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Investigation on disease incidence, distribution and diversity of *Begomoviruses* infecting cucurbitaceous crops in North Bengal, India

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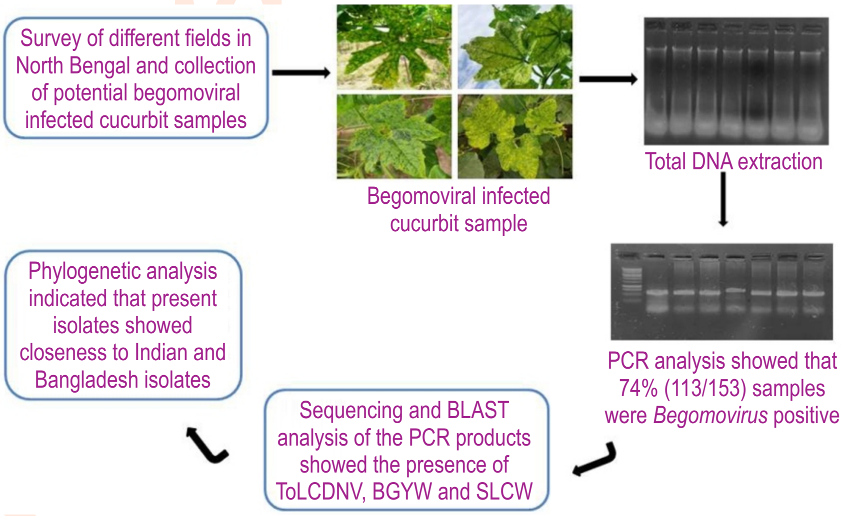
Abstract

Aim: To study the disease incidence and diversity of *Begomovirus* infection and crop loss due to spread of virus across different districts of North Bengal.

Methodology: Several fields in different districts of North Bengal were surveyed and potential begomoviral-infected samples were collected for further analysis. Total DNA extraction, PCR analysis, sequencing and phylogenetic analysis were conducted.

Results: During 2019-2021, ten fields of each district were surveyed and begomoviral occurrence was observed in almost every field. The disease incidence in different districts varied from 15-45%. The highest disease (42.5%) incidence of was observed in the Darjeeling district while the lowest (15%) was recorded from Alipurduar district. The maximum crop loss due to *Begomovirus* was 64-100% in bitter gourd, 40-80% in cucumber and 20-30% in wax gourd. 153 samples from 6 different cucurbits were collected and analysed. PCR using AV494/AC1048 primers that amplified coat protein region (~550bp) showed 74% of the samples as positive. Upon sequencing and BLAST analysis, ToLCNDV, SLCCV and BGYVV were detected. In phylogenetic analysis, the isolates clustered in three separate clades based on *Begomovirus* species irrespective of the hosts. Present isolates showed more closeness to Indian and Bangladesh isolates.



Interpretation: The documentation of disease severity caused by *Begomovirus* is of extreme importance for food security of the people. Present study revealed that *Begomovirus* is present in all the crop field studied. The results indicated an urgent need for the management of the viruses to save crops.

Key words: *Begomovirus,* Cucurbitaceous crops, Disease incidence, North Bengal

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Introduction

India is a vast country with a diverse agro climate, which makes it possible to grow a wide variety of crops and it is the second largest in the world in terms of crop production (Gulati *et al.,* 2022). Indian agriculture sector contributes about 16.5% of total GDP and generates employment for 42.3% of the population in India. India is also rich in biodiversity of vegetables and is the primary/secondary center of origin of many vegetables (Gulati and Juneja, 2022). Vegetables are a key source of nutritional security in India, primarily due to high yield in a short time. Cucurbitaceous crops are rich source of nutrition and have several medicinal values. Different types of cucurbits are cultivated in India which account for 5.6 % of the total vegetable production (Rolnik and Olas, 2020). According to FAO, India produces 10.52 ton per ha of cucurbits which are grown on 4,290,000 ha of cropped area (Sharma, 2023). According to 2021-2022 census, West Bengal produces 344.674 metric tons of cucumber in 25.298 ha area and 356.213 metric tons of pumpkin in 21.772 ha area.

North Bengal referring to the northern region of West Bengal is considered to be one of the key agro-ecological niches of India. Being located between Eastern Himalaya and Indo-Burma vegetational hotspots, it also serves as one of the important areas for the cultivation of several agricultural and horticultural crops (Rawal *et al.,* 2013). The climatic condition of this region is also conducive for viral attack leading to crop loss. It is important to recognize and control crop pathogens for successful cultivation of crops. Crops, which are prone to viral diseases include cucurbits, potato, tomato, papaya, chilli and common beans (Saha *et al.,* 2014). The rapid expansion of agriculture throughout the world has resulted in the emergence, increase and spread of new and minor diseases to newer areas. Among the diseases, insect-transmitted viruses are important (Inoue-Nagata *et al.,* 2016).

Begomoviruses have a vast host range and most of the hosts are economically important crop plants. Begomoviruses have emerged as a group of destructive viruses due to their high recombination capacity and ability to acquire extra DNA components (Qureshi *et al.,* 2022). They are exclusively transmitted by whitefly, *Bemisia tabaci* which is considered as one of the most invasive pests and virus vectors (Fiallo-Olivé *et al.,* 2020). Factors leading to the diversity and spread of begomoviruses include interaction with satellite DNAs that are gathered throughout their evolution, their ability to recombine the genomes, continuous movement of their insect vectors to different regions of the world, polyphagia of its vectors and numerous wild plants that act as reservoir (Fiallo-Olivé and Navas-Castillo, 2023). Begomovirus (Family: Geminiviridae) is the largest and most prevalent genus of plant-infecting DNA virus (Malathi, 2017). Their genome structure consists of a single-stranded circular DNA which remains enclosed in a twinned quasi-icosahedral particle. Begomoviruses contain either a monopartite or a bipartite genome (Zerbini *et al.,* 2017). Bipartite begomoviruses have two separately encapsidated genomic components, known as DNA-A (~2.7 kb) and DNA-B (~2.6 kb) whereas the monopartite begomoviruses contain only DNA-A (~2.7 kb) (Fiallo-Olivé *et al.,* 2021). Additional small-circular single stranded DNAs may be found along with most of the monopartite begomoviruses and some bipartite begomoviruses. These small circular DNAs are called alphasatellites and/or betasatellites (Lozano *et al.,* 2016). Studies on the occurrence and distribution of *Begomovirus* infecting cucurbit crops from the present study area are limited. Hence, the objective of the study was to perform a comprehensive analysis of the distribution and genetic diversity of begomoviruses infecting cucurbit crops across North Bengal, India.

Materials and Methods

Sample collection: During the cropping seasons of 2019-2021, several fields in different locations were surveyed in different districts of North Bengal (Fig. 1). Around 153 samples from 6 different cucurbit crops (cucumber, pumpkin, sponge gourd, snake gourd, wax gourd and bitter gourd) that showed potent begomoviral symptoms like leaf yellowing, blistering, mosaic pattern, puckering, leaf deformation and vein clearing were collected during the time of survey (2019-2021) from the fields studied. Based on observations of probable virus infections, the disease severity was recorded using a scale (ranging from 0-4) following the method of Song *et al.* (2004) with minor modifications. Details of the scale used are as follows:

0- No infection; 1- Slight infection with about 25% of full scale; 2- Moderate infection, about 50% disease; 3- Severe infection, 75% show infection; 4- Complete infection, 100% are infected;

The percentage of disease incidence was calculated by the formula:

$$\text{Disease incidence (\%)} = \frac{(\Sigma \text{ scale x number of plants / fields infected})}{(\text{highest scale x total number of plants / fields})} \times 100$$

DNA extraction, PCR, sequencing and sequence analysis: Total DNA of the collected samples was extracted by cetyltrimethylammonium bromide (CTAB) method following the protocol of Haible *et al.,* 2006. PCR was performed using Begomovirus specific primers AV494/AC1048 (Khan *et al.,* 2007; Samarakoon *et al.,* 2012) that amplified the coat protein (CP) region (~550bp). PCR mix of 25 µl reaction mixture containing 5 µl (5×) Taq DNA buffer, 1 µl (10 mM) dNTPs, 1.5 µl (25 mM) $MgCl_2$, 2 µl DNA template, 0.5 µl (10 µM) primers (both forward and reverse) and 0.125 µl (5u/µl) Taq DNA polymerase was prepared. The remaining volume was adjusted using sterile distilled water. PCR amplification, was carried out as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min and final extension for 10 min at 72°C. The obtained amplicons were analysed in 1% agarose gels under UV-transilluminator (Genei, Bangalore, India). The purified amplicons were cloned in pGEM T-easy vector following the method of Sambrook and Russel (2001). The cloned products were sent for sequencing to Bioserve Biotechnologies (India) Private Limited., Hyderabad (India). The obtained sequences were subjected to Basic Local Alignment Search Tool-nucleotide

(BLASTn) analysis for sequence similarity and identity with the other sequences that were already submitted in the GenBank. A phylogenetic tree using MEGA was created by Neighbor-joining method through Kimura-2 parameter with Bootstrap value of 1000 (Tamura *et al.,* 2013). Sequence identity matrix was created by SDTv1.2 (Muhire *et al.,* 2014).

Results and Discussion

About 153 samples from 6 different cucurbitaceous crops were collected from five districts (Darjeeling, CoochBehar, Malda, Uttar Dinajpur, Alipurduar) of North Bengal (Fig. 1). Symptoms such as leaf yellowing, puckering, deformation, leaf curling, vein clearing, yellowing, mosaic and blistering were observed across different fields in different districts of North Bengal. The symptoms observed in cucurbits were leaf mosaic, puckering and blistering, yellowing and vein clearing symptoms in cucumber; leaf yellowing and mosaic in pumpkin; mosaic and deformation in bitter gourd; vein clearing and deformation in wax gourd; puckering and blistering in sponge gourd; leaf yellowing and mosaic in snake gourd (Fig. 2).

Viral occurrence was observed in almost every field visited, however the disease incidence varied. Ten fields in each district were visited and based on the severity of symptoms in each field the existing disease incidence was calculated. The results showed that disease incidences were 42.5% in Darjeeling district, 27.5% in Malda district, 15.0% in Alipurduar district, 32.5% in Uttar Dinajpur district and 22.5% in CoochBehar District (Fig. 3). The highest disease incidence was recorded from Darjeeling (42.5%), while the lowest was recorded from Alipurduar district (15.0%). Based on field symptomatology and effect on crop production across different districts, the highest disease incidence was noted in bitter gourd (75.0%), followed by cucumber (50.0%) and wax gourd (27.5%). Similar reports of disease incidence of 100% in bitter gourd (Devi *et al.,* 2022) and 50-60% in cucumber (Venkataravanappa *et al.,* 2021) have been reported from different states of India like Uttar Pradesh,

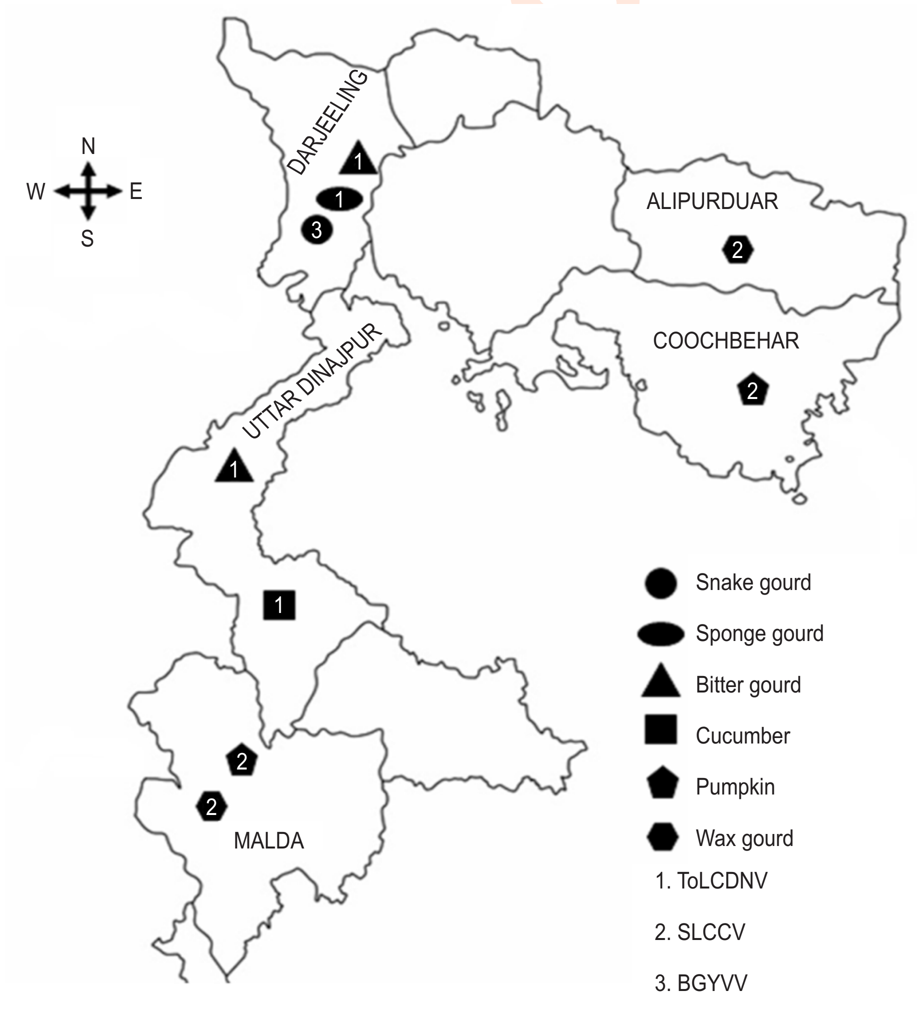

Fig. 1: Map of North Bengal showing areas of sample collection and *Begomovirus* distribution pattern

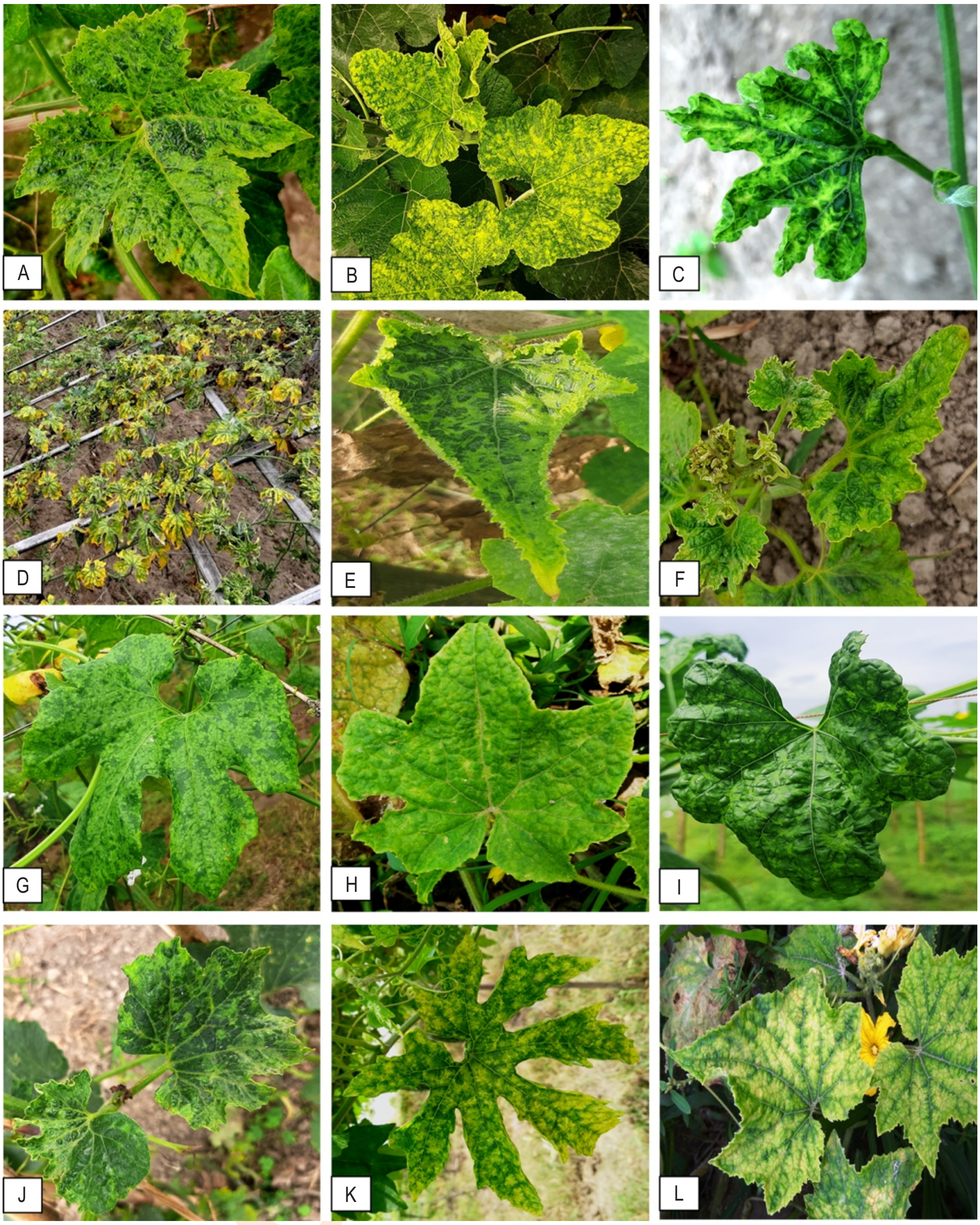

Fig. 2: Observed symptoms of different cucurbitaceous crops of North Bengal: Mosaic in Wax gourd (A); Pumpkin (B); Bitter gourd (K); Snake gourd (G); Cucumber (E); Deformation in Bitter gourd (C); Wax gourd (F, J); Puckering and blistering in Cucumber (H); Sponge gourd (I); Yellowing and vein clearing in Cucumber (L) and Severely infected field view of Bitter gourd (D).

Himachal Pradesh and Tamil Nadu. In the present study, the crop loss due to *Begomovirus* was found to be 64-100% in bitter gourd, 40-80% in cucumber and 20-30% in wax gourd. Cent percent crop loss in bitter gourd (Devi *et al.,* 2022) has earlier been reported from India. Huge yield loss ranging from 70-100% in cucurbits due to *Begomovirus* have also been reported from different countries like Philippines (Neoh *et al.,* 2023), Lebanon (Sobh *et al.,* 2012) and Thailand (Charoenvilaisiri *et al.,* 2020).

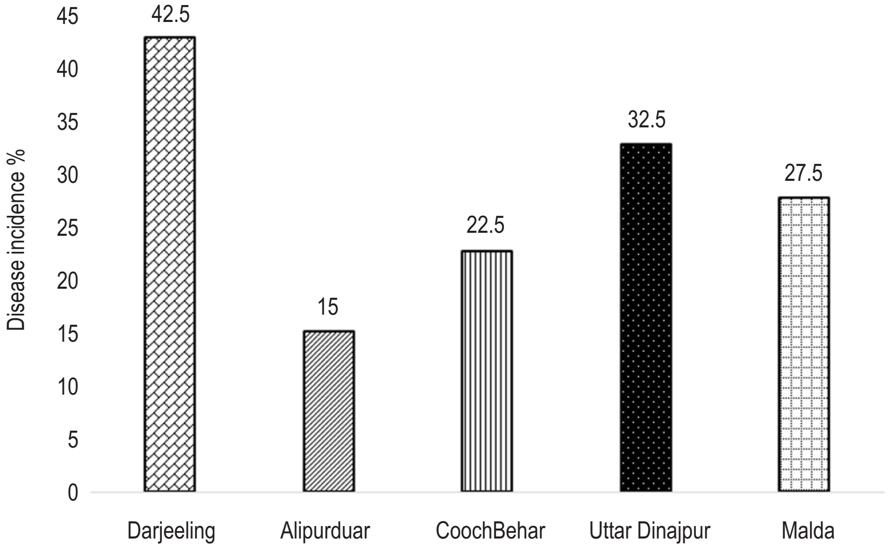

Fig. 3: Percentage of disease incidence across different districts of North Bengal.

PCR of the total DNA extracted from 153 samples were done. Samples [74% (113/153)] were found to be infected with *Begomovirus* and showed a positive PCR amplification (~550 bp). Based on symptom severity, the host plant and place of collection, isolates were selected and around 50% of the begomoviral positive isolates were sequenced (to serve as a representative isolate). BLASTn analyses of the obtained sequences were done using coat protein (CP) sequence. The *Begomovirus* species varied from different host and different area. All the sequences obtained showed 92-97% similarity with ~550 bp of CP gene of *Begomovirus* sequences submitted in the GenBank. Isolates obtained from sponge gourd, cucumber and bitter gourd (OL2523073, MN207009, MN207007, MN207010) showed >96% similarity with *Tomato leaf curl New Delhi virus* (ToLCDNV). Isolates from pumpkin and wax gourd (OP377758, MN215885, OP377761, OL649770) showed >97% similarity with *Squash leaf curl china virus* (SLCCV). Isolate from snake gourd (MN207008) showed 97.55% similarity with *Bitter gourd yellow vein virus* (BGYVV) (Table 1).

For phylogenetic analysis, coat protein sequences of *Begomovirus* from different countries of Asia, available in the GenBank were obtained. Upon analysis, three clades were formed for three *Begomovirus* species *i.e.,* SLCCV (Clade A), BGYVV (Clade B) and ToLCNDV (Clade C). Present isolates clustered in clades based on their species of *Begomovirus* irrespective of the hosts from which they were isolated. Pumpkin (MN215885|SLCCV, OP377758|SLCCV), wax gourd (OP377761|SLCCV, OL649770|SLCCV) clustered with SLCCV (Clade A). Snake gourd (MN207008|BGYVV) clustered with BGYVV (Clade B). Bitter gourd (MN207010|ToLCDNV, MN207007|ToLCDNV), sponge gourd (OL649767|ToLCDNV) and cucumber (MN207009|ToLCDNV) isolates clustered with ToLCNDV (Clade C). In this study, it was also observed that the isolates showed closer relationship with the Bangladesh isolates rather than Indian isolates or isolates from any other Asian countries (Fig. 4). In Clade A (SLCCV), the isolates formed a separate cluster along with Bangladesh isolate however isolates from India and Pakistan formed a separate cluster. Similar result was also seen in Clade C (ToLCNDV) where the isolates again showed a separate clustering along with Bangladesh isolates while the isolates from India, Pakistan and Thailand were noted to form separate clusters. Clade B (BGYVV) showed clustering of present isolates with isolates from India and Bangladesh. The results of phylogenetic analysis were also supported by sequence identity matrix, which is a two dimensional colour-coded matrix based on sequence pairwise identity (Fig. 5).

Isolates of ToLCNDV showed evolutionary closeness to Indian and Bangladesh isolates. ToLCNDV, has been reported, to cause severe economical damage (Moriones *et al.,* 2017). ToLCNDV, have been reported from different vegetable and fiber crops (Zaidi *et al.,* 2016). Till date, the incidence of ToLCNDV was limited to Asian countries, which were mostly reported from India, Bangladesh and Pakistan in the Indian subcontinent, and few from Taiwan, Indonesia and Thailand. However, recently ToLCNDV has been expanding its host range and spreading to different geographical locations (Yazdani-Khameneh *et al.,* 2016). ToLCNDV was initially isolated from crops belonging to Solanaceae family, but many reports from cucurbit crops have also been published (Ito *et al.,* 2008; Yazdani-Khameneh *et al.,* 2016). Present isolate BGYVV also formed group, showing closeness to Indian and Bangladesh isolates. Bitter gourd yellow vein virus (BGYVV) have been reported from several countries like Sri Lanka (Bandaranayake *et al.,* 2014), Pakistan (Tahir *et al.,* 2010a) and India (Mohanan and Sharma, 2020). Sequence comparisons revealed that the inter-specific recombination between Tomato leaf curl New Delhi virus and Tomato leaf curl

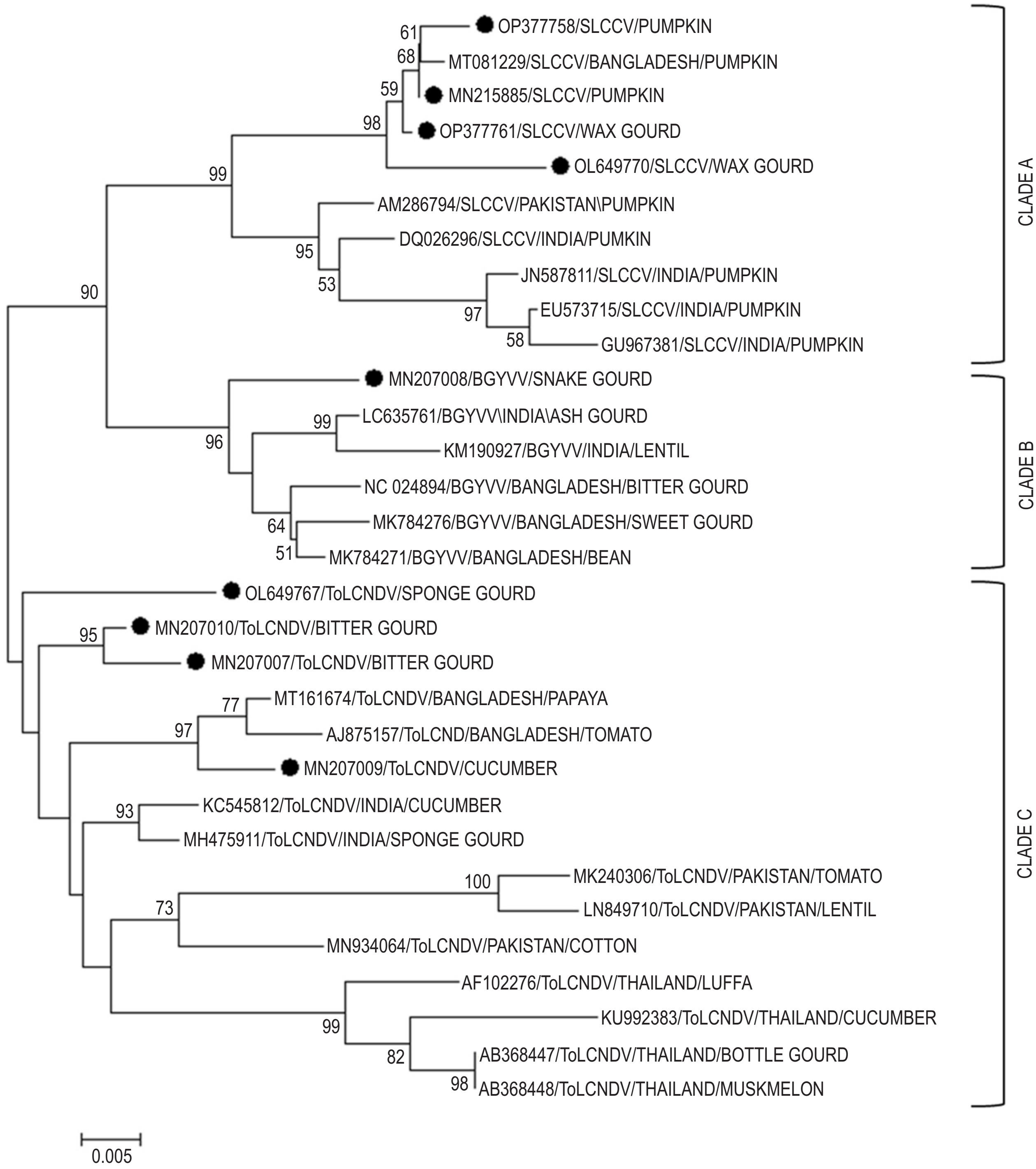

Fig. 4: Phylogenetic analysis of the CP sequences of *Begomovirus* isolates. Phylogenetic tree was generated by neighbour-joining method with bootstrap values of 1000. Values at nodes designate the percentage of bootstrap (out of 1000 replicates) which are indicated if values are greater than 50. GenBank accession numbers, virus species and place of collection is mentioned at the end of each branch. Our isolates are indicated by ●

Bangladesh virus led to the emergence of BGYVV Tahir *et al.* (2010a). Phylogenetic analysis indicates that present isolates of SLCCV clustered with itself along with the Bangladesh isolate irrespective of the host. Squash leaf curl China virus (SLCCV) is emerging as an economically important virus (Bragard *et al.,* 2013). SLCCV has been reported from different cucurbit hosts. SLCCV infecting squash (Cucurbita pepo) reduced production in India and Pakistan and resulted in huge economic losses

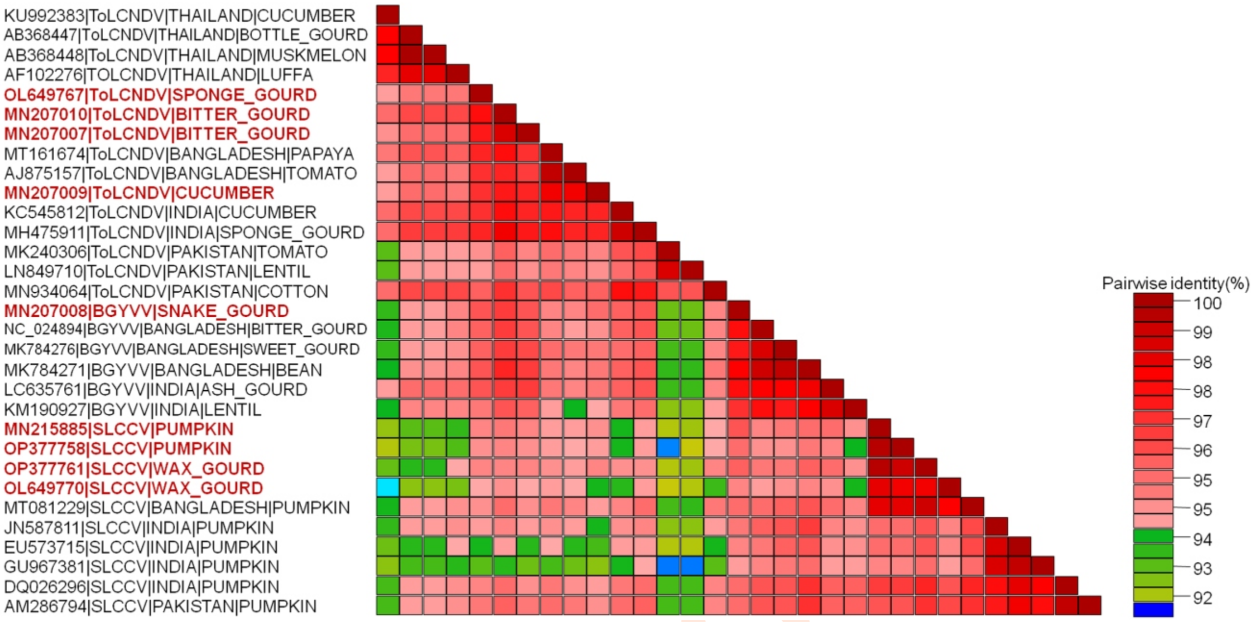

Fig. 5: Sequence identity matrix of *Tomato* leaf *curl China* Virus (ToLCDNV), *Squash leaf curl China virus* (SLCCV) and *Bitter gourd yellow vein virus* (BGYVV) isolates. Percent identity corresponds to the color matrix which is indicated on the side of the figure.

Table 1: List of Begomoviral isolates with assigned code, visual symptoms and host range distributed in five districts

Virus	Host plant	Representative Isolate code	Symptoms	Accession no.	% identification from BLASTn analysis	Place of collection
Tomato leaf curl New Delhi virus	Sponge gourd	Sp-Phn-01	PC, BL	OL649767	96.66	Darjeeling
	cucumber	Cu-Isl-01	PC, YL, MS	MN207009	97.16	Uttar Dinajpur
	Bitter gourd	Bi-Isl-01	DF, MS	MN207007	97.88	Uttar Dinajpur
	Bitter gourd	Bi-Phn-01	MS	MN207010	97.3	Darjeeling
Squash leaf curl china virus	Pumpkin	Pu-Mal-01	MS	OP377758	99.43	Malda
	Pumpkin	Pu-CoB-01	MS	MN215885	97.38	CoochBehar
	Wax gourd	Wg-Mal-01	DF	OP377761	99.81	Malda
	Wax gourd	Wg-Apd-01	MS	OL649770	98.07	Alipurduar
Bitter gourd yellow vein virus	Snake gourd	Sn-Phn-01	MS	MN207008	97.55	Darjeeling

SYMPTOMS: Deformation: DF; Leaf curling: LC; Mosaic: MS; Puckering: PC; Vein clearing: VC; Blistering: BL; Yellowing: YL

(Saritha *et al.,* 2011; Tahir *et al.,* 2010b). SLCCV have also been reported from cucurbits like pumpkin (Vo *et al.,* 2022), ash gourd (Riyaz *et al.,* 2015) and melon (Wu *et al.,* 2020). 100% crop loss in *Benincasa hispida* due to SLCCV was reported from Tamil Nadu, India (Riyaz *et al.,* 2015). *Begomovirus* has become an important constraint to crop production leading to devastating crop loss (Pandey *et al.,* 2021). The vast host range, high recombination, ability to acquire extra DNA components, have helped begomoviruses to emerge as a group of destructive viruses (Saeed *et al.,* 2017). Mutation, reassortment and recombination in begomoviruses result in genetic variation that have led to increased infection rates, virulence, expanded host range, evolution and ability to adapt in changing environment (Nigam *et al.,* 2021).

The spread in *Begomovirus* population may also be responsible for increase in *Bemisia tabaci* population, a vector for Begomovirus (Gilbertson *et al.,* 2015). In India, a large number of begomoviruses have been identified infecting various crops (Manju *et al.,* 2018). At present, cucurbits are infected by several *Begomovirus* species all over the world (Malathi, 2017). Around 24 species of *Begomoviruses* and their variants are reported to infect cucurbits globally. Vast diversity is being observed among the *Begomoviruses* infecting cucurbits (Kumari *et al*., 2022). In the present study, CP genes were used for the identification and phylogenetic analysis. Variation in CP suggests evolution of *Begomovirus.* CP plays a key role in viral movement and vector transmission in plants. Although CP proteins have evolved differently in different host and different geographic areas, selection pressure of specific region has more influence rather than host in the evolution of CP proteins (Mondal *et al.,* 2019). In phylogenetic analysis, the isolates of this study also showed similar results. They showed more closeness toward the Indian and Bangladesh isolates. Additionally, the clustering was irrespective of the hosts. This also shows the possible trans-boundary movement of the virus which might occur through plant materials or its insect vectors (Jones *et al.,* 2019).

Small or medium scale farmers usually practice polyculture or diverse crop cultivation where different crops are grown in small area. This practice leads to cross contamination, infection and disease spreading across different crops grown (Iverson *et al.,* 2014; Kiran *et al.,* 2021; Loomis, 2022). A preliminary identification of *Begomovirus* can help to describe (provisionally) the probable evolution and diversity of *Begomovirus.* The global seed trade also plays a key role in increasing *Begomovirus* evolution, as it facilitates species to reach new areas and introduce vectors to new hosts (Subiastuti *et al.,* 2019). High disease incidence have been observed in different districts of North Bengal may be due to warm and humid tropical climate, which is favorable for the survival of whitefly. Polyphagous nature of the vector and diverse crop cultivation and sustenance of *begomovirus* in several hosts may also be the cause of high disease incidences.

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