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# Molecular characterization of *Chilli veinal mottle virus* infecting king chilli (*Capsicum chinense* J.) in north eastern region of India

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**Abstract**
King chilli (*Capsicum chinense* Jacq.) is being grown in varied climatic conditions of North East region (NER) of India throughout. As chilli's having wide geographical distribution, king chilli is exposed to many pathogens and particularly to *Chilli veinal mottle virus* (ChiVMV) that limit its production. Present study, conclusively reported the 52.00-72.85% relative occurrence of the ChiVMV in NER India during 2019-20 and association of virus with different typical symptoms like cupping of leaf lamina and mottling, vein banding and puckering, shoe's string of leaf in king chilli samples. Further, two ChiVMV Manipur isolates upon mechanical inoculation showed significantly difference in symptom expression, indicating wide pathogenic diversity among them. Based on partial nuclear inclusion protein a (NIa) sequence, analysis of two ChiVMV isolates from Manipur indicated genetic homogeneity among them, but distinctiveness from ChiVMV isolates reported from the other parts of India and other countries. ChiVMV isolates from Manipur (MNP1 and MNP2) segregated to a distinct cluster and clustering nearest to the isolates from Taiwan (OK181760) and China (MT974520). The present finding may be helpful for characterization of virus and its sustainable management through the deployment of genetic resistance targeted against viruses.

**Keywords:** King chilli, *Chilli veinal mottle virus*, nuclear inclusion protein a North East India

## 1. Introduction
King chilli is an important commercial spice and vegetable crop of family *Solanaceae* (Nightshade family) and certified as one of the hottest chilli in the World by the Guinness World Records in 2006, measuring 1,001,304 Scoville Heat Unit (SHUs), beating the "Mexican red savanna habaneros" (5,77,000 SHUs) (Bosland *et al*., 2007) [2], and at the same time it possess a pleasant and palatable aroma. A number of landraces of this chilli are noted in the north-eastern (NE) region of India (Kumar *et al.*, 2011) with different local names such as Naga chilli in Nagaland, Bhut Jolokia in Assam and U-Morok in Manipur (Verma *et al*., 2013) [15]. Among the five cultivated species of *Capsicum*, *viz*., *Capsicum annum, Capsicum baccatum, Capsicum chinense, Capsicum frutescens* and *Capsicum pubescens*, Naga King Chilli belongs to species *chinense.* The existing climatic condition of the NE India has provides unique conformity with evolution and flourishing of diverse chilli landraces which are now preferably cultivated in the NE region. Since the NE region of India is recognized as a hot-spot for chilli diversity (Sarpras *et al*. 2016) [11].

Different landraces of king chilli grown in the state of NE India are infected by biotic stresses, particularly viral diseases, leading to reduction of production and productivity of king chilli in North East. The varied disease symptom expression in the chilli landraces and lack of exact identification of associated viral pathogens has been a hurdle in their management. Among the various potyviruses infecting chilli, ChiVMV is the most significant and destructive virus present throughout eastern Asia and in some African countries. The occurrence of ChiVMV in Naga chilli (*C. chinense* Jacq.) in Meghalaya of NE India was first reported by Banerjee *et al*. (2014) [1] based on mechanical transmission assay, transmission electron microscopy, RT-PCR and sequence analysis. The infectivity of ChiVMV has been reported from various countries including Korea (Ha *et al*., 2013) [3], Indonesia (Taufik *et al*., 2005) [13], Papua New Guinea (Davis *et al*., 2002), China (Wang *et al*., 2006) [16], West and East Africa (Womdim *et al*., 2001) [7]. ChiVMV is a positive-sense single-stranded RNA (+ssRNA) virus which belongs to the member of the genus *Potyvirus*, family *Potyviridae* (King *et al*., 2011) [4].

About 30% of the existing plant viral diseases is caused by potyvirus and its extent of infection is economically hazardous to various crops including pepper. The virus is transmitted by the aphid vectors in a non-persistent, non-circulative manner. The transmission to most host is promptly proficient by mechanical inoculation. However, it has not transmitted through seeds (Ng and Falk, 2006) [6].

Agro-climate of NE region is characterized by high humidity and prolonged rainfall which provide congenial conditions for the rapid growth and multiplication of aphid vectors, thereby providing an opportunity for infection round the year. Further, the level of diversity in pathogenic behaviour of ChiVMV prevalent in the NE region can be correlated with diversity at the genetic level. In the present study a number of virus infected king chilli were sampled from different king chilli growing region of Manipur and other parts of NE states and characterized biologically as well as by molecular methods.

## 2. Materials and Methods

The king chilli leaf samples were collected from randomly from different part of king chilli growing areas of the Manipur to collect the symptomatic and asymptomatic leaves in order to determine the incidence of ChiVMV based on molecular conventional PCR during 2019-2020. Fresh leaf samples of king chilli plants, exhibiting characteristic symptoms of viral infection like dark green mottling, mosaic, mottling, puckering, chlorosis, vein banding, shoe's string, leaf distortion and stunting were collected. Each sample from different plants were kept in samples zip bags in ice boxes during the survey, and later at 4 $^0$C in a refrigerator for molecular detection though RT-PCR.

### 2.1 Detection of ChiVMV infection through RT-PCR method in surveyed king chilli leaf samples

Total RNA was isolated from symptomatic and asymptomatic leaves samples were carried out using RNeasy plant Mini kit (Qiagen, Germany) and first strand complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase (Promega, Madison, USA) as per manufacturer's protocol. cDNA was synthesis by mixing, 8 µl RNA (~100 ng), µl of total RNA for each sample with 1 µl reverse primer (10µM) of ChiVMVNia-F and incubated at 70 $^0$C for 5 min. Then 6 µl 5X buffer (Promega, Madison, WI, USA), 6 µl nuclease free molecular grade water, 2.5 µl dNTP mix (2.5 mM each) (Promega, Madison, WI, USA), 0.5 µl RNasin® Ribonuclease inhibitor (Promega, Madison, WI, USA) and 1 µl M-MLV reverse transcriptase (2500U) (Promega, Madison, WI, USA) were added to the mixture of primer and RNA. The reaction mixture was incubated at 42 $^0$C for 60 min. The cDNA was stored at −80 $^0$C for further analysis.

The PCR reaction was performed using ChiVMV specific primer pairs ChiVMVNia-R/F (Accession no. AJ237843) to amplify a 1050 bp region of Nuclear Inclusion A Protein (NIa) of ChiVMV. 25 µl of reaction mixture containing 2.5 µl of 5x taq buffer, 1 µl of Mgcl2, 1µl dNTP mix (2.5 Mm each), 1µl each of forward primer and reverse primer and 0.5 µl of Taq. Polymerase and sterile distilled water to make up the volume. Thermocycling used for amplification of NIa gene of ChiVMV were 94 $^0$C for 4 min followed by 30 cycles of 94 $^0$C for 1 min, 56 $^0$C for 1 min 20 s and 72 $^0$C for 1 min 20 s with final extension of 72 $^0$C for 10 min. The resulting RT-PCR products were electrophoresed in 1.8% agarose gel containing ethidium bromide and visualized under UV illumination.

King chilli samples exhibiting different symptoms of viral infection were detected by RT-PCR-based indexing using the ChiVMV specific primer pairs ChiVMVNia-R/F in order to determine the pathogenic diversity. Infected chilli leaves sap of selected isolates in phosphate buffer (0.2 M, pH 7.4) in 1:2 w/v dilution, was mechanically inoculated on five-six young healthy chilli plants at 2–3 leaf stages by rubbing carborundum powder as an abrasive to study the pathogenic diversity. Then, inoculated plants were maintained in insect-proof glass house at a temperature of 18–24 $^0$C with relative humidity of 75–80% and observed symptoms developed after 2 to 28 days post-inoculation. The different symptomatic king chilli samples from different king chilli growing groves of Manipur were selected for analysis of genetic diversity of ChiVMV isolates. Purified amplified products of NIa gene from each isolate were sequenced (Promega, USA) using forward and reverse primers from Eurofins, Banglore and GCC, Kolkata, India and obtained sequences were assembled for further analysis.

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 ChiVMV isolates. The consensus sequences obtained from each fragment were assembled using CLUSTAL X. By using Bioedit Sequence Alignment Editor 7.1.3 (Hall, 1999), Sequence identity matrix for a pairwise combination of aligned sequences was calculated. The Phylogenetic inference was drawn using MEGAXI based on the clustering of similar sequences to determine the evolutionary relationship. The evolutionary distances were computed using the p-distance method. Trees were constructed with phylogeny test by bootstrapping 1000 replicates. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993) [12].

## 3. Results

Typical symptoms of yellow mosaic, leaf mottling, puckering, shoe-string, vein banding, and severe curling of leaves were observed in king chilli plants during the survey (Fig.1). The ChiVMV disease incidence of about 52.00-72.85% was found among the different locations surveyed. Since, the present study was made to identify the virus associated with the observed typical symptoms. All the tested symptomatic king chilli samples showed positive infection of ChiVMV in RT-PCR assay with amplification of partial sequence of 1050 bp and no amplification in asymptomatic leaf samples collected from different regions of NE India.


**Fig 1:** Symptomatic diversity of ChiVMV as evident from diverse symptoms observed at different groves of Manipur, **a** infected king chilli samples from Sekta, Imphal east district, **b** infected king chilli samples from Sirarakhong, Ukhrul district

Biological transmission studies on king chilli revealed the pathogenic diversity among the selected 2 ChiVMV isolates (ChiVMV-MNP1 and MNP2) with the symptom of thinning of lamina, leaf deformation with shoestring after 19-24 days of inoculation. Positive amplification of 1050 bp was also observed in mechanically inoculated king chilli. Symptom expression of two ChiVMV isolates (ChiVMV-MNP1: Sekta, Imphal East district and ChiVMV-MNP1: Sirarakhong, Ukhrul district) showed their different pathological characters. ChiVMV-MNP1 showed the symptoms of leaf deformation, thinning of lamina and shoestring. Symptoms of leaf puckering and mild dark green islands produced by isolate ChiVMV-MNP2. This indicated pathogenic diversity of ChiVMV isolates prevalent in the king chilli groves of Manipur (Fig.2 and 3).

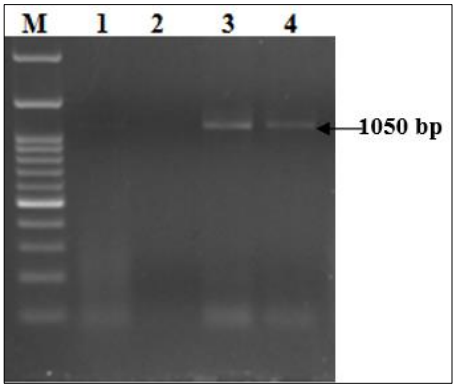


**Fig 2:** RT-PCR detection of ChiVMV in infected king chilli plant sample using ChiVMV- primer pair (ChiVMVNia-R/F), where, M: 100 bp GCC ladder, Lane 1,2: Healthy plant sample and Lane 3 and 4:ChiVMV infected plant sample

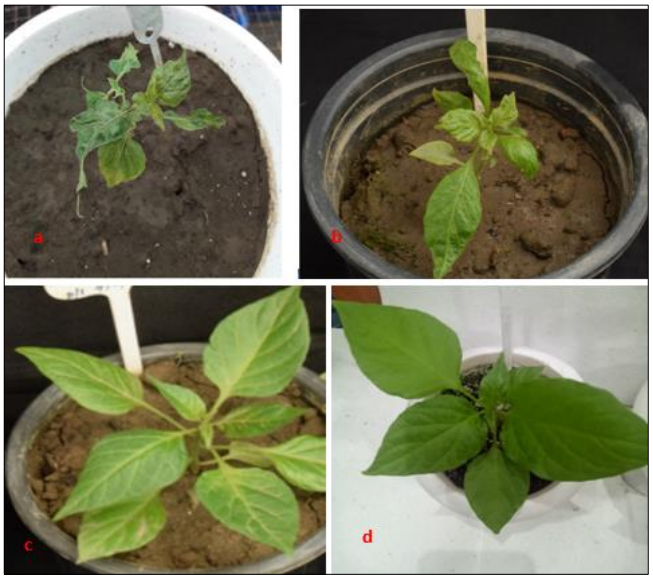

**Fig 3:** Pathogenic diversity of ChiVMV isolates as studied by mechanical inoculation of selected isolates on king chilli. a Symptoms symptoms of leaf deformation, thinning of lamina and shoestring produced by isolate ChiVMV-MN1: Sekta, Imphal East district b. Symptoms of leaf puckering and mild dark green islands produced by isolate ChiVMV-MNP2: Sirarakhong, Ukhrul district, c mock-inoculated king chilli plant; d un-inoculated healthy king chilli plant. Symptoms observed in the photographs were after 24 days post-inoculation

### 3.1. Genetic diversity of ChiVMV isolates from Manipur

ChiVMV isolates (MNP1 and MNP2) were characterized for their genetic diversity based on the 1050 bp region of nuclear inclusion A protein fragment. These two Manipur isolates shared maximum similarity of 89-90.09% nucleotide identity with other ChiVMV sequences available in GenBank database. Maximum Likelihood method phylogenetic analysis of these ChiVMV isolates with the earlier reported isolates from India and other parts of the world showed genetically distinct from other isolates. ChiVMV isolates from Manipur (MNP1 and MNP2) segregated to a distinct cluster (which were genetically homogenous within the cluster), nearest to which were the isolates from Taiwan (OK181760) and China (MT974520) (Fig. 4). The analysis indicated genetic homogeneity among ChiVMV isolates occurring in Manipur region of NE India but distinctiveness from ChiVMV isolates reported from the other parts of India.

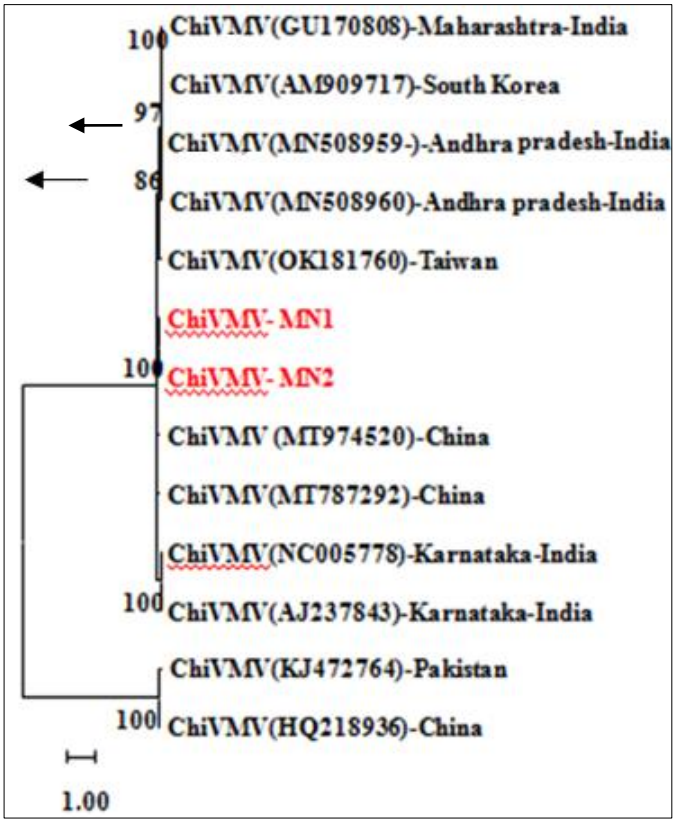


**Fig 4:** Phylogenetics tree based on partial nucleotide sequences of NIA gene (1050 bp) of two Manipur isolates of ChiVMV (ChiVMV-MNP1 and MNP2: marked with reddening) with other ChiVMV isolates from India and other parts of the world. Phylogenetic inference was drawn using Maximum Likelihood method in Mega XI software. The bootstrap of 1000 replicates values are showed next to the branches when less than 5%. the scale bar represents a genetic distance of 1.00

## 4. Discussion

The rich biodiversity of chilli in North East region of India has a significant contribute to chilli genetic resources of the country. The region have unique landraces which led it to be described as hot spot of chilli biodiversity (Purkayastha *et al*. 2012) [9]. Biotic constraints particularly symptoms akin to viral infection has long been recorded as a major hurdle to the successful production of these chillies in the region. High incidence of ChiVMV was recorded through visual observations of symptoms which could be conclusively indicated the association of ChiVMV with decline of chilli plantation. Prakash *et al*. (2002) [8] reported the association of

ChiVMV with viral-like which was agreed with the present study. The wide diversity of pathogenic characteristic of ChiVMV isolates from Manipur in present finding is similar with the earlier finding of (Tsai *et al*. 2008) [14]. The present study reported that ChiVMV isolates from Manipur region of NE India segregated in a cluster which was different from the cluster comprising of ChiVMV isolates from other parts of India and the remaining world. Sanabam *et al*., (2018) [10] indicated genetic homogeneity of five ChiVMV isolates from Manipur based on partial coat protein (CP) sequence analysis, but distinctiveness from ChiVMV isolates reported from the other parts of India.

## 5. Conclusion

King chilli is exposed to many biotic stress, particularly to *Chilli veinal mottle virus* (ChiVMV) that limit its production. The accurate detail of viral disease complex in king chilli in Manipur remains unknown. Since, there is prerequisite requirement of identifying and characterization the king chilli associated viruses Present study, conclusively reported the 52.00-72.85% of wide occurrence of the ChiVMV in NER India. Further, analysis of two ChiVMV isolates from Manipur indicated genetic homogeneity among them, but distinctiveness from ChiVMV isolates reported from the other parts of India and other countries. The present finding may be helpful for characterization of virus and its sustainable management through the deployment of genetic resistance targeted against viruses.

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