Coat protein sequence shows that *Cucumber mosaic virus* isolate from geraniums (*Pelargonium* spp.) belongs to subgroup II[†]

NEERAJ VERMA*, B K MAHINGHARA, RAJA RAM and A A ZAIDI

Plant Virus Lab, Floriculture Division, Institute of Himalayan Bioresource Technology, Palampur 176 061, India

**Corresponding author (Fax, 91-1894-230433; Email, neer_ali@yahoo.com)*

A viral disease was identified on geraniums (*Pelargonium* spp.) grown in a greenhouse at the Institute of Himalayan Bioresource Technology (IHBT), Palampur, exhibiting mild mottling and stunting. The causal virus (*Cucumber mosaic virus*, CMV) was identified and characterized on the basis of host range, aphid transmission, enzyme linked immunosorbent assay (ELISA), DNA-RNA hybridization and reverse transcription polymerase chain reaction (RT-PCR). A complete coat protein (CP) gene was amplified using degenerate primers and sequenced. The CP gene showed nucleotide and amino acid homology up to 97%–98% and 96%–99%, respectively with the sequences of CMV subgroup II. The CP gene also showed homologies of 75%–97% in nucleotide and 77%–96% in amino acid with the CMV Indian isolates infecting various crops. On the basis of sequence homology, it was concluded that CMV-infecting geraniums in India belong to subgroup II.

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1. Introduction

Geranium (*Pelargonium* spp.) of family Geraniaceae is a high value aromatic crop yielding essential oil used for various purposes and some of the pelargonium plant species are the largest selling garden ornamental crop of the world. The crop is reported to be infecetd by a number of pests and diseases (Rao *et al* 2000) including a number of viruses (Franck and Loebenstein 1995). Vegetative propagation of the crop results in perpetuation of viruses from one generation to other, causing decrease in yield and quality of the plants.

Cucumber mosaic cucumovirus (CMV) of family *Bromoviridae* is one of the most important widespread viruses in the world infecting the largest number (approximately 1000) of plant species. The genome of the virus consists of plus-sense single stranded three RNAs (RNA 1, RNA 2 and RNA 3) and a subgnomic RNA (RNA 4) which is encoded by the 3′-half of RNA 3 (Palukaitis *et al* 1992) and which is involved in encapsidation (Suzuki *et al* 1991).

A number of CMV strains reported from all over the world have been placed in to two subgroups I and II on the basis of serology (Wahyuni *et al* 1992; Hu *et al* 1995; Ilardi *et al* 1995), nucleic acid hybridization (Owens and Palukaitis 1988), gene sequences (Owens *et al* 1990; Szilassy *et al* 1999) and restriction fragment length polymorphism (RFLP) (Rizos *et al* 1992; Sialer *et al* 1999). CMV subgroup I has recently been subdivided into IA and IB on the basis of gene sequences available for CMV strains

Keywords. CP gene sequence; *Cucumber mosaic virus* (CMV); ELISA; Geranium; phylogeny; RT-PCR

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Abbreviations used: CMV, *Cucumber mosaic virus*; CP, coat protein; ELISA, enzyme linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction.

and phylogenetic analysis (Palukaitis and Zaitlin 1997; Roossinck *et al* 1999; Roossinck 2002). Further, Asian strains of CMV have been placed in subgroup IB (Palukaitis and Zaitlin 1997).

On the basis of coat protein gene sequences, various viruses have been grouped. Such as in case of CMV isolates A, Dat and H (Srivastava and Raj 2004), Or (Verma *et al* 2005), Gera (Verma *et al* 2004), Al (Verma *et al* 2005); *Prunus necrotic ringspot virus* isolates (Hammond 2003) and *Turnip mosaic virus* isolates (Ohshima *et al* 2002).

CMV infection on *Pelargonium* spp. has been reported from Italy (Quacquarelli and Gallitelli 1979), France (Albouy and Morand 1976), German Democratic Republic (Schmatz and Kegler 1979), Denmark (Paludan 1975, 1976), Poland (Korbin and Kaminska 1998) and is in the list of the EPPO (1992) Certification Scheme. These reports are mainly based on symptoms, host range, morphology and serological studies.

The present studies have been carried out to characterize the CMV strain Gera (CMV-Gera) up to genomic level using coat protein (CP) gene analysis found infecting geraniums (*Pelargonium* spp.) exhibiting mosaic, mottling and stunting. These were grown in the green houses of IHBT, Palampur.

2. Materials and methods

2.1 *Mechanical and insect transmission*

Young virus infected leaves of geranium (*Pelargonium* spp.) were macerated with chilled 0.01 MTris-HCl buffer, pH 7.8–8.0, containing 0.01 M sodium sulphite and 0.1% cysteine hydrochloride in a prechilled mortar with pestle. The slurry thus obtained was squeezed through double layered muslin cloth followed by low-speed centrifugation (10,000 rpm for 5 min). The supernatant thus obtained was mechanically inoculated to a number of plants viz. *Chenopodium album*, *C. amaranticolor*, *C. quinoa*, *Cucumis sativus*, *Nicotiana benthamiana*, *N. clevelandi*, *N. glutinosa*, *N. megalosiphon* and *N. tabacum* cv. Samsun NN. The culture of CMV-Gera was maintained on *N. glutinosa* plants for further studies.

Healthy virus-free *Myzus persicae* and *Aphis gossypii* reared on healthy *C. sativus* and *N. benthamiana*, respectively were used for the insect transmission as per the methods described by Noordam (1973).

2.2 *Enzyme linked immunosorbent assay*

CMV-specific antibodies along with alkaline phosphatase-linked antibodies procured from DSMZ (Germany) were used for enzyme linked immunosorbent assay (ELISA) as per the manufacturer's instruction and as described by Verma *et al* (2005).

2.3 *RNA isolation*

Total RNA from virus-infected geranium and mechanically inoculated *N. glutinosa* leaves were isolated using RNAqueous (Ambion, USA). Viral RNA was also isolated by disrupting the particles by 1% SDS followed by extraction with phenol-chloroform method (Sambrook *et al* 1989).

2.4 *Reverse transcription polymerase chain reaction*

Reverse transcription polymerase chain reaction (RT-PCR) was performed to amplify the complete CP gene of CMV-Gera with some flanking region using total RNA (approximately 5 μg) and viral RNA (approximately 2–3 μg) both as template using cucumovirus group-specific primers as described by Choi *et al* (1999) that gave an amplification of approximately 940 bp containing CP gene (657 bp).

First strand cDNA synthesis and PCR were carried out as described by Sharma *et al* (2005).

2.5 *Cloning and sequencing of PCR amplicons*

The PCR-amplified DNA fragment was cut from the gel and eluted using gel extraction kit (QBIOGENE). Eluted DNA was cloned in p-GEM Teasy vector (Promega, USA) as per the manufacturer’s instruction. The ligated products were transformed in competent cells of *Escherichia coli* strain DH5α with resistance to ampicillin. The transformed clones were selected on LBA plates containing 100 μg/ml ampicillin. The clones were checked using restriction enzymes *Eco*RI and *Pvu*II, the sites of which were present in vector.

Restriction enzyme digested positive clones (four in numbers) were sequenced using Sanger’s dideoxy chain termination separately in an automated sequencer (ABI Prism 310, Applied Biosystems, USA). For sequencing SP6 and T7 primers (1.66 pm) were mixed with purified plasmid DNA and the reaction was carried out using Big Dye Terminator Sequencing v3 seque-ncing kit (Applied Biosystems, USA). The sequences were matched with their corresponding electropherograms.

2.6 *Sequence analysis*

The CP gene sequence obtained has been submitted to EMBL Database (accession No AJ866272) and was

compared with those of various CMV strains belonging to subgroup I (A and B) and II and also with the CP gene sequences of CMV Indian isolates (table 1).

Multiple nucleotide and deduced amino acid (using Expasy Translation tool, www.us.expasy.org/tools/dna.html) alignments with the available sequences were carried out using CLUSTALW programme version 1.82 (Higgins *et al* 1994, www.ebi.ac.uk./clustalw/) and Multalin programme version 5.4.1 (Corpet 1988, www.prodes.toulouse.inra.fr/multalin/multalin). The alignment files created by ClustalW were bootstrapped 1000 times for generating neighbour-joining phylogenetic tree using Tree Explorer. *Tomato aspermy virus* (TAV) CP gene (accession No. AJ550020) was used as outsource.

Table 1. Coat protein gene sequences of various *Cucumber mosaic virus* strains used for comparison

Subgroup	Strain	Accession number	Origin
IA	KM	AB004780	Japan
IA	Fny	D10538	USA (NY)
IA	C	D00462	USA (NY)
IA	Kor	L36251	Korea
IA	Sny	U66094	Israel
IA	Ny	U22821	Australia
IA	FT	D28487	Japan
IA	FC	D10544	USA
IA	OL	AJ890464	India
IA	Ll	AJ831578	India
IA	Lt	AJ890465	India
IB	C7-2	D42079	Japan
IB	2A1-A	AJ271416	USA
IB	As	AF013291	Korea
IB	Tfn	Y16926	Italy
IB	IA-3a	AB042294	Japan
IB	NT9	D28780	Taiwan
IB	Oahu	U31220	USA
IB	Tai	D49496	Taiwan
IB	Phym	X89652	India
IB	D	AF281864	India
IB	H	AF350450	India
II	Trk7	L15336	Hungary
II	Q	M21464	Australia
II	S	AF063610	USA
II	LS	AF127976	USA
II	SP103	U10923	USA
II	m2	AB006813	Japan
II	Sn	U22822	Australia
II	Wem	L40953	Unknown
II	AL	AJ585086	India

3. Results

3.1 *Transmission studies*

The virus was found to be mechanically transmissible to the plants used in host range studies. All the *Chenopodium* spp. used in the study evoked chlorotic lesions on the inoculated leaves. Young expanded cotyledons of *C. sativus* inoculated with the virus evoked local lesions and after 12–15 days of inoculation the plants became systemically infected showing mosaic. All the virus inoculated *Nicotiana* spp. showed mosaic symptoms after 16–24 days of inoculation.

M. persicae and *A. gossypii* transmitted the virus in non persistent manner within 2–3 min of inoculation. About 90% of *N. glutinosa* plants were found to be infected with the virus. The presence of the virus was also confirmed by RT-PCR.

3.2 *ELISA*

A high contrast of colour (yellowish green) developed by the CMV infected plants in ELISA. The mean absorbance values at 405 for negative and positive controls were 0.023 and 0.438, respectively while for positive samples, the values ranged between 0.346–0.433.

3.3 *RT-PCR and sequence analysis*

Gel electrophoresis of RT-PCR amplicons revealed an expected size of approximately 940 bp (figure 1). The amplicons were successfully cloned in p GEM-T Easy vector. Restriction digestion of plasmid DNA isolated from the selected colonies gave positive confirmation of several clones having the inserts. On sequencing it was found to have some flanking region of RNA 3 at both 5′ and 3′ ends of CP gene. The complete CP (657 bp, 218 aa) including initiation and termination codons has been deposited in EMBL Database (accession No. AJ866272).

Sequence analysis of CP of CMV-Gera revealed 75–77% and 76%–78% homology with reference to nucleotides and amino acids, respectively of CMV strains of subgroup IA while with CMV strains of subgroup IB it showed 75%–77% and 74%–78% homology with reference to nucleotides and amino acids, respectively. CMV-Gera showed a high homology with the strains of CMV subgroup II (97%–98% in nucleotide and 96%–99% in amino acids). That clearly showed that our CMV-Gera isolate belong to CMV subgroup II. Our isolate showed a very high homology and sequence conservation in terms of amino acid (99%) with the Q strain (subgroup II). The differences in nucleotides of CMV-Gera and Q were also present at various nucleotide positions due to replacement of either

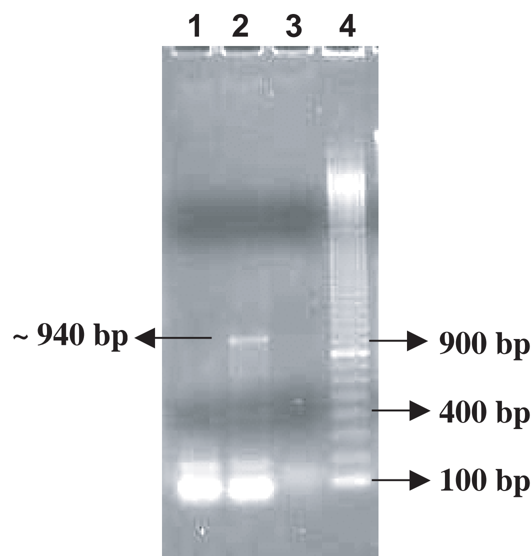

Figure 1. Gel photograph of RT-PCR amplicons. Lanes 1 and 3 contain CMV negative samples (*Nicotiana glutinosa*), lane 2 contains CMV positive Geranium (DNA band of approximately 940 bp) and lane 4 contains DNA ladder (100 bp).

purines with pyrimidines (five places), purine to purine (at one place) or pyrimidine to pyrimidine (one place) viz. at positions number 71and 639 (A is replaced by T), 189, 441 and 564 (C is replaced by T), 383 (T is replaced by C) and 384 (G is replaced by A). The changes of nucleotides at position numbers 71, 383 and 384 resulted in the differences at two amino acid positions with Q strain viz. 54 [isoleucine (I) a hydrophobic amino acid is present in place of asparagine (N) (which is polar) and 128 (valine (V)) is replaced by similar amino acid alanine (A)] (figure 2).

Amino acid and nucleotide sequence homology was also carried out with complete CP gene sequences of CMV infecting various crops in India. CMV-Gera showed 75%–97% and 77%–96% homology with respect to nucleotide and amino acid, respectively. The CMV-Gera showed very high sequence conservation with the AL isolate (97% nucleotide and 96% amino acid homology) that belongs to CMV subgroup II. The differences in the nucleotides of CMV-Gera and AL lie at the positions viz. 122 (A replaced by G); 123 and 131 (G replaced by A); 157 and 383 (T replaced by C); 159, 192 and 639 (A replaced by T); 170 (G replaced by C); 190 (G replaced by T); 195 and 228 (C replaced by A); 207, 441 and 564 (C replaced by T); 238 (T replaced by A). These changes leads to the replacements of amino acids in CMV-Gera CP viz. arginine (R) 41 lysine (K), K44R (both K and R are positively charged but K is hydrophobic), threonine (T) 57 serine (S) (both T and S are polar but T is hydrophobic), cysteine (C) 64 glycine (G) (both are similar amino acid i.e. hydrophobic), K 65 asparagine (N), K76N (K is hydrophobic and N is polar), isoleucine (I) 80 phenylalanine (F) (both are hydrophobic but I is aliphatic and F is aromatic) and A128V (both A and V are similar). With other CMV Indian strains our Gera isolate differed at various positions but two differences viz. threonine (T) is present at 12th position and at 28th position it lacks serine (S) (both T and S are polar but T is hydrophobic).

A phylogenetic tree (figure 3) constructed using CP gene amino acid alignment of various strains of CMV and TAV as an out-group also favours the results of sequence similarity of CMV-Gera with CMV subgroup II. Clear clusters of CMV subgroups IA, IB and II formed in the dendrogram.

4. Discussion

CMV has the broadest host range among the plant viruses and has been reported infecting geranium from various parts of the world. But no efforts have been done to classify the virus. Our attempt for this study was to classify and characterize CMV up to genomic level and to find out any similarity or variability with the other strains.

The virus was able to transmit mechanically on the plants used in the study very efficiently and produced the systemic and local symptoms characteristic of CMV. Both the aphid species (*A. gossypii* and *M. persicae*) transmitted the virus in nonpersistent manner and approximately 90% of the aphid inoculated plants produced systemic mosaic.

ELISA test used in testing the plants (either mechanically inoculated or naturally infected) for the presence of virus produced satisfactory results. Most of the time the plants were also checked by RT-PCR.

Sequence analysis of CMV-Gera isolate infecting geranium revealed a very high amino acid homology with the strains of CMV sub group II especially with Q strain (99%). The virus also showed a very high amino acid homology (96%) with the Indian CMV-AL strain. Such a high sequence homology of CMV-Gera with the CMV subgroup II strains suggests a common origin of the virus. Despite the different geographical region and specific crop, there is less variation in the CP gene of these isolates.

Various cucumovirus isolates have been classified on the basis of RNA 3 sequences e.g. CMV (Roossinck *et al* 1999; Deyong *et al* 2005) and *Peanut stunt virus* (Hu *et al* 1997). Therefore, these isolates can be used to group the newly discovered cucumovirus on the basis of gene sequence data.

The main differences in the properties of amino acids with Q strain viz. I54N and with Indian CMV-AL strain viz. R41K, K44R, T57S, K65N, K76N and I80F may have some impact on the coat protein orientation, symptom expression, transmission etc. which need to be further studied.

An interesting thing is that the length of CP gene of CMV-Gera remained same (657 bp, 219 aa) as with other

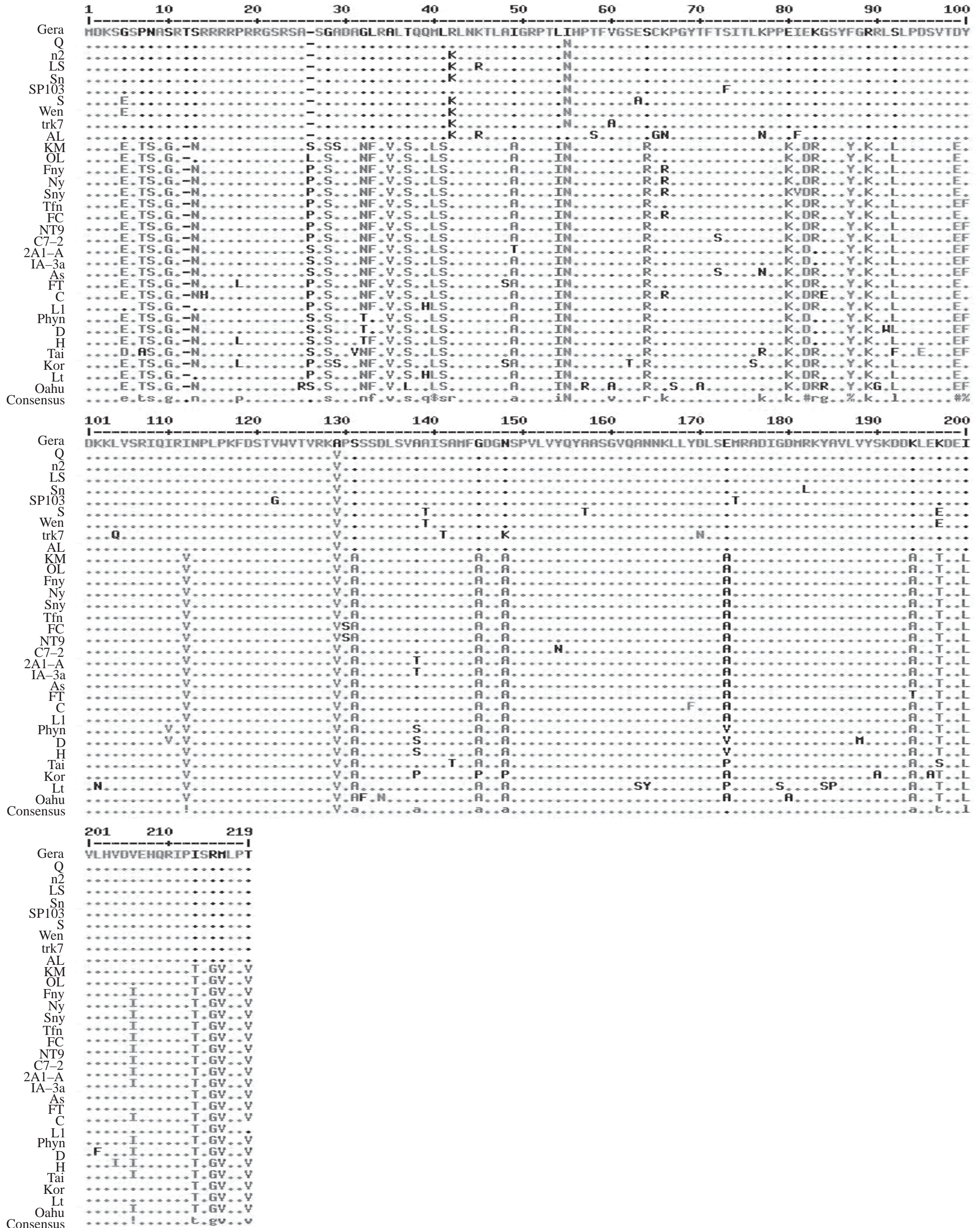

Figure 2. Amino acid sequence alignment of coat protein of CMV-Gera isolate with that of CMV strains of subgroups I (A and B), II and Indian isolates (Phym, D, H, OL, Ll, Lt and AL) using Multalin programme 5.4.1.

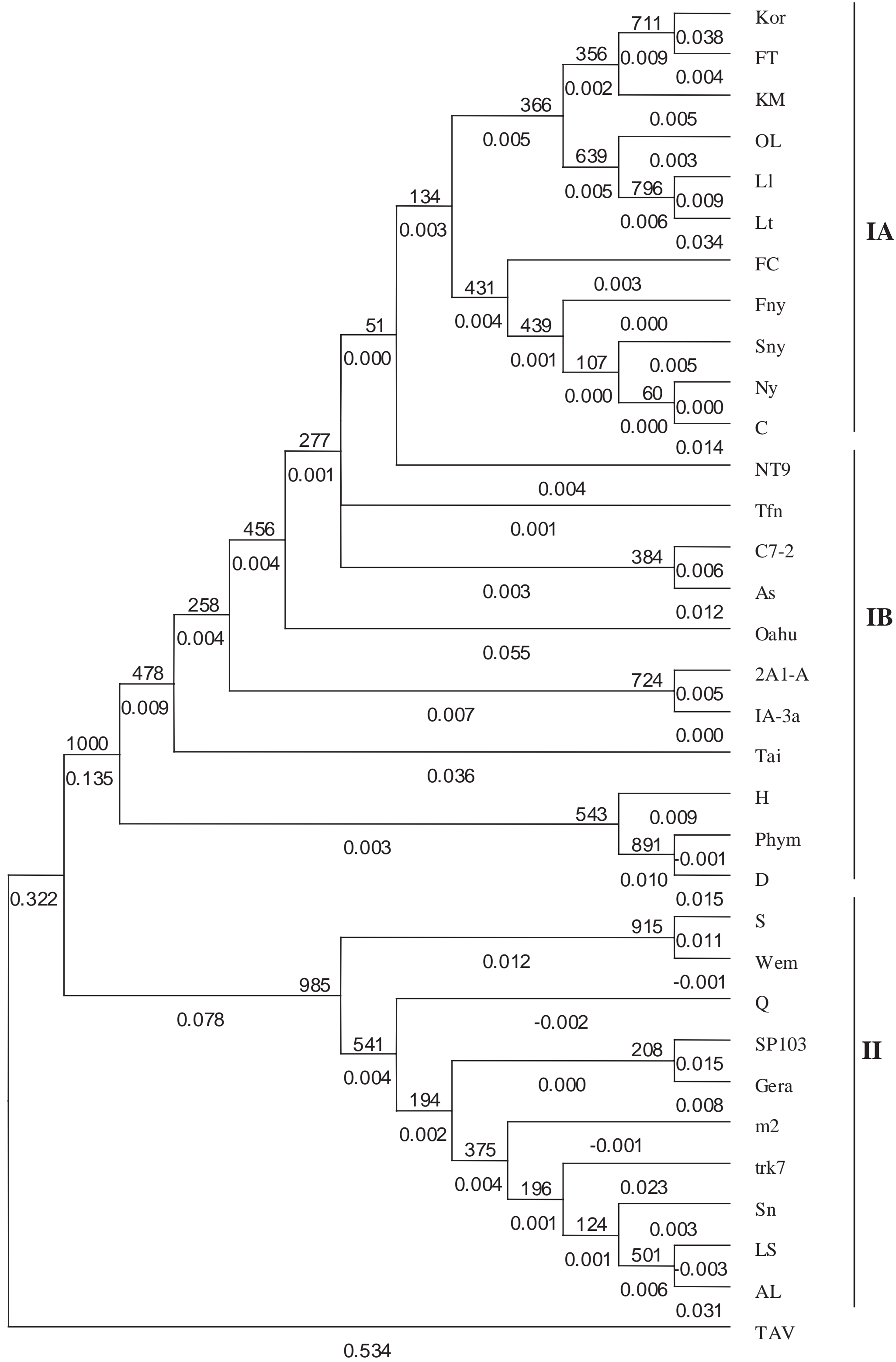

Figure 3. Phylogenetic relationship of CMV-Gera with the strains of CMV subgroups I (A and B), II and Indian strains based on the amino acid alignment using ClustalW version 1.82 through TreeExplorer. *Tomato aspermy virus* (TAV) (Acc. No. AJ550020) was used as an outgroup. The bootstrapping and branch length values are above and below the joining lines, respectively.

reported CMV isolates since an extra T (threonine) is present at 12th position but at 28th position it lacks serine (S) that may have some impact on coat protein properties.

Also, these results are in contrast with the earlier reports (Srivastava and Raj 2004) that CMV isolates/strains belong to subgroup IB since our results clearly showed that CMV-Gera isolate belongs to subgroup II.

Since geranium is an important medicinal and garden crop, it becomes necessary to characterize the virus/es infecting the crop so that effective control measures or quarantine steps can be developed to minimize the losses caused by viruses. Due to high genetic similarity the CMV CP gene can also be used to develop transgenic geraniums that will be useful for the growers.

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