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PARTIAL CHARACTERIZATION OF AN ISOLATE OF CUCUMBER MOSAIC VIRUS FROM ISMAILIA GOVERNORATE

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ABSTRACT

Cucumber mosaic virus (CMV) was isolated from naturally infected cucumber plants *Cucumis sativus* L. grown in various garden and greenhouses of Ismailia Governorate , Egypt exhibiting systemic mosaic , blistering, fruit malformation and stunted plant growth and identified by biological, serological and molecular analysis. The isolated virus gave positive reaction with CMV antiserum but not with antibodies of *WMV* and *SqMV* using DAS-ELISA .*CMV* was able to infect different host plant species including squash , pumpkin , pepper, bean , *Chenopodium amaranticolor* and cowpea , showing foliar symptoms of mosaic, deformations and necrotic and chlorotic ring spots, that resemble those induced by *CMV*. SDS-PAGE test showed various distinguishable sole novel protein bands in four cucumber cultivars infected with CMV but not in healthy one . RT-PCR, with the primer CMV1 and CMV2 for CMV-*cp*. gene , yielded 422 base pair DNA fragments. The following sequences were used in the comparison: Brazil (AF418577), China (FJ403473), New Zealand (AY861395) and India (AJ810260). The partial nucleotide sequence alignment, showed (95%) homology between *CMV* New Zealand isolate and *CMV*-Egyptian isolate .The sequence alignment also indicated that the *CMV* isolate is far from similarity with India *CMV*, showing (92%) according to the phylogenetic homology tree , which were 92 to 99% identical to those of *CMV*- Egypt isolate.

Keywords: *Cucumis sativus* L., *Cucumber mosaic virus (CMV)*, Molecular characterization , Detection and RT-PCR.

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INTRODUCTION

About twenty economically important viruses were detected in cucumber (*Cucumis sativus* L.) crops **(Brunt *et al.*, *1996*)**. *Cucumber mosaic virus* (CMV) is reported to infect 1287 plant species. CMV was detected in leguminous, ornamental and tomato plants **(Zitikaite, 1999).**, is one of the most common plant viruses of substantial agricultural importance, infecting more than 1,000 plant species **(Van Regenmortel *et al.*, 2000).** CMV the type species of the genus *Cucumovirus*, family *Bromoviridae* , CMV is a single-stranded positive-sense tripartite genome RNA virus. CMV isolates have been described in several hosts **(Maciel-Zambolim *et al.*, 1990, 1994; Dusi *et al.*, 1992; Duarte *et al.*, 1994; Boari *et al.*, 2000; Eiras *et al.*, 2000, 2001; Colariccio *et al.*, 1987, 1996, 2002).** *CMV* are considered to be very important in Africa general and Egypt specially as for their geographical distribution, pathogenic variation and yield loss. The main purpose of this study was isolation and identification of *CMV* using ELISA , distinguishable sole bands in both healthy and diseased leaves for which it could be used according to it is presence as sign for the virus infection by SDS-PAGE test and RT-PCR technique . The following sequences were used in the comparison: Brazil (AF418577), China (FJ403473), New Zealand (AY861395) and India (AJ810260) **(Takeshita *et al.*, 2001; Colariccio *et al.*, 2002 and Eiras *et al.*, 2002).**

MATERIALS & METHODS

Serological diagnosis of the virus isolate:

Samples of cucumber plants exhibiting systemic mosaic and stunting , reduced fruit yield and deformation were collected from different fields of Ismailia Governorate. DAS-ELISA technique was used for virus detection as described by **Clark and Adams (1977)** using the antiserum specific for *CMV* and compared with those of *WMV* and *SqMV*. All viruses tested in triplicate using conventional double antibody sandwich enzyme-linked immuno-sorbent assay (DAS-ELISA) according to the manufacturer's instructions (Sanofi-Santi animal, France). Optical density was measured at λ= 405 nm in an ELISA micro well reader (using Dynatech Immunoassay MR 7000). Samples with an absorbency of at least twice that the healthy controls were considered as a positive for the presence of virus.

Virus samples and natural hosts:

Plant samples which gave positive reaction in the direct ELISA test with CMV were used as a source of the virus . Inoculum was prepared and used to inoculate the following indicator hosts *Chenopodium amaranticolor* Coste & Reyn and *Vigna unguiculata* L. cv. Borma . To obtain virus isolate in a pure form, the single local lesion technique was followed according to **(Kuhn, 1964).** Cucumber (*Cucumis sativus* L.), Squash (*Cucurbita pepo* L.), Pumpkin (*Cucurbita maxima*), pepper (*Capsicum annuum* L.) and Bean (*Phaselous vulgaris*) known as a systemic hosts for CMV were inoculated with the virus. Inoculated plants were kept in separate cages, as a source of virus infection.

CMV protein marker:

Total soluble protein extraction:

The leaves of four cucumber cultivars (Dina, Razena, Prince and Riea) were collected from infected and healthy plants and ground to flour in a mortar by using liquid nitrogen. Total soluble proteins were extracted in SDS reducing buffer, (store at room temperature) composed of Deionized water (38 ml), 0.5 M Tris -HCl -pH 6.8 (10 ml), Glycerol (8 ml), 10 % (w/v) SDS (16 ml), 2-mercapto-ethanol (4ml) and 1% (w/v) Bromophenol blue (4ml) until became total volume 80 ml . The sample was diluted at least 1:4 with sample buffer and the extract was centrifuged at 10.000 rpm for 20 minutes . Then 10 µl of total soluble protein was taken for electrophoresis.

Protein analyses:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 10% acrylamide slab gels following the system of **Laemmli. (1970).** Separating gels composed of 0.75M Tris – HCl pH8.8, 10% SDS, 0.025% of N,N,N,N-tetramethylenediamine (TEMED) and 30% ammonium persulfate. Stacking gels contained 0.57M Tris-HCl pH6.8, 10% SDS, 0.025% TEMED and 30% ammonium persulfate. Electrode buffer contained 0.025M Tris, 0.192M glycine, 0.1% SDS and pH8.3. Electrophoresis was carried out with a current of 25 mA and 130 volts per gel until the bromophenol blue marker reached the bottom of the gel after 3hrs. After electrophoresis, the silver staining method for protein described by **Sammons, *et al.* (1981)** was used. This method of staining is sensitive and detects as little as 2mg of protein in single band . Then the

gels were photographed. Using SDS-PAGE marker for molecular weight 14.000- 29.000-66.000 Cat. Number: WM-SDS-70L 1mg/ml .

Total RNA extraction and RT-PCR:

EZ-10 Spin Column Total TNA Minipreps Super Kit (BIO BASIC INC) was used to extract the total RNA from infected and uninfected cucumber leaves according to the kit procedure. A newly designed oligonuclotide specific CMV1 (5'GCCGTAAGCTGGATGGACA A3').CMV2(5`TATGATAAGAA GCTTGTTTCGCG 3`) primers **(Wylie *et al.*, 1993),** were used for RT-PCR amplification of CMV coat protein gene. cDNA (One step RT-PCR) was amplified as described by (AB gene, UK). PCR was carried out in the Biometra T Gradient thermo cycler with one initial denaturation cycle at 94 °C for 2 min, followed by 30 cycles of amplification with temperature profiles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C. Thirty cycles were followed by one final extension cycle at 72 °C for 7 min.

PCR analysis:

The amplified DNA was electrophoresed on 1% agarose gel with 1xTAE buffer, stained with ethidium bromide and photographed using (Gel Doc 2000 Bio-RAD).The molecular weight of the PCR products were determined by comparison with DNA markers, 100bp ladder (AB gene, UK),

Sequencing and phylogenetic analysis:

The RT-PCR product amplified with CMV1 and CMV2 primer was used as template using the same primer. The reaction was performed in a final volume of 50 µl consisting of 1x PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM MgCl2), 200 µM dNTPs, 1 µl of DNA target from CMV PCR product, 10 µM of each primers.,2.5 untis of *Taq* DNA polymerase (5 Units / µl, AB gene, UK). The conditions for amplification were a denaturation step at 94°C for 2 min followed by 30 cycles of 1min at 94°C,1 min at 50°C and 2 min at 72°C, with a final extension of 10min at 72°C. Reactions were cycled in a thermocycler Biometra T Gradient. PCR products were purified with GFX^{TM} PCR purification kit (Amersham Pharmacia Biotech Inc. USA).The sequence was carried out in Macrogen Inc, gene link DNA Sequencing service, Seoul, Korea.

The nucleotide sequence of the coat protein was compared and analyzed using DNAMAN Sequence Analysis Software

(Lynnon BioSoft .Quebec, Canada) comparison with the *CMV* isolates previously characterized and available in the Gene Bank was achieved. The phylogenetic analysis and the homology rate were also carried out.

RESULTS & DISCUSSION

Serological diagnosis of the virus isolate:

The identity of the virus isolate was confirmed by DAS-ELISA technique using kits supplied from SANOFI. Positive reaction was obtained with *CMV* specific antisera **(Anonymous, 1998).**

Virus samples and natural hosts:

Infected cucumber plants with *CMV* showed viral symptoms of systemic mosaic, blistering, fruit malformation and stunted plant growth. All samples gave positive reaction with CMV and were susceptible to cucumber mosaic viral infection with nearly the same degree of sensitivity. *CMV* was able to infect different host plant species including Squash (*Cucurbita pepo* L.) , Pumpkin (*Cucurbita maxima*), Pepper (*Capsicum annuum* L.), Bean (*Phaselous vulgaris*) , *Chenopodium amaranticolor* and Cowpea (*Vigna unguiculata*) , showing foliar symptoms of mosaic, deformations and necrotic and chlorotic ring spots after inoculation , that resemble those induced by *CMV*. Symptoms started to appear 7-14 days after inoculation . Cucumber plants, showed systemic mosaic after 8 days from inoculation with extracts from the positive reacted samples **(Figure 1).** This virus was isolated in previous studies from cowpea and other hosts, by several investigators in different countries (**Daniels and Campbell, 1992; (Hu *et al.*, 1995; Carrère *et al.*, 1999; Takeshita *et al.*, 2001 and Eiras *et al.*, 2000, 2001, 2002).**

CMV protein marker:

Electrophoresed and densitometric analysis of protein bands by SDS-PAGE are illustrated in **Figure (2)** .The results tabulated in **Table (1)** showed various distinguishable sole bands in diseased leaves for which it could be used according to it is presence as sign for the CMV infection. Inference brought about in this table hinted to consideration that it would be acceptable if we assume there is a possible correlation between the virus infection and the novel protein bands presence and showed summary for protein bands markers suggested for detection of CMV infection in different cucumber leaves cultivars (Dina , Razena , Prince and Riea) were presented in disease leaves and absented in healthy. These bands named novel protein bands were distinguished in molecular weight between 70 and 25 Kd in infected four cucumber

cultivars . These bands maybe create in infected lanes result effect the virus in plant metabolism and response of plant for CMV infection . Percentage of amount of protein (% Amt.) in Dian and Razena cv. more than % Amt. in Prince and Riea cv. these result illustrated that susceptibility Dian and Razena cv. to CMV infection in excess of Prince and Riea . These results are in agreement with those reported by **Ahmed, Sayda (2001).** Data in this result confirmed that the protein markers consistently produced in different Cucumber leaves cultivars might suggesting for the detection of CMV infection . In synopsis , during the investigation regarding percentage determined in each band. **Sang &Joo (1992) and Ahmed, Sayda (2001).**

Reverse transcription-polymerase chain reaction RT-PCR:

The RT-PCR was used for detection of *CMV* coat protein (*cp*) gene in infected cucumber . PCR fragment of correct size 422bp was amplified with the primer CMV1 and CMV2 for CMV-*cp*. gene. Agarose gel electrophoresis analysis of the amplified PCR products is demonstrated in **Figure (3).** However, no product was amplified from healthy cumber plants using the same procedure.

Sequence analyses:

DNA amplified product from cucumber plants representing Cucumer mosaic virus *CMV* show 442bp in **Figure (3).** PCR product using CMV1 and CMV2 primer was purified with GFX^{TM} purification kit. A multiple alignment was done along with sequences previously obtained which have already been used as reference sequences in other studies and from the Gene bank sequence data. **(Eiras *et al.*, 2004; Farreyrol *et al.*, 2004; Deyong *et al.*, 2005 and Sun, 2008).** The following sequences were used in the comparison: Brazil (AF418577), China (FJ403473), New Zealand (AY861395) and India (AJ810260). The partial nucleotide sequence alignment **Figure (4)** showed (95%) homology between *CMV* New Zealand isolate and *CMV*-Egyptian isolate . The sequence alignment also indicated that the *CMV* isolate is far from similarity with India *CMV*, showing (92%) according to the phylogenetic homology tree **Figure (5).**

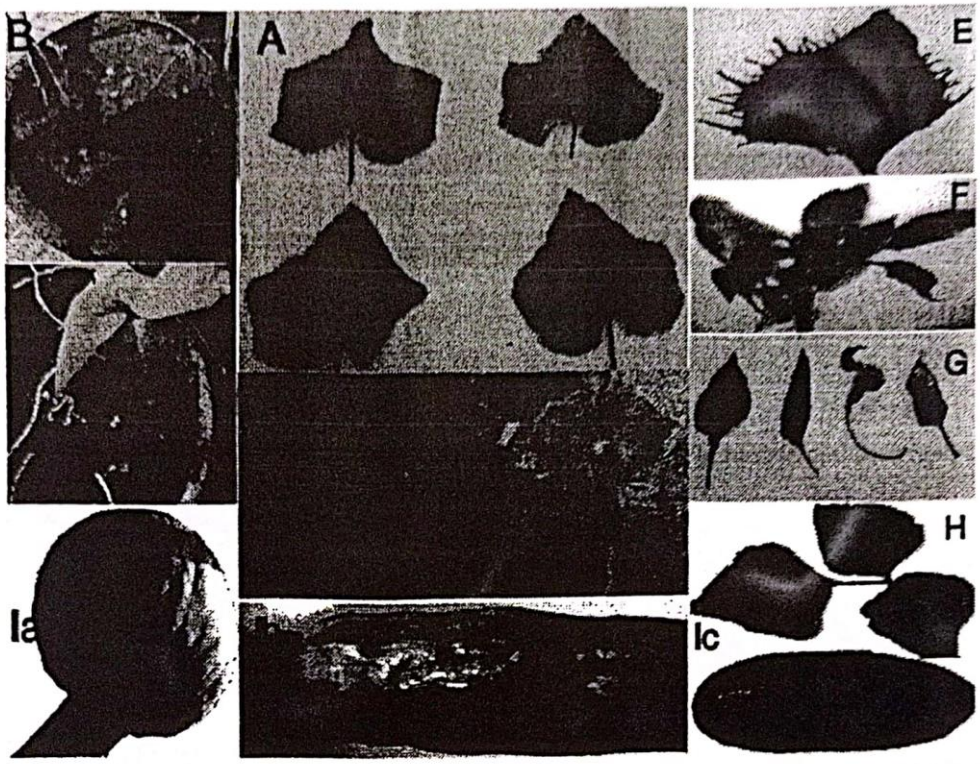

Figure 1. Symptoms caused by CMV showing a wide range of discoloration and fruit malformation . (A) Systemic mosaic on *C. sativus* , (B) Necrotic ring spot on *Ch. amaranticolor* , (C) Chlorotic ring spot on *V. unguiculata* , (D) Mosaic on *C. maxima* , (E) Chlorotic blotching on *C. pepo* , (F &G) Mosaic and Leaf deformation on *C. annuum* , (H) Mosaic and blisters on *Ph. vulgaris* and (Ia,Ib, Ic)Mosaic and fruit malformation on pumpkin , squash and cucumber fruits respectively.

Table (1). Hypothesized protein markers for the detection of CMV infection in different cucumber leave cultivar.

Bands present in diseased and absent in healthy						
Cultivars	**Bands No.**	**M.W**	**% Amt.**	**Bands No.**	**M.W**	**% Amt.**
Dina	16	70	16.43	45	25	11.14
Razena	15	71	12.88	46	25	11.2
Prince	14	71	9.75	47	25	9.93
Riea	13	71	9.79	48	25	10.24

M.W = Molecular weight (K.Da), %Amt = Percentage of Amount of protein

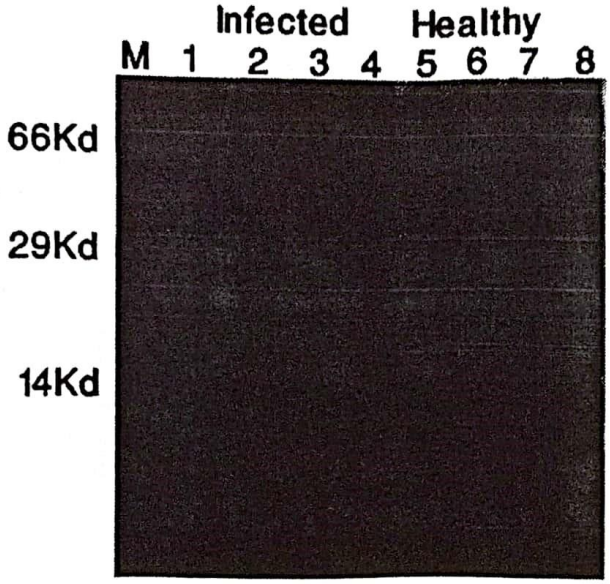

Figure 2. Sodium dodcyle sulphate– polyacrylamide gel electrophoresis (SDS-PAGE) of infected and non infected cucumber leaves cultivars infected with CMV. Lanes 1,2,3,and 4 (infected leaves):Lane 1 = cv. Dina , Lane 2 = cv. Razena Lane 3= cv. Prince, Lane 4= cv. Riea . Lanes 5,6,7 and 8 (non-infected leaves) Lane M = SDS-Marker .

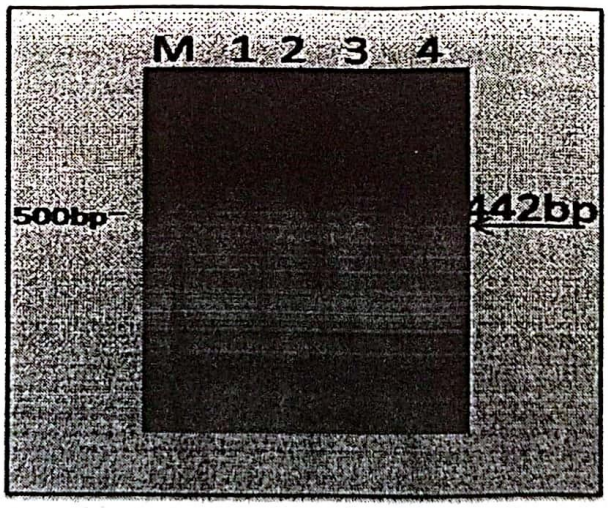

Figure 3. Agarose gel electrophoresis analysis of amplified *CMV- cp* gene fragment Lanes (1 to 4). RT-PCR products of four CMV samples showing amplified *CMV-cp*, Gene fragment of the correct size 442bp (arrow) in lanes (3). Lane (1,2 and 4) healthy squash. M: 100bp ladder (AB gene, UK),

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CMV-Egypt          TATCCTTTGCCG..ATTTGATTCTACCGTGTGGGTGACGG      38
CMV-Brazil         AATCCTTTGCCGAAAATTTGATTCTACCGTGTGGGTGACAG     40
CMV-China          AATCCTTTGCCGAAAATTTGATTCTACCGTGTGGGTGACAG     40
CMV-India          AATCCTTTGCCGAAATTTGATTCTACtGTGTGGGTGACGG      40
CMV-New Zealand    AATCCTTTGCCGAAATTTGATTCTACCGTGTGGGTGACGG      40

CMV-Egypt          TCCGTAAAGTTCCTGCCTCCTCGGACCTGTCCGTTTCTGC      78
CMV-Brazil         TCCGTAAAGTTCCTGCCTCCTCGGACTTATCCGTTGCCGC      80
CMV-China          TCCGCAAAGTTCCTGCCTCATCGGACTTATCCGTTACcGC      80
CMV-India          TCCGTAAAGTTCCTGCCTCCTCGGACCTGTCCGTTTCcGC      80
CMV-New Zealand    TCCGTAAAGTTCCTGCCTCCTCGGACCTGTCCGTTACCGC      80

CMV-Egypt          CATCTCTGCTATGTTCGCAGACGGAGCCTCACCGGTACTG     118
CMV-Brazil         CATCTCTGCTATGTTTGCGGACGGAGCCTCACCGGTACTG     120
CMV-China          CATCTCTGCTATGTTCGCGGACGGAGCCTCACCGGTACTG     120
CMV-India          CATCTCTGCTATGTTCGCGGACGGAGCCTCACCAGTACTG     120
CMV-New Zealand    CATCTCTGCTATGTTTTGCgGACGGAGTCTCACCGGTACTG    120

CMV-Egypt          GTTTATCAGTACGCCGCATCCGGAGTCCAAGCTAACAACA     158
CMV-Brazil         GTTTATCAGTAtGCCGCATCtGGAGTCCAAGCCAACAACA     160
CMV-China          GTcTATCAGTACGCCGCATCCGGAGTCCAAGCCAACAAtA     160
CMV-India          GTTTATCAGTATGCCGCATCCGGAGTTCAAGCTAACAACA     160
CMV-New Zealand    GTTTATCAGTACGCCGCATCCGGAGTTCAAGCTAACAACA     160

CMV-Egypt          AACTGTTGCATGATCTTTCGGCGATGCGCGCTGATATTGG     198
CMV-Brazil         AATTGTTGTATGATCTTTCGGCGATGCGCGCTGATATaGG     200
CMV-China          AACTGTTGtATGATCTTTCGGCGATGCGCGCTGATATTGG     200
CMV-India          AATTGTTGtATGATCTTTCGGTGgTGCGCGCTGATATTGG     200
CMV-New Zealand    AATTGTTGTATGATCTTTCTGCGATGCGCTCTGATATTGG     200

CMV-Egypt          CGACATGAGAAAGTACGCCGTACTCGTGTATTCAAAAGAC     238
CMV-Brazil         TGACATGAGAAAGTACGCCGTCCTCGTGTATTCAAAAGAC     240
CMV-China          CGACATGcGAAAGTACGCCGTTCTCGTGTATTCAAAAGAC     240
CMV-India          TGACATGAGAAAGTACGCCGTGCTCGTGTATTCAAAAGAC     240
CMV-New Zealand    CGACATGAGAAAGTACGCCGTACTCGTGTATTCAAAAGAC     240

CMV-Egypt          GATGCACTCGAGACAGATGAACTAGTACTTCATGTCGACA     278
CMV-Brazil         GATGCGCTCGAGACgGACGAGCTAGTGCTTCATGTTGACA     280
CMV-China          GATGCTCTCGAGACGGATGAGTTAGTACTTCATGTCGACA     280
CMV-India          GATGCGCTCGAGACgGATGAGCTAGTACTTCATGTCGACA     280
CMV-New Zealand    GATGCACTCGAGACGGATGAGCTAGTACTTCATGTCGACA     280

CMV-Egypt          TTGAGCACCAACGTATTCCCACATCTGGGGTGCTCCCAGT     318
CMV-Brazil         TCGAGCACCAACGcATTCCCACATCTGGGGTGCTCCCAGT     320
CMV-China          TCGAGCACCAACGcATTCCCACATCTGGGGTGCTCCCAGT     320
CMV-India          TTGAGCACCAACGcATTCCCACATCTGGAGTGCTCCCAGT     320
CMV-New Zealand    TTGAGCACCAACGTATTCCCACATCTGGGGTGCTCCCAGT     320

CMV-Egypt          TTGAACTCGTGTTTT.CCAGAACCCTCCCTCCGATTTCTG     357
CMV-Brazil         CTGATTcCGTG..TTCCCAGgATCCTCCCTCCGATTTCTG     358
CMV-China          TTGAACTCGTGTTTTCCCAGAAtCCTCCCTCCGAC.TCTG     359
CMV-India          TTGAACTCGTGTTTT.CCAGAACCCTCCCTCCATTTTCTG     359
CMV-New Zealand    TTGAACTCGTGTTTT.CCAGGACCCTCCCTCCGATTTCTG     359

CMV-Egypt          TGGCGGGAGCTGAGTTGGCAGTGTTGCTATAAACTGTCTG     397
CMV-Brazil         TGGCGGGAGCTGAGTTGGCAGTtcTGCTATAAACTGTCTG     398
CMV-China          TGGCGGGAGCTGAGTTGGtAGTaTTGCTATAAACTGcCTG     399
CMV-India          aGGCGGGAGCTGAGTTGGtAGTGTTaCTATAAACTGcCTG     399
CMV-New Zealand    TGGCGGGAGCTGAGTTGGtAGTaTTGCTCTAAACTaTCTG     399
    Consensus            GGCGGGAGCTGAGTTGG AGT  T CT TAAACT  CTG

CMV-Egypt          AAGTCACTAAACGCTTTGCGGTGAACGGGTTGTCCACCGC     437
CMV-Brazil         AAGTCACTAAACGTTTTACGGTGAACGGGTTGTCCATCCA     438
CMV-China          AAGTCACTAAACGCTTTGCGGTGAACGGGTTGTCCATCCA     439
CMV-India          AAGTCACTAAACGCTTTGCGGTGAACGGGTTGTCCATCCA     439
CMV-New Zealand    AAGTCACTAAACGCGTTGCGGTGAAACGC                428

CMV-Egypt          CCTTA                                        442
CMV-Brazil         GCTTA                                        443
CMV-China          GCTTA                                        444
CMV-India          G                                            440
                   Consensus
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Figure 4. Sequence alignment of Cucumer mosaic virus *CMV* coat protein gene sequences. Brazil (AF418577), China (FJ403473), New Zealand (AY861395) and India (AJ810260).

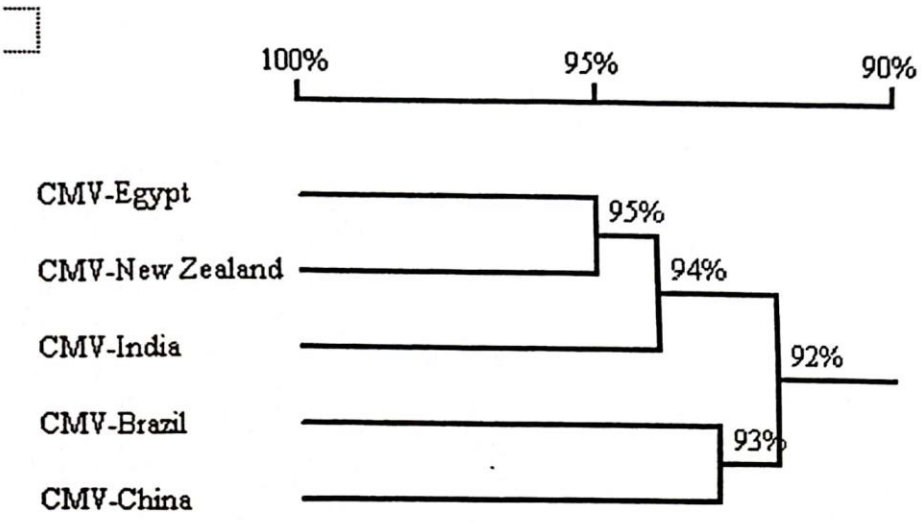

Figure 5. The phylogenetic homology tree based on multiple sequence alignments of the Cucumer mosaic virus *CMV* coat protein gene with reference to international isolates

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