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Detection and molecular characterization of cucumber mosaic virus infecting chilli

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Abstract
CMV (cucumber mosaic virus) is the most important and devastating virus infecting chilli (*Capsicum annuum*. L). Leaf sample from chilli plants expressing systemic mosaic and mottling symptoms, typical to CMV infection was collected and designated as CMV PS1 isolate. The CMV isolate was mechanically inoculated to chilli and *Nicotiana glutinosa* plants. For molecular characterization, total RNA was isolated from infected plants and used for carrying out RT-PCR with CMV coat protein (CP) specific primers. PCR amplification generated an expected CP amplicon of 1.1 kb, which was further cloned and sequenced. The CMV CP nucleotide sequence showed 93.91 to 99.19 per cent identity with other CMV isolates infecting different crops worldwide. The sequence demarcation graph and phylogenetic analysis showed close association of CMV PS1 isolate with CMV isolate Ho (MN340998.1) belonging to subgroup IB prevailing in Indian conditions.

Keywords: Cucumber mosaic virus, molecular characterization, coat protein, phylogeny

1. Introduction
Chilli (*Capsicum annuum* L.) is an important export-oriented vegetable and commercial spice crop in the world. It belongs to the nightshade family, *Solanaceae* (Walsh and Hoot, 2001) [16]. Worldwide, it is used in almost every cuisine as a spice for its pungency, flavour and colour. Due to its prime importance in the human diet, pharmaceutics and cosmetic industry, chilli has been extensively cultivated across different countries in the world. Viral diseases are a major threat for chilli production resulting in low fruit quality and quantity. More than 65 viral diseases have been reported to infect chilli worldwide (Devi and Devi, 2020) [5]. Among these, cucumber mosaic virus (CMV) is reported as a more serious pathogen causing up to 100 per cent yield loss in marketable fruits and sometimes resulting in abandoning the fields prior to harvest (Khan *et al*., 2006) [8].
CMV has a tripartite, positive sense, ssRNA genome with each segment designated as RNA1, RNA2 and RNA3 and at least two subgenomic RNAs, RNA4 and RNA4A, which are transcribed from RNA3 and RNA2, respectively (Palukaitis *et al*., 1992) [11]. RNA 1 and 2 codes for components of the replicase complex and RNA2 also codes for the 2b protein, which is suppressor of gene silencing. RNA3 encodes 3a protein, essential for the virus movement and the coat protein (CP), which is expressed from subgenomic RNA4 (Conti *et al*., 1997) [4].
The CP has an important role not only in the formation of the viral particles but also in virus movement, transmission by aphid vectors and symptom expression (Kumari *et al*., 2013) [10]. Based on their biological, serological and molecular properties, CMV strains can be divided in two subgroups, I and II, the former being further divided into subgroups IA and IB. The subgroup IB is suggested to contain the 'Asian strains' whereas other members of subgroup I have been kept under subgroup IA (Roossinck *et al*., 2002) [13].
CMV infected chilli plants express the symptoms of vein clearing, mosaic mottling, yellow discoloration, leaf deformation, leaf narrowing or shoe string, stunted growth, whitish streaks on green fruits and reduced fruit size (Bhadramurthy *et al*., 2009; Ashwathappa *et al*., 2021) [2, 1] and ultimately result in yield reduction and considerable economic losses. Understanding the genetic variations within viral populations is important for tracking virus evolution, predicting the emergence of new strains and designing effective control strategies. Molecular characterization of CMV helps in assessing the genetic diversity and it is vital to understand prevalence of particular strain present the respective area. Here we report molecular

characterization of the CMV subgroup IB isolate.

2. Material and Methods

2.1 Source and maintenance of the virus isolate

Leaf sample from chilli plants showing typical symptoms of mosaic, mottling and leaf narrowing or shoe string was collected from the naturally infected field located at Zonal Agricultural Research Station (ZARS), GKVK, Bengaluru and was macerated in a cooled mortar and pestle with 0.05 M potassium phosphate bufer (pH 7.0) and 0.02% 2-β-mercaptoethanol at a rate of 2 mL/g of leaf tissue. To remove debris, crushed sap was filtered through a double-layered muslin cloth and celite powder (600 mesh at 0.025 g/mL sap) was used as an abrasive during mechanical sap inoculation. The inoculum was applied to the upper surface of chilli (cv. Arka meghana) and *N. glutinosa* leaves and gently rubbed in one direction with a small piece of absorbent cotton. Excess inoculum on the leaves was rinsed away with a fine stream of distilled water after 5 min. Inoculated plants were kept in an insectproof glasshouse to monitor symptom manifestation.

2.2 PCR amplification and cloning of CMV CP gene

Total RNA was extracted from CMV infected chilli leaf samples collected from chilli plants inoculated with CMV isolate and maintained in the glasshouse by using a modified phenol-chloroform and lithium chloride (LiCl) method, following the procedures outlined by Khairul-Anuar *et al.* (2019) [7]. The quality of the RNA was checked on 1.2 per cent agarose gel and quantified by Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). cDNA synthesis of CMV CP was done using specific reverse primer cited in the previous study by Ashwathappa *et al.* (2021) [1]. Initially, 5 µg total RNA with reverse primer specific to CP of CMV (10 pmol/ µl) was taken separately and incubated at 70°C for 5 min, followed by addition of 4 µL 5X RT reaction buffer, 2.5 µL 10 mM dNTP mixture and 4 µL of DEPC treated autoclaved water and 0.5 µL PrimeScript™ Reverse Transcriptase enzyme (200 U/ µL) in a total reaction of 20 µL. Subsequently, the reaction was carried out at 42 °C for 60 min, terminated by incubating at 70 °C for 10 min.

PCR amplification was performed using CP specific primers, the details on primers used, PCR cycle conditions and the anticipated amplicon size is provided in table 1. The PCR reaction mixture (20 µL) consisted 10 µL of 2x PCR master mix (Emerald Amp, TaKaRa, Japan), 5 µL of nuclease free water, 1.5 µL of 10 pmol/µL each of forward and reverse primers. PCR amplification was carried in an Eppendorf thermal cycler. Four microliters of PCR product was electrophoresed on one per cent agarose gel stained with ethidium bromide and visualized under gel documentation system. The amplified PCR product of CMV CP was purified and ligated into pTZ57R/T vector (Fermentas, Germany) as per the manufacture's protocol. Three positive clones were selected, plasmids were isolated using NucleoSpin® Plasmid Isolation kit (TakaraBio, USA) and sequencing was done using M13F/R primers at Eurofins Genomics India Pvt. Ltd, Bangalore, India.

Table 1: Details of CMV CP gene specific primers and PCR conditions used in the current study

Primer sequences (5' to 3')	PCR conditions	Product size
F-GTAATCTTACCACTGTGTGTGTG R-CAGATTTGTCCATGACTCGACTC	Initial denaturation: 94 °C for 3 min Denaturation: 94 °C for 1 min Annealing: 67 °C for 1 min Extension: 72 °C for 1 min Final extension: 72 °C for 10 min Number of cycles: 35	1.1 (kb)

2.3 Sequence analysis

The CMV CP gene sequence of PS1 isolate obtained after sequencing was subjected to BLASTn search to identify similar sequences in the National Centre for Biotechnology Information (NCBI) database, Maryland, United States. Sequences displaying the highest identity were retrieved from NCBI GenBank, aligned using the BioEdit program (Hall, 1999) [6] and used to calculate pairwise percent identity with the CMV PS1 isolate utilizing the Sequence Demarcation Tool version 1.2 (SDTv1.2). Phylogenetic tree was constructed by analysing the sequences using the Neighbor-Joining based on Kimura 2 parameter model (Kimura, 1980) [9] with complete gap deletion and resampled with 1000 bootstrap replications in MEGA X software to explore the relationships among various strains. Papaya ringspot virus (PRSV) was used as outgroup for phylogenetic analysis.

3. Results

3.1 Mechanical inoculation

CMV-PS1 isolate was successfully mechanically transmitted on to *Capsicum annuum* (cv. Arka meghana) and *N. glutinosa* plants. Inoculated plants expressed systemic mosaic and mottling symptoms after 10-15 days post inoculation of virus (Fig. 1).

3.2 PCR amplification and molecular characterization of CMV CP

The cDNA obtained was subjected for PCR amplification of CMV using CP specific primers and resulted in expected amplicon of size 1.1 kb (Fig.2). The amplified fragment was successfully cloned and sequenced bi-directionally, the consensus sequence was deposited in NCBI, GenBank (Accession number OR996343).

Comparative nucleotide sequence analysis was performed by comparing the CP sequence of CMV PS1 isolate with the corresponding CP region of 15 different CMV isolates shown highest identity during the BLAST search and one PRSV isolate sourced from the NCBI database. The sequence comparison revealed a shared identity of 93.91 to 99.19 per cent between the current CMV CP isolate CP gene sequence with other CMV CP sequences previously reported from various regions such as Egypt (KX014666.1), Brazil (MK387174.1), South Korea (KC527768, KC527762.1, KC527757) and different parts of India (Table 2). Additionally, a comparison was made between CMV PS1 isolate and 15 other CMV CP gene sequences of different CMV isolates obtained from the NCBI database using SDTv1.2 (Fig. 3). The pairwise identity of the query sequence with the retrieved consensus sequences is provided in table 2. Subsequent phylogenetic analysis of the CMV PS1 CP gene

sequences, alongside selected reference CMV isolates (Table 2) indicated that the CMV PS1 isolate clustered closely with the cucumber mosaic virus isolate Ho (MN340998.1) infecting chilli in India (Fig. 4).

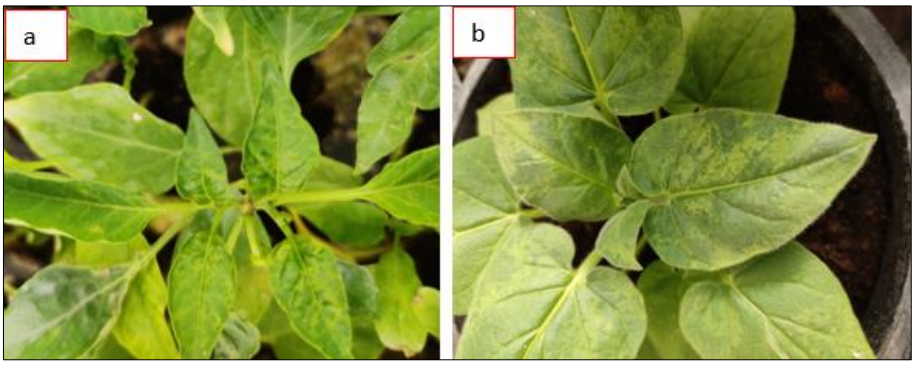

Fig 1: Symptoms induced by the CMV PS1 isolate on chilli (a) and *N. glutinosa* (b)

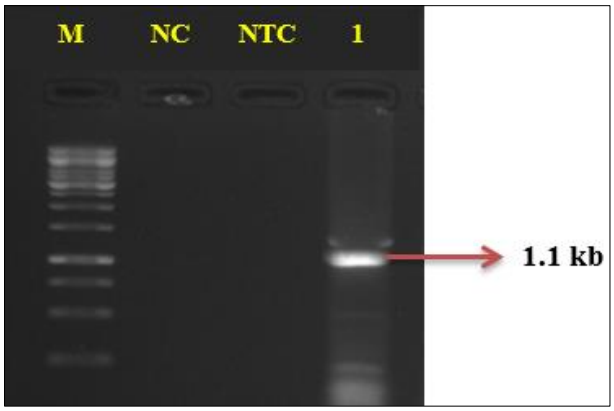

Fig 2: PCR amplification of coat protein (CP) gene of CMV PS1 isolate using specific primers. M-Marker (1kb), NC-Negative control, NTC-Non template control, 1-Infected sample

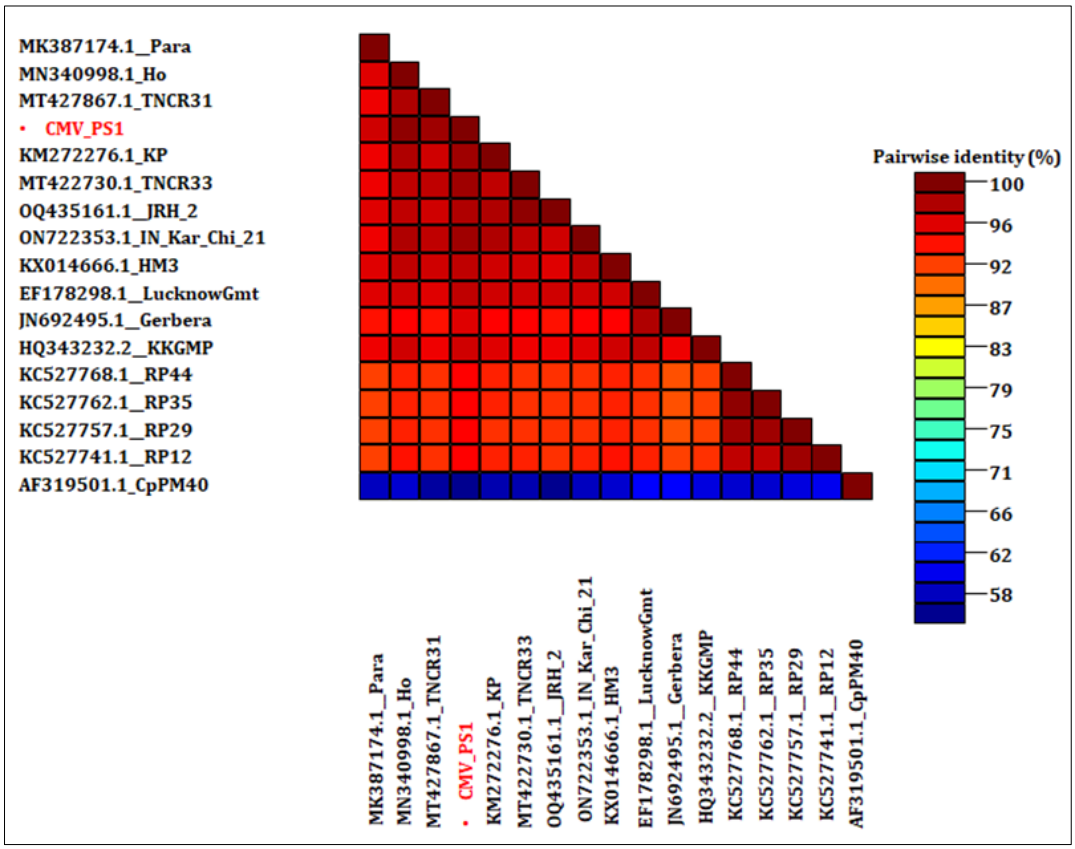

Fig 3: Graphical representation of percentage pairwise genomic scores and nucleotide identity plot of coat protein gene of CMV PS1 infecting chilli with reference sequences using Sequence Demarcation Tool (SDTv1.0)

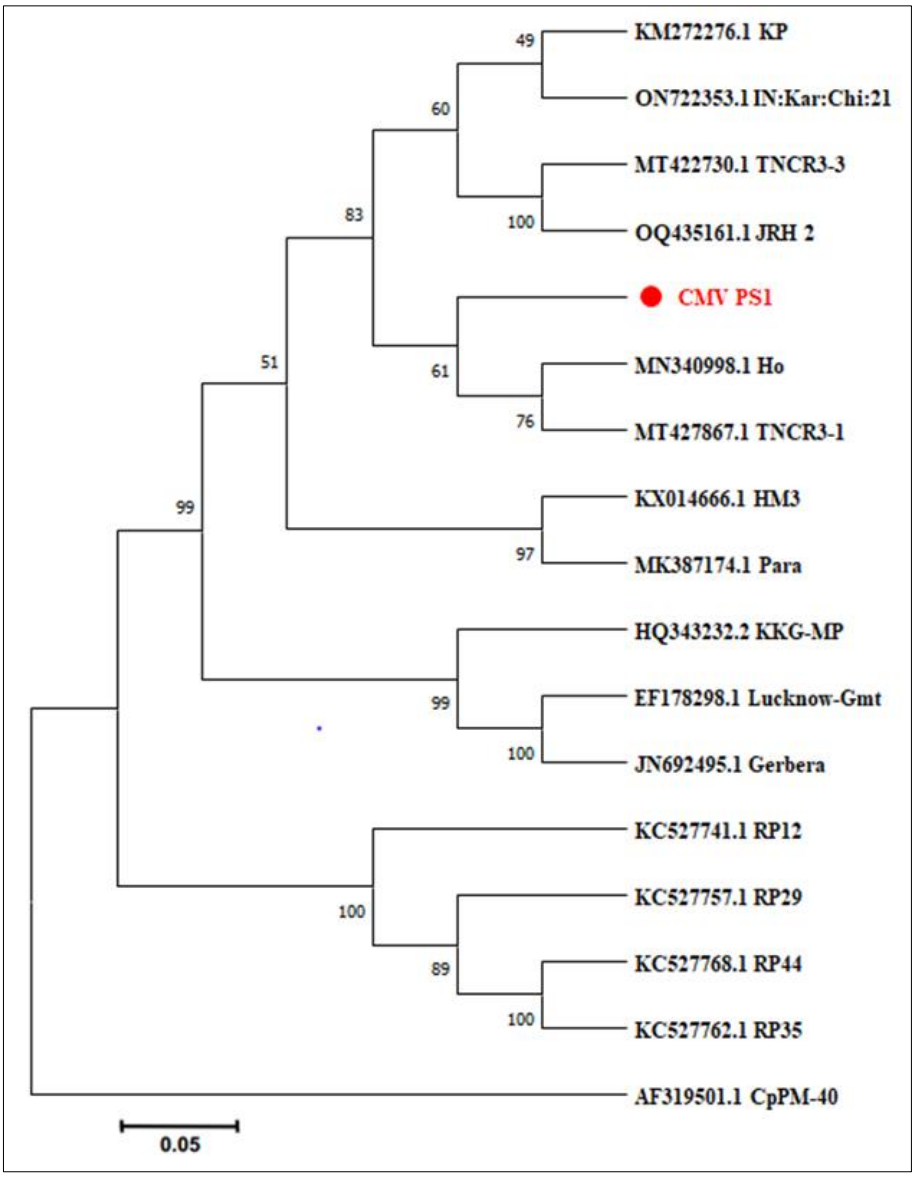

Fig 4: Neighbor-Joining tree constructed with CMV CP nucleotide sequences depicting the phylogenetic relationship of CMV PS1 isolate with other CMV isolates/strains

Table 2: The CMV CP gene sequences employed in the phylogenetic analysis and percent nucleotide identity of CMV PS1 isolate with other CMV nucleotide sequences retrieved from NCBI

Acc. No.	Virus	Strain/Isolate	Host	Origin	Percent identity
MN340998.1	CMV	Ho	Chilli	India	99.19
KM272276.1	CMV	KP	Chilli	India	98.98
MT427867.1	CMV	TNCR3-1	Chilli	India	98.66
MT422729.1	CMV	TNCR3-2	Chilli	India	98.55
ON722353.1	CMV	N:Kar:Chi:21	Chilli	India	98.34
KX014666.1	CMV	HM3	Tomato	Egypt	97.46
OQ435161.1	CMV	JRH 2	Chilli	India	97.83
EF178298.1	CMV	Lucknow-Gmt	Banana	India	96.95
MK387174.1	CMV	Para	*Arachis pintoi*	Brazil	96.74
HQ343232.2	CMV	KKG-MP	Brinjal	India	96.25
JN692495.1	CMV	Gerbera	Gerbera	India	96.04
KC527768	CMV	RP44	Chilli	South Korea	94.41
KC527762.1	CMV	RP35	Chilli	South Korea	94.11
KC527757	CMV	RP29	Chilli	South Korea	93.91
AF319501	PRSV	-	Papaya	Mexico	-

4. Discussion

CMV is the most devastating virus with a wider host range. Depending on the host plant and strain/isolate, CMV is known to induce variable symptoms, such as necrotic or chlorotic lesions, mild to severe mosaic, stunting, leaf deformation and shoestring formation (Kumari *et al*., 2013) [10]. Both chilli and

N. glutinosa plants inoculated with CMV PS1 isolate took up the infection and induced systemic mosaic and mottling symptoms. Understanding genetic structure and their evolutionary mechanisms is an important aspect of managing CMV and the risk of emerging new strains (Revathy and Bhat, 2017; Garcia and Fraile, 2013) [12, 3]. Prevalence of several diseases caused by CMV subgroups IA and II are present worldwide, while subgroup IB is restricted to Asia only (Roossinck, 2002) [13]. In the present study, we investigated the CP genetic structure of CMV PSI isolate infecting chilli. Based on sequencing and phylogenetic relationships, CMV PSI isolate was found to be closely related to CMV subgroup IB. Ashwathappa *et al*. (2021) [1] performed phylogenetic analysis of RNA1, RNA2 and RNA3 of CMV-Ko isolate with subgroup IB isolates and showed close association of CMV-Ko isolate with CMV originating from India, Europe and East Asian isolates characterized previously. Likewise, our findings align with the findings of Vinodhini *et al*. (2020) [15], who revealed the association of TN CMV CP isolates with subgroup IB clustered with Indian chilli isolates (HM348784 and KM272275) and black pepper isolate (KU947031) along with other members of subgroup IB.

One of the most critical forces in the evolution of *Cucumovirus* is recombination (Bonnet *et al*. 2005) [3]. The evolutionary constrain imposed on the virus may inflict apparent variation in amino acids and nucleotides. Hence, the variation in the genetic composition can occur due to recombination or reassortment leading to the emergence of new phenotypes and interspecies transmission. Evolution of virulent viral strains by recombination has devastating effect on the host population. Molecular characterization and SDT analysis contribute to our understanding of plant virus dynamics, which is essential for developing effective management strategies and ensuring global food security.

5. Conclusion

The findings obtained from PCR amplification of CMV CP and subsequent nucleotide identity analysis and phylogenetic investigations confirm the presence of CMV and its close association with CMV subgroup IB in chilli. Evolution of virulent viral strains by recombination has devastating effect on the host population. Therefore, complete genome analysis is vital for the better conception about genetic structure and evolution of CMV.

6. Acknowledgement

6.1 Conflict of interests

The authors declare that they have no competing interests.

6.2 Human and animal rights

This article does not contain any studies with human and animal subjects performed by any of the authors.

6.3 Informed consent

Informed consent was obtained from all individual participants included in the study.

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