Genetic diversity of *Chilli veinal mottle virus* infecting different chilli landraces in North East India indicates the possibility of transboundary movement of virus

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Abstract

Diverse chilli genetic resources of North East India occupy a unique niche in the Nation's chilli gene pool. Widely cultivated chilli landraces (King chilli: *Capsicum chinense*, bird eye chilli: *Capsicum frutescens* and *Capsicum annuum*) of North East India in general and Manipur in particular suffer from decline complex due to high incidence of viral diseases. With this background and the known prominent distribution of *Chilli veinal mottle virus* (ChiVMV) in Asian region, we studied its prevalence and association with diverse symptoms of chilli landraces. Molecular indexing of samples from 40 chilli plantation groves of Manipur using reverse transcription (RT)-PCR targeting the coat protein (CP) region of ChiVMV genome revealed a high incidence and wide prevalence. Out of a total of 127 chilli samples collected from different groves of Manipur, 81 chilli samples (63.78%) were positive for ChiVMV. Interestingly, ChiVMV infection rate was comparatively higher in the hilly groves (69.23% samples positive) compared to the valley groves (60% samples positive). Present study through the extensive surveys and molecular indexing work, conclusively reported the association of ChiVMV with diverse symptoms like cupping of leaf lamina with mottling, vein banding and puckering in different chilli landraces. Further, five representative ChiVMV isolates sampled from different groves of Manipur upon mechanical inoculation showed significant variation in symptom expression, indicating wide pathogenic diversity among them. Partial coat protein (CP) sequence analysis of five ChiVMV isolates from Manipur although indicated genetic homogeneity among them, but distinctiveness from ChiVMV isolates reported from the other parts of India. Phylogenetic clustering of ChiVMV isolates from Manipur near Chinese isolates rather than other Indian isolates suggested the possibility of transboundary movement.

Keywords *Chilli veinal mottle virus* · King chilli · Pathogenic diversity · North East India

Introduction

Chilli (*Capsicum* spp.) has high genetic diversity in the bio-diverse region of North East (NE) India. Unique aroma, taste and enriched capsaicin content of NE India's chillies (Sarpras et al. 2016) play a prominent role in the rural economy of this region in general and Manipur in particular. Soil physiography and climatic condition of the NE India has unique conformity with evolution and flourish of diverse chilli landraces which are now preferably cultivated in the region. King chilli (*Capsicum chinense*), known

as *Umorok* in Manipuri language is popular for its unique aroma, taste, high capsaicin content and thus recognized as one of the hottest chillies on the planet (Bosland and Baral 2007). Different variants of *Capsicum annuum* (landrace *Morok atekpa*), bird eye chilli (*Capsicum frutescens*) landraces viz. *Uchi-morok*, *Leihao-morok* and *Chingpi-morok* (synonymous *Morok-masingkha*) also comprise of other economically important chilli genetic resources of the NE region. The biotic stresses, particularly viral diseases, are often a major limitation in the chilli plantation being the major cause of lower productivity. Varied disease symptom expression in the chilli landraces and lack of exact identification of associated viral pathogens has been a hurdle in their management. Infection of *Cucumber mosaic virus* (CMV) on chilli from Darjeeling hills (Biswas et al. 2005) and *Potyvirus*, *Cucumovirus*, *Tospovirus* from Assam (Talukdar et al. 2017) has been reported using serological techniques,

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however, their exact molecular identity is not known. Based on the sequencing of a genomic fragment, association of chilli leaf curl virus (CLCuV) with *Capsicum chinense* was also reported (Adluri et al. 2016). Recently, first authentic molecular evidence for the infection of *Chilli veinal mottle virus* (ChiVMV) in *Capsicum chinense* from low to moderate temperature geographical areas of NE India was provided (Banerjee et al. 2014). ChiVMV was suspected to be responsible in bringing havoc in the chilli plantations in Manipur also, where similar low to moderate temperature conditions prevails. ChiVMV belong to the genus *Potyvirus*, which comprised of more than 30% of known plant viruses (King et al. 2011). Disease symptom associated with ChiVMV infection, i.e., mottle and mosaic was first reported from Malaysia in 1947 (Brunt et al. 1996). Its infection has been reported from various countries including Korea (Ha et al. 2013), Indonesia (Taufik et al. 2005), Papua New Guinea (Davis et al. 2002), China (Wang et al. 2006), West and East Africa (Womdim et al. 2001). ChiVMV is naturally transmitted by different species of aphid vectors in a non-persistent manner. 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 this study, an attempt was made to study the prevalence of ChiVMV in different chilli landraces of NE India (Manipur region) and analysing their pathogenic and genetic diversity, which will have applications in routine indexing of the chilli saplings and developing a management strategy.

Materials and methods

Extensive grove-wise surveys were conducted to collect the symptomatic and asymptomatic leaves from different chilli groves representing different locations of Manipur covering hills slopes, valley areas, home gardens, poly houses, etc. (Table 1). A total of 127 numbers of samples from 40 chilli growing groves covering all the district of Manipur were collected and brought to laboratory for further testing. Different chilli samples which were indexed include king chilli (landrace *Umorok*), bird eye chilli (landraces *Uchi-morok*, *Leihao-morok* and *Chingpi-morok*) and *Capsicum annum* (landrace *Morok atekpa*). Collected samples were cryopreserved at -80°C . Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's instructions. First strand complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase (Promega, Madison, USA) using total RNA as template and antisense primer, targeting the coat protein region of *Chilli veinal mottle virus*. Reverse

transcription (RT)-PCR amplification of coat protein (CP) gene of ChiVMV genome was performed using primer pair reported by Zhao et al. (2014). Thermocycling used for amplification of CP gene of ChiVMV were 95°C for 5 min followed by 30 cycles of 94°C for 45 s, 59°C for 1 min 50 s and 72°C for 2 min with final extension of 72°C for 10 min.

To determine the pathogenic diversity, chilli samples exhibiting diverse symptoms of viral infection were subjected to RT-PCR-based indexing using the ChiVMV-specific primers (Zhao et al. 2014). The results of RT-PCR indexing were correlated with symptom diversity. For determining the pathogenic diversity, mechanical inoculation of selected isolates was done using standard method with carborundum powder as an abrasive. Inoculums were prepared by crushing infected chilli leaves in phosphate buffer (0.2 M, pH 7.4) in 1:2 w/v dilution, which was mechanically inoculated on six young healthy chilli plants (2–3 leaf stages) by rubbing. Inoculated chilli plants were maintained in insect-proof glass house at a temperature of $18\text{--}24^{\circ}\text{C}$ with relative humidity of 75–80%. Symptoms were recorded from 2 to 28 days post-inoculation. The initial inoculum was tested free from the cross-contamination of other chilli viruses using PCR techniques. All the inoculated plants were also tested for other non-targeted viruses using PCR.

For analysis of genetic diversity of ChiVMV isolates, five isolates originating from different chilli groves of Manipur, both valley and hills (ChiVMV-Mnp1: Kakyai, Bishnupur district; ChiVMV-Mnp2: Shantipur, Imphal West district; ChiVMV-Mnp3: Sirarakhong, Ukhrul district; ChiVMV-Mnp4: Langathel, Thoubal district; ChiVMV-Mnp5: Lukhambi, Tamenglong district) sampled from symptomatic king chilli and bird eye chilli were selected. These isolates were found exhibiting distinct pathogenic behaviour. RT-PCR amplicons representative of different ChiVMV isolates were purified and 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 ChiVMV isolates. The consensus sequences obtained for each fragment were assembled using CLUSTAL X. The phylogenetic inference was drawn using 432 bp-long conserved CP gene fragment of ChiVMV isolates employing MEGA 6 software (Tamura et al. 2013) and the evolutionary distances were computed using the *p* distance method. Trees were constructed with phylogeny test by bootstrapping 1000 replicates.

Results

High incidence of ChiVMV

Amplification of 1166 bp genomic fragment of ChiVMV (comprising of CP gene) in RT-PCR from the symptomatic

Table 1 RT-PCR-based detection of ChiVMV in the samples collected from different chilli groves

Sl no.	Locations	District	Chilli cultivar ^a	Number of samples	ChiVMV positive	ChiVMV negative
1	Sinam village	Imphal East	KC (2)	2	—	2
2	Ishikha	Imphal East	KC (3)	3	2	1
3	Maiba Khul	Imphal East	KC (3)	3	—	3
4	Sambei	Imphal East	KC (2)	2	2	—
5	Sil-heipung	Imphal East	KC (3)	3	3	—
6	Uchekon Takhok	Imphal East	KC (2)	2	—	2
7	Sailen village	Imphal East	KC (2) + MA (1)	3	3	—
8	Andro	Imphal East	KC (2) + MA (2)	4	2	2
9	Danjan/Wakan	Imphal East	KC (3) + MA (3)	6	6	—
10	Maklang	Imphal West	KC (4)	4	4	—
11	Iramsiphai	Imphal West	KC (1)	1	1	—
12	Lamlongei	Imphal West	KC (2)	2	1	1
13	Mayang Imphal	Imphal West	KC (4) + MA (1)	5	—	5
14	Shantipur	Imphal West	KC (4)	4	3	1
15	Uchiwa Huiyen	Thoubal	KC (1) + LM (1)	2	2	—
16	Kakching Lamkhai	Thoubal	KC (4)	4	3	1
17	Nongpok Sekmai	Thoubal	KC (2) + LM (1)	3	—	3
18	Nungthar	Thoubal	KC (2)	2	—	2
19	Chandrakhong	Thoubal	KC (4)	4	2	2
20	Keipharok	Thoubal	KC (4)	4	—	4
21	Langngathel	Thoubal	KC (3) + MA (2)	5	5	—
22	Kakyai	Bishnupur	KC (3)	3	3	—
23	Ngarian	Bishnupur	KC (3)	3	2	1
24	Keinao	Bishnupur	KC (1)	1	1	—
25	Phunal Maring	Chandel	KC (2)	2	2	—
26	Lukhambi	Tamenglong	KC (2) + UM (2)	4	4	—
27	Leimatak	Tamenglong	KC (4) + MA (2)	6	4	2
28	Molkon	Senapati	KC (2) + UM (2)	4	3	1
29	Paodel Basti	Senapati	KC (2)	2	—	2
30	Kotlen	Senapati	KC (2) + MA (3) + UM (2)	7	4	3
31	Santing	Senapati	KC (3)	3	—	3
32	Thumnaopokpi	Senapati	KC (1)	1	1	—
33	Sirarakhong	Ukhrul	KC (4)	4	4	—
34	Boharam	Ukhrul	KC (1)	1	1	—
35	Yeosam	Ukhrul	KC (2) + UM (2)	4	3	1
36	Singngat	Churachandpur	KC (2) + MM (5)	7	5	3
37	Mualtam	Churachandpur	UM (3)	3	3	—
38	Lamzang	Churachandpur	UM (1)	1	1	—
39	Ijeizog	Churachandpur	KC (1)	1	—	1
40	Phaimol	Churachandpur	KC (1) + MA (1)	2	2	—
Total				127	81	46

^aSampled chilli cultivars are mentioned as KC, king chilli/*Umorok* (*Capsicum chinense*); MA, *Morok atekpa* (*Capsicum annuum*); LM, *Leihao-morok* (*Capsicum frutescens*); UM, *Uchi-morok* (*Capsicum frutescens*); MM, *Morok-masingkha* (*Capsicum frutescens*). The numbers in parenthesis represents the number of samples of respective cultivar

chilli samples confirmed the association of ChiVMV (Gel image not shown). Seasonal surveys indicated, high infection rate of ChiVMV during the period of July–November

(period spanning from rainy season to just before the chilling winters). During chilling winters and pre-monsoon months, the infection rate of ChiVMV under field conditions was

less. All the tested chilli landraces showed positive infection of ChiVMV in RT-PCR although exhibited differences in the symptomatic expression (Fig. 1). The bird eye chilli landraces were observed to exhibit mild symptoms as compared to king chilli and other landraces under field conditions. A total of 127 chilli leaf samples were tested, out of which 81 samples (i.e., 63.78% of the total sample) were found positive for ChiVMV infection. The present findings showed high prevalence of ChiVMV in major chilli growing areas of Manipur (Table 1). Interestingly, ChiVMV infection rate was comparatively higher in the hilly groves (69.23% samples positive) compared to the valley groves (60% samples positive). This indicated the comparatively high infection of ChiVMV in the hills (areas with low temperature regimes).

Pathogenic diversity of ChiVMV infection

Varied symptoms of ChiVMV infection in different chilli landraces were observed in the present study. ChiVMV-infected chilli leaf samples prominently showed symptoms of shoestring, vein banding, narrowing and cupping of leaf lamina (Fig. 1). Neglected chilli plantations from different locations were observed to exhibit the characteristic symptoms of small leaves (Fig. 1) and overall stuntedness of plants. King chilli plantations at various sites showed more severe and diverse symptoms due to ChiVMV infection. ChiVMV-infected king chilli plants at different

locations showed the symptoms of severe puckering, thinning, shoestring and cupping of leaf lamina in addition to severe mottling and green–yellow mosaic. Infected king chilli plants were stunted as compared to healthy plants (Fig. 1). ChiVMV infection was also observed on bird eye chilli plants (Fig. 1) with varied symptom severity. Difference in symptomatic expression was recorded in bird eye chilli samples with prominent symptoms of thinning of lamina in samples from Molkon, Senapati district while mild puckering was the most prominent symptoms in the samples collected from Lamzang, Churachandpur district. Interestingly, the king chilli plants exhibiting the symptoms of green white mosaic leaf lamina were not having ChiVMV infection which might be due to the infection of other pathogens (data not shown). Biological transmission studies on king chilli revealed the pathogenic diversity among the selected five different ChiVMV isolates (ChiVMV-Mnp1 to Mnp5) with most isolates exhibiting the symptom of thinning of lamina and leaf deformation. Symptom expression of these five ChiVMV isolates collected from different chilli groves of Manipur (ChiVMV-Mnp1: Kakyai, Bishnupur distt, ChiVMV-Mnp2: Shantipur, Imphal West distt, ChiVMV-Mnp3: Sirarakhong, Ukhrul distt, ChiVMV-Mnp4: Langathel, Thoubal distt, Manipur and ChiVMV-Mnp5: Lukhambi, Tamenglong distt) revealed their distinctive pathological behaviour (Fig. 2). ChiVMV-Mnp1 produced typical shoestring and thinning of leaf lamina. Isolates

Fig. 1 Symptomatic diversity of ChiVMV as evident from diverse symptoms observed at different groves of Manipur, **a** infected king chilli samples from Leimatak, Tamenglong district, **b** infected king chilli samples from Shantipur, Imphal West district, **c** infected bird eye chilli samples from Molkon, Senapati district, **d** infected king

chilli samples from Kakyai, Bishnupur district, **e** Infected king chilli samples from Singngat, Churachandpur district, **f** infected king chilli samples from Lukhambi, Tamenglong district, **g** infected bird eye chilli from Lamzang, Churachandpur district

ChiVMV-Mnp2 and ChiVMV-Mnp5 produced differential degree of mottling and puckering on the leaves of inoculated king chilli plants. ChiVMV-Mnp3 upon inoculation exhibited typical symptoms of mottling and dark green islands on leaves (Fig. 2). In contrast, ChiVMV-Mnp4 produced leaf cupping and puckering symptoms. This indicated pathogenic diversity of ChiVMV isolates prevalent in the chilli groves of Manipur.

Genetic diversity of ChiVMV isolates

Five representative isolates of ChiVMV isolates (Mnp1–Mnp5) sampled from different groves of Manipur were characterized for their genetic diversity based on the conserved 432 bp fragment of CP gene. For conserved CP gene fragments, they shared maximum similarity with ChiVMV sequences available in GenBank database with a query coverage of more than 85%, which confirmed viral identity of the respective amplicons. Neighbour-joining (NJ) phylogenetic analysis of these ChiVMV isolates with the earlier reported isolates from India and other parts of the world indicated genetic distinctiveness. All the five ChiVMV isolates from Manipur (Mnp1–Mnp5) segregated to a distinct cluster (which were genetically homogenous with in

the cluster), nearest to which were the isolates from Italy and China (Fig. 3). 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.

Discussion

India is a major contributor of chilli products in the world. As per FAO report, India produced 12.66 lakh tons of chillies during 2016 (<http://www.fao.org>). North East region of India encompassing rich biodiversity has a significant share in country's chilli genetic resources. The unique landraces of the region have led it to be described as hot spot of hot pepper biodiversity (Purkayastha et al. 2012). The capsaicin content in most of the cultivated Indian chilli species is 1%, in contrast to the widely adapted *Capsicum chinense* landraces which have capsaicin content of 2–4% (Mathur et al. 2000; Sanatombi and Sharma 2008). In addition, diverse landraces of bird eye chilli (*Capsicum frutescens*) and *Capsicum annuum* are also grown in the region. Biotic constraints particularly symptoms akin to viral infection has long been recorded as a major hurdle to the successful

Fig. 2 Pathogenic diversity of ChiVMV isolates as studied by mechanical inoculation of selected isolates on king chilli (*Capsicum chinense*). **a** Symptoms of leaf deformation, thinning of lamina and shoestring produced by isolate ChiVMV-Mnp1 (sampled from Kakyai, Bishnupur distt, Manipur); **b** symptoms of leaf mottling, puckering and deformation produced by isolate ChiVMV-Mnp2 (sampled from Shantipur, Imphal West distt, Manipur); **c** symptoms of severe mottling and dark green islands produced by isolate

ChiVMV-Mnp3 (sampled from Sirarakhong, Ukhrul distt, Manipur); **d** symptoms of leaf cupping and puckering produced by isolate ChiVMV-Mnp4 (sampled from Langathel, Thoubal distt, Manipur); **e** symptoms of green mottling and deformation on leaves produced by isolate ChiVMV-Mnp5 (sampled from Lukhambi, Tamenglong distt, Manipur); **f** mock-inoculated king chilli plant; **g** un-inoculated healthy king chilli plant. Symptoms observed in the photographs were after 28 days post-inoculation

Fig. 3 Genetic relationship of ChiVMV isolates from Manipur (ChiVMV-Mnp1 to Mnp5: marked with green circle) with other ChiVMV isolates from India and other parts of the world based on partial nucleotide sequences of CP gene (432 bp). Phylogenetic inference was drawn using neighbour-joining (NJ) method in Mega 6 software. Bootstrap support values from a NJ search with 1000 replicates are shown at the nodes of the branches. The branches showing bootstrap values less than 50% were collapsed

production of these chillies in the region. 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). NE region being contiguous to the Eastern Asian region provided a strong base for the possible prevalence of pathogenically and genetically diverse ChiVMV isolates. Extensive surveys and indexing of chilli samples of different landraces for ChiVMV infection from different groves of Manipur (a state in Indo-Myanmar region with highest genetic diversity of king chilli) conclusively indicated the association of ChiVMV with decadence of chilli plantation. 63.78% of the total samples were tested positive for ChiVMV infection in RT-PCR indicating wide prevalence of this virus. Chilli plantations in the hilly ecosystem, showed higher infection rate of ChiVMV compared to those in valley areas of the region. This might be due to the fact that chilli is being planted in close vicinity of forest areas from where the primary infection might be carried through aphid vectors. Association of ChiVMV with viral-like symptoms was earlier reported from Eastern Uttar Pradesh (Prakash et al. 2002).

Detection of ChiVMV in the popular chilli landraces of *Capsicum chinense*, *Capsicum frutescens* and *Capsicum annuum* has shown their susceptibility to this *Potyvirus*. Based on the severity of field reaction, *Capsicum frutescens* landraces showed less severe symptoms compared to *Capsicum chinense* and *Capsicum annuum* landraces. Chilli

cultivars tolerant to the region's biotic stress will be very helpful in tackling viral disease prevailing in this region. Screening of diverse chilli accessions for identification of resistant or tolerant lines will be helpful in sustainable chilli production with better life span.

Present finding of biological transmission study gave a clear evidence of wide diversity in the pathogenic behaviour of ChiVMV isolates prevalent in the chilli groves of Manipur. Existence of pathotype variants of ChiVMV in the present study come corollary to earlier reports (Moury et al. 2005; Tsai et al. 2008). Despite having contrasting pathogenic behaviour, the sequence analysis using a conserved CP gene fragment (432 bp) could not reveal the genetic diversity among the ChiVMV isolates. The genetic heterogeneity among the characterized ChiVMV isolates might be due to the strong selective constraint on CP protein (King et al. 2011). The ChiVMV isolates of 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 the geographically isolated region of NE India segregated in a cluster which was very distinct to the cluster comprising of ChiVMV isolates from other parts of India. The genetic closeness of ChiVMV isolates characterized in the present

study with the isolates from China indicated possible transboundary 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 Manipur, presents a high chance of transboundary transmission of viral pathogen from neighbouring areas. However, further full genome characterization of prevalent ChiVMV isolates from this region and their pathogenicity analysis will decipher the actual evolution of this virus in biodiversity hot spot region of NE India and support in its transboundary movement. This is the first report on widespread occurrence of ChiVMV infection on different landraces of chilli in NE India and their biological and genetic characterization.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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