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Genetic Characteristics of *Citrus Tristeza Virus* Isolates from Cultivated Citrus in China Based on Coat Protein Gene

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Abstract

Citrus tristeza virus (CTV) is an important citrus pathogen causing considerable economic loss to citrus production. Knowledge on genetic evolutionary of the CTV population in China remains limited. In this study, 1439 samples were collected from nine citrus-producing areas of China. The coat protein (*CP*) genes of CTV were amplified by RT-PCR, and sequenced to analyze the genetic evolution. Analysis of the base composition showed an AU preference pattern, with the GC content was lower than AU content. Nine CTV populations were clustered into one clade in neighbor-joining (NJ) tree, indicative of a close phylogenetic relationship among the populations in China. Analysis of molecular variation (AMOVA) revealed that 77.72% genetic variations of CTV populations were observed among populations, with an F_{ST} value of 0.223. The values of d_N/d_S and neutrality test of *CP* gene were ranged from 0.016 to 0.082 and −1.377 to 1.456, respectively, the results suggesting that all of nine CTV populations were relatively constantly maintained under purifying selection. Our study demonstrated the genetic characteristics and molecular evolution relationship of CTV populations in China, and provided a theoretical basis for scientific control of CTV.

Keywords

Citrus Tristeza Virus, Cultivated Citrus, Coat Protein Gene, Genetic Evolution

1. Introduction

As one of the most important fruit crops in the world, citrus is widely cultivated

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in China. According to statistics, as early as 2008, citrus planting area and output in China were ranked first in the world. But for a long time, plant pathogens such as *Citrus tristeza virus* (CTV), *Huanglongbing* (HLB), *Citrus tatter leaf virus* (CTLV) have posed a huge impact on the healthy development of citrus industry in China. CTV, a member of the genus *Closterovirus* in the family *Closteroviridae*, has been widely occurred in citrus-producing areas of China [1] [2]. Twenty-five years ago, Pappu *et al.* [3] had reported the complete genome sequence of a mild strain of T30. Subsequent studies showed that certain nucleotide differences existed among different CTV isolates [4]. Whole genome sequencing of different CTV isolates indicated that CTV genome contained a single-stranded positive-sense RNA (+ssRNA) of around 19226~19296 nt in length, which was the largest genome among the known plant viruses [5]. CTV genome consists of 12 open reading frames (ORF), the *CP* gene and *CPm* gene together code for the coat protein, in which the *CP* gene encodes a 25 KDa main coat protein, and the *CPm* gene encodes a 27 KDa subcoat protein [6]. The coat protein of virus is often used as a key gene for molecular detection of plant viruses because of its highly conservative [7]. Noticeably, the full-length or partial core sequence of the *CP* gene has been widely used to analyze the genetic diversity and molecular sequence characteristics of plant viruses, such as *Tobacco mosaic virus* (TMV), *Lettuce mosaic virus* (LMV), *Ornithogalum mosaic virus* (OrMV), *etc* .[7] [8] [9].

Due to the transportation of virus-carrying seedlings and the transmission of aphids, CTV has widely occurred in major citrus-producing areas in China. The complexity and diversity of hosts and habitats, as well as the mixed infection of viruses have further caused the occurrence of viral genome recombination mutations. In addition, mutation and recombination are common evolutionary factors in genetic diversity and variation in populations of plant RNA viruses, and are known as the preliminary source of variation in populations [10]. Previous studies on CTV from cultivated citrus mostly focused on organization structure, molecular evolution, and CTV-host interactions [11] [12] [13], but there is few system information on the occurrence, distribution, and genetic evolution of the CTV populations in China. Hence, in this study, larger samples of CTV isolates from nine major citrus-production areas in China were collected. The *CP* gene sequence of each sample was used to analyze the nucleotide composition, genetic evolution, as well as the molecular variation. This study will provide a theoretical basis for scientific prevention and control of CTV in citrus production.

2. Materials and Methods

2.1. Sample Collection

In 2018-2019, a total of 1439 fresh citrus leaf samples with no obvious symptoms were collected from commercial orchards in the main citrus-producing areas of nine provinces in China (**Figure 1**). Two leaves at each of four directions of citrus trees (planted more than ten years old) were collected as one sample referring to

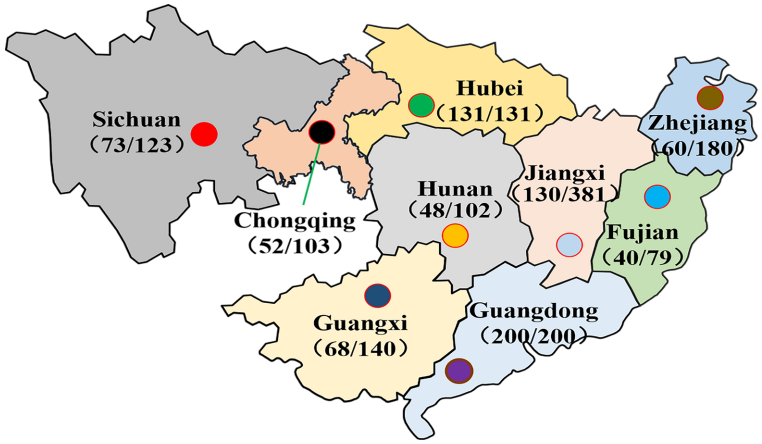

Figure 1. Geographical distribution and CTV infections in nine major citrus-producing areas in China. The numbers of CTV-positive samples versus the total number of collected samples were presented bellow each location.

the methods of Garnsey *et al.* [14], 1439 citrus samples were tested with direct tissue blot immunoassay (DTBIA) to identify positive CTV samples.

2.2. Methods

Total RNAs were extracted from CTV-positive samples using TRIzol reagent (TaKaRa, Beijing, China) according to the manufacturer's instructions. First strand cDNA was synthesized by M-MLV reverse transcriptase (TaKaRa, Beijing, China). The primers CP1: (5'-ATGGACGACGAAACAAAG-3') and CP3: (5'-TCAACGTGTGTTGAATTT-3') were used for the amplification of *CP* (672 bp) gene of CTV isolates as described previously [15]. The amplicons for each gene were purified and sequenced from both directions at Sangon Biotech Co., Ltd. (Shanghai, China). After analyzed using Contig Express software, each sequence was compared to the calibrated sequence of corresponding gene fragments of a known CTV isolates in GenBank.

2.3. Sequence Analysis

Base composition, non synonymous mutation (d_N) and synonymous mutation (d_S) were calculated by MEGA X software [16]. In order to determine the geographical relationship between CTV populations, neighbor-joining (NJ) phylogenetic tree was constructed using MEGA X software [16] with Nei's genetic distances. *CP* gene fragments of CTV isolates VT (U56902) [17], T3 (KC525952), T30 (AY260651) [18], T36 (U16304) [5], NZRB-TH30 (FJ525434) [19], NuagA (AB046398) [20], Kpg3 (HM573451) [21], HA16-5 (GQ454870) [22], SY568 (AF001623) [23], and B165 (EU076703) [24], and one isolate of *Beet yellow virus* (BYV: NC_001598) [25] from GenBank were included for analysis, in which the BYV isolate was used as out-group. Nucleotide diversity (*Pi*), haplotype diversity index (*Hd*), Fu and Li's *D*, Fu and Li's *F*, Tajima's *D* and nucleotide mismatch distribution were calculated using DNA SP V5 software [26]. The analysis of molecular variance (AMOVA) was calculated by Arleqin version 3.0 software

[27].

3. Results

3.1. CTV Infection in Different Citrus Producing Areas

A total of 1439 citrus samples from nine locations were tested by DTBIA, 802 of which were CTV-positive. The overall infection rate was 55.73%, and the infection rate in each citrus-producing area was ranged from 33.33% to 100%. To be clear, the infection of CTV in Zhejiang was the lowest, while all the samples collected in Hubei and Guangdong Provinces were detected to be CTV-positive, with an infection ratio of 100% (**Figure 1**). The results showed that CTV was widely spread and presented a severe threat to citrus production in China.

Thirty CTV isolates were randomly selected from nine major citrus-producing areas for sequencing and sequence analysis, and a total of 270 CTV sequences were obtained. The selected sequences were all 672 bp in length and deposited in GenBank as HB (Hubei, 1-30, accession numbers: OK082161-OK082190), FJ (Fujian, 1-30, accession numbers: OK082191-OK082220), GD (Guangdong, 1-30, accession numbers: OK082221-OK082250), HN (Hunan, 1-30, accession numbers: OK082251-OK082280), CQ (Chongqing, 1-30, accession numbers: OK082281-OK082310), SC (Sichuan, 1-30, accession numbers: OK082311-OK082340), GX (Guangxi, 1-30, accession numbers: OK082341-OK082370), ZJ (Zhejiang, 1-30, accession numbers: OK082371-OK082400), and JX (Jiangxi, 1-30, accession numbers: OK082401-OK082430).

3.2. Base Composition Analysis

Multiple comparisons of the *CP* sequences derived from nine CTV populations identified 450 conserved sites, 222 variable sites, 179 parsimony information sites and 43 singleton sites. The average GC content of amplified *CP* sequences was 44.32%, which was much lower than AU content (**Figure 2**), showing the

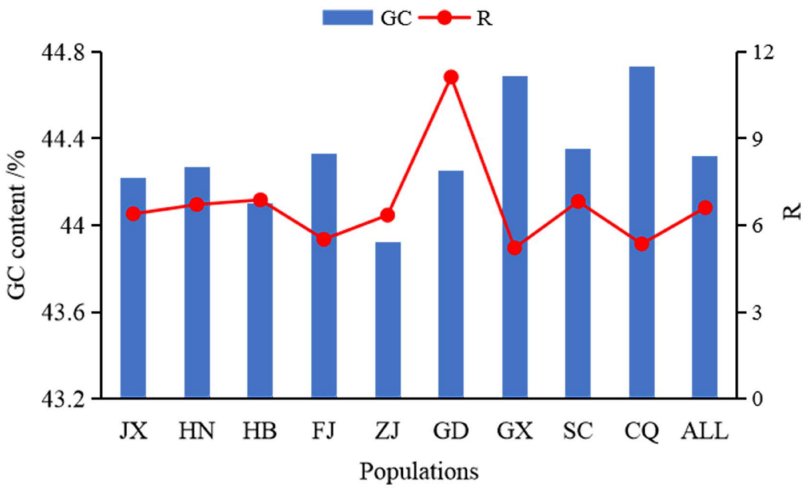

Figure 2. Base composition analysis of CTV populations based on the *CP* gene. R is the ratio of transitions to transversions.

characteristics of AU preference. The GC content varied among the selected populations, ranging from 43.33% content in ZJ population to 44.73% in CQ population, but no statistical significance was found. The ratio R of transformation to transversion of nine CTV populations was 5.217 - 11.131, with an average of 6.611.

3.3. Phylogenetic Analysis

The NJ phylogenetic tree constructed using the *CP* sequences showed that the nine CTV populations and other 10 reported CTV isolates were randomly clustered regardless of geographic origins (**Figure 3**). However, the nine CTV populations were divergent from CTV isolates derived from T30, kpg3, and HA16-5, suggested that these three CTV isolates did not prevalently occur in China. In addition, populations from different geographic origins were clustered together, indicating that the genetic relationship among the CTV populations was relatively close, and the geographical correlation with foreign isolates is poor.

3.4. Analysis of Molecular Variation

Based on AMOVA, only 22.28% of the total variations were from among populations, while 77.72% were from within populations for *CP* gene (**Table 1**). The variance components among populations were 3.84, and the variance components within populations accounted for 13.396. The value of F_{ST} of 270 CTV isolates total population were 0.223 (P = 0.000), suggesting a low level of geographical population differentiation in CTV population for *CP* gene (**Table 1**).

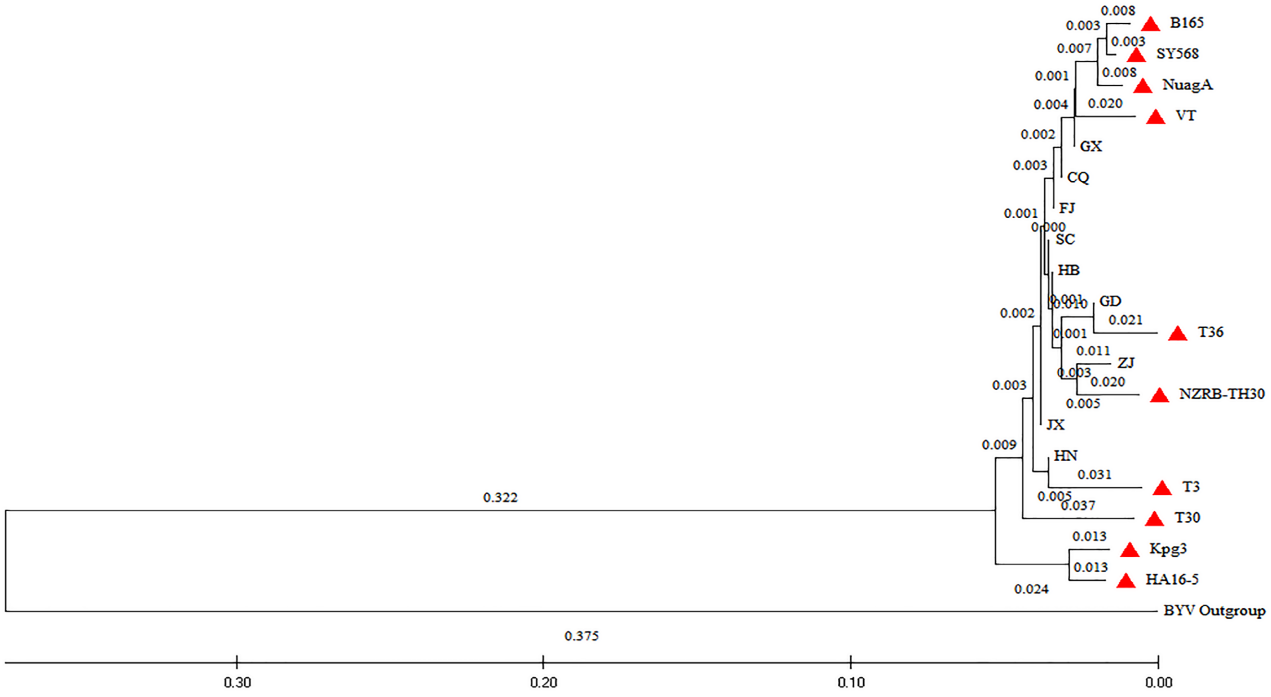

Figure 3. Construction of NJ phylogenetic tree of CTV population based on *CP* gene. CTV isolates from GenBank were marked with red triangles.

3.5. Genetic Diversity Analysis

The total nucleotide diversity index (*Pi*) and Haplotype diversity index (*Hd*) within sequences of *CP* gene for total CTV population were 0.050 and 0.999, respectively. The *Pi* of *CP* gene was ranged from 0.027 to 0.057, with no obvious difference among those CTV populations (**Table 2**). Among them, the genetic diversity of HN CTV population is the most abundant, while that of GD population is low.

3.6. Natural Selection Pressures

The analysis on the selection pressure of CTV population showed that, the calculated values of non-synonymous mutation (d_N) of nine CTV populations were lower than the calculated values of synonymous mutation (d_S), and the ratios of d_N/d_S of nine CTV populations ranged from 0.016 to 0.082 (**Table 2**). There was no evidence for positive selection among nine CTV populations, as the ratios of d_N/d_S between sequences were less than 1, indicating the negative selection. Among the nine CTV populations, the lowest ratios of d_N/d_S of 0.016 were observed in GD CTV population, while highest ratios of 0.082 were found in ZJ populations.

3.7. Demographic History

In total nine CTV populations, the values of Fu and Li's *D*, Fu and Li's *F*, and

Table 1. AMOVA analysis among CTV populations based on *CP* gene.

Source of variation	*d.f.*	Sum of squares	Variance components	Percentage of variation (%)	Fixation index
Among populations	8	1028.656	3.840	22.28	
Within populations	261	3496.300	13.396	77.72	F_{ST} = 0.223 P = 0.000
Total variances	269	4524.956	17.23532		

Table 2. Genetic diversity, neutral test and selection pressure analysis of CTV populations based on *CP* gene.

Population	Nucleotide diversity (*Pi*)	Haplotype diversity (*Hd*)	Fu and Li's *D*	Fu and Li's *F*	Tajima's *D*	d_N	d_S	d_N/d_S
JX	0.054 ± 0.003	0.998	0.145 (P > 0.1)	0.061 (P > 0.1)	−0.134 (P > 0.1)	0.014 ± 0.003	0.223 ± 0.026	0.060
HN	0.057 ± 0.002	0.956	0.797 (P > 0.1)	0.962 (P > 0.1)	0.846 (P > 0.1)	0.012 ± 0.003	0.259 ± 0.032	0.046
HB	0.042 ± 0.002	1	−0.102 (P > 0.1)	−0.182 (P > 0.1)	−0.257 (P > 0.1)	0.010 ± 0.002	0.173 ± 0.021	0.058
FJ	0.045 ± 0.004	1	−0.925 (P > 0.1)	−0.949 (P > 0.1)	−0.559 (P > 0.1)	0.012 ± 0.003	0.191 ± 0.024	0.063
ZJ	0.029 ± 0.005	0.993	−1.155 (P > 0.1)	−1.377 (P > 0.1)	−1.182 (P > 0.1)	0.009 ± 0.002	0.110 ± 0.016	0.082
GD	0.027 ± 0.003	0.998	1.102 (P > 0.1)	1.260 (P > 0.1)	0.994 (P > 0.1)	0.002 ± 0.001	0.127 ± 0.021	0.016
GX	0.033 ± 0.004	0.993	−0.162 (P > 0.1)	−0.477 (P > 0.1)	−0.882 (P > 0.1)	0.008 ± 0.002	0.132 ± 0.017	0.061
SC	0.037 ± 0.003	0.993	1.456 (P<0.05)	1.418 (P > 0.1)	0.683 (P > 0.1)	0.006 ± 0.002	0.161 ± 0.023	0.037
CQ	0.036 ± 0.003	1	−0.796 (P > 0.1)	−0.840 (P > 0.1)	−0.537 (P > 0.1)	0.009 ± 0.003	0.146 ± 0.021	0.062
ALL	0.050 ± 0.001	0.999	−1.112 (P > 0.1)	−1.003 (P > 0.1)	−0.587 (P > 0.1)	0.011 ± 0.003	0.216 ± 0.024	0.051

Tajima's *D* index of neutrality test were ranged from −1.377 to 1.456, with no statistical difference (P > 0.05). In addition, among three tested method with CTV isolates of HN, GD, and SC CTV population, and Fu and Li's *D* and *F* test values for JX CTV population were negative, but none of them were significant (**Table 2**).

In order to further determine the dynamic history of CTV population, the mismatch distribution of CTV population was analyzed (**Figure 4**). The results showed that nine geographical populations and the whole CTV population were multimodally distributed. This may suggest that CTV populations do not experience population expansion events, and the population size tends to be stable.

4. Discussion

In this study, a total of 1439 samples were collected from nine commercial citrus orchards covering all major citrus pockets in China. After detection by DTBIA, 802 samples were identified as CTV-positive. The overall incidence rate of CTV was 55.73% (**Figure 1**), which suggested that the CTV was commonly occurred in China, probably transmitted by the frequent transportation of virus-carrying seedlings. The base composition analysis indicated that the GC content of 270 nucleic acid sequences of *CP* gene was between 43.33% and 44.73%. The AU preference characteristics may promote adaptation of CTV in citrus host, and play important role in host–virus interaction and pathogenicity [28].

The complete genome sequencing has given us a new perspective to understand the occurrence and characterization of CTV, but there are only limited numbers of whole genome sequences available from certain regions. Therefore, comparisons and phylogenetic analyses of easily obtained and widely available *CP* gene sequences have widely been used for molecular characterization, as well as typing of CTV isolate from different geographical origins, various host, and biological properties [15] [29] [30]. The phylogenetic analysis revealed that nine CTV populations clustered together with seven foreign isolates (VT, T3, 30, T36, NZRB-TH30, NuagA, SY568 and B165) in a sub-group (**Figure 3**), showing a poor correlation between genetic divergence and geographic origin. Similar results were obtained with AMOVA, representing by 77.72% variations were observed from within populations and the F_{ST} (0.223) were lower than 0.25 [31]

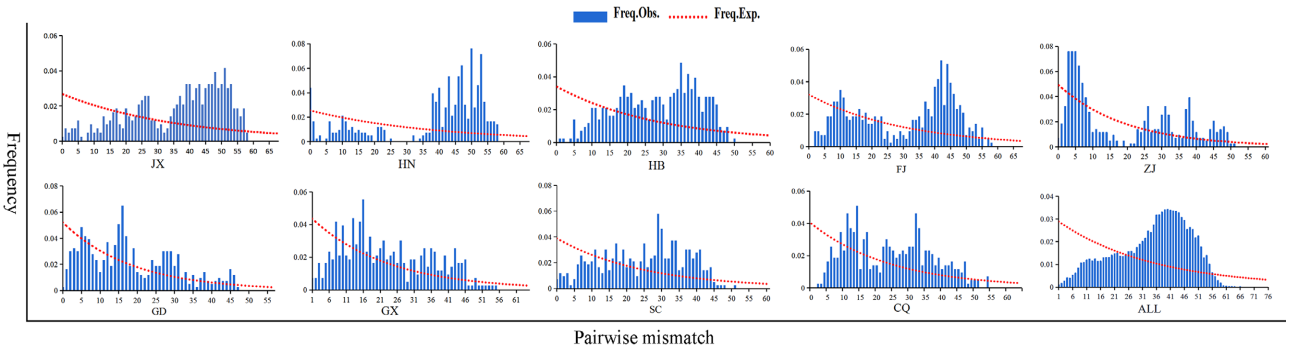

Figure 4. Pairwise mismatch distribution of CTV population based on *CP* gene.

(**Table 1**). This phenomenon might be caused by four reasons: 1) CTV transmitted by many kinds of aphid vectors, and the large species and quantity provided conditions for the spread of CTV in cultivated citrus to a certain extent; 2) CTV was characterized by a slow evolution at a rate of 1.58×10^{-4} nt per site per year [32], along with the fact that *CP* gene at the 3' end was highly conservative [33]; 3) the frequent transactions of CTV-carrying seedlings in each region provided a basis for genetic exchange among the CTV populations; 4) the geographical environmental factors might not be able to drive the genetic differentiation of CTV in China.

With the analysis of genetic diversity on the sequences of *CP* gene, the values for nucleotide diversity index (*Pi*) were ranged from 0.027 to 0.057 (**Table 2**). Interestingly, the *Pi* values obtained with *CP* gene in this study was similar with that of CTV isolates from cultivated citrus in India [34]. In context of the long distance and the geographical barrier between China and India, the genetic exchange level of those CTV populations was expected to be low. Therefore, it is more likely that the level of genetic variation remained relatively stable during a long period of evolution for these nine CTV populations.

The results of natural selection test indicated that *CP* gene was under purification selection in all nine CTV populations (**Table 2**). Davino *et al.* [35] analyzed that the d_N/d_S of Italian *cucumber mosaic virus* (CMV) was less than 1, which was speculated to be a regulatory ability of virus in order to adapt to the host defense mechanism. In this study, the purification selection of CTV was an advantageous mutation of the virus in order to adapt to survival, and further improved its adaptability by accumulating advantageous mutations. In addition, CTV isolates from nine populations showed at equilibrium because three neutrality test statistics (Fu & Li's *D*, Fu & Li's *F*, and Tajima's *D*) were non-significant, and the mismatch distributions were multimodal (**Table 2** and **Figure 4**). Indeed, virus population expansion was associated with the host quantity [36]. The large citrus production in China is the possibly reasons for the CTV populations maintained relatively constant.

5. Conclusion

The genetic characteristics of nine CTV populations from China were analyzed basing on the *CP* genes. The CTV populations from different provinces had no significant genetic differentiation and remained relatively stable, possibly due to the relatively high level of genetic exchanges among CTV populations. These nine CTV populations were found under purification selection, which might prevent the occurrence of deleterious mutations and keep the genetic variations at low level in each CTV population. Our results could provide a theoretical reference for the genetic evolution of CTV.

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Conflicts of Interest

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

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