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Characterization and complete nucleotide sequencing of *Pepper Mild Mottle Virus* infecting Bell Pepper in India

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Abstract Capsicum (*Capsicum annuum* L var. *grossum* Sendt) commonly known as bell pepper or sweet pepper, is one of the most economical agricultural crop grown under both open and polyhouse conditions. The presence of *Pepper mild mottle virus* (PMMoV) from different districts of Himachal Pradesh was confirmed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and RT-PCR using coat protein (CP) gene amplification. The first complete genome sequence of Indian isolate of PMMoV (HP-1) was elucidated and compared with other members of *Virgaviridae* family and PMMoV isolates. Sequence homology, multiple alignment and phylogenetic analysis on the basis of nucleotide and amino acid sequences showed that PMMoV-HP1 isolate is more closely related to the PMMoV-J, the Japanese isolate. Based on CP gene amino acid sequence analysis, the PMMoV-HP1 isolate showed 100 per cent identity with P_{12} pathotypes (capable of breaking L^2 gene mediated resistance in capsicum). This is the first report of the PMMoV complete genome organization intercepted in India.

Keywords Genome · Tobamovirus · Pepper mild mottle virus · Pathotype

Introduction

Capsicum (*Capsicum annuum* L. var. *grossum* Sendt) one of the most popular and highly remunerative crops is cultivated in most parts of the world, especially in temperate regions of Central and South America, European countries, tropical and subtropical regions of Asian continent mainly in India and China. India has an average annual production of 0.9 million tons from an area of 0.885 million hectare with a productivity of 1266 kg per hectare which accounts for one fourth of world production of capsicum (Sreedhara *et al*. 2013).

Capsicum is vulnerable to the attack of various fungal, bacterial and viral pathogens around the world but viral diseases take heavy toll of the crop under open as well as protected cultivation causing both yield and quality losses. About 13 viruses *viz*., *Cucumber mosaic virus* (CMV), *Potato virus Y (*PVY), *Pepper mottle virus* (PepMoV), *Pepper severe mosaic virus* (PeSMV), *Pepper mild mottle virus* (PMMoV), *Tomato yellow leaf curl virus* (TYLCV), *Pepper veinal mottle virus* (PVMV), *Tomato spotted wilt virus* (TSWV), *Tobacco mosaic virus* (TMV), *Pepper yellow mosaic virus* (PepYMV), *Groundnut ringspot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV), and *Pepper ring spot virus* (PepRSV) are known to infect capsicum in different parts of the world (Gracia *et al.* 1968; Kang *et al*. 1973; Lockhart & Fischer 1974; Villalon 1975; Ong

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et al. 1980; Wetter *et al.* 1984; Sharma *et al*. 1993). Among these, PMMoV, a *Tobamovirus* is posing threat to capsicum cultivation due to its highly contagious nature and long survival in soil.

PMMoV was first recognized as the latent strain in USA (McKinney 1952), and then in Europe as a distinct strain (Wetter *et al*. 1984), is now distributed worldwide (Lamb *et al*. 2001) which includes Australia (Pares 1985), Taiwan (Green & Wu 1991), Egypt (Othmann 1991), Oregan (Hamm *et al*. 1995), North America (Beczner et al. 1997; Adkins *et al*. 2001), Japan (Honda & Kameya-Iwaki 1991; Ikegashira *et al*. 2004), Georgia (Martinez-Ochoa *et al*. 2003), China (Xiang *et al*. 1994; Wang *et al*. 2006), North Africa (Mnari-Hattab & Ezzaier 2006), Panama (Herrera-Vasquez *et al*. 2009), Brazil (Lima *et al*. 2011), and Turkey (Caglar *et al*. 2013). Moreover, Colson *et al*. (2010) detected PMMoV RNA in the food products, human stools and its association was also correlated with immune responses, fever, abdominal pains and pruritus in humans.

PMMoV, a member of genus *Tobamovirus*, is a rigid rod shaped virus belonging to family *Virgaviridae* (King *et al*. 2011). The virus is highly contagious and like other tobamoviruses also transmitted through seed and soil (Komuro & Iwaki 1969; Lanter *et al*. 1982; Brunt *et al*. 1996; Tan *et al*. 1997; Ikegashira *et al*. 2004; Toyoda *et al*. 2004). This virus can survive in crop debris including roots in soil, and contaminated equipments etc. Soil transmission during transplanting or seed transmission is considered to be the primary source of infection (Greenleaf 1986). The decontamination of contaminated field is extremely difficult as long as green pepper is continuously cultivated (Ikegashira *et al*. 2004). Seed borne infection in commercially available green pepper seeds has been reported by many workers (Tosic *et al*. 1980; Toyoda *et al*. 2004). This virus frequently results in significant crop losses or reduction both under field and greenhouse plantings (Jarret *et al*. 2008). Initially the pathogen causes mild foliar symptoms and many times it remains unnoticed until more evident symptoms appear on fruits resulting high yield losses. So far five pathotypes *viz*., P_0, P_1, P_{12} , P_{123}, and P_{1234} of PMMoV have been recognized on the basis of their ability to break *L* gene-mediated resistance (conferred by L^1, L^{1a}, L^2, L^3, and L^4), allelic forms of the same locus on chromosome 11 (Boukema IW 1984; Sawada *et al*. 2004). A new resistance gene designated as *Hk* imparting resistance to *Tobamovirus*, has been discovered in capsicum by Sawada *et al*. (2005).

In Himachal Pradesh (HP), Bell pepper commonly known as "Shimla mirch" brings remunerative returns to the farmers and grown on an area of 2,447 ha with production and productivity of 31,810 t and 13.00 t/ha, respectively (Anonymous 2013). In recent years, this crop is becoming very popular as an off-season crop under polyhouse conditions. In HP, India, PMMoV was first intercepted by Sharma & Patiyal (2011) in polyhouse grown capsicum hybrids and the popularization of the polyhouse venture in the state has led to the escalated incidence of the virus. Recent surveys of polyhouses constructed in different districts of HP have shown that PMMoV is the most prevalent and destructive pathogen of polyhouse. In the present study, genome of Indian isolate of PMMoV has been sequenced and its evolutionary relationship with other members of family *Virgaviridae* has been discussed.

Materials and Methods

Infected capsicum plants exhibiting symptoms like mosaic, mottle, leaf and plant deformations were collected from various regions of HP under polyhouse conditions. The plants were indexed for the presence of different viruses *viz*., CMV, PepMoV, PMMoV, TMV and *Tomato mosaic virus* (ToMV) through DAS-ELISA (Bioreba, Switzerland) using polyclonal antibodies (Clark & Adams 1977) and the samples found positive for PMMoV antiserum were maintained on susceptible bell pepper variety "California wonder" through sap inoculation using standard leaf rub method. The virus was mechanically inoculated on California wonder, *Datura stramonium* and *Nicotiana glutinosa* plants at 3-4 leaf stage. The disease symptoms were studied on the artificially inoculated plants. Virus preparations were further subjected to transmission electron microscopy using leaf dip method described by Brandes (1964). The identity of the virus as PMMoV was confirmed by RT-PCR using coat protein gene (CP gene) specific primers (F5'CCAATGGCTGACAGATTACG-3' and R5'CAACGACAACCCTTCGATTT-3') and finally one isolate HP-1 was used for full genome sequence determination.

Total RNA was isolated from 100 mg of infected plant leaves using Trizol as per manufacturer instructions (Life Tech). The integrity of total RNA was analyzed on 1 per cent denaturing gel. Leaves from uninoculated healthy plants exhibiting negative reaction with PMMoV

Fig. 1 Symptoms produced by PMMoV-HP1 isolate on healthy susceptible plants of "California Wonder" upon artificially inoculation. 1: mild mosaic, 2: leaf puckering, mild mottling and mosaic, 3: leaf deformation, 4: lumpy and deformed fruit

antiserum in DAS-ELISA were used as negative control. For RT-PCR, cDNA was synthesized using 1 μg of total RNA and oligod$T_{(10\text{-}18)}$ primer in 10 μl reaction using MMuLV reverse transcriptase (USB) as per manufacturer instructions. RT-PCR amplification based on CP region was carried out in 25 μl reaction volume using 2.5 μl of 10x Taq buffer, 1 μl of 25 $MgCl_2$, 2.5 μl of 2mM dNTPs mix each, 1μl of 10 μM each CP gene specific primer, 0.2 μl of 5U/μl Taq polymerase (Banglore Genei). The final volume was adjusted using nuclease free water. The amplification was performed in GeneAmp PCR system 9700 (Applied Biosystems) with initial denaturation of 94°C for 4 min followed by 35 cycles of 94°C for 15 sec, 48°C for 40 sec and 72°C for 1 min with a final extension

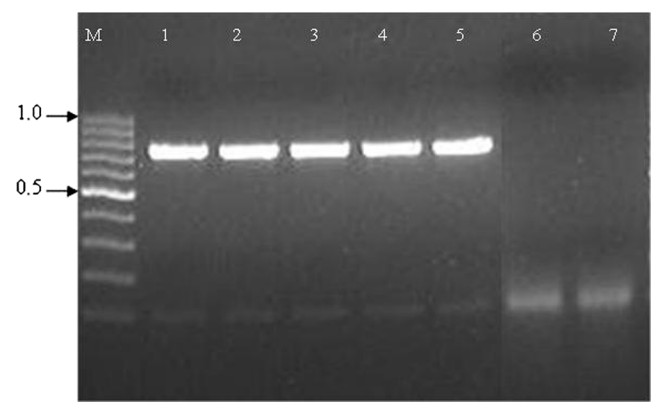

Fig. 2 RT-PCR based confirmation of PMMoV using primer pair specific to coat protein region. M: 100bp DNA ladder, Lane 1-5: represents ampilcons of ~730 bp in infected plants, Lane 6 -7: represents healthy plants of capsicum

of 7 min at 72°C. PCR products were electrophoresed on 1.2% agarose gel stained with ethidium bromide and visualized in gel documentation system (Alpha Innotech, USA).

For complete sequencing of the genome, ten primer pairs were designed based on full genome sequence available in NCBI database (www.ncbi.nlm.nih.gov) using primer3 software (Table S1). For RT-PCR, the reaction was performed in 50 μl volume using master mix (Phusion High-Fidelity PCR NEB), containing 1 μl of 10 μM each gene specific primer, 2.5 μl of cDNA. The final volume was adjusted with nuclease free water. Amplification was performed in GeneAmp PCR system 9700 (Applied Biosystems) with initial denaturation of 94°C for 4 min followed by 35 cycles of 94°C for 15 sec, annealing at 45°C for 40 sec and extension at 72°C for 1 min with a final extension of 7 min at 72°C.

For the determination of complete sequencing of 5' and 3' ends, RACE-PCR was carried out using SMART-RACE based method as per the manufacturer instructions (Clontech). The amplicons were cloned into pGEM-T Easy vector system (Promega) and both the strands of recombinant plasmids were custom sequenced. The complete nucleotide sequence of each amplicon was deduced and BLAST analyzed (www.ncbi.nlm.nih.gov/blast). The sequences were also

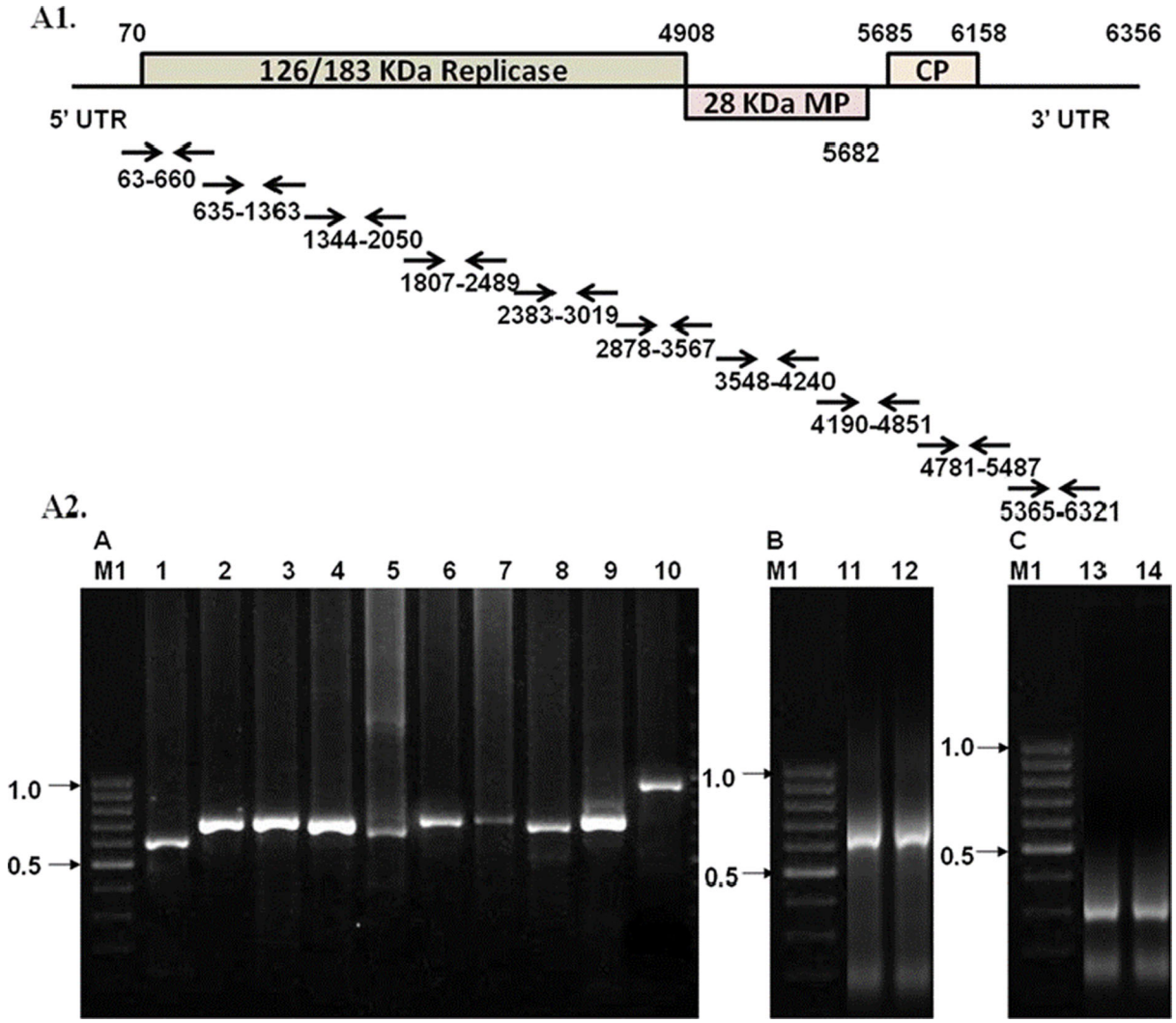

Fig. 3 A consensus PMMoV- HP1 genome map with primers positions used for full genome sequencing (A1) and A2 represents PCR based amplification of PMMoV genome using RT-PCR (A) and RACE PCR (B&C) respectively. M1: 100 bp DNA ladder, Lane 1-10: Represents RT-PCR based amplification of different regions corresponding to the genome of PMMoV, B & C: Represents the 5' UTR and 3' UTR amplification using RACE PCR, respectively

checked manually using bioEdit software (Altschul *et al*. 1997). The different fragments were aligned together and complete genome sequence was submitted to NCBI GenBank vide accession number KJ631123. The multiple alignment of the present isolate was done using ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/) and phylogenetic trees were constructed using MEGA5 program (Tamura *et al*. 2011) using the Neighbor-Joining method.

Results

The appearance of symptoms after sap inoculation indicated the viral nature of the pathogen. The inoculated plants showed mild mottling after about 8-10 days of inoculation followed by leaf deformation, yellow/ green mosaic and chlorosis of the leaves (Fig. 1). However, the symptoms were more prominent on young leaves. The infected plants remained stunted and developed small, distorted and lumpy fruits with colour variations. The virus produced chlorotic local lesions on *Datura stramonium* and *Nicotiana glutinosa.*

The identity of the virus as PMMoV was confirmed by DAS-ELISA using polyclonal antibodies ELISA kits, where a clear reaction was obtained with the PMMoV antiserum against controls. Electron microscopy of the symptomatic leaf tissue using crude sap extracts from infected leaves of "California wonder" showed the presence of short rigid rod shaped virus particles measuring about 300 nm in length similar to *Tobamovirus* particles. In RT-PCR, amplicon of ~730 bp corresponding to CP (Fig. 2) were obtained in the infected samples while no amplification was observed in healthy plants.

PCR amplification of various genome regions of PMMoV-HP1 isolate (genbank accession number KJ631123) using virus specific primers (Table S1) revealed that the viral genome consist of 6356 nucleotides (nt) comprising of 5' UTR, coding sequences and 3' UTR (Fig 3). The 5' UTR of the virus positioned upstream of the first open reading frame (ORF) is comprised of 69 nt and 3' UTR consists of 198 nt (6159-6356). The coding region contains four long ORFs typical of tobamoviruses. The first ORF (126K protein) is between nt 70-3423, terminates with an amber read through codon, so that ORF2 (183K protein) finishes at nt 4908. The ORF3 encodes cell to cell movement protein gene (28K protein), consists of 774 nt starting at nt position 4909 without any nucleotide insertion after the stop codon of ORF2 and finishes at nt 5682. The CP gene of 17.2K protein size (ORF4) starts at nt 5685, two nucleotides after the stop codon of ORF3 and finishes at nt 6158. The CP gene codes for 157 amino acids.

The phylogenetic tree constructed by using MEGA5 software categorised the present isolate along with other

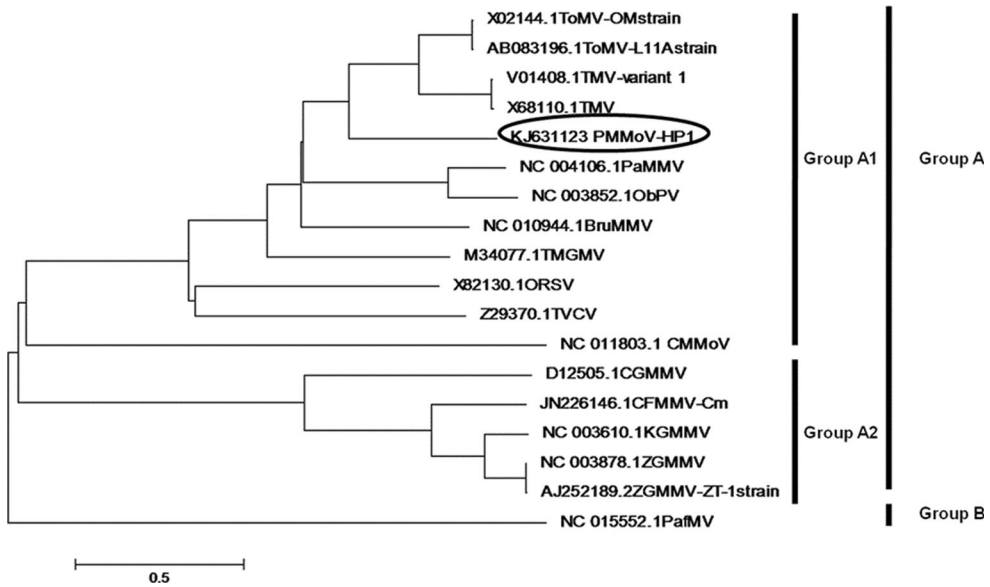

Fig. 4 Phylogenetic tree showing relationship of PMMoV-HP1 with other viruses of family *Virgaviridae* at nucleotide level using MEGA5 software

Table 1 Pairwise similarity index of PMMoV-HP1 complete nucleotide sequence with other PMMoV isolates

	AB276030.1 PMMoVL4BV-P1234	AJ308228.1 PMMoV-Ia-P123	AB550911.1 PMMoV-BR-DF01-P12	AY859497.1 PMMoV-CN-P12	AB254821.1 PMMoV-Iw-P12	M81413.1 PMMoV-S-P12	AB000709.2 PMMoV-J-P12	AB069853.1 PMMoVC-1421-P12	AB113117.1 PMMoVTPO-2-19-P12	AB113116.1 PMMoVPa18-P12	AB126003.1 PMMoV-Kr-P12	KJ631123 PMMoV-HP1
AB276030.1PMMoVL4BV-P1234	-	94.73	97.14	97.11	99.56	97.40	97.47	97.45	97.33	97.33	99.59	96.88
AJ308228.1PMMoV-Ia-P123		-	94.32	94.26	94.66	94.56	94.59	94.57	94.48	94.48	94.71	94.15
AB550911.1PMMoV-BR-DF01-P12			-	99.18	97.28	99.45	99.54	99.53	99.4	99.43	97.17	98.91
AY859497.1PMMoV-CN-P12				-	97.17	99.48	99.58	99.56	99.4	99.43	97.15	98.91
AB254821.1PMMoV-Iw-P12					-	97.47	97.54	97.52	97.39	97.39	99.59	96.95
M81413.1PMMoV-S-P12						-	99.87	99.86	99.7	99.73	97.42	99.18
AB000709.2PMMoV-J-P12							-	99.98	99.83	99.86	97.48	99.28
AB069853.1PMMoVC-1421-P12								-	99.81	99.84	97.47	99.26
AB113117.1PMMoVTPO-2-19-P12									-	99.97	97.34	99.13
AB113116.1PMMoVPa18-P12										-	97.34	99.17
AB126003.1PMMoV-Kr-P12											-	96.93
KJ631123PMMoV-HP1												-

members of the family *Virgaviridae* (Fig 4). Two major groups formed in this analysis, first comprised of all members of the family *Virgaviridae* except *Passion fruit mosaic virus* (PafMV) which constituted the second group. The group A was further divided into two clusters A1 and A2 accommodating *Brugmansia mild mottle virus* (BruMMV), *Cactus mild mottle virus* (CMMoV), *Cucumber fruit mottle mosaic virus* (CFMMV), *Cucumber green mottle mosaic virus* (CGMMV), *Kyuri green mottle mosaic virus* (KGMMV), *Obuda pepper virus* (ObPV), *Odontoglossum ringspot virus* (ORSV), *Paprika mild mottle virus* (PaMMV), ToMV, TMV, *Tobacco mild green mosaic virus* (TMGMV), *Turnip vein-clearing virus* (TVCV) and *Zucchini green mottle mosaic virus* (ZGMMV), respectively. The present isolate was grouped in the first sub cluster in the neighbourhood of ToMV, TMV, PaMMV, ObPV, BruMMV, TMGMV, ORSV, TVCV whereas the viruses *viz.*, CGMMV, CFMMV, KGMMV and ZGMMV were placed in the second sub cluster.

The complete nucleotide sequence similarity between PMMoV-HP1 isolate and other PMMoV isolates ranged between 94-99 per cent (Table 1). Pair wise comparison of PMMoV-HP1 isolate with all 11 complete PMMoV genomes available in public databases revealed that the per cent identity of the present isolate was maximum with PMMoV-J (99.28%) with 46 nt differences distributed throughout the genome and minimum with PMMoV-Ia (94.15%) indicating its possible evolution with the Japanese isolate. There was a difference of only 11 amino acids (aa) in the coding regions again showing maximum variation in regions except CP which is most important for differentiation of PMMoV pathotypes due to its ability to overcome the *L* gene mediated resistance in capsicum.

The phylogenetic tree constructed, grouped all PMMoV isolates into two main clusters placing PMMoV-HP1 isolate along with ten isolates identified into different pathotypes whereas only one isolate PMMoV-Ia was placed in the second group (Fig 5). The present isolate was placed in the first sub cluster in the neighbourhood of PMMoV-J, PMMoV-C1421, PMMoV-Pa18, PMMoV-TPO-2-19, PMMoV-S, PMMoV-CN, and PMMoV- BRDF01 whereas three isolates *viz.*, PMMoV-Iw, PMMoV-L4BV, PMMoV-Kr were placed together in the second sub cluster. In this analysis we found that isolates representing different countries (PMMoV-S, PMMoV-CN, PMMoV-BRDF01) were placed in separate clades except Japanese isolates. The mutations in the CP gene of the tobamoviruses are advocated for the breakdown of the *L* gene mediated resistance and the PMMoV pathotypes have been differentiated on the basis of variations in aa sequence. In an attempt to place the present isolate in a given pathotype group, CP aa sequence of the test isolate was compared with known pathotypes. Pair wise comparison of the CP aa sequences (Table 2) revealed 100% similarity of the present isolate with pathotype PMMoV P_{12}.

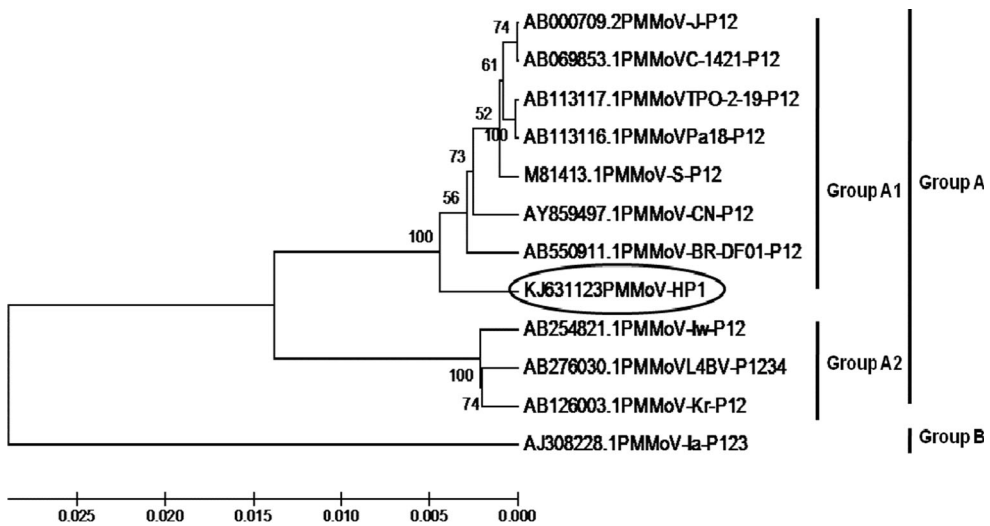

Fig. 5 Phylogenetic analysis based on full genome sequence of PMMoV- HP1 (KJ631123) and other PMMoV isolates using Mega5 software

Table 2 Pairwise similarity index of PMMoV-HP1 coat protein with other PMMoV isolates representing pathotypes P_{1234}, P_{123} and P_{12}

	AB276030.1PMMoVL4BV-P_{1234}	EF422083-PMMoV-Is-P_{1234}	EF432637PMMoV-Is-P_{123}	AJ308228.1PMMoV-Ia-P_{123}	AB550911.1PMMoV-BR-DF01-P_{12}	EF434393PMMoV-Is-P_{12}	KJ631123PMMoV-HP1
AB276030.1PMMoVL4BV-P_{1234}	-	96.18	96.82	95.54	98.73	98.73	98.73
EF422083-PMMoV-Is-P_{1234}		-	98.73	98.09	97.45	97.45	97.45
EF432637PMMoV-Is-P_{123}			-	98.09	97.45	97.45	97.45
AJ308228.1PMMoV-Ia-P_{123}				-	96.82	96.82	96.82
AB550911.1PMMoV-BR-DF01-P_{12}					-	100	100
EF434393PMMoV-Is-P_{12}						-	100
KJ631123PMMoV-HP1							-

Discussion

The symptomatology and transmission of the present isolate confirmed the viral etiology of the disease and its serological relationship with PMMoV antiserum established its identity as PMMoV. The electron microscopy of the virus preparations revealed the association of short rigid rod shaped particles, typical of *Tobamovirus*. The identity of the virus was further confirmed by RT-PCR using CP gene specific primers. The symptoms of the present isolate resembled with PMMoV symptoms described by different workers across the world (Wetter *et al.* 1984; Beczner *et al.* 1997; Antignus *et al.* 2008; Sevik 2011).The results pertaining to identification of test virus on the basis of DAS-ELISA and RT-PCR using the CP specific primers were in conformity with Wetter *et al.* (1984); Beczner *et al.* (1997); Kalman *et al.* (2001); Ikegashira *et al.* (2004); Mnari-Hattab & Ezzaier (2006); Herrera-Vasquez *et al.* (2009); Lima *et al.* (2011); Caglar *et al.* (2013); Zheng *et al.* (2013); Rong *et al.* (2013).

The genome of PMMoV-HP1 isolate comprised of 6356 nt having four ORFs typical of tobamoviruses and other PMMoV reported from other parts of the world (Alonso *et al.* 1991; Kirita *et al.* 1997; Velasco *et al.* 2002; Hagiwara *et al.* 2002; Yoon *et al.* 2005; Ichiki *et al.* 2005; Wang *et al.* 2006; Genda *et al.* 2007; Hamada *et al.* 2007; Oliveira *et al.* 2010). The present isolate shared maximum identity with PMMoV-J (Japenese) isolate and its comparison with the present isolate revealed 46 single nucleotide mutations where 37 were the transitions (21 out of 37 between pyrimidines C-T and 16 between purines A-G) followed by 10 transversion mutations (3 G-T, 6 A-T and 1 A-C). These mutations were non-silent and resulted in 11 amino acid differences: seven in the 126/183 KDa protein, three in the 28 KDa movement protein. There was no difference in 17.2 KDa coat protein. The maximum identity *i.e.* more than 99 per cent between the Japanese and the present isolate suggests that the two are derived from same origin and might have entered India through import of infected seed. *Capsicum annuum* L was introduced to Spain during fifteenth century, from where its cultivation spread rapidly to Europe. California wonder is the representative cultivar of *Capsicum annuum* L. cultivated across the world. This cultivar was introduced in Japan in mid twentieth century from America (Kirita *et al.* 1997) whereas, in India, it was introduced from Brazil by Portuguese. The path of *Capsicum annuum* L.

might explain the long stable journey of PMMoV from Japan to India. The capsicum seed in India is imported from different countries like China, Japan, Netherlands, USA and Euorpe and the seed import might have facilitated the introduction of present isolate in India. This could be the reason for the placement of the present isolate in one cluster along with the representative isolates of Brazil, China, Japan and Spain. However, this aspect still needs further confirmation by involving more number of isolates

The results of phylogenetic analysis were similar to that obtained by Oliveira et al. (2010) where like the present isolate, PMMoV-BRDF01 found to be clustered with one Spanish (PMMoV-S), four Japanese (PMMoV-J, PMMoV-C1421, PMMoV-Pa18, PMMoV-TPO-2-19) and one Chinese isolate (PMMoV-CN). It has been reported that the ability of the tobamoviruses to overcome *L* gene-mediated resistance (L^1, L^{1a}, L^2, L^3 and L^4) in capsicum determines their pathogenic behaviour (P_0, P_1, P_{12}, P_{123} and P_{1234}) and the most important component of tobamoviruses responsible for this is the CP gene. The *Tobamovirus* CP is the elicitor of *L* gene-mediated resistance and act as a determinate in assigning a viral pathotype (Berzal-Herranz *et al*. 1995; Tsuda *et al*. 1998; Dardick *et al*. 1999; Hamada *et al*. 2002; Genda *et al*. 2007). It has been demonstrated that mutations in the CP gene are associated with breakdown of resistance inferred by the *L* gene (Tsuda *et al*. 1998; Gilardi *et al*. 1998, 2004; Hamada *et al*. 2002, 2007; Genda *et al*. 2007). Thus, based on CP gene homology, the present isolate has been tentatively identified as pathotype PMMoV P_{12} as aa sequence of the CP region showed 100 per cent similarity with that of pathotypes PMMoV-P_{12} reported by Alonso *et al*. (1991), Kirita *et al*. (1997), Hagiwara *et al*. (2002), Ichiki *et al*. (2005), Yoon *et al*. (2005), Wang *et al*. (2006), Oliveira *et al*. (2010). However, authentic validation as pathotype P_{12} requires phenotyping on the differential hosts (*L* alleles). In conclusion, this study constitutes the first report on characterization of any PMMoV isolate from India and its relationship with particular pathotype. The information generated in this study will facilitate the work on identification of effective sources of resistance in the capsicum germplasm and its use in breeding for resistance as no information is available on any aspect of this virus from India at least.

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Conflict of Interest There is no conflict of interest among authors.

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