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Research Article

MOLECULAR DETECTION AND IDENTIFICATION OF TOMATO LEAF CURL NEW DELHI VIRUS ASSOCIATED WITH YELLOW MOSAIC DISEASE OF RIDGE GOURD (*Luffa accutangula* L.)BASED ON COAT PROTEIN GENE

HURAKADLI MANJUNATH S.*, RANGASWAMY K.T., BASAVARAJ S., BHAGYASHREE M., NAGARAJU N. AND PRAMEELA H.A.

Department of Plant pathology, University of Agricultural Sciences, GKVK, Bengaluru, Karnataka 560065, India
*Corresponding Author: Email-mshurakadli@gmail.com

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Abstract- A severe yellow mosaic disease on ridge gourd with a significant disease incidence was observed during the survey at different locations of southern dry zone of Karnataka in the year 2013-2014. The disease consisted of yellow mosaic accompanied with slight curling of leaves and the whitefly (*Bemisia tabaci*) population was also observed in the field during survey. The characteristic yellow mosaic disease symptoms and whitefly population indicated the possibility of begomovirus infection. PCR was carried out using the total DNA isolated from infected leaf samples and a pair of begomovirus specific primers which resulted in the expected size (~750 bp) amplicon indicated the presence of begomovirus. For further identification of the begomovirus, the PCR amplicons were cloned and sequenced. The sequence data analysis revealed highest of 93-92% similarities with several isolates of *Tomato leaf curl New Delhi virus* (*ToLCNDV*) at nucleotide levels. The phylogenetic analysis also showed closest relationships of the causal virus with various variants of *ToLCNDV*. Based on highest sequence similarities and closest relationships with *ToLCNDV*, the virus isolated from ridge gourd was considered as an isolate of *Tomato leaf curl New Delhi virus*.

Keywords- *Tomato leaf curl New Delhi virus* (*ToLCNDV*), Yellow mosaic disease, Ridge gourd, Coat protein gene.

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Introduction

Ridge gourd (*Luffa acutangula* L. Roxb) is one of the most important vegetable crop cultivated throughout India. It belongs to *Cucurbitaceae* family and is popularly known as angled gourdor Kalitori. The word "Luffa" is derived from Arabic word, which means the spongy characteristics of the mature fruit [1]. Ridge gourd is cultivated in the tropics and subtropics of the world for its tender edible fruits bothon commercial scale and in kitchen gardens. It is a popular vegetable both as spring summer and rainy season crop [2]. It is cultivated in India, Philippines, Myanmar, Indonesia, Taiwan, Srilanka and Malaysia.

Cucurbits suffers from many fungal, bacterial and viral diseases. Among viral diseases, yellow mosaic begomovirus has been considered as an important limiting factor in cucurbits productivity. During the survey in the year 2013-14, a severe yellow mosaic disease was observed on ridge gourd in different locations of southern Karnataka, India. The incidence of disease was significant and symptoms consisted of severe yellow mosaic accompanied with slight curling on leaves and infected plants had small and lesser fruits as compared to healthy ones. Losses due to yellow mosaic disease ranged from 4.7 to 36% per cent depending on the cultivar susceptibility and time of infection. The disease incidence sometimes reaches 100 per cent [3]. Characteristic symptoms of cucurbits yellow mosaic disease includes leaf curling, yellow spot on the newly emerged leaves, chlorosis and mosaic, vein banding and severe mosaic mottling [4].Begomovirus has emerged as serious problem in several cucurbits since 1980s. So far, only two different begomo virus species *Squash leaf curl China virus* (*SLCCNV*) and *ToLCNDV* are known to affect cucurbits in India.

The begomovirus diseases have been detected in cucurbitaceous crops, such as bottle gourd, bitter gourd, cucumber, ivy gourd, musk melon sponge gourd, pumpkin and water melon through PCR methods using primers of putative CP gene of Tomato leaf curl New Delhi Virus [5-8].

The yellow mosaic disease of ridge gourd was found to be caused by *Tomato leaf curl* New Delhi virus *(ToLCNDV). Tomato leaf curl New Delhi virus* (*ToLCNDV*) (genus Begomovirus, family Geminivirdae) is the most important viral pathogen in tomato in India [9]. *ToLCNDV* a bipartite begomovirus containing DNA-A and DNA-B. The DNA A encodes for the coat protein (CP), replication initiation protein (Rep),replication enhancer (REn), and transcriptional activator protein (TrAP), whereas DNA-B encodes for intra- and intercellular movement proteins which play a role in translocation of the virus particles. *ToLCNDV* is predominantly transmitted by whitefly (*Bemisia tabaci*) in the tropical and subtropical regions of the world, causing severe disease and leads to enormous yield loss in dicot plants including pepper, tomato, cassava, beans, cotton and cucurbits.

All begomo viruses encode a coat protein (CP) in which packages, all the genomic and satellite molecules are present. The CP acts as the coat of the virus particles and is essential for virus transmission from diseased to healthy plants by *B. tabaci.* The CP is highly conserved amongst the begomo viruses originating from the same geographical region and thus it has been adapted to transmission by local vector population [10]. The CP is therefore an essential component of begomovirus survival and has been used widely to characterize and establish the relationships of many begomo viruses [11].

Here, we report the association of *Tomato leaf curl New Delhi virus* (*ToLCNDV*) with yellow mosaic disease of ridge gourd based on PCR detection and

characterization of the causal virus using the begomovirus coat protein (CP) gene specific primers and sequence analysis of ~750bp amplicon obtained from infected samples of *Luffa accutangula* collected from southern districts of Karnataka. Phylogenetic analysis also reveals that yellow mosaic disease causing virus in ridge gourd is an isolate of *Tomato leaf curl New Delhi virus.* To the best of our knowledge, this is the first record of a ToLCNDV variant infecting ridge gourd and causing enormous yield loss to the crop in Karnataka, India.

Materials and Methods

Survey and Collection of Plant samples

Surveys were carried out during the 2013-2014 to know the per cent disease incidence of yellow mosaic disease of ridge gourd in Southern Karnataka, India; The disease was diagnosed in the field based on symptoms exhibited on plants. The per cent disease incidence was assessed by recording the number of plants showing disease symptoms, out of the total number of plants examined by using the formula mentioned below [12]. Diseased leaf samples of ridge gourd plants showing typical symptoms of begomovirus infection were collected during survey. The leaves from the top of the plants that showing yellow mosaic symptoms were selected for sampling. The samples were put in plastic bags, labelled and stored in -80^{0}C at virology lab, UAS, Bangalore. These samples were used for total DNA extraction and screened for the presence of begomovirus using begomovirus specific primers [13].

Total DNA extraction

The total genomic DNA was extracted from leaf tissues of healthy plants and YMV infected ridge gourd plants by C-TAB method. Infected plant tisue (150 mg) was ground in a pre-sterilized pestle and mortar using C-TAB buffer(100 mMTris (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% CTAB and 0.2% Mercaptoethanal) and transferred to sterile eppendorf tube and incubated for 1 hour in water bath at 65ºC. The supernatant was collected into eppendorf tubes. To this added equal volumes of chloroform and Isoamyl alcohol (24:1) and mixed by vertexing. Then the tubes were centrifuged at 13,000 rpm for 10 min. The supernatant was collected and the DNA was precipitated by mixing with 0.1 volume of 7.5M ammonium acetate and 0.6 volume of chilled isopropanol then incubated at -20ºC for overnight. After incubation, the tubes were taken out and centrifuged at 13,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol and again centrifuged at 13,000 rpm for 10 min, the supernatant was discarded, vaccum dried for 10 min and re-suspended in 50 µl $T_{10}E_{0.1}$ buffer.

Amplification of viral DNA by Polymerase Chain Reaction (PCR)

PCR was performed using a pair of begomovirus specific primers. The forward and reverse primers were: Deng A 5' TAATATTACCKGWKGVCCSC -3' and Deng B 5'-TGGACYTTRCAWGGBCCTTCACA -3', respectively [13]. And also pair of primers designed from the coat protein gene region of a well characterized begomovirus, such as; P1F-5'-ATGGCGAAGCGACCAGC-3' and P1R-5'-TTAATT TGTTACGCAA TCATA -3'[14]. PCR was performed in 25 µl of reaction mixture using 12.5 µl Fermentas 2x master mix, 0.5 µl of 3 U/ µl of Taq DNA polymerase, 2 µl of each primers, 4 µl of DNA template and finally volume was made with sterile distilled water. The conditions for amplification are; 1 cycle of 94°C for 2 min , 35 cycles of 94°C for 1 min, 55°C for 2 min ,72°C for 3 min and 1 cycle of 72°C for 10 min [14]. The amplified PCR products were separated on 1% agarose gel in 1x TBE buffer. The banding pattern was documented in gel documentation system.

Elution, sequencing and phylogenetic analysis

DNA from agarose gel in TBE buffer was extracted and purified by using QIA quick gel extraction kit (Cat. No. 28704; Qiagen, Germany) according to the instructions given by the manufacturer. Eluted product was sent to the National Centre for Biological Sciences (NCBS), Bengaluru, for sequencing by primer walking method. Obtained sequences were aligned and joined together to get full length sequence using 'nucleotide blast' at basic blast programmes and 'align two (or more) sequences' at specialized blast programmes freely assessing 'Basic Local Alignment Search Tool (BLAST)' at the National Centre for Biotechnology Information (NCBI)(http://www.ncbi.nlm.nih.gov/). Sequences of other begomo viruses used for comparison were taken from the NCBI database [Table-2]. The phylogenetic neighbor-joining trees and evolutionary analysis were conducted using MEGA 6.06 software package [15]. Robustness of trees was determined by bootstrap sampling of multiple sequence alignment with 1000 replications.

Results and Discussion

Disease incidence and Natural symptoms

Survey carried out from 2012-2013 in southern dry tract of Karnataka revealed that occurrence of the disease on ridge gourd range between 37.5 to 86.0 per cent. There are many reports on begom viruses infecting wide range of cucurbits from different parts of the country. They are likely to be a major threat to the cucurbits production in the future. The present study confirmed with the occurrence of begomovirus on bottle gourd in Delhi and Haryana varied from 4.7 to 36% reported by Sohrab, et al. [3], on bitter gourd in Gorakhpur up to 20% reported by Tiwari, et al. [16]. The differences in incidence of disease in areas surveyed might be due to variation in the source of inoculum, vector population, crop and weather condition. High level of incidence at certain locations might be due to the abundant source of inoculum. Results of this study has epidemiological significance for monitoring and management of the disease.

During the course of survey in different parts of southern Karnataka in the present study, a variety of symptoms were recorded on *Luffa acutangula* plants under field conditions. The most characteristic symptoms in the early stage of the disease were yellow mosaic followed by curling of leaves [Fig-1]. However, in case of severity of the disease, yellow mosaic with puckering, extensive chlorosis, stunting of plants was and malformation of fruits was also noticed. Similar observations were obtained by Tiwari, et al. [4], typical yellow mosaic and leaf curling symptoms on sponge gourd and bitter gourd observed during survey at Gorakhpur, Uttar Pradesh.

PCR amplification of Coat protein gene and their sequence identities

Polymerase chain reaction technique was employed to confirm the association of *Begomovirus* with yellow mosaic disease through amplification of PCR product approximately 560 bp CP gene fragments using Begomovirus group specific universal primers [13]. A band of approximately 560 bp corresponding to viral coat protein was consistently amplified from total DNA template extracted from infected ridge gourd plants [Fig-2]. No such virus specific products were obtained with DNA template extracted from healthy leaf material.This indicates that the causal agent of ridge gourd yellow mosaic disease is a begomovirus.

To identify the begomovirus responsible for causing the yellow mosaic disease of ridge gourd, PCR was carried out with virus specific complete coat protein primers [14].The PCR product of 760 bp corresponding to coat protein (CP) gene was amplified from YMV infected ridge gourd plants which was absent in healthy plants [Fig-3]. The amplified product was eluted and sequenced.

The sequences obtained were subjected to BLAST of NCBI. BLAST search analysis of the sequence from ridge gourd virus isolate showed 93% nucleotide identity with *Tomato Leaf Curl New Delhi Virus* (*ToLCNDV*)-isolate TC-309 (KF551576.1) and *ToLCNDV- PkT* (AF448058.2), 92% nucleotide identity with *ToLCNDV-Bottlegourd* (DQ272540.2), *ToLCNDV-Severe strain* (U15015.2), *ToLCNDV- India: UP: Bahraich: Luffa cylindrica* (KC207815.1), *ToLCNDV-Mild strain* (U15016.1), *ToLCNDV-AVT1* (AY428769.1), *ToLCNDV- Murcia* (KF749224.1), *ToLCNDV- Pumpkin: New Delhi* (AM286434.1), *ToLCNDV-Almeria 661* (KF749223.1), *ToLCNDV- Spain-Almeria* (KF891468.1), *ToLCNDV-Chilli* (GU831540.1), and *ToLCNDV- India :Delhi:Cucumis*n (KC545812.1), 91% nucleotide identity with *ToLCNDV-Luffa* (AF102276.1) and potato isolate of *ToLCNDV* (DQ272541) [Table-1]. Based on highest sequence similarities with *ToLCNDV*, the virus isolated from ridge gourd was considered as an isolate of *Tomato leaf curl New Delhi virus*.

The present investigation compared with Sohrab, *et al*. [17] who reported the ToLCNDV, which is associated with yellow mosaic disease of luffa and pumpkin. The PCR amplified products of coat protein gene (750 bp) from Luffa and pumpkin

isolates were separately cloned and sequenced. The BLAST result showed highest homology with *Tomato leaf curl New Delhi virus* and designated as ToLCNDV-Luffa. Comparison of the nucleotides sequence of Luffa: Del isolate (AY309957) with selected begomo viruses showed 96.1% nucleotide identity with ToLCNDV-Luffa (AF102276) followed by 95.1% identity with ToLCNDV-[Svr] (U15015) and 87.7% with SqLCV-[Pum:Del] (AY686500) was observed.

Fig-1 Different types of symptoms of YMV on ridge gourd
Development of yellow spots on leaves (A),Yellow mosaic with green and yellow patches(B),Yellow mottling (C),Downward leaf curling (D),Complete chlorosis of leaves with severe yellow mosaic symptoms(E), Mishapen fruits(F).

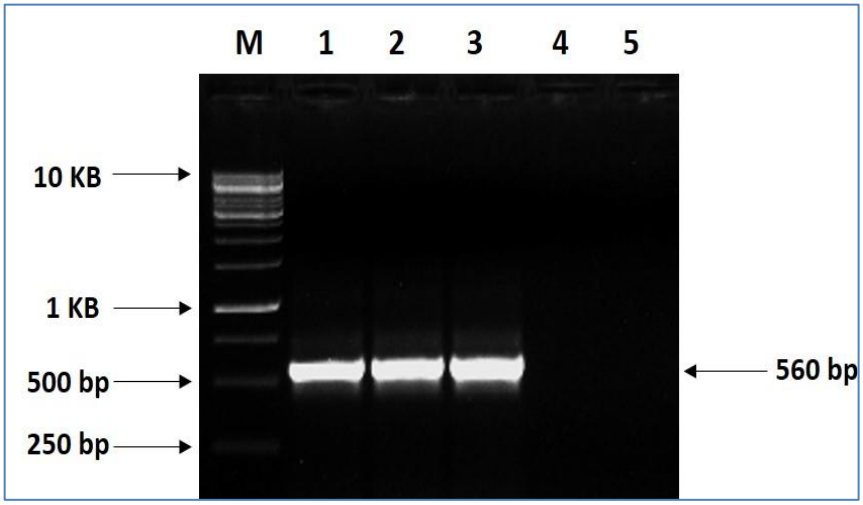

Fig-2 PCR detection of YMV using begomovirus group specific primers. M =1kb Marker , 1, 2, 3 = YMV infected Ridge gourd, 4 = Healthy Ridge gourd, 5= Water control

Table-1 *Nucleotide sequence identity of yellow mosaic virus- ridge gourd isolate with other begomoviruses by BLAST analysis.*

Homology with the nucleotide sequence in GenBank	Abbreviation	% Homology	Accession Number
Tomato leaf curl New Delhi virus isolate TC309	***ToLCNDV*-TC309**	93	KF551576.1
Tomato leaf curl New Delhi virus - [PkT5/6] segment DNA-A,	***ToLCNDV- PkT***	93	AF448058.2
Tomato leaf curl New Delhi virus from bottle gourd	***ToLCNDV-Bottlegourd***	92	DQ272540.2
Tomato leaf curl New Delhi virus-Severe	***ToLCNDV-Sev***	92	U15015.2
Tomato leaf curl New Delhi virus : India: UP: Bahraich: Luffa cylindrica	***ToLCNDV-*** India: UP: Bahraich: Luffa cylindrica	92	KC207815.1
Tomato leaf curl New Delhi virus-Mild	***ToLCNDV-Mild***	92	U15016.1
Tomato leaf curl New Delhi virus isolate ToLCNDV-AVT1	***ToLCNDV-AVT1***	92	AY428769.1
Tomato leaf curl New Delhi virus - Murcia 8.1 segment DNA-A,	***ToLCNDV-Murcia***	92	KF749224.1
Tomato leaf curl New Delhi virus-[Pumpkin: New Delhi]	***ToLCNDV-*** Pumpkin: New Delhi	92	AM286434.1
Pumpkin yellow vein mosaic virus coat protein	***PYMV-CP***	91	AY686500.1
Tomato leaf curl New Delhi virus isolate Almeria 661	***ToLCNDV-*** Almeria 661	92	KF749223.1
Tomato leaf curl New Delhi virus isolate ToLCNDV-Spain-Almeria	***ToLCNDV-*** Spain-Almeria	92	KF891468.1
Tomato leaf curl New Delhi virus-Chilli pepper isolate DHO5 coat protein gene, complete cds	***ToLCNDV-Chilli***	92	GU831540.1
Tomato leaf curl New Delhi virus AV1 gene :coat protein, isolate 26	***ToLCNDV-isolate_26***	92	AJ810365.1
Tomato leaf curl New Delhi virus :India :Delhi:Cucumis: DNA-A,	***ToLCNDV-India :Delhi:Cucumis***	92	KC545812.1
Tomato leaf curl New Delhi virus-[Luffa] complete cds	***ToLCNDV-Luffa***	91	AF102276.1
Tomato leaf curl New Delhi virus from potato	***ToLCNDV-Potato***	91	DQ272541.

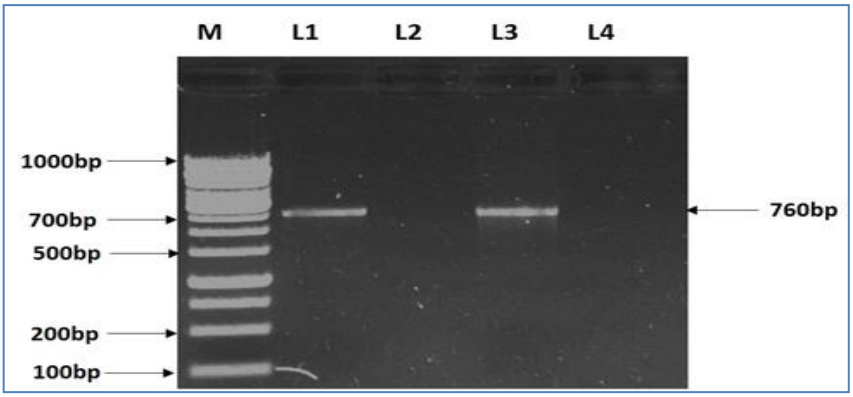

Fig-3 PCR Amplification of coat protein gene of yellow mosaic begomo-virus infecting Ridge gourd. M =Marker, L L1 & L3 - YMV infected ridge gourd, L2 - Healthy ridge gourd, L4 - Water control

Table-2 *List of begomoviruses used in the phylogenetic analysis for comparision with yellow mosaic begomovirus coat protein gene sequences*

Geminivirus species used	Abbreviation	Gene bank accession No
Tomato leaf curl New Delhi virus isolate TC309	*ToLCNDV*-TC309	KF551576.1
Tomato leaf curl New Delhi virus - [PkT5/6] segment DNA-A,	*ToLCNDV- PkT*	AF448058.2
Tomato leaf curl New Delhi virus from bottle gourd	*ToLCNDV-Bottlegourd*	DQ272540.2
Tomato leaf curl New Delhi virus-Severe	*ToLCNDV-Sev*	U15015.2
Tomato leaf curl New Delhi virus : India: UP: Bahraich: Luffa cylindrica	*ToLCNDV-* India: UP: Bahraich: Luffa cylindrica	KC207815.1
Tomato leaf curl New Delhi virus-Mild	*ToLCNDV-Mild*	U15016.1
Tomato leaf curl New Delhi virus isolate -AVT1	*ToLCNDV-AVT1*	AY428769.1
Tomato leaf curl New Delhi virus - Murcia 8.1 segment DNA-A,	*ToLCNDV-Murcia*	KF749224.1
Tomato leaf curl New Delhi virus-[Pumpkin: New Delhi]	*ToLCNDV-* Pumpkin: New Delhi	AM286434.1
Pumpkin yellow vein mosaic virus coat protein	*PYMV-CP*	AY686500.1
Tomato leaf curl New Delhi virus isolate Almeria 661	*ToLCNDV-* Almeria 661	KF749223.1
Tomato leaf curl New Delhi virus isolate ToLCNDV-Spain-Almeria	*ToLCNDV-* SpainAlmeria	KF891468.1
Tomato leaf curl New Delhi virus-Chilli pepper isolate DHO5 coat protein gene, complete cds	*ToLCNDV-Chilli*	GU831540.1
Tomato leaf curl New Delhi virus AV1 gene :coat protein, isolate 26	*ToLCNDV-isolate_26*	AJ810365.1
Tomato leaf curl New Delhi virus :India :Delhi:Cucumis: DNA-A,	*ToLCNDV-India :Delhi:Cucumis*	KC545812.1
Tomato leaf curl New Delhi virus-[Luffa] complete cds	*ToLCNDV-Luffa*	AF102276.1
Tomato leaf curl New Delhi virus from potato	*ToLCNDV-Potato*	DQ272541.
Afrcan cassava mosaic virus-Uganda severe	ACMV-UGSvr	AF126802.1
Cucurbit leaf curl virus	CuLCuV	AF224760.1
Maize streak virus-A[South Africa]	MSV-A[SA]	Y00514.2
Pumpkin yellow vein mosaic virus-[Jessore] AV2 gene, isolate 2	PYVMV-Jes	AM087113.1
Tobacco leaf curl Kochi virus-[KK]	TbLCK-[KK]	AB055009.1
Tomato golden mosaic virus-yellow vein	TGMV-Yv	K02029.1.1
Tomato leaf curl Bangalore virus	ToLCBV-B1	Z481182.1.1
Tomato leaf curl Bangalore virus-[Ban4]	ToLCBV-[Ban4]	AF165098.1
Tomato leaf curl Bangalore virus-[Ban5]	ToLCBV-[Ban5]	AF295401.1
Tomato leaf curl Bangalore virus-[Kolar]	ToLCBV-[Kol]	AF428255.1
Tomato leaf curl Gujarat virus	ToLCGV	AY190291.1
Tomato leaf curl Philippines virus	ToLCPV	AB050597.1

Phylogenetic analysis

The phylogenetic tree was constructed by MEGA6 programme based on nucleotide sequence data of YMV of ridge gourd with other selected isolates of ToLCNDV and other begomo viruses [Table-2]. The phylogenetic dendrogram of coat protein gene sequence of YMV ridge gourd isolate are shown in [Fig-4]. The begomovirus under the study and other selected begomo viruses formed two major clusters. The major Cluster-I grouped all the ToLCNDV isolates and cluster II grouped all isolates of tomato leaf curl virus and other begomo-viruses. Phylogenetic analysis of coat protein gene sequences of YMV revealed that YMV-ridge gourd isolate (RGYMV Bangalore) clustered with ToLCNDV-strains. So, isolate under study considered as an isolate of ToLCNDV on the basis of phylogenetic analysis. From these results, we concluded that ToLCNDV is also associated with yellow disease of ridge gourd. The present study compared with Tiwari *et al*., (2012) reported that phylogenetic analysis of yellow mosaic virus infecting *Luffa cylindrica* clustered with the strains of Tomato leaf curl New Delhi virus (ToLCNDV).

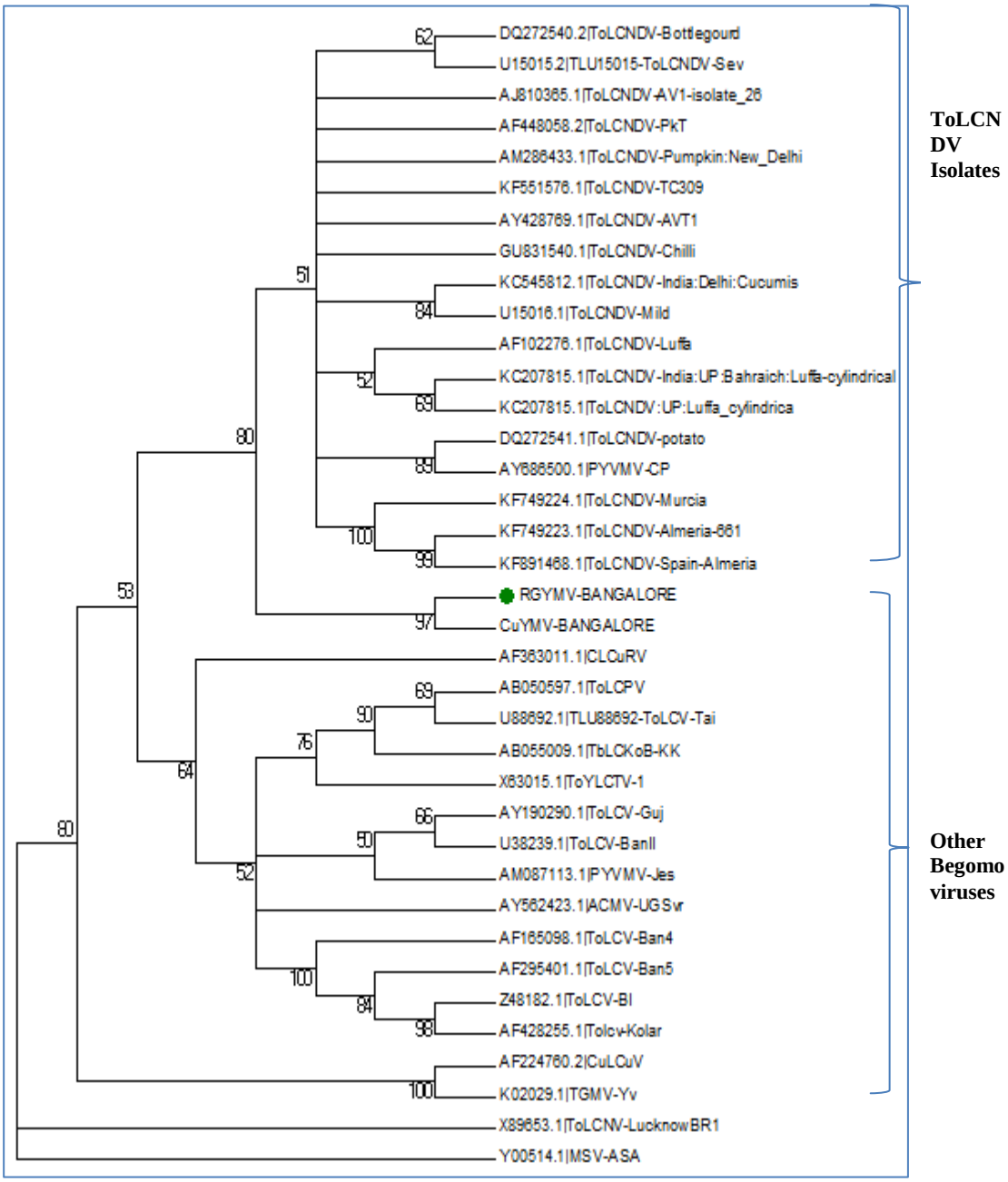

Fig-4 Phylogenetic tree obtained from comparison of complete nucleotide sequence of coat protein of ridge gourd yellow mosaic virus with other begomo-viruses from database. The dendrograms are calculated using neighbour-joining algorithm of MEGA 6.06 version. Numbers at nodes indicate percentage bootstrap confidence scores (1,000 replications).

CP genes represent the most conserved gene in the family Geminiviridae and CP sequences can be used as preliminary virus identification, or to infer geographic and vector relationship. On the basis of the various studies, it can be concluded that the yellow mosaic virus disease of ridge gourd prevailing in southern karnataka are caused by *Tomato Leaf Curl New Delhi Virus (ToLCNDV)*.

ToLCNDV is an emerging problem in numerous crops and widely distributed in India, Pakistan, Philippines and Thailand. Although, *ToLCNDV* is a major viral pathogen in solanaceous vegetables, it has emerged in several cucurbitaceous vegetables, bottle gourd, bitter gourd, cucumber, ivy gourd, long melon, pumpkin, and watermelon in northern India and chayote in north-western India. In Thailand, *ToLCNDV* has been reported to infect bottle gourd, cucumber and muskmelon[18]. The present study for the first time established association of ToLCNDV with the ridge gourd yellow mosaic disease based on coat protein gene sequence. Under field conditions, we have observed yellow mosaic symptoms in ridge gourd plant

resulting severe form of disease in the southern Karnataka in successive years. The previous and present studies further show that *ToLCNDV* has emerged in several cucurbits in India, however its prevalence in the farmers field needs further investigation.

Conclusion

Coat protein (CP) genes represent the most conserved gene in the family Geminiviridae and CP sequences can be used as preliminary virus identification, or to infer geographic and vector relationship. On the basis of the various studies, it can be concluded that the yellow mosaic virus disease of ridge gourd prevailing in southern Karnataka is caused by *Tomato Leaf Curl New Delhi Virus (ToLCNDV)*.

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Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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