Molecular Characterization of Two Isolates of Wild Tomato Mosaic Virus and Chilli Veinal Mottle Virus Co-infecting Chilli Pepper in China

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Research Article

Keywords: Wild tomato mosaic virus, Chilli veinal mottle virus, co-infection, recombination, phylogenetic analysis

Posted Date: April 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-442269/v1

Abstract

The present study reports observation of a field chilli pepper disease consisting of a co-infection with two potyviruses: Wild tomato mosaic virus Dehong isolate (WTMV-Dh) and Chili veinal mottle virus Dehong isolate (ChiVMV-Dh). We obtained the complete genome sequences of these two viruses by NGS sequencing. The WTMV-Dh is 9,598 nucleotides (nt) in length and encodes a complete polyprotein of 3,075 amino acids (aa). The polyprotein of WTMV-Dh shares 76.1–82.6% nt and 85.3–89.5% aa identities with the other three WTMV isolates reported previously. The ChiVMV-Dh is 9688 nt in length and encodes a complete polyprotein with 3, 089 aa. The polyprotein of ChiVMV-Dh shares 80.8–92.2% nt and 85.3–95.6% aa identities with the other ChiVMV isolates reported previously. Following phylogenetic analysis based on the polyprotein sequences of other potyviruses, WTMV-Dh clustered with the Vietnam strain WTMV-Laichau while ChiVMV-Dh clustered with several ChiVMV Sichuan isolates. Evaluation of the recombination events within the WTMV and ChiVMV subgroups indicated that some putative recombination events occurred in critical regions. These regions include the N-terminal of HC-Pro and P1 region of WTMV-Dh, CP and the P3 to CI region of ChiVMV-Dh, which may be new evidence of adaptive evolution of potyviruses.

Background

Chilli pepper (*Capsicum annuum* L.) is an economically important crop in the world. The production of chilli pepper is hampered by susceptibility to numerous plant pathogens, including potyviruses [1, 2]. Potyviruses form a large group of aphid transmitted plantviruses of the genus *Potyvirus*. The potyviruses virion is a flexuous filamentous capsid with a length of 700–900 nm and a diameter of 11–13 nm. The genome of potyviruses consists of a single-stranded RNA (ssRNA) with a size of about 9.7 Kb, two untranslated regions in 5'UTR and 3'UTR, and a single major open reading frame (ORF) encoded a single large polyprotein. This polyprotein is cleaved into 10 functional proteins as described previously [3-5].

Chili veinal mottle virus (ChiVMV), a member of the genus *Potyvirus*, was first reported in West Malaysia in 1979 [6] after which it has been widely reported in many Asian and East African nations as an important pathogen of chilli crops [7-10].

Wild tomato mosaic virus (WTMV), also a member of the genus *Potyvirus*, was first shown to infect wild tomatoes in Vietnam in 2008 [3]. Later, the disease was reported to infect various crops, including tobacco, wild eggplant, and *Solanumnigrum* L. in Guangdong, Hainan and Sichuan regions of China [11-14]. At present, however, only three complete genome sequences have been reported, including isolates from wild tomato, tobacco, and *Solanumnigrum* L.

In July 2018, a chilli pepper sample that showed virus-like symptoms, including mosaicism, yellow mottling and shrinkage (Fig.1a), was collected in Dehong, southwest of Yunnan Province. For negative staining and transmission electron microscopy (TEM) observation, symptomatic leaves were ground to obtain a crude sap and follow the steps as previously described [15]. Results revealed potyvirus-like filamentous particles with a size of approximately 11 ´ 760 nm on the saps of symptomatic leaves (Fig.1b).

To further identify the virus species infecting the chilli pepper sample, the complete viral sequence was determined by transcriptome next-generation sequencing technology (NGS). Total RNA was extracted from symptomatic leaf tissues using the mirVanamiRNA Isolation Kit (Thermo Fisher Scientific, Madison, WI, USA), following the manufacturer's protocol. The ribosomal RNA was removed and the cDNA library was constructed using the TruSeq Stranded Total RNA LT-with Ribo-Zero Plant (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Then, libraries were sequenced on the Illumina sequencing platform (IlluminaHiSeq X ten) to generate 150 bp paired-end reads. The clean reads were *de novo* assembled into transcript using the Trinity software (version:trinityrnaseq_r20131110) by the paired-end method [16]. Virus identification from the transcriptome data was made by BlastX search against the virus reference database (http://www.ncbi.nlm.nih.gov/genome/viruses/) [17], which is optimized for highly similar sequences with the threshold E-value of 10^{-5}. All virus-associated contigs were aligned to the respective reference viral genome using the Bowtie2 program [18].

NGS sequencing generated total of 24,598,402 raw reads, and 24,183,806 clean reads with length 150 nucleotides (nt) after removing low quality reads with the quality control Q30>95.45%. Finally, a total of 12,377 unigene reads (>300bp) were obtained by *de novo* assembly. After BlastX search against NR databases, the contig DN4369_c0_g1_i1 with a length of 9598 nt was mapped to the WTMV complete sequence and named WTMV-Dh (NCBI accession No. MT793717). Another contig, DN4168_c0_g1_i1 with a length of 9688 nt, was mapped to the ChiVMV entire sequence and named ChiVMV-Dh (NCBI accession No. MT787292). To analyze the accumulation level of the NGS reads of each of the virus, the complete sequence of WTMV-Dh and ChiVMV-Dh were used as references for BLAST searches of viral reads from the clean reads pool. The result showed that a total of 793,404 reads (3.28%) were mapped to WTMV-Dh, whereas 215,871 reads (0.89%) were mapped to ChiVMV-Dh. According to the respective FPKM values, the complete sequence of WTMV-Dh is 8.19E+04, which is much higher than the value of ChiVMV-Dh (Table 1).

Sequence analysis showed that the complete sequence of WTMV-Dh contains a complete polyprotein ORF, but 11 nt on the 5'UTR and 44 nt on the 3'UTR were missing, compared with other WTMV isolates. The polyprotein ORF encodes 3,075 amino acids, and the polyproteinis predicted to cleave into 10 matureproteins, like other WTMV isolates [5, 14] (S1 Fig.). Multiple sequence alignments were conducted by Meg Align (Lasergene7.1) for analyses of sequence identity. The result showed the region encoding the WTMV-Dh polyprotein shares the highest amino acid (aa) sequence identity (89.5% aa) with the Vietnam strain Laichau (DQ851495.1) (Table 2). Comparison of the nucleotide and amino acid sequences of individual proteins/regions showed that 5'UTR and P1 of the WTMV subgroup are hypervariable, sharing a low identity of 48.6% to 71.9% nt in 5'UTR, and 50.0% to 59.7% aa in P1. The 6K1 of WTMV-Dh was found to share low nucleotide homology (74.7%) but high amino acid identity (98.1%) with the Sn and XC-1. Meanwhile, we showed that the P3 of WTMV-Dh shares both low nucleotide homology (71.6%–72.2%) and amino acid identity (70.6%–71.2%) with the strains Sn and XC-1 (Table 2). To evaluate the recombination events, the Recombination Detection Program, version 4 (RDP4) had been done based on 22 potyviruses sequences that are closely related to WTMV (the sequence information is shown in Fig. 2). The results revealed a total of three putative recombination events of WTMV-Dh (Table 4). One of the recombination regions detected from nucleotide 1049 to 1494 of WTMV-Dh forms part of the HC-Pro N-terminal and Laichau was predicted as the major parent while XC-1 as the minor parent. ther two recombination events were detected in the 5'UTR and P1 regionsfrom nucleotides 48 to 162, and 166 to 342 of the WTMV-Sn. For the recombinant WTMV-Sn, the minor parent was identified as WTMV-Dh, while XC-1 was identified as the major parent. Phylogenetic

comparison (MEGA 6.06) of WTMV-Dh to other potyviruses based on polyprotein amino acid sequence showed that the WTMV subgroup is divided into two branches. In the first branch, WTMV-Dh clustered with the Vietnam strain Laichau together, while the second branch was composed of the other two Sichuan strains (Fig. 2).

Sequence analysis showed that the complete sequence of ChiVMV-Dh contains a complete polyprotein ORF but lacks 39 nt on the 3'UTR. The polyprotein is composed of 3,089 amino acids and is expected to cleave into 10 mature proteins, similar to other ChiVMV isolates [5] (S1 Fig). The coding region of the ChiVMV-Dh polyprotein is more closely related to Sichuan strains than Yunnan strains. The polyprotein shares 95.6%, 95.3%, and 95.3% aa sequence identity with the Sichuan strains Yp8 (KC711055), Pp4 (KC711056), and LZ (MK405594), respectively, and 90.4% aa sequence identity with the Yunnan strain YN-t (JX088636). But ChiVMV-Dh shared the highest identity with YN-t in the 5'UTR (80.2% nt) and P1 (84.4% nt, 82.7% aa) regions compared with other ChiVMV isolates (Table 3). The 6K1 of ChiVMV-Dh shared a low nucleotide homology but the highest amino acid identity with YN-t (100% aa). Among all the ChiVMV isolates, the P3 of ChiVMV-Dh shared the least homology with the YN-t isolate (76.6% nt, 76.5% aa). The recombination events were examined by RDP4 based on 22 potyviruses sequences downloaded from the NCBI (the sequence information is shown in Fig. 2) that are closely correlated with ChiVMV-Dh. As shown in Table 4, a total of four putative recombination events related to ChiVMV-Dh were found. One of the putative recombination events was detected in CP and the 3'UTR, from nucleotide 8828 to 9781 of ChiVMV-Dh. The major parent was predicted as Dzh-Qyg, while YN-t was shown to bethe minor parent. The other three putative recombination events were detected in the same region of ChiVMV that covered parts of P3, CI, and 6K1. All the recombinants were from Indian and Pakistan isolates, and ChiVMV-Dh was assumed to be the minor parent, while the Korean isolate AM90971 was predicted as the major parent. Phylogenetic analysis was conducted based on the polyprotein amino acid sequence to determine the phylogenetic relationship of ChiVMV-Dh with other potyviruses. The resultant neighbor-joining tree divided all ChiVMV isolates into two distinct clades, and ChiVMV-Dh clustered in the clade2 group. The ChiVMV clade2 has two branches, and ChiVMV-Dh clustered with Sichuan strains, while YN-t clustered in another branch (Fig. 2).

To evaluate the biological characteristics of the two viruses, *Chenopodium quinoa* was mechanically inoculated with the viruses using co-infected chilli pepper samples. Subsequently, single local lesions were separated three times to obtain purified virus isolates [19]. Purified virus isolates were identified as either ChiVMV-Dh or WTMV-Dh by RT-PCR amplification using ChiVMV and WTMV specific primers (S2 Table) and sequencing, respectively. The ChiVMV-Dh and WTMV-Dh strains were then single or mix inoculated to chilli pepper using the methods above. Fourteen days post-inoculation (dpi), different symptoms were observed on chilli pepper, and RT-PCR was conducted to detect virus species (Fig. 3). The result showed WTMV-Dh induced mosaicism and deformity in developing leaves and yellowing of inoculated leaves. Meanwhile, ChiVMV-Dh induced shrinkage and yellowing of developing leaves, and veinal yellowing of inoculated leaves. Co-inoculated chilli pepper showed yellowing and mosaicism on developing leaves and yellowing and necrotic spotting on inoculated leaves.

In conclusion, we reported the co-infection of chilli pepper with WTMV and ChiVMV under natural conditions for the first time. We also report the complete genome sequence of WTMV and ChiVMV from chilli pepper. By co-inoculation experiment, the symptoms of these two viruses on chilli pepper under single-infection and co-infection were determined. It was found that the co-infection symptoms of WTMV and ChiVMV on chilli pepper are a combination of the single-infection symptoms and show more severe symptoms than single-infection. This finding indicates that co-infection of these two viruses may play a synergistic effect and increase virulence.

Recombination events are considered to be a significant source of genetic diversity and enhance host adaptability of plant viruses. Co-infection facilitates genetic exchange and recombination between two viruses. Although we did not find direct recombination events between WTMV-Dh and ChiVMV-Dh, some recombination events were found within the WTMV or ChiVMV subgroups. The recombination events include P1, P3 to CP, and HC-Pro region.

Previous reports have indicated the P1 region is related to host adaptation and definesthe host range [20, 21]. Another study found recombination events in the P1 region of WTMV-Sn, but the minor parents of WTMV-Sn are unknown [14]. Herein, analysis of a WTMV-Dh recombination event found that WTMV-Dh may be a putative minor parent of WTMV-Sn in the P1 region.

Also found were recombination events of ChiVMV-Dh from P3 to the CI region, as the minor parent of isolates originated from India and Pakistan. Previous studies have found that Indian and Pakistan isolates of ChiVMV are closely related to Chinese isolates. Thus, a transboundary movement of infected chilli seedlings or other host plants is speculated [22, 23]. However, our study indicated that the ChiVMV isolates from India and Pakistan are more likely to be recombinant viruses and that ChiVMV-Dh may serve as an intermediary between East Asian and South Asian ChiVMV isolates.

HC-Pro is a multifunctional protein composed of three functional regions, and the N-terminal region is necessary for aphid transmission [24, 25]. We blasted the complete aminoacid sequence of HC-Pro in WTMV-Dh and ChiVMV-Dh. The results showed that the HC-Pro N-terminal regions of these two viruses differ substantially, but the central and C-terminal regions are highly conserved. Overall, the HC-Pro regions of the two viruses share 59%, 79.5% and 93.63% sequence identity in the N-terminal (1–100 aa), central (101–300 aa) and C-terminal (301–457 aa) regions, respectively. Therefore, we speculated the differences in the HC-Pro N-terminal sequences of WTMV-Dh and ChiVMV-Dh might be involved in the affinity with aphid species.

We also found a recombination event in the HC-Pro N-terminal region of WTMV-Dh. The major parent was Laichau, while the minor parent was the Sichuan isolate XC-1. This result further indicates that the N-terminal of HC-Pro is a hot spot for recombination and variability, which may participate in the adaptive evolution of aphid transmission.

Declarations

Acknowledgements

The study was supported by the National Natural Science Foundation of China (31660508), the Science and Technology Program of Yunnan Province (2018FB028, 2018FA020), National Natural Science Foundation of China-Yunnan Joint Fund Key Project (U1802235), Yunnan Provincial Academician Workstation of Fang Rongxiang and Yunling Scholar (Zhongkai Zhang).

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Tables

Table 1

Analyses for read coverage of reads mapping to WTMV-Dh and ChiVMV-Dh in co-infected samples.

Coding region	ChiVMV-Dh reads number	ChiVMV-Dh FPKM	WTMV-Dh reads number	WTMV-Dh FPKM
Complete sequence	215871	2.20E + 04	793404	8.19E + 04
P1	14731	2.05E + 04	54309	7.61E + 04
Hc-pro	29797	2.72E + 04	99328	9.08E + 04
P3	18026	2.19E + 04	65638	7.90E + 04
6K1	531	4.11E + 03	1104	8.54E + 03
CI	35334	2.29E + 04	148485	9.64E + 04
6K2	153	6.47E + 02	364	2.79E + 03
VPg	7350	1.61E + 04	27650	6.05E + 04
NIa	15697	2.71E + 04	52927	9.14E + 04
NIb	32137	2.59E + 04	122457	9.86E + 04
CP	18996	2.76E + 04	55931	8.16E + 04

Table 2

Nucleotide (nt) and amino acid (aa) sequence homologies (%) of the individual coding sequences of WTMV-Dh to those of other WTMV isc

Virus	Polyprotein (nt/aa)	5'UTR (nt)	P1 (nt/aa)	HC-Pro (nt/aa)	P3 (nt/aa)	6K1 (nt/aa)	CI (nt/aa)	6K2 (nt/aa)	VPg (nt/aa)	NIa (nt/aa)	NIb (nt/aa)
WTMV-Laichau (DQ851495.1)	82.6/89.5	58.9	64.5/59.7	82.8/95.2	81.9/81.8	88.9/100	86.1/96.0	87.4/94.3	84.1/93.1	84.4/95.5	83.4/91.
WTMV-Sn (MK070541)	76.6/85.9	71.9	60.2/57.0	81.0/94.3	72.2/70.6	74.7/98.1	77.8/90.8	78.6/90.6	76.2/88.9	79.8/93.4	76.8/90.
WTMV-XC-1 (KM401435.1)	76.1/85.3	48.6	55.5/50.0	81.2/94.1	71.6/71.2	74.7/98.1	78.0/91.0	78.6/90.6	76.2/88.4	79.6/93.0	77.1/89.

Table 3

Nucleotide (nt) and amino acid (aa) sequence homologies (%) of the individual coding sequences of ChiVMV-Dh to those of other ChiVM

Virus	Polyprotein (nt/aa)	5'UTR (nt)	P1 (nt/aa)	HC-Pro (nt/aa)	P3 (nt/aa)	6K1 (nt/aa)	CI (nt/aa)	6K2 (nt/aa)	VPg (nt/aa)	Nla (nt/aa)	N (n
ChiVMV-Yp8 (KC711055)	92.2/95.6	76.7	81.4/78.7	92.0/97.6	93.3/94.8	95.7/98.1	93.6/98.6	92.2/94.1	94.2/95.3	93.9/98.3	9
ChiVMV-LZ (MK405594)	92.1/95.3	76.1	81.9/78.7	92.3/97.8	93.0/94.8	94.4/98.1	93.1/98.0	91.5/94.1	93.9/95.3	93.7/96.7	9
ChiVMV-Pp4 (KC711056)	92.1/95.3	75.3	82.1/79.0	91.8/97.6	93.1/93.6	94.4/98.1	93.4/98.4	92.2/94.1	93.7/94.8	93.9/98.3	9
ChiVMV-YN-t (JX088636)	83.1/90.4	80.2	84.4/82.7	85.9/95.4	76.6/76.5	82.1/100	81.9/91.3	73.9/86.3	83.2/91.6	82.8/89.7	8
ChiVMV-GD (KU987835)	81.8/88.5	53.1	64.8/59.3	81.1/92.6	81.9/80.8	80.9/94.4	83.0/92.4	81.0/90.2	85.5/94.8	86.6/96.7	8
ChiVMV-WC (GQ981316)	81.6/88.3	52.9	67.4/58.7	80.5/92.3	81.9/80.8	79.0/88.9	83.2/92.5	79.7/90.2	85.3/94.8	86.1/96.7	8
ChiVMV(LN832362)	81.3/88.0	52.6	63.0/56.7	80.7/92.8	81.0/79.9	76.5/94.4	83.2/92.9	79.1/88.2	85.9/94.2	86.8/95.9	8
ChiVMV (AJ972878)	81.3/86.1	52.2	63.4/56.7	81.0/91.7	81.8/80.8	79.0/94.4	82.9/86.6	78.4/88.2	86.4/95.3	86.8/95.9	8
ChiVMV(AM909717)	81.5/86.4	51.3	63.7/56.7	80.9/91.7	81.7/80.5	79.0/94.4	82.8/86.4	78.4/88.2	86.6/94.8	86.5/95.5	8
ChiVMV(NC005778)	80.8/85.3	51.0	60.0/54.3	80.7/92.6	81.8/81.1	85.2/96.4	83.2/84.7	78.4/88.2	87.3/93.7	87.5/95.9	8
ChiVMV-PK (MN207122)	84.1/88.5	52.9	64.2/58.3	82.1/93.2	89.4/91.0	87.7/96.3	87.5/88.8	77.8/88.2	85.6/88.4	88.8/97.5	8
ChiVMV-Ch-Jal(GU170807)	82.4/87.1	51.0	63.7/58.0	78.6/91.0	88.4/87.5	88.3/98.1	86.3/88.2	75.2/86.3	83.3/84.7	87.2/97.5	8
ChiVMV-Ch-War(GU170808)	82.3/86.7	51.0	63.0/57.3	78.8/92.1	87.8/86.3	87.7/98.1	86.4/86.9	71.9/80.4	80.2/85.8	86.5/95.0	8
ChiVMV-HN (KR296797)	81.4/87.2	54.1	62.9/56.7	80.1/91.5	81.2/79.7	77.2/94.4	83.4/91.1	79.7/88.2	86.0/94.2	87.1/95.9	8
ChiVMV(AJ237843.3)	80.9/85.4	51.0	60.0/54.3	80.7/92.6	81.8/81.1	85.2/94.4	83.2/84.7	78.4/88.2	87.3/93.7	87.5/95.9	8

Table 4

Summary of predicted recombination events identified by RDP4

Recombinant	Parents		Begin	end	P-value						
	Major	Minor			RDP	GENECONV	BootScan	MaxChi	Chimaera	SiScan	3Seq
WTMV-Dh	WTMV-Laichau	WTMV-XC-1	1049	1494	2.56×10^{-9}	-	1.474×10^{-09}	4.873×10^{-03}	1.909×10^{-02}	1.633×10^{-02}	9.666×10^{-07}
WTMV-Sn	WTMV-XC-1	WTMV-Dh	48	162	8.417×10^{-22}	1.679×10^{-17}	3.085×10^{-43}	1.828×10^{-02}	-	1.202×10^{-10}	1.179×10^{-12}
WTMV-Sn	WTMV-XC-1	WTMV-Dh	166	342	4.794×10^{-32}	4.091×10^{-17}	1.313×10^{-24}	3.776×10^{-12}	3.869×10^{-9}	7.956×10^{-6}	1.179×10^{-12}
ChiVMV-Dh	ChiVMV-Dzh-Qyg	ChiVMV-YN-t	8828	9781	3.187×10^{-19}	3.519×10^{-04}	7.333×10^{-18}	2.684×10^{-08}	3.458×10^{-09}	5.639×10^{-09}	5.898×10^{-13}
ChiVMV-Ch-Jal	AM90971	ChiVMV-Dh	3038	4897	3.711×10^{-14}	-	5.008×10^{-13}	2.368×10^{-06}	2.627×10^{-06}	8.707×10^{-13}	6.871×10^{-06}
ChiVMV-Ch-War	AM90971	ChiVMV-Dh	3326	4426	3.711×10^{-14}	-	5.008×10^{-13}	2.368×10^{-06}	2.627×10^{-06}	8.707×10^{-13}	6.871×10^{-06}
ChiVMV-PK	AM90971	ChiVMV-Dh	3678	4214	3.711×10^{-14}	-	5.008×10^{-13}	2.368×10^{-06}	2.627×10^{-06}	8.707×10^{-13}	6.871×10^{-06}

Figures

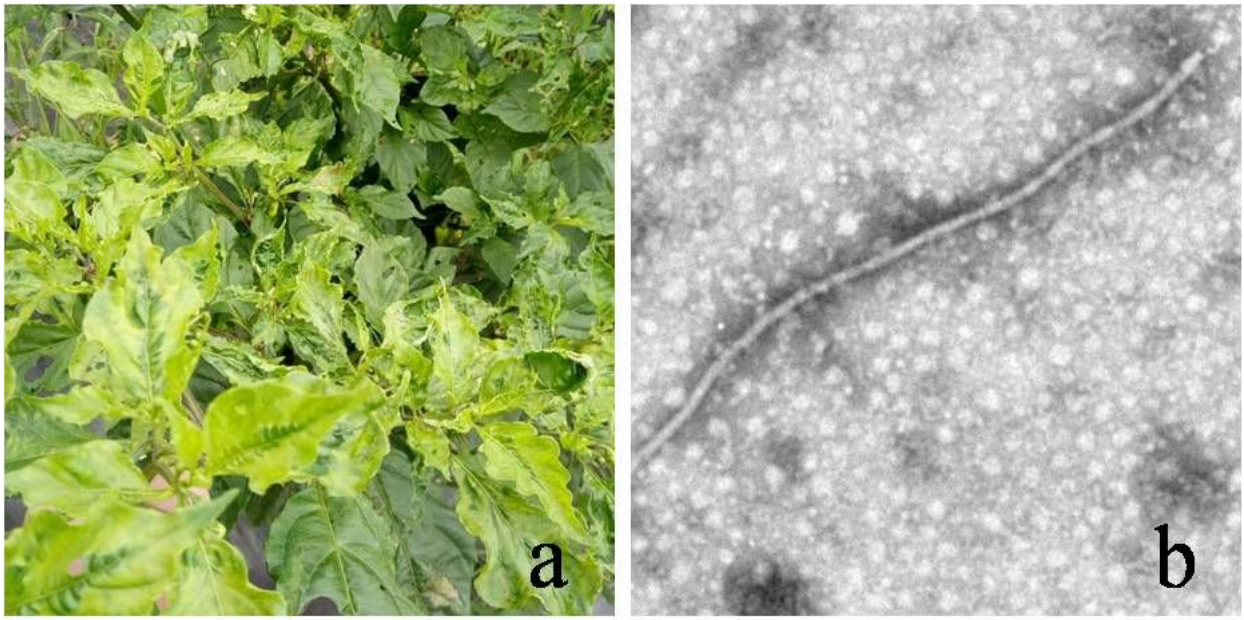

Figure 1

Symptomatic of chilli pepper disease and virus morphology. a, Chilli pepper leaves showed mosaic, mottle and shrinkage; b. Virus particles were observed in the symptomatic leaf tissue of the chilli pepper sample.

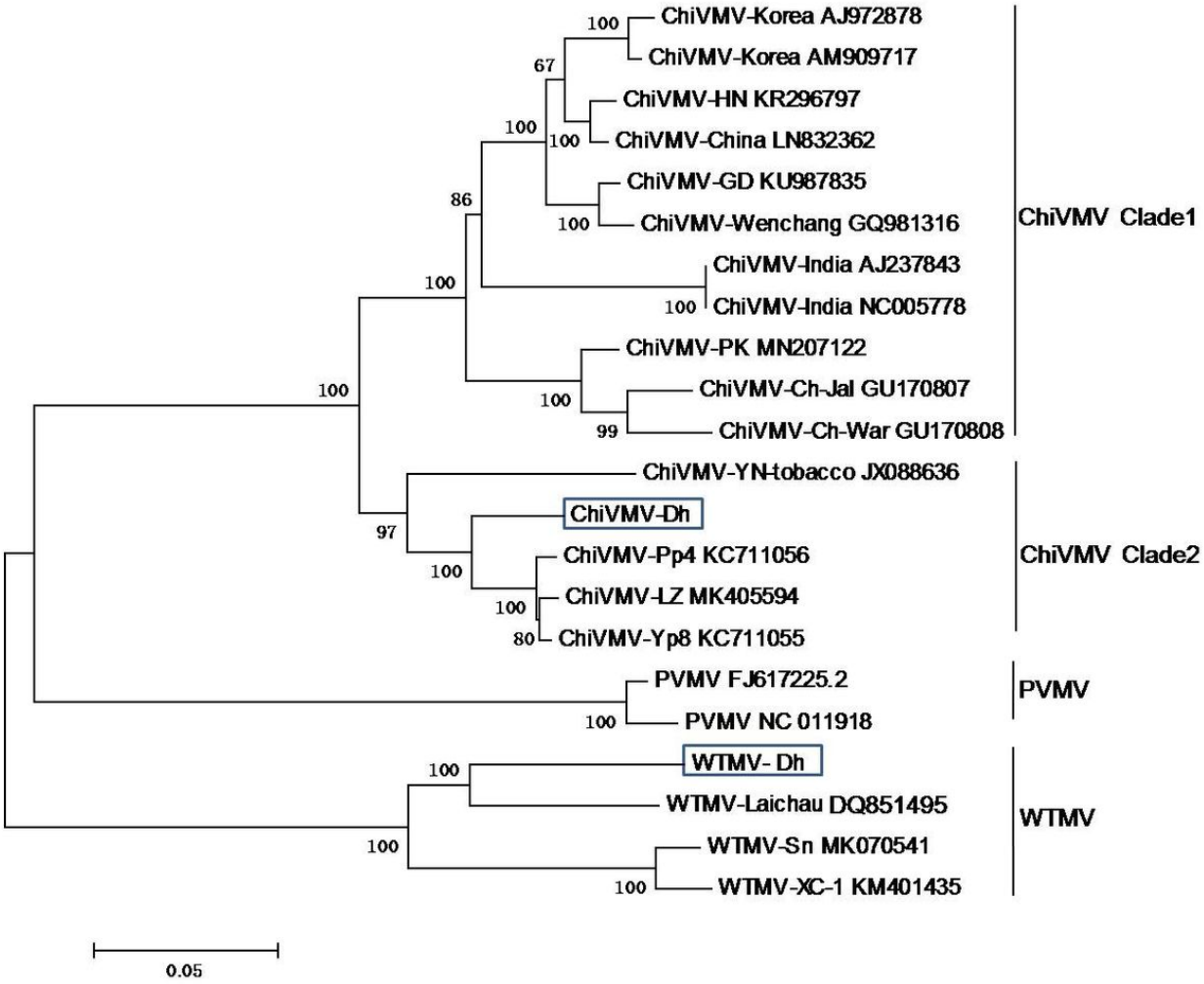

Figure 2

Phylogenetic trees of potyviruses based on polyprotein amino acid sequences. ChiVMV-Dh and WTMV-Dh isolates are boxed. Bootstrap values on the branches represent the percentage of 1,000 bootstrap replicates

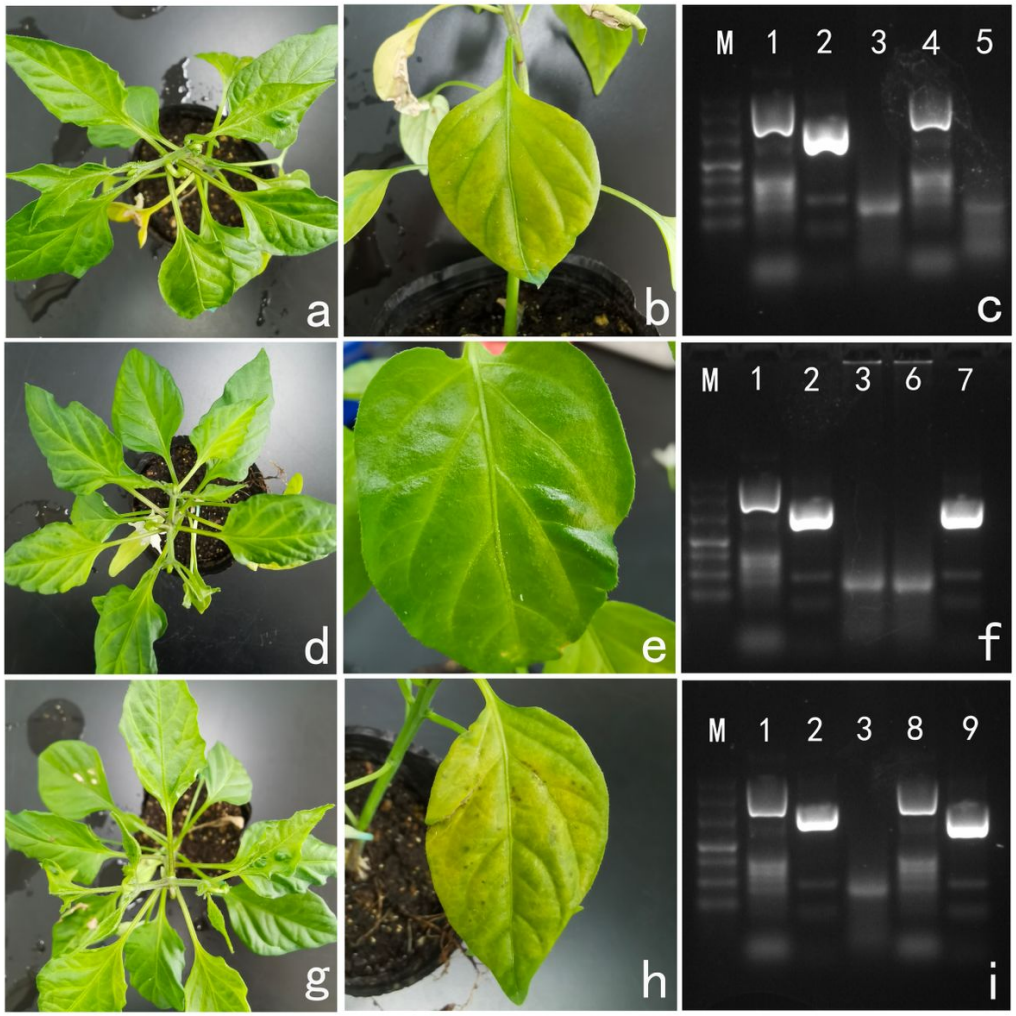

Figure 3

Symptoms of single/co-inoculation of WTMV-Dh and ChiVMV-Dh on chilli pepper at 14 days post-inoculation. a. a. WTMV-Dh symptoms on developing leaves; b. WTMV-Dh symptoms on inoculated leaves; c. Detection WTMV-Dh by RT-PCR; d. ChiVMV-Dh symptoms on developing leaves; e. ChiVMV-Dh symptoms on inoculated leaves; f. Detection of ChiVMV-Dh by RT-PCR; g. co-infection symptoms of WTMV-Dh and ChiVMV-Dh on developing leaves; h. co-infection symptoms of WTMV-Dh and ChiVMV-Dh on inoculated leaves; i. Detection of WTMV-Dh and ChiVMV-Dh by RT-PCR. M: Marker1. WTMV positive control2. ChiVMV positive control; 3. Negative control4, 5, 6, 7, 8 and 9. Detection WTMV and ChiVMV respectively by use pepper samples in the parallel left picture

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- S1Fig.jpg
- SupplementaryTable1.docx
- ChilliveinalmottlevirusisolateDh.fas
- WildtomatomosaicvirusisolateDh.fas