



Association of Tomato Leaf Curl New Delhi Virus, Betasatellite, and Alphasatellite with Mosaic Disease of Spine Gourd (*Momordica dioica* Roxb. Willd) in India

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Background: Spine gourd (*Momordica dioica* Roxb. Willd) is one of the important cucurbitaceous crops grown across the world for vegetable and medicinal purposes. Diseases caused by the DNA viruses are becoming the limiting factors for the production of spine gourd reducing its potential yield. For the commercial cultivation of the spine gourd, propagation material used by most of the growers is tuberous roots and stem cuttings, which in turn results in an increased occurrence of the mosaic disease. There is a need for understanding the causal agent; through characterization of which will lead to the designing management strategies for the spine gourd mosaic disease control.

Objectives: Characterization of a begomovirus and its satellites associated with mosaic disease on spine gourd.

Materials and Methods: Total DNA was extracted from spine gourd samples exhibiting symptoms typical to the begomovirus infection (mosaic mottling, leaf curl) and was tested by PCR using begomovirus specific primers. Furthermore, the complete genome of begomovirus (DNA A, DNA B, alpha satellite, and beta satellite) was amplified by rolling circle amplification (RCA) method.

Results: The full-length sequences of DNA A, DNA B, alpha satellite, and beta satellite isolated from symptomatic spine gourd were determined. The full length genomes (DNA A and DNA B) of the Tomato leaf curl New Delhi Virus (ToLCNDV) infecting spine gourd were compared with the other begomovirus genomes available in the data base. The sequence analysis has revealed that DNA A and DNA B components of the begomovirus infecting spine gourd share 95.4-96.2 and 86.7-91.2% identical sequence (i.e., nucleotide (nt) identity) with that of ToLCNDV infecting potato and cucurbits in the Indian subcontinent isolates reported earlier (available in GenBank), respectively. Further, alpha satellite and beta satellite were also detected in the begomovirus infected spine gourd samples. The recombination analysis of the DNA A, DNA B, beta satellite, and alpha satellite of the begomovirus infecting spine gourd showed the associated begomovirus and satellite DNAs were driven from the different begomoviruses, leading to emergence as a new variant of the begomovirus infecting spine gourd.

Conclusions: The commercial cultivation of the spine gourd by most growers depends on the tuberous roots and stem cutting. The occurrence of begomovirus in spine gourd gives an alarming signal against utilization of such infected plant materials in the crop breeding and improvement programs. Using the clean virus-free vegetative propagation material is considered as one of the most important methods for controlling viral diseases. The study is highly useful for detection of the begomovirus infecting spine gourd in the detection of the virus infection in the clonally propagated planting material.

Keywords: Alphasatellite; Betasatellites; Begomovirus; Recombination

1. Background

Spine gourd (*Momordica dioica* Roxb. Willd) is a perennial, rhizomatous, dioecious, climbing creeper which belongs to the family of *Cucurbitaceae* and commonly is known as Kakrol. It is originated from Indo-Malayan region and distributed throughout India, China, Nepal, Bangladesh, Myanmar, Pakistan, and Sri Lanka (1). In India, the crop is being widely cultivated in Orissa, Maharashtra, Karnataka, Andhra Pradesh, Bihar, and West Bengal for its good taste and high nutritional value. Generally, spine gourd is largely cultivated through vegetative propagation and is less propagated by seeds (2). The edible fruits contain a high amount of protein, carbohydrate, fiber, moisture, ash, iron, calcium, phosphorus, thiamine, riboflavin, and niacin (3). Apart from nutritional value, all parts of spine gourd have medicinal properties to cure various diseases and disorders in human being. It has a huge demand in the market, but still remains underutilized (4, 5) and commercially under-exploited due to its vegetative mode of propagation, dioecious nature, low percentage of the seed germination, and a long period of seed dormancy (4). The commercial propagation of the spine gourd mainly depends on the tuberous roots followed by stem cuttings. The Spine gourd plants showing symptoms typical to the begomoviruses' infection in the cucurbits is emerging as the major constraint for its production and making the availability of the healthy planting material difficult in the country. Begomoviruses are single-stranded DNA viruses belonging to the family *Geminiviridae* with the morphology of geminate particles and are transmitted by the whitefly (*Bemisia tabaci*). Begomovirus could either have a monopartite or bipartite genome. The bipartite begomoviruses have two genome components referred to as DNA A and DNA B. The DNA A component contains five open reading frames (ORFs) encoding pre-coat protein, coat protein in the virion strand, as well as DNA replication-associated proteins in the complementary strand (6). The DNA B component contains two ORFs and encodes factors required for inter and intra-cellular movement in the host plants (7). Furthermore, based on the genome organization, genetic diversity, and geographical distribution, it has been further divided into two groups; the old world (OW) (Europe, Africa, Asia and Australia) and the new world (NW) (America) begomoviruses. The NW begomovirus is evaluated to be bipartite with lack of AV2 ORF in the DNA A component. Whereas both bipartite and monopartite begomovirus in the OW encodes AV2 ORF. The monopartite begomoviruses have a single genome analogous to the DNA A of the bipartite viruses with the association of additional ssDNA molecules known as betasatellites and/or alphasatellites (DNA1) (8). Betasatellites are approximately half the genome size of their helper begomoviruses and are required for inducing typical

disease symptoms in their original hosts (9). Alphasatellites are self-replicating circular ssDNA molecules and depend on the helper virus for movement, encapsidation, vector transmission, and play no role in symptom induction (10).

2. Objective

Spine gourd is consumed by tribal groups living around the natural forest areas, especially at higher altitudes, where the native folks consume it as a daily vegetable. The spine gourd did not gain much popularity until it was discovered to have a high nutritional and medicinal value, which helps the development of body towards natural immunity from many common ailments. The disease caused by the begomovirus is the major constraint for production of the healthy planting material as well as its production in the country. The virus infected plants are exhibiting severe mosaic, mottling, and leaf curl symptoms. The disease incidence is ranged from 50-60% across different farmer's fields at Varanasi, Uttar Pradesh state of India. Therefore, the current study was undertaken to characterize the probable begomovirus and its associated satellites with the mosaic disease of the spine gourd in India.

3. Materials and Methods

3.1. Virus Source

Five leaf samples of spine gourd exhibiting mosaic, mottling, leaf curl, and distortion symptoms (Fig. 1A-1D) and two samples from non-symptomatic plants were collected during 2014-15 from different farmer's fields of the Varanasi, Uttar Pradesh state, India.

3.2. DNA Isolation, PCR-Mediated Amplification, and Sequencing

Total DNA was extracted from symptomatic and non-symptomatic leaf samples using CTAB (11). The presence of begomovirus infection in the spine gourd was tested by PCR using begomoviruses specific degenerative primers (12). The full-length genomic DNA components were amplified from the virus-infected samples using a TempliPhi illustration amplification kit (GE Healthcare, Piscataway, NJ). The resulted rolling circle amplification (RCA) product was digested with *Bam*H1 (DNA-A) and *Xba*I (DNA-B) restriction endonucleases for isolation of the monomeric units of the genome, cloned into pUC19 plasmid (13), and were transformed into the competent *Escherichia coli* DH5 α strain. Restriction digestion was performed for confirming recombinant clones. Similarly, to identify the association of the sample with satellite genomes, the total DNA was amplified using betasatellite (DNA- β) (14) and alphasatellite (15) specific primers. The amplified PCR products were ligated into pTZ57R/T vector (INSTA cloning kit, Thermo Fisher Scientific Inc., PA). Recombinant

clones were identified by restriction endonuclease digestion for the presence of the cloned products. The selected clones were sequenced with an automated

sequencing ABI PRISM 3730 (Applied Biosystems) at Amnion DNA Sequencing facility, Bengaluru, Karnataka, India.



Figure 1. Spine gourd plant showing (A) a mild mosaic, (B) severe mosaic, (C) and (D) drying of the fruits under natural conditions.

3.3. Sequence Analysis and Detection of Recombination Events

The sequences obtained were initially analyzed using the Vector NTI Advance™ 9 software to remove vector sequences. Further, the sequences were verified for the presence of all begomovirus specific Open reading frames (ORFs) using NCBI ORF finder and conserved nonanucleotide sequence. The selected begomovirus species (Table S1), betasatellites (Table S2) and alphasatellites (Table S3) accessions showing highest percentage sequence identity/similarity/homology with the current sequences in the study were retrieved from the GenBank for analysis. The retrieved sequences were aligned with the present isolate using the MUSCLE method in SDT version 1.2 (16) and percent pair-wise nt identities were generated. The phylogenetic tree was generated by MEGA 7 software (17) using the neighbour-joining method with 1,000 bootstrapped replications to estimate evolutionary distances between all pairs of the sequences. Split-decomposition trees were constructed with 1,000 bootstrap replicates based on parsimony splits as implemented in SplitsTree version 4.11.3 with default settings (18). Recombination analysis was carried out using the recombination detection program (RDP), GENECOV, Bootscan, Max Chi, Chimera, Si Scan, 3Seq which are integrated in the RDP4 (19). The default RDP settings with 0.05 *P*-value cut off

throughout and standard Bonferroni correction were used.

4. Results

4.1. Genome Organization of DNA A Component of Begomovirus

The spine gourd leaf samples showing symptoms typical to the begomovirus infection were showed positive PCR amplification to begomovirus specific primers and no amplification was observed from non-symptomatic samples. Further, the full genome components (DNA A and DNA B) were amplified and the resultant amplified products of 2.7 kb were cloned into pUC19 plasmid. The representative ten recombinant plasmids were sequenced and confirmed by BLAST analysis. The DNA A sequences of the five begomovirus isolates characterized from spine gourd showed 99.8-100 percent nt sequence identity among themselves indicating that they belong to a single species, based on species demarcation criteria for the begomoviruses; 91% nt sequence identity for complete genome (20). Therefore, one representative from the begomovirus isolate of SPYG1 was selected for amplification of the DNA A and DNA B as well as alpha and betasatellite of the begomovirus infecting spine gourd.

4.2. Sequence Identities of the DNA A and DNA B Components with Other Begomoviruses

The DNA A and DNA B component of the begomovirus isolate SPYG1 were determined to be 2745 nt and 2696 nt in length, respectively and were deposited in the GenBank database (Accession no.: KY780213 and KY780214). The SDT analysis of the DNA A component of the isolate SPYG1 infecting spine gourd showed the highest nt identity of 95.4-96.2 per cent with the isolates of the Tomato Leaf Curl New Delhi virus (ToLCNDV) infecting potato (EF043231, EF043230, AY286316, AM850115) in the India (Table 1), which is followed by the isolates of ToLCNDV reported on cucurbits (83.8 -84.2%), chilli (93.7-95.8%), tomato (92.8-94.9%), and eggplant (93.3%) from India (Table 1). While DNA B-like sequence showed the highest

level of nucleotide identity of 86.7-91.2% with the isolates of ToLCNDV infecting cucurbits crops (AM286435, AB330080, AY939924, HM989846, JN208137, KC545813, DQ020490) in the Indian subcontinent, which are summarized in the database (Table 2). Based on the current species demarcation criteria for the begomoviruses (91% nt sequence identity among complete genome) (20), the begomovirus isolate (SPYG1) with more than 95 percent nt sequence identity with ToLCNDV-pot is considered as a variant of the ToLCNDV. This result was well supported by the phylogenetic analyses of the both DNA A and DNA B components of the SPYG1 isolate by grouping with Indian isolates of ToLCNDV infecting tomato, potato, and cucurbits crops in the India and China (Fig. 2A and 2B).

Table 1. The pairwise percent of nucleotide identities between the genomic components and amino acid sequence identities of the encoded genes from the ToLCNDV-(IN: SPYG1: Var: 15) with the components and genes of the selected begomoviruses available in the databases.

Begomovirus#	Genome DNA A	IR	Gene (percentage amino acid sequence identity)						
			AV2	CP	Rep	TrAP	REn	AC4	AC5
ToLCNDV-Cucurbits (14)*	93.6-96.1	80.3-94.9	86.6-95.5	96.0-98.0	85.1-98.0	76.2-96.2	78.6-94.1	77.5-91.3	65.2-90.6
ToLCNDV-Potato (4)*	95.4-96.2	96.3-100	94.6-96.4	97.2-98.4	95.5-96.3	88.4-89.9	35.2-89.7	94.8-100	87.5-92.5
ToLCNDV-Tomato (11)*	92.8-94.9	90.9-96.0	90.1-95.5	95.7-97.6	93.9-95.8	87.7-92.8	82.3-93.3	86.2-91.3	67.0-90.0
ToLCNDV-Chilli (4)*	93.7-95.8	92.7-96.0	92.8-95.5	97.2-98.0	94.1-97.2	90.6-96.4	90.4-94.1	89.6-96.5	85.7-88.8
ToLCNDV-Eggplant (1)*	93.3	93.8	93.7	96.8	94.1	90.6	91.1	86.2	-
ToLCPaV-Tomato (2)*	84.1-85.1	79.2-82.1	66.9-70.4	90.6	87.4-87.7	78.4	79.4-80.1	84.4-86.2	56.5
ToLCPaV-Cucurbits (24)*	83.8-84.2	77.0-81.4	65.3-71.3	87.1-91.4	84.3-87.1	72.9-78.4	73.7-80.8	73.1-87.9	57.1
SLCCNV-Pumpkin (5)*	87.0-90.4	79.4-82.0	90.1-91.9	94.1-97.6	88.9-91.9	67.6-69.7	61.7-81.1	75.8-79.3	81.9-86.9
MYMIV (2)	66.2-67.2	42.2-42.9	43.3-41.5	73.1-74.3	70.1-70.4	37.6-42.1	37.5	27.2-28.2	11.1

* Numbers of sequences from the databases were used in the comparisons.

IR- Intergenic region

#The species are indicated as, *Tomato leaf curl New Delhi virus* (ToLCNDV), *Tomato leaf curl Palampur virus* (ToLCPaV), *Squash leaf curl China virus* (SLCCNV), *Mungbean yellow Mosaic Indian virus* (MYMIV). For each column, the highest value is underlined.

Table 2. The pairwise percent of the nucleotide identities between the genomic components and amino acid sequence identities of the encoded genes from the ToLCNDV-(IN: SPYG1: Var: 15) with the components and genes of the selected begomoviruses available in the databases.

Begomovirus#	Genome DNA B ^a	IR ^a	Gene (percentage amino acid sequence identity)	
			BV1 ^b	BC1 ^b
ToLCNDV-Tomato (13)	82.0-89.8	51.8-86.4	75.7-97.0	87.3-94.3
ToLCNDV-Potato (6)	87.1-87.4	77.2-84.0	93.2-94.4	92.1-92.8
ToLCNDV-Chilli (4)	85.0-90.0	75.3-85.3	93.2-94.7	87.3-92.8
ToLCNDV-Cucurbits (9)	86.7-91.2	64.3-86.2	80.5-98.1	92.1-95.0
ToLCPaV-Tomato (3)	68.5-69.1	52.0-52.9	78.3-79.1	88.2-89.6
ToLCPaV-Cucurbits (12)	68.2-68.9	44.9-54.7	77.9-79.1	86.1-89.3
SLCCNV-Pumpkin (4)	62.1-62.7	47.0-49.1	72.7-72.3	86.1-88.6
ToLCNDV-Okra (1)	886	783	958	928
MYMIV (2)	41.9-42.1	26.8-27.8	25.0-25.2	38.7-39.0

^aNucleotide identity; ^b Amino 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* (ToLCPaV), *Squash leaf curl China virus* (SLCCNV), *Mungbean yellow Mosaic Indian virus* (MYMIV). For each column, the highest value is underlined.

Table 3. The percentages of nucleotide and amino acid sequence identities between betasatellite of the spine gourd (DNAβ) and betasatellites of other begomoviruses

Betasatellites*	Complete sequence of DNAβ (percentage NSI)	Percentage amino acid sequence identity of βC1 gene
ToLCJoB (17)	81.7-87.0	85.7-100
ToLCBDB (14)	61.1-63.5	38.7-61.1
ChiLCB (7)	60.4-62.9	58.7-60.3
ToLCPnB (2)	70.6-71.7	67.4-73.8
ToLCuB (2)	61.6-63.1	57.7-57.1
CroYVMB (3)	53.7-54.5	46.0-49.2
RaLCB (1)	561	523

* Numbers of sequences from the databases used in the comparisons.

#The species are indicated as *Tomato leaf curl Joydepur betasatellite* (ToLCJoB), *Tomato leaf curl Bangladesh betasatellite* (ToLCBDB), *Tobacco leaf curl Patna betasatellite* (ToLCPnB), *Tomato leaf curl betasatellite* (ToLCB), *Croton yellow vein mosaic betasatellite* (CroYVMB), *Radish leaf curl betasatellite* (RaLCuB). For each column, the highest value is underlined.

Table 4. Percentages of the nucleotide or amino acid sequence identities between alphasatellite of spine gourd and alphasatellite of the other begomoviruses

Alphasatellites#	Complete sequence of DNAD1 (percentage NSI)	Percentage amino acid sequence identity of Rep gene
NVLP (7)	72.2-76.7	<u>62.5-93.9</u>
CLCuBuVD1 (3)	74.7-75.3	<u>93.2-93.9</u>
AEVD1 (2)	72.6-75.7	88.5-91.7
ToCSVD1 (2)	72.0-72.9	90.0-90.1
SiLCNVD1 (2)	69.9-70.2	88.2-88.5
CyTLCuD1 (3)	69.6-70.0	79.0-89.2
ToLCKVD1 (1)	<u>76.9</u>	<u>93.9</u>
CLCuVD1 (1)	71.2	86.6
OLCuDD1 (1)	74.8	91.1
BYVMVD1 (1)	74.9	91.1
PaLCuVD1 (1)	70.7	86.3
ToLCYCNDV1 (1)	69.8	86.9
SnLCuKVD1 (1)	74.5	84.4
SiLCVD1 (1)	69.6	90.1

The species are indicated as, Nanovirus-like particle (NVLP), Cotton leaf curl Burewala alphasatellite (CLCuBuVD1), Ageratum enation alphasatellite (AEVD1), Tobacco curly shoot alphasatellite (ToCSVD1), Sida yellow vein China alphasatellite (SiLCNVD1), Cyamopsis tetragonoloba leaf curl alphasatellite (CyTLCuD1), Tomato leaf curl Karnataka alphasatellite (ToLCKVD1), Cotton leaf curl alphasatellite (CLCuVD1), Okra leaf curl virus alphasatellite (OLCuDD1), Bendhi yellow vein mosaic alphasatellite (BYVMVD1), Papaya leaf curl alphasatellite (PaLCuVD1), Tomato yellow leaf curl China virus (ToLCYCNDV1), Sunflower leaf curl Karnataka alphasatellite (SnLCuKVD1), Sida leaf curl alphasatellite (SiLCVD1). For each column, the highest value is underlined.

ORF wise, the amino acid (aa) sequence identities at the protein level showed the highest aa sequence identities with the different isolates of ToLCNDV, the regions *viz.* pre-coat (AV2), coat protein (CP), C4, and C5 shared maximum amino acid identity with the ToLCNDV infecting potato. Whereas, Rep (C1), TrAP (C2), and REEn (C3) regions shared the most identity with the ToLCNDV infecting chilli (Table 1) in the DNA A component.

In the IR region, the identity of begomovirus (i.e., clone SPYG1) was more than 96% with the intergenic regions (IRs) of the reported ToLCNDV infecting potato isolates (Table 1). The length of IR is 275 nt and similar to those of bipartite begomoviruses reported so far for the DNA A component.

Similarly, individually encoded proteins of the DNA B component were compared. The highest amino acid (aa) sequence similarities were found for movement protein (80.5-98.1%) and nuclear shuttle protein (92.1-95.0%) with that of ToLCNDV infecting cucurbitaceous crops (Table 2). The length of the IR is 311 nt and similar to those of ToLCNDV isolates available in the database and shared 51.8-86.4 percent identity with the ToLCNDV isolates infecting tomato.

4.3. Genome Organization of the Alpha and Betasatellite and Sequence Affinities to Other Alpha and Betasatellite

The alphasatellite (DNA D1) and betasatellite (DNA β 1) isolated from infected spine gourd were determined to be 1388 nt (Acc NO. MG571523) and 1360 nt (Acc. NO. MG571522) in length, respectively.

The alphasatellite have a single large ORF with a coding capacity of 315 aa in the virion-sense (coordinates 89-1036) (21). The sequence showed the maximum nt sequence identity (76.9%) with Tomato leaf curl Karnataka alphasatellite (ToLCKVD1, Acc. NO JX570736) (Table 4). Since the alphasatellite sequence

was showing very low levels of nt sequence identity (less than 77%) with other isolates reported earlier and there is no proposed species demarcation threshold for classification of the alphasatellites, the present alphasatellite will be named as spine gourd mosaic alphasatellite.

The phylogenetic analysis of this studied genome component shown in Figure 3A indicated that the alphasatellite isolated from spine gourd segregated separately from all other satellites infecting different crops. Similarly, betasatellite sequence in the current study contains all the features typical to the betasatellites reported so far (12, 14) and showed maximum nt identity of 81.7 to 87 percent with the tomato leaf curl Jodeypur betasatellite (ToLCJoB) isolates originating from the Indian subcontinent infecting different crops (Table 3). Based on the recently proposed species demarcation threshold for betasatellites (22), the betasatellite identified here is an isolate of ToLCJoB infecting chilli and tomato, which is well supported by phylogenetic analysis (Fig. 3B).

4.5. Neighbor-Net and Recombination Analysis

A neighbor-network, Pairwise Homoplasy Index (PHI) test and breakpoint analysis for the recombination was carried out for nt sequences of the different begomoviruses genomic components (DNA A, DNA B, betasatellite, and alphasatellite) along with the sequences of the virus isolated from the spine gourd. This revealed that SPYG1 isolate was evolved through recombination.

The RDP 4 analysis indicated the evidence of recombination in ToLCDNV (SPYG1) infecting spine gourd with most of the DNA A fragments derived from ToLCNDV, Squash leaf curl China virus (SLCCNV) and Tomato leaf curl Palampur virus (ToLCPaV) to emerge as a new strain of ToLCNDV infecting spine gourd (Table S4, Fig. 4).

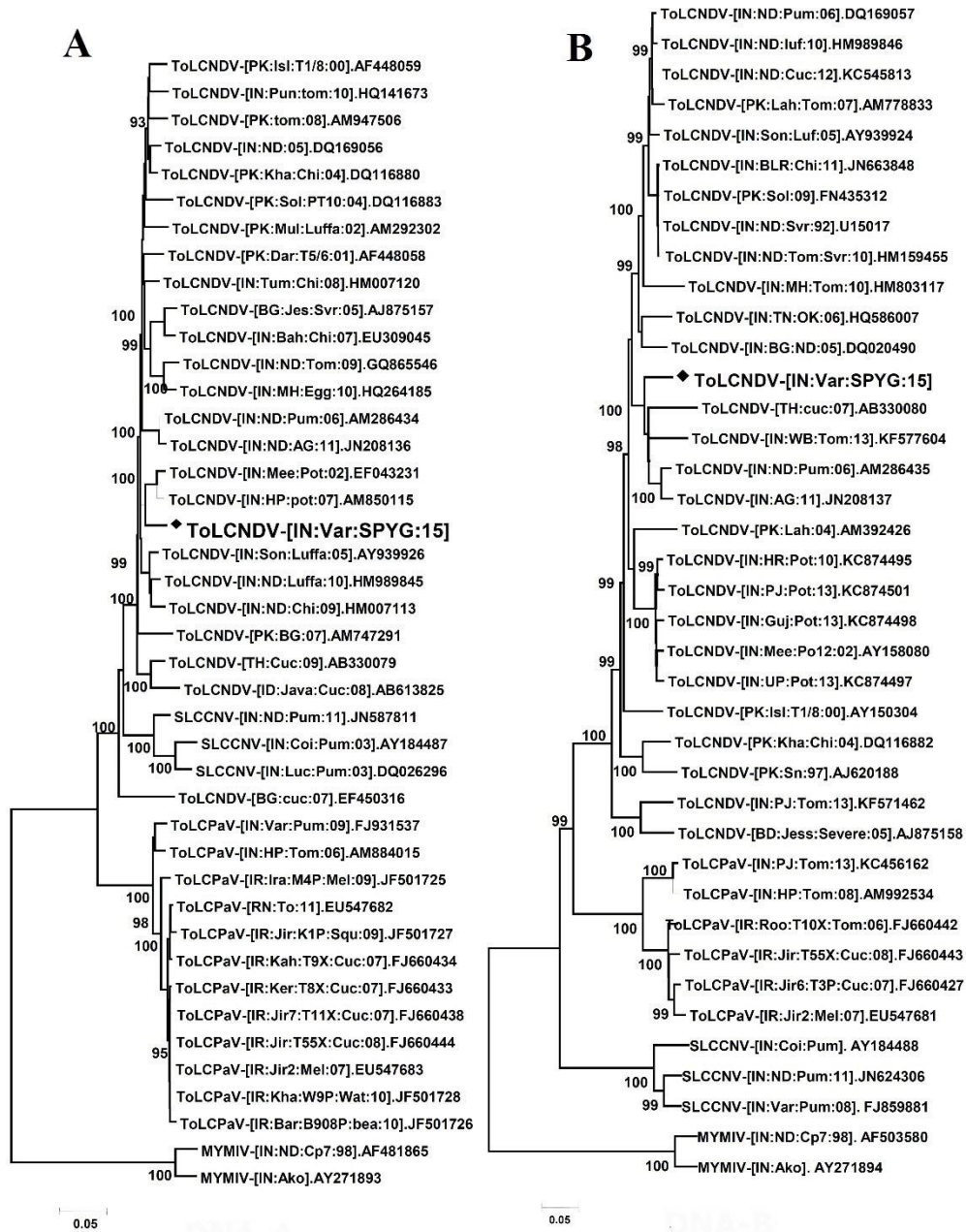


Figure 2. Phylograms were constructed using the neighbour joining (NJ) method. (2A) The DNA-A sequence of the begomovirus (SPYG1 clone) and (2B) the DNA-B sequence of the begomovirus (SPYG1 clone) associated with the severe mosaic disease of the spine gourd in India. Branches corresponding to the partitions reproduced in the less than 50% bootstrap replicates are collapsed. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 Replicates) is shown below the branches.

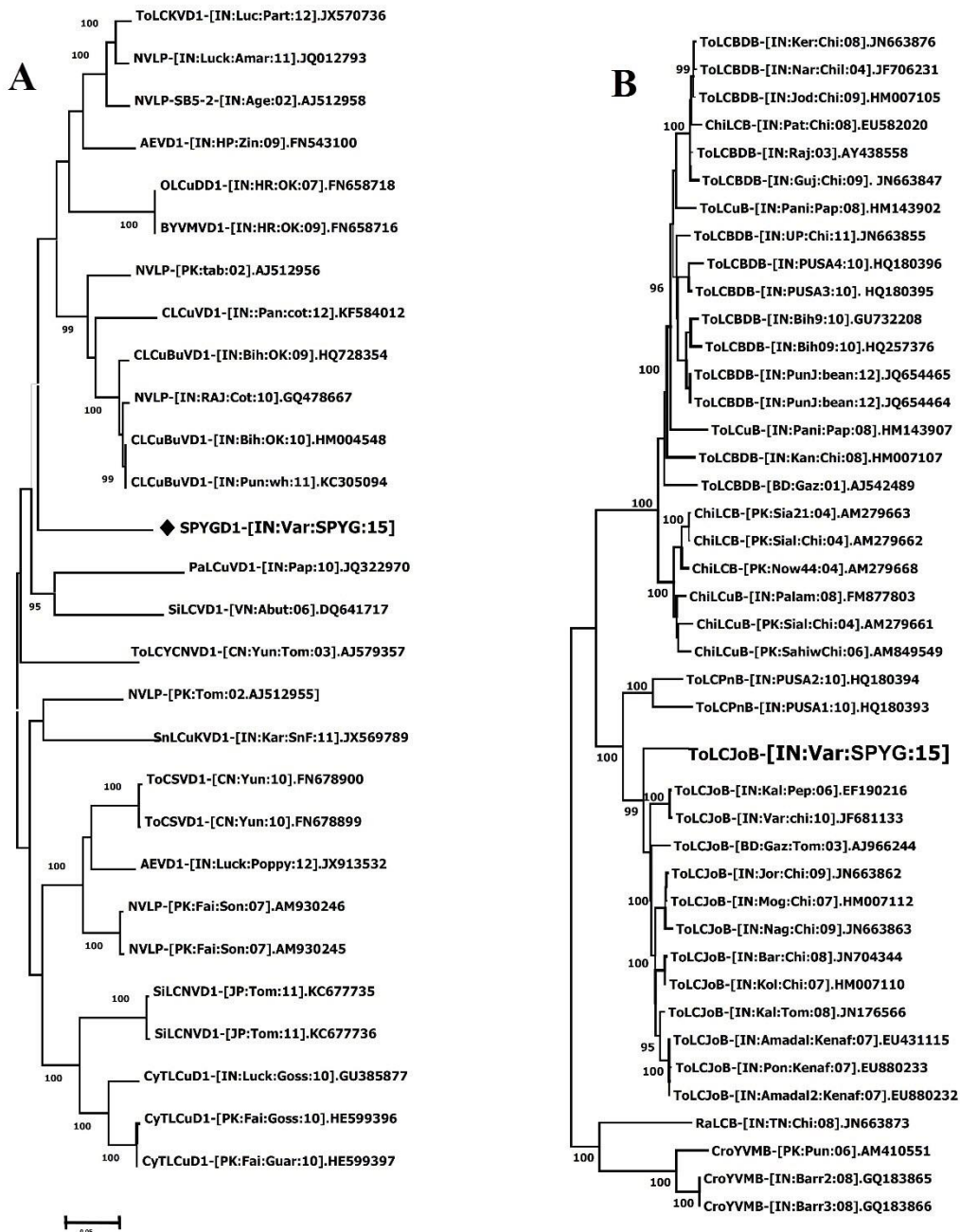


Figure 3. Phylograms were constructed from the aligned complete nucleotide sequences of (3A) Alphasatellite (DNA D1) and (3B) betasatellite (DNA β) associated with severe mosaic disease of spine gourd in India using the NJ method. The horizontal distances are proportional to the sequence distances; vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1000 replicates was performed and the bootstrap percent values more than 50 are numbered along branches.

In case of DNA B sequence, most part of BV1 (movement protein) and BC1 (nuclear shuttle protein) is derived from ToLCNDV and ToLCPaV infecting tomato, okra, and melons. The IR region is derived from the Mungbean yellow mosaic India virus (MYMIV) and ToLCNDV (Table S4, Fig. 4). The RDP analysis indicated that evidence of recombination in betasatellite suggestive of the most parts of betasatellite DNA descended from Tobacco leaf curl Patna betasatellite (ToLCPnB), Tomato leaf curl Bangladesh betasatellite (ToLCBDB), and Tomato leaf curl Joydebpur

betasatellite (ToLCJoB) infecting different solanaceous, and malvaceous crops in India. The analysis of the alpha-satellite indicated that most of satellites DNA descended from Cotton leaf curl alphasatellite (CLCuVD1), Cyamopsis tetragonoloba leaf curl alphasatellite (CyTLCuD1), Okra leaf curl alphasatellite (OLCuDD1), and Sida leaf curl alphasatellite (SiLCNVD1) satellites (Table S3) are associated with cotton, guar, okra, and tomato infecting begomoviruses, respectively.

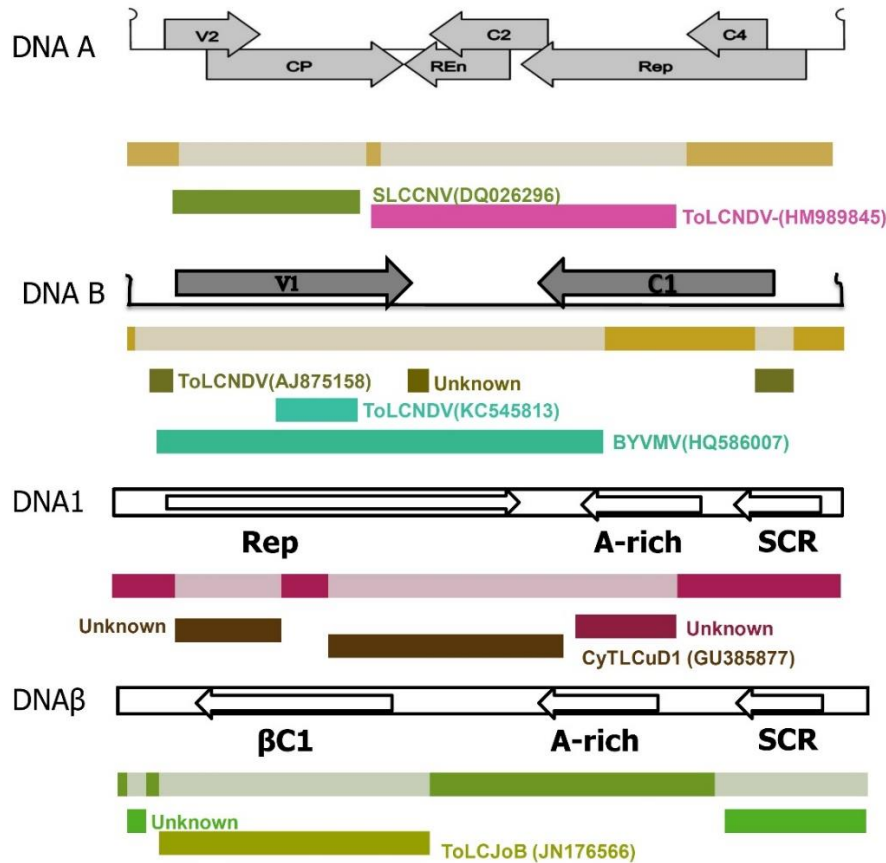


Figure 4. Analysis of recombination for ToLCNDV isolate isolated from spine gourd: The begomoviruses, alphasatellites and betasatellites acronyms given are squash leaf curl China virus (SLCCNV), tomato leaf curl new Delhi virus (ToLCNDV), cyamopsis tetragonoloba leaf curl alphasatellite (CyTLCuD1), Tomato leaf curl joydebpur betasatellite (ToLCJoB), sequence of indeterminate origin is indicated as “unknown”. The box below at the top of the diagram indicates the approximate position recombination is occurring in the genome of the begomoviruses.

5. Discussion

Spine gourd is one of the cucurbitaceous crops and mosaic disease associated by begomoviruses is a limiting factor for its production. The ToLCNDV was initially identified on solanaceous crops in India (23), since then it has been reported across many countries and known to infect 43 plant species belong to the different families (24-26). The frequency of the newly emerging strains of the ToLCNDV on several cultivated and non-cultivated plant species are increasing in the recent years, indicating that the virus poses a serious threat to vegetables belonging to the family Cucurbitaceae, Euphorbiaceae, Solanaceae, Malvaceae and Fabaceae throughout the world (26). Generally, the betasatellites are found associated with the old world (OW) monopartite begomoviruses (10) and not in bipartite begomoviruses with the two exceptions, MYMIV (27, 28) and ToLCNDV (29) infecting mung bean and tomato, respectively in India. In the present study, for the first the time, we have characterized ToLCNDV bipartite virus with both betasatellite and alphasatellite associated with the mosaic disease of the spine gourd in India. The tripartite interaction between ToLCNDV and betasatellite in tomato was well proved through agro-inoculation of the cloned DNA A, DNA B, and betasatellites in the *N. benthamiana* and tomato (30). It

was evident that the plants co-inoculated with DNA A, DNA B, and betasatellites will enhance the symptom severity in the *N. benthamiana* and tomato directly increases the helper viral DNA A and DNA B replication levels in the host cells.

The betasatellite associated with spine gourd mosaic disease is identified as ToLCJoB, which is also associated with the heterologous begomoviruses infecting chilli (31, 32), tomato (33), and kenaf (34) plants in India. The trans-replication of ToLCNDV and defective betasatellites has been shown for many distinct begomovirus species/betasatellite combinations (30, 34-36). The other bipartite begomoviruses confirmed to trans-replicate betasatellite are Sri Lankan cassava mosaic virus (36), Tomato yellow leaf curl Thailand virus (37), and the New World bipartite Cabbage leaf curl begomovirus (38). These observations clearly indicated that there is no distinct demarcation between monopartite and bipartite begomoviruses for their ability to trans-replicate betasatellites in the genome. The nt sequence of alphasatellite isolated from spine gourd showed less than 77 per cent identity with the previously reported alphasatellites, hence we proposed the name spine gourd mosaic alphasatellite. The exact role of alphasatellites has not been fully understood.

However, alphasatellites attenuate the disease symptoms and involve in the maintenance of the low level of betasatellite accumulation (39). It was also shown that the Rep of alphasatellites can suppress RNAi pathway in monopartite begomovirus disease complexes (40). The PHI value in the present study has strongly supported the presence of recombination in SPYG1 isolate. The different methods used for recombination breakpoint analysis provided strong evidence for the presence of the past recombination events in most of the genome components. The role of such overlapping recombination between different isolates or different species in adaptation to spine gourd may be an interesting aspect that needs to be resolved. Such inter and intra-species recombinations are the predominant feature of begomovirus evolution (8) and have been implicated in the emergence of new begomovirus species and adaptation in new hosts in the agricultural system (40).

The commercial cultivation of spine gourd by most of the growers is depends on the tuberous roots and stem cutting. The occurrence of the mosaic disease on spine gourd gives an alarming signal against utilization of such virus infected planting materials in the crop breeding and improvement program. The technique developed here will be highly useful to detect the virus infection in clonally propagated plants such as spine gourd.

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Conflicts of Interest

The authors declare that they have no competing interests.

Ethical Approval

This article does not contain any studies with the human participants performed by any of the authors.

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