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Molecular characterization of *Cucumber mosaic virus* infection in chilli (*Capsicum annuum* L.) and its phylogenetic analysis

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Abstract
Cucumber mosaic virus (CMV) is the most serious viral pathogen affecting chilli (*Capsicum annuum* L.) worldwide. The proper diagnosis of virus infection using visual observations alone makes the management of CMV very difficult. In order to find the occurrence, field samples exhibiting the symptoms of necrosis, leaf malformation, stunting and reduction in plant size were collected. To study the nature of CMV infection, samples were diagnosed by reverse transcriptase polymerase chain (RT-PCR). Total RNA was isolated from leaves of infected plants and used in RT-PCR reaction with a primer specific to CMV coat protein region. The results revealed that a viral amplicon of expected 800 bp size were obtained from infected plants and phylogenetic relationship determined by the comparison of coat protein gene nucleotide sequences with other CMV isolates reported from India and worldwide. Sequence analysis of the coat protein gene had nucleotide similarity of 96 to 94 per cent with known strains of CMV. The multiple sequence alignment revealed that high homology between the isolates, further confirms the placement of the chilli strain of CMV in a single subgroup with India isolates. The proper diagnosis of CMV infection through molecular approaches could help in timely adoption of management strategy.

Keywords: Chilli, *Cucumber mosaic virus*, coat protein gene and diversity analysis

Introduction
Chilli (*Capsicum annuum* L.) a genus in Solanaceae family known as red pepper, is an important condiment cum vegetable crop in India. Capsicum is native to Central and South America consisting of thirty species, however solely 5 species including of *Capsicum annuum* has been demonstrated for their commercial importance. As per the latest statistics, India produced around 8 lakh tonnes of dry chilli from an area of 9.3 lakh hectares. The major chilli producing states of India are Maharashtra, Karnataka Andhra Pradesh and Tamil Nadu. As similar to other crops, chilli is affected by several pathogens and pest which will cause comprehensive loss in production. Among the pathogens, roughly 10 viruses encountered in commercial chilli fields, become a serious threat affecting the yield [1-5]. Several viruses had reportedly to be infected the chilli, among these *Cucumber mosaic virus* (CMV) is found to be a serious pathogen causing mosaic disease which exhibit wide pattern of symptoms including mottling, mosaic, yellow discolouration, vein clearing, leaf deformation and shoestring leaves. In addition, CMV has a wider range of host plants including weed species and crops with widespread distribution throughout the world [6-7]. The CMV infection causes 60 per cent yield loss in chilli if infects the crop during early stage [8]. *Cucumber mosaic virus* (CMV), is a member of the genus Cucumovirus in the family Bromoviridae, has wide host range, infecting more than 1,200 plant species [9]. The CMV genome comprising of three positive sense, single stranded RNAs consisting of RNA1, RNA2 and RNA3. The gene for encapsidation of virus is found in the subgenomic RNA (RNA4) encoded by RNA3 [10]. Further, CMV isolates have been placed into two subgroups I and II, on the basis of serology, nucleic acid hybridization and gene sequences. CMV subgroup I have been recently divided into IA and IB on the basis of gene sequences available for CMV strains and phylogenetic analysis. Furthermore, Asian strains of CMV have been placed into subgroup IB [11]. Generally, CMV is transmitted by several species of aphid in a nonpersistent manner [12]. Sometimes, CMV infection favour the synergistic effect with other viruses in chilli plants. For example, CMV and *Pepper mottle*

virus as coinfection in chilli produces nonlethal symptoms under greenhouse conditions [13]. However, plant virus infection may alter the morphology and physiology of the plants which affects the behaviour of host plant resistance and attractiveness for insects.

Previously, CMV has been identified only through morphological and symptom based studies in chilli and other vegetable crops. So far, no study has been found on the molecular profiling of protein sequence to discriminate the phylogeny of the pathogen to identify the proper management strategies. Recently, plant virologists have focused their attention to develop transgenic or enhanced host plant resistance against plant viruses through molecular approaches worldwide. The enhancement of host plant resistance appears as one of the best options because it offers an easy, cheap and sustainable technology to farmers, i.e., improved cultivars. In this prospects, field observation of characteristic virus symptoms are the primary detection techniques for the virus infection followed by the different serological and molecular tools for further endorsement. It is need to be addressed to understand the biological and molecular properties of emerging CMV infecting pepper in India, since a detailed study on the disease has not been taken up in India. Even the occurrence of CMV has been reported from many vegetable crops in India, only limited reports are available on the biological and molecular characterization and their exact identification remains unaddressed. Hence, the present study was aimed to detect the CMV through molecular characterization and phylogenetic relationship of CMV using the coat protein gene as they represent the most important genes in virus.

Materials and Methods

Collection of Isolates

Field survey was conducted in Coimbatore districts of Tamil Nadu during growing seasons of 2017-18 to document the virus diseases. The per cent disease incidence was recorded by counting the total number of plants and virus infected plants in each and every field of different areas. The plant samples showing characteristic CMV like symptoms were collected separately for further analyses. Ten samples from each field in each place were collected in zip-loc bags and labeled. Samples were transported to the laboratory on ice and kept at 4 °C.

Total RNA extraction, cDNA synthesis and amplification of CP gene

Total RNA approximately, 50 µg/µl were extracted from 100 mg leaves of infected chilli using Trizol plant extraction kit (Sigma chemicals, USA) according to the manufacturers protocol and resuspended in 50 µl nuclease free water. The total RNA isolated from the virus infected field chilli samples were subjected to PCR in 50µl reaction volume containing cDNA and 2 units of enzyme mix and with primers specific to CMV (F1-TTCTCCGCGAGTTAGC; CMV R1-CGTAAGCTGGATGGAC) corresponding to coat protein gene of CMV to amplify the complete coding region of CP genes. The PCR settings comprised of 35 cycles of amplification including denaturation at 94 °C for 2 min, annealing at 52-60 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR reaction was carried out in Eppendorf Mastercycler Gradient ES. The amplified products were analyzed on 1% agarose gel, stained with ethidium bromide and photographed under UV-gel doc system (Alpha imager).

Cloning, sequence alignment and phylogenetic analysis

The amplicon of coat protein gene fragment was purified using QIAGEN gel extraction kit (Qiagen Inc., Chatsworth, CA, USA) and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) following the manufacturer's instructions and transformed into *Escherichia coli* DH5α by following standard molecular biology procedures[14]. Plasmid DNA was isolated from the potential recombinant clones using Wizard plus DNA purification kit (Promega, Madison, WI) according to the manufacturer's protocol and the positive clones were identified by restriction digestion analysis using *EcoRI* enzyme. The three independent clones were sequenced from both orientations for each fragment separately. The sequences were then edited using the BIOEDIT Software. Sequence similarity search of the GenBank database was done using the Basic Alignment Search Tool (BLAST) program. The amino acid sequences of CP gene of CMV was translated from the consensus nucleotide sequence using the EMBOSS Transeq program. Both the nucleotide and amino acid sequences were then aligned with selected sequences using the CLUSTAL W program [15-16]. Phylogenetic analysis was done on MEGA 7 and trees were created using the neighbour-joining method [17-18]. The robustness of the trees was determined by bootstrap using 1,000 replicates. *Peanut stunt virus* was used as a reference out-group member of the genus Cucumovirus for rooting the phylogenetic tree.

Result and Discussion

The survey was conducted in major chilli growing tracks of Tamil Nadu, a highest per cent disease incidence of CMV was recorded in Madampatti area of Coimbatore district of Tamil Nadu state. The severe disease symptom of CMV infection was observed under field condition in young plants, before the flowering stage. During the field survey for recording the incidences of CMV, plant showed the diagnostic symptoms of virus infections were mosaic, leaf distortion, vein chlorosis and stunting. The plant samples were collected from symptomatic and asymptomatic (apparently healthy) plants and brought to the laboratory under ice cold conditions for further analysis. Rashid *et al* [19] found the wider range of symptoms from the field infected chilli, which exhibits complex symptoms viz., mosaic, mottle, leaf distortion, vein chlorosis and stunting. Similarly, Govardhan Rao and Ramana [20] also reported the occurrence of CMV in chilli with the characteristic symptoms of severe mottling, vein cleaning, vein banding, leaf curling, plant stunting, few flowers and fruits under field conditions. Plant age at infection, virus strain, vectors involved and environmental conditions that prevailed in the season might be the reasons for variation in symptoms. In order to characterize CMV, cDNA derived from the extracted RNA of chilli samples collected from field were used for PCR amplification of CP genes of CMV. The amplified PCR products from infected samples with an amplicon size of approximately 800 bp were excised and cloned into pGEM-T easy vector (Fig 1). The independent clones were selected and confirmed through restriction analysis using EcoRI. The independent clones were sequenced in both the orientations using universal M13 primers. The gene sequence was edited using BIOEDIT software and the full length nucleotide sequence was obtained. The sequence analysis of these clones shared above 94 per cent nucleotide sequence identities to sequence available in NCBI database. The nucleotide sequence analysis using NCBI BLAST confirmed the association of *Cucumber Mosaic virus* (CMV). The CP gene sequence of CMV isolate

was submitted in NCBI Genbank database bearing the Accession No. MN943095.

Analysis of CP gene sequence of CMV were compared with corresponding genes from known isolates at the nucleotide and amino acid sequence levels. The CMV sequence of three isolates our sequences had had maximum of 96.50 and per cent nucleotide homology with Chilli CMV of Indian isolate (AJ810260). This was followed by sequence of our isolates had 96.04 nucleotide homology with CMV from chilli and Jasmine isolates (KF12906 and KJ74601), respectively. The evolution of new strains has complicated the diagnosis, which requires a combination of bioassay, serological and molecular assays. To simplify the identification and differentiation of various groups, a competitive (single antisense and multiple sense primers) reverse transcription-polymerase chain reaction (RT-PCR) is used [21]. Phylogenetic analysis revealed chilli strain has very high homologies between the CMV strains of other crops and confirmed the formation of single subgroup (Fig 2). Multiple sequence alignment revealed a near perfect homology between the nucleotide sequence of the chilli strain and the nucleotide sequences of other strains. Similarly, Biswas *et al*. [7] amplified the coat protein (CP) gene of the CMV using RT-PCR with CP gene specific primers, cloned and sequenced. Sequence analysis revealed that, CP gene showed homologies of 95-99% at nucleotide level with the sequences of CMV subgroup II and the phylogram assigned that the CMV isolate infecting chilli-pepper is clustered well with the other analyzed isolates belonging to subgroup II. Jain *et al*. [22] reported the occurrence of natural infection of TSV in chillies for first time in India and identified the virus by RT-PCR by using TSV-CP gene specific primers with an amplicon size of 717 nucleotides long and could encode a protein of 238 amino acids. Comparative amino acid sequence analysis revealed that the virus infecting chillies shared very high levels of similarity at both nucleotides (98-99%) and amino acid (98%) levels, with the corresponding region of TSV isolates. In conclusion, infection of *Cucumber mosaic virus* from chilli has been confirmed through characterization of CP gene using PCR analysis. This could help in the early detection of virus infection under field condition in future.

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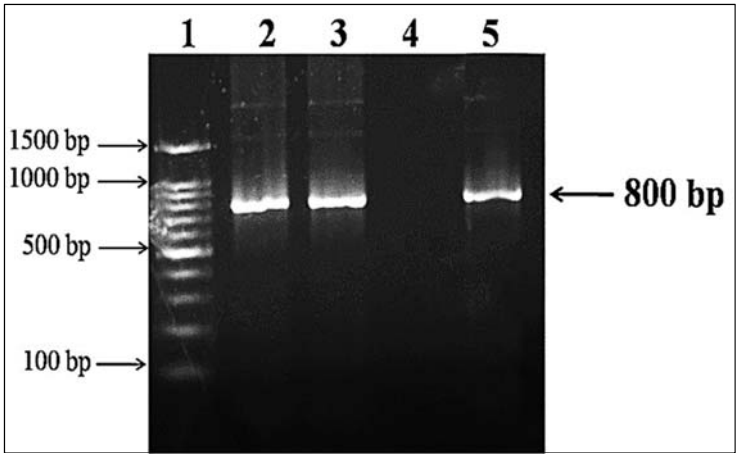

Fig 1: Amplification *Cucumber mosaic virus* from infected samples. Lane 1-100 bp ladder; Lane-2 & 3-Infected sample; Lane-4-Healthy control; Lane-Positive control

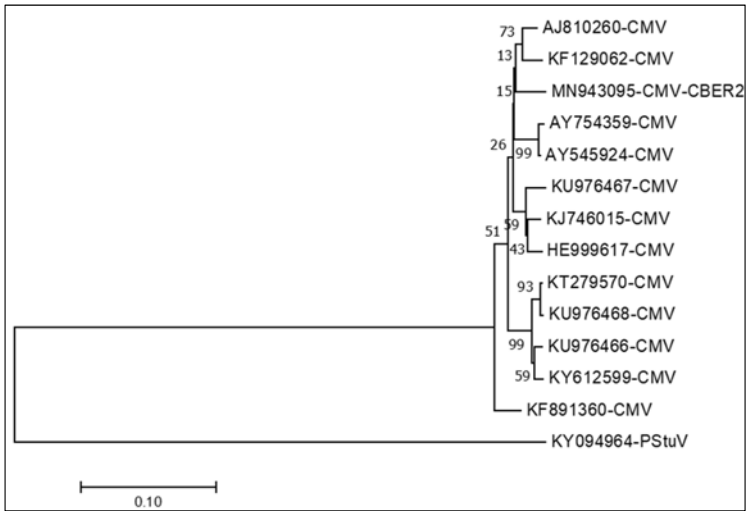

Fig 2: Neighbour-joining phylogenetic tree based on the nucleotide sequences of CP gene of CMV and *Peanut stunt virus* is defined as an out-group.

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