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Molecular Detection and Characterization of Tomato Leaf Curl New Delhi Virus Causing Mosaic Disease on Bitter Gourd in Tamil Nadu, India

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ABSTRACT

Bitter gourd plants showing symptoms of mosaic, mild leaf curl with blistering from major bitter gourd areas of Tamil Nadu were found to be infected with *Begomovirus* by PCR using universal *Begomovirus* degenerate primer (Deng 540/541). From the blast result, it is identified as *Tomato leaf curl New Delhi virus* (ToLCNDV). Coat protein region of the begomovirus infected four samples collected from Coimbatore and Pollachi area were amplified using new primer pair (GK ToLCV F/R) shared a nucleotide and amino acid identity of more than 98% towards the ToLCNDV from Asia (India and Pakistan) reported from cucurbits. From the phylogenetic analysis it is understood that, the *Begomovirus* infecting bitter gourd in Tamil Nadu is ToLCNDV since they are clustered with the ToLCNDV and were well supported on the branches of the Begomoviruses infecting cucurbits in India. This is the confirmed molecular evidence for the occurrence of *Tomato leaf curl NewDelhi virus* on bitter gourd in Tamil Nadu.

Key words *Bitter gourd; Cucurbits; Begomovirus; Virus disease; Mosaic*

Bitter gourd (*Momordica charantia* L.) is a cucurbitaceous vegetable which is also known as bitter melon is grown extensively in India and throughout south-east Asia. It is considered as one of the major vegetable crop in Tamil Nadu and grown in an area of about 1122 ha. Major bitter gourd growing belt in Tamil Nadu are Coimbatore, Salem, Dharmapuri, Dindigul, Tiruppur and Kanyakumari. Besides it is consumed as food for its excellent source of minerals, vitamins, dietary fibers and antioxidant, it is also having medicinal properties. It is being used in treating the diabetic patient, also it is known to possess antiviral, antimalarial and immune booster activity. It is highly beneficial in treating blood disorders, liver disorders, eye problems, alcohol detoxification, piles, psoriasis and respiratory disorders (Anonymous, 2014).

Naturally, bitter gourd has been reported to be infected by many viruses, which affects its cultivation. RNA viruses *viz*., *Cucumber mosaic virus* (Takami *et al.,* 2006), *Papaya ring spot virus* (Chin, and Ahmad, 2007), *Zucchini yellow mosaic virus* (Fukumotu, *et al.,* 1993), *Zucchin tigre mosaic virus* (Romay, *et al*., 2014) and *Watermelon silver mottle virus* (Tokashiki, and Yasuda, 1991) are found to infect the bitter gourd. Similarly viruses *viz*., *Bitter gourd yellow mosaic virus* (Rajinimala, *et al.,* 2005), *Indian cassava mosaic virus* (Rajinimala, and Rabindran, 2007), *Pepper leaf curl Bangladesh virus* (Raj, *et al.,* 2010), *Squash vein yellowing virus* and *Cucurbit leaf crumple virus* (Adkin, *et al.,* 2008) and *Tomato leaf curl New Delhi virus* (Tiwari, *et al*., 2010) are the begomoviruses infecting bitter gourd has been reported. During the survey conducted in the year 2012-14, a severe mosaic disease was observed in Coimbatore, a major bitter gourd growing area of Tamil Nadu, India. The disease incidence was significant and symptoms consisting of severe mosaic accompanied with blistering and slight curling on the leaves were observed. The infected plants had small and lesser fruits compared to the healthy ones. With this background, an attempt had been made to find out the virus associated with the mosaic disease of bitter gourd in Tamil Nadu from the samples collected from Coimbatore region.

MATERIALS AND METHODS

Survey and Collection of plant samples

Surveys were conducted during 2012-2014 in different bitter gourd growing regions of Coimbatore to study the virus diseases associated with bitter gourd. During our survey, bitter gourd samples from Thondamuthur and Pollachi regions of Tamil Nadu showing mosaic, blistering and mild leaf curl were collected along with healthy plant samples and brought to the laboratory in the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore for molecular analysis of the associated virus with the

Table 1. Percentage identity of nucleotide and amino acid sequences of CP gene encoded by ToLCNDV of Bitter gourd in Tamil Nadu with those other selected *Begomovirus* for the study

Accession	Virus	TN THON BG 1		TN POL BG 1		TN POL BG 2		TN POL BG 4	
		nt	aa	nt	aa	nt	aa	nt	aa
FN645905	*Tomato leaf curl New Delhi virus* [Bottle gourd: 4-RCA-AI-F]	98.3	100.0	97.6	98.8	98.5	100.0	97.6	98.8
AM747291	*Tomato leaf curl New Delhi virus* [Bittergourd: Mn-05]	98.4	100.0	97.5	98.8	98.3	100.0	97.5	98.8
KC513822	*Tomato leaf curl New Delhi virus* [Poppy: Lucknow]	97.1	99.6	96.4	98.4	97.1	99.6	96.2	98.4
KJ862841	*Bitter gourd yellow vein virus* [BD12C8]	97.5	98.8	96.7	97.6	97.4	98.8	96.8	97.6
KM190927	*Bitter gourd yellow vein virus* [Kanpur-Lentil]	97.0	99.6	96.3	98.4	97.2	99.6	96.3	98.4
AM491590	*Bitter gourd yellow vein virus* [Pakistan:Lahore: BGAL4]	96.6	98.4	96.1	97.2	97.0	98.4	95.9	97.2
AY396151	*Squash leaf curl China virus* [Pumpkin: Lucknow]	96.4	99.6	96.3	98.4	96.6	99.6	95.8	98.4
DQ026296	*Squash leaf curl China virus* [Pumpkin: Lucknow]	96.4	99.6	96.3	98.4	96.6	99.6	95.8	98.4
AM286794	*Squash leaf curl China virus* [*Cucurbita pepo*: Lahore: CPoAL4]	95.9	98.8	95.8	97.6	96.1	98.8	95.3	97.6
JN587811	*Squash leaf curl China virus* -[Pum:IARI]	95.8	97.6	95.3	96.4	95.7	97.6	95.2	96.4
KC171648	*Squash leaf curl China virus* [GZ01]	89.2	95.7	88.9	94.9	89.1	95.7	88.5	94.9
EU543562	*Squash leaf curl China virus* [Wax Gourd:Nakhon Pathom]	89.6	92.6	89.3	91.8	89.5	92.6	88.9	91.8
AM260206	*Squash leaf curl China virus*[G25]	89.3	96.4	88.9	95.7	88.9	96.4	88.4	95.7
AY686500	*Pumpkin yellow vein mosaic virus*	96.3	98.8	96.1	97.6	96.7	98.8	95.7	97.6
FJ931537	*Tomato leaf curl Palampur virus* [Varanasi]	84.9	91.0	84.9	90.6	84.5	91.0	84.4	90.6
KF663700	*Tomato leaf curl Palampur virus* [TC238]	84.8	90.6	84.8	90.2	84.9	90.6	84.3	90.2
KC904968	*Tomato leaf curl Palampur virus* [Lahore]	84.6	90.2	84.6	89.8	84.8	90.2	84.1	89.8
GQ225738	*Tomato leaf curl Palampur virus* [Var:Pum:08:1]	84.6	90.6	84.3	89.4	84.5	90.6	83.7	89.4
JQ911767	*Ageratum enation virus* [*Ageratum* sp.]	80.6	92.6	80.1	91.8	80.6	92.6	79.7	91.8
GQ268327	*Ageratum enation virus* [Gorakhpur]	80.6	92.2	80.1	91.4	80.6	92.2	79.7	91.4
GU170806	*Tomato leaf curl virus* [To-KLR.1]	78.0	87.9	77.8	87.1	78.0	87.9	77.5	87.1
KC713784	*Tomato leaf curl virus* [MPT :Coimbatore]	76.4	81.3	75.7	80.5	76.4	81.3	75.5	80.5
JX547015	*Tomato leaf curl virus* [Antiyur:Erode]	75.1	78.2	74.6	77.4	75.1	78.2	74.2	77.4
EF197941	*Pumpkin yellow mosaic virus* [MP1]	90.2	94.1	90.0	93.3	90.0	94.1	89.6	93.3
EF035481	*Pepper leaf curl Bangladesh virus*	81.1	92.6	80.6	91.8	81.1	92.6	80.2	91.8
AF314531	*Pepper leaf curl Bangladesh virus*	81.1	93.3	80.6	92.6	81.1	93.3	80.2	92.6
DQ116881	*Pepper leaf curl Bangladesh virus*	78.9	91.0	78.8	90.2	79.7	91.0	78.4	90.2
AF423180	*Indian cassava mosaic virus*-[Tri]	78.2	89.8	77.9	89.1	78.2	89.8	77.3	89.1
AJ575819	*Indian cassava mosaic virus* [Ker2: Adivaram 2]	78.2	90.2	77.9	89.4	78.2	90.2	77.3	89.4
AY998122	*Indian cassava mosaic virus* [Kolli hills]	78.2	89.4	77.9	88.7	78.2	89.4	77.3	88.7
KM269359	TN THON BG 1	100.0	100.0	98.7	98.8	99.0	100.0	98.8	98.8
KM269360	TN POL BG 1	98.7	98.8	100.0	100.0	98.7	98.8	98.7	98.8
KM269361	TN POL BG 2	99.0	100.0	98.7	98.8	100.0	100.0	98.4	98.8
KM269362	TN POL BG 4	98.8	98.8	98.7	98.8	98.4	98.8	100.0	100.0

nt – Nucleotide aa – amino acid

symptoms. The samples were stored at -80°C for further studies. Disease incidences were estimated by recording symptomatic and non-symptomatic plants in the field randomly at ten different locations with one square meter each (Sohrab, *et al*., 2010).

Total DNA extraction and PCR amplification

The total DNA was extracted from 100 mg of the infected leaf tissue as well as healthy leaf samples using CTAB method described by Doyle and Doyle (1990). The genomic DNA quality and quantity was checked on 0.8% agarose gel and stored at -20°C for further use. For preliminary screening, PCR amplification has been carried out with *Begomovirus* specific degenerate primer pair of Deng 541: 5' TAAT ATTACCKGWKGVCCSC 3' Deng 540: 5' TGGACYTTRCAWGGBCCTTCACA 3' to amplify a part of the movement protein and coat protein (CP) genes of DNA-A (Deng, *et al*., 1994).

PCR was carried out with the master mix (Smart Prime, India) in 50µl reaction volume containing 25 µl master mix, 5µl (10 pmole) each of forward and reverse primers and 5µl (50ng) of template DNA. Amplified PCR products were cloned in pGEMT vector (Promega, USA) and sequenced. With the help of the resultant sequences, a new degenerate primer pair GKToLCV F: ATGKYGAAGC GACCAGCMGA; GKToLCV R: CGCCCKCMGAYTGGGMTTTTCTT were designed with the help of *Begomovirus* sequences available in the NCBI database in order to amplify the entire coat protein gene. PCR was made with the following conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 90 sec. Resultant product has been cloned, sequenced and submitted in the NCBI database.

Sequence comparison and Phylogenetic analysis

The sequences of virus isolates under this study were compared with the sequences of selected begomoviruses obtained from the GenBank database (Table-1). Multiple sequence alignment was done using Clustal W (www.ebi.ac.uk) followed by phylogenetic analysis using MEGA 6.0 (www.megasoftware.net) and constructed with the neighbor-joining algorithm, bootstrapped with 1000 replicates. Similarity percentage were calculated for the viruses under this study with other genbank accessions using BIO-EDIT.

RESULTS AND DISCUSSION

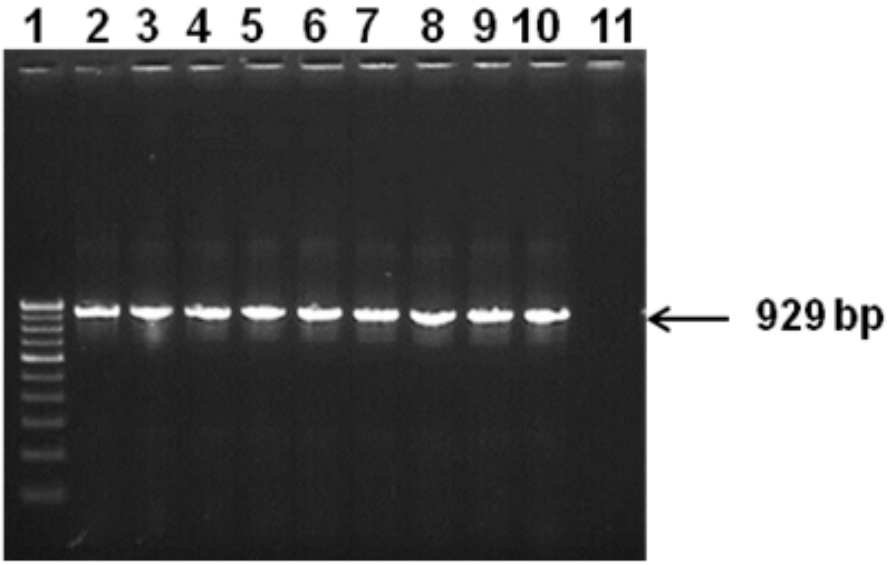
During our survey in different bitter gourd growing areas of Coimbatore in the present study, symptoms such as mosaic, slight leaf curl with blistering (Fig. 1) were recorded on bitter gourd plants under field conditions with a disease incidence ranging from 65-80%. Virus incidence on bitter gourd plants ultimately leads to less number of fruits with reduced fruit size compared to healthy plants.

Detection and Characterization of virus

DNA extracted from the symptomatic and non-symptomatic leaves of bitter gourd were subjected to PCR for preliminary screening for the presence of *Begomovirus* using Deng 540/541 primer pairs. Symptomatic leaves showed an amplification of ~510bp

a. Mosaic with slight leaf curl of bitter gourd leaves b. Blistering on bitter gourd leaves

Fig. 1. Symptoms of ToLCNDV on bitter gourd in Tamil Nadu



1 – 100bp Marker 2,3 – TN THON BG 1 4,5 – TN POL BG 1
6,7 – TN POL BG 2 8,9 – TN POL BG 4 10 – Positive control
11 – Healthy control

Fig. 2. Gel photograph showing amplification of ToLCNDV using specific primer pairs GK ToLCV F/R

but not in non-symptomatic leaves which confirms the presence of *Begomovirus* associated with the mosaic diseases of bottle gourd. Amplified products were cloned in pGEMT vector. Two clones per ligation were sequenced and the clones shared 99.6% nucleotide sequence identity between them and sequences were analyzed. In a BLAST search, sequence shares 98 % similarity towards ToLCNDV.

With the help of the sequences in NCBI database having maximum similarity towards the virus isolates of the present study new set of primers (GKToLCV F/R) were designed in such a way that to cover the complete coat protein gene (771nt) of ToLCNDV and SLCCNV to a amplification size of 929 bp. Amplifed product were cloned and sequenced (Fig. 2).

BLASTn analysis of nucleotide sequence data of the complete coat protein gene of our ToLCNDV isolates TN THON BG1 (KM269359), TN POL BG1 (KM269360), TN POL BG2 (KM269361) and TN POL BG4 (KM269362) revealed a maximum identity of 98% with ToLCNDV isolates FN645905, AM747291 and KC513822 reported from bottle gourd, bitter gourd and poppy respectively from Asia (India and Pakistan). In the nucleotide analysis, isolate from Coimbatore shared a maximum identity of 98.4 % towards the ToLCNDV (AM747291) of Pakistan and remaining all the three isolates from Pollachi shared a maximum identity of 97.6-98.5 towards the ToLCNDV (FN645905) from India on bottle gourd. Further nucleotide alignment analysis revealed 98.8 – 100% identity towards ToLCNDV (FN645905 and AM747291) at amino acid level followed by *Bitter gourd yellow vein virus* (BGYVV). All these isolates shared 98.4 to 99 % identity among themselves at nucleotide and amino acid level. Phylogenetic analyses were done using the Molecular Evolutionary Genetics Analysis tool with 1000 replicates bootstrapping and a dendrogram was generated with the Neighbour joining method and viewed by the NJ plot program. NCBI GenBank accession numbers of different begomoviruses infecting cucurbits in India were used for coat protein (CP) sequence comparisons and phylogenetic analysis. The phylogenetic analysis of nucleotide sequence of ToLCNDV infecting bitter gourd in Tamil Nadu formed a separate cluster with ToLCNDV isolates from Asia (India and Pakistan) along with BGYVV (Fig. 3).

Bitter gourd is an economically important vegetable crop in Indian subcontinent. The cultivation of bitter gourd becomes cumbersome due to the virus incidence in Tamil Nadu. In this study, bitter gourd samples were collected from the major bitter gourd growing areas of Tamil Nadu (Coimbatore and Pollachi) showing mosaic, slight leaf curl and blistering of leaves. In PCR investigation, samples showing symptoms were positive

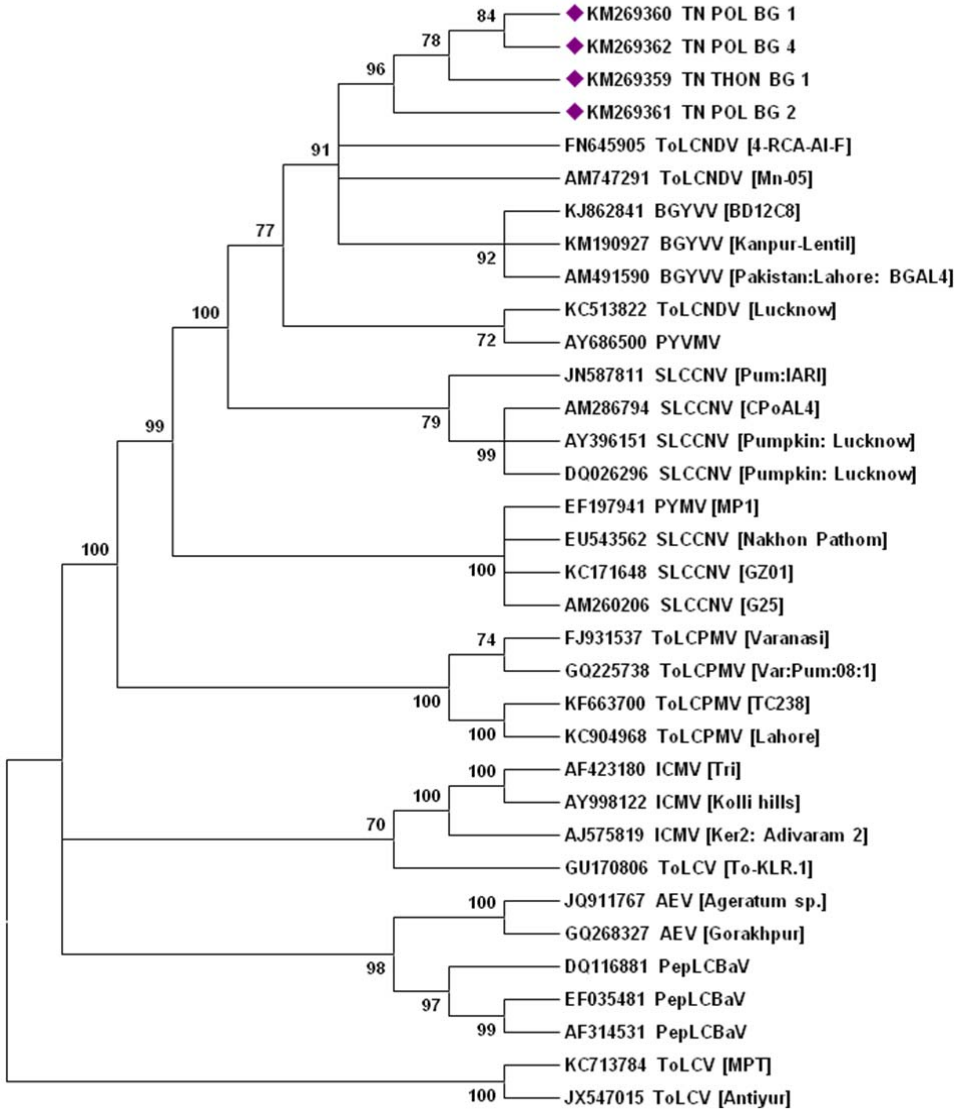


Fig. 3. Phylogeny of complete nucleotide sequence of CP region of ToLCNDV infecting bitter gourd in Tamil NAdu with other *Begomovirus* isolates. The tree was generated using the Neighbour-joining method in MEGA 6. A bootstrap analysis with 1000 replicates was performed and bootstrap percent values more than 70 are numbered along branches.

for the preliminary screening of the association of begomovirus with the degenerate primer. Similarly, Saha, *et al.* (2014) confirmed the association of begomovirus with the tomato leaf curl disease on tomato in the samples collected from Brahmaputra valley of Assam.

Since the coat protein genes of the begomoviruses are most conserved, for the demarcation of begomoviruses at species level, differences in coat protein have been used (Brown, *et al*., 2001; John, *et al*., 2006; Santoso, *et al*., 2008; Samad, *et al*., 2009; Haider, *et al*., 2007). Hence, sequence analysis of the complete coat protein amplified by using the new set of primers obtained 98% and 100% identity at nucleotide and aminoacid levels towards ToLCNDV. 1-1.2% variation was found among the sequences in location wise comparison. Based on the highest sequence identity and close phylogenetic relationship towards the ToLCNDV, the isolates of begomovirus infecting bitter gourd under this study belongs to ToLCNDV

(Fauquet, *et al.*, 2008). In this study, sequence information of CP gene provides sufficient information about the association of ToLCNDV with the mosaic disease of bitter gourd in Tamil Nadu. To the best of our knowledge, this is the first molecular characterization of ToLCNDV associated with mosaic

disease of bitter gourd in Tamil Nadu.

Association of different begomoviruses *viz*., *Bitter gourd yellow mosaic virus* (Rajinimala, *et al.,* 2005), *Indian cassava mosaic virus* (Rajinimala and Rabindran, 2007), *Pepper leaf curl Bangladesh virus* (Raj, *et al.,* 2010) and *Tomato leaf curl New Delhi virus* (Tiwari, *et al*., 2010) were reported from different parts of India on bitter gourd. Initially ToLCNDV was found to be infecting only solanaceous vegetables, but now it is an emerging problem for many agricultural and horticultural crops in India and neighboring countries (Hussain, *et al.,* 2005; Maruthi, *et al.,* 2005; Ito *et al.,* 2008). In addition, ToLCNDV is found to infect many weeds *viz*., *Eclipta prostrata* (Haider, *et al.,* 2006) and *Croton bonplandianum* (Reddy, *et al.,* 2005) suggested that these weed species harbour ToLCNDV near the vicinity of the cropping area of economically important crops. Moreover the cultivation of solanaceous vegetables *viz*., tomato, chilli throughout the year and monocropping of the cucurbitaceous vegetables made the crop host availability for the survival, perpetuation and dissemination of ToLCNDV throughout the year.

ToLCNDV is being transmitted by whitefly in semipersistent manner, the high disease incidence may be due to prevalence of whitefly vector with its polyphagous nature, increase of global temperature and intensive crop cultivation made the virus availability in the cropping area throughout the year on their alternative hosts. Since precise detection of causal agent is an important step in devising management strategies, present work will be a preliminary step for developing the integrated management strategies for the control of virus disease on bitter gourd.

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