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Molecular evidence for the association of *Cucumber mosaic virus* causing mosaic and leaf deformation of *Pisum sativum* in Western Uttar Pradesh, India

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Abstract. *Cucumber mosaic virus* associated with the mosaic, leaf deformation and stunting of *Pisum sativum* was identified as a member of subgroup IB on the basis of the capsid protein gene sequence.

Sweet pea (*Pisum sativum* L., family Leguminaceae) is a major consumable food crop in the tropical and subtropical regions of the world including India. Pea crop growing in the experimental plots, gardens and fields around Aligarh Muslim University, Aligarh (Western Uttar Pradesh) India, were found naturally infected, exhibiting mosaic, leaf deformation and stunting of the entire plant. The disease affected fruit yield and was prevalent. The incidence varied in different experimental fields from 22–37%. Infected plants were collected from three field locations in Aligarh and were maintained in an insect proof glasshouse.

Leaf dip preparations of the infected pea leaves revealed the presence of 28 nm isometric particles, typical of *Cucumber mosaic virus* (CMV). Symptomatic leaf tissue was ground in 0.1 M phosphate buffer (1 : 1, w/v pH 7.0) and squeezed through double layered muslin cloth. The filtrate was mechanically inoculated to several indicator species. Necrotic lesions developed on *Gomphrena globosa*, *Chenopodium amaranticolor*, *C. murale* and *Spinacia oleracea* within 7–10 days post inoculation (dpi). Systemic mosaic symptoms developed (20–25 dpi) on *Nicotiana tabacum* cv. White Burley, *N. tabacum* cv. Samsun-NN, *N. glutinosa*, *N. benthamiana*, *N. rustica* and *Cucumis sativus.* The pea isolate was vector-transmitted using healthy *Myzus persicae* and *Aphis gossypii* in a non-persistent manner to *N. tabacum* cv. White Burley, *N. glutinosa*, *N. benthamiana* and *Cucumis sativus* following the method of Noordam (1973). The presence of the virus in inoculated hosts was confirmed by enzyme linked immunosorbent assay (ELISA) (Verma *et al*. 2005) using CMV specific antiserum (PVAS242a, American Type Culture Collection, USA) and alkaline phosphatase linked secondary antibodies (DSMZ, Germany). The mean absorbance at 405 nm for negative and positive controls were 0.023 ± 0.003 and 0.438 ± 0.002, respectively, whereas in infected samples absorbance ranged between $0.346–0.433 \pm 0.002$.

For molecular identification of CMV, total RNA was isolated from three naturally infected pea plants each collected from three different location and healthy leaves of pea using RNAqueous (Ambion, USA). First strand cDNA (cDNA) was synthesised using total RNA as template, CMV-CP specific reverse primer 5′-GCATGGTACCTCAAACTGG GAGCAC-3′ and RTase (200 U, MBI Fermentas, USA) following the manufacturer's procedure. Complementary DNA was amplified in GeneAmp PCR9700 system thermal cycler (Applied Biosystems, USA) in 50 μL reaction mixture containing 10 ng cDNA as template, 0.2 μM forward (5′-GCA TTCTAGATGGACAAATCTG AATC-3′) and aforesaid reverse primer and 1.5 U *Taq* DNA polymerase (Invitrogen, USA). Following the initial 5 min denaturation, 30 cycles of amplification were performed with denaturation at 94°C for 1 min, annealing at 45°C for 1 min and elongation at 72°C for 1 min followed by a final elongation of 10 min at 72°C. Electrophoresis in 0.8% agarose gel at 5 V/cm revealed a ~650 bp product from each of the infected pea plants collected from the three different locations.

The amplified product from one location was gel purified (Au-Prep Sigma gel extraction kit) and cloned into pGEM-T easy vector system (Promega, USA). Randomly, 10 ampicillin positive clones were screened through restriction digestion and two clones were sequenced in an automated sequencer (ABI Prism 310). For sequencing SP6 and T7 primers were mixed with purified plasmid DNA and the reaction was carried out using Big Dye Terminator Sequencing V3 sequencing kit, Applied Biosystem, USA. The sequence data obtained was submitted to EMBL Database (Accession EU140547).

Maximum (93–94%) amino acid identity was obtained with CMV subgroup IB when analysed with BLAST in NCBI GenBank. Lower (89–90%) identity was with subgroup IA and 74–77% identity was with subgroup II (Table 1). Multiple nucleotide and deduced amino acid alignments with the available sequence were carried out using CLUSTAL-W program v. 1.82 (Higgins *et al*. 1994) and the aligned files were bootstrapped 100 times generating a neighbour-joining phylogenetic tree using Tree-Explorer. The pea isolate grouped with a clade of Indian CMV strains of

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Table 1. Percentage nucleotide (nt) and amino acid (aa) identities of *Cucumber mosaic virus* (pea isolate under study, Accession: EU140547) with various CMV strains and other members of *Cucumoviruses* available in NCBI GenBank database

CMV, *Cucumber mosaic virus*; TAV, *Tomato aspermy virus*; PSV, *Peanut stunt virus*

Accessions	Natural host	Abbreviation	Location	Sub-group	% identities at the level of nt	% identities at the level of aa
AM158321	*Musa paradisiaca*	Ban-Delhi	India	IB	96	94
EF178298	*Musa paradisiaca*	Ban-Lucknow	India	IB	94	93
DQ910858	*Musa paradisiaca*	Ban-Lucknow2	India	IB	94	93
EF608461	*Piper nigrum*	KPS10-Thailand	Thailand	IB	93	94
AY560556	*Piper nigrum*	SG1-Thailand	Thailand	IB	94	94
AJ810259	*Capsicum annuum*	KS44-Thailand	Thailand	IB	92	93
EF593025	*Rauvolfia serpentina*	Rau- Lucknow	India	IB	92	94
DQ640743	*Musa paradisiaca*	Ban-Maharashtra	India	IB	92	93
AF350450	*Hyoscyamus muticus*	Henbane-Lucknow	India	IB	92	93
D10538	Not known	CMV-fny	USA	IA	89	90
D12499	Not known	CMV-Y	Japan	IA	88	89
D00385	Not known	CMV-O	Japan	IA	88	70
M21464	Not known	CMV-Q	Australia	II	72	77
L15336	Not known	CMV-Trk	Hungary	II	71	74
EF153735	*Chrysanthemum mmorifolium*	TAV-Chrysanth	India	Out group	46	39
NC_002040	*Pisum sativum*	PSV-Pea	China	Out group	44	42

subgroup IB. Our biological, serological and molecular studies confirmed the pea virus to be CMV of subgroup IB (Fig. 1).

In India, pea has been reported to be infected by *Pea common mosaic virus* (Sreenivasan and Nariani 1966), *Pea top necrosis virus* (Roy and Gupta 1977), *Pea necrotic mosaic virus* (Sharma and Gupta 1978), *Tomato spotted wilt virus* and *Bean yellow mosaic virus* (Rishi and Hampton 1987). A literature survey has also revealed the occurrence of CMV on pea causing mosaic disease, which has been characterised only at the biological and serological level (Rao *et al*. 1995). To the best of our knowledge this is the first report characterising the CMV pea isolate at the molecular level.

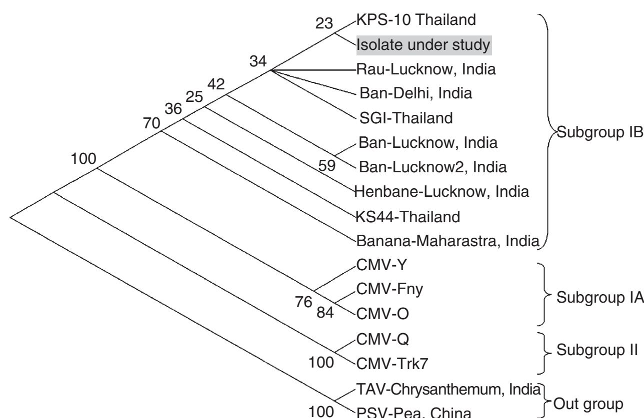

Fig. 1. Phylogenetic analysis of CMV pea isolate with the established CMV strains of subgroup 1A and II of *Cucumoviruses* using MEGA 4. The evolutionary history was inferred using the neighbour-joining methods. The percentage of replicate trees in which the associated isolates clustered together in the bootstrap test (100 replicates) is shown next to the branches and distances were computed using Poisson Correction methods. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 215 positions in the final dataset.

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