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Molecular Characterization of Begomoviruses Causing Tobacco Leaf Curl Disease in different Parts of Karnataka

GURUDEVI V. NAVALI AND K. T. RANGASWAMY
Department of Plant Pathology, College of Agriculture, UAS, GKVK, Bengaluru - 560 065
Email: gvnavali5017@gmail.com

ABSTRACT

Molecular characterization of the 6 different isolates from major tobacco growing areas of Karnataka through sequencing of 1.2kb amplicon corresponding to 4 major genes *viz.*, coat protein (AV1) gene and replication initiation (AC1) gene partial, replication enhancer gene (AC3) and transcription activator gene (AC2) complete. The obtained sequences were subjected to NCBI BLAST search. TbLCV isolates from Davanagere, Mysuru and Belagavi showed 91 per cent of sequence similarity with the tomato leaf curl Karnataka virus (KX219744.1), tomato leaf curl Katnataka virus-Bangalore isolate (FJ514798.1) and Tomato leaf curl Joydebpur virus (DQ673859.1), respectively. Mandya and Shivamogga isolates showed 91 per cent of sequence similarity with Indian cassava mosaic virus (Z24758.1) and Indian cassava mosaic virus strain Salem (KU550960.1), respectively. Whereas, Chamarajanagar isolate showed 87 per cent sequence similarity with tobacco leaf curl Thailand virus (DQ871221.1). Phylogenetic analysis of these isolates revealed that, they formed 3 clusters, wherein Davanagere, Mysuru and Belagavi were grouped in one cluster showing highest sequence similarity with the Tomato leaf curl virus (ToLCV), Mandya and Shivamogga formed another cluster with Indian cassava mosaic virus (ICMV). Chamarajanagar isolate formed a separate cluster with Tobacco leaf curl Thailand virus (TbLCTV-Thailand). This is the first report of association of three begomoviruses with the TbLCV disease in Karnataka.

Keywords : Tobacco leaf curl virus, tobacco, isolates, major gene

TOBACCO (*Nicotiana* spp.) belonging to family Solanaceae is one of the most important and widely grown cash crop in both tropics and subtropics in the world. More than 70 species of tobacco are known, the chief commercial crop is *Nicotiana tabacum* and the more potent variant, *N. rustica* is also used around the world. *Nicotiana tabacum*, an annually grown herbaceous plant. Its leaves are commercially grown in many countries and processed into tobacco. India is the second largest producer of tobacco (761.32 T) after China and shares about 11.42 per cent of world total production from an area of 4.49 lakh ha followed by Brazil (FAOSTAT, 2016). Tobacco contains the phytochemicals *viz*., nicotine, anatabine, anabasine. All parts of the plant contain nicotine, which can also be extracted and used as an insecticide. Apart from many other uses the juice of the leaves used as an insect repellent. It has medicinal uses *viz.*, first aid for stings, cuts remove intestinal parasites, skin rashes, eczema and rheumatism, toothaches and toothpaste (Anon., 2016). Tobacco is affected by several diseases *viz*., Damping off, Frog eye leaf spot, Fusarium wilt, wild fire disease, root knot disease and viral diseases like tobacco mosaic virus (TMV), tobacco leaf curl virus (TbLCV), tobacco necrosis virus (TNV) and tobacco streak virus (TSV) *etc*. Among these viral diseases, tobacco leaf curl disease is also gaining importance next tobacco mosaic virus and tobacco streak virus which is a limiting factor on crop growth and yield.

For the first time tobacco leaf curl disease appeared in Indonesia and India in 1912 (Pal and Tandon, 1937). Tobacco leaf curl virus (TbLCV) causes the most destructive disease of tobacco (*Nicotiana tabacum*) in India and substantial yield losses. Affected plants remain stunted, their leaves develop vein thickening, curling, veinal depressions and profuse enations. The virus is transmitted in nature by the whitefly vector *Bemisia tabaci*.

The incidence of disease caused by TbLCV geminivirus, in ten tobacco growing areas of India ranged from 1.2 to 77 per cent. The highest incidence of disease was observed in Andhra Pradesh (77%)

followed by Gujarat (59%), Karnataka (17%), Bihar (11.6%) and West Bengal (5.4 %) (Valand *et al*., 1992).

Tobacco leaf curl causing virus belongs to the genus *Begomovirus,* family Geminiviridae. The begomoviruses have either monopartite genome having one circular ssDNA component of about 2.7 kb designated as DNA-A or bipartite genomes having two similar size components designated as DNA- A and DNA-B. The DNA A genome contains four major genes encoding coat protein (CP), replication initiator protein (Rep), transcriptional transactivator protein and replication enhancer protein. The DNA B contains two genes encoding proteins required for movement of virus, host range and pathogenicity.

So far, only one begomovirus species, Tomato leaf curl Bangladesh virus was reported in India (Singh *et al*., 2011). Viruses evolve continuously and there may be variations in the sequences due to several reasons like mutations. Knowledge of sequence diversity among isolates of a virus and their distribution has the potential to deepen our understanding of viral origins, development, dispersion and disease etiology. This information would be useful in developing effective virus disease management practices including Pathogen derived resistance. The present paper reports the sequence comparison analysis to ascertain the genetic diversity among tobacco leaf curl isolates from Karnataka.

Material and Methods

Survey, collection and maintenance of isolates

Tobacco seedling were raised in pro-trays containing coir pith for 45-60 days. Later these seedlings were transplanted to plastic pots containing soil + sand + FYM (1:1:1) and were maintained inside insect proof cages at Department of Plant Pathology glasshouse. Surveys were carried out during the 2016-2017 and 2017-2018 in major tobacco growing areas of Karnataka, India to know the per cent disease incidence of leaf curl disease. The disease was diagnosed in the field based on visual 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. Diseased leaf samples of tobacco plants showing typical symptoms of begomovirus infection were collected and maintained under glasshouse. These collected isolates were inoculated to healthy tobacco seedlings with whiteflies. These inoculated plants were kept in insect proof glasshouse and observed for symptom expression.

Meanwhile, the top leaves of the plants showing symptoms were selected for total DNA extraction and screened for the presence of begomovirus using begomovirus universal primers (Deng *et al*., 1994).

DNA extraction

The total genomic DNA was extracted from leaf tissues of healthy and leaf curl infected tobacco plants of different isolates by C-TAB method. Infected plant tissue (150 mg) was ground in a pre-sterilized pestle and mortar using C-TAB buffer (100 mM Tris (pH8.0), 1.4 M NaCl, 20 mM EDTA, 2 per cent CTAB and 0.2 per cent Mercaptoethanal) and transferred to sterile Eppendorf tube and incubated for 1 hour on water bath at 65 ºC. The supernatant was collected into new Eppendorf tubes. To this equal volumes of chloroform and Isoamyl alcohol (24:1) mixture was added 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 -20 ºC 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 per cent ethanol and centrifuged at 13,000 rpm for 10 min, the supernatant was discarded, vacuum dried for 10 min and re-suspended in 50 µl $T_{10}E_1$ buffer.

Amplification of viral DNA by Polymerase Chain Reaction (PCR). PCR was performed using a pair of begomovirus universal primers. The forward and reverse primers were: Deng A 5'-TAATATTACCKGWKGVCCSC -3' and Deng B 5'-TGGACYTTRCAWGGBCCTTCACA -3', respectively (Deng *et al*., 1994) and also pair of primers designed for 4 major genes of TbLCV *viz.*, coat protein (AV1) gene and replication initiation (AC1) gene partial, replication enhancer gene (AC3) and transcription activator gene (AC2) complete such as;

MK-F -5'- ATATCTGCAGGGNAARATHTG GATGGA -3' and MK R-5'- TGGACTGCAGA CNGGNAARACNATGTGGGC -3'. 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, 2 µl of DNA template and finally volume was made with sterile distilled water. The conditions for amplification are; 1 cycle of 94 °C for 3 min , 35 cycles of 94 °C for 1 min, 55 °C for 1.5 min, 72 °C for 2 min and 1 cycle of 72 °C for 10 min. The amplified PCR products were resolved on 1 per cent agarosegel 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 QIAquick gel extraction kit (Cat. No. 28704; Qiagen, Germany) by following the instructions given by the manufacturer. Eluted product was sent to private company Eurofins Pvt. Ltd. Benagluru for sequencing. The amplicons sequence results were analysed using NCBI BLAST (National Centre for Biological Information-Basic Local Alignment Search Tool) search, followed by multiple sequence alignments using Clustal W (Larkin *et al.,* 2007) and BioEdit Sequence Alignment editor (version 7.0.5.3) (Hall, 1999) to determine per cent sequence similarities with other sequences and with reference sequences which showed maximum similarities in the BLAST search (Table I). Based on the per cent sequence similarities, phylogenetic tree was generated by Mega 7.0.26 software (Kumar *et al*., 2016) using the neighbour joining method with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously. Phylogenetic treewas constructed using the neighbour joining algorithm of Clustal X.

Results and Discussion

Symptomatology and confirmation of leaf curl infection in isolates

After 10-15 days of inoculation with different isolates of TbLCV, plant expressed characteristic leaf curl symptoms in glasshouse condition and similar results were obtained by Singh *et al.*, 2011 who reported that affected plants developed distinct yellowing and leaf curl symptoms. Polymerase chain reaction (PCR) was employed for the confirmation

Table I

List of begomoviruses used in the phylogenetic analysis for comparison of leaf curl disease of tobacco

Begomovirus species used	Abbreviation	Gene bank accession No.
Tomato leaf curl Karnataka virus	ToLCKV	KX219744.1
Tomato leaf curl virus Bangalore isolate SZ73	ToLCBV-SZ73	KY420142.1
Tomato leaf curl Karnataka virus India:Punjab	ToLCKV-Panjab	FJ514798.1
Tomato leaf curl Karnataka virus isolate TC289	ToLCKV-TC289	KF551578.1
Tomato leaf curl Joydebpur virus	ToLCJV-Joydebpur	DQ673859.1
Tobacco curly shoot virus isolate CNBD: Tha	TbCSV-CNBD: Tha	KM383756.1
Datura leaf curl virus isolate Sudan Datura	DLCV-Sudan Datura	MF402919.1
Indian cassava mosaic virus- AR10	ICMV-AR10	Z24758.1
Jatropha leaf curl virus isolate Gujarat	JLCV- Gujarat	KM411359.1
Indian cassava mosaic virus strain Salem	ICMV- Salem	KU550960.1
Indian cassava mosaic virus-Ker2 isolate Adivaram	ICMV-Adivaram	AJ575819.1
Tobacco leaf curl Thailand virus	TbLCTV-Thailand	DQ871221.1
Tobacco leaf curl Yunnan virusY161	TbLCV-Y161	AJ566744.1

of begomovirus using universal primers where all the 6 isolates (Mysuru, Mandya, Chamarajanagara, Shivamogga, Davanagere and Belagavi) yielded 550 bp amplicon on gel electrophoresis compared with positive control (Tomato leaf curl virus) whereas desired amplicon was not amplified in healthy samples (Plate 1). The results confirmed that begomovirus was

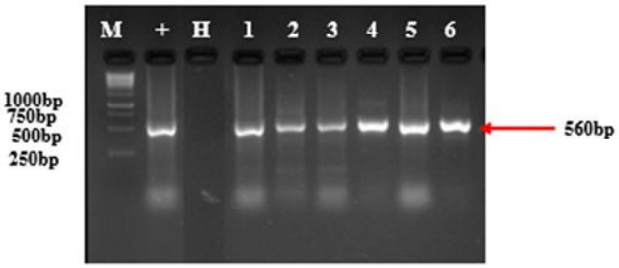

Plate 1:PCR amplification of different tobacco leaf curl isolate with Universal Deng primers

M: 1kb Ladder, +: +ve control (Tomato leaf curl virus) H: Healthy tobacco, 1, 2, 3, 4, 5 and 6: Mysuru, Davanagere, Belagavi, Mandya, Shivamogga and Chamarajanagar leaf curl infected isolates of tobacco

the causal agent of the tobacco leaf curl virus disease. These results were on par with work done by Deepa (2016) who used PCR technique to detect tobacco leaf curl virus disease using Deng primers, which amplified the product of approximately 0.5 kb size. Similarly, Sinha *et al*. (2011) used the PCR technique for the detection of the pepper leaf samples collected from the plants showing typical curling symptom which was infected with pepper leaf curl virus (PLCV) for the presence of virus in diseased plant samples and the infected leaf DNA samples produced a typical 550 bp amplification product and confirmed it to be a begomovirus.

Molecular characterization of the isolates

PCR was carried out to these 6 isolates with specific primers, to identify the taxonomic group of begomovirus responsible for causing the leaf curl disease in tobacco, where all 6 isolates yielded characteristic 1.2 kb amplicon on gel electrophoresis compared with positive control and the desired amplicon was not present in healthy samples (Plate 2). These results were on par with Roy *et al*., 2013 who reported that all the seven symptomatic samples yielded an expected 1.2 kb amplicon, whereas no such amplification was obtained from asymptomatic plants, thus, indicating the association of a begomovirus with

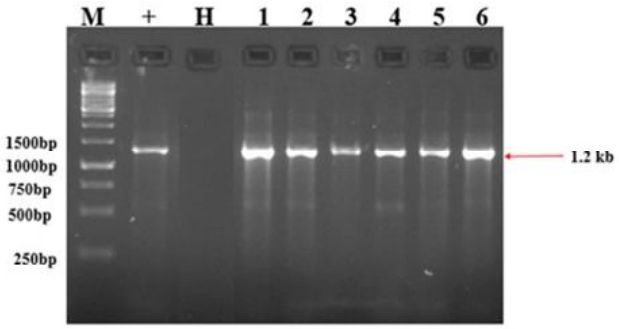

Plate 2: Gel electrophoresis of 4 major genes [coat protein (AV1) gene and replication initiation (AC1) gene partial, replication enhancer gene (AC3) and transcription activator gene (AC2) complete] different tobacco leaf curl isolates from Karnataka

M: 1kb Ladder, +: +ve control (Tomato leaf curl virus) H: Healthy tobacco, 1, 2, 3, 4, 5 and 6: Mysuru, Davanagere, Belagavi, Mandya, Shivamogga and Chamarajanagar leaf curl infected isolates of tobacco

symptomatic samples of Tomato leaf curl New Delhi virus in Ash Gourd (*Benincasa hispida*) germplasm showing a severe yellow stunt disease in India.

Sequences of 3 isolates, Davanagere, Mysuru and Belagavi showed 91 per cent sequence similarity with the Tomato Leaf curl Karnataka virus (KX219744.1), Tomato leaf curl Katnataka virus-Banglore isolate (FJ514798.1) and Tomato leaf curl Joydebpur virus (DQ673859.1), respectively. Mandya and Shivamogga isolates showed 91 per cent sequence similarity with Indian cassava mosaic virus (Z24758.1) and Indian cassava mosaic virus strain Salem (KU550960.1), respectively, whereas, Chamarajanagar isolate showed 87 per cent sequence similarity with Tobacco leaf curl Thailand virus (DQ871221.1). Paximidis *et al*., 2001 reported that mixture of begomoviruses in leaf curl infected tobacco plants in Karnataka, South India, where coat protein gene (CP) and common region (CR) of TbLCV-Kar1 and TbLCV-Kar2 which are not reported earlier in the tobacco, but may be distinct begomoviruses that could have arisen through recombination events during mixed infections. Phylogenetic comparison demonstrated no significant homology between the Indian tobacco begomoviruses and a tobacco-infecting begomovirus from Zimbabwe, showing that as these two are different geminiviruses, there is a geographic basis for phylogenetic relationships rather than an affiliation with tobacco as a host.

Singh *et al*., 2012 who reported that tobacco leaf curl was associated with other begomovirus and identified them as a strain of Radish leaf curl virus (RaLCV) based on nucleotide sequence of the viral genome (EU194914) and it was the first report of yellow leaf curl disease of tobacco, and the association of RaLCV with a disease of tobacco.

Nucleotide sequences of the different virus isolates were downloaded from NCBI database (Table I) and aligned using BioEdit Sequence Alignment editor (version 7.0.5.3) to determine per cent sequence similarities with other sequences and with reference sequences which showed maximum similarities in the blast search and phylogenetic tree was constructed using Mega 7.0.26 software. The begomovirus of different isolates form 3 clusters where Davanagere, Mysuru and Belagavi were grouped in one cluster showing highest sequence similarity with the Tomato leaf curl virus (ToLCV), whereas Mandya and Shivamogga form another cluster with Indian cassava mosaic virus (ICMV) and Chamarajanagar isolate form a separate cluster with Tobacco leaf curl Thailand virus (TbLCV) (Fig. 1).These results indicated that

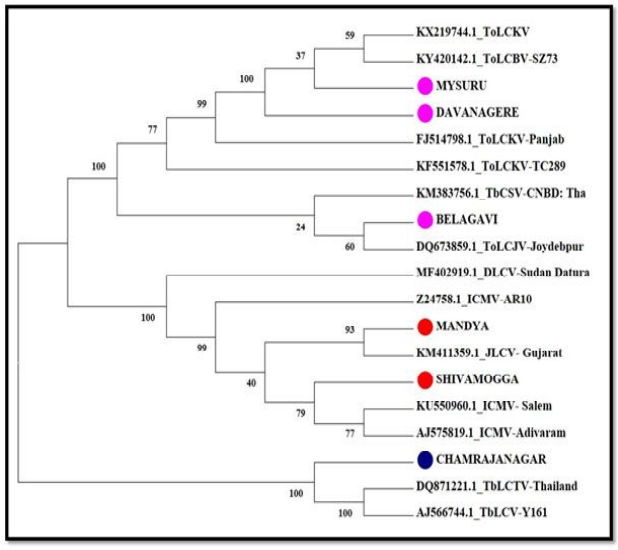

Fig. 1: Neighbor-joining tree of nucleotide sequence 4 major genes [coat protein (AV1) gene and replication initiation (AC1) gene partial, replication enhancer gene (AC3) and transcription activator gene (AC2) complete] of tobacco leaf curl isolates of Karnataka with other begomoviruses with 1000 boot strap replications

in Karnataka tobacco leaf curl virus disease known to be caused by different begomoviruses.

Diversity of the 6 TbLCV disease isolates

The nucleotide identity matrix of the 6 TbLCV isolates with other begomoviruses from the NCBI ranged from 61.0 to 94.5 per cent (Table II). Mysuru TbLCV isolate showed highest nucleotide identity matrix of 94.8 per cent with ToLCKV and lowest with DLCV Sudan Datura of 61 per cent. Mandya TbLCV isolate showed highest maximum identity matrix of 89.4 per cent with Shivamogga TbLCV isolate and least with 61.9 per cent with Mysuru TbLCV isolate.

Chamarajanagar TbLCV isolate showed highest nucleotide identity matrix of 79.2 per cent with TbLCV Y161 and lowest with 61 per cent with Mysuru TbLCV isolate. Shivamogga TbLCV isolate showed highest nucleotide identity matrix of 89.4 per cent with Mandya TbLCV isolate and lowest with 63.6 per cent with ToLCKV. Davanagere TbLCV isolate showed highest nucleotide identity matrix of 90.4 per cent with ToLCKV and least with 65.2 with DLCV Sudan Datura. Belagavi TbLCV isolate showed highest nucleotide identity matrix of 86.6 per cent with Davanagere TbLCV isolate and lowest with 63.1 with Chamarajanagar TbLCV isolate. The results indicated that the nucleotide identity matrix of all the 6 TbLCV isolate varied from one isolate to another isolate, it is due to association of the different virus in different location in the Karnataka.

Similarly Barboza *et al*. (2018) studied the diversity and distribution of begomoviruses in commercial tomato and sweet pepper fields from different agricultural production systems of the major growing regions of Costa Rica. A total of 651 plants were randomly sampled from greenhouses and open field crops during 2011 and 2012 in three different geographical locations. The bipartite begomoviruses, Tomato yellow mottle virus, Tomato leaf curl Sinaloa virus and Pepper golden mosaic virus and the monopartite begomovirus Tomato yellow leaf curl virus were detected in the collected samples. The complete genome of isolates from each species was cloned and sequenced. The frequency of detection of these four begomoviruses in the analysed samples ranged from 0 to 9 per cent, the presence and the prevalent virus varied largely according to the geographical location, the host (tomato and pepper) and the production system.

TABLE II

Nucleotide identity matrix of the 6 TbLCV isolates of Karnataka with other begomoviruses

Seq->	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
TbLCV Thailad	ID	75.2	73.2	63.9	65.4	65.5	63.6	64.3	63.7	66.0	67.4	66.6	95.2	62.1	73.3	78.7	73.4	67.7	65.1
ICMV AR10	75.2	ID	82.2	76.0	80.6	82.0	65.5	66.2	65.7	67.2	67.6	67	74.2	63.9	86.6	76.4	88.0	69.0	66.7
DLCV Sudan Datura	73.2	82.2	ID	70.0	74.0	75.0	62.1	62.8	62.0	64.2	65.4	64.5	73.8	61.0	78.6	74.7	79.3	65.2	63.3
JLCV Gujarat	63.9	76	70.0	ID	87.3	85.7	70.6	70.8	70.9	73.4	72.4	72.1	63.6	69.3	77.5	66.1	77.2	73.3	70.6
ICMV Salem	65.4	80.6	74.0	87.3	ID	94.2	71.7	72.5	71.8	75.3	74.7	75.0	65.2	70.1	78.6	66.5	82.9	74.6	72.4
ICMV Adivaram	65.5	82.0	75.0	85.7	94.2	ID	71.2	71.9	70.9	74.3	74.6	74.3	64.5	69.2	76.3	66.4	79.6	73.7	71.5
ToLCKV	63.6	65.5	62.1	70.6	71.7	71.2	ID	92.6	97.1	79.8	83.1	81.2	63.2	94.8	63.5	62.2	63.6	90.4	81.3
ToLCKV Panjab	64.3	66.2	62.8	70.8	72.5	71.9	92.6	ID	92.6	81.5	82.7	82.5	63.8	92.3	64.2	63.5	64.3	85.4	78.6
ToLCBV SZ73	63.7	65.7	62.0	70.9	71.8	70.9	97.1	92.6	ID	79.8	83.1	81.4	63.2	94.5	63.6	62.1	63.7	90.0	81.2
ToLCJV Joydepur	66.0	67.2	64.2	73.4	75.3	74.3	79.8	81.5	79.8	ID	83.6	83.9	65.2	78.0	66.5	64.6	66.8	83.5	83.4
ToLCKV TC289	67.4	67.6	65.4	72.4	74.7	74.6	83.1	82.7	83.1	83.6	ID	86.2	66.8	81.0	64.6	64.9	65.2	86.0	81.2
TbCSV CNBD: Tha	66.6	67.0	64.5	72.1	75.0	74.3	81.2	82.5	81.4	83.9	86.2	ID	65.8	79.1	66.1	64.1	65.6	84.4	83.7
TbLCV Y161	95.2	74.2	73.8	63.6	65.2	64.5	63.2	63.8	63.2	65.2	66.8	65.8	ID	61.6	73.0	79.2	72.9	67.2	64.0
Mysuru	62.1	63.9	61.0	69.3	70.1	69.2	94.8	92.3	94.5	78.0	81.0	79.1	61.6	ID	61.9	61.0	62.4	87.3	78.5
Mandya	73.3	86.6	78.6	77.5	78.6	76.3	63.5	64.2	63.6	66.5	64.6	66.1	73.0	61.9	ID	76.8	89.4	66.8	65.4
Chamrajanagar	78.7	76.4	74.7	66.1	66.5	66.4	62.2	63.5	62.1	64.6	64.9	64.1	79.2	61.0	76.8	ID	76.1	66.1	63.1
Shivamogga	73.4	88.0	79.3	77.2	82.9	79.6	63.6	64.3	63.7	66.8	65.2	65.6	72.9	62.4	89.4	76.1	ID	67.3	65.4
Davanagere	67.7	69.0	65.2	73.3	74.6	73.7	90.4	85.4	90.0	83.5	86.0	84.4	67.2	87.3	66.8	66.1	67.3	ID	86.6
Belagavi	65.1	66.7	63.3	70.6	72.4	71.5	81.3	78.6	81.2	83.4	81.2	83.7	64.0	78.5	65.4	63.1	65.4	86.6	ID

Based on the molecular characterization 1.2 kb amplicon corresponding to 4 major genes *viz.*, coat protein (AV1) gene and replication initiation (AC1) gene partial, replication enhancer gene (AC3) and transcription activator gene (AC2) complete and diversity analysis of the 6 TbLCV isolates, it can be concluded that, 3 different begomoviruses are responsible for causing tobacco leaf curl virus (TbLCV) disease *viz.*, Tomato Leaf curl Karnataka virus, Indian cassava mosaic virus and Tobacco leaf curl Thailand virus in Karnataka.

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