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MOLECULAR CHARACTERIZATION OF *CHILLI VEINAL MOTTLE VIRUS* INFECTING KING CHILLI (*CAPSICUM CHINENSE* JACQ.) OF NORTH EAST INDIAN STATE ASSAM

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Abstract– King chilli crop of Assam, India infected with Chilli veinal mottle virus (ChiVMV) belongs to genus potyvirus of the family potyviridae suffered from necrosis of leaves, leaf puckering, filiform leaves and eventually plant death. King chilli crop particularly suffers from decline complex due to high incidence of viral diseases. Prominent distribution of Chilli veinal mottle virus (ChiVMV) in King chilli growing region of Jorhat and Golaghat districts of Assam state were considered to study the prevalence and association with symptoms associated. Molecular indexing of samples from 30 chilli plantation orchards of Jorhat and Golaghat districts using reverse transcription (RT)-PCR targeting the coat protein (CP) region of ChiVMV genome revealed a high incidence and wide prevalence of the virus. Out of a total of 96 chilli samples collected from different orchards of Jorhat and Golaghat districts, 41 chilli samples (42.70%) were positive for ChiVMV. From Golaghat out of 34 samples 14 were found positive to ChiVMoV (41.17%), whereas in Jorhat out of 62 samples 27 were found positive to the virus (43.54%). Present study through the extensive surveys and molecular indexing work, conclusively reported the association of ChiVMV with diverse symptoms likemosaic/mottling, vein banding, leaf puckering /rugose/leaf distortion, narrow leaf lamina, shoe-string of leaves/leaf curling and stunted growth in King chilli. ChiVMV isolate from Jorhat, Assam were characterized based on sequencing of coat protein (CP) gene and phylogenetic analysis. Sequence analysis of CP gene showed that ChiVMV from Jorhat shared 100% identity with China isolates. This was reported as the first record of ChiVMVincidence in King chillicrop of Assam.

INTRODUCTION

Chilli (*Capsicum* spp.) is genetically highly bio-diverse in the region of North East (NE) India. Exceptionally aromatic taste and enhanced capsaicin content of North East India's chillies play a significant role in the economy of the rural people of this region. Kingchilli (*Capsicum chinense* Jacq.) is an interspecific hybrid chilli pepper occurring naturally as well as cultivated in Northeast India especially in Arunachal Pradesh, Assam, Nagaland and Manipur (Adluri *et al.* 2016). It is known by different vernacular names in various states of the region. It is indigenous to the Northeast region of India and possess a number of variants (Kumar *et al.,* 2011; Murmu *et al.* 2014) with different local names such as Naga chilli in Nagaland, Bhut Jolokia in Assam, and U-Morokin Manipur (Sanatombi *et al.,* 2010; Verma *et al.,* 2013). King chilli or Bhut jolokia in Assamese local language (*Capsicum chinense. L*), is considered as one of the hottest chilli in world is extensively grown in the fields of Assam. It is the fifth hottest chilli in the world rated at more than 1 million Scoville heat units (SHUs). India produces 1492.14MT of chillies annually with an average national productivity at 1.92MT/ha. The north eastern part of the country has an area of 43.10 thousand hectare under chilli with a production of 39.25 MT, of which a portion is contributed by Bhut jolokia (IHB, 2014). Here it is to be noted that although it is an important spice crop of north east India, it is mostly cultivated in an unorganized sector in different parts of the region. Therefore, there are no authentic estimates available on the area and production (Meghvansi *et al.,* 2010).

Owing to the high commercial potential of Bhut Jolokia, the crop is seen to easily infested by various diseases including fungal, bacterial as well as viral

diseases in the north eastern region. It has been observed that the infestation by viruses is highest about 60% as compared to fungal (10%) and bacterial infection (3%) in Assam (Talukdar *et al.* 2017). Different researches have shown over time that the crop is susceptible to different viruses which include Cucumber mosaic virus (CMV), Potato virus Y (PVY) (Talukdar *et al.,* 2015; Baruah *et al.*, 2016), Tomato Spotted Wilt Virus (TSWV) (Talukdar *et al.,* 2015), Chilli leaf curl virus (ChLCV) (Talukdar *et al.*, 2015; Baruah *et al.*, 2016) and Groundnut Bud Necrosis Virus (GBNV) (Baruah *et al.*, 2016). Bhut jolokia has also been found to be susceptible to Chilliveinal mottle virus as reported from Meghalaya (Sanabam *et al.,* 2018; Banerjee *et al.,* 2014). The plants are at times infected by a single virus or collectively by different viruses thereby forming a viral complex (Baruah *et al.,* 2016) which reduces the production and productivity to greater extent. Analysis of bhut jolokia samples collected from different regions of north east India has shown a higher incidence of Cucumber mosaic virus (CMV) around 37.97% and Chilliveinal mottle virus (ChiVMV) around 21.51% as compared to other begomoviruses and tospoviruses (Chanu *et al.*, 2017).

Most pepper viruses are distributed worldwide with the exception of *Chilli veinal mottle virus* (ChiVMV), *Pepper severe mosaic virus*, *Pepper veinal mottle virus* and *Pepper mottle virus*. These have been reported only in certain geographic areas. ChiVMVwas first reported on chilliin Malaysia (Ong and Ting, 1977). By far the largest is the genus *Potyvirus*, containing over 100 species which have particles >700 nm long and are transmitted by aphids in a non-persistent manner. The aphids, white fly and thrips are the major which besides sucking the sap of the plant parts, also act as vectors of virus diseases like mosaic and leaf curl due to which the crop suffers heavy losses (Singh *et al.*, 1998)

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 (Riechmann *et al.,* 1992). It is readily sap transmissible to a narrow range of hosts and is transmitted by aphid in a non persistent manner. The genome of ChiMV consists of a ploy A tail at 3′ end and its 5′ end is covalently linked to a viral VPg protein. The genome consists of two open reading frames which encode 11 proteins in which coat protein gene is required for virion assembly, cell-to-cell and systemic movements (Rojas *et al.,* 1997; Quenouille *et al.,* 2013). ChiVMV is mainly transmitted by aphids from one plant to another in a non-persistent manner (Ravi *et al.,* 1997). These factors make the viruses almost ubiquitous and contribute to the higher rates incidence.

Extremely limited information and experimental data are available on the occurrence of ChiVMV virus complex in bhut jolokia of Assam. Taking this background into account the proposed investigation was carried out in order to gain an understanding molecular detection and characterization of ChiVMV isolates associated with King chilles in Assam using RT-PCR technique coupled with sequence analysis. Along with this molecular charecterization an attempt was made to identify the virus associated with the characteristic symptoms.

MATERIALS AND METHODS

An overall pocket-wise King chilli orchard surveys were carried out to collect the symptomatic King chilli leaves from different orchards representing different locations of Jorhat and Golaghat districts of Assam covering valley areas, home stead gardens etc. during the year 2018-2019 (Table 1). The suspected plants in various locations were observed carefully and different types of symptoms in the infected plants were recorded. During the field survey leaves and young shoots of King chilli plants were examined for presence of insect vectors. Twenty plants were considered in a particular location and recorded aphid population as high (> 20 aphids/twig), medium (> 10 aphids/ twig) and low (<10 aphids/ twig). A total of 4-8 symptomatic samples were collected from each pockets surveyed and samples were stored in plastic bags and brought to the laboratory after which they were processed for virus testing. Collected symptomatic leaves were kept in RNA later and stored at '- 80 °C' for future purpose. 98 numbers of samples from 20 different locations covering Jorhat and Golaghat districts and brought to Plant Virology Laboratory, Department of Plant Pathology, AAU, Jorhat for rmolecular analysis of the leaf tissue samples using RT PCR tool.

Total RNA was isolated from Bhut Jolokia leaf tissue samples using standard Tri-Reagent method. Tri-Reagent method or commonly known as TRIzol method was carried out using RNAiso plus reagent from Takara Clonetech containing 38 per cent phenol. Total RNA from virus infected Bhut Jolokia

leaf tissue samples were used for reverse transcription. A 20 µl reverse transcription (RT) mixture was prepared by following the protocol of TaKaRaPrimeScript reverse transcription kit. 1µl of viral RNA was used in these reactions while sterile water was used in no template control. The RT mixture was reverse transcribed at 50 °C for 30 minutes and then at 70 °C for 15 minutes (Cool it in ice). The cDNA thus obtained was used for performing further PCR reactions. The cDNA thus obtained was subjected to PCR amplification using using 5' *CAGGAGAGAGTGTTATGCTG* 3' and 5' *TTTTTTTTTTTTTTTTAACGCCAACTATTG* 3' as forward primer and reverse primers respectively for detection of *ChiVMoV* (Chanu *et al.,* 2017). Thermocycling used for amplification of CP gene of ChiVMV one cycle of initial denaturation at 95 °C for 2 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at primer specific temperature for 1 minute, extension at 72 °C for 1 minute and 30 seconds and a final extension at 72 °C for 5 minutes. After completion of the PCR reaction all PCR amplicons were resolved on 1.5 % agarose gel in 1X TBE, stained with 0.06 µl/ml ethidium bromide and visualized under UV light in Gel documentation system (BIORAD). The amplified PCR product was send to Bioserve Biotechnologies India Pvt. Ltd. Hyderabad, India for sequencing. Sequencing was done in both directions using forward and reverse primers.

For defining symptomatic study of ChiVMoV infection in King chilli, mechanical inoculation of was done using standard method with celite powder as an abrasive onto King 30-35 days old king chilli seedlings. Inoculums were prepared by crushing infected King chilli leaves in phosphate buffer (0.2M,pH7.4) in 1:1w/vdilution, which was mechanically inoculated on three young healthy 30-35 days old king chilli plants at 2–3 leaf stages by rubbing. The inoculated leaves were washed with a jet of water to remove the traces of celite. Inoculated King chilli plants were maintained in insect-proof greenhouse under controlled conditions. All the inoculated plants were also tested for ChiVMoV infection using RTPCR tool. The plants were labelled and kept for observation under insect proof conditions and were observed at weekly intervals post- inoculation.

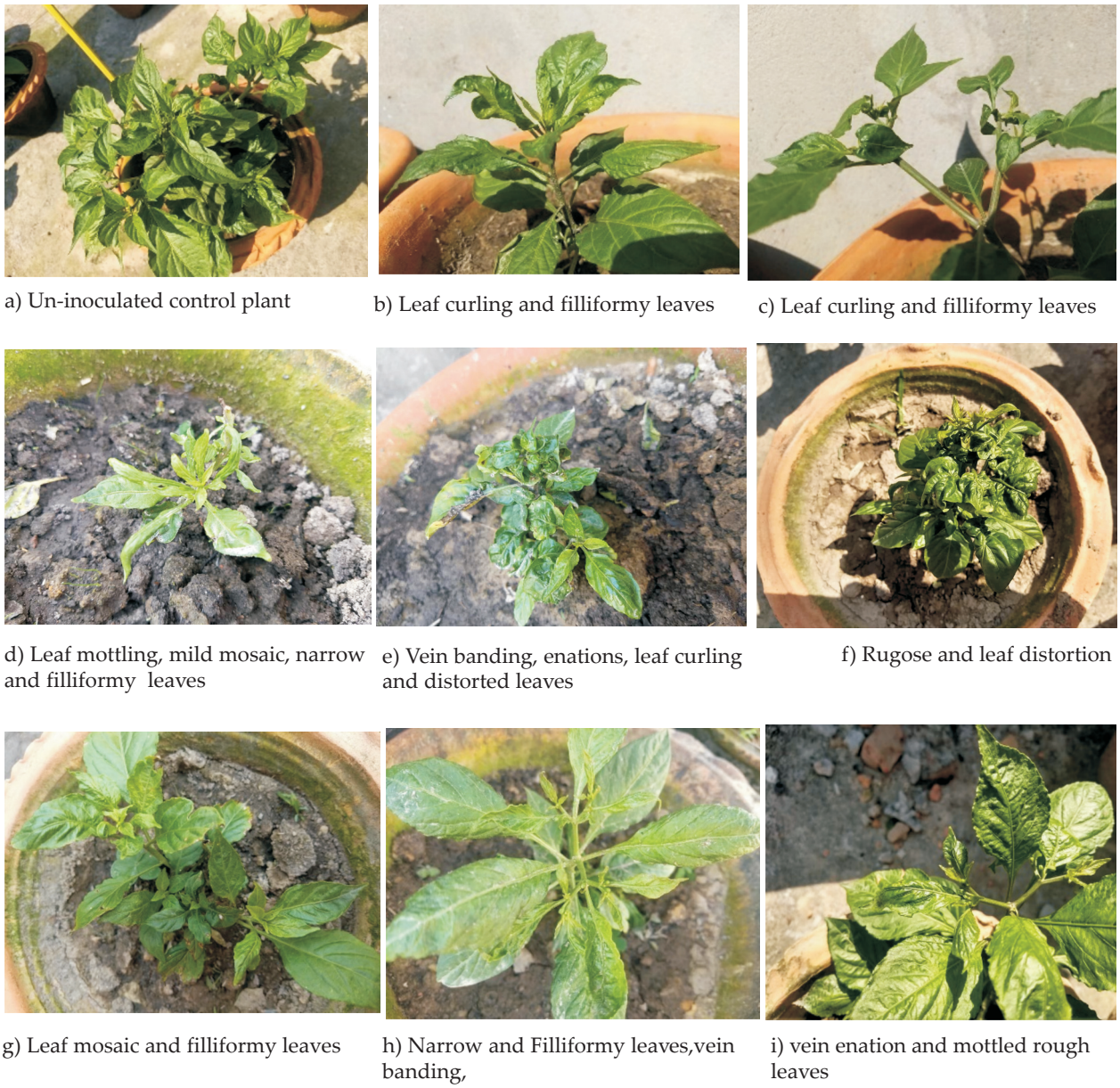
For analysis of genetic diversity of ChiVMV in Jorhat and Golaghat districts of Assam RT-PCR amplicons of ChiVMV isolates were sequenced. Basic local alignment search tool (BLAST) available in the NCBI (http://www.ncbi.nlm.nih.gov/)was used for putative identification of the obtained nucleotide sequences of genomic fragments of ChiVMVisolates. The consensus sequences obtained for each fragment were assembled using CLUSTAL X. The phylogenetic inference was drawn using 1160 bp-long conserved CP gene fragment of ChiVMV isolates employing MEGA6 software and the evolutionary distances were computed using the *p* distance method.

Study on specific symptoms associated with ChiVMoV

Research survey were led in different King chilli growing locations of Upper Brahmaputra Valley Zone specifically in Jorhat and Golaghat districts of Assam. Based on Typical symptoms viral disease infection viz. of leaf mottling and mosaic, leaf curling, puckering, shoe-string, vein banding etc. leaf tissue samples from all the locations were examined and recorded. In order to characterize the symptoms of selected representative ChiVMV infected King chilli symptoms biologically, isolates showing and found positive using RT PCR were mechanically inoculated to host (king chilli) plants. Twenty five numbers of king chilli samples inoculated representing different locations and symptoms were recorded following 20 days of inoculation. Inoculated plants producing symptoms were further confirmed by RT- PCR. Symptomatic king chilli leaf tissue samples collected from the green houses and observed virus specific amplification of 1160 bp in RT-PCR assay. No amplification was found in asymptomatic leaf samples collected from green house grown plants. Non- inoculated control plants did not show any symptoms (Fig1:a). Among the symptoms mild mosaic and mottling along with vein banding was very common. Leaf mottling along with mild mosaic, vein banding, leaf lamina narrowing and distortion of leaves were identified symptoms for ChiVMoV in the mechanically inoculated representative samples using molecular methods (Fig.1:b-i). In all the surveyed locations (Table 1) the prevalence of aphid vectors were observed.

Molecular detection

ChiVMV was successfully detected using reverse transcription PCR based method. ChiVMV was detected in 20 locations. A total of 96 numbers of



a) Un-inoculated control plant

b) Leaf curling and filliformy leaves

c) Leaf curling and filliformy leaves

d) Leaf mottling, mild mosaic, narrow and filliformy leaves

e) Vein banding, enations, leaf curling and distorted leaves

f) Rugose and leaf distortion

g) Leaf mosaic and filliformy leaves

h) Narrow and Filliformy leaves,vein banding,

i) vein enation and mottled rough leaves

Fig. 1. Veinal mottle symptom in mechanically inoculated King chilliplants

symptomatic and asymptomatic chilli leaf samples were tested for ChiVMV infection through RT-PCR using primers targeting coat protein (CP) region of viralgenomes, out of which 41 numbers of samples were found positive to ChiVMoV infection (Table 1). The study established the existence of ChiVMV in King Chilli plantation of Assam. The present study also conclusively reported the association of ChiVMV in bringing havoc to the chilli. The viral origin of specific amplicons was confirmed by sequencing.

Coat protein genomic sequence and phylogenetic analysis of ChiVMV Jorhat isolate

A partial coat protein genomic sequence of ChiVMV isolates was obtained by Reverse Transcrition PCR (RT-PCR) using gene specific primers. The coat protein genomic sequence of ChiVMV was determined to be 1160nt in length (Figure 2). The nucleotide homology ChiVMV jorhat, Teok and Golaghat isolates was deposited in gene bank (Gene bank Accession number MK584555.1, MW584703 and MW584704) and the results were analyzed. The

Table 1. RT PCR based detection of ChiVMoV in Jorhat and Golaghat districts of Assam

Sl. No.	District	Month of survey	Total samples	ChiVMV positive	ChiVMV negative	Symptoms	Insect vectors
1	Jorhat	Patiya Gaon	7	2	5	1,5,6****	**
2	Jorhat	Kasogoral Gaon	6	3	3	2,3	**
3	Jorhat	Hahsara	6	5	1	2,5,6	*
4	Jorhat	Sipahikhula	5	2	3	1,3	***
5	Jorhat	Khonamukh	3	1	2	3,6	**
6	Jorhat	Kaliapani Block	4	1	3	2,1	*
7	Jorhat	BongalPukhuri	4	2	2	5,4	**
8	Jorhat	Choladhara	3	1	2	6,1	**
9	Jorhat	Alengmora	6	1	5	4,2	***
10	Jorhat	Dogaon	4	1	3	6,5,1	**
11	Jorhat	Malouali	4	2	3	5,2,4	*
12	Jorhat	Club Road	5	2	3	4,1,3	**
13	Jorhat	Barbheta	5	4	1	3,2	*
14	Golaghat	Jamuguri	6	4	2	2,6,5	***
15	Golaghat	Herheri	5	3	2	1,3,2	***
16	Golaghat	Tenganigaon	6	2	4	5,3	***
17	Golaghat	No.1 Tamuli Gaon	5	1	4	6,1,5	*
18	Golaghat	Chubongaon	4	1	3	2,3,4	**
19	Golaghat	Bengenakhuwachariali	3	1	2	4,6	*
20	Golaghat	Budhbari	5	2	3	1,3.5	**

****Mosaic/mottling: 1; Vein banding:2; leaf puckering /rugose/leaf distortion:3; Narrow leaf lamina:4; Shoe-string of leaves/leaf curling: 5; Stunted growth: 6

(*) = Low, (**) = Medium, (***) = High

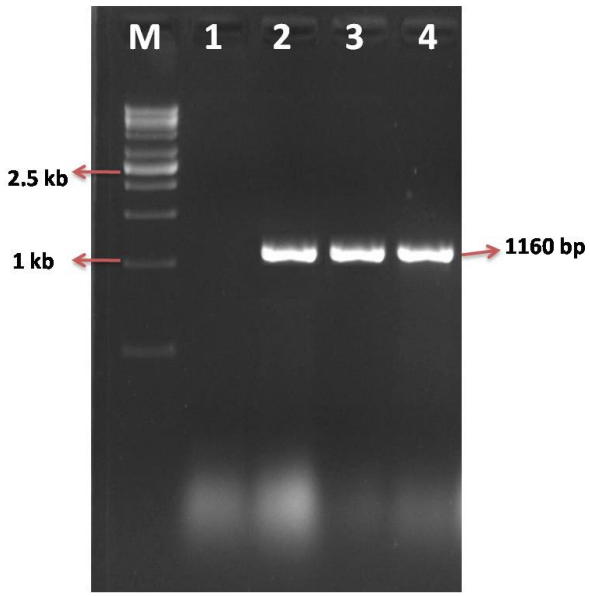


Fig. 2. Molecular detection and analysis of diseased Bhut Jolokia plants collected from Assam,India. Agarose gel electrophoresis showing amplification of 1160 nt CP gene, with lane M: 1 kb ladder, lane 1: healthy control, 2,3 and 4- ChiVMV isolates of Jorhat, Teok and Golaghat infecting Bhut Jolokia crop.

results analyze that nucleotide homology of coat protein gene of ChiVMV jorhat isolate showed the percent identity of 99-97% of ChiVMV isolates of North China. All the three isolates i.e., Jorhat, Teok and Golaghat are found to be in same cluster (Figure 3). ChiVMV Isolates of Assam (i.e Jorhat, Teok and Golaghat were found phylogenetically related to ChiVMV isolates from Liaoning, China (MG674070.1, MG674072.1, MG674073.1 and MG674075.1) forming one genogroup. The percent identity was 99.70-99.80 percent of these Liaoning, China isolates with ChiVMV, Jorhat isolate. The isolated ChiVMV samples from Assam is showing close relatedness with isolates of south-west china forming a different cluster but with close proximity (KC11056.1, HQ317867.1, MK405594.1….. KC693766.1 and HQ317868.1). Isolates from Bangalore was forming a different geno group isolated from Jorhat sequence 84.79-85 percent identity with ChiVMV Jorhat isolate. ChiVMV Isolate from Rawalpindi, Punjab (MF773493.1) was showing 90.52 percent identity with Jorhat isolate, but it is forming the same genogroup with isolates form Yunnan, China (JXD88636.1) whereas the isolates of Bengaluru, Karnataka and Maharastra are forming a different cluster.

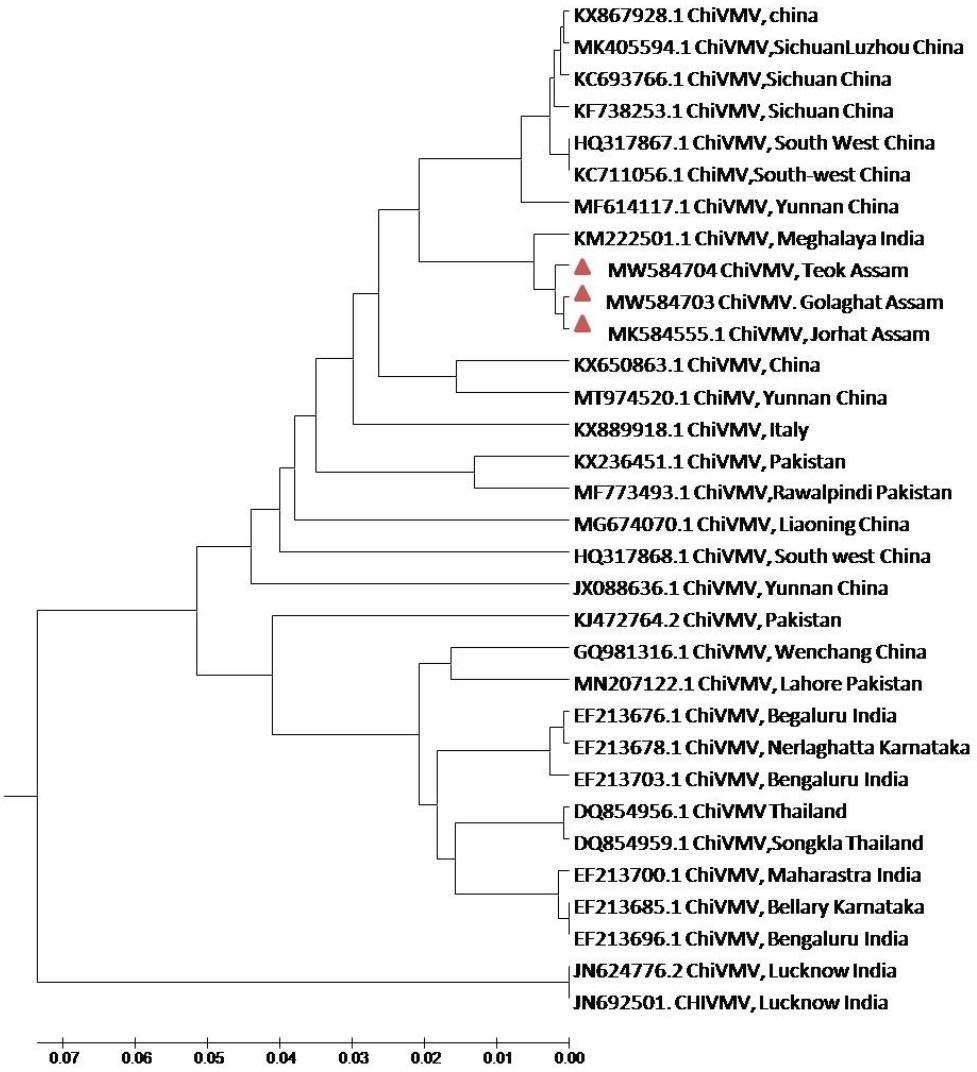


Fig. 3. Phylogenetic analysis of Jorhat, Golaghat and Teok isolates of Chilli veinal mottle virus

DISCUSSION

Association of ChiVMV with viral-like symptoms was earlier reported from different parts of India (Prakash *et al.,* 2002; Anindya *et al.,* 2004; Joseph and Savithri, 1999 and Ravi *et al.,* 1997). The virus incidence in Naga chilli is an emerging problem in North-east India. The current study confirmed the incidence of ChiVMV in Naga chilli. Disease symptoms such as mottling, vein banding, narrowing and distortion of leaves followed by stunted growth were observed in *C. chinense* plants growing in different king chilli fields of Assam. Among the viruses infecting chilli and sweet pepper, ChiVMV of the *Potyvirus* genus, CMV of the *Cucumovirus* genus, ChiLCV of the *Begomovirus* genus are considered to be the most important (Reddy and Reddy, 2010). Reports are available on the survey of viral disease incidence in Bhutjolokia from Assam based on symptomatology, transmission, host range and serological assays (Baruah *et al.,* 2016; Talukdar *et al.,* 2015, 2017). Molecular method have also been used for the detection and confirmation of viruses including ChiVMV (Banerjee *et al.* 2014) in Meghalaya and ChiLCV (Baruah *et al.* 2016) in Assam. Talukdar *et al.* (2015, 2017) reported the viral disease incidence on Bhutjolokia in Assam based on DASELISA to be 52%, 52% and 42% for *Cucumovirus*, *Potyvirus* and *Tospovirus* respectively. Baruah *et al.* (2016) also detected four viruses in Bhutjolokia viz., CMV, *Potato virus* Y (PVY), *Groundnut Bud Necrosis Virus*

Table 2. Coat protein genomic sequence accession numbers with locations used in phylogenetic analysis of ChiVMV Jorhat isolate

Sl.No.	Accession	Location	Percent identity with ChVMoV Jorhat
1.	MG674070.1	Liaoning, China	99.80
2.	MG674072.1	Liaoning, China	99.80
3.	MG674073.1	Liaoning, China	99.70
4.	MG674075.1	Liaoning, China	99.70
5.	LT899999.1	West Bengal, India	94.57
6.	LT900000.1	West Bengal, India	93.80
7.	KC711055.1	Sichuan China	93.66
8.	HQ317867.1	Sichuan China	93.87
9.	HQ317867.1	Sichuan China	93.87
10.	KF738253.1	Sichuan China	93.53
11.	MF773493.1	Rawalpindi Punjab	90.52
12.	HQ317868.1	Sichuan China	90.14
13.	EF213696.1	Bangalore, India	85.00
14.	EF213702.1	Bangalore, India	85.00
15.	EF213679.1	Bangalore, India	84.96
16.	EF213675.1	Bangalore India	84.88
17.	EF213684.1	Bangalore India	84.79
18.	MH373395.1	Manipur, India	87.24
19.	MH373396.1	Manipur, India	87.24
20.	MH373397.1	Manipur, India	87.24
21.	MH373398.1	Manipur, India	87.24
22.	MH373399.1	Manipur, India	87.24

(GBNV) and ChiLCV with disease incidence of 55.0%, 36.0%, 44.9% and 42.5%. Due to high level of viral disease incidence in all in all the cultivated locations, an attempt was made to identify the ChiVMoV associated with the crop. With biological assay a symptomatic study of ChiVMoV infection on King chilli crop was carried out along with an overall survey and detection of ChiVMoV in Jorhat and Golaghat districts of Assam. Similar study was carried out by Banerjee *et al.* 2014 reported ChiVMV infection on Naga chilli from Meghalaya indicating association of this particular virus in chilli decline in the North east India. High incidence of viral infection as recorded through visual observations of symptoms and recent report of ChiVMV infection on Naga chilli from Meghalaya indicated the possibility of its association with chilli decline in the NE region (Banerjee *et al.* 2014). Sanabam *et al.*, 2018 reported that, NE region being contagious to the Eastern Asian region provided a strong base for the possible prevalence of pathogenically and genetically diverse ChiVMV isolates. Previous researches revealed genetic heterogeneity among the characterized ChiVMV isolates using CP gene fragment might be due to the strong selective constraint on CP protein (King *et al.*, 2011). The ChiVMV isolates of North East Indian state Manipur grouped in a single genetic cluster which is corollary to earlier reports of the geographic adaptation being the reason for ChiVMV as reported earlier by geographic clustering of ChiVMV isolates from seven regions of Asia (Tsai *et al.*, 2008).

The overall clustering pattern of ChiVMV isolates from other parts of India and the remaining world indicated existence of high-genetic diversity even for conserved CP gene fragment used in the present study. ChiVMV isolates from Assam (Accession number MK584555.1, MW584703 and MW584704) segregated in a cluster which was very distinct to the cluster comprising of ChiVMV isolates China isolates. Study with the isolates from China indicated possible trans-boundary movement. Changing agro-climatic condition has been a major driving force in the distribution of insect vectors, resulting in certain diseases coming above the threshold level. Geographical location of North East India especially, presents a high chance of trans-boundary transmission of viral pathogen from neighboring areas. However, further full genome characterization of prevalent ChiVMV isolates from this region is to be done. Present study along with previous studies concluded that the ChiVMoV is widely associated with King chilli crop of Assam

with high degrees of disease incidence.

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