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Molecular Identification of *Tomato Leaf Curl New Delhi Virus* Associated with Mosaic Disease of Pumpkin from Central India

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Abstract

The *Tomato leaf curl New Delhi virus* is an emerging disease of pumpkin associated with mosaic disease in central, India. Symptomatic pumpkin leaf samples from different locations in the Bhopal district were collected and confirmed for begomovirus disease by PCR using a begomovirus coat-protein gene-specific primer. All five samples were amplified, expecting ~800 bp of amplicons of associated begomovirus; these amplicons were purified and sequenced for identification of begomovirus species associated with mosaic disease on the pumpkin. The consensus nucleotide sequence data were submitted to the NCBI database under the accession numbers OQ320768, OQ320770, OQ320774, OQ116977, and OQ116978. The understudy begomovirus isolates showed the highest 95–97% nucleotide sequence identities and close phylogenetic relationships with several isolates of *Tomato leaf curl New Delhi virus*. To our knowledge, this was the first record of the *Tomato leaf curl New Delhi virus* associated with the mosaic disease of pumpkin in Central India.

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Pumpkin;
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Introduction

Pumpkins (*Cucurbita pepo* L., C. *Maxima* L. & C. *Moshchata* Duch.) are important vegetable crops in home-garden and commercial across the world. The pumpkin crop is one of the most popular summer vegetable crops grown on commercial scale worldwide. The crop is also widely grown in China, America, Mexico, and India. In terms of area and production, pumpkin is India's second most important cultivated vegetable crop in the *Cucurbitaceae* family. In India, it is mostly grown in Madhya Pradesh, Uttar Pradesh, Chhattisgarh, West Bengal, Odisha, Karnataka, Tamil Nadu, Kerala, and Bihar. In India, the cultivated area is about 1.10 lakh ha, production is 23.12 million tonnes, and 21.01 tonnes/ha productivity during 2021-22 (agricoop.nic.in).

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Begomoviruses (*Geminiviridae family*) are circular single-stranded DNA viruses with twinned (geminate) morphology and contain either monopartite (DNA-A) or bipartite (DNA-A and DNA-B) genomes approx. 2.6kb to 2.8kb in size. Begomovirus is transmitted by vector *Bemisia tabaci* (Genn.), an emerging plant virus pathogen in the family Geminiviridae, which inflicts diseases on a variety of crops in tropical and subtropical areas.[1] Pumpkin is a common host of various begomoviruses.[2,3,4,5,6,7] In northern and southern India, mosaic and leaf curl diseases caused by begomoviruses, are rising problems because they affect the production and yield of pumpkin crops. The current study aims to determine the prevalence and distribution of begomovirus infections in Central India especially in Madhya Pradesh state. Symptomatic mosaic leaf samples were collected for the detection and identification of associated begomovirus on pumpkin crops. The data collected will be crucial for the improvement of virus-resistant varieties/cultivars and effective management plans for begomovirus infections affecting pumpkin crop species in Central India.

Materials and Methods

Investigation for Disease Incidence

The survey was conducted from different locations in the Bhopal district. In each location, symptomatic and healthy plant leaves were collected.

Genomic Extraction and Detection of Begomovirus using Polymerase Chain Reaction

The method described by Dellaporta *et al.* (1983) was used to extract the total DNA from leaf samples taken from both symptomatic and healthy plants. To amplify the coat-protein gene of begomovirus, the polymerase chain reaction (PCR) was carried out using total DNA as a template and a set of begomovirus degenerate CP region primers: CPIT-I/ CPIT-T.[8] The PCR was performed in 25 µl volume, containing template DNA (100 ng), 10X Taq buffer with 25 mM $MgCl_2$, 10 mM dNTPs, 25 pmol primers (each), and Taq DNA polymerase (250 U, DSS Takara Bio India Pvt. Ltd., New Delhi). The reactions were performed in a BIO-RAD T-100 Thermal cycler at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 47°C for 1 min, and 72°C for 1:30 min. The cycling was succeeded by a final extension for 5 min at 72°C. The PCR amplicons were analysed by 1.2% agarose gel electrophoresis and the expected size was eluted using Wizard SV Gel and PCR Clean-up System Promega, USA.

Sequencing and Sequence Analysis

The eluted product was sequenced commercially by Barcode Biosciences Pvt. Ltd, Bengaluru (India) using both the forward and the reverse primers. BLAST (Basic Local Alignment Search Tool) search analysis of nucleotide sequence of the virus isolates was conducted using Blastn with sequences available in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE). The Genomatix alignment tool and the Sequence Demarcation Tool (SDTv1.2) were used for the pairwise identity comparison of virus isolates under study with the sequences of selected begomovirus obtained from the GenBank database.[9,10] Sequences under study were aligned using the ClastalW algorithm and MEGAv11; bootstrapping for 1000 replicates was used for generating phylogenetic trees.[11,12]

Result

Disease Symptoms and Effectiveness of Transmission

During the year 2022, in five fields in Bhopal District, Madhya Pradesh, India (two fields at Berkhedi bazyaft (23.177° N, 77.340° E), and one-one sample collected field at Shyampur (23.341° N, 77.426° E), Arwaliya (23.343° N, 77.412° E), and Saket Nagar (23.208° N, 77.456° E)), virus-like symptoms were observed in pumpkin crops. In each field visited infected leaf samples showing Severe Mosaic, Mosaic, and leaf curling were collected (Figure 1).

Genomic Extraction and Detection of Begomovirus using PCR

Total genomic DNA was isolated by the Dellaporta method from symptomatic and asymptomatic leaf samples to confirm the association of begomovirus with leaf curl and mosaic disease of pumpkin crops.[13] This method yielded a good quality & quantity of DNA preparation as checked by taking its best O.D. at 260/280 is 1.8 and concentration also checked by the 1.2% agarose gel electrophoresis. DNA samples were subjected to PCR using the begomovirus coat-protein gene-specific primers for DNA-A: CPIT-I/CPIT-T8 primers amplified coat protein (AV1) gene. The PCR products were analyzed by electrophoresis in 1.2% agarose gel.

As expected, bands of ~800 bp (CPIT-I/CPIT-T). The PCR amplicon of ~800 bp of the PCR reaction was gel eluted through Wizard SV Gel and PCR Clean-Up System (Promega Pvt. Ltd. USA), and eluted products sequenced from Barcode Biosciences Pvt. Ltd, India. The obtained sequence data of the complete coat protein gene (AV1) were deposited in the NCBI GenBank database under accession numbers OQ320768, OQ320770, and OQ116978 in C. *maxima*, OQ320774 in C. *moshchata*, and OQ116977 C. *pepo*.

Fig. 1: Healthy and Symptomatic leaves of pumpkin: (A) Healthy leaf (B) Leaf curling (C) Severe Mosaic (D) Mild Mosaic (E) Leaf curling with severe mosaic (F) Mosaic.

Sequencing and Sequence Analysis

BLASTn analysis of the understudy pumpkin virus isolates coat protein gene (Acc. No. OQ320768, OQ320770, OQ320774, and OQ116977, OQ116978) showed 97.67-99.74% identities with each other and showed the highest 95-97% sequence identity with isolates of *Tomato leaf curl New Delhi virus* (ToLCNDV: MH577015, KF551576) in tomato and 70-95% sequence identity with *Squash leaf curl China virus* (SLCCNV: AY396151, LC511776, MW248689), *Pumpkin yellow vein mosaic virus* (PYVMV: AY686500), *Tomato leaf curl Palampur virus* (ToLCPalV: FJ931537, GQ225738), *Squash leaf curl Philippines virus* (SLCPHV: AB085793, DQ866135), *Squash leaf curl virus* (SLCV: KY652743); and *Chilli leaf curl virus* (ChiLCV: MN119490).

Table 1: The Genomatix DiAlign programme was used to check similarity at nucleotide (nt) and amino acid (aa) of coat-protein begomovirus isolates under study (OQ116977, OQ116978, OQ320768, OQ320770, and OQ320774) with selected begomoviruses.

S. No.	Acc. No	Virus	Host	Location	OQ320768		OQ320770		OQ320774		OQ116977		OQ116978	
					AV1		AV1		AV1		AV1		AV1	
					nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
1	MH577015	ToLCNDV	Tomato	Anand, Gujarat	95	98	95	98	95	98	93	90	94	95
2	KF551576	ToLCNDV	Tomato	Junagad, Gujarat	95	98	95	98	95	98	92	90	95	95
3	MW538661	ToLCNDV	Ridge gourd	India	95	98	95	98	95	98	93	90	94	95
4	AM286434	ToLCNDV	Pumpkin	India: New Delhi	94	98	93	98	93	98	92	89	94	95
5	HQ840736	ToLCNDV	Coccinia grandis	Lucknow	95	98	93	98	93	98	92	90	94	95
6	EU366163	ToLCNDV	Luffa acutangula	Gorakhpur	95	99	95	98	95	99	92	90	94	96
7	AM286433	ToLCNDV	Pumpkin	New Delhi, India	94	98	93	97	93	98	92	89	94	94
8	MW399221	ToLCNDV	Tomato	India	94	98	94	97	94	98	92	89	94	94
9	MW382288	ToLCNDV	Tomato	India	94	98	94	97	94	98	92	89	94	94
10	KC545812	ToLCNDV	Cucumis sativus	New Delhi	94	98	94	97	94	98	92	89	94	94
11	EU439261	ToLCNDV	Luffa cylindrica	Gorakhpur, India	95	98	94	98	94	98	91	89	94	95
12	JN129254	ToLCNDV	Pumpkin	IARI, New Delhi	95	98	94	97	94	98	91	89	93	94
13	MW248655	ToLCNDV	Squash	Malaysia	94	98	93	97	93	98	90	89	93	94
14	KT948072	ToLCNDV	*Cucurbita pepo*	Pakistan	95	98	95	98	95	98	92	90	94	95
15	KF749225	ToLCNDV	Zucchini	Spain	91	97	91	97	91	97	87	89	90	95

16	LC596382	ToLCNDV	Zucchini	Spain	91	97	91	97	91	97	87	89	90	95
17	FJ931537	ToLCPalV	Pumpkin	Varanasi	83	91	83	90	83	90	80	84	82	89
18	GQ225738	ToLCPalV	Pumpkin	Varanasi, Uttar Pradesh	83	90	83	90	83	90	80	83	83	88
19	DQ026296	SLCCNV	Pumpkin	Lucknow	95	99	94	98	94	99	90	90	93	96
20	EU573715	SLCCNV	Pumpkin	Varanasi	94	98	94	98	94	98	90	89	93	95
21	AY184487	SLCCNV	Pumpkin	Coimbatore, Tamil Nadu	94	98	93	98	93	98	89	89	92	95
22	AY396151	SLCCNV	Pumpkin	Lucknow	95	99	94	98	94	99	90	90	93	96
23	GQ225735	SLCCNV	Pumpkin	Varanasi, Uttar Pradesh	93	98	93	98	93	98	90	89	92	95
24	LC511776	SLCCNV	*Cucurbita maxima*	Indonesia	90	96	90	96	90	96	85	87	88	93
25	MW248689	SLCCNV	Squash	Malaysia	89	96	88	96	88	96	85	87	86	93
26	AB085793	SLCPHV	*Cucurbita moschata*	Philippines	89	95	89	94	89	94	85	87	88	92
27	DQ866135	SLCPHV	Pumpkin	Taiwan: Tainan	88	94	88	94	88	94	84	86	87	92
28	AY686500	PYVMV	Pumpkin	India	94	98	94	98	94	98	91	89	93	96
29	KY652743	SLCV	Pumpkin	Timor-Leste	88	94	88	94	88	94	82	86	87	91
30	MN119490	ChiLCV	*Cucurbita maxima*	Oman	72	80	72	80	72	80	70	75	72	78

*In every column, the maximum value is highlighted

Abbreviation

ToLCNDV (*Tomato leaf curl New Delhi virus*), ToLCPalV (*Tomato leaf curl Palampur virus*), SLCCNV (*Squash leaf curl China virus*), SLCPHV (*Squash leaf curl Philippines virus*), PYVMV (*Pumpkin yellow vein mosaic virus*), SLCV (*Squash leaf curl virus*), and ChiLCV (*Chili leaf curl virus*).

Pairwise sequence comparisons (By Genomatix DiAlign Tool) showed the highest 95% similarities to the ToLCNDV and 70-72% ChiLCV (Table 1). The AV1 (Coat-protein) gene of begomovirus isolates was compared with other begomoviruses available in the GenBank database, and pairwise identity scores were calculated using the SDTv1.2. The SDT analysis showed that isolates OQ320768, OQ320770, and OQ320774 had maximum nucleotide (nt) similarities (95%); OQ116977 had maximum nt similarities (93%), and OQ116978 had maximum similarities (94%) with ToLCNDV. The isolates individual coat-protein gene (AV1) was compared with the AV1 of different begomovirus. The analysis showed that AV1 shared the highest 80-99% amino-acid (aa) similarities with ToLCNDV from India. This isolates species was also supported by a two-dimensional color-coded matrix of pairwise identity scores of the AV1 gene of ToLCNDV generated by SDTv1.2 (Figure 2).

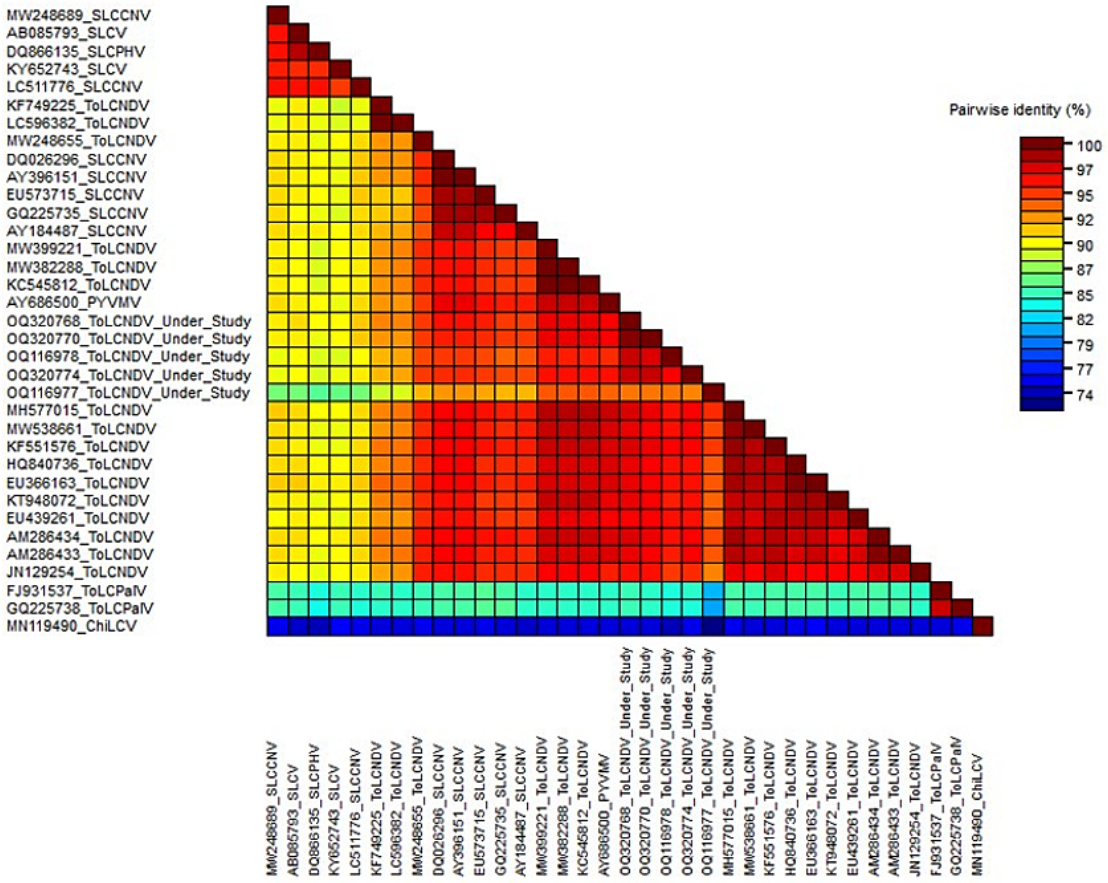

Fig. 2: Using the Sequence Demarcation Tool, a two-dimensional color-coded matrix of pairwise identity scores of begomovirus isolates was constructed. (http://web.cbio.uct.ac.za/SDT).

To determine the evolutionary relationship of the understudy begomovirus isolates (OQ320768, OQ320770, OQ320774, OQ116977, and OQ116978), the phylogenetic trees were constructed using the MEGAv11 using the neighbor-joining method with 1000 bootstraps replicates with selected begomoviruses that were found to be closely related by BLASTn (Figure 3). The understudy begomovirus isolates (OQ320768, OQ320770, OQ320774, OQ116977, and OQ116978) showed the closest relationship with the ToLCNDV (MH577015) on tomato plant from Anand, Gujarat, India and shared distinct relationships with other begomovirus species reported in tomato, pumpkin, Zucchini and other plant species from India and abroad.

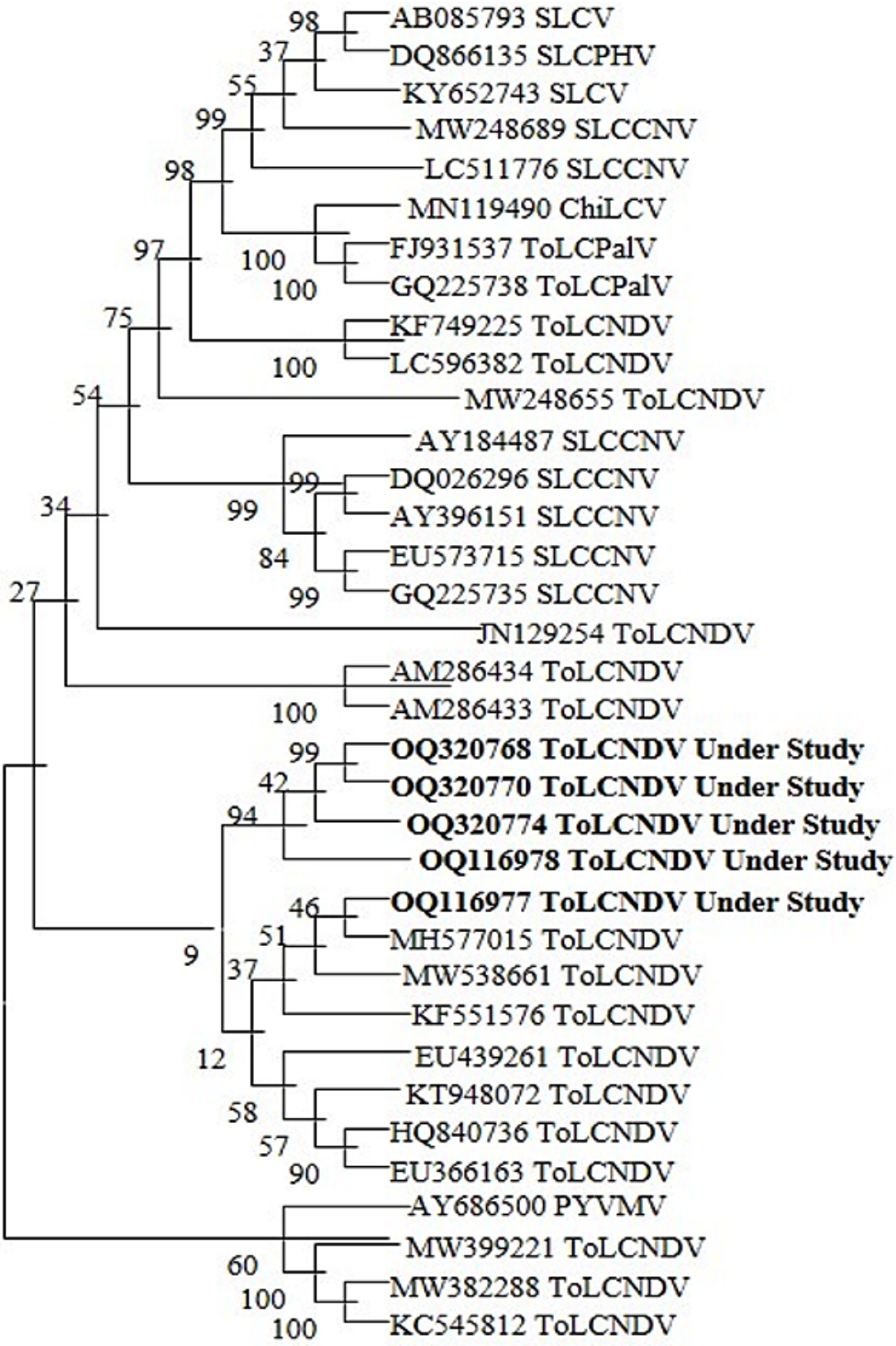

Fig. 3 : Using the Neighbor-joining method, the phylogenetic trees constructed from aligned AV1 nucleotide sequences of pumpkin isolates with other begomoviruses. Bootstrap analysis with 1000 replicates was performed, and bootstrap percentages greater than 0.50 are on branches.

Discussion

During the summer, rainy, and winter seasons in various areas of India, pumpkin is one of the most extensively grown cucurbitaceous vegetables. The pumpkin crop is affected by many insects and diseases caused by fungi, bacteria, and viruses. Globally, agriculture is severely impacted by begomoviruses spread by whiteflies because of their variety, capacity for recombination, fluctuating host ranges, and severe disease symptoms14. In the current investigation, leaf samples of C. *pepo*, C. *maxima*, and C. *moshchata* that had mild to severe mosaic and leaf curling were collected from the field and examined by PCR for the presence of the begomovirus using a primer that was specific to the coat-protein gene of the begomovirus. Further begomovirus-positive pumpkin samples were sequenced and submitted in Genebank database. The complete coat protein gene sequences of understudy virus isolate showed the highest similarity with isolates of ToLCNDV and infecting tomato (MH577015; KF551576) and ridge gourd (MW538661) from India.

An emerging problem of ToLCNDV affects a number of crops in India, Pakistan, the Philippines, Thailand, Italy, and China.[15,16,29] However, ToLCNDV is the most important viral pathogen in the solanaceae family, it has been observed in northern, north-western and southern India infecting a variety of cucurbitaceous crops, including pumpkin, ridge gourd, bottle gourd, long melon, watermelon, ivy gourd, bitter gourd, and cucumber.

In the early 1940s, a yellow vein disease infestation was first identified in crops from northern India.[2] The causal virus is designated as “Pumpkin Yellow Vein Mosaic Disease” (PYVMD) and the occurrence of this disease was first reported from New Delhi,[23],[15] Pune,[3] Maharashtra,[3] Uttar Pradesh,[24,27] West Bengal,[25] Kerala,[26] Karnataka,[4,6] Coimbatore,[5] Assam[7] and Bihar.[28]

On the basis of the highest sequence identities, pairwise similarities, and closest phylogenetic relationship understudy virus isolate was identified as an isolate of *Tomato leaf curl New Delhi virus* on the pumpkin. To our knowledge, this was the first recode of *Tomato leaf curl New Delhi virus* associated with mosaic disuse of pumpkin from Central India.

Pumpkin plant species are perennial climber crops grown all over India, especially in the Northern and Central parts. The molecular data presented here provide useful information on the occurrence of ToLCNDV in pumpkin plant species from Central India. Therefore further studies are required to know the role of climate change in favor of the persistence of vector and spread of begomovirus and the development of infectious clones to screen the begomovirus disease on pumpkins. Further, more molecular studies are required in the future for the characterization of associated begomoviruses on the basis of their complete genome and to develop management strategies to control the mosaic disease of pumpkins to improve their quality and production of pumpkin crops in India.

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Conflict of interests

There is no conflict of interest.

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