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Genetic variability of *tomato leaf curl new delhi virus* infecting cucumber in sub-Himalayan plains in Eastern India

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Abstract Cucumber (*Cucumis sativus*, *Cucurbitaceae*) is a vegetable crop widely cultivated in India.Disease survey was conducted from December, 2016 to March, 2017in different locations of sub-Himalayan plains in Eastern India. During survey, leaf curl disease of cucumber was found to be one of the most serious threats in cucumber production. Based on disease symptoms, thirty infected and five healthy leaf samples were collected. Twenty out of thirty infected samples were found positive for begomovirus infection upon PCR with begomovirus specific universal primers. Amplified products were cloned, sequenced and four representative sequences (one from each location) were submitted to GenBank. BLAST analysis of the isolates showed 97-99% nucleotide identity with *Tomato leaf curl New Delhi virus* (ToLCNDV) infecting cucumber from Bangladesh. A phylogenetic tree was constructed and a two-dimensional colour-coded sequence identity matrix was generated to find out the relationship between present ToLCNDV isolates and other isolates of India and neighboring countries. From the present study it was evident that the present isolates were closely related to Bangladeshi isolates rather than other Indian isolates.This might becorrelated to different factors like-availability of vectors, weather condition and agricultural practices in this area are similar with the neighbor country Bangladesh.

Keywords: *Cucumis sativus*, ToLCNDV, PCR, Sub-Himalayan plains, Phylogenetic analysis

Introduction

Tomato leaf curl New Delhi virus (ToLCNDV) is an economically important bipartite *Begomovirus* belonging to the family *Geminiviridae*. Begomoviruses can have bipartite or monopartite genomes. The genome of bipartite begomoviruses consists of two circular single-stranded DNA molecules of about 2.5-2.7 kb, referred to as DNA-A and DNA-B. Monopartite

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begomoviruses have a single genomic DNA that is homologous to the DNA-A of the bipartite genome (Brown *et al.,* 2015).

Cucumber is a low-calorie food and contains high levels of vitamin K, flavonoids (apigenin, luteolin, quercetin, and kaempferol), antioxidants (beta carotene, vitamin C and vitamin B) and lignins (Mukherjee *et al.,* 2013). In India during 2016-17, the production of cucumber was 1,142,000 metric tons in a total area of 78,000hectare (Government of India, 2017). Several begomovirus diseases have been reported in cucumber so far, which includes *Tomato leaf curl virus* (Raj and Singh, 1996) and *Tomato leaf curl Palampur virus* (Raj *et al.,* 2011) in northern India; and ToLCNDV, *Tomato leaf curl Karnataka virus, Mesta yellow vein mosaic virus, Tomato severe leaf curl virus* and *Pepper golden mosaic virus* (Suresh *et al.,* 2013) in western India. Cucumber was also reported to be infected by *Tomato yellow leaf curl virus* in Indonesia (Sohrab *et al.,* 2017) and *Tomato leaf curl New Delhi virus* in Indonesia (Mizutani *et al.,*2011) and Sri Lanka (Bandaranayake *et al.,* 2016).

However, *Begomovirus* infecting cucumber in eastern part of India has never reported. The cucumber planted fields in sub-Himalayan plains of Eastern India were surveyed, where leaf curl symptoms were prominent. The objective was to identify leaf curl-associated *Begomovirus* through PCR based methods and to study the genetic variability of the virus within sub-Himalayan plains of Eastern India.

Materials and methods

Survey, disease incidence and collection of diseased samples

During December, 2016 and March, 2017 a survey was conducted in cucumber fields of sub-Himalayan plains of Eastern India.Thirty infected and five healthy leaf samples were collected with leaf curling (upward and downward), leaf yellowing, leaf mosaic, vein clearing and leaf distortion symptoms.In severe cases the plants showed stunted growth (Figure 1). Disease incidences were estimated following the method of Sohrab *et al.* (2010).

Total DNA extraction and PCR amplification

Total DNA was extracted from the infected and healthy leaf samples following the method of Haible *et al.* (2006). Total DNA, isolated from both healthy and infected samples were run on 1% agarose gel, observed on UV-transilluminator and stored at -20℃ for further use. For detection of *Begomovirus*, PCR amplification was done using *Begomovirus* specific primers

DengA (5′-TAATATTACCKGWKGVCCSC-3′) and DengB (5′-TGGACYTTRCAWGGBCCTTCACA-3′) to amplify the movement protein (AV2) and partial coat protein(CP) genes of DNA-A (Reddy *et al.*, 2005).

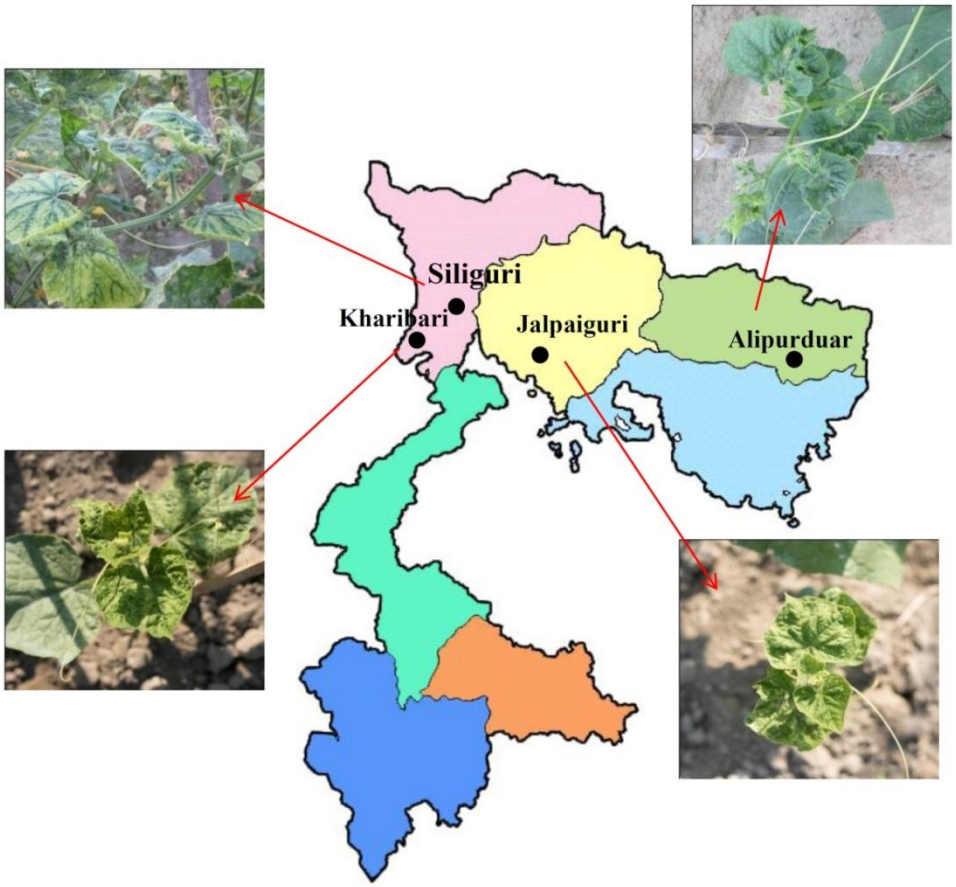

Figure 1. Map of Northern West Bengal and regions of collection of infected plant samples from four different spots of North Bengal. The black dots (●) showing the collection spots along with place of collection

Sequencing and phylogenetic analysis

The purified PCR products were cloned into pGEM-T vector following the method of Sambrook and Russel (2001) and the clones were sent to Chromous Biotech Pvt. Ltd. for sequencing. The nucleotide sequences were aligned using ClustalW (Thompson *et al.*, 1994). After BLAST analysis the sequences were deposited to GenBank. Sequence identity matrix was generated

using SDT 1.2 (Muhire *et al.*, 2014) and a phylogenetic tree was generated by neighbour-joining method and Kimura-2 parameter in MEGA 6.0 (Tamura *et al.*, 2013).

Results

Survey and diagnosis of viral disease

During initial survey about 46-54% of the crops were found to be symptomatic in different cucumber growing fields of sub-Himalayan plains of Eastern India (Table 1). Total DNA was extracted from all the leaf samples collected and were tested for the presence of *Begomovirus* through PCR with'DengA' and 'DengB' primers. Twenty out of thirty infected samples showed amplicon of ~530 bp. None of the healthy leaf samples of the cucumber growing fields produced this specific amplicon (Figure 2). Amplified products were cloned, sequenced and four representative sequences (one from each location) were submitted to GenBank [Acc.Nos.MG721012(JPG-01), KY807530 (SLG-01), KY783746(SLG-02) and MG721013 (ALP-01)]. BLAST analysis revealed that the obtained sequences of the samples JPG-01, SLG-01 and SLG-02 showed similarity with Bangladesh isolates infecting tomato with nucleotide identity of 97% (Acc. No. MG721012), 98 % (Acc.No.KY807530) and 99% (Acc.No. KY783746) respectively. ALP-01showed 99% nucleotide identity with Bangladesh isolate infecting cucumber (Acc. No. EF450316).

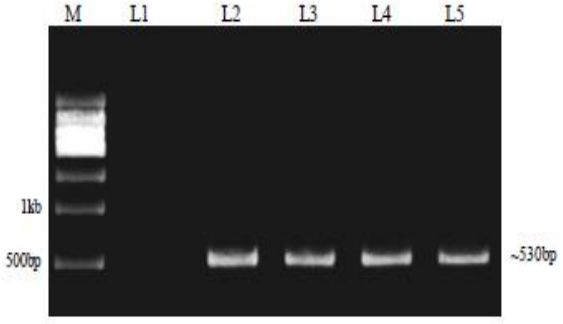

Figure 2. Amplicons of four positive isolates of ToLCNDV on 1% agarose gel. [M= 500bp DNA ladder; L1= Control; L2= SLG-01 (Siliguri) ToLCNDV isolate; L2= SLG-02 (Kharibari) ToLCNDV isolate; L3= ALP-01 (Alipurduar) ToLCNDV isolate and L4= JPG-01 (Jalpaiguri) ToLCNDV isolate]

Table 1. Samples collected from sub-Himalayan plains of Eastern India with sample codes, associated symptoms, % disease index, status of PCR with *Begomovirus* specific primer and GenBank Accession No. of the representative isolates

Place of collection	Sample code	Symptoms[/1]	% Disease index	Status of PCR with *Begomovirus* specific primer[/2]	Representative Isolate (Acc. No.)
	ALP-01	LC, LD, St		+	
	ALP-02	LC, YM		+	
	ALP-03	St, LD, LC		-	
Alipurduar,West	ALP-04	LC, LD, St		+	ALP01
Bengal,India	ALP-05	YM, St, no fruit	46	-	(MG721013
	ALP-06	St, YM		+	)
	ALP-07	LC, St		+	
	ALP-08	SL		-	
	JPG-01	LC, LD, St, YM		+	
	JPG-02	LC, YM		+	
	JPG-03	St, LD, LC, no fruit		-	
	JPG-04	LC, LD, St		+	
Jalpaiguri, West	JPG-05	YM, St, no fruit		+	JPG01
Bengal,India	JPG-06	St, YM, LD	50.20	+	(MG721012
	JPG-07	LC, St		-	)
	JPG-08	YM, St, no fruit		-	
	JPG-09	SL		-	
	SLG-02	LC, LD, St, YM		+	
	SLG06	LC, YM, St		+	
	SLG-05	St, LD, LC, no fruit		-	
	SLG-09	LC, LD, St		+	
Siliguri, West	SLG-08	YM, St, no fruit		-	SLG02
Bengal, India	SLG-10	St, YM, LD	54.10	+	(KY783746)
	SLG-11	LC, St, LD		+	
	SLG-12	YM, St, no fruit		-	
	SLG-13	SL		-	
	KHR-01	LC, LD, St, YM		+	
	KHR-02	LC, YM, St		-	
	SLG-01	St, LD, LC		+	
	KHR-04	LC, LD, YM		-	
Kharibari, West	KHR-05	YM, St, no fruit	53.50	+	SLG01
Bengal, India	KHR-06	St, YM		+	(KY807530)
	KHR-08	LC, St, LD		+	
	KHR-09	SL		-	
	KHR-10	SL		-	

[/1] LC= Leaf curl; LD= Leaf deformation; St= Stunted growth; YM= yellow mosaic; SL= Symptom less.
[/2]+ = PCR positive; - = PCR negative

Phylogenetic analysis

Altogether 80 isolates of ToLCNDV in worldwide were taken into consideration for phylogenetic analysis (Figure 3). The isolates formed several small clusters. Almost all the ToLCNDV isolates reported from nearby geographical locations clustered together. The phylogenetic tree consisted of 6 major clusters. The isolates of the present study are present in two different clusters. However, all the isolates of the study showed close relationship withBangladeshi isolates .The phylogenetic analysis was also supported by the two dimensional colour-coded identity matrix where four present isolates [ALP-01(MG721013), JPG-01 (MG721012), SLG-02(KY783746) and SLG-01(KY807530)] showed similar colour pattern i.e., they had the highest nucleotide similarity with that of Bangladeshi isolates (Figure 4).

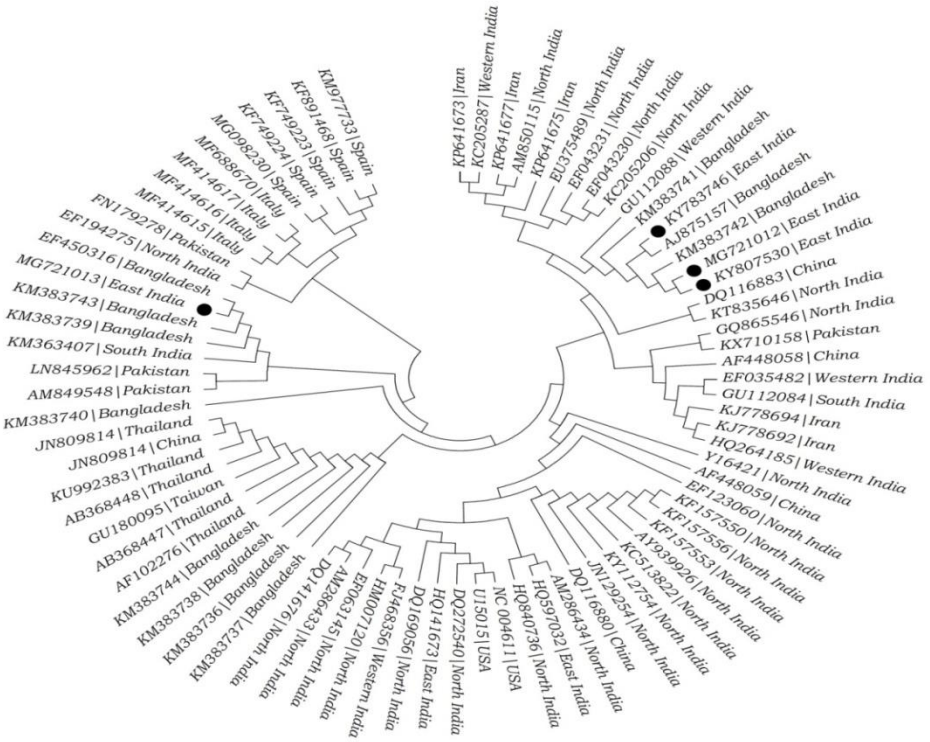

Figure 3. Phylogenetic relationship of ToLCNDV taken from different geographical locations. In each case, the reference isolates are mentioned with Accession number. The neighbor-joining tree with 1,000 boot strap replicates was prepared using MEGA version 6.0 after multiple alignment of the coat protein gene sequences using CLUSTALW. Sequencesof the present study are represented with round black dots (●)

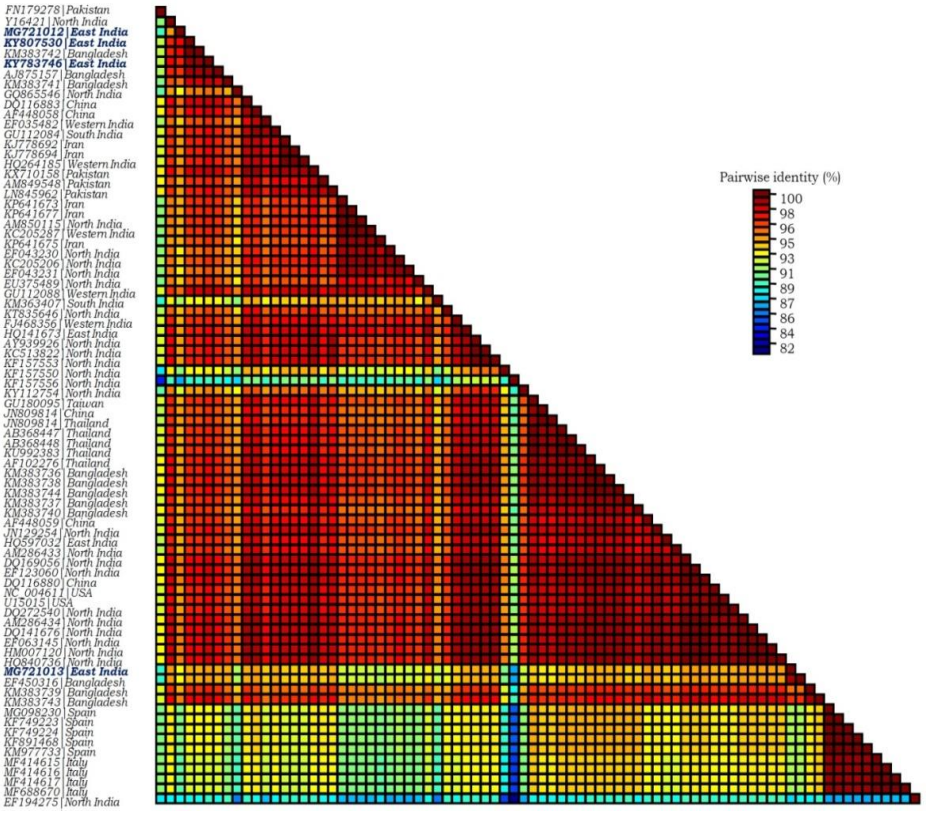

Figure 4. Nucleotide sequence identity matrix of the ToLCNDV isolates of the present study along with other ToLCNDV isolates from GenBank. Identity percentages are indicated on the right side corner of the matrix

Discussion

The present study revealed the occurrence of disease caused by *Begomovirus* in cucumber from sub-Himalayan plains of Eastern India. During the initial survey, symptoms of leaf curling, yellowing, mosaic, vein clearing and stunted growth were found to be very common in this region. These symptoms are generally associated with *Begomovirus* infection as reported by several workers (Raj *et al.,* 2010, 2011; Phaneendra *et al.,* 2012; Tiwari *et al.,* 2008, 2010, 2012; Sohrab *et al.,* 2017). *Begomovirus* has been found to be a limiting factor in cucumber production in Saudi Arabia (Sohrab *et al.,* 2017) and in Thailand (Ito *et al.,* 2008). Since, the disease symptoms along with literature reports indicated *Begomovirus* infection, further tests based on PCR were conducted to confirm and identify the *Begomovirus* present in the infected cucumber plants. PCR amplification with universal *Begomovirus* primers which amplifies the partial coat protein gene produced the expected amplicons

indicating the occurrence of this virus in two-third of the symptomatic leaf samples. A coat protein gene is considered to be the most conserved of the viral genes and the sequencing of this region is recognized as sufficient for the initial identification of begomoviruses (Fauquet and Stanley, 2003). This has been used in the diagnosis of *Begomovirus* infection in cucurbits by several authors (Sohrab *et al.,* 2006; Singh *et al.,* 2007; Raj *et al.,* 2010, 2011; Tiwari *et al.,* 2008, 2010, 2012). In the present study, sequencing of the PCR products and subsequent BLAST analysis identified the sequences to be that of ToLCNDV. Various crops in India, Pakistan, Thailand and Bangladesh are reported to be attacked by ToLCNDV (Zaidi *et al.,* 2017; Venkataravanappa *et al.,* 2019; Suresh *et al.,* 2013). It has been reported for the first time in India from tomato and later on from various crops like Chili, cucurbits, potato, papaya, bitter gourd and cotton (Dharmendra *et al.,* 2011). Sequence identity matrix of the present study also revealed genetic relatedness between the present isolates and other ToLCNDV isolates reported from Bangladesh. This was further confirmed when the Bangladeshi isolates clustered together with the present isolates in the phylogenetic tree. Thus, the possible risk of transboundary movement of the virus through plant materials or insect vectors was evident. Many begomoviruses are considered as important quarantine pests in the world (Hamilton, 2000).

Similar type of clustering of ToLCV has also been reported by several workers worldwide (Reddy *et al.,* 2011; Bandaranayake *et al.,* 2016; Fortes *et al.,* 2016; Zaidi *et al.,* 2016; Yazdani-Khameneh *et al.,* 2016). The ToLCV-coat protein gene analysis may provide valuable information to the recent occurrence of tomato leaf curl disease in sub-Himalayan region of West Bengal. It concluded that high disease incidence may be attributed to prevalence of whitefly vector, warm tropical climate supporting year round survival of the whitefly, intensive cultivation of crops and polyphagous nature of the whitefly serving path of sustenance of begomovirus in alternative hosts (Saha *et al.,* 2014). The threat of begomoviral spread to the eastern part of India has been taken into consideration and this may be correlated to different factors like weather condition, tomato livestock import-export and agricultural practices that are operational in the study area. In the present communication, on the basis of sequence analysis of the coat protein gene, the genetic relatedness of the begomoviruses infecting cucumber was inferred.

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