. limon* cultivar**	**Collection site**	**Total number of reads mapping to references**	**RB**	**Kpg3/ SP/ T3***	**HA 16-5**	**VT**	**AT-1**	**B165**
15-4023	Limoneira 8A Lisbon	EEAOC	319135	24.2	60.7†	14.8	-	-	-
15-4034	Frost. Eureka		1000573	43.7	44.1†	12.1	-	-	-
15-4037	Frost Eureka	Finca Santa Isabel	750547	24.4	67†	8.5	-	-	-
15-4040	Genova EEAT		783243	35.7	50.6†	13.5	-	-	-
15-4043	Genova EEAT		560235	17.3	71†	11.5	-	0.1	-
15-4046	Limoneira 8 A Lisbon		650729	38.2	45.5†	16.2	-	-	-
15-4049	Frost. Eureka	Finca El Naranjo	566832	24.9	50†	25	-	-	-
15-4051	Frost. Lisbon	Finca Monte Grande	664469	14.1	71.1†	14.7	-	-	-
15-4055	Limoneira 8 A Lisbon		361570	33	62†	5	-	-	-
15-4057	Frost. Eureka		715537	65.6†	29.2	5	-	-	-
15-4058	Frost Lisbon		435444	46.2	35.3†	17.8	0.5	-	-
15-4060	Genova EEAT		551144	20.6	65.7†	13.7	-	-	-
15-4062	Frost Lisbon		168543	12	71.9†	15.8	-	0.2	-

* This part of the CTV genome does not resolve amongst Kpg3 or SP or T3 genotypes.

Table 3: Symptoms observed on various citrus indicator hosts inoculated with CTV isolates from lemon from this study observed 7th month post-inoculation. Symptom rating scale: 0 = **no** reaction; 0.5 = very mild reaction; 1 = mild reaction; 2 = moderate reaction; and 3 =severe reaction. Values provided for each replicate separately.

	Symptom rating on the each of four replicates of various indicator hosts							
	Mexican lime			**Duncan**			**Sour orange**	**Sweet orange**
	Leaf symptoms	**Stunting**	**Stem pitting**	**Seedling yellows**	**Stunting**	**Stem pitting**	**Seedling yellows**	**Orange Stem pitting**
15-4040	3-3-3-3	0-1-2-2	1-2-2-2	0-0-0-0	0-0-2-2	0-0-0-0	0-0-0-0	0-0-0-0
15-4060	2-2-3-3	0-0-0-2	0,5-0,5-2-3	0-0-0-0	0-0-3-3	0-0-0-0	0-0-0-0	0-0-0-0
15-4049	3-3-3-3	1-1-1-2	1-1-2-2	0-0-0-0	1-1-2-2	0-0-1-1	0-0-0-0	0-0-0-0
15-4057	3-3-3-3	1-1-1-1	1-2-2-3	0-0-0-0	0-0-1-1	0-0-0,5-0,5	0-0-0-0	0-0-0-0
15-4058	1-1-3-3	0-1-2-3	0,5-0,5-0,5-0,5	0-0-0-0	0-1-2-3	0-0-0-0	0-0-0-0	0-0-0-0

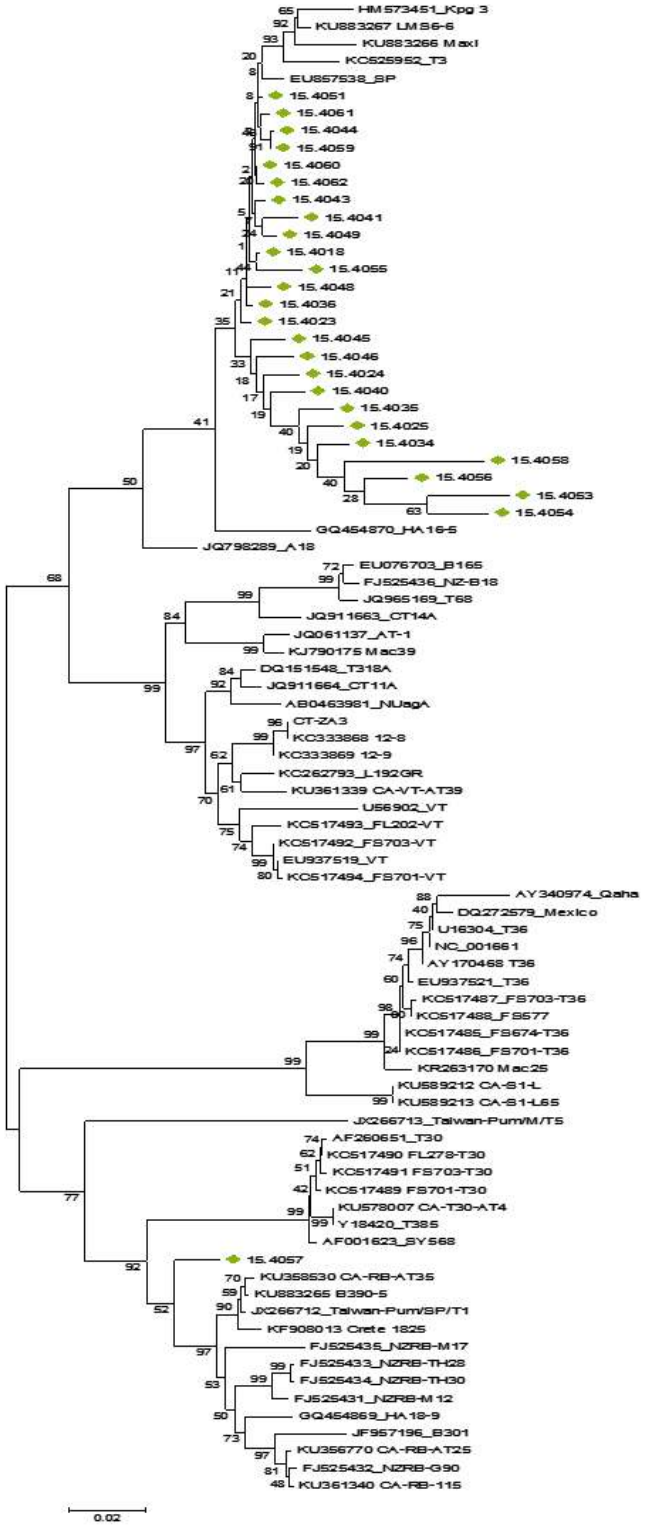

Figure 1: Neighbour-joining dendrogram produced using the Maximum Composite Likelihood Model (MCL), displaying the relationships between the direct nucleotide sequences of the p33 gene amplicons derived from lemon samples collected in Argentina. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

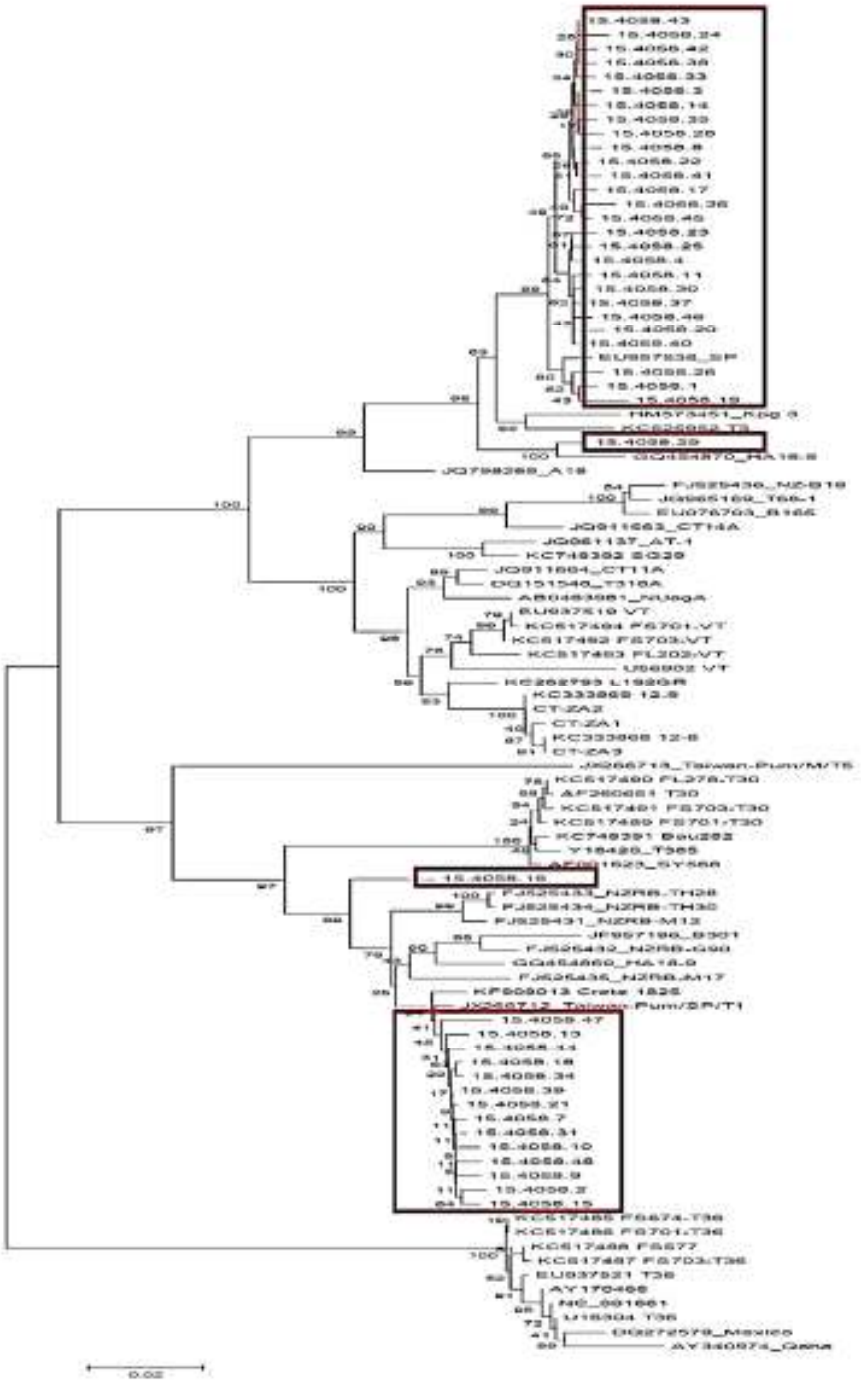

Figure 2: Dendrogram of maximum composite likelihood analysis (bootstrapping of 1000) of nucleotide sequencing of multiple clones (n = 43) (in red blocks) of the p33 gene amplicon in sample 15-4058 compared against cognate regions of known CTV genotypes. The nucleotide sequence of each clone is annotated 15.4058-#, where # is the number of the clone.