Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

Nightshade (*Solanum nigrum*), an intermediate host between tomato and cucurbits of *Tomato leaf curl New Delhi virus*

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Summary. Geminiviruses infect many crop plants, and are limiting factors for vegetable crop production. Begomoviruses (*Geminiviridae*) cause typical symptoms of leaf curling and puckering in nightshade (*Solanum nigrum*), a seasonal weed in Bihar, India. To investigate if nightshade was an intermediate host for begomovirus, virus DNA was extracted and characterized. The DNA-A of the virus yielded 2737 nt and DNA-B yielded 2706 nt. The intergenic region (IR) showed a conserved nonanucleotide sequence that potentially forms a stem-loop structure. The genomic sequence of DNA-A shared 94% identity with that of *Tomato leaf curl New Delhi virus* (ToLCNDV)-ivy gourd isolate. However, the sequence of DNA-B showed 95% identity with a bitter gourd isolate. PCR-based detection revealed the presence ToLCNDV in bottle gourd, pumpkin, sponge gourd, and bitter gourd. The IR sequences of the viruses isolated from these cucurbits and tomato were 100% identical. Whitefly-mediated transmission of the virus to cucurbits and tomato from nightshade was also demonstrated. These results indicate that nightshade may act as reservoir of ToLCNDV, and is involved in developing epidemics in cucurbit species. The strain of ToLCNDV has probably adapted from solanaceous to cucurbitaceous hosts. This is the first report of ToLCNDV infecting nightshade in India, highlighting this virus as a possible cause of disease epidemics in economically important cucurbits.

Keywords. *Begomovirus*, genetic diversity, leaf curl.

OPEN ACCESS

Citation: M. Ansar, A.K. Agnihotri, T. Ranjan, M. Karn, Srinivasaraghavan A, R.R. Kumar, A.P. Bhagat (2021) Nightshade (*Solanum nigrum*), an intermediate host between tomato and cucurbits of *Tomato leaf curl New Delhi virus*. *Phytopathologia Mediterranea* 60(3): 409-419. doi: 10.36253/phyto-12745

Accepted: July 13, 2021

Published: November 15, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Assunta Bertaccini, Alma Mater Studiorum, University of Bologna, Italy.

INTRODUCTION

Weed plants possess ecological adaptability, and are found throughout the world. They are sources and reservoirs of viruses infecting many economically

important crops. Weeds are also alternative hosts, where economically important pathogens can survive between crop cycles (Mubin *et al*., 2010; Papayiannis *et al*., 2011; Wyant *et al*., 2011; Jyothsna *et al*., 2013). For example, whitefly-transmitted geminiviruses (*Begomovirus*; *Geminiviridae*) are known to infect economically important crops as well as weed hosts (Seal *et al*., 2006; Mubin *et al*., 2009; Ansar *et al*., 2019; Agnihotri *et al*., 2019). *Tomato leaf curl New Delhi virus* (ToLCNDV) is an important bipartite geminivirus in *Begomovirus*, which infects approx. 43 different plant species in the Indian subcontinent, West Asia, and Europe (Moriones *et al*., 2017). The virus affects plant species in the *Cucurbitaceae*, *Fabaceae*, *Malvaceae*, *Euphorbiaceae*, and *Solanaceae* (Hussain *et al*., 2004; Ito *et al*., 2008; Naimuddin *et al*., 2016). ToLCNDV is an emerging virus in the Mediterranean basin. Alternative hosts of ToLCNDV were include *Ecballium elaterium*, *Datura stramonium*, *Sonchus oleraceus*, and *Solanum nigrum* (Miguel *et al*., 2019).

ToLCNDV is a bipartite virus that has two genomic DNAs, DNA-A and DNA-B. There are six open reading frames (ORFs) in DNA-A encoding six proteins that assist in replication, transcription, pathogenesis, and encapsidation. The DNA-B has two ORFs with products that play a major role during the movement of the virus through host plasmodesmata. Both DNA segments share a common region involved in the replication of DNA-B by a DNA-A-encoded replication initiator protein (Padidam *et al*., 1995; Hanley-Bowdoin *et al*., 2013).

In India, a great variety of vegetable crops are grown throughout each year to meet food demands. Tomato is mainly grown during the Rabi season (winter) in northern India, and several cucurbits, including bottle gourd (*Lagenaria siceraria*), pumpkin (*Cucurbita maxima*), sponge gourd (*Luffa cylindrica*), and bitter gourd (*Momordica charantia*), are commonly grown in this region. These crops can be infected by several monopartite and bipartite begomoviruses that induce typical curling and mosaic symptoms. More than 25 weed species grow in crops of the two main vegetables (tomato and cucurbits). Nightshade (*Solanum nigrum* L.), a seasonal weed, grows abundantly associated with tomato and remains in fields up to the peak of the cucurbit season. Nightshade plants have puckered leaves, a symptom previously and commonly attributed to insect feeding. Leaf puckering and mild leaf curling observed in nightshade at the Vegetable Research Farm, Bihar Agricultural University, Sabour, India, suggested an alternate host of ToLCNDV, possibly acting as a green bridge for the virus.

The present study investigated the causal virus of nightshade leaf distortions, and explored the possibility of host shift between two major families of vegetable crops.

MATERIALS AND METHODS

Collection of plant samples, DNA extraction, and PCR for virus detection

Symptomatic leaves of nightshade plants (n = 23) that showed leaf puckering and curling were collected along with symptomless leaves from the experimental vegetable field, at Bihar Agricultural University, Sabour, India, during 2016 to 2018. Total genomic DNA was extracted using a GeneJet DNA isolation kit (ThermoFisher). DNA was also extracted from curled and mosaic leaves of bitter gourd, mosaic leaves of sponge gourd and pumpkin, and mottled leaves of bottle gourd and tomato. PCR was carried out on the DNA samples using the whitefly-transmitted geminivirus-specific primer Deng541F/540R (Deng *et al*., 1994) to detect virus DNA. The PCR program was executed in Surecycler-8800 (Agilent) with the following steps: preheating at 94°C (3 min); 30 cycles of denaturation at 94°C (30 sec), annealing at 53°C (30 sec), extension at 72°C (1 min); and final extension at 72°C (10 min). This PCR was carried out with Dream Taq Green Master Mix (2×) (ThermoFisher) in total 25 µl reaction, which consisted of 2 µl template DNA (25 ng μL^{-1}), 1 µL each primer (20 pmol), 12.5 µL 2× master mix, and 8.5 µL nuclease-free water. Similarly, PCR was also carried out for DNA-B using the specific primer pair PVL1v2040/PCRc1 (Rojas *et al*., 1993). Amplified products were visualized using 1% agarose gel electrophoresis with 1× TAE buffer containing 0.1% ethidium bromide. The gel was examined under a documentation system (UV Tech). Five PCR-amplified products (each 15 µL) of nightshade were directly sequenced using Deng541F/540R primer (10 µL) at Xcelris Lab Ltd, Ahmedabad, India.

Rolling circle amplification and cloning

The DNA extracted from three symptomatic nightshade leaves was subjected to full genome amplification using the rolling circle (RCA) method and the REPLI-g Mini Kit (QIAGEN GmbH). The RCA products were digested with the five restriction enzymes *Sac*I, *Bgl*I, *Pst*I, *Eco31*I, and *Hind*III (ThermoFisher), to obtain a linearized fragment. The digested products were visualized on 1% agarose gel to select the ≈2.7 kb fragments. These were purified using a gel extraction kit (ThermoFisher), cloned into pJET/1.2 blunt cloning vector using the CloneJET cloning kit (ThermoFisher), and were then sequenced at Eurofins Genomics, Bengaluru, India.

Analysis of full genome sequences

The obtained sequences were assembled and analyzed using Bioedit (version 7.2) software. Multiple sequence alignments and phylogenetic trees were made using MEGA X. Both the sequences of ToLCNDV were submitted to GenBank under the acc. No.s MH465599 and MH465600 for, respectively, the genomes of the DNA-A and DNA-B. All ORFs were determined and compared with the representative sequences listed in Table 1, using available software at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The sequences that showed maximum identity with the viral DNA sequences isolated from nightshade were selected for pairwise percent nucleotide identity using ClustalW (http://www.genome.jp/tools/clustalw/).

Amplification of the intergenic region and its sequence analysis

For the intergenic region (IR) amplification, PCR was carried out on the extracted DNAs reported above. The PCR steps were as follows: initial denaturation at 94°C (3 min); 35 cycles of denaturation at 94°C (30 sec), annealing at 54°C (30 sec), extension at 72°C (1 min); final extension at 72°C (10 min). The reaction mixture (25 µL) was prepared with Taq Mix (2×) (Sisco Research Laboratories), which consisted of 2 µL template DNA (25 ng μL^{-1}), 1 µL each of forward and reverse primer (20 pmol), 12.5 µL 2× mix, and 8.5 µL nuclease-free water. A specific primer pair of ToLCNDV-IR F 5'TGCCTTCGAACTGGATGAG3' and ToLCNDV-IR R 5'CCTACGCGATGTGTGAGT3' was designed from the 2.7 kb whole genome of ToLCNDV-nightshade (IR-F 2386-2404, IR-R 579-596). The primer pair was targeted to amplify the IR. From each amplified sample, two PCR products were directly sequenced. The sequences obtained were trimmed and aligned in MEGA X along with the full genome sequence of the nightshade ToLCNDV isolate. Multiple sequence alignments of the viral sequences obtained in the present study and other related viruses was carried out with Genomatix Software Suite v3.10 (http://www.genomatix.de) using default settings.

Table 1. Percentages of nucleotide and amino acid sequence identities between ToLCNDV-nightshade and isolates of other related viruses.

Virus: Host: Location	NCBI GenBank Acc. No.	% identity of whole genome (DNA-A)	% identity in different ORFs						
			AV1	AV2	AC1	AC2	AC3	AC4	AC5
ToLCNDV-Nightshade: India	MH465599	[X]100/2737	[Y]100/771 [Z]100/256	100/329 100/109	100/1086 100/361	100/420 100/139	100/411 100/136	100/294 100/97	100/486 100/161
ToLCNDV-Ivy gourd: India	KY780201	94/2737	93/771 94/256	97/330 100/109	94/1086 93/361	97/420 95/139	99/411 98/136	97/296 95/97	94/363 87/161
BGVMV-Bitter gourd: Bangladesh	KJ862841	93/2737	93/771 95/256	93/339 99/112	94/1086 93/361	97/420 97/139	96/411 96/136	97/296 96/97	-
ToLCNDV-Bitter gourd: India	KY780207	93/2737	92/771 94/256	95/339 100/112	92/1086 91/361	97/420 97/139	98/411 97/136	95/296 90/97	93/486 85/161
BGVMV-Lentil: India	KM190927	93/2740	92/774 95/256	93/342 100/113	93/1086 92/361	96/420 98/139	97/411 95/136	97/296 92/97	93/363 84/120
ToLCNDV-Bottle gourd: India	FN645905	93/2737	94/771 95/256	94/339 97/112	93/1086 91/361	96/420 96/139	95/411 94/136	96/296 92/97	96/225 91/74
BGVMV-Bitter gourd: Pakistan	AM491590	93/2732	92/771 94/256	94/339 98/112	93/1086 82/361	97/420 96/139	97/411 95/136	98/126 41/41	--
ToLCNDV-Poppy: India	KC513822	91/2739	95/771 98/256	94/339 96/112	86/1086 88/361	95/420 93/139	93/411 88/136	79/177 67/58	--
ToLCNDV-Chili: India	KU196750	90/2739	95/771 95/256	94/339 96/112	86/1086 87/361	94/420 92/139	93/411 88/136	79/177 67/58	--
ToLCNDV-Ridge gourd: India	HM989845	90/2739	95/771 96/256	93/339 94/112	86/1086 88/361	95/420 92/139	94/411 88/136	79/177 67/58	96/486 92/160
ToLCNDV-Pumpkin: Pakistan	KT948072	90/2739	92/771 96/256	93/339 95/112	86/1086 87/361	95/420 92/139	12/411 88/136	79/177 67/58	--

[X] % nucleotide identity/total nucleotides in DNA-A
[Y] % nucleotide identity/total nucleotides in the ORF
[Z] % amino acid identity/total amino acids in the ORF

Genetic recombination and phylogenetic relationships

The DNA-A and DNA-B sequences of the nightshade ToLCNDV isolate were used to determine the recombination events with the Recombination Detection Program (RDP4 v.4.36), using default settings (Martin *et al*., 2010). To make the phylogenetic tree, MEGA X software was used, with a bootstrap value of 1000 replicates, and all missing data and gaps were removed (Tamura *et al*., 2013). Thirty sequences of DNA-A and DNA-B of begomoviruses were selected to construct the tree.

Whitefly-mediated transmission in different host plants

Whitefly-mediated transmission of ToLCNDV was tested from nightshade to tomato and four cucurbit plant species. Non-viruliferous colonies of whiteflies (*Bemisia tabaci*) were raised on caged eggplant seedlings, as described by Muniyappa *et al*. (2000). The status of the virus-free colony was verified using PCR on randomly collected whiteflies. Total DNA was extracted using the DNeasy DNA kit (Qiagen) following the manufacturer's protocol. PCR was carried out using Deng541F/540R and ToLCNDV-AF 5'TACGATCTTGTCCGAGATCTCA3', ToLCNDV-AR 5'ACCCAGGTCCTTAAGTACCT3' (DNA-A) primers. A 25 µL reaction mixture, containing 12.5 µL of Dream Taq Green Master Mix (2×), 1 µL of each primer, 2 µL DNA, and 8.5 µL of nuclease-free water, was used for both sets of primers (Deng541F/540R and ToLCNDV-AF/ToLCNDV-AR). The reactions were run in an Eppendorf Nexus thermocycler, with the cycling parameters described above for the Deng541F/540R primer pair, except that the annealing temperature for the ToLCNDV-AF/ToLCNDV-AR primer pair was set at 54°C. Healthy seedlings of nightshade, tomato, bottle gourd, pumpkin, sponge gourd, and bitter gourd were grown separately in insect-proof cages. Eighteen plants of each species were grown in separate trays and replicated in five trays. The transmission assessment was carried out in three different sets of experiments. Non-viruliferous whiteflies (25 ± 2) were released on caged infected nightshade plants for 16 h of acquisition feeding. After that, ten whiteflies were collected and held for 6 h of fasting. They were then released on caged healthy plants. Whitefly-infested nightshade seedlings served as experimental control plants. The whiteflies were then allowed 48 h of inoculation feeding and were killed by spraying with 0.5% Thiamethoxam 25 WG (Syngenta). Inoculated plants with whiteflies were inspected for up to 30 d for symptom appearance.

Field and PCR monitoring for ToLCNDV in nightshade and cucurbits

For field-grown tomato plants, disease incidence (DI) was monitored from the first fortnight of February to July 2016–2018 (12 observations). Plants of nightshade and the cucurbits bottle gourd, pumpkin, sponge gourd, and bitter gourd were also observed for DI. For the cucurbits, 120 plants of each species were marked and monitored every 15 d. The percentage of symptomatic nightshade plants was assessed using 1 m^2 quadrat in each cucurbit plot. Plants that had curled and mosaic leaf symptoms were counted and tagged to record DI. During each observation, 18-26 leaves were collected from different plants of each species and processed for DNA extractions and PCR. Two sets of primers ToLCNDV-AF/ToLCNDV-AR and ToLCNDV-BF 5'ATTATGTATGGTTAAGCGATG3' and ToLCNDV-BR 5'GCGGCCAATATGTCAA3' (DNA-B) were used to confirm the presence of, respectively, DNA-A and DNA-B of ToLCNDV. The reaction (25 µL) was prepared with Taq Mix (2×) (Sisco Research Laboratories), with the PCR program set as described above. The annealing temperature for ToLCNDV-BF/ToLCNDV-BR was set at 48°C.

RESULTS

Presence of ToLCNDV in symptomatic nightshade leaves

The PCR amplification using the whitefly-transmitted geminivirus specific primer Deng541F/540R to detect the presence of virus DNA provided an amplified fragment of ≈530 bp in 16 out of 23 nightshade DNA samples. Of these, five PCR products were sequenced directly, and the NCBI database was searched for the best match of these sequences using BLAST (https://blast.ncbi.nlm.nih.gov). The BLAST results showed that these sequences matched with the sequences of ToLCNDV isolates (data now shown). Subsequently, presence of the virus was further confirmed in all five samples by performing a PCR using DNA-B-specific primers PVL1v2040/PCRc1, which produced a fragment of the expected size, i.e., ≈600 bp.

Genome organization of ToLCNDV

The linearized viral genome produced by *Sac*I digestion was 2737 nt, and its sequence was submitted to the NCBI database under the acc. No. MH465599. Analysis using BLASTn and ORF finder showed that the isolated virus genome had organization typical of geminiviruses,

and consisted of seven ORFs. Of these, two ORFs (AV1 and AV2) were in virion sense, and five (AC1, AC2, AC3, AC4 and AC5) were in the complementary sense separated by IR and a putative stem-loop structure within a conserved nonanucleotide sequence. The DNA fragment obtained by *Pst*I digestion was 2706 nt and had the two ORFs BV1 and BC1 (NCBI database acc. No. MH465600).

DNA-A sequence comparison of the nightshade ToLCNDV isolate with other viruses

The virus isolated from nightshade leaves was named as ToLCNDV-Ns (nightshade), as its genomic organization was identical with that of a ToLCNDV isolate. The DNA-A sequence of ToLCNDV-Ns was compared with that of other ToLCNDV isolates to determine the sequence identity (Table 1). In pairwise alignment, the DNA-A of ToLCNDV showed 94% identical with that of ToLCNDV-Ivy gourd isolate (NCBI database acc. No. KY780201) and 93% identical with that of five other isolates (NCBI database acc. No.s KJ862841, KY780207, KM190927, FN645905, and AM491590). In a general comparison of ORF, the AV1 gene was 771 nt encoding 256 aa as the other viruses used under this study. ToLCNDV-Ns showed a maximum nucleotide identity of 95% with three isolates of ToLCNDV (NCBI database acc. No.s KC513822, KU196750, HM989845). Of these, it showed 98% aa identity only with NCBI database acc. No. KC513822. The AV2 gene was 329 nt and encoded a 109 aa protein. This gene also showed maximum identity (97%) with the corresponding gene of NCBI database acc. No. KY780201. Although nucleotide identity differed across different isolates, 100% identity was found at amino acid sequence level with an ivy gourd isolate (NCBI database acc. No. KY780201), a bitter gourd isolate (NCBI database acc. No. KY780207), and a BGVMV lentil isolate (NCBI database acc. No. KM190927). The AC1, AC2, and AC3 genes with, respectively, 1086 nt/361 aa, 420 nt/139 aa, and 411 nt/136 aa, showed similarity with the corresponding genes of all the isolates used in this study. In ToLCNDV-Ns, the AC4 gene is 294 nt, whereas it is 296 nt in five isolates NCBI database acc. No.s KY780201, KJ862841, KY780207, KM190927, and FN645905. Furthermore, it is 177 nt in NCBI database acc. No.s KC513822, KU196750, HM989845, and KT948072. The AC5 gene (291 nt) was present in only five of the leaf curl viruses used in this comparison study, and its maximum identity was 96% with two isolates (NCBI database acc. No.s FN645905, HM989845), and 94% with NCBI database acc. No. KY780201.

IR sequence analysis

The IR sequence of ToLCNDV-Ns was identified from the 2.7 kb genome, and was aligned with the IR sequences of viruses infecting bottle gourd, pumpkin, sponge gourd, bitter gourd, and tomato. The conserved nonanucleotide sequence in the hairpin-loop (TAATATTAC) was examined, which is distinctive in *Begomovirus* of the family *Geminiviridae* (Fontes *et al*., 1994). The TATA box was recognized in the IR sequences of all isolates (Figure 1). The IR sequence of ToLCNDV-Ns was 100% identical with that of isolates from four cucurbits and tomato. However, it showed 86% to 89% identity with the IR sequences of ToLCNDV isolates from pumpkin (NCBI database acc. No. KT948072), ridge gourd (NCBI database acc. No. HM989845), chili (NCBI database acc. No. KU196750), and poppy (NCBI database acc. No. KC513822). The IR was 272 nt in three isolates, and 274 nt in four isolates.

Genetic recombination and phylogenetic relationships

The DNA-A of ToLCNDV-Ns (NCBI database acc. No. MH465599) was examined for recombination events. Two events in the DNA-A sequence were detected. ToLCNDV bottle gourd (NCBI database acc. No. FN645905) and bitter gourd (NCBI database acc. No. KJ862841) isolates were identified as major parents, with the first recombination breakpoint at residues 435–1035 nt. A second breakpoint was detected at 1368-1968 nt, with major isolate parents being the NCBI database acc. No.s KY780201 (ivy gourd), KJ862841, KY780207 (bitter gourd), and FN645905 (bottle gourd) isolates (Table 2). The minor parents in both recombination events were unknown. These recombination events were detected in the AV1, AV2, AC1, AC2, AC3, and AC5 genes. A putative recombination event detected in DNA-B of ToLCNDV (NCBI database acc. No. MH465600) was in the IR, with breakpoints at residues 128-168 nt. For this event, ToLCNDV NCBI database acc. No. KC545813 (cucumber) was recognized as major parent, and FN435312 (tomato) was recognized as the minor parent.

The DNA-A sequences of ToLCNDV and other begomoviruses were subjected to phylogenetic analysis. This showed that DNA-A sequences of begomoviruses and ToLCNDV-Ns (NCBI database acc. No. MH465599) were grouped with those of ToLCNDV isolates that mainly infect cucurbit hosts (clade G). There were two subclades with parallel evolution. The sequence of ToLCNDV probably evolved earlier, whereas that of the other four isolates (NCBI database acc. No.s KJ862841, AM491590, KY780201, and KY780207) probably evolved at a later stage. The DNA-B of ToLCNDV-Ns was like-

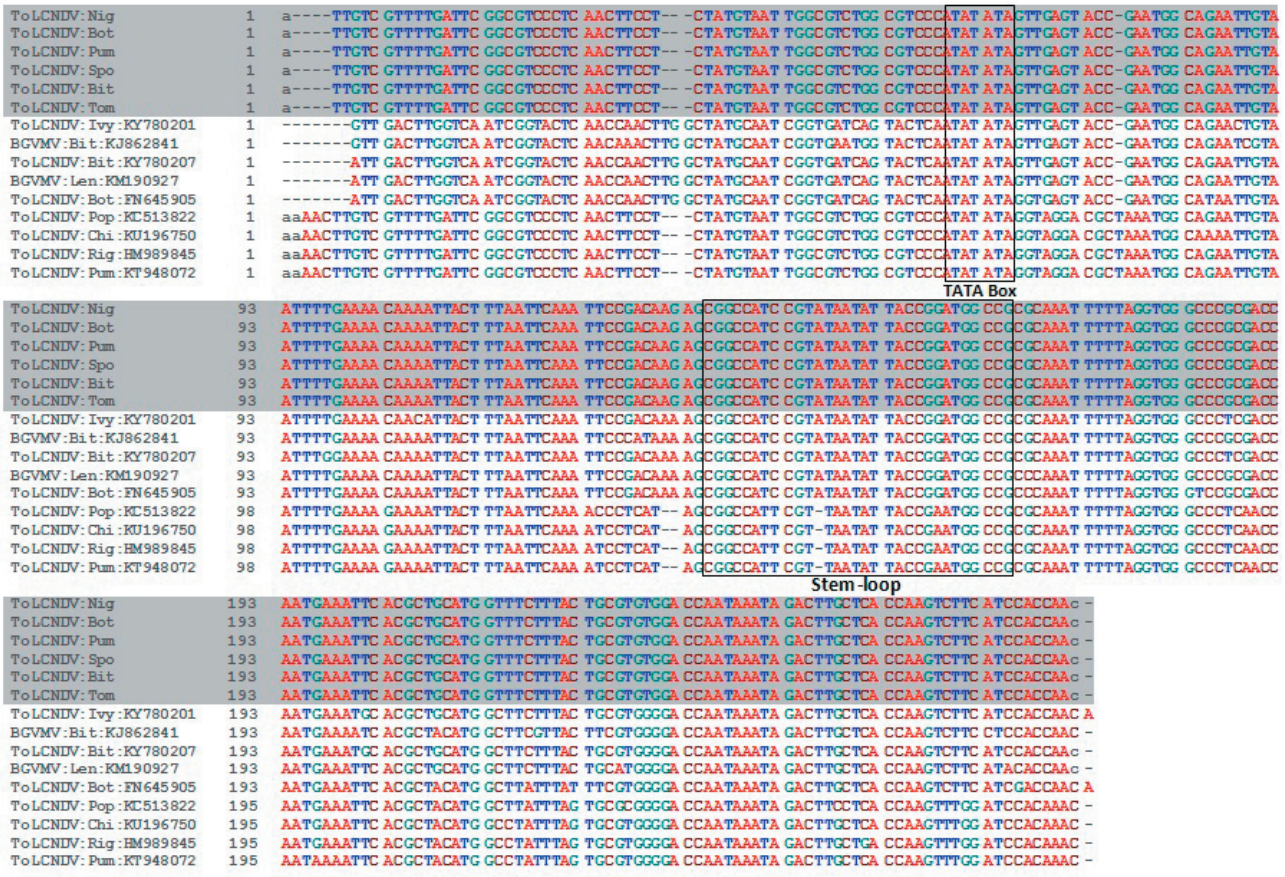

Figure 1. Multiple sequence alignment of IR sequences of ToLCNDV infecting nightshade, and other host plants (Nig = -nightshade, Bot = -bottle gourd, Pum = -pumpkin, Spo = -sponge gourd, Bit = -bitter gourd, Rig = ridge gourd, Ivy = -ivy gourd, Len = -lentil, Pop = -poppy, Tom = tomato, and Chi = -chili).

Table 2. Breakpoint analyses of DNA-A and DNA-B components and the putative parental sequences of ToLCNDV (nightshade), with respective *P*-values

Break points (begin-end	Minor parental sequence(s)	Major parental sequence(s)	RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan
DNA-A								
435-1035	Unknown (HM98984)	FN645905 KJ862841	3.75×10^{-10}	-	5.82×10^{-08}	1.46×10^{-03}	1.88×10^{-06}	5.42×10^{-04}
1368-1968	Unknown (HM989845) Unknown (KC513822) Unknown (KU196750) Unknown (HM007113) Unknown (KT948072)	KY780201 KJ862841 KY780207 FN645905	4.44×10^{-11}	3.59×10^{-06}	3.14×10^{-04}	8.45×10^{-06}	2.72×10^{-05}	2.80×10^{-40}
DNA-B								
128-168	FN435312	KC545813	1.435×10^{-03}	-	-	-	-	-

ly to be ancestral, and the corresponding sequences of other viruses probably diverged later. Other bipartite begomoviruses clustered into species-specific clades; for example, *Mungbean yellow mosaic India virus* (clade A), *Horsegram yellow mosaic virus* (clade B), *Rhyncosia yellow mosaic virus* (clade C), *Dolichos yellow mosaic virus*

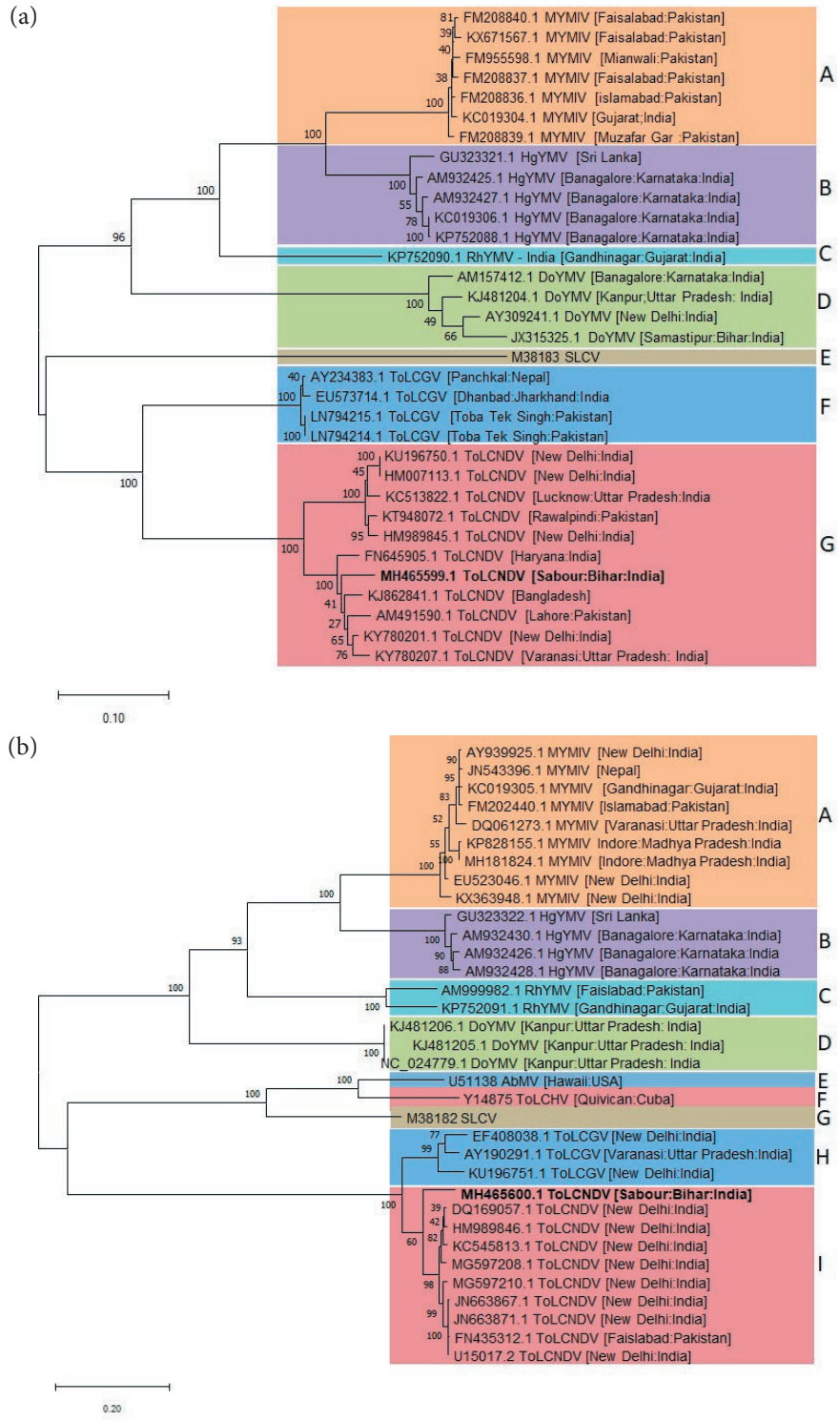

Figure 2. (a) Phylogenetic analysis of *Tomato leaf curl New Delhi virus* (ToLCNDV) DNA-A (present study, nightshade) with other leaf curl and mosaic begomoviruses, using the Maximum-likelihood method in MEGA X with bootstrap (1000 replicates). Clades A: *Mungbean yellow mosaic India virus* (MYMIV), B: *Horsegram yellow mosaic virus* (HgYMV), C: *Rhyncosia yellow mosaic virus* (RhYMV), D: *Dolichos yellow mosaic virus* (DoYMV), E: *Squash leaf curl virus* (SLCV), F: *Tomato leaf curl Gujarat virus* (ToLCGV), and G: ToLCNDV **(b)** Phylogenetic analysis of DNA-B of ToLCNDV (present study, - nightshade) with other reported begomoviruses using the Maximum-likelihood method in MEGA X with bootstrap (1000 replicates). Clades A: MYMIV, B: HgYMV, C: RhYMV, D: DoYMV, E: Abutilon mosaic virus (AbMV), F: Tomato leaf curl Hainan virus (ToLCHV), G: SLCV, H: ToLCGV, and I: ToLCNDV.

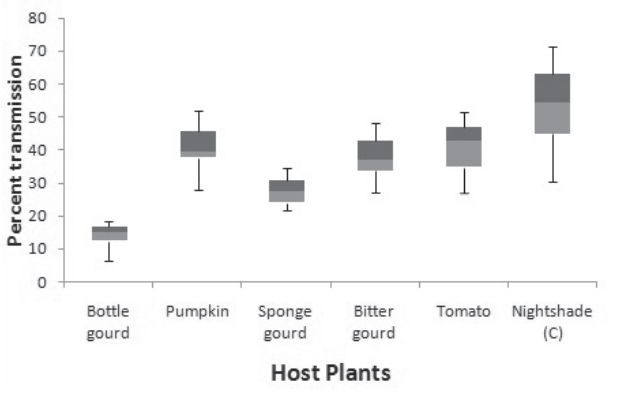

Figure 3. Box plots of mean percent whitefly-mediated transmission of ToLCNDV from nightshade to nightshade (experimental control, C) and different cucurbit hosts.

(clade D), and *Tomato leaf curl Gujarat virus* (clade F), were grouped into clades distinct from that of ToLCNDV (Figure 2a). In the analysis of DNA-B sequences, the present study isolate (MH465600) was also grouped with other ToLCNDV isolates (clade I), which were different from other bipartite begomoviruses (Figure 2b).

ToLCNDV whitefly transmissibility

The transmission experiments showed that ToLCNDV was acquired by whiteflies from infected nightshade plants, and was transmitted to bottle gourd, pumpkin, sponge gourd, and bitter gourd, tomato, and to nightshade (experimental controls). Virus transmission to pumpkin and bitter gourd was as efficient as that to tomato ($P < 0.05$). Lower rates of transmission were observed in bottle gourd (mean = 14.3%) and sponge gourd (28.2%); Figure 3). The symptoms appeared 18 to 25 d after inoculation, and were leaf curl, leaf puckering, and mosaic. Each typical symptom was confirmed by PCR using the primers ToLCNDV-AF/ToLCNDV-AR. Amplification was observed from each symptomatic plant (data not shown).

Field and PCR monitoring for ToLCNDV in nightshade and cucurbits

Symptom appearance in field-grown nightshade plants was noticed in mid February 2016-2018, with a progressive increase until June, and the overall average DI was 16.2%. DI was noticed in the first fortnight of April (except for bottle gourd), with mild leaf mottle and curling. In subsequent observations, symptoms of yellow mosaic, mottling, and curled leaves were increasingly expressed. Bottle gourd remained in the field until July with severe mottling symptoms. More than 77% of the symptomatic plants were infected with ToLCNDV. Pumpkin and bitter gourd developed mild to severe mosaic symptoms by June. Sponge gourd exhibited typical yel-

Host plants	February		March		April		May		June		July	
	Obs-1	Obs-2	Obs-3	Obs-4	Obs-5	Obs-6	Obs-7	Obs-8	Obs-9	Obs-10	Obs-11	Obs-12
Nightshade	+	+	+	++	++	+++	++	+++	+++	+++		
Bottle gourd						+	+	++	++	+++	++++	+++++
Pumpkin					+	+	++	+++	++++	++++		
Sponge gourd					+	++	+++	+++	++++	++++	+++++	
Bitter gourd					++	++	++	+++	++++	+++++		

Obs: Observation

Suspected virus disease incidence (symptomatic appearance)

Tomato leaf curl New Delhi virus PCR positive samples: + 10-15%, ++ >15-25%, +++ >25-35%, ++++>35-45%, +++++ >45%

DI <5% | DI 5-15% | DI 15-25% | DI 25-40%

Figure 4. Appearance and incidence of leaf curl symptoms in nightshade weeds and different cucurbit hosts during months of 2016-2018.

low spots, with 38% DI in the first fortnight of July (Figure 4). In earlier observations (first fortnight of March), 10%–15% of the nightshade samples were positive for ToLCNDV. In subsequent observations, the percentage of infected samples increased. In the late maturity phase, means of 58.3% the bitter gourd samples and 64.0% of sponge gourd were positive for ToLCNDV (Figure 4).

DISCUSSION

Begomoviruses widely affect economically important crops that include more than 300 species (Zerbini *et al*., 2017). These viruses are vectored by whiteflies, and they incite severe symptoms in dicot plants in tropical and subtropical regions (Kumari *et al*., 2010; Singh *et al*., 2012; Inoue-Nagata *et al*., 2016, Agnihotri *et al*., 2018). In the Indian subcontinent, leaf curl diseases are widely distributed in various crops, and cause severe crop and economic losses (Khan *et al*., 2006; Senanayake *et al*., 2007; Zehra *et al*., 2017). ToLCNDV is an important bipartite *Begomovirus*, which affects a diverse range of crops. This virus has expanded its host range, and has spread to new geographical regions including West Asia and the western Mediterranean basin (Fortes *et al*., 2016; Yazdani-Khameneh *et al*., 2016). How begomoviruses adapted to new hosts has become an important area of research.

In the present study, a strain of ToLCNDV is reported to be associated with leaf curl disease in nightshade in the north-eastern part of the Indo-Gangetic plains. Based on PCR detection approx. 70% symptomatic nightshade samples were shown to be infected with begomoviruses, and later confirmed to be infected with ToLCNDV. This virus also infected cucurbit crop plants growing in association with the nightshade weeds. Infections in nightshade probably resulted from infections in previously grown tomato crops, which were severely infected with ToLCNDV. Cucurbits are commonly grown during the summer season (April-July) after harvesting tomato crops (October-March). Between these two important crops, nightshade is a potential reservoir host for the virus.

The strain of the virus studied here was shown to transmit from tomato to cucurbits through nightshade. The IRs of the virus strains from nightshade, tomato, and four cucurbits were identical, and the sequences of these isolates showed high identity with those from some isolates from pumpkin and ridge gourd available in the GenBank database. The recombination analysis indicated that the major parents of these virus isolates were strains that commonly infect cucurbits, particularly bottle gourd and bitter gourd. The whitefly-mediated transmission of these viruses from nightshade to tomato and four cucurbits was also demonstrated, indicating the probable leaf curl disease cycle in this region. In previous studies, ToLCNDV was shown to infect cucumber, melon, and zucchini squash (Mnari-Hattab *et al*., 2015; Panno *et al*., 2016). Nightshade was has also been reported to be infected by *Tomato leaf curl Joydebpur virus* (ToLCJV) along with betasatellite, which provides the link between tomato and chili crops (Ansar *et al*., 2018).

The confirmed presence of ToLCNDV and ToLCJV in nightshade indicates the probable role of this weed as reservoir for viruses that affect different economically important crops. However, a comprehensive detection program is required to determine presence of other viruses in the studied host species, which may cause economic losses. A reservoir host eradication programme with an integrated control approach may be beneficial for related economically important vegetable crops.

ACKNOWLEDGEMENTS

This research was supported by the Science and Engineering Research Board, Department of Science and Technology, Government of India, Young Scientist Scheme-YSS/2015/000923. The paper was developed under BAU communication number 895/201029. The assistance provided by Dr Vihang Ghalsasi for manuscript revision is greatly appreciated.

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