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Molecular Characterization of Tobacco Mosaic Virus Infecting Chilli and its Infectivity Test on *Nicotiana* Species

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Abstract

Tobacco mosaic virus (TMV) is a major pathogen that causes significant losses in vegetable crops worldwide. Leaf sample was collected from chilli plants exhibiting chlorotic mosaic patterns and mottling symptoms characteristic of TMV infection. The virus was mechanically transmitted to *Nicotiana tabacum* plants for maintenance and was designated as the TMV Bengaluru isolate. For molecular characterization, total RNA was extracted from the infected *N. tabacum* plants and subjected to reverse transcription polymerase chain reaction (RT-PCR) using TMV coat protein (CP)-specific primers. The PCR amplification yielded a CP gene amplicon of 700 bp, which was subsequently cloned and sequenced. Nucleotide sequence analysis of the TMV CP gene revealed 90.00–99.72 per cent identity with TMV isolate reported from India. Further analysis using the Sequence Demarcation Tool and phylogenetic studies indicated a close relationship between the TMV Bengaluru isolate and Indian TMV isolate (GenBank accession: JQ895560). The TMV Bengaluru isolate demonstrated the ability to infect chilli highlighting its potential to adapt, spread and establish in diverse plant hosts.

Keywords : Tobacco mosaic virus, Coat protein, Phylogeny, Nucleotide identity, Tobacco

Tobacco mosaic virus (TMV) is one of the first viruses to be discovered and is a highly infectious plant virus (Scholthof *et al*., 2022). It is a type member of the genus, *Tobamovirus* and family *Virgaviridae*. The TMV genome consists of positive-sense single-stranded RNA (ssRNA) molecule of 6.4 kilonucleotide (knt) that is packaged in a non-enveloped rod-shaped coat approximately 300 nm in length. Its genome consists of four ORFs, encoding a 126 kDa replicase component that has methyl-transferase (MT), helicase (Hel) and RNA silencing suppressor domains (Vogler *et al*., 2007 and Stobbe *et al*., 2012) a 183 kDa protein which is a read-through product of the 126 kDa protein ORF and has an additional RNA-dependent RNA polymerase (RdRp) domain, a movement protein (MP) and a coat protein (CP) (Harries *et al*., 2009).

The symptoms of TMV are primarily reported on tobacco, but can also infect different hosts in the *Solanaceae* family inducing diverse symptoms (Yin *et al*., 2010). It is also known to infect numerous weeds (Korbecka-Glinka *et al*., 2021) and ornamental species (Alexandre *et al*., 2000). TMV infected tobacco plants showed veinal discoloration on young leaves,

followed by a mottling or mosaic pattern of light and dark-green areas, blistering and fern-shaped leaves (Nolla, 1938). TMV is transmitted through direct contact of healthy plants with infected plants, contaminated tools or seeds (Gergerich & Dolja, 2006 and Knapp & Lewandowski, 2001).

Despite a comprehensive understanding of the virus's structure and transmission mechanisms, TMV continues to inflict significant economic losses worldwide surpassing $100 million annually (Chen *et al*., 2022). TMV causes heavy yield losses in tobacco, tomato and pepper worldwide (Gao *et al*., 1994; Palloix *et al*., 1994; Xu *et al*., 1994; Sikora *et al*., 1998 and Kavyashri *et al*., 2018). Yield reductions of up to 90 per cent have been estimated on pepper (Escudero, 1996) and up to 34 per cent losses in tomato (Giri and Mishra, 1991) due to TMV and its association with other viruses. Understanding the genetic variations within viral populations is crucial for monitoring virus evolution, predicting the emergence of new strains and developing effective control strategies. The current study focuses on the molecular characterization of TMV, offering significant insights into its genetic diversity and enhancing the understanding of the prevalence of specific isolates in the respective area.

Material and Methods

Source and Maintenance of the Virus Isolate

Chilli plants showing typical symptoms of mosaic pattern with light and dark green areas and mottling was collected from the naturally infected field located at Zonal Agricultural Research Station (ZARS), GKVK, Bengaluru. The sample was macerated in a cooled mortar and pestle with 0.05 M potassium phosphate buffer (pH 7.0) and 0.02 per cent 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 *Nicotiana tabacum* leaves and gently rubbed in one direction with a piece of absorbent cotton. Excess inoculum on the leaves was rinsed away with distilled water after five to ten minutes (Vinaykumar *et al*., 2018). Inoculated plants were kept in glasshouse to monitor symptom manifestation. The TMV culture was maintained as stock in the glasshouse at Department of Plant Pathology, UAS, GKVK, Bengaluru for further study and designated as TMV Bengaluru isolate.

PCR Amplification and Cloning of TMV CP Gene

The infected plant samples of TMV collected from the mechanically inoculated tobacco plants maintained in the glasshouse along with the healthy leaf samples were used for RNA extraction by using a modified phenol-chloroform and lithium chloride (LiCl) method, following the procedures outlined by Khairul-Anuar *et al*. (2019) and Sajeevan *et al*. (2014). The quality of the RNA was checked on 1.2 per cent agarose gel and quantified by Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). The cDNA synthesis of TMV was prepared using specific reverse primer. Initially, 5 µg total RNA with reverse primer specific to TMV (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 mMdNTP mixture and 4 µL of DEPC treated autoclaved water and 0.5 µL Prime Script TM 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 TMVCP 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 using the Proflexthermal cycler (Carlsbad, California, United States). 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

TABLE 1

Details of TMV CP gene specific primers and PCR conditions used in the current study

Primer sequences (5' to 3')	PCR conditions	Product size
F-CAGGATTCWTCATCAGTYAATTACTG R-CAGAKAGYGAKGCTACCAG	Initial denaturation: 94°C for 3 min Denaturation: 94°C for 45 sec Annealing: 55°C for 45 sec Extension: 72°C for 1:30 min Final extension: 72°C for 10 min Number of cycles: 35	700 bp

of TMV 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 at Eurofins Genomics India Pvt. Ltd., Bengaluru, India.

Sequence Analysis

The TMV CP gene sequence obtained 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 score were retrieved from NCBI GenBank, aligned using the BioEdit programme (Hall, 1999) and pairwise per cent identity of the TMV Bangalore isolate was calculated utilizing the Sequence Demarcation Tool version 1.2 (SDTv1.2). Phylogenetic tree was constructed using the Neighbour-Joining method based on Kimura 2 parameter model (Kimura, 1980) with complete gap deletion and resampled with 1000 bootstrap replications in MEGA X software to explore the relationships among various strains. Tomato brown rugose fruit virus (ToBRFV) and odontoglossum ringspot virus (ORSV) were used as out group for phylogenetic analysis.

Recombination Analysis

In order to know the possible recombination events in TMV Bangalore isolate, neighbor-net analysis was performed with 1000 bootstrap replicates using Splits-Tree version 5.3.0 (Huson and Bryant, 2006). The recombination break point analysis was performed using RDP5 (Martin *et al.,* 2020) package version 5.101 employing six different algorithms (Recombination Detection Programme (RDP), GENECONV, Max Chi, Chimaera, Si Scan and 3Seq). Recombination events detected by at least three algorithms in the reconstructed viral genomes were considered as significant. Viral sequences used for phylogenetic analyses were used for the detection of recombination.

TMV Inoculation to *Nicotiana* Species under Glasshouse Conditions

The TMV culture maintained as stock culture at Department of Plant Pathology, GKVK, Bengaluru was used for mechanical inoculation. The virus infected leaf samples were mechanically inoculated onto the leaves of *N. glutinosa* and *N. benthamiana*, as described earlier. The inoculated plants were maintained in an insect-proof glasshouse for symptom expression. Some plants were mock inoculated without the virus, which were used as a negative control.

RESULTS AND DISCUSSION

Mechanical Inoculation of TMV Isolate

TMV isolate obtained from chilli was successfully transmitted to *Nicotiana tabacum* plants. The inoculated plants shown systemic infection with expression mosaic mottling symptoms after ten days post inoculation of virus (Plate 1), which resembling those reported by Kumar *et al*. (2011). These

Plate 1 : Symptoms induced by the tobacco mosaic virus isolate on *Nicotiana tabacum*

symptoms arise due to TMV-induced disruption of chloroplast development and photosynthetic activity, which severely impacts plant health and productivity (Beachy, 1999 and Ding *et al*., 1996).

Total RNA isolated from *N. tabacum* was subjected for PCR amplification using TMV CP gene specific primers. The resulted PCR amplicon of 700 bp size was obtained (Plate 2) were cloned and sequenced. Molecular characterization of plant viruses allows for the precise identification of viral isolates and strains. It provides insights into the virus diversity, evolution and their pathogenic mechanisms (Barba *et al*., 2014). The obtained nucleotide sequence of coat protein region of TMV was compared with other selected

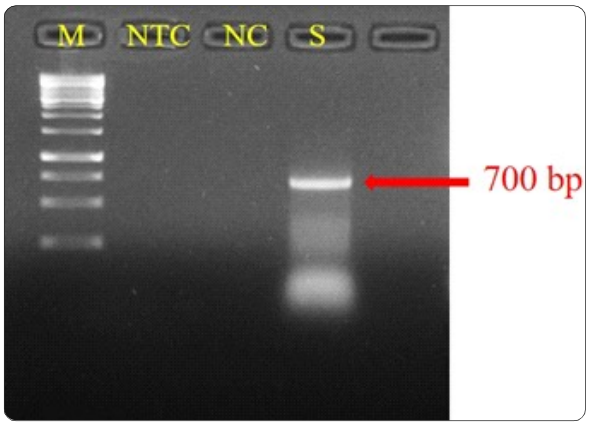

Plate 2 : Agarose gel showing PCR amplification of TMV coat protein gene from the leaf sample of *N.tabacum* inoculated with the TMV Bengaluru isolate. Lane M-Marker (1kb), NTC-Non-template control, NC-Negative control, S-Coat protein of TMV

tobamovirus isolates from GenBank database. The analysis revealed more than 95 per cent identity with several TMV isolates infecting different crops reported from various regions worldwide, including China, India, South Korea, France, USA, Germany, Brazil, Spain and Italy (Table 2). These results were also confirmed using Sequence Demarcation Tool are shown in Table 2 and Fig. 1.

Subsequently phylogenetic analysis of the TMV CP gene sequences with selected reference TMV isolates provided further evidence of a shared evolutionary origin or geographic linkage, as indicatedby close clustering with the tobacco mosaic virus isolate India (JQ895560.1) (Fig. 2). Our results are in alignment with the studies of Cherian *et al.* (1999) which revealed genetic variation among TMV isolates from different geographic regions contributing to TMV's adaptability and worldwide distribution. Kumar *et al.* (2013) focused on the characterization of TMV isolates in India and their relationships with global isolates. The TMV isolates from Southeast Asia, including Indonesia when compared with global sequences formed distinct clusters and subgroups within TMV populations, suggesting regional adaptations while maintaining global connectivity (Hidayat *et al*., 2019).

TMV isolate in the current study exhibited a high degree of genetic similarity to isolates from both European and Asian countries, indicating its global distribution and adaptability (Wang *et al*., 2016). Phylogenetic analysis helps to trace the evolutionary origins and relationships between different plant viruses, offering a deeper understanding of how viruses adapt to different plant species and environments (Gorbalenya and Lauber, 2017).

A neighbor-net network analysis (using Splits Tree version 4.11.3) was conducted for the TMV Bengaluru isolate, along with other retrieved sequences from NCBI. The analysis revealed a tree-like structure with straight branches, indicating the absence of potential recombination events (Fig. 3). This result was further validated by recombination break point analysis using

TABLE 2

TMV and other selected isolates available in the NCBI GenBank used in study for analysis

Accession No.	Virus	Strain/isolate	Host	Origin	Per cent Identity
JQ895560.1	TMV	India	*Glycine max*	India	99.72
HE818412.1	TMV	Beipiao	*Nicotiana tabacum*	China	99.58
HE818411.1	TMV	Changle-9	*Nicotiana tabacum*	China	99.30
HE818448.1	TMV	Xifeng	*Nicotiana tabacum*	China	99.30
HE818459.1	TMV	Zhenfeng	*Nicotiana tabacum*	China	99.16
HE818452.1	TMV	Xunyang	*Nicotiana tabacum*	China	99.02
AF395127.1	TMV	Fujian	Tobacco	China	98.75
HE818422.1	TMV	Fenggang-1	*Nicotiana tabacum*	China	98.75
HE818435.1	TMV	Ludian	*Nicotiana tabacum*	China	98.61
PQ032045.1	TMV	Sru-1	*Pennisetumpurpureum*	China	98.61
HE818449.1	TMV	Xingren-1	*Nicotiana tabacum*	China	98.33
AJ011933.1	TMV	Hangzhou	*Viciafaba*	China	98.19
OR082758.1	TMV	DSMZ PV	*Nicotiana tabacum*	Germany	98.19
HE818410.1	TMV	Chengjiang	*Nicotiana tabacum*	China	98.05
OK149218.1	TMV	Cpa	*Carica papaya*	China	98.05
NC_001367.1	TMV	Goelet	Tobacco	USA	98.05
JX993906.1	TMV	SXFQ	*Solanumlycopersicum*	China	98.05
MK087763.1	TMV	A	*Nicotianabenthamiana*	Spain	98.05
MH595919.1	TMV	Harbin-1	Tobacco	China	98.05
V01409.1	TMV	USA variant2	-	USA	98.05
AB369275.1	TMV	Pet	*Nicotianabenthamiana*	South Korea	98.05
HE818460.1	TMV	Guangyuan	*Nicotiana tabacum*	China	97.91
HE818442.1	TMV	Songtao-2	*Nicotiana tabacum*	China	97.91
OQ953825.1	TMV	France	Tobacco	France	97.91
HE818457.1	TMV	Yongren-1	*Nicotiana tabacum*	China	97.91
HE818432.1	TMV	Jianshi-2	*Nicotiana tabacum*	China	97.77
KF972427.1	TMV	Ancestor	Tobacco	Spain	97.77
HE818444.1	TMV	Tianzhu-1	*Nicotiana tabacum*	China	97.63
KF972430.1	TMV	WT-L3	Tobacco	Spain	97.63
HE818455.1	TMV	Yihan-1	*Nicotiana tabacum*	China	97.63
MN912489.1	TMV	Lages	*Physalis peruviana*	Brazil	96.52
HE818413.1	TMV	Bijie	*Nicotiana tabacum*	China	97.36
HE818447.1	TMV	Wuxi	*Nicotiana tabacum*	China	97.22
AB354955.1	TMV	Kyunggi	*Impatiens balsamina*	South Korea	97.08
ON156784.1	TMV	Ta9	*Nicotiana tabacum*	Italy	96.94
D63809.1	TMV	Saga	-	Japan	93.75
AF126505.1	TMV	IISc	*Solanumlycopersicum*	India	91.80
EU152113.2	TMV	Aligarh	*Solanumlycopersicum*	India	90.00
PQ271631.1	ToBRFV	FJ-1	*Solanumlycopersicum*	China	81.79
U34586.1	ORSV	Singapore 1	-	Singapore	71.30

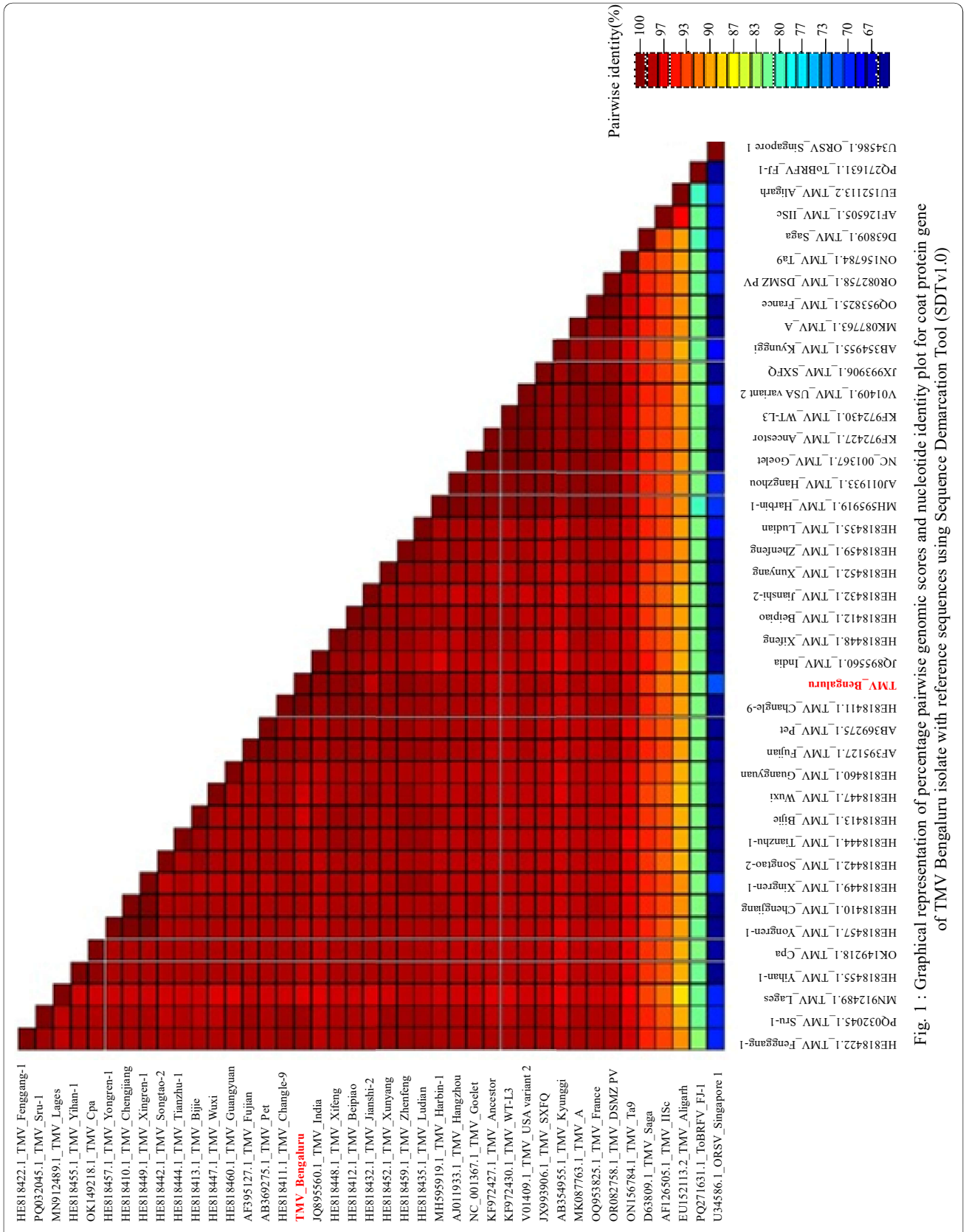

Fig. 1 : Graphical representation of percentage pairwise genomic scores and nucleotide identity plot for coat protein gene of TMV Bengaluru isolate with reference sequences using Sequence Demarcation Tool (SDTv1.0)

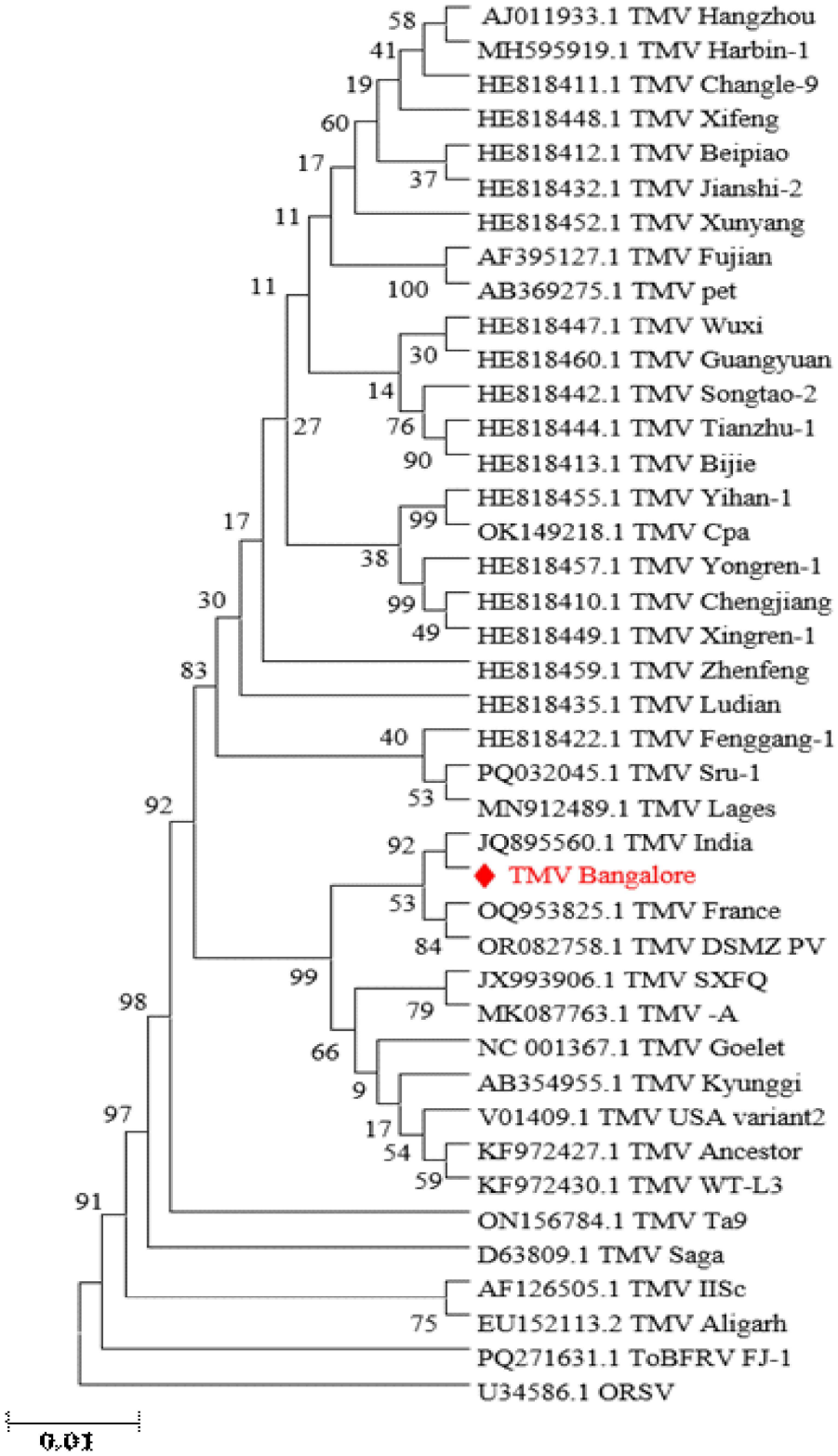

Fig. 2 : Phylogenetic tree constructed for coat protein gene sequence of TMV Bengaluru isolate along with other TMV isolates sequences retrieved from NCBI GenBank using Neighbor joining algorithm with 1000 bootstrap replicates. Horizontal distances are proportional to sequence distances; vertical distances are arbitrary

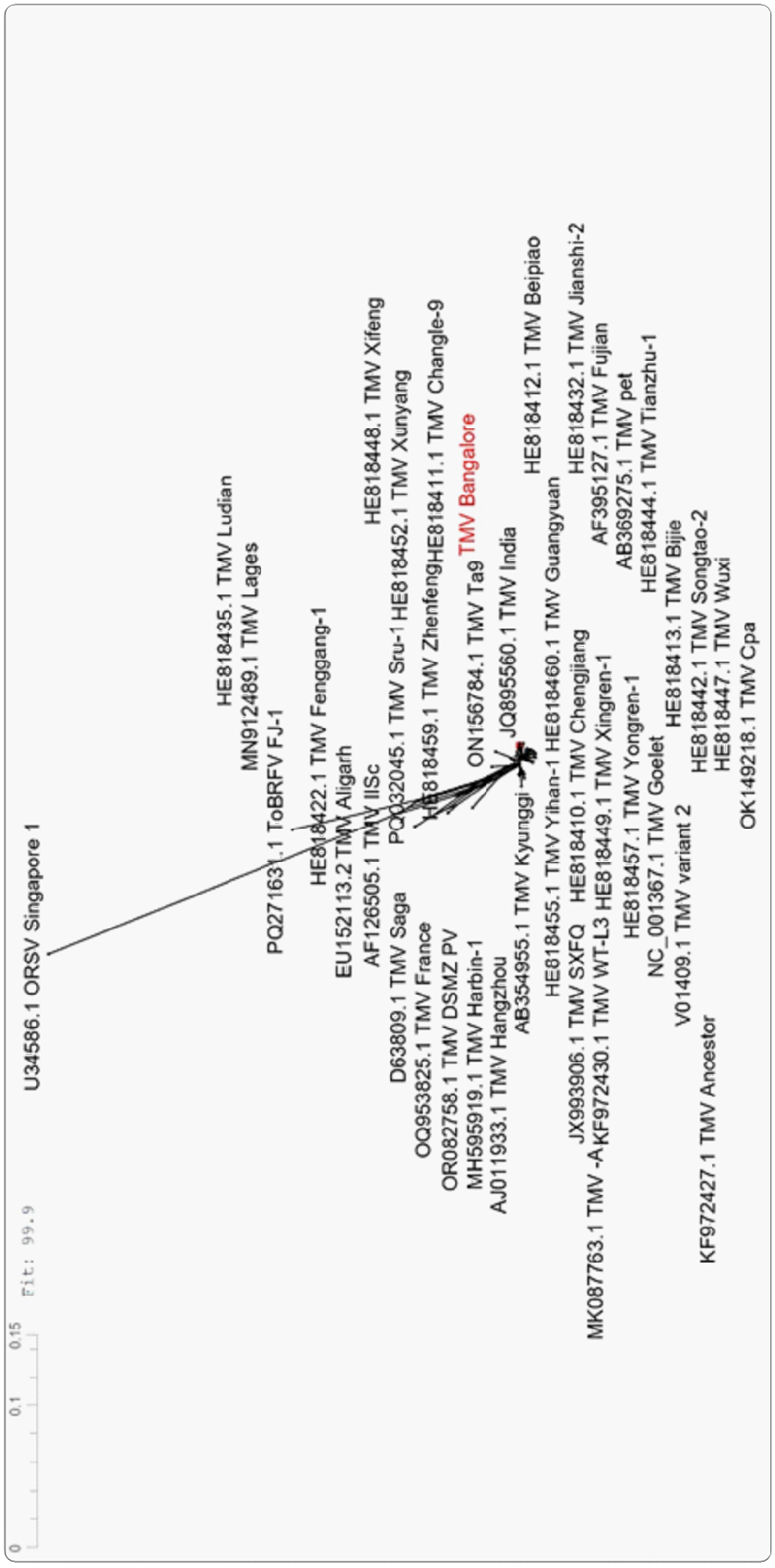

Fig. 3: A neighbor-net constructed for TMV Bengaluru isolate with other selected 40TMV isolates from NCBI database by using Split-Tree v5.3.0

Plate 3 : TMV Bengaluru isolate induced symptoms on *Nicotiana glutinosa* upon mechanical inoculation under glasshouse condition

RDP4 software, which also showed no recombination events. The absence of recombination in the TMV Bengaluru isolate suggests that the virus primarily evolves through mutation and selective pressure rather than genetic exchange with other viral strains. Fraile and Garcia-Arenal (2010) highlighted the evolutionary dynamics of plant viruses, emphasizing the limited role of recombination in TMV's genetic diversity. These findings supported the genetic stability of TMV and the rarity of recombination events in its evolutionary process (Gibbs and Ohshima, 2010).

Symptoms Induced by TMV on Different *Nicotiana* Species

The symptoms induced by TMV vary significantly among different *Nicotiana* species inoculated with the virus, reflecting the diverse interactions between the virus and its host plants. *N. glutinosa* exhibited a hypersensitive response to TMV inoculation, characterized by localized necrotic lesions at the site of infection (Plate 3). This species is recognized as local lesion host for the TMV, which is in correlation with Lewis *et al*. (2005). The hyper sensitive response effectively restricts viral movement, preventing systemic spread, showcasing a robust form of resistance mediated by the plant's genetic makeup (Holmes, 1938 and Dawson, 1999).

In *Nicotiana benthamiana*, TMV infection typically induced systemic symptoms such as chlorosis, mosaic patterns, leaf curling and stunted growth (Plate 4).

Plate 4 : TMV Bengaluru isolate induced symptoms on *Nicotiana benthamiana* upon mechanical inoculation under glasshouse condition

TMV is known to induce symptoms like mild curling and yellowing of leaves during the early stages of infection (Spitsin *et al*., 1999). The systemic nature of TMV symptoms in *N. benthamiana* is due to the virus ability to move *via* plasmodesmata and the phloem, infecting distant tissues after initial local replication. The viral movement protein and coat protein are critical for facilitating this spread, contributing to the characteristic systemic symptomatology (Beachy and Zaitlin, 1977).

The variation in TMV symptoms highlights the complex interplay between TMV and its host plants. It implicates the importance of host genetic factors in determining the outcome of infection, making it a valuable area of study for understanding viral pathogenesis and developing resistant crop varieties.

The present study underscores the molecular characterization and phylogenetic analysis of the TMV Bangalore isolateand its evolutionary relatedness to globally distributed TMV isolates. Symptom expression studies on *Nicotiana* species further supported the infectivity and host adaptation potential of the TMV Bangalore isolate. These findings highlight the genetic conservation of TMV CP sequences worldwide and emphasize the importance of continuous monitoring and molecular characterization of TMV isolates to better understand their evolution, host interactions and epidemiology for effective management strategies.

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