Nucleotide

GenBank

# Cucumber mosaic virus isolate CMV/Bangladesh/2019 segment RNA3, complete sequence

GenBank: MT795916.1

FASTA Graphics

Go to:

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            complete sequence.
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VERSION     MT795916.1
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            Viruses; Riboviria; Orthornavirae; Kitrinoviricota; Alsuviricetes;
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  AUTHORS   Nahiyan,A.S.M., Paul,T.K., Eusufzai,T.K., Hasan,M.M., Islam,S. and
            Ansarey,F.H.
  TITLE     Molecular Identification and Characterization of Cucumber Mosaic
            Virus Isolate Associated with Mosaic Disease of Cucumber in
            Bangladesh
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2227)
  AUTHORS   Nahiyan,A.S.M., Paul,T.K., Eusufzai,T.K., Hasan,M.M., Islam,S. and
            Ansarey,F.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUL-2020) Advanced Seed Research and Biotech Center
            (ASRBC), Advanced Chemical Industries (ACI) Ltd., House 18/B, Road
            126, Gulshan Avenue, Dhaka North, Dhaka 1212, Bangladesh
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     2221 gagacca
//
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RESEARCH ARTICLE

# Molecular characterization of *Cucumber mosaic virus* subgroup II isolate associated with cucumber in Bangladesh

**Tridib Kumar Paul[1] · Mohammad Mahmood Hasan[1] · Tasnin Khan Eusufzai[1] · Md. Moniruzzaman Hasan[1] · Saiful Islam[1] · Fareyzul Haque Ansarey[1] · Mohammad Nurul Islam[2] · Abu Shamim Mohammad Nahiyan[1]**

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## Abstract

*Cucumber mosaic virus* (CMV) associated with severe cucumber (*Cucumis sativus* L.) mosaic disease was identified in leaf samples collected from 13 districts of Bangladesh during the period 2017–2020. Leaves and fruits with typical mosaic disease symptoms were observed under field conditions. Infected leaves were first screened by DAS-ELISA and the infection by CMV was further confirmed through RT-PCR employing primers specific to the conserved region and coat protein of CMV RNA3. The full length amplified RNA3 was cloned and sequenced. Sequence analysis showed that the RNA3 sequence of the isolate had the highest (98%) similarities with an Indian isolate (CMV-Palampur). Based on the highest sequence identities and close phylogenetic relationship, the causal virus of mosaic disease in cucumber was identified as an isolate of CMV belonging to subgroup II. All the conserved motifs of CMV RNA3 at both nucleotide and amino acid level were found as well. Recombination analysis showed that the probable parents of this isolate are CMV-Tsh and CMV-CTL strains from China. This virus strain might have got introduced in Bangladesh through imported seeds of Cucumber from other Asian countries. To the best of our knowledge, this is the first-ever report of incidence of CMV subgroup II from Bangladesh. The isolate is designated as CMV-BD.

**Keywords** *Cucumber mosaic virus* · Recombination analysis · RT-PCR · DAS ELISA · Subgroup II

## Introduction

*Cucumber mosaic virus* (CMV), a single-stranded positive-sense RNA plant virus, belongs to the genus Cucumovirus of the family Bromoviridae. It is distributed worldwide and has a very broad host range of about 1200 species causing severe damage in some of the most economically important species under *Solanaceae* and *Cucurbitaceae* families (Roossinck et al. 1999). CMV is a tripartite virus having three plus-sense, single-stranded RNA molecules, encased in separate particles. RNA1 and RNA2 encode for the protein 1a and 2a respectively, which form the replicase complex (Palukaitis et al. 1992). N-terminal region of 1a protein contains a putative methyltransferase domain (Rozanov et al. 1992) and the C-terminal region shows sequence similarity to viral helicases (Gorbalenya et al. 1989). RNA2 encodes for another protein 2b, which is expressed from the sub-genomic RNA4 A and acts as a suppressor of gene silencing (Li et al. 1999). This protein plays a role in the long-distance movement of the virus (Wang et al. 2004) and also behaves as a pathogenicity determinant (Shi et al. 2002). Another study showed that this suppressor protein is indirectly involved in aphid transmission as well (Ziebell et al. 2011). Lastly, the genomic segment of CMV, RNA-3, contains 2 genes, namely, 3a encoding cell to cell movement protein (MP) and 3b encoding coat protein (CP) (Roossinck et al. 2000). The coat protein is required for encapsidation of the genomic RNAs into virus particles (Ng and Perry 2004) and affecting symptom expression (Suzuki et al. 1995).

CMV strains are divided into two major subgroups, termed, subgroup I and subgroup II based on serological typing and sequence similarity of genomic RNA (Palukaitis

Tridib Kumar Paul, Mohammad Mahmood Hasan and Tasnin Khan Eusufzai contributed equally to this work.

✉ Abu Shamim Mohammad Nahiyan
nahiyan80@hotmail.com

[1] Advanced Seed Research and Biotech Centre, ACI Ltd., Dhaka 1212, Bangladesh

[2] Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka, Dhaka 1000, Bangladesh

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et al. 1992). Subgroup I is further divided into IA and IB based on the diversity of the CP gene and the 5′ untranslated (UTR) region of RNA3 (Roossinck et al. 1999). There are 92–94% similarities present in the nucleotide sequence of the CMV strains within the subgroup IA and IB. Though subgroup IA and II have a worldwide distribution, IB is restricted to Asia only (Roossinck 2001).

Cucumber is widely cultivated in Bangladesh for substantial economic return. In 2018–19 Bangladesh produced 73,220 Mt of cucumber from approx. 10,000 ha of land with an average yield potential of 7.2 Mt/ha (BBS 2019). However, this production rate is quite low in comparison with other major cucumber growing countries (Lutfa et al. 2019). One of the major reasons for such low productivity of cucumber in Bangladesh is the widespread infection of CMV resulting in mosaic disease (Akhter et al. 2019). Due to the hot and humid conditions of sub-tropical Bangladesh, the disease is more widespread than in many parts of the world. This disease has been creating immense economic losses to the cucurbit growers, ultimately to the whole nation because of the production of misshapen and mottled fruits. To date, no CMV resistant/tolerant variety from Bangladesh is reported. Othercucurbitaceous vegetables widely in Bangladesh are sweet gourd/summer squash (*Cucurbita moschata*), bottle gourd (*Lagenaria siceraria*), white gourd/wax gourd (*Benincasa hispida*), pumpkin (*Cucurbita maxima*), bitter gourd (*Momordica charantia*) and ridge gourd (*Luffa acutangula*). Viral disease symptoms (yellow mosaic spot) frequently appear in these cucurbitaceous vegetables throughout the country but there is no data on infection of these cucurbits by CMV isolates (Akhter et al. 2019).

Detection of plant viruses and their molecular characterization is a prerequisite for developing virus-resistant or tolerant crops. To date, only one report is available on the molecular detection of CMV in eggplants from Bangladesh (Bagewadi et al. 2015). Earlier CMV was reported from cucumber plants, however, it was limited to serological identification only (Akhter et al. 2008). Here, an attempt was made to characterize the CMV isolate prevalent in Bangladesh.

## Materials and methods

### Collection of diseased leaf samples and DAS-ELISA

Leaves showing disease symptoms (mosaic pattern of green and yellow on the leaf blade and fruit, stunted fruit) were collected from 13 different locations of northern and southern parts of Bangladesh, namely, Panchagar, Nilphamari, Thakurgaon, Dinajpur, Jamalpur, Tangail, Manikganj, Dhaka, Gazipur, Munshiganj, Brahmanbaria, Cumilla, and Feni (Fig. 1). Altogether 130 leaf samples showing mosaic pattern (alternation of yellow- and green-coloured blocks on the leaf blade) and suspected to be infected by CMV were collected. For narrowing down the sample size and better specificity, the collected samples were subjected to Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA) using CMV Complete kit 480 (BIOREBA, Switzerland) following the manufacturer's instructions. The leaves were preserved at − 80 °C in a freezer until further experiments.

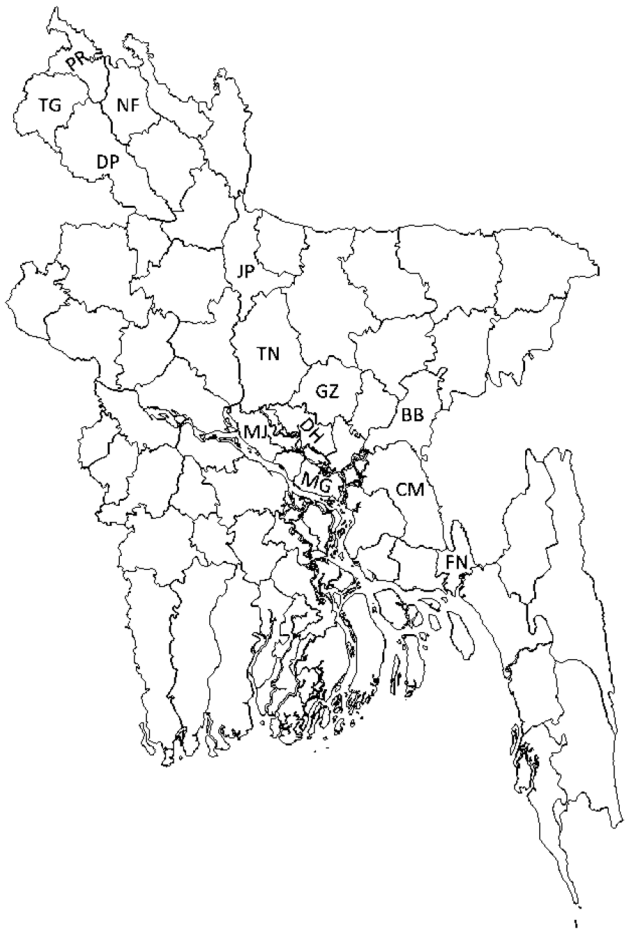


**Fig. 1** Map of Bangladesh showing different district from where CMV infected leaf samples were collected. The locations being visited for sample collection is marked with letters. *PR* Panchagar, *NF* Nilphamari, *TG* Thakurgaon, *DP* Dinajpur, *JP* Jamalpur, *TN* Tangail, *GZ* Gazipur, *DH* Dhaka, *MJ* Manikganj, *MG* Munshiganj, *BB* Brahmanbaria, *CM* Cumilla, *FN* Feni

### RNA extraction

Total RNA was extracted from infected leaf tissue (100–200 mg) using Plant RNA Purification Mini Kit provided by Thermo Fisher Scientific, USA according to manufacturer's instructions. The isolated RNAs were stored at − 80 °C until further use.

**Table 1** List of primers used in this study for the identification of CMV with respective product size

| Primers | | | Primer sequence | Tm (°C) | Expected amplicon (bp) | References |
|---|---|---|---|---|---|---|
| Specification | Name | | | | | |
| RNA3 specific primer | CMV_RNA3 | F | 5′-GTAATCTTACCACTTTCTTT-3′ | 49 | ~ 2214 | Kumari et al. (2013) |
| | | R | 5′-TGGTCTCCTTATGGAGAACCTG-3′ | | | |
| Conserved region RNA3 | CMV_F | F | 5′-GTAGACATCTGTGACGCGA-3′ | 54 | 540 | De Blas et al. (1994) |
| | | R | 5′-GCGCGAAACAAGCTTCTTATC-3′ | | | |
| Coat protein | CMV_CP | F | 5′-TTGAGTCGAGTCATGGACAAATC-3′ | 59 | 678 | Lin et al. (2004) |
| | | R | 5′-AACACGGAATCAGACTGGGAG-3′ | | | |

## RT-PCR amplification and gel electrophoresis

Isolated RNAs were subjected to 2 step RT-PCR amplification with respective primers (Table 1). cDNA synthesis of the isolated RNAs was carried out using the RevertAid First Strand cDNA Synthesis kit (Thermofisher Scientific, USA) according to the provided manual. Human GAPDH RNA was used as a positive control. This RNA with specific primers was provided with the kit that generates a product of 496 bp.

The cDNA pool was then subjected to PCR amplification in a thermocycler machine. The PCR was carried out using Q5® High-Fidelity DNA Polymerase (New England Biolabs, USA). For this purpose, 1 µl of cDNA was used. The PCR mixture was subjected to one cycle of initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 49/54/59 ºC (based on primers mentioned in Table 1) for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 7 min in a Thermocycler (BIO-RAD C1000 Touch thermocycler). After completion of the reaction, products were kept at 4 °C before gel electrophoresis.

## Cloning and sequencing

An expected amplicon of ~2200 bp obtained with RNA3 specific primers was eluted using a QIAquick gel extraction kit (Qiagen, USA) according to the instructions given by the manufacturer. The eluted products were ligated into the pGEM®-T Easy Vector System (Promega Corporation, USA) according to the Manufacturer's instructions. After ligation, plasmids were transformed into competent cells of *E. coli* DH5α followed by standard molecular biology procedures (Sambrook 1989). One of the positive clones was sequenced from both ends. The DNA sequence was determined by fluorescent dideoxy chain terminator technology in an automated DNA sequencer (ABI PRISM 3130xl genetic analyzer) using ABI prism Big Dye Terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems, USA).

## Sequence analysis

The obtained complete sequence of RNA3 genome assembled by aligning on overlapping regions using BioEdit software version 7.0 (https://bioedit.software.informer.com/7.0/). The obtained nucleotide was subjected to the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov). Nucleotide (nt) and amino acid (aa) sequence alignments were performed using the CLUSTALW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic relationships between these isolates were inferred from the aligned nucleotide sequences by the Molecular Evolutionary Genetic Analysis version 10.0.5 (MEGA X) (https://www.megasoftware.net/). The tree was constructed following the Neighbour-joining method with 1000 non-parametric bootstrap replicates (Tamura et al. 2007). Percentage homology study with other CMV strain of subgroup I and II was performed by the Genomatix DiAlign program (Morgenstern 1999). To find the ORF in the RNA3 sequence the nucleotide sequence was translated into the amino acid sequence using the Expasy translation tool (https://web.expasy.org/translate/) and then subjected to the ORF finder program of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). The translated amino acid sequences were used to compare the amino acid substitutions with the MP and CP sequences of other strains available in the NCBI database. The sequences were aligned using the Clustal Omega Multiple Sequence Alignment program (https://www.ebi.ac.uk/Tools/msa/clustalo/).

## Recombination analysis

To detect possible recombination between different CMV isolates, automatic recombination scans of sequence alignments was carried out using the RDP4 program (Martin et al. 2015). In total, 7 recombination detection methods were implemented and included RDP (Martin et al. 2004, 2015), Bootscan (Salminen et al. 1995), GENECONV (Padidam et al. 1999), MaxChi (Posada and Crandall 2001), Chimaera (Posada and Crandall 2001), Siscan (Gibbs et al. 2000) and 3SEQ (Boni et al. 2007). The program was run using the

**Table 2** The DAS-ELISA result reflecting infection frequency in samples collected from different locations of Bangladesh

| Location | ELISA test result | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Panchagar[a] | + | + | + | + | + | + | + | + | + | - |
| Dinajpur[a] | + | − | − | + | + | − | + | + | + | + |
| Thakurgaon | − | − | − | + | + | − | − | − | − | − |
| Nilphamari | + | − | + | + | + | − | − | − | − | − |
| Jampalpur[a] | − | + | − | + | + | + | + | + | + | − |
| Tangail[a] | + | − | + | + | + | + | + | + | − | − |
| Brahmanbaria | − | − | − | − | + | + | − | − | − | − |
| Manikganj | − | + | + | + | − | − | + | − | − | − |
| Gazipur[a] | − | + | + | + | + | − | + | + | + | + |
| Dhaka[a] | + | + | + | − | + | + | + | + | + | − |
| Cumilla | − | − | − | + | − | − | − | − | − | − |
| Munshiganj[a] | − | + | + | + | + | + | + | + | − | + |
| Feni | − | + | − | − | − | − | − | − | + | − |

'+' presence of CMV infection, '−' absence of CMV infection

[a]Infection frequency greater than 50%

default settings plus the Bonferroni corrected P value cut-off (a = 0.05). The results obtained in the recombination analysis by RDP were confirmed using a boot scanning method (Salminen et al. 1995).

## Results

### Screening of CMV infected samples

Among the total collected samples, 69 samples tested positive for the presence of CMV in DAS-ELISA. The infection rate was found much frequent in Panchagar, Dinajpur, Jamalpur, Tangail, Gazipur, Dhaka and Munshiganj (Table 2). All the samples that were positive in DAS-ELISA for CMV infection also showed a positive band in 2-step RT-PCR amplification, however, 39 samples showed prominent bands and the rest of them were light. A540 bp band was observed when the CMV_F primer was used (Fig. 2). It amplified the conserved region of RNA3 that contained an intergenic region (IR) and a portion of the CP. For another primer, CMV_CP, an amplicon of 678 bp was observed (Fig. 2). This amplicon was the sequence that encodes coat protein. The presence of these 2 desired bands confirmed the presence of CMV in the infected leaf samples. Following the RT-PCR confirmation of the presence of CMV, the full-length RNA3 (~ 2200 bp) was amplified from infected leaf tissues using the CMV_RNA3 primer. Upon gel electrophoresis, a ~ 2200 bp band was observed (Fig. 3). Hence, the amplification of the RNA3 of CMV was successful.

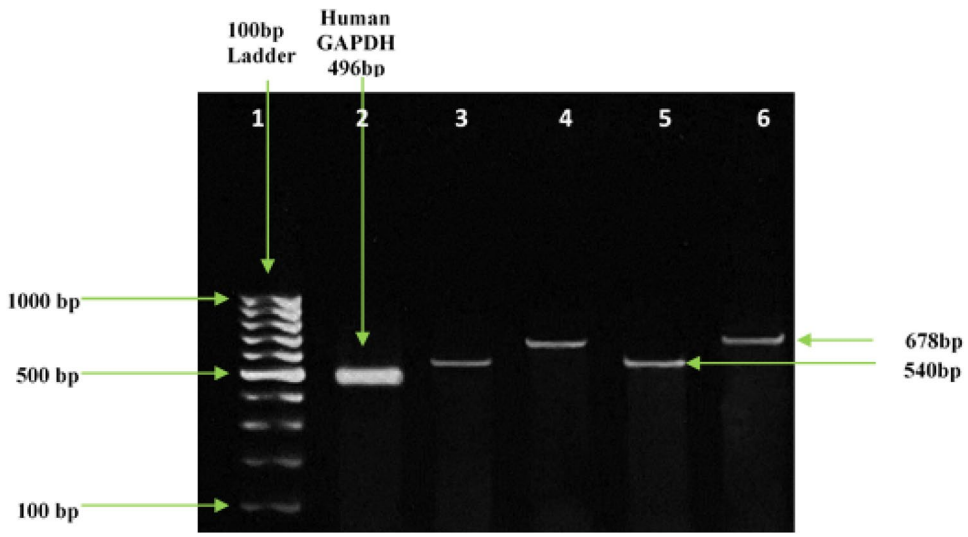


**Fig. 2** Agarose gel electrophoresis of amplified conserved region and coat protein of RNA3. Lane 1: DNA 100 bp ladder, lane 2: positive control (Human GAPDH) and lane 3–6: different representative experimental samples. Lane 3 and 5 showing amplification of conserved region and Lane 4 and 6 showing amplification of CP region of RNA3

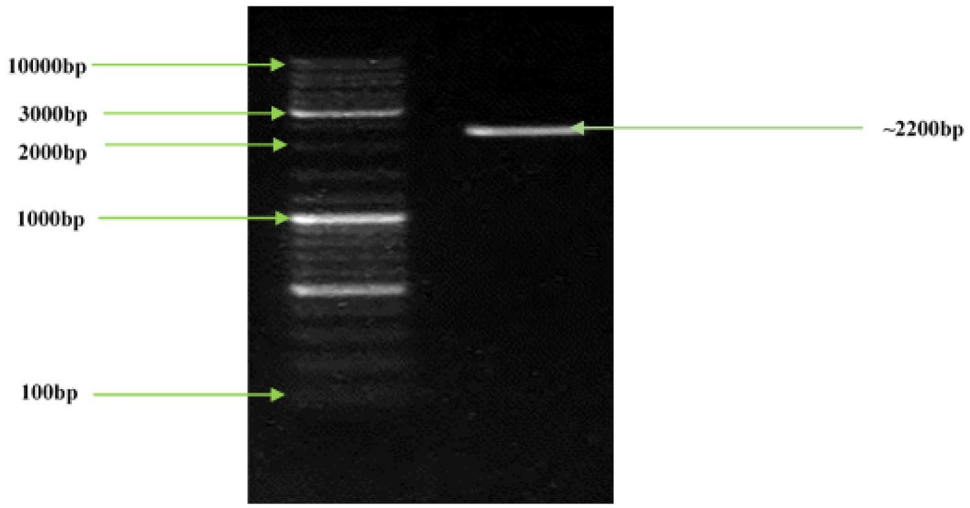


**Fig. 3** Agarose gel electrophoresis of the amplified full length RNA3 sequence. Lane-1 contains 1 kb ladder and lane-2 shows ~ 2200 bp band obtained using RNA3 specific CMV_ RNA3 primer

## Analysis of the RNA3 sequence of the CMV isolate

### Phylogenetic tree construction

Sequencing results provided a nucleotide sequence of 2227 bp which was subjected to the NCBI nucleotide BLAST program. The BLAST result showed that the subject had similarity with RNA3 of other CMV strains at the nucleotide level and the highest (98%) similarity was observed with HE583224 (Palampur-India). This sequence was submitted to the NCBI database (accession number: MT795916). To understand the phylogenetic relationship, a total of 29 sequences of RNA3 molecule of CMV belonging to different subgroups, i.e., subgroup IA, IB and II were downloaded. These 29 sequences along with the current RNA3 sequence and an out-group sequence, *Peanut Stunt Virus* RNA3 full sequence (accession number: U15730), were subjected to multiple sequence alignment. The aligned sequences were then used for developing a phylogenetic tree. Three clusters were formed from the 30 sequences which indicated that the subgroups of CMV were IA, IB and II (Fig. 4). The tree determined that the current isolate had the closest relationship with the 'HE583224-strain Palampur-India' within subgroup II. This confirmed that the CMV isolate of the current study belongs to belonged to subgroup II and was termed as CMV-BD.

### ORF finding

The ORF finder program suggested that the RNA3 sequence of CMV-BD consisted of 2 ORFs, namely, MP and CP (Fig. 5). The first ORF consisted of the start codon (AUG) at 97 nucleotides (nt) position and the termination codon (UAG) at 936 nt positions. The start codon of the second ORF was situated at the 1249 nt position and had a terminator codon (UAG) in the 1905 nt position. Therefore, the MP gene consisted of 840 nt that translated to 279 aa residues and the CP consisted of 657 nt that translated to 218 aa residues. These 2 ORFs were separated by a 312 nt long intergenic region (IR) and flanked by a 5′-untranslated region (5′ UTR) and a 3′ UTR of 96 and 322 nucleotides, respectively.

### Amino acid substitution

At the amino acid level, the MP of CMV-BD had several substitutions when compared to other strains of the world (Fig. 6). The MP of CMV-BD had 3 substitutions with the Palampur-India strains at 18 (S/T), 42 (S/G) and 273 (S/P) positions and 3 substitutions with TN at 18 (S/T), 158 (F/Y) and 253 (N/S) positions. The CP of CMV-BD showed 100% similarities with the Palampur-India strain. However, several substitutions were observed with other strains. For instance, with the TN strain, 3 substitutions were present at 10 (S/G), 41 (K/R) and 106 (R/H) positions. The matrix for pairwise alignments was obtained using the DiAlign 2 program (Morgenstern 1999). Genomatix Dialign analysis of the complete RNA3 sequence of CMV-BD against some selected strains of CMV belonging to subgroup IA, IB and II revealed its highest 98–96% similarities with Palampur, TN, LS, Tsh, IPO, and Q strains of CMV of subgroup II reported from India, Japan, USA, China, Netherland, and Australia, respectively. It had 95% similarity with strain Tag-china from subgroup II. The isolate showed the highest 78% similarities with Pepo, Y, Mf, Ns, Fny, and Leg strains belonging to subgroup IA reported from Japan, South Korea, Hungary, and the USA. Whereas 77–76% similarities were found with the SD, IA, CS, ND, CTL, and Ix strains of CMV belonging to the subgroup IB reported from China, Indonesia, India, and the Philippines. This Genomatrix DiAlign program also analyses sequence similarity within the different segments of complete RNA3 sequence, e.g., MP, CP, IR, 5′ UTR and 3′ UTR both at nucleotide and aa level (Table 3).

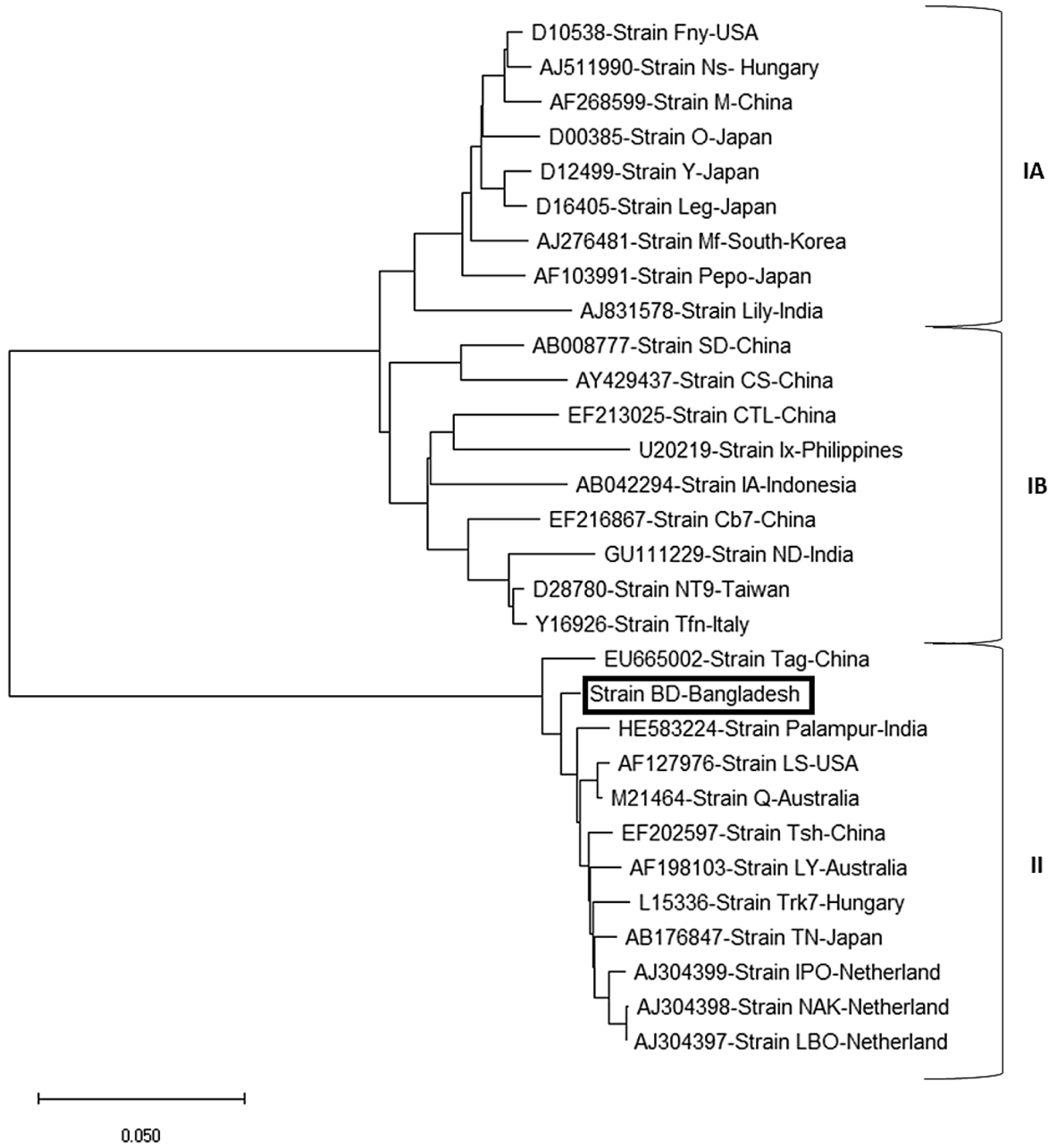


**Fig. 4** Phylogenetic placement and relationship of CMV-BD with other CMV isolates worldwide. Neighbor joining tree prepared by MEGA X showing phylogenetic relationship of CMV from different parts of the world based on RNA3 nucleotide sequence. Distance scale represents the percentage of genetic variation among strains

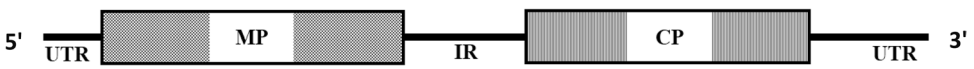


**Fig. 5** Diagrammatic representation of the RNA3 of CMV showing its ORFs. Two ORFs (MP & CP) are flanked by 5′-UTR and 3′-UTR. There is an intergenic region (IR) between the 2 ORFs

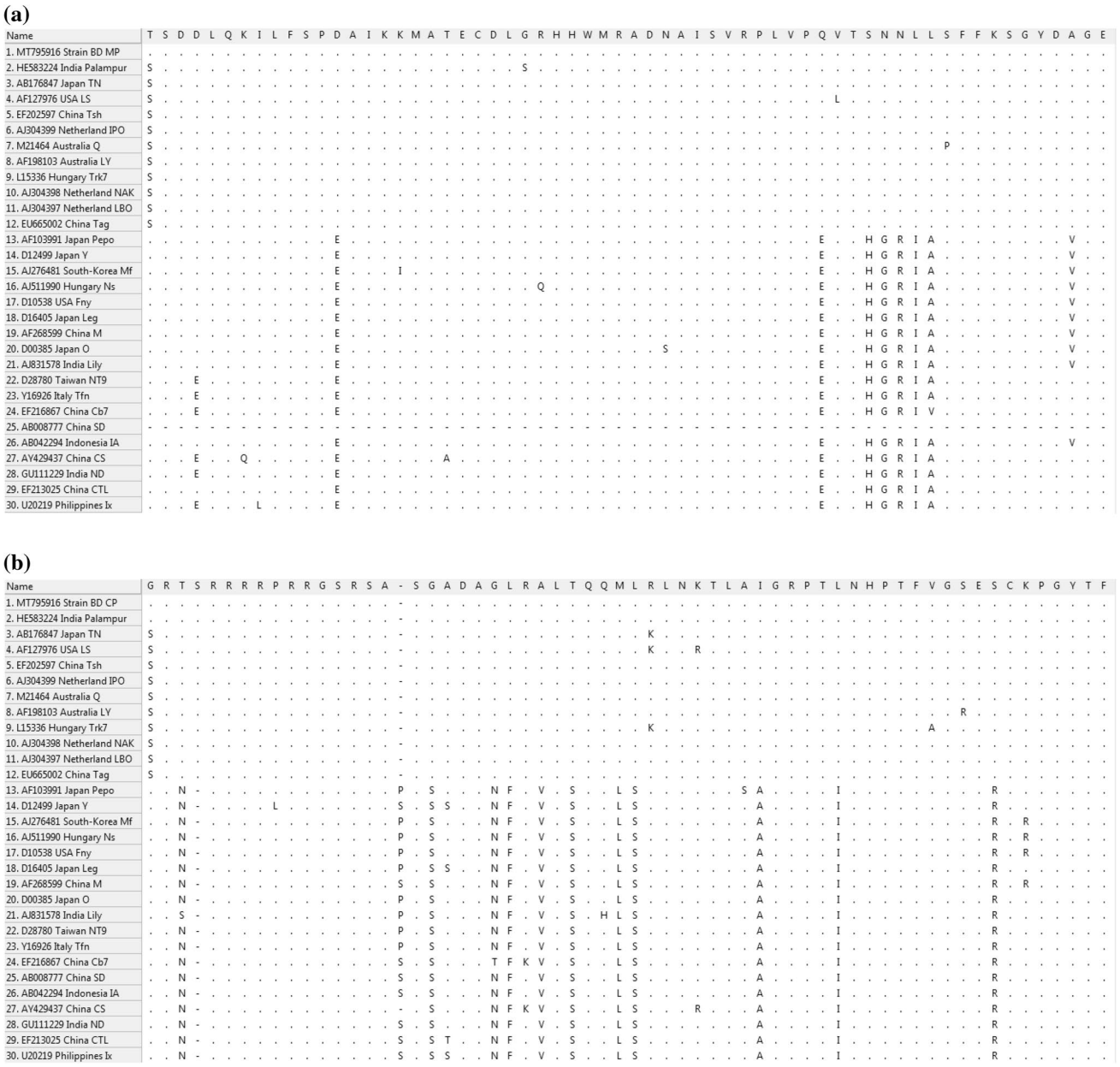


**Fig. 6** Multiple sequence alignment portion of **a** movement protein (aa position 18–79) and **b** coat protein (aa position 10–71) of CMV RNA3 at amino acid level showing amino acid substitutions between CMV-BD and other strains of the world

## Recombination analysis

Recombination analysis of CMV-BD along with 29 other strains was studied using the full-length sequence of RNA3. Recombination analysis using the RDP4 tool predicted that there was a recombination event between 1224 and 1254 nt positions of RNA3 sequence of CMV-BD isolate. The RDP4 program detected the 'EF202597-Tsh' and 'EF213025-CTL' isolates as the possible major and minor parental sequences, respectively (Fig. 7). Besides, recombination analysis was carried out for CMV-Palampur strain which showed that 'EU665002-Tag' and 'AB042292-IA' are the major and minor parents respectively (Figure not shown).

## Discussion

CMV was first identified in New York in 1916 and till now it has been isolated from more than 500 plant species (García-Arenal and Palukaitis 2008). identification. The CMV is mainly divided into 2 subgroups, e.g., subgroup-I and subgroup-II. Subgroup-I is further divided into subgroups

**Table 3** RNA3 sequence-based percentage homology value of *Cucumber mosaic virus* (CMV) isolate (CMV-BD) at nucleotide (nt) and amino acid (aa) level with respect to various isolates of CMV reported worldwide obtained by Genomatix DiAlign program

| Accession no. | Country | Strain/isolate | Sub-group | RNA3 | MP | | CP | | IR | 5′ UTR | 3′ UTR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | nt | aa | nt | aa | | | |
| HE583224 | India | Palampur | II | 98 | 98 | 98 | 98 | 99 | 93 | 98 | 98 |
| AB176847 | Japan | TN | II | 96 | 97 | 98 | 96 | 98 | 98 | 98 | 98 |
| AF127976 | USA | LS | II | 96 | 97 | 98 | 96 | 97 | 98 | 98 | 98 |
| EF202597 | China | Tsh | II | 96 | 98 | 98 | 97 | 98 | 98 | 98 | 96 |
| AJ304399 | Netherland | IPO | II | 96 | 97 | 98 | 96 | 96 | 97 | 97 | 98 |
| M21464 | Australia | Q | II | 96 | 97 | 98 | 96 | 98 | 98 | 98 | 98 |
| AF198103 | Australia | LY | II | 96 | 98 | 97 | 97 | 98 | 98 | 97 | 94 |
| L15336 | Hungary | Trk7 | II | 96 | 97 | 97 | 96 | 95 | 98 | 97 | 97 |
| AJ304398 | Netherland | NAK | II | 96 | 97 | 97 | 96 | 95 | 97 | 97 | 97 |
| AJ304397 | Netherland | LBO | II | 96 | 97 | 97 | 96 | 95 | 97 | 97 | 97 |
| EU665002 | China | Tag | II | 95 | 97 | 97 | 95 | 98 | 98 | 97 | 90 |
| AF103991 | Japan | Pepo | IA | 78 | 77 | 84 | 71 | 83 | 81 | 45 | 50 |
| D12499 | Japan | Y | IA | 78 | 75 | 83 | 72 | 83 | 81 | 45 | 50 |
| AJ276481 | South-Korea | Mf | IA | 78 | 77 | 83 | 72 | 83 | 82 | 46 | 51 |
| AJ511990 | Hungary | Ns | IA | 78 | 75 | 83 | 70 | 83 | 81 | 45 | 51 |
| D10538 | USA | Fny | IA | 78 | 77 | 83 | 69 | 83 | 80 | 51 | 52 |
| D16405 | Japan | Leg | IA | 78 | 76 | 83 | 71 | 83 | 83 | 42 | 47 |
| AF268599 | China | M | IA | 77 | 77 | 83 | 71 | 81 | 81 | 43 | 47 |
| D00385 | Japan | O | IA | 77 | 77 | 83 | 71 | 83 | 84 | 51 | 47 |
| AJ831578 | India | Lily | IA | 76 | 58 | 67 | 69 | 84 | 81 | 51 | 50 |
| D28780 | Taiwan | NT9 | IB | 78 | 76 | 83 | 74 | 83 | 61 | 44 | 52 |
| Y16926 | Italy | Tfn | IB | 78 | 76 | 83 | 75 | 83 | 60 | 51 | 55 |
| EF216867 | China | Cb7 | IB | 78 | 76 | 84 | 70 | 83 | 62 | 43 | 55 |
| AB008777 | China | SD | IB | 77 | 77 | 81 | 72 | 82 | 67 | 46 | 52 |
| AB042294 | Indonesia | IA | IB | 77 | 77 | 82 | 74 | 83 | 59 | 43 | 59 |
| AY429437 | China | CS | IB | 77 | 75 | 82 | 75 | 81 | 59 | 42 | 54 |
| GU111229 | India | ND | IB | 77 | 69 | 75 | 75 | 83 | 58 | 44 | 53 |
| EF213025 | China | CTL | IB | 77 | 74 | 83 | 73 | 83 | 70 | 43 | 63 |
| U20219 | Philippines | Ix | IB | 76 | 74 | 82 | 70 | 82 | 55 | 44 | 57 |

IA and IB. To identify the group to which a CMV isolate belonged, most of the scientists focus on the RNA3 genome which contains MP and CP (Babu et al. 2014; Bagewadi et al. 2015; Dong et al. 2018; Khan et al. 2011; Kumar et al. 2014, 2015). In our study, we also used primers specific to different regions of CMV RNA3. Before performing RT-PCR we did DAS-ELISA to narrow down the sample volume. Using the primers that amplify the conserved region of RNA3 (comprising partial regions of both IR and CP), 540 bp product was obtained. An amplicon of 678 bp was seen for the CP specific primers which were exactly similar to the findings reported by De Blas et al. (1994) and Lin et al. (2004). The presence of these 2 regions confirmed the presence of CMV in our collected samples, therefore, we took our study further to derive the full-length sequence of RNA3 molecule and characterize the CMV isolates of this study.

Full-length amplification of RNA3 using RNA3 specific primers yielded a band of approximately 2200 bp and the sequencing result revealed the sequence length to be 2227 bp and the original authors who used this set of primers reported ~2214 bp in length (Kumari et al. 2013). The sequence was subjected to nucleotide BLAST and the highest similarity (98%) was found with the Palampur-India strain. This sequence along with 29 other RNA3 sequences of different CMV strains across the world was used for multiple sequence alignment and phylogenetic tree construction. The tree suggested that our isolate belonged to subgroup II. Works carried out in India on the identification of CMV in different crops recorded the presence of CMV strains belonging to all the subgroups, i.e., subgroup IA (Dubey and Aminuddin 2008), subgroup IB (Kumar et al. 2015; Madhubala et al. 2005; Srivastava et al. 2004) and subgroup II (Khan et al. 2011; Kumari et al. 2013; Sudhakar et al. 2006).

We studied the homology of RNA3 molecule between different isolates of the world at both nt and aa level and analyzed the sequence further for the conserved motifs that are found in all the CMV strains. Our data from the Genomatix

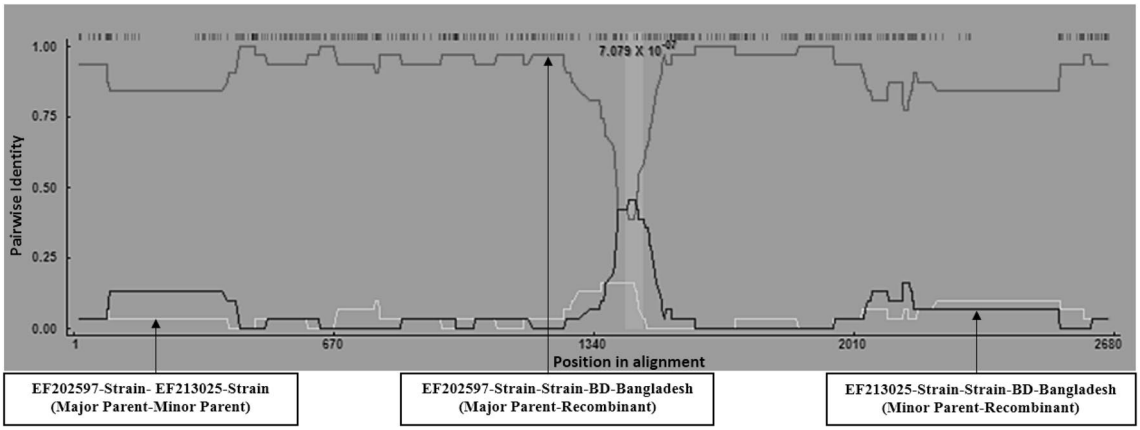


**Fig. 7** Recombination analysis using RDP4 tool: Plot showing the results of the RDP analysis. RDP4 program detected the 'EF202597-Tsh'and 'EF213025-CTL' isolates as putative major and minor parent respectively for CMV-BD isolate

DiAlign program (Table 3) suggested that the MP, CP, 5′ UTR and 3′ UTR were more conserved with the Palampur-India strain each with 98% similarity. In terms of IR, 93% similarity was observed. Though there was 7% dissimilarity in the IR region no change was observed in the core promoter sequence of CP that lies in the same region. In the IR region, the conserved motif 'GGT TCA ATT CC' was found as well (McGarvey et al. 1995). Interestingly, the CMV-BD strain had 98–97% similarities in the IR with the other isolates of subgroup II, however, the overall similarity was 2% less with all other strains than the Palampur strain. When compared with the isolates of subgroup I, the overall similarity dropped to 78–76%. Maximum homology between the isolates of subgroup I and II was present in the MP and CP sequences and the greatest diversity was observed in the IR, 5′ UTR and 3′ UTR. Such variation in these regions of subgroups I and II are previously reported (Swapna et al. 2011). Overall high homology in the MP and CP sequences at aa level is to maintain the structure and function of the proteins because defective mutants are reported to be faulty in aphid transmission (Ng and Perry 2004).

The 5′ UTR and 3′ UTR regions of CMV-BD showed a high level of similarity with other isolates of sub-group II (Table 3). Sequence alignments of complete RNA3 sequences of CMV-BD isolate also revealed that all the common features of CMV belonging to the 5′ UTR and 3′ UTR were present in the CMV-BD strain, for illustration, the presence of conserved TG tract in 5′ UTR which was reported earlier by Boccard and Baulcombe (1993). In the 3a ORF the motif 'CAA CAG TCC TC' and another highly conserved region 'TCC AGC TTA CGG CTA AAA TGG TCA GTC G' in 3′ UTR, were also obtained as in other CMV strains (McGarvey et al. 1995).

At the amino acid level, the CMV-BD strain had the highest homology in the MP and CP with other isolates of subgroup II. In movement protein, maximum strains of both subgroup I and II held T at position 6 whereas, CMV-BD had P like Palampur, TN, LS and Q strains. Such 6T > P substitution was not observed in subgroup I strains. At position 18, S was conserved in subgroup II isolates and the case of subgroup I, it was substituted by T. Interestingly, CMV-BD had T in this position even it belongs to subgroup II. A similar observation was observed at position 273 where CMV-BD had P. At this position, P was conserved in subgroup I and in subgroup II it was conserved with S. The amino acid substitution 51 N > K and 240I > F in the MP of Fny strain is reported to be associated with reduced movement of CMV (Gal-On et al. 1996; Kaplan et al. 1997). The CMV-BD strain had N in position 51. In position 240, CMV-BD had L like all the other strains of subgroup II whereas, the strains of subgroup I showed that I was conserved in position 240. Such data supported that the MP of CMV-BD is fully functional. At position 158, only CMV-BD and Palampur strains had Y whereas, all other isolates of subgroups I and II had F. In CP of CMV, at 16 position, arginine was present which is conserved in all other CMV strains including CMV-BD. This arginine at 16 position is highly conserved and has a critical role in viral RNA and CP interactions (Perry et al. 1998; Schmitz and Rao 1998). Amino acid residue at position 129 is also proven to be critical in symptom determination, e.g., pale green mosaic phenotype (E and D), necrotic phenotype (K, H, I, L and V), and chlorosis (C, S, and Q) (Mochizuki and Ohki 2011). In CMV-BD, V was observed in position 129 and necrotic regions were also observed in the collected leaf samples of this study which supports the evidence of Mochizuki and Ohki (2011). At position 10, G was conserved in all the strains of subgroup I. In the same position, S was conserved in subgroup II strains. Though CMV-BD and Palampur strains are in subgroup II it showed G in position 10 like subgroup I. Data showed that R is

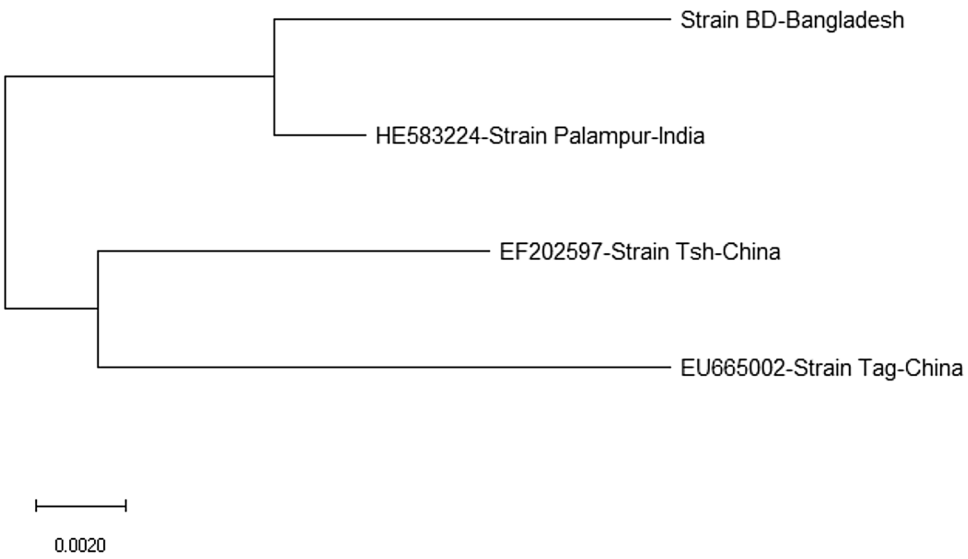


**Fig. 8** Phylogenetic placement and relationship of BD, Palampur, Tsh and Tag strains of CMV. Neighbor joining tree prepared by MEGA X showing phylogenetic relationship of these 4 strains based on RNA3 nucleotide sequence. Distance scale represents the percentage of genetic variation among strains

conserved at position 107 in all strains except for CMV-BD and Palampur where there was an R > H substitution. Another 219T > V substitution was observed in CMV-BD which was abundant in subgroup I.

The recombination analysis revealed that there had been a recombination event between 1224 nt and 1254 nt of CMV-BD and the program elucidated that the major and minor parents were Tsh and CTL strains respectively from China. Since the CMV-BD strain showed the highest similarity with the CMV-Palampur strain, we also performed the recombination analysis for the later one. The result showed that the major and minor parents are Tag and IA strains from China and Indonesia respectively (data not shown). Considering the major parents of CMV- BD and CMV-Palampur strains, it is suggested that both of them might have got introduced from China. However, for further understanding, we constructed a phylogenetic tree using the full-length RNA3 nt sequence of BD, Palampur, Tsh and Tag strains. The phylogenetic tree (Fig. 8) showed that the Tsh and Tag were closely related.

Previously, all strains of India were reported to be in subgroup I but recently the existence of subgroup II is being reported as well and this may be due to the import of propagating materials from other countries (Dubey and Aminuddin 2008). The cucumber seed market of Bangladesh is entirely dependent on import from other Asian regions. Based on the aforementioned information and our data, we propose 2 probable routes of introduction of CMV subgroup II in Bangladesh. The first one is directly through the imported cucumber seeds from different Asian countries and the second one is from China to Bangladesh through India. To date, two studies were conducted to identify CMV in Bangladesh and the earliest one was only based on I-ELISA in different vegetables including cucumber, but they failed to classify the subgroups of the isolates (Akhter et al. 2008). Recently, another study identified CMV in *Solanum melongena* following RT-PCR in Bangladesh and they claimed it to be in the subgroup IB (Bagewadi et al. 2015). However, to the best of our knowledge, this is happening to be the first report on molecular identification of CMV from cucumber plants in Bangladesh and our results revealed that it belonged to subgroup II. This finding along with the previous report (Bagewadi et al. 2015), prove that there is the existence of multiple CMV strains in Bangladesh belonging to both subgroups I and II.

## Conclusions

This first report on the RT-PCR based molecular detection and characterization of CMV from the infected cucumber plants from Bangladesh will provide insights towards the future detection of CMV from cucumber in other regions of Bangladesh as well as in other vegetable crops. This data would be helpful in future crop improvement programs of Bangladesh particularly in developing resistant cucumber varieties following RNAi and CRISPR/CAS technology.

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## Declarations

**Conflict of interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

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