

Detection and characterization of tomato leaf curl New Delhi virus association with mosaic disease of ivy gourd (*Coccinia grandis* (L.) Voigt) in North India

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Received: June 16, 2017; **Revised:** November 16, 2017; **Accepted:** November 29, 2017; **Published online:** December 21, 2017

Abstract: Sixteen ivy gourd (*Coccinia grandis* (L.) Voigt) plant samples showing severe mosaic symptoms were collected from New Delhi and Varanasi (Uttar Pradesh) in India. Begomovirus infection was confirmed by PCR using begomovirus-specific primers. Amplified PCR products (1.2 kb fragments) were cloned and the sequence was characterized. Based on sequence analysis, begomovirus associated with the majority of ivy gourd samples (16) was found to be a member of a bipartite begomovirus species, which is closely related to tomato leaf curl New Delhi virus (ToLCNDV). Therefore, two samples of ivy gourd, IVG1-ND and IVG2-Var, were selected for full-length genome (DNA-A and DNA-B-like sequence) amplification by the rolling circle DNA amplification (RCA) method. Sequence analysis performed using the Species Demarcation Tool (SDT) program revealed that they share 89.5-91.3% (IVG1-ND) and 93.4-96.8% (IVG2-Var) nucleotide (nt) identity with the DNA-A-like sequence of ToLCNDV isolated from cucurbits and chilli, respectively. The IVG1-ND and IVG2-Var isolates shared 90% nt identity among themselves, indicating that they are two different strains of ToLCNDV. Similarly, SDT analysis of the DNA-B-like sequence of IVG1-ND and IVG2-Var exhibited showed 82.7-93.3% nt identity with the DNA-B-like sequences of ToLCNDV infecting cucurbits. The recombination analysis of DNA-A and DNA-B-like sequences showed that the greater part of their genome most likely originated from previously reported begomoviruses that are known to infect chilli and cucurbits through recombination.

Key words: begomoviruses; ivy gourd; recombination; phylogenetic analysis; perennial crop

INTRODUCTION

Ivy gourd (*Coccinia grandis* (L.) Voigt) is an important vegetable and medicinal plant from the Cucurbitaceae family. It is distributed in tropical Asia, Africa, Pakistan, India and Sri Lanka [1,2]. The fruits are used as a green vegetable [2], while different parts of the plants are utilized in traditional medicine for treating jaundice, diabetes, wound healing, ulcers, stomach ache, skin disease, fever, asthma and cough. The leaf constituents possess hypoglycemic, hypolipidemic

and antioxidant properties [3]. Ivy gourd is a good source of vitamin A, β -carotene and proteins. However, its production is hampered by many fungal and viral diseases. Among the viral pathogens, begomoviruses play a major role in reducing fruit yield of ivy gourd. There are several begomoviruses reported in cucurbitaceous crops in India, including tomato leaf curl New Delhi virus (ToLCNDV) [4,5], squash leaf curl China virus [6], pepper leaf curl Bangladesh virus [7], Mesta yellow vein mosaic virus [8], Indian cassava mosaic virus [9], tomato leaf curl Palampur

virus [10], ageratum enation virus [11] and Coccinia mosaic virus [12]. Among these viruses, ToLCNDV has become a major limiting factor for the production of many crop plants [13]. The emergence of new strains of ToLCNDV resulting from recombination enables this virus to infect new hosts, resulting in huge economic losses. ToLCNDV was first reported from India on solanaceous crops [14], but it was found to be infecting more than forty diverse plant species in Pakistan, India, Bangladesh, Iran, Sri Lanka, Malaysia, Taiwan, Thailand, Indonesia, Tunisia, Spain and Italy [13]. ToLCNDV is a bipartite begomovirus and it consists of two genome components, DNA-A and DNA-B. DNA-A contains the AV1 and AV2 genes in the virion sense strand and AC1, AC2, AC3 and AC4 in the complementary strand. DNA-B contains the BV1 gene in the sense strand, and the BC1 gene in the complementary strand [14].

Being a perennial crop, ivy gourd can serve as a potential inoculum reservoir for viruses, as well as being affected itself by the viruses. With this backdrop, the current study aimed to characterize the begomovirus infecting ivy gourd from Uttar Pradesh and New Delhi, India. The presented results should be helpful in improving our understanding of the expanding host range of ToLCNDV and its inoculum reservoirs in terms of its management. In the current study, the incidence, symptomatology, detection and characterization of begomovirus infecting ivy gourd are presented and discussed.

MATERIALS AND METHODS

Virus isolates

Symptomatic ivy gourd plant samples exhibiting severe mosaic in leaves, vines, roots and fruits were collected during 2013-2014 from the fields of Varanasi and Mirzapur, Uttar Pradesh (six ivy gourd symptomatic samples from each location); four samples of virus-infected ivy gourd leaves, roots and vines were also collected from Pusa campus, IARI, New Delhi, India. One asymptomatic ivy gourd sample from each field was also collected. Parts of the collected roots and vines were used for molecular characterization, while the remaining roots and vines were planted under controlled conditions to maintain the virus.

DNA isolation, PCR-mediated amplification and sequencing

Total DNAs were extracted from 100 mg of leaves tissue, roots, stem (vine) and fruits of infected and healthy plants using the cetyl trimethylammonium bromide (CTAB) method described by Doyle and Doyle [15]. DNA extracted from asymptomatic ivy gourd plant samples served as negative controls in PCR amplification. DNA extracted from Bendi yellow vein mosaic disease affected bhendi leaf sample served as a positive control in PCR amplification [16]. The presence of a begomovirus infection in ivy gourd was confirmed by PCR using begomovirus group-specific primer pair 2395F/680R [16]. Two representative samples (IVG1-ND and IVG2-Var) were selected from each location for full-length genome amplification (DNA-A- and DNA-B-like sequences) by the rolling circle amplification (RCA) method using an Illustra TempliPhi amplification kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's recommendations. The resulting products were digested with different restriction endonucleases (*Bam*HI, *Eco*RI and *Hind*III) to provide a 2.8-kb linear DNA fragment. Among the different enzymes, *Bam*HI gave the maximum length of 2.8-kb linear DNA fragments, which were cloned into *Bam*HI-linearized pUC19 plasmid [17]. The ligated products were transformed into a competent DH5 α strain of *Escherichia coli*. Colony PCR followed by restriction digestion with *Bam*HI and *Sca*I was performed to confirm recombinant clones. The confirmed clones were sequenced in both orientations in the Eurofin Genomic India Pvt. Ltd DNA Sequencing facility, Bengaluru, Karnataka, India.

Sequence analysis and detection of recombination events

Sequences were assembled and verified for the presence of begomovirus specific open reading frames (ORFs), using the NCBI ORF finder. Sequence similarity searches were performed by comparing a sequence to all sequences available in the GenBank database using BLASTn [18]. Sequences showing the highest identity scores (Supplementary Table S1) with the present isolates were aligned using the Muscle method in Species Demarcation Tool Version 1.2 (SDT) [19], and the percent pairwise identity of the identified se-

quences and representative sequences from the database were generated. Phylogenetic trees were generated by MEGA 7 software [20] using the maximum likelihood method with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously. Recombination analysis was carried out using the recombination detection program (RDP), GENECOV, Max Chi, Chimera, Si Scan and 3Seq, integrated in the Recombination Detection Program 4 (RDP4) [21], with closely related isolates of ToLCNDV infecting different cucurbits and solanaceous crops and other representative viruses present in the GenBank database (NCBI). Default RDP settings with 0.05 P-value cut-off throughout and standard Bonferroni correction were used.

RESULTS

Detection of begomovirus in ivy gourd

By visual inspection, the most commonly observed symptoms were mosaics, blistering, reduction in leaf size and stunted growth of plants (Fig. 1), with the incidence of disease up to 60 and 70% in New Delhi and Uttar Pradesh, respectively. Total DNA isolated from 16 ivy gourd samples (12 from Uttar Pradesh and 4 from New Delhi) were amplified by PCR using begomovirus-specific primers. A PCR amplicon of a 1.2-kb fragment was cloned and sequenced and showed more than 95% nt identity with ToLCNDV. Therefore, only two samples (IVG1-ND and IVG2-Var) were selected for complete characterization by the RCA method. Sequence alignments using the Muscle method in SDT Version 1.2 showed that the begomovirus clones from India share only 90% nt identity among themselves, indicating that these two ivy gourd isolates (IVG1-ND and IVG2-Var) are two different strains of ToLCNDV



Fig. 1. Ivy gourd plant showing severe mosaic (A) and mosaic and mottling symptoms under natural conditions (B).

infecting cucurbits and chilli in the Indian subcontinent according to the presently applicable species demarcation criteria for begomoviruses [22].

Genome organization of DNA-A-like sequence of begomovirus

The DNA-A-like sequences of ivy gourd clones IVG1-ND (KY780201) and IVG2-Var (KY780202) were determined to be 2739 nt in length and resembled the typical genome organization of Old World bipartite begomoviruses with potentially encoded six conserved ORFs: AV2 (precoat protein), AV1 (coat protein) in sense orientation, and AC3 (replicase enhancer protein), AC2 (transcriptional activator protein), AC1 (replication associated protein), AC4 (C4 protein) and AC5 (C5 protein) in antisense orientation, with the capacity to encode proteins of predicted molecular mass of 11.05 kDa or more.

SDT analysis of the DNA-A-like sequence of ivy gourd clone IVG1-ND (2739 nt, GenBank accession number KY780201) showed the highest nt identity of 89.5-91.3% with ToLCNDV infecting different cucurbits in the Indian subcontinent. The DNA-A-like sequence of the ivy gourd clone IVG2-Var (2739 nt, GenBank accession number KY780202) showed a maximum nt identity of 93.4-96.8% with an isolate of ToLCNDV infecting chilli in India (Table 1a). Comparison of the DNA-A-like sequence of the ivy gourd clone IVG1-ND with other isolates of ToLCNDV that infect different crops revealed that the nt identity ranged from 88.8-90.4% with tomato, 89.4-90.5% with chilli and 89.4-89.9% with potato. Similarly, the ivy gourd clone IVG2-Var identity ranged from 93.1-94.4% with tomato, 88.5-93.5% with cucurbits and 93.8-94.0% with potato (Table 1a). Based on the current species demarcation criteria for begomoviruses (91% nucleotide sequence identity) [22], the begomovirus clone IVG1-ND isolated from ivy gourd is a new strain of ToLCNDV infecting cucurbits, whereas the clone IVG2-Var is an isolate of ToLCNDV infecting chilli in India.

The phylogenetic tree based on the comparison of the complete nucleotide sequences of the DNA-A-like sequence of ivy gourd clones (IVG1-ND and IVG2-Var) was characterized in this study. All sequences of begomoviruses identified previously on cucurbits, sola-

Table 1a. Percentage nucleotide sequence identities for pairwise comparisons between the complete sequences (DNA-A) and intergenic regions (IR) of the virus isolates from ivy gourd obtained in this study with selected begomoviruses available in the databases.

Begomovirus species [#]	ToLCNDV [IN:ND:IVG1:04]		ToLCNDV [IN:Var:IVG2:05]	
	DNA-A	IR	DNA-A	IR
ToLCNDV-Cucurbits (14*)	<u>89.5-91.3</u>	74.9-81.5	88.5- 93.5	81.2- 90.4
ToLCNDV-Potato (4*)	89.4-89.9	<u>86.4-87.5</u>	93.8 – 94.0	79.4-82.3
ToLCNDV-Tomato (11*)	88.8-90.4	70.9-83.4	93.1- 94.4	70.6-90.4
ToLCNDV-Chilli (4*)	89.4-90.5	79.0-82.7	<u>93.4-96.8</u>	85.4-90.1
ToLCNDV-Eggplant (1*)	89.1	80.5	92.9	87.2
ToLCPV-Tomato (2*)	75.1-81.8	72.7-75.3	86.4-86.9	<u>88.2-92.2</u>
ToLCPV-Cucurbits (24*)	81.1-82.3	72.5-74.6	85.7- 86.9	87.5-91.9
SLCCNV-Pumpkin (5*)	84.7-86.6	77.2-80.6	87.2-90.7	83.7-86.2
MYMIV (2*)	67.6-68.4	61.8-62.8	66.1-67.0	59.2-60.0

*Numbers of sequences from the databases used in the comparisons. IR – Intergenic region. #The species are indicated as tomato leaf curl New Delhi virus (ToLCNDV), tomato leaf curl Palampur virus (ToLCPV), squash leaf curl China virus (SLCCNV), tomato leaf curl Gujarat virus (ToLCGuV), mungbean yellow mosaic Indian virus (MYMIV). For each column the highest value is underlined.

Table 1b. Percentage of amino acid sequence identities of encoded genes of DNA-A from the ToLCNDV isolated from ivy gourd and genes of closely related begomoviruses selected in the databases.

Begomoviruses	CP (AV1)		V2		Rep (AC1)		TrAP (C2)		REn (C3)		C4		C5	
	IVG1	IVG2												
ToLCNDV-Cucurbits (14*)	<u>96.8-98.4</u>	<u>96.8-98.0</u>	<u>91.7-96.3</u>	<u>90.1-96.4</u>	<u>83.3-89.1</u>	<u>92.2-98.3</u>	<u>88.4-94.2</u>	<u>91.3-97.8</u>	84.5-89.7	91.9-96.3	<u>55.1-77.5</u>	<u>77.5-96.5</u>	88.3-90.0	<u>58.3-95.6</u>
ToLCNDV-Potato (4*)	97.6-98.0	94.9-96.0	92.6-94.4	92.6-93.7	86.7-87.2	94.4-95.0	87.0-88.4	89.9-91.3	86.0-86.7	90.3-94.1	60.3-65.5	87.9-91.3	84.1-89.1	88.1-90.0
ToLCNDV-Tomato (11*)	77.7-98.2	77.3-97.6	70.6-95.5	72.3-95.5	80.8-87.2	79.2-96.1	54.4-92.8	57.4-94.2	63.4-89.7	61.9-95.5	60.3-63.7	84.4-91.3	80.8-92.3	55.9-90.6
ToLCNDV-Chilli (4*)	98.0- 98.2	97.6-98.0	94.4-95.4	93.7-96.3	86.9-88.0	95.0-97.5	90.6-92.8	92.0-97.6	<u>86.7-90.4</u>	<u>91.9-98.5</u>	60.3-68.9	86.2-96.4	<u>83.3-92.5</u>	87.5-89.4
ToLCNDV-Eggplant (1*)	98.0	97.6	93.5	95.5	86.7	95.2	92.8	92.0	86.7	92.6	62.0	84.4	-	-
ToLCPaV-Tomato (2*)	91.0-91.2	90.0-90.6	74.3-76.1	77.0-77.6	81.1-81.4	90.5-90.8	79.0-79.1	78.0-78.4	77.9-78.6	78.6-79.4	62.0-63.7	91.3-93.1	57.0-57.5	55.0-55.9
ToLCPaV-Cucurbits (24*)	90.2-91.7	85.5-91.4	73.3-76.1	76.7-79.4	80.6-81.1	88.9-90.3	77.6-79.8	76.9-79.1	77.2-79.4	77.9-80.1	62.0-65.1	86.2-93.1	57.0-57.3	55.0-55.4
SLCCNV-Pumpkin (5*)	95.3-98.1	<u>94.5-98.0</u>	91.7-94.4	93.7-95.5	80.0-82.8	88.9-92.2	70.1-73.1	71.6-73.8	75.7-77.9	78.6-83.0	53.4-55.1	72.4-74.1	86.6-91.6	83.2-88.1
MYMIV (2*)	72.2-74.2	71.8-73.0	42.0-42.2	42.8-44.6	69.0-70.2	70.0-70.3	44.4-45.9	45.1-45.9	39.0-39.5	38.0-39.5	54.6-55.6	50.0-51.7	23.0-23.7	24.0-24.1

*Numbers of sequences from the databases used in the comparisons. #The species are indicated as tomato leaf curl New Delhi virus (ToLCNDV), tomato leaf curl Palampur virus (ToLCPV), squash leaf curl China virus (SLCCNV), mungbean yellow mosaic Indian virus (MYMIV). For each column the highest value is underlined.

naceous crops in the Indian subcontinent and other selected begomovirus sequences available in the database are shown in Fig. 2A. Characterized ivy gourd clones (IVG1-ND and IVG2-Var) were segregated with earlier characterized isolates of ToLCNDV infecting chilli and bitter melon in the Indian subcontinent (Fig. 2A).

ORF-wise sequence identities of IVG1-ND and IVG2-Var at the protein level exhibited the highest identity with isolates of ToLCNDV infecting cucurbits in four regions: AV2, CP, C1 and C2. However, REEn (C3) and the C5 region in IVG2-Var showed maximum amino acid identity with an isolate of ToLCNDV infecting chilli and cucurbits (Table 1b). The intergenic region (IR) of ivy gourd clones IVG1-ND (86.4-87.5%) and IVG2-Var (88.2-92.2%) had more homology with the IRs of ToLCNDV and ToLCPV (tomato leaf curl Palampur virus) infecting potato and

tomato, respectively (Table 1a). The length of the IR is 272 nt and is similar to those of other bipartite begomoviruses reported so far. The IR encompasses an absolutely conserved hairpin structure containing a nonanucleotide sequence (TAATATTAC) that marks the origin of virion-strand DNA replication. Two repeated sequences known as “iterons” (GGTGTGTC nt positions 2605-2610 and 2633-2638) were detected adjacent to the TATA box in both ivy gourd isolates (IVG1-ND and IVG2-Var).

Genomic organization and affinities of the DNA-B-like sequence

The complete DNA-B-like sequence of IVG1-ND and IVG2-Var clones was determined in both orientations and was found to be 2694-2695 nt in length.

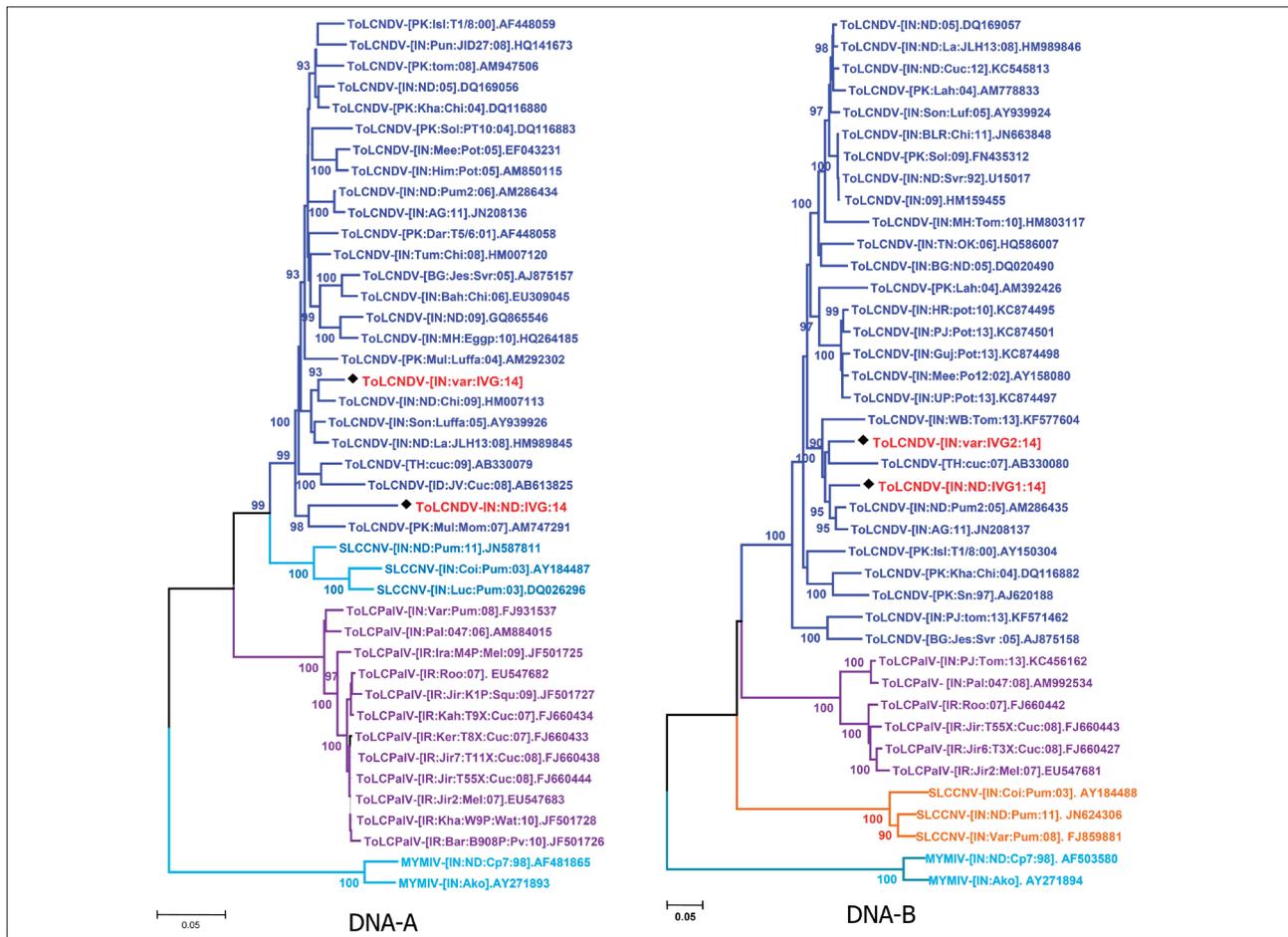


Fig. 2. Dendrograms were constructed using the maximum likelihood method. **A** – DNA-A sequence of begomoviruses (IVG1-ND& IVG2-Var). **B** – DNA-B sequence of begomoviruses (IVG1-ND& IVG2-Var) associated with severe mosaic disease of ivy gourd from two distinct geographical regions in India. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown below the branches. The begomoviruses acronyms given are: tomato leaf curl New Delhi virus (ToLCNDV), tomato leaf curl Palampur virus (ToLCPV), squash leaf curl China virus (SLCCNV) and mungbean yellow mosaic Indian virus (MYMIV). The database accession number in each case is given. Isolate and strain descriptors are as given in reference 22 in the text.

The sequences are available in the database under the accession numbers of KY780203 and KY780204. In order to compare the cloned sequences of ivy gourd clones (IVG1-ND and IVG2-Var) with the published begomovirus sequences, sequence identity searches were performed using the BLAST algorithm. Extensive comparison and SDT analysis showed that these clones shared the highest nt identity with ToLCNDV (82.7-93.3% nucleotide sequence identity), which infects cucurbits in the Indian subcontinent (Table 2). Comparison of the DNA-B-like sequence of ivy gourd clone IVG1-ND with other isolates of ToLCNDV showed nt identity of 82.3-89.3% with tomato,

85.3-89.3% with chilli and 86.8-87.5% with potato. Similarly, ivy gourd clone IVG1-Var had an nt identity of 83.6-90.2% with tomato, 86.2-89.6% with chilli and 87.6-88.1% with potato (Table 2). These results are well supported by phylogenetic analysis, which showed that the clones IVG1-ND and IVG2-Var are closely clustered with ToLCNDV infecting cucurbits in India and China (Fig 2b). The IR of the clones shared 74.4-96.9% nt identity with ToLCNDV isolates infecting cucurbits. The length of the IR in both isolates is 310 nt and is similar to those of ToLCNDV isolates available in the database. Within the intergenic region, incomplete direct repeats of an iteron

Table 2. Percentages of nucleotide identities between the genomic component (DNA-B) and amino acid sequence identities of encoded genes from the ToLCNDV infecting ivy gourd with the components and genes of selected begomoviruses available in the databases.

Begomoviruses	IVG1				IVG2			
	DNA-B ^a	IR ^a	BV1 ^b	BC1 ^b	DNA-B ^a	IR ^a	BV1 ^b	BC1 ^b
ToLCNDV-Cucurbits (9*)	<u>85.8-94.4</u>	<u>74.4-96.9</u>	<u>89.5-97.3</u>	93.5-94.6	<u>86.3-93.6</u>	<u>75.6-90.9</u>	<u>89.5-97.3</u>	93.5-94.6
ToLCNDV-Potato (6*)	86.8-87.5	81.9-83.2	91.4-93.6	93.5-94.3	87.6-88.1	83.7-86.0	91.4-93.6	93.5-94.3
ToLCNDV-Tomato (13*)	82.3-89.3	83.0-91.0	86.8-93.6	85.0-93.5	83.6-90.2	82.0-89.3	86.8-93.6	85.0-93.1
ToLCNDV-Chilli (4*)	85.3-89.3	85.9-86.7	92.5-93.6	88.6-93.2	86.2-89.6	85.0-86.2	92.5-93.6	88.6-93.2
ToLCPV-Cucurbits (12*)	73.8-75.6	70.8-78.4	77.6-78.3	89.3-90.3	74.4-76.5	67.7-73.6	77.6-78.3	89.3-90.2
ToLCPV-Tomato (3*)	74.4-75.6	70.8-74.0	70.5-70.8	88.9-90.7	75.5-76.4	67.7-68.9	70.5-70.8	88.9-90.6
SLCCNV-Pum (4*)	72.5-73.2	71.7-73.6	70.5-70.8	86.4-88.9	73.3-73.4	73.5-74.8	70.5-70.8	86.4-88.9
ToLCNDV-Okra (1*)	89.3	84.3	92.9	<u>96.7</u>	88.1	84.7	92.9	<u>96.7</u>
MYMIV (2*)	58.8-59.5	58.4-70.1	29.0-29.2	43.3-44.5	58.2-58.9	58.1-57.8	29.0-29.2	43.3-44.52

*Numbers of sequences from the databases used in the comparisons. ^aNucleotide identity; ^bAmino acid identity. BV1 – nuclear shuttle protein gene, BC1 – movement protein gene. #The species are indicated as tomato leaf curl New Delhi virus (ToLCNDV), tomato leaf curl Palampur virus (ToLCPV), squash leaf curl China virus (SLCCNV), mungbean yellow mosaic Indian virus (MYMIV). For each column the highest value is underlined.

(GGTGTC) were detected adjacent to the TATA box of the Rep promoter and probable Rep binding motifs, which bind in a sequence-specific fashion to iterated DNA motifs (iterons) functioning as essential elements for virus-specific replication.

Analysis of the complete DNA-B-like sequence of IVG1-ND and IVG2-Var clones showed a typical genome organization similar to other bipartite begomoviruses with two ORFs. One is on the virion strand BV1 (movement protein), and the other is on the complementary strand BC1 (nuclear shuttle protein), possessing the capacity to encode proteins of a predicted molecular mass of 30 kDa or more. When individually encoded proteins were compared, the highest amino acid sequence similarities (89.5-97.3%) of movement protein was with ToLCNDV infecting cucurbits and nuclear shuttle protein (96.7%), with BYVMV infecting okra, respectively (Table 2).

Recombination detection analyses

Ivy gourd isolates (IVG1-ND and IVG2-Var) were subjected to recombination analysis using Recombination Detection Program 4 [21]. The obtained results indicate that the DNA-A-like sequence of IVG1-ND and IVG2-Var had a recombinant molecule arising from cucurbit- and chilli-infecting begomoviruses (ToLCNDV and ToLCPV) from India (Fig. 3). RDP classified ToLCNDV and ToLCPV as the major as well as minor parents that donate the initial fragment of IR, REP, AC4 and CP to the DNA-A-like sequence of IVG1-ND and IVG2-Var to infect ivy gourd. In IR,

the iteron sequence of both isolates IVG1-ND and IVG2-Var remained the same as in the other strains of ToLCNDV infecting cucurbits. Similarly, in the case of the DNA-B-like sequence, the greatest part of BV1 (movement protein) was derived from ToLCNDV infecting cucurbits and tomato (Fig. 3). Out of nine, six algorithms used by RDP4, RDP, GENECONV, MaxChi, Chimera, SiScan and 3Seq strongly support the recombinant origin of this virus in DNA-A-like sequences of IVG1-ND and IVG2-Var. However, in the DNA-B-like sequence only the IVG1-ND isolate had a recombinant origin, which is strongly supported by all the methods (Supplementary Table S2).

DISCUSSION

The begomoviruses transmitted by *Bemisia tabaci* are becoming the major constraint in the production of many crops belonging to different taxonomic classes. Diseases caused by one of its member, ToLCNDV, are emerging as a prime challenge to the cultivation of many agricultural crops in Pakistan, India, Bangladesh, Iran, Sri Lanka, Malaysia, Taiwan, Thailand, Indonesia, Tunisia, Spain and Italy [13]. The virus was first identified in tomato [14] and subsequently in different solanaceous vegetables [23,24]. The host range of the virus is very broad and known to infect many cucurbitaceous vegetables, such as bottle gourd, bitter melon, cucumber, long melon, pumpkin, ridge gourd and watermelon in northern and north-western India [25-28]. The losses incurred by this virus amount to 100% in cucurbits crops [29]. A disease incidence

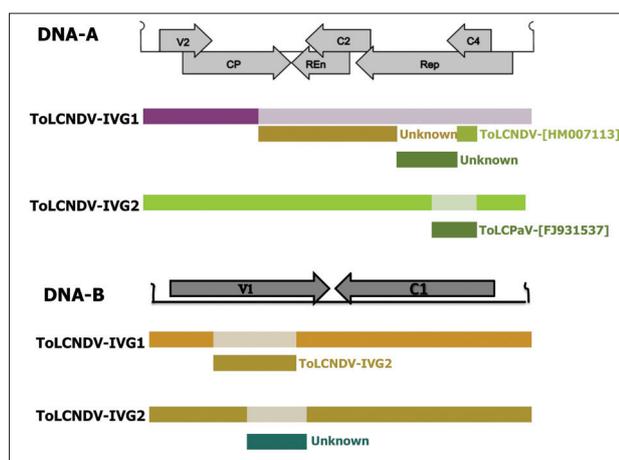


Fig. 3. Analysis of recombination for ToLCNDV isolates isolated from ivy gourd. The begomovirus acronyms given are tomato leaf curl New Delhi virus (ToLCNDV) and tomato leaf curl Palampur virus (ToLCPV). A sequence of indeterminate origin is indicated as “unknown”. The box below at the top of the diagram indicates the approximate position recombination is occurs in the genome of the begomovirus.

up to 70% with a similar begomovirus infection in other cucurbits was observed in ivy gourd, indicating its devastating effect. The detection of ToLCNDV in the ivy gourd samples collected from Varanasi, Mirajpur and New Delhi, and their subsequent genome sequencing and analysis in the current study adds it to the list of its expanding host range. As ivy gourd is a vegetative propagated crop with a perennial character, the ToLCNDV detected is an emerging challenge for its cultivation, apart from the plant serving as a reservoir for the virus inoculum. The PCR diagnostic and viral genome sequencing of the samples collected from two different locations confirmed that the mosaic disease of ivy gourd is associated with two different strains of bipartite begomoviruses (ToLCNDV) infecting cucurbits and chilli in the Indian subcontinent. The symptoms on ivy gourd were similar to those induced by begomoviruses infecting other cucurbits reported worldwide.

Analysis of the CR sequences of both DNA-A- and DNA-B-like sequences of the ivy gourd clones IVG1-ND and IVG2-Var revealed that they possessed more homology with an isolate of ToLCNDV infecting tomato, eggplant and potato. The IR contains a predicted stem-loop sequence with a conserved non-nucleotide sequence (TAATATTAC) in the loop, which can be found in the majority of geminiviruses

characterized to date and marks the origin of virion-strand DNA replication [30,31].

The iteron sequences of both DNA-A- and DNA-B-like sequences identified in the stem loop regions of IVG1-ND and IVG2-Var were identical to the ToLCNDV infecting tomato, eggplant and potato. Iterons are recognition sites for sequence-specific binding of the cognate REP protein [32]. Depending on the viral species, modifications with respect to sequence, size or number of iterons, or even a point mutation in these motifs can adversely affect Rep binding, both *in vivo* and *in vitro* [33,34]. We failed to detect the association of betasatellites and alphasatellites, suggesting that mosaic disease in ivy gourd is caused by an Old World begomovirus with bipartite genomes.

Recombination is the most important factor in the emergence and evolution of begomoviruses across the world [35-37]. Recombination analysis of the virus isolates in the present study suggests that ToLCNDV infecting ivy gourd is a recombinant variant of previously reported viruses. To be considered as a recombinant event in the RDP program, the event should have a significant result in at least three of the methods employed in the RDP program [21]. Evidence suggests that DNA-A- and DNA-B-like sequences of the ivy gourd isolates IVG1-ND and IVG2-Var descended from two distinct viruses, ToLCNDV and ToLCPV, that infect cucurbits and tomato in the Indian subcontinent. Recombination is a rapid process to create new genomes with adaptive advantages, which could accelerate their evolution, favoring expansion of the host range and therefore the emergence of novel diseases [37,38].

Ivy gourd is a perennial crop and propagated clonally by stem cuttings and tuberous roots, and for commercial cultivation most growers depend on such means of vegetative propagation. Once stem cuttings are infected by viruses, they can act as reservoirs for the mobilization of viruses to other cucurbitaceous crops. The occurrence of mosaic disease on ivy gourd presents an alarming signal against the utilization of such planting materials in crop improvement programs. Although there are many techniques available to eliminate virus infection in clonally propagated plants, the techniques must be thoroughly standardized for this crop for the complete elimination of the

virus. The other major concern is the international exchange of germplasm whereby viruses are introduced into areas where they are absent; this is one of the major reasons for the expanding host range of begomoviruses [39]. Therefore, further investigation into the occurrence, distribution and diversity of begomoviruses is needed for the successful management of these viruses in cucurbit crops.

Acknowledgments: The research was supported by the project: "Consortium platform on vaccines and diagnostics", funded by the Indian Council of Agricultural Research, Government of India, New Delhi, India.

Author contributions: All the authors participated in the research and article preparation. VV designed the experiments and drafted the article. CNLR designed the study, participated in the acquisition and analysis of the data and revision of the manuscript; SS participated in the collection of the data. SKS revised the manuscript. MKR guided and designed the study and revised the article. All authors approved the submitted version of the article.

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

REFERENCES

- Cooke CIET. Flora of Presidency of Bombay. London: Taylor and Francis; 1903. p. 645.
- Sastri BN. The Wealth of India, A dictionary of raw material and industrial products. CSIR New Delhi. Publication and Information Directorate; 1950. 413 p.
- Tamilselvan N, Thirumalai T, Elumalai EK, Balaji R, David E. Pharmacognosy of *Coccinia grandis*: a review. Asian Pac J Trop Biomed. 2011;1:299-2.
- Sohrab SS, Mandal B, Pant RP, Varma A. First reports of association of tomato leaf curl New Delhi virus with yellow mosaic disease of *Luffa cylindrica* in India. Plant Dis. 2003;87:1148.
- Tiwari AK, Snehi SK, Singh R, Raj SK, Rao GP, Sharma PK. Molecular identification and genetic diversity among six begomovirus isolates affecting cultivation of cucurbitaceous crops in Uttar Pradesh, India. Arch Phytopathol Plant Prot. 2012;45:62-72.
- Muniyappa V, Maruthi MN, Babiha CR, Colvin J, Briddon RW, Rangaswamy KT. Characterization of pumpkin yellow vein mosaic virus from India. Ann Appl Biol. 2003;142:323-31.
- Raj SK, Snehi SK, Khan MS, Tiwari AK, Rao GP. First report of pepper leaf curl Bangladesh virus strain associated with bitter melon yellow mosaic disease in India. Australasian Plant Dis Notes. 2010;5:14-16.
- Khan JA, Siddiqui MK, Singh BP. The association of Begomovirus with bitter melon in India. Plant Dis. 2002;86:3.
- Rajinimala N, Rabindran R. First report of Indian cassava mosaic virus on bitter melon (*Momordica charantia* L.) in Tamil Nadu, India. Australasian Plant Dis Notes. 2007;2:81-82.
- Namrata J, Saritha RK, Datta D, Singh M, Dubey RS, Rai AB, Rai M. Molecular characterization of tomato leaf curl Palampur virus and pepper leaf curl betasatellite naturally infecting pumpkin (*Cucurbita moschata* L.) in India. Indian J Virol. 2010;21:128-32.
- Raj SK, Snehi SK, Khan MS, Tiwari AK, Rao GP. Molecular identification of an Ageratum enation virus isolate associated with mosaic disease of pointed gourd (*Trichosanthes dioica* L.) in India. Phytoparasitica. 2011;39:497.
- Nagendran K, Satya VK, Mohan Kumar S, Karthikeyan G. Molecular characterization of a distinct bipartite Begomovirus species infecting ivy gourd (*Coccinia grandis* L.) in Tamil Nadu, India. Virus Genes. 2016;52(1):146-51.
- Zaidi SSEA, Martin DP, Amin I, Farooq M, Mansoor S. Tomato leaf curl New Delhi virus: a widespread bipartite begomovirus in the territory of monopartite begomoviruses. Molecular Plant Pathology. 2017;18:901-11.
- Padidam M, Beachy RN, Fauquet CM. Tomato leaf curl geminivirus from India has a bipartite genome and coat protein is not essential for infectivity. J Gen Virol. 1995;76:249-63.
- Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. Focus. 1990;12:13-15.
- Venkataravanappa V, Reddy CNL, Jalali S, Krishna Reddy M. Molecular characterization of distinct bipartite begomovirus infecting bhendi (*Abelmoschus esculentus* L.) in India. Virus Genes. 2012;44:522-35.
- Venkataravanappa V, Swarnalatha P, Reddy CNL, Neha Chauhan, Krishna Reddy M. Association of recombinant chilli leaf curl virus with enation leaf curl disease of tomato: a new host for chilli begomovirus in India. Phytoparasitica. 2016;44:213-23.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403-10.
- Muhire BM, Varsani A, Martin DP. SDT: A virus classification tool based on pairwise sequence alignment and identity calculation. PLoS One. 2014;9:e108277.
- Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870-74.
- Martin DP, Murrell B, Golden M, Khoosal A, Muhire B. RDP4: Detection and analysis of recombination patterns in virus genomes. Virus Evol. 2015;1:vev003.
- Brown JK, Zerbini FM, Navas-Castillo J, Moriones E, Ramos-Sobrinho R, Silva JCF, Fiallo OE, Briddon R, Hernandez ZC, Idris A, Malathi VG, Martin DP, Rivera BR, Ueda S, Varsani A. Revision of Begomovirus taxonomy based on pairwise sequence comparisons. Arch Virol. 2015;160:1593-19.
- Arguello-Astorga GR, Ruiz-Medrano R. An iteron-related domain is associated to motif 1 in the replication proteins of geminiviruses: identification of potential interacting amino acid-base pairs by a comparative approach. Arch Virol. 2001;146:1465-85.
- Hanley-Bowdoin L, Settleage SB, Orozco BM, Nagar S, Robertson D. Geminivirus: models for plant DNA replication, transcription, and cell cycle regulation. Cri Rev Pl Sci. 1999;18:71-6.

25. Seal SE, Jeger MJ, Van den Bosch F, Maramorosch K, Shatkin AJ, Thresh JM. Begomovirus evolution and disease management. *Adv Virus Res.* 2006;67:297-16.
26. Prasanna HC, Rai M. Detection and frequency of recombination in tomato-infecting begomoviruses of South and Southeast Asia. *Virology J.* 2007;4:111.
27. Venkataravanappa V, Prasanna HC, Reddy CNL, Krishna Reddy M. Evidence for two predominant viral lineages, recombination and sub population structure in begomoviruses associated with yellow vein mosaic disease of okra in India. *Plant Pathol.* 2015;64:508-18.
28. Garg ID, Paul-Khurana SM, Kumar S, Lakra BS. Association of a geminivirus with potato apical leaf curl in India and its immune-electron microscopic detection. *J Indian Potato Assoc.* 2001;28:227-32.
29. Varma A, Malathi VG. Emerging geminivirus problems: A serious threat to crop production. *Ann App Biol.* 2003;142:145-64.
30. Khan MS, Raj SK, Singh R. First report of tomato leaf curl New Delhi virus infecting chilli in India. *Plant Pathol.* 2006;55:289.
31. Mandal B, Mandal S, Sohrab SS, Pun KB, Varma A. A new yellow mosaic disease of chayote in India. *Plant Pathol.* 2004;53:797.
32. Shorab SS, Mandal B, Ali A, Varma A. Molecular diagnosis of emerging begomovirus diseases in cucurbits occurring in northern India. *Indian J Virol.* 2006;17:88-95.
33. Sohrab SS, Mandal B, Ali A, Varma A. Chlorotic curly stunt: a severe begomovirus disease of bottle gourd in northern India. *Indian J Virol.* 2010;21:56-63.
34. Tiwari AK, Sharma PK, Khan MS, Snehi SK, Raj SK, Rao GP. Molecular detection and identification of tomato leaf curl New Delhi virus isolate causing yellow mosaic disease in bitter gourd (*Momordica charantia* L.), a medicinally important plant in India. *Med Plants.* 2010;2:117-23.
35. Arguello-Astorga GR, Guevara-Gonzalez LR, Herrera-Estrella LR, Rivera-Bustamante RF. Geminivirus replication origins have a group-specific organization of iterative elements: a model for replication. *Virol.* 1994;203:90-100.
36. Chatterji A, Chatterji U, Beachy R, Fauquet C. Sequence parameters that determine specificity of binding of the replication-associated protein to its cognate site in two strains of tomato leaf curl virus-New Delhi. *Virol.* 2000;273:341-50.
37. Orozco BM, Gladfelter HJ, Settlege SB, Eagle PA, Gentry RN, Hanley-Bowdoin L. Multiple cis elements contribute to geminivirus origin function. *Virol.* 1998;242:346-56.
38. Gloria Lozano, Helena P, Trenado, Rodrigo A, Valverde, Navas-Castillo J. Novel begomovirus species of recombinant nature in sweet potato (*Ipomoea batatas* L.) and *Ipomoea indica*: taxonomic and phylogenetic implications. *J Gen Virol.* 2009;90:2550-62.
39. Varma A, Mandal B, Singh MK. Global emergence and spread of whitefly *Bemisia tabaci* transmitted geminiviruses. In: Thompson WMO, editor. *The whitefly, Bemisia tabaci* (Homoptera: Aleyrodidae) interaction with Geminivirus infected host plants. Springer Netherlands; 2011. p. 205-92.

Supplementary Data

Supplementary Table S1. GenBank accession numbers of selected begomovirus sequences from the database used in this study for analysis of DNA-A and DNA-B components. Available at: <http://serbiosoc.org.rs/sup/supt1.pdf>

Supplementary Table S2. Details of recombination between ToLCNDV and other begomoviruses detected using RDP4. Available at: <http://serbiosoc.org.rs/sup/supt2.pdf>