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Molecular diagnosis of Chilli veinal mottle virus causing mosaic in Capsicum (*Capsicum annum* var. grossum Sendt)

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ABSTRACT: Viral diseases are considered as the major limiting factors in Capsicum (*Capsicum annum* var. grossum Sendt) production and *Chili veinal mottle virus* (ChiVMV) is one of the important viruses, which decreases yield significantly. Surveys were conducted during *rabi* 2015-16 to determine the prevalence of incidence of mosaic disease in major capsicum growing districts namely, Chikkaballapura, Kolar, Bengaluru rural and Ramanagar. The per cent incidence of mosaic disease based on symptoms in field was noticed highest in Ramanagar (54.85 %) and the least incidence of mosaic disease was observed in Chikkaballapura (26.85 %). The symptoms includes yellowing, mosaic and mottling and the virus was preliminarily confirmed by DAC-ELISA. Among the 40 samples, 30 samples were reacted positively with ChiVMV antiserum and later it was maintained in *Datura metel* and the viral genome was analysed. Further, the diagnosis of virus causing mosaic disease in capsicum by RT-PCR, revealed that the percentage of nucleotide homology of ChiVMV-Kolar (KOL-1) isolate found to be 93.81 per cent and the lowest nucleotide homology of ChiVMV-KOL-1 isolate found 78.31 per cent and percentage of nucleotide homology of Chikkaballapura (CHB-1) isolate was found to be 93.97 per cent and the lowest nucleotide homology of ChiVMV-CHB-1isolate found to be 81.96 per cent.

Keywords: *Chili veinal mottle virus, Capsicum annum*, ELISA, RT-PCR and phylogenetic

INTRODUCTION

Capsicum (*Capsicum annum* L var. Grossum Sendt) also called bell pepper is an important vegetable crop known for its nutritional and commercial purposes. Among various biotic constraints in the production of bell pepper, viral diseases play a major role. Bell pepper is highly susceptible to natural infection by a large number of viruses such as Potyviruses *viz.,* potato virus Y (PVY), pepper veinal mottle virus (PVMV), pepper vein banding virus (PVBV), Chilli veinal mottle virus (ChiVMV), pepper mottle virus (PMV), tobacco etch virus (TEV) (Caranta *and* Palloix,1996). Among all the viruses, Chilli veinal mottle virus (ChiVMV) is the predominant virus with >50 per cent incidence which reduce the yield by 50 to 80 per cent. The incidence of ChiVMV infection may cause yield loss up to 95 per cent and 30 per cent in sweet chilli and small chilli, respectively based on field surveys conducted by Green *et al*., (1999) in 16 Asian countries. ChiVMV was found to be the most prevalent virus with increased incidence of 19.6 per cent as compared to previous surveys and monitoring in Pakistan (Shah *et al.,* 2009). Yield reductions of more than 50 per cent have been reported when the crop became infected at an early growth stage (Ong *et al*., 1980). Chilli veinal mottle virus (CVMV) is flexuous filamentous particle and a member of the *potyvirus* genus, is endemic virus in hot pepper, mainly in Asian countries (Green and Kim, 1991). It is readily sap transmissible to a narrow range of hosts including *Capsicum annuum, C. frutescent, Lycopersicon esculentum, Solanum melongena, Datura stramonium, Nicotiana* spp. and *Chenopodium* spp. (Green *et al*., 1999). Of late, ChiVMV was also detected outside Asia, in Tanzania, by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Nono-Womdim *et al*., 2001). Recently, molecular approaches such as amino acid sequence identity of the CP gene and nucleotide sequence identity of the full-length viral RNA have become the accepted method for accurate diagnosis and even for the taxonomy of potyviruses (Fauquet *et al*., 2005). Modern techniques such as electron microscopy and serology when combined with the molecular techniques will confer a greater measure of reliability in the identification of viruses and virus strains. The present paper reveals severity of the disease in the field, diversity of different viruses and molecular characterization of the most prevalent virus including virus sequences and phylogenetic relationships of the ChiVMV isolates of Southern Karnataka with the already reported isolates of ChiVMV.

MATERIALS AND METHODS

Survey: A roving survey was conducted during 2015-16 to determine the incidence of mosaic disease in major capsicum growing districts of Southern Karnataka (Chikkaballapura, Kolar, Bengaluru rural and Ramanagar). Plants were observed for the typical symptoms *viz.,* yellowing, mosaic symptoms, mottling *etc.* During the process, type of symptoms was recorded at different fields and samples were collected. For each one acre of field, five random sites (10m X 10m) were selected and the average disease incidence was calculated.

Diagnosis of capsicum mosaic disease caused by ChiVMV

Serological Assay: The samples collected from the field during survey were subjected to serological assay using CMV and ChiVMV antiserum adopting the DAC-ELISA procedure (Hobes *et al*., 1987).

Bio-assay and culture maintenance

The leaves showing mosaic symptoms were collected from the fields of Kolar, Chikkaballapura, Bengaluru rural and Ramnagar districts. The isolates were confirmed by ELISA for the ChiVMV and the one showing the highest virus titre was inoculated on capsicum (Plate 1) and further on *Datura metel*. The culture was maintained by frequent re-inoculation by sap inoculation method on *D. metel* (Plate 2).

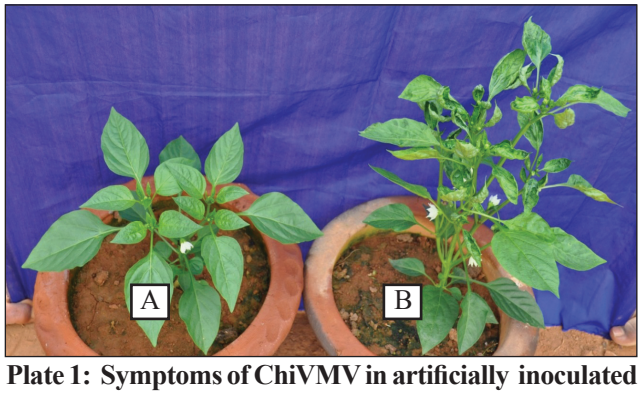

Plate 1: Symptoms of ChiVMV in artificially inoculated capsicum seedlings A. Healthy B. Infected

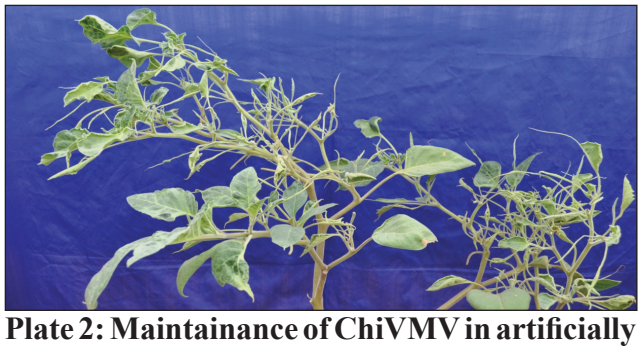

Plate 2: Maintainance of ChiVMV in artificially inoculated *Datura metel*

Molecular diagnosis

RNA isolation and cDNA synthesis

Frozen tissue sample approximately 0.5-1.0 g of tissue was ground into fine powder in a pre-chilled mortar and pestle and the ground tissue was transferred into a pre-chilled 50 ml conical tube and 5-10 ml of TRIzol reagent (1ml of TRIzol/100 mg of tissue) was added. Contents were mixed well with vortex and 1 ml of the mixture was transferred into labeled, *RNase*-free 1.5 ml tube to incubate for 5 min at room temperature. Chloroform 200 µl per ml of TRIzol was added and vortexed for 20 seconds and incubated the contents for 10 min. at room temperature and centrifuged at 13,200 rpm for 10 min. at 4°C. The aqueous phase was carefully transferred into a new RNase-free 1.5 ml tube without disturbing the other phases and the tube was placed on ice as soon as transferred. Equal volume of isopropanol was added and mixed gently by inverting 2-3 times and incubated it on ice for 30 min and centrifuged at 13,200 rpm for 20 min. at 4°C. A very small pellet the total RNA was visible at the bottom of the tube. The supernatant was decanted and allowed to stand upside down on kimwipes (Kim tech science, Canada) for 5 min. The pellet was washed with 75 per cent ethanol (500 µl). The liquid was decanted and the inside of the tube was wiped to dry with a clean Kimwipe, without touching the pellet. The pellet was resuspended in 500 µl of *RNase*-free water and was incubated on ice for an hour and pipetted occasionally for dissolving the pellet. The content was centrifuged at 13,200 rpm for 20 min at 4°C and the supernatant was transferred into a new *RNase*-free 1.5 ml tube. It was precipitated with 10 per cent 3M sodium acetate and equal volume of isopropanol. Contents were incubated on ice for 1 hr or overnight at -80°C. Centrifuged at 13,200 rpm for 20 min at 4°C. The final pellet was resuspended into 50 µl of *RNase*-free water and was stored at -80°C.

The total RNA was treated to remove DNA using Turbo DNA-freeTMkit (cat#AM1907 Ambion, USA) as per the manufacturer's instructions. In order to eliminate the genomic DNA, 20 µg of total RNA was digested with *RNase*-free *DNase* I and finally the total RNA was precipitated into desired volume of water. The quantity and quality of total RNA was checked using Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The total RNA was subjected to Nanodrop ND-1000 using RNase-free water as blank: absorbance was recorded at 260/280 and 260/230. Further, the sample was fractionated over a formaldehyde agarose gel. Absence of genomic DNA contamination was subsequently confirmed by PCR with total RNA as template (Caldana *et al*., 2007).

Table 1. Average per cent disease incidence of capsicum mosaic disease in different districts in Southern Karnataka

District	Per cent disease incidence	
	Average	Range
Kolar	32.99	14.85-47.42
Chikkaballapura	20.25	7.99-26.85
Ramanagar	27.42	26.28-54.85
Bengaluru rural	29.24	27.42-36.56

Reaction mixture for RT-PCR: 10 µl total RNA, 5 lM RT primer, 2 µl 10x RT buffer, 0.8 µl 25X dNTP Mix, 1 µl MultiScribeTM reverse transcriptase and 1 µl RNAse inhibitor (Applied Biosystems).The 10 µl reactions were incubated in an Applied Biosystems Thermocycler in a 96 well plate for 10 min. at 25^{0}C, 120 min. at 37^{0}C, 5 min. at 85^{0}C and then held at 4^{0}C. All reverse transcriptase reactions, including no template controls and RT minus controls were run in duplicate. The efficiency of cDNA synthesis was assessed by normal PCR amplification of control 18S rRNA genes. Only cDNA preparations that generated sharp bands were selected for further experiment.

First strand cDNA synthesized by reverse transcription was amplified by Polymerase Chain Reaction. PCR reaction mixture was of 25 µl. The primer used in the study was forward primer WEICN-F (5' CCCCGYTTSCTMTCYGGMAGNTC 3') and reverse primer M4T-R (5' CGAGCACGACTTTTTTTTTTTTT 3'). The PCR amplification was carried out in a thermal cycler with the following conditions; initial- denaturation at 94^{o}C for 3 min. followed by 94^{o}C for 30 sec. annealing at 55^{o}C followed by extension at 72^{o}C for 1 min. 30 sec. with final extension at 72^{o}C for 20 min.

Analysis of RT-PCR product by Agarose gel electrophoresis

The amplified product was analysed by agarose gel electrophoresis with ethidium bromide based staining along with markers. Agarose (1 per cent) gel was casted in TBE buffer (1X) in a horizontal gel frame (Hoefer HE99X 18 x 30 cm Amersham Bioscience Pvt. Ltd. USA); products were visualized by incorporating 1 µl (10 mg/ml) ethidium bromide per 10 ml of gel solution and viewed in a gel documentation system (Syngene Pvt. Ltd. USA).

The procedure followed for gel electrophoresis was as follows

The perspex tray and comb were thoroughly cleaned with 70 per cent alcohol using tissue paper and the ends of the perspex tray were sealed with spacers and comb was inserted. Agarose gel of 1 per cent concentration was prepared by adding 1 g agarose to 100 ml of TBE (1x) buffer (EDTA 0.5 M at pH 8) and the resulting solution was boiled by putting the flask in microwave oven and then allowed to cool to 60 ^{0}C. Ethidium bromide (5 µl of conc. 5 mg/ml) was added to the gel and mixed gently and the gel was poured into the tray and air bubbles were removed by using pipette. When the gel was completely set, tape was removed and the gel was placed into the electrophoresis tank. Approximately 500 ml of TBE (1x) buffer was poured into the electrophoresis tank, enough to cover the gel to a depth of 5 mm. Comb was removed carefully. About 1/10th volume of loading dye (6x) bromophenol blue dye was added to DNA samples and mixed by gentle tapping and spinning for 2-3 sec in a micro centrifuge. PCR samples were loaded onto the wells and the power supply of about 90 V was provided to run the gel. The power supply was switched off when loading dye was about 2 cm from positive end, and the gel was removed from the gel apparatus. The gel was viewed and photographed by using gel documentation system (UVI, Tech England).

Sequencing of amplified product

The PCR amplified product was sequenced using M-13 primers employing primer walking technique at Chromous Biotech Pvt. Ltd., Bangalore.

Sequence analysis of ChiVMV

The sequences were subjected for analysis through Vecscreen service available in NCBI website. The available sequence information from fragments was subjected to analysis using BLAST algorithm available at http:/www.ncbi.nim.nih.gov. The BLASTn algorithm was used for searching homology sequence at nucleotide level, whereas BLASTp algorithm at amino acid level. Further, sequences were assembled using Bioedit. Sequences analysis and comparison of ChiVMV to the reference sequences were performed using http:/www.ncbi.nim.nih.gov. The complete ChiVMV or polyprotein

sequences and CP sequences of other ChiVMV isolates and other Potyviruses were downloaded from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Multiple nucleotide sequence alignments were performed with CLUST W and a phylogenetic tree was reconstructed by the maximum-likelihood method using MEGA 7.

RESULTS AND DISCUSSION

Survey: The average per cent disease incidence in Kolar district was 32.99 and it ranges from 14.85 to 47.42 per cent. In Chikkaballapura district the average per cent disease incidence was 20.25 and it ranged from 7.99 to 26.85 per cent and in Ramanagar district the average per cent disease incidence was 27.42 and it ranged from 26.28 to 54.85 per cent and in Bengaluru rural district the average per cent disease incidence was 29.24 and it ranged from 27.42 to 36.56(Table 1). The disease intensity was varied from region to region mainly because of diversity in cultivars used and plant protection practices that the farmers followed.

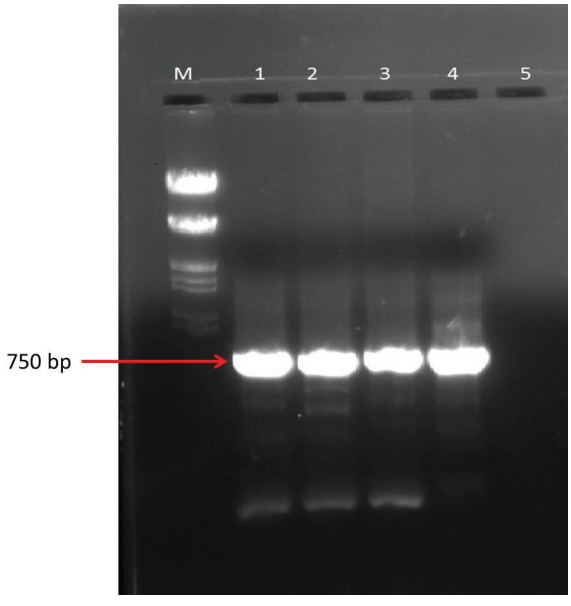

Plate 3. Agarose gel showing the RT- PCR amplification of ChiVMV infecting capsicum by using the CP gene specific primer.

Lane M: Lambda DNA/ECOR1+HindIII Marker

Lane 1: Capsicum mosaic sample from Kolar

Lane 2: Capsicum mosaic sample from Chikkaballapura

Lane 3: Inoculated sample

Lane 4: Positive control (*Datura metel*)

Lane 5: Apparently healthy sample

Serological survey: The samples brought from the field were subjected to serological assay using CMV and ChiVMV antiserum adopting the DAC-ELISA procedure (Hobes *et al*., 1987), among the 40 samples, 30 samples were reacted positively with ChiVMV antiserum and the ten samples collected from Chikkaballapura district have reacted with CMV antiserum(data not shown).

Bio assay and culture maintenance: The isolates confirmed for ChiVMV by ELISA and one showing the highest virus titre was maintained on *Datura metel* (Plate1 and 2).

Molecular diagnosis

PCR detection: The cDNA synthesized by reverse transcription was amplified by Polymerase Chain Reaction using the specific primers. The resultant product was a amplicon of 750 bp (Plate 3). The amplified product was sent for sequencing. The sequence so obtained was used for blast analysis and further study. The alignment of the sequence was done using Clustal W and the dendrogram was constructed using MEGA 7 (https;//www.megasoftware.net).

The results of the sequencing are furnished in Table 2. The results revealed that highest percentage of nucleotide homology of ChiVMV KOL-1 isolate found to be with accession EF213688.1 (CHM15 Raichur) and ChiVMV CHB-1 was 93.37 per cent with accession GU170808.1 (ChiVMV-Ch-War Maharashtra) with chilli veinal mottle virus peppers in India. While the lowest nucleotide homology of ChiVMV KOL-1 isolate was found to be 78.31 per cent with accession KJ472764.1 (ATIPK Pakistan) and ChiVMV CHB-1 was 81.96 per cent with accession KJ472764.1 (ATIPK Pakistan)(Table 2).

The phylogenetic analysis with MEGA 7 showed six clusters (Figure 1). The 1st cluster includes isolates from Trichy, CH34 (TN) with nucleotide identity of 88.81 per cent with ChiVMV Kolar isolate and 88.88 per cent with Chikkaballapura isolate,

The nucleotide sequence similarity of isolates *viz.,* ChiVMV KOl-1 isolate ranged from 93.81per cent to 78.31per cent and ChiVMV CHB-1 isolate ranged from 93.37per cent to 81.96 per cent with the other Gen bank accessions used for the analysis. ChiVMV isolates are more closely related to each other molecularly, regardless of symptoms. Similar results were reported by Chachulska *et al*., 1997, Gorsane *et al*., 2001, Kerlan *et al*., 1999 and Reddy *et al*., 2004.

Table 2. Nucleotide sequence similarity of ChiVMV in capsicum- Kolar isolate and Chikkaballapura isolate, Karnataka with selected Genbank accessions

Subject Id	Code of Accession IDs	Kolar isolate-1 (%)	Chikkaballapura isolate-1 (%)
ChiVMV isolate CHM15 Raichur Karnataka	EF213688.1	93.81	89.83
ChiVMV isolate Be16 Bellary Karnataka	EF213683.1	93.64	89.83
ChiVMV isolate S7 Sherewad Maharashtra	EF213700.1	90.00	90.26
ChiVMV isolate CH34 Trichy Tamil Nadu	EF213679.1	88.81	88.88
ChiVMV isolate CHL46 Kodaly Kerala	EF213687.1	88.48	88.20
ChiVMV isolate CHL40 Trissur Kerala	EF213681.1	88.47	88.97
ChiVMV isolate PM1 Bangalore Karnataka	EF213703.1	88.46	88.63
ChiVMV isolate TN15 Coimbattore Tamil Nadu	EF213697.1	88.39	88.45
ChiVMV isolate G2 Guntur Andhra Pradesh	EF213694.1	88.39	88.45
ChiVMV isolate ChiVMV-Ch-War Maharashtra	GU170808.1	87.05	93.37
ChiVMV isolate SRt8 Thailand	DQ854958.1	84.97	88.54
ChiVMV isolate UB32 Thailand	DQ854957.1	84.02	88.41
ChiVMV isolate China1 China	DQ854950.1	83.28	88.26
ChiVMV isolate P1037 Taiwan	DQ854942.1	83.13	88.10
ChiVMV isolate ATIPK Pakistan	KJ472764.1	78.31	81.96
ChiVMV isolate BP Thailand	DQ854954.1	85.37	89.62
ChiVMV isolate ChiVMV-VN/C2 Vietnam	DQ925441.1	86.35	87.58
ChiVMV isolate RAJ Rajastan	KJ000073.1	87.81	93.97
ChiVMV isolate CHL42 Trissur Kerala	EF213686.1	87.04	88.11
ChiVMV isolate ChiVMV-VN/C4 Vietnam	DQ925443.1	86.85	87.84
ChiVMV Kolar isolate	-	100	89

The analysis of the phylogenetic trees from partial polyprotein (partial Nib and entire coat protein), coat protein and 3' 11TR sequences of 32 ChiVMV isolates with other potyviruses was performed and the ChiVMV isolates were well separated from the out group taxa and formed a monophyletic group. This major phyletic group formed within potyvirus genus comprising of Plum pox virus (PPV), Tobacco vein banding mosaic virus (TVbMV), pepper veinal mottle virus (PVMV) and ChiVMV isolates based on amino acid sequences and with nucleotide sequences, clustering of ChiVMV isolates was with Scallion mosaic virus (ScaMV), Carnation veinal mottle virus (CarMV) and pepper veinal mottle virus (PVMV) (Gorsane *et al*., 2001). The phylogenetic trees were identical with polyprotein, CP and 3 '-UTR sequences with minor variation. Based on amino acid (73.8 to 78%) and nucleotide sequence (68.6 to 71.6%) pepper veinal mottle virus is the closest relative of ChiVMV. The clustering of CINMV as a separate group along with PPV or PRSV in the phylogenetic analysis using CP sequences or polyprotein sequence has been earlier reported, which is in agreement with the present phylogenetic grouping (Anindya *et al*., 2004, Adams *et al*., 2004; Moury *et al*., 2005). The phylogenetic grouping of the ChiVMV isolates in the potyviridae grouping also was similar to other potyviridae members grouping (Chen *et al*., 2002; Shi *et al*., 2005; Adams *et al*., 2004; Chen *et al*., 2001).

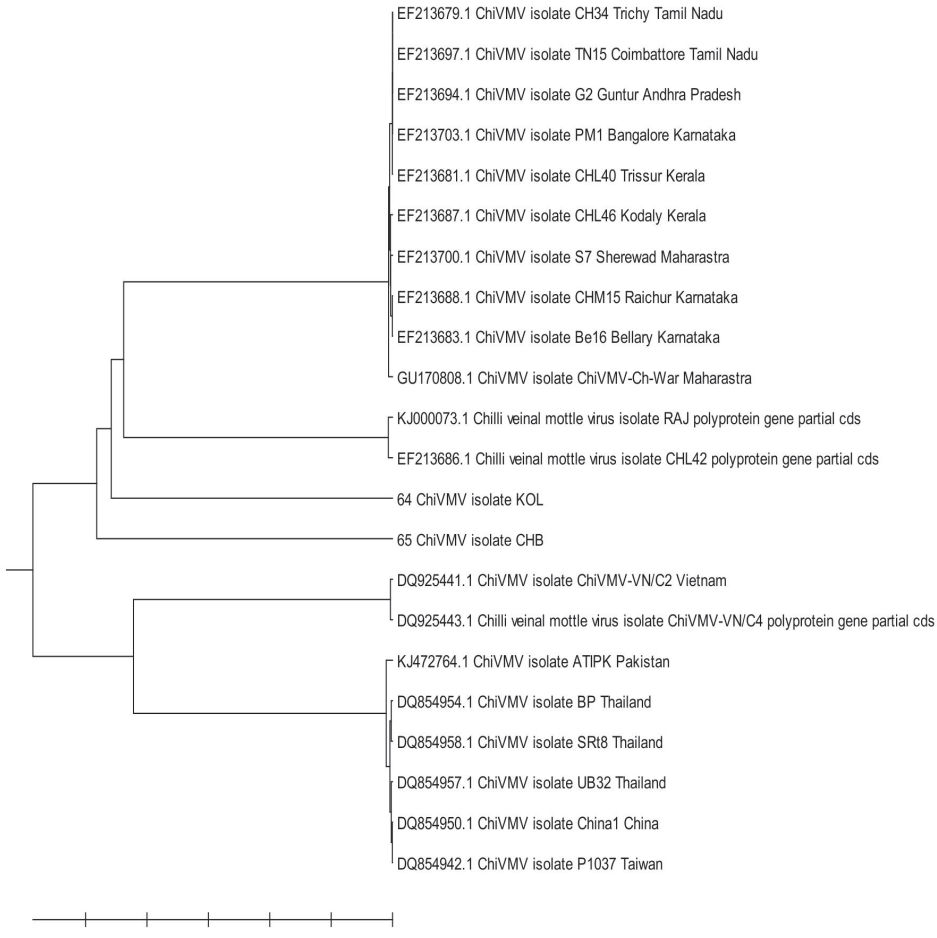

Fig.1. Dendrogram showing phylogenetic relationship of ChiVMV isolate from Kolar and Chikkaballapura with other genebank accessions

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