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RESEARCH ARTICLE

Genetic recombination and diversity analysis reveal novel strain of *Citrus tristeza virus (Closterovirus tristezae)* in Khasi mandarin growing region of Assam

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Abstract

Northeast (NE) India, a hotspot for citrus biodiversity, is facing a serious threat from the *Closterovirus tristezae* (CTV). A devastating CTV transmitted by the brown citrus aphid (*Toxoptera citricida*). This pathogen has led to the decline of over one million citrus trees across India, threatening the region's citrus industry. CTV is characterised by flexuous, filamentous virions (2000×11 nm) and a positive-sense, single-stranded RNA genome (~19.3 kb) encoding 12 open reading frames (ORFs) and approximately 19 putative proteins. To assess the prevalence and genetic diversity of CTV, a systematic survey was conducted in Khasi mandarin (*Citrus reticulata*) orchards across four districts of Assam: Kamrup Metro, Karbi Anglong, Kamrup Rural and Goalpara. Infected trees exhibited a spectrum of disease symptoms, including decline, chlorosis, leaf yellowing, poor growth and stunting. Disease incidence was determined using Direct Antigen Coated-Enzyme Linked Immunosorbent Assay (DAC-ELISA) and percent disease incidence, revealing 68.2 % overall infection rate. Genetic characterization of 19 CTV isolates, based on a 404-nt fragment of the 5' ORF1a, unveiled substantial sequence variability, with pairwise nucleotide identities ranging from 85–100 %. Phylogenetic reconstruction grouped these isolates into five distinct genogroups, underscoring significant intra-farm genetic diversity within citrus orchards. Recombination analysis using RDP4 software identified multiple recombinant isolates (*BHKM-1, ASKM-1, ASKM -2, MKM-2 and RTKM-1*), with BHKM-6 as the major parent and MB-3 as a minor parent contributing to recombination events. These findings provide critical insights into the genetic landscape of CTV in Northeastern (NE) India, emphasising the need for targeted disease management strategies to mitigate further citrus decline.

Keywords: citrus decline; disease incidence; genetic diversity; phylogenetic analysis; recombinant

Introduction

Citrus (*Citrus* spp.), one of the most significant fruit crops of the family *Rutaceae*, is cultivated in over 140 countries worldwide (1). In India, citrus is a prominent fruit crop for the nation's economy and is ranked third among the major fruit crops after banana and mango. The NE region of India is considered a natural home for different citrus species (2-4). CTV is a significant contributor to the citrus decline in citrus-growing regions worldwide, responsible for the death of millions of citrus trees over the past 70 years (5, 6). The virus spreads through infected propagative materials and is locally transmitted by the brown citrus aphid (*Toxoptera citricida*) semi-persistently (7). CTV is a phloem-restricted, flexuous, filamentous and the longest known plant virus, with particle dimensions of 2000 × 11 nm. It belongs to the genus *Closterovirus* (Family: Closteroviridae). It has a positive-sense single-stranded RNA genome of 19.3 kb, comprising 12 open reading frames (ORFs) that potentially encode at least 19 putative proteins (8, 9).

The virus infects nearly all citrus species, their cultivars and related plants (10). CTV infection typically induces symptoms such as the decline of citrus species grafted on sour orange rootstock, yellowing and growth cessation in sour orange, lemon and grapefruit and stunting and stem pitting, leading to reduced fruit yield and quality across various citrus species (11). Commercially cultivated citrus fruits include mandarins (*Citrus reticulata*), sweet oranges (*C. sinensis*), acid limes (*C. aurantifolia*), sweet limes (*C. limetta*) and lemons (*C. limon*). CTV is prevalent across all citrus-growing regions of India: Northeast, South, Northwest and Central, with disease incidence estimated to range from 10 % to 90 %, affecting nearly all key citrus species, cultivars and hybrids (12). CTV poses a significant threat, in the Northeast state, with *Toxoptera citricida*, its most efficient vector, being widespread (6). Khasi mandarin (KM) holds the maximum commercial value in Assam, Manipur, Meghalaya and other NE states (13). However, the productivity of mandarin in the NE states is 5.86 MT/ha which is lower than the national productivity of 11.08 MT/ha (9).

The genetic diversity of CTV isolates in various citrus-growing regions worldwide has been well-documented in previous studies (14, 15). A total of 20 complete genomes of CTV isolates are available in the NCBI GenBank database, revealing significant sequence variation that groups them into eight genogroups; T36, VT, T3, RB; T68, T30, HA16-5 and S1 (16, 17). Recombination events contributing to the emergence of divergent CTV isolates have been documented in earlier studies (18). Genetic diversity among Indian CTV isolates has been investigated using a limited number of isolates and partial sequence data (16-19). Studies indicate that Indian isolates are closely related to the VT genotype, as determined by the analysis of the 5´ ORF1a fragment and multiple molecular markers (15). They identified greater sequence variability in CTV isolates between nucleotide positions 1082–1484 compared to other regions of the 5´terminal sequence. Furthermore, analysis of the 5´ ORF1a fragment and coat protein (CP) gene of CTV isolates from the Darjeeling hills in NE India (20) and the Delhi region (21) revealed distinct phylogroups, highlighting the presence of genetically diverse CTV isolates within the country.

Understanding the genetic variation and geographical distribution of CTV variants is crucial for unravelling the virus's epidemiology, ensuring precise diagnostic techniques and formulating sustainable long-term management strategies. The genetic diversity of CTV is largely attributed to recombination events, which facilitate the emergence of novel variants with potentially altered virulence, host specificity and transmission dynamics. Although recombination has been widely recognised as a major factor in the diversity of CTV (15, 22, 23), research efforts in this domain remain limited, particularly in Assam, where citrus cultivation plays a vital role in the agricultural economy. Previous studies have primarily focused on broad genetic analyses, but a comprehensive examination of recombination events in the ORF1a region of the CTV genome is scarce. Given the importance of ORF1a in viral replication and pathogenesis, a deeper investigation into recombination events using the 5′ ORF1a fragment could provide valuable insights into viral evolution, strain emergence and potential changes in pathogenicity. Furthermore, there is a lack of studies correlating these recombination patterns with specific host plant responses and vector transmission efficiency, leaving a critical gap in understanding the epidemiological consequences of such genetic exchanges. Our study aims to bridge this knowledge gap by providing empirical evidence of recombination between divergent CTV sequences in Assam, which could enhance disease management strategies and contribute to developing resistant citrus cultivars.

Materials and Methods

Survey, collection and maintenance of CTV isolates

Surveys were conducted in four Khasi mandarin-growing districts of Assam: Kamrup Metro, Karbi Anglong, Kamrup Rural and Goalpara, to study the incidence of CTV and collect samples. Citrus trees exhibiting symptoms such as stunted and poor growth associated with decline syndrome, leaf yellowing and vein clearing were targeted for sample collection. Twigs from symptomatic trees in each orchard were collected and transported to the laboratory for detection and molecular assays (Fig. 1). Disease incidence was calculated using the standard method for PDI:

$$Percent\ disease\ incidence\ (PDI) = \frac{No.of\ infected\ samples}{\text{No. of samples tested}} \times 100$$

Virus isolates

Several isolates of CTV were collected from the KM growing region of Assam and the presence of CTV was confirmed by the DAC-ELISA protocol described earlier (23). Five twigs containing leaves from each selected tree were cut, placed in a polythene bag, sprayed with water and transported to the laboratory. These twigs were grafted onto rough lemon rootstocks and successful unions were kept in insect-proof chambers of the nursery, Sundarban Nursery, Dhupdhara, Goalpara, Assam and greenhouse, Advanced Centre for Plant Virology (ACPV), ICAR-Indian Agricultural Research Institute (ICAR-IARI), New Delhi (Fig. 2).

RNA extraction, cDNA synthesis, molecular cloning of CTV sequences

Total plant RNA was extracted from tender bark tissues using the SV Total RNA isolation system (Promega, Madison, USA). First-strand cDNA synthesis was performed with M-MLV reverse transcriptase (Promega, Madison, USA), following the standard protocol (20). For amplification of viral sequences, Polymerase Chain Reaction (PCR) was conducted using the forward primer KLM488F and the reverse primer KLM491R, targeting a 404-nt fragment of the 5′ ORF1a gene of the CTV genome. The resulting amplicons were purified with the QIA quick PCR purification kit (Qiagen, Maryland) and cloned into a T&A cloning vector (RBC, UK). Clones were propagated in *E. coli* DH5α strain following standard procedures. The cloned viral DNA was sequenced using M13 forward and reverse primers in an automated sequencer (ABI 3011, Chromous Biotech Pvt. Ltd., Bangalore, India). Two clones from each isolate were sequenced and consensus sequences were used for subsequent analyses.

Sequence analysis

Several CTV isolates collected from citrus-growing regions across India were analysed genetically, focusing on the 5′ ORF1a fragment (1076 to 1479-nt positions of the T36 reference sequence). The analysis revealed extensive genetic diversity within the Indian CTV populations, with virus variants distributed across all the geographically distinct citrus growing zones. Additionally, evidence of recombination events within the CTV genomes was documented, highlighting the dynamic evolution of the virus in India. The corresponding sequences of international and previously reported Indian CTV isolates were used to compare the present CTV isolates. The multiple sequence alignments were carried out using the software Clustal W, version 1.6 (24). Phylogenetic tree was constructed using the MEGA 11 software by maximum likelihood method (25). Sequence identity matrix was generated using Sequence Demarcation Tool (SDT) version 1.2 (26). The putative recombination events were identified using Recombination Detection Program (RDP4) version 4.55 implementing seven algorithms, RDP, GeneConv, Bootscan, MaxChi, Chimera, SiScan and 3Seq using default parameter values for the different detection programs (27).

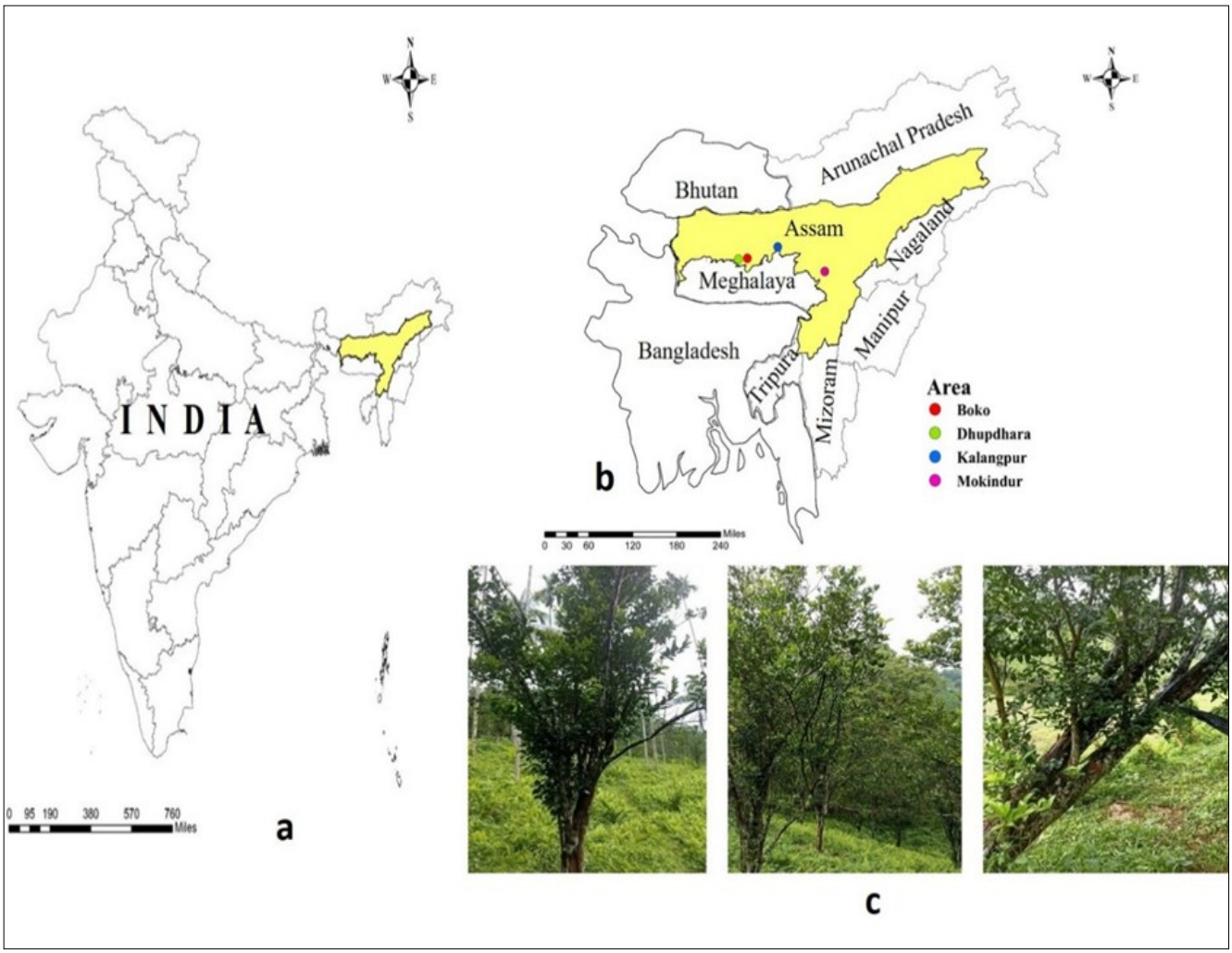

Fig. 1. Map of India showing KM growing states in NE India. (a) Location of Assam state in NE India. (b) Magnified images of Assam state (highlighted in yellow colour); different colour dots indicating the location of sample collection; line borders indicating neighboring states/ countries. (c) KM orchards.

Fig. 2. KM sample grafted on rough lemon maintained in an insect-proof greenhouse.

Results

Survey, symptomatology and collection of virus infected citrus samples from Assam

ELISA test showed that KM orchards were infected by CTV. Out of the 44 citrus samples, 30 samples were CTV-infected with a total PDI of 68.2 %. The highest PDI was recorded in Goalpara district (90 %) and the lowest in Karbi Anglong (50 %). The OD values measured via ELISA, expressed as fold increases (X fold) over the negative control, showed clear variation among the surveyed NE Indian states. In Kolangpur (Kamrup Metro) recorded a 2X fold increase, Mokindur (Karbi Anglong) and Boko (Kamrup Rural) each showed a 4X fold increase, while Dhupdhara (Goalpara) exhibited the highest OD value of 9X fold (Table 1).

Molecular cloning and sequencing of fragment of the 5'ORF1a

Citrus samples that tested positive for CTV through DAC-ELISA were classified as distinct CTV isolates from different KM-growing districts of Assam. Among 23 isolates analysed, 19 were found to be CTV-infected, while four were identified as disease-free (Fig. 3). The PCR products of the desired length were obtained from all the present CTV isolates. Of these, the PCR products of five CTV-isolate (Sl. No. 1 to 5 from Table 2) were cloned and the remaining products were sent directly for sequencing by outsourcing (ABI 3011, Chromous Biotech, Bangalore). The clones of five CTV isolates were confirmed by standard colony PCR. Two positive clones from each CTV isolate were sent for sequencing by outsourcing. The consensus CTV sequences were identified, aligned and analysed.

Sequence analysis of CTV isolates

In the present study, pair-wise sequence analysis using 5'ORF1a gene fragment of the present 19 isolates and the previously reported Indian and international CTV isolates showed overall 80 -100 % nt identities among them. A range of 85-100 % nt identity was observed amongst the present Indian CTV isolates (Fig. 4b), whereas 80-98 % nt identity was observed amongst the present and previously reported Indian CTV isolates. Phylogenetic tree analysis revealed that the 19 CTV isolates were segregated into five distinct genogroups (Fig. 4a). Among these, seven isolates clustered with the recognised CTV genotype T30. Another set of isolates grouped within the fourth genogroup alongside the recognised CTV genotype VT/Kpg3. The remaining isolates formed a fifth genogroup, clustering with previously identified Indian CTV isolates D1 and D2.

Recombination analysis

To identify the recombination events, sequences of 5'ORF1a gene fragment of all the present CTV isolates were analysed using recombination-detection program, RDP4 (Fig. 5). The RDP4 detected BHKM-1, ASKM-1, ASKM-2, MKM-2, RTKM-1 for as recombinants with a breakpoint positioned at 382-404 nt for BHKM-1 and 382-51 for ASKM-2, MKM-2, RTKM-1 supported by maximum probability, 2.949×10^{-4} detected by 3Seq and 1.167×10^{-3} GENECONV algorithm (Table 3). Recombination analysis of present isolates indicated that CTV isolates BHKM-6 was the major parent and MB-3 was the minor parent contributing to recombination events.

Table 1. Incidence of virus and virus-like diseases in KM orchards in different areas of NE India

State	District	Location	Symptoms	Confirmed by	
				CTV	
				ELISA	PDI
				OD Value (X-Fold) *	Plant infected/ Plant tested (%)
Assam	Kamrup Metro	Kolangpur	H, Vcl, Mg	2	7/10 (70)
	Karbi Anglong	Mokindur	H, Vfl, Pg, Mg	4	6/12 (50)
	Kamrup Rural	Boko	H, Vcl, Ly, Pg, Mg	4	8/12 (66.6)
	Goalpara	Dhupdhara	H, Vy, Mg	7	9/10 (90)
			Total		30/44 (68.2 %)

H: Healthy; Pg: Poor Growth; Chl: Chlorosis; St: Stunting; Vy: Vein yellowing; Vcl: Vein clearing; Vfl: Vein flecking; Ly: Leaf yellowing; Mg: Medium growth; CPGSAS: College of Post Graduate Studies and Agricultural Sciences; *X fold compared to healthy control; OD values: 1.2-1.9 for infected Khasi mandarin samples; 0.17-0.25 for healthy control (healthy Khasi mandarin) and 0.14- 0.24 for buffer controls

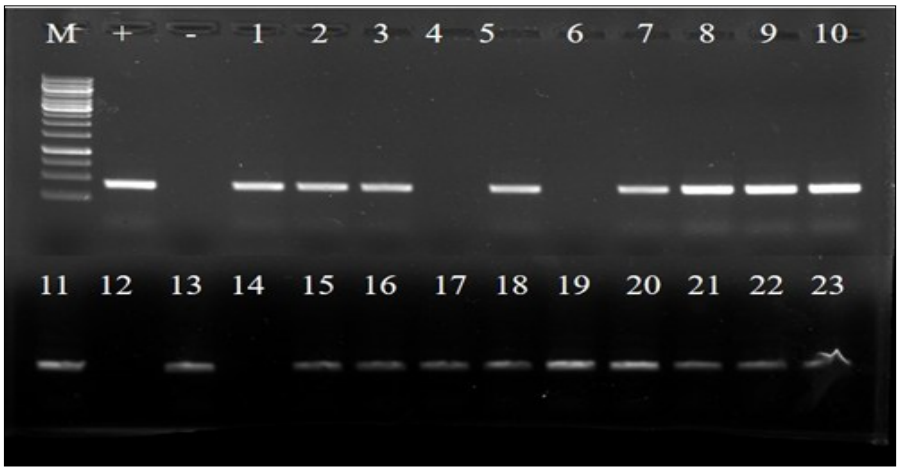

Fig. 3. Agarose gel electrophoresis showing PCR amplification of on 5'ORF 1a fragment (404 nt) of CTV of Assam. Lane M:1kb ladder; + positive control; - Healthy Lane 1: MKM-2; Lane 2: AKM-1; Lane 3: SRKM-1; Lane 4: MKM-1; Lane 5: SRKM-4; Lane 6: MKM-3; Lane7: SRKM-6; Lane8: RTKM -1; Lane9: RTKM-2; Lane10: KTKM-3; Lane11: KTKM-4; Lane12: SRKM-5; Lane13: ASKM-2; Lane14: SRKM-2; Lane15: BHKM-1; Lane16: BHKM-2; Lane17: BHKM-3; Lane 18: BHKM-4; Lane 19: BHKM-6; Lane 20: BHKM-7; Lane 21:BHKM-9; Lane 22: BHKM-10; Lane23: ASKM-1.

Table 2. Source of KM twigs collected from different orchards in Assam for identification of pathogen-free mother stock

Sn.	Sample	District	Village	Age (plant)	Symptoms	ORF1a	Accession number
1	MKM-2	Kamrup Metro	Borkasarong	72-74	H, Ly, Mg	+	PQ568321
2	AKM-1	Kamrup Metro	Borkasarong	67-68	H, Mg, Vcl	+	PQ568320
3	SRKM-1	Kamrup Metro	Kolangpur	68-69	H, Mg, Pg	+	PQ568322
4	SRKM-4	Kamrup Metro	Kolangpur	62-63	H, Mg, Vcl	+	PQ568323
5	SRKM-5	Kamrup Metro	Kolangpur	66-67	H, Mg, Vfl	+	PQ568324
6	SRKM-6	Kamrup Metro	Kolangpur	73-74	H, Ly, Mg	+	PQ568325
7	RTKM-1	Kamrup Metro	Mangursila	72-74	H, Mg, Pg	+	PQ568326
8	RTKM-2	Kamrup Metro	Mangursila	73-74	H, Mg, Vfl	+	PQ568327
9	KTKM-3	Karbi Anglong	Mokindur	72-74	H, Mg, Ly	+	PQ568328
10	KTKM-4	Karbi Anglong	Mokindur	65-66	H, Mg, Pg	+	PQ568329
11	ASKM-1	Kamrup Metro	Kolangpur	67-68	H, Mg, Ly	+	PQ568318
12	ASKM-2	Kamrup Metro	Kolangpur	68-69	H, Mg, Vfl	+	PQ568319
13	BHKM-1	Kamrup Rural	Boko	69-70	H, Mg, Ly	+	PQ568311
14	BHKM-2	Kamrup Rural	Boko	68-69	H, Mg, Pg	+	PQ568312
15	BHKM-3	Kamrup Rural	Boko	69-70	H, Mg, Ly	+	PQ568313
16	BHKM-4	Kamrup Rural	Boko	64-65	H, Mg, Pg	+	PQ568314
17	BHKM-6	Kamrup Rural	Boko	66-67	H, Mg, Vfl	+	PQ568315
18	BHKM-7	Kamrup Rural	Boko	60-61	H, Mg, Ly	+	PQ568316
19	BHKM-9	Kamrup Rural	Boko	62-63	H, Mg, Pg	+	PQ568317
20	*BHKM-10	Kamrup Rural	Boko	69-70	H, Gg	-	------
21	*MKM-1	Kamrup Metro	Borkasarong	73-74	H, Gg	-	------
22	*MKM-3	Kamrup Metro	Borkasarong	65-66	H, Gg	-	------
23	*SRKM-2	Kamrup Metro	Kolangpur	62-63	H, Gg	-	-------

H: Healthy; Pg: Poor growth; Chl: Chlorosis; St: Stunting; Vy: Vein yellowing; Vcl: Vein clearing; Vfl: Vein flecking; Ly: Leaf yellowing; Mg: Medium growth; Gg: Good growth; CPGSAS: College of Post Graduate Studies and Agricultural Sciences; *X fold compared to healthy control; OD values: 1.2-1.9 for infected KM samples; 0.17-0.25 for healthy control (healthy KM samples) and 0.14- 0.24 for buffer control

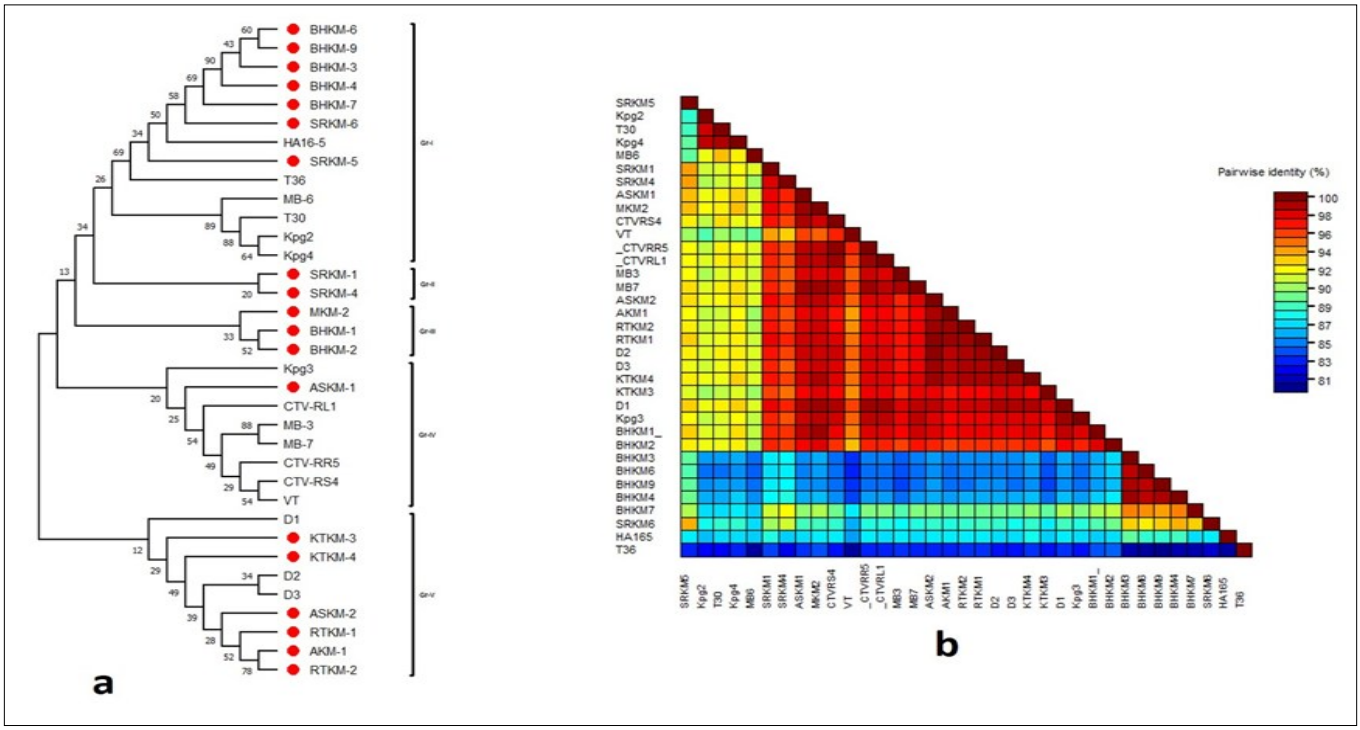

Fig. 4. Phylogenetic relationship and sequence identity matrix of present CTV isolates along with other CTV isolates (a). Phylogenetic relationships among CTV isolates using maximum likelihood parameter (1000 bootstrap) based on sequences of 5′ORF1a gene fragment of CTV genome; the present isolates are highlighted in bold font and genogroups are marked in the right panel of the figure; VT, T36, T30, T3, T68-1, RB-G90 and HA16-5 are the representative isolates of seven International recognized genotypes; Others are Indian isolates characterized earlier. (b). Colour-coded pair-wise percent nucleotide identity matrix of CTV isolates based on sequences of 5′ORF1a gene fragment; each colour cell represents a percent identity score between two CTV isolates (one indicated horizontally to the left and the other vertically at the bottom). A coloured key indicates the correspondence between pair-wise identities and colours displayed in the matrix.

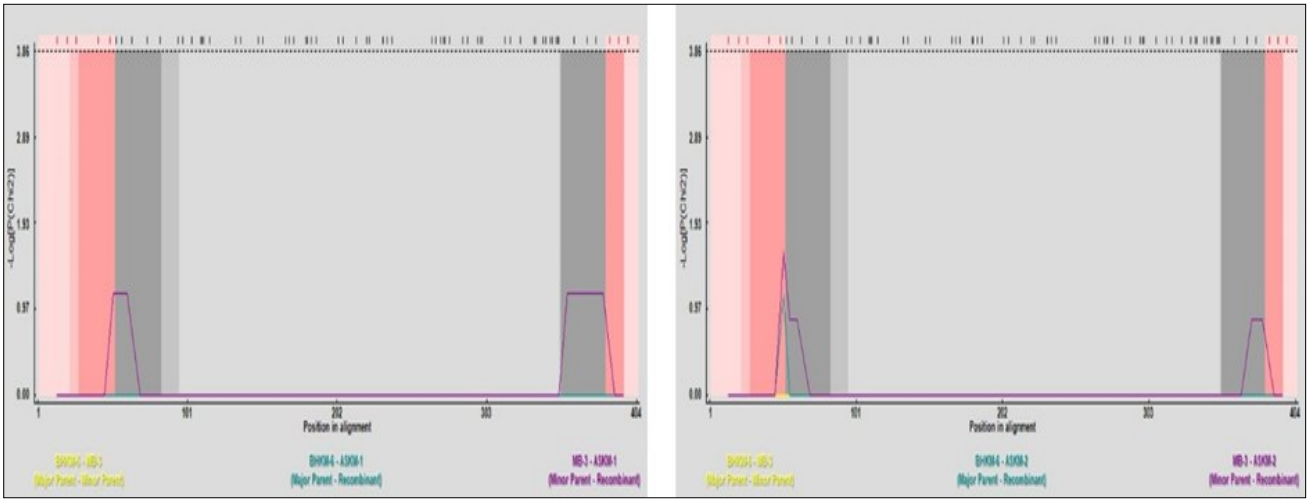

Fig. 5. Recombination analysis using sequences of 5′ORF1a gene fragment of Indian and international CTV isolates by RDP4. Graphical representation of recombination event in isolate ASKM-1 and MKM-2 using chimera algorithm. Position of sequence alignment and log P value has been represented in X and Y axis, respectively. Sequence with recombination origin is indicated by deep shaded area.

Table 3. Recombination events in 5′ ORF1a gene fragment sequences of present CTV isolates detected by recombination-detecting program RDP4

Recombinant isolate	Donor (Major/Minor)	Breakpoint (nt)	Algorithm	Average P-value
BHKM-1	BHKM-6/MB3	382-404	GENECONV	1.167×10^{-03}
			3Seq	2.949×10^{-04}
ASKM-1	BHKM-6/MB3	382-51	GENECONV	1.167×10^{-03}
			3Seq	2.949×10^{-04}
ASKM-2	BHKM-6/MB3	382-51	GENECONV	1.167×10^{-03}
			3Seq	2.949×10^{-04}
MKM-2	BHKM-6/MB3	382-51	GENECONV	1.167×10^{-03}
			3Seq	2.949×10^{-04}
RTKM-1	BHKM-6/MB3	382-51	GENECONV	1.167×10^{-03}
			3Seq	2.949×10^{-04}
KTKM-4	BHKM-6/MB3	382-51	GENECONV	1.167×10^{-03}
			3Seq	2.949×10^{-04}

Discussion

The findings from this study demonstrate that CTV is widespread in Khasi mandarin orchards in Assam. Among the 44 samples of KM citrus tree collected across various districts, 30 tested positives for CTV infection using DAS-ELISA, indicating an overall CTV disease incidence of 68.2 % in the region. Similar decline symptoms, characterized by chlorosis, poor growth and stunting in mandarin trees across orchards in NE India, including Assam, have previously been attributed to CTV (28-30). This highlights the urgent need for advanced molecular diagnostic approaches for accurate and rapid detection of CTV, as diagnosis based on visible symptoms is unreliable due to the influence of climatic conditions (e.g., temperature, RH, rainfall etc.), citrus species and the presence of different CTV strains.

Our results are consistent with earlier reports of severe CTV infections in citrus-growing regions of Assam (30). Phylogenetic analysis of the present 19 CTV isolates segregated them into five distinct genogroups. Seven isolates clustered with the recognized T30 genotype, another group aligned with the VT/Kpg3 genotype and the remaining isolates grouped with previously identified Indian isolates D1 and D2, indicating the presence of five different CTV genotypes. Intra-farm genetic diversity among CTV isolates within individual citrus farms has been previously reported (31-34). For instance, three distinct CTV genotypes were found in the ICAR-IARI, New Delhi (32), while (33, 34) identified two CTV genotypes (Kpg3/VT and B165) in farms at ICAR-IARI Regional Station, Kalimpong and the Indian Institute of Horticultural Research, Bangalore, as well as VT and T30 genotypes at ICAR-IARI Regional Station, Pune.

The present study and earlier reports confirm that intra-farm diversity of CTV genotypes is common in citrus farms across India. Recombination analysis of present isolates indicated that CTV isolates BHKM-6 was the major parent and MB-3 was the minor parent contributing to recombination events. Evidence for such recombination events in the evolution of divergent CTV isolates in India has been documented previously (33, 34). The northeast region of India exhibits a high incidence of CTV infection, accompanied by extensive genetic variability. Identifying these distinct groups can facilitate the development of group-specific primers to accurately and rapidly detect predominant virus genogroups. A distribution map of CTV and its variants across India will provide valuable insights into the epidemiology of the disease and aid in designing effective management strategies. Given the high disease incidence and the widespread distribution of CTV-infected planting materials to farmers, implementing sanitation practices and replanting with virus-free propagative materials are crucial to mitigate economic losses in citrus cultivation.

Conclusion

Assessing the genetic diversity, identification and distribution of CTV variants in citrus-growing regions of India is crucial for understanding its molecular epidemiology and devising effective management strategies. This study highlights the importance of sequence analysis and phylogenetic relationships among various CTV isolates, which can significantly contribute to developing improved diagnostic methods. By identifying conserved sequences within specific CTV genogroups, researchers can design targeted primers, enhancing early detection and disease monitoring. The study also underscores the alarming prevalence of citrus decline in NE India, where mandarin orchards experience disease incidences as high as 68.2 %, threatening the region's citrus industry. Given that CTV primarily spreads through infected planting material and the brown citrus aphid, implementing stringent measures such as the use of disease-free plantlets, regular field inspections and effective aphid control programs is imperative for mitigating disease transmission. Additionally, future research should delve deeper into whole-genome recombination patterns, investigate the influence of environmental factors on viral diversity and assess the potential effects of climate change on the evolution and spread of CTV. A comprehensive understanding of these aspects will aid in formulating sustainable disease management strategies, ensuring the long-term viability of citrus production in India.

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Authors' contributions

Methodology, investigation, data analysis and original draft preparation was done by HK. Conceptualisation, investigation, data analysis, editing and supervision was performed by KKB, HK and EM. Editing and supervision was done by SS, DDC, LP, BRM.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no competing interests.

Ethical issues: None

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