

# Severe *Citrus tristeza virus* Isolates from Eastern Mexico Are Related to the T36 Genotype Group

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## Abstract

*Citrus tristeza virus* (CTV) outbreaks have been reported in the main citrus growing region of Mexico in the past four years. Recently, in eastern Mexico (the major citrus-growing region in the country), severe CTV isolates have been detected. However, the molecular identity of observed isolates remains unestablished. This research was undertaken to elucidate the molecular characterization of CTV populations spreading in this region and to compare it with phylogeny of existing isolates. Genotyping of 32 collected isolates was performed using reverse-transcription polymerase chain reaction (RT-PCR) with sequence analysis of the coat protein (CP) gene, putatively associated with pathogenicity. This protein is a 25 kDa major capsid protein, which forms a long virion body coating 95% of the particle length. A comparative sequence analysis was performed using CTV sequences from different geographical origins already published and deposited in the GenBank databases. Phylogenetic analysis showed that the degree of sequence divergence among isolates correlated with their pathogenicity. Based on the sequencing results, the collected isolates were categorized as mild or severe phylogenetic clusters, each being genetically distinct. The severe group was associated with either a-like or with a T36-like genotype. The latter group matched with the quick decline and stem pitting drastic symptoms observed in the field. This study identified the presence of severe CTV isolates related to the T36-like genotype

and to the cause of quick decline and stem pitting in sweet orange propagated on sour orange rootstock. Knowledge derived from these analyses could serve to design management strategies for this disease and to understand the current epidemic outbreak in scenarios where the most efficient vector is present.

## Keywords

CTV, Epidemiology, Severe Isolates, Stem Pitting, Quick Decline, Coat Protein Gene

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## 1. Introduction

The *Citrus tristeza virus* (CTV), a member of the genus *Closterovirus* in the family *Closteroviridae*, is the causal agent of one of the most destructive viral diseases of citrus and is responsible for the death of over 100 million trees over the last century [1] [2]. The disease is a concern in Mexico, where about 568,188 citrus hectares are grown in 24 states yielding 7.8 million tons of fruit [3]. CTV spread occurs by introduction of infected plants or propagation material, and by several aphid species that transmit the virus semipersistently [4]. The brown citrus aphid [*Aphis citricidus* Kirkaldy (Syn. *Toxoptera citricida*)], considered the most efficient vector of CTV [4] [5] [6] [7], entered the country in 2000 through the Yucatan Peninsula and by 2007 it was already in Veracruz, the major citrus-growing region in the country [8].

CTV virions are filamentous particles of about  $2000 \times 11$  nm, with two coat proteins of 25 kDa (major capsid protein, CP) and 27 kDa (minor capsid protein, CPm) that cover approximately 95% and 5% of the virions, respectively [9] [10]. Its genome is monopartite, composed of a simple RNA chain of  $\approx 19.3$  kb and is organized in 12 open reading frames (ORFs) encoding at least 19 proteins [11]. Natural CTV infection occurs as mixtures of genomic variants, which occur in trees exposed to constant re-infestation of aphids [12] [13].

Worldwide, CTV has been reported to infect almost all citrus species, including their hybrids, and can cause various symptoms depending on the virus strain, the host and scion/rootstock combinations [14]. The most common severe symptoms of CTV are: 1) quick decline or death of trees grafted on sour orange (*Citrus aurantium* L.) rootstock; 2) stem pitting of the scion; and 3) seedling yellows on sour orange, lemon and grapefruit. In the field conditions, strains that not induce visible symptoms on commercial citrus varieties grafted on tolerant rootstock have been considered mild isolates, whereas strains inducing stem pitting, regardless of the rootstock, are considered as severe isolates [1]. CTV isolates are classified into six major genotype groups or strains, namely T3, T30, T36, T68, VT and RB. The classification is based on the nucleotide sequence similarity of the ORF1a [15], which shows levels of sequence identity between CTV variants between 72.3% and 90.3%.

In Mexico, the presence of severe CTV type variants is indicated in some

zones of the states of Tamaulipas and Baja California [16]. In these regions, detection of the T30 isolate, widely spreading in the citrus growing areas, has predominated [17] [18] [19] [20] [21]. In Veracruz, the occurrence of CTV structural changes within the spectrum of severe type isolates is reported by other authors [22]. In this region, the epidemiological condition of CTV, defined by the presence of the most efficient vector *Aphis citricidus*, and the predominance of the most CTV-susceptible rootstock (sour orange) in more than 90% of the orchards, prompts the need to establish the identity of reported isolates and to study their epidemiological behavior. The objective of this study was to determine and molecular features of CTV isolates spreading in Veracruz using RT-PCR and sequencing of the CP region.

## 2. Materials and Methods

### 2.1. Virus Isolates

Samples from CTV-infected plants were collected between 2016 and 2017 along two transects in northern Veracruz, principal citrus-growing state of Mexico. The sampling was directed at trees with symptoms of decline, stem pitting and branch death. From each tree, young shoots were obtained from the four cardinal points at 1.50 m from the ground level. The collected material was stored at 4°C until processed in the laboratory.

### 2.2. RNA Extraction

Total RNA was extracted using 0.1 g of mid ribs from CTV infected leaves were ground in 500 µL of saline buffer (NaCl 1.4M, Tris-HCl 0.1M pH 8.0) following the protocol by Harris [23]. Subsequently, 600 µL of 2% CTAB, 0.5% β-mercaptoethanol in RNase free water was added, mixed by vortexing and incubated at 55°C for 30 min. Next, 400 µL of phenol: chloroform: isoamyl alcohol (25:24:1) were added, mixed by vortexing and centrifuged at 14 000 rpm for 10 min at 4°C. From the aqueous phase 500 µL were transferred to a new microcentrifuge tube; 1/10 of a volume of ammonium acetate one volume of isoamyl alcohol was added and incubated at -20°C for 10 min. Afterwards, the samples were centrifuged at 14,000 rpm for 5 min, the top layer was discarded and the pellets washed with 1 mL of 70% ethanol and centrifuged again at 14,000 rpm for 1 min. After centrifugation, the liquid was discarded and the pellets dried at room temperature before suspending in 30 µL of RNases-free water [5]. The quantity and quality of the RNA were determined by spectrophotometry (Thermo Scientific NanoDrop™ 2000).

### 2.3. Amplification of the CP Genomic Region

For amplification of the CP genomic region (672 bp), reverse transcription polymerase chain reaction (RT-PCR) with set of primers IRA1 5'ATGGACGAC-GAAACAAAGAAATTG3' and IRA2 5'GCTCAACGTGTGTAA3' [16] was used. To generate the cDNA, approximately 200 ng of total RNA were denatured

at 95°C for 5 min. Afterwards, 1 µL of 10 µM of each primer were added, along with 1 µL of 10 mM MgCl<sub>2</sub>, 0.4 µL of 10 mM dNTPs, 0.2 µL (40 U) of RNaseOUT (Invitrogen, Carlsbad, CA, USA), and 0.2 µL (200 U) of M-MLV reverse transcriptase (Promega, Madison, WI), up to a final volume of 6 µL and incubated at 42°C for 40 min.

The CP genes were amplified by PCR from the cDNA by combining 2 µL of cDNA, 2.5 µL of 10X Buffer, 1.25 µL of 50 mM MgCl<sub>2</sub>, 0.5 µL of 10 mM dNTPs, 0.25 µL of 10 µM each primer, 0.125 µL (1.25 U) of Taq DNA Polymerase (Promega, Madison, WI) and RNase-free water to a final volume of 25 µL. The amplification was carried out in a T100 thermocycler (BioRad, Hercules, CA) and consisted from 1 cycle at 95°C for 1 min, 35 cycles of 1 min at 94°C, 1 min 55°C, and 1 min at 72°C followed by a period of final extension of 5 min at 72°C.

## 2.4. Sequencing and Phylogenetic Analysis

PCR products were resolved in 1% agarose gels, then were purified utilizing a commercial kit, and sequenced by an external company (Macrogen, Seoul, Korea). DNA Sequences were manually edited using PreGap and Gap [24] to obtain consensus sequences.

The consensus sequences were aligned and manually edited using MUSCLE [25]. The sequences were analyzed with maximum parsimony (MP) (PAUP4.0a167) [26] and Bayesian Inference (BI) (MrBayes v.3.2) [27] to infer phylogenies. MP analysis was carried out utilizing 1000 random-sequence-addition replicates and tree-bisection and reconnection (TBR) with branch swapping, excluding gaps and non-informative characters, leaving a total of 586 positions in the final matrix. Nodes support was determined for bootstrap values with 1000 replicates. BI analysis was performed with ten million generations, where four MCMCs (Markov Chain Monte Carlo) were run simultaneously. The branch lengths were calculated as the average rates of change at each branch of the largest posterior probability trees. Phylogenetic trees were visualized and edited using Mesquite v3.61 [28].

Accession numbers for previously reported CTV nucleotide sequences used in this study were: T36 (U166034), T30 (AF260651), VT (U56902), T385 (Y18420), SY568 (AF001623), NUagA (AB011185), CTV (DQ272579), NZ-M16 (EU857538), NZ-B18 (FJ525436), TAM11 (AF342890 and AY652895), NL8 (AF342891 and AY652892), VER2-2 (AF342892), QR2753-1 (AF342893 and AY652893), BC15-1 (AF342894 and AY652899) MichL9A11/9-1 (AF342895), Colima 1997 (AY649491) y Yucatán 2000 (AY649492).

## 3. Results

### 3.1. Symptoms of Severe CTV in Sweet Orange Trees

Typical CTV symptoms on sweet orange trees were observed in all trees sampled in the study. The severe symptoms of CTV isolates were identified only on sour orange rootstock. These symptoms varied from a vein clearing, death of branches, stem pitting of the scion that reduces yield and fruit quality to a gradual tree de-

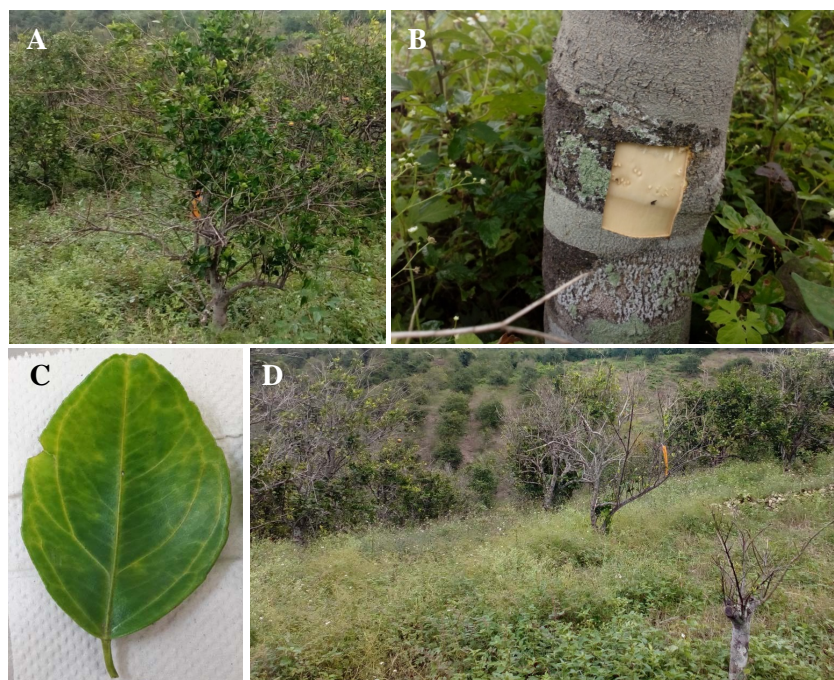
clining (**Figures 1(A)-(D)**).

### 3.2. Phylogeny of CTV Isolates

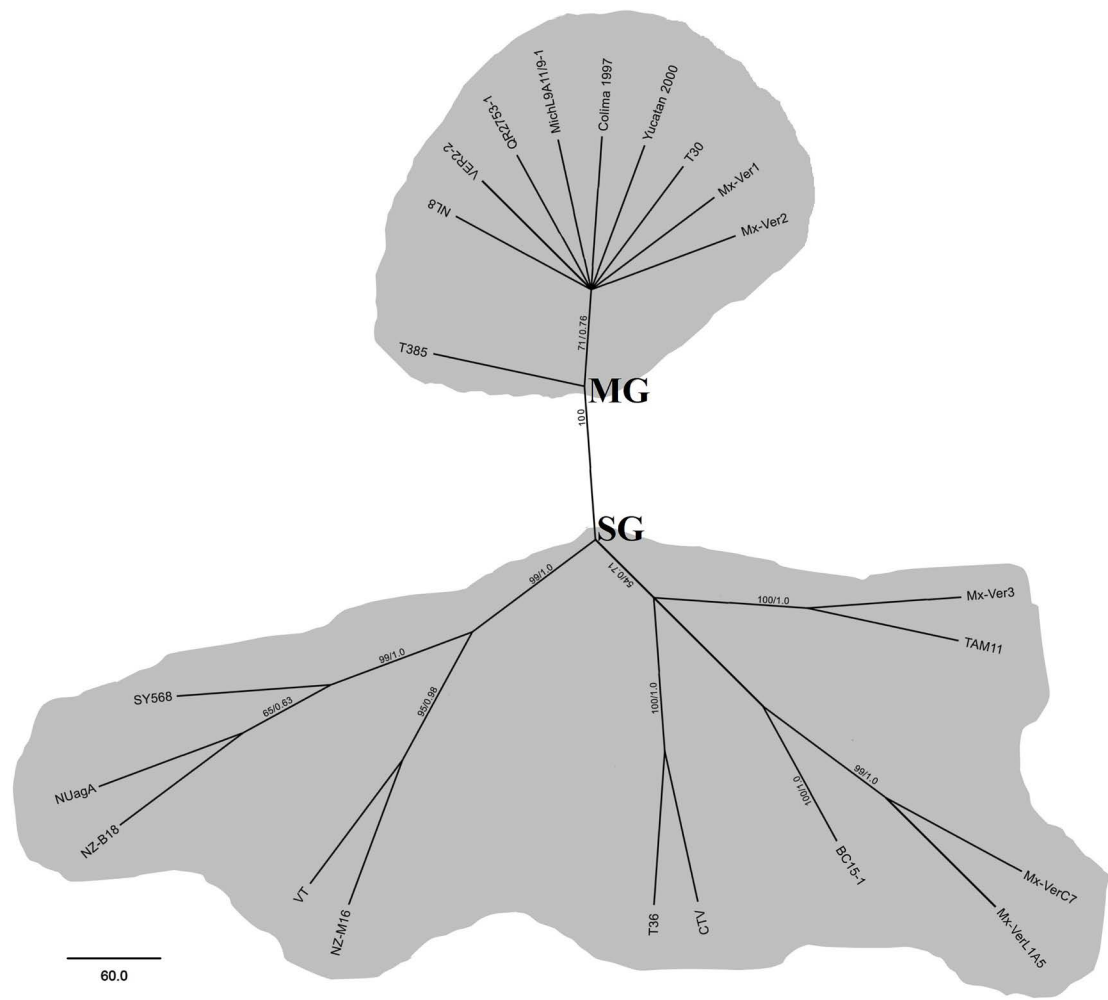
In total, a dataset of 586 nucleotides of the CP region of the CTV genome were determined. Alignments of this genomic region indicated that variations at nucleotide level were simple nucleotide changes. Overall similarities for the CP region among CTV Mexican isolates ranged from 86.7% to 99.8% at the nucleotide level (78 and 1 nucleotides, respectively). However, the sequences of these strains clustered into two groups that had very high sequences homology (99.8% identical nucleotides, only one transition T × C) such as Mx-Ver1 and Mx-Ver2 and less sequences homology (91% identical nucleotides) such as Mx-VerC7, Mx-VerL1A5 and Mx-Ver3 (data not shown).

The maximum parsimony and Bayesian inference method were used to generate the phylogenetic tree for the CP region including the following full length CTV sequences: T36 [11] and T30 from Florida [29], VT from Israel [30], T385 from Spain [31], SY568 from California [32], NUagA from Japan [33], CTV from Mexico [34], NZ-M16 and NZ-B18 from New Zealand [35]; and CP sequences from Mexico: TAM11, NL8, VER2-2, QR2753-1, BC15-1, MichL9A11/9-1, Colima 1997 and Yucatán 2000 [16].

In the phylogenetic tree a very consistent and defined cluster was observed with a bootstrapping over 99, which included the highly homologous Mexican isolates (Mx-Ver1 and Mx-Ver2) as well as isolates T30, Yucatán 2000, Colima 1997, MichL9A11/9-1, QR2753-1, VER2-2, NL8 and T385 (**Figure 2**).



**Figure 1.** Symptoms of severe CTV in sweet orange (*Citrus sinensis*). (A) Death of branches; (B) Stem pitting; (C) vein clearing; (D) Quick decline. In each scenario, CTV presence was confirmed as positive by RT-PCR and was related to the T36-like genotype.



**Figure 2.** Maximum Parsimony analysis (MP) of *Citrus tristeza virus* using coat protein (CP) gene sequences. Phylogenetic tree was generated using PAUP as indicated in Materials and Methods. A similar topology was generated using Bayesian inference (BI) with MrBayes. Only one of the seven most parsimonious trees (length = 171) is shown. The consistency and retention indices were 0.7572 and 0.9000, respectively, while the homoplasy index was 0.2428. Bootstrap support values (>60%) based on 1000 replicates and Bayesian posterior probabilities (>0.6) based on ten million generations are indicated at the internodes in the order MP/BI.

On the other hand, CTV isolates Mex-Ver3, Mex-VerC7, Mx-VerL1A5, NUagA, VT, SY568, NZ-B18, NZ-M16, TAM11, BC15-1, CTV and T36, which induce severe symptoms, showed a more disperse phylogenetic distribution (**Figure 2**). The comparative analysis revealed that the severe Mexican isolates Mex-Ver3, Mex-Ver7 and Mx-VerL1A5, tended to be clustered away from the group formed by SY568, NUagA, NZ-B18, VT and NZ-M16, although not enough to be considered a separate cluster.

#### 4. Discussion

The focus of this study was to characterize the molecular profile of CTV isolates from the northern region of Veracruz, Mexico and to examine their phylogenetic relationships with reported CTV isolates. The epidemic outbreak of severe

CTV symptoms in sweet orange in northern Veracruz, where around 90% of the orchards are established with the combination of sweet orange/sour orange susceptible to the disease, is a real and worrisome situation that has epidemiological implications, particularly if CTV isolates are differentially transmitted by aphids [36] [37] [38]. The main vector (*Aphis citricidus*) has been present in Veracruz since 2007 [8], so our hypothesis is that the vector may be selecting and spreading severe isolates, that already exist in the pool of regional isolates [5] [39].

The phylogenetic relationships among different CTV isolates from Mexico, analyzed by sequencing of the CP genomic region, also allowed comparison of Mexican isolates with those reported in the GenBank database.

To establish the degree of homogeneity in the virus population, the sequences alignments of the Mexican isolates were compared with isolates of the six distinct genotypes and whose complete genome sequences are known: a severe quick decline isolate T36 from Florida-USA [11], grapefruit stem pitting and a decline isolate VT from Israel [30], a sweet orange stem pitting and seedling yellow isolate SY568 from California-USA [32], a seedling yellow isolate NUagA from Japan [33], a quick decline and stem pitting isolate CTV from Mexico [34], a mild stem pitting isolate in sweet orange NZ-M16 from New Zealand [35], a severe stem pitting and seedling yellows isolate NZ-B18 from New Zealand [35], a severe vein cleaning, severe stem pitting and quick decline isolate TAM11 from Mexico, a severe vein cleaning and moderate stem pitting in Mexican lime isolate BC15-1 from Mexico [16] and eight mild isolates, T385 from Spain, T30 from Florida-USA [24] and NL8, VER2-2, QR2753-1,, MichL9A11/9-1, Colima 1997 and Yucatán 2000 from Mexico [16].

The results of the present study showed that clustering of isolates highly correlates with their symptom severity. The overall branching pattern of the phylogenetic tree was highly similar for all the mild isolates, including two of the Mexican isolates, forming a single cluster. This high similarity is not surprising since the mild isolate T30 from Florida, is widely distributed in the citrus areas of Mexico [18] [40]. However, a different situation was observed for the severe isolates, where a unique branch could not be identified for them. In this way, the severe isolates were located more dispersed in the tree and grouped with the severe isolate T36 from Florida, which causes vein cleaning, severe stem pitting and declining.

Our results are in agreement with several reports that suggest a considerable degree of sequence variation in CTV isolates. The sequencing of the CP region from seven CTV isolates from Florida, allowed their classification into three groups, I (severe-like T36), II (severe-like T3) and III (mild) represented by sequences of T36, T3 and T30, respectively, with discernible differences at the nucleotide level among the CTV isolates. This grouping was confirmed by adding an additional four virus isolates from several countries since all sequences could be assigned to one of the three types previously established [41].

Another report compared the predominant sequence variants of CP gene from eight CTV isolates of different geographic origin and pathogenicity [16].

As a result of the phylogenetic analyses, the CTV isolates were separated in two groups: first (mild group) including the mild isolates and second (severe group) including the severe isolates. This last group was loosely more disperse but included all symptomatic isolates, maybe as result of recombination events [42]. When we compared the five Mexican isolates with those eight reported for CP [16], the divergence among the CTV isolates was similar forming two clearly distinct groups: mild and severe. Although the data presented in this work are based on a limited number of CTV isolates from northern Veracruz, they clearly indicated the existence of a severe-like T36 genotype associated to the symptoms commonly occurring in this region.

## 5. Summary

In summary, this study is the first attempt to the molecular characterization of CTV isolates from reports of severe symptoms of CTV in Veracruz by the official sector of Mexico [43]. The study established that severe isolates of CTV observed in that region are related to the T36 genotype group, both molecularly and by symptomology. The findings suggest the need for determining the population structure of CTV in other citrus-growing regions of the Gulf of Mexico and the Yucatan Peninsula. In the latter region, in particular, such a need should be a priority due to the historical presence of *Aphis citricidus*, putatively the most important vector of CTV, and due to the disease status in terms of economic importance to the state. Information from the analysis reported herein could be useful to design management strategies for CTV and to understand the current epidemic outbreak under scenarios where the effective aphid vector is present.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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