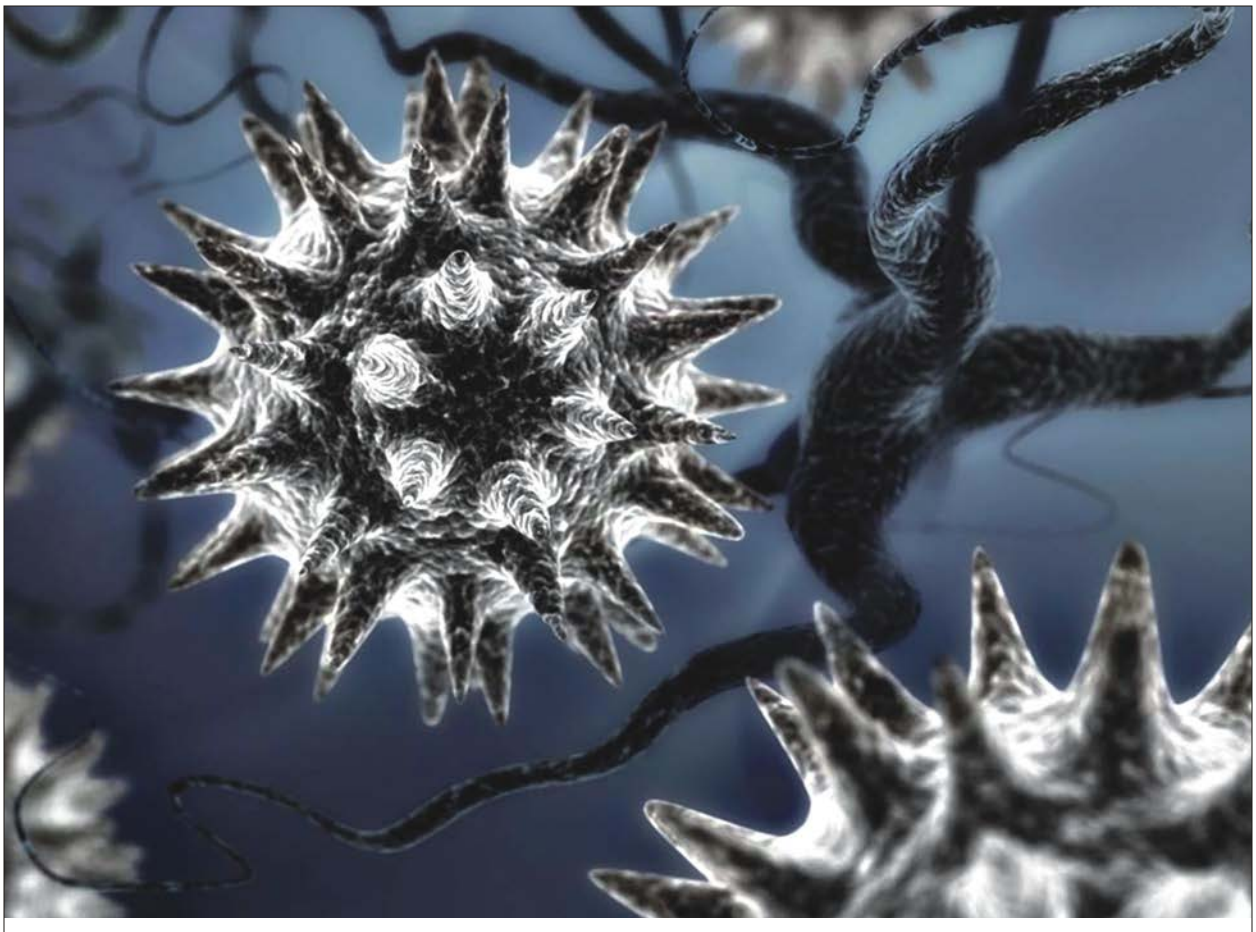

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Intraspecific Diversity of *Cucumber mosaic* Cucumoviridae in Egypt

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ABSTRACT

Cucumber mosaic virus (CMV) was isolated from naturally infected Banana, Geranium and Gladiolus and exhibited different external symptoms. The three CMV isolates were transmitted by sap inoculation with 6, 5 and 5 out of 10 inoculated *N. glutinosa* plants as well as by aphids with 6, 5 and 4 out of 10 inoculated Cucumber plants, respectively. These isolates exhibited different morphological characters of single local lesion on *Chenopodium amaranticolor* and had different sap stability (TIP, DEP and LIV) *in vitro*. The differential host reactions were differed among these isolates in incubation period and external symptoms; the amplified cp-gene RNA of confirm the diversity of CMV isolates. It was proved that CMV could involve variation of infection due to one more than CMV strains. It has been declared that the complementary role of biological diagnosis addition, in addition to molecular diagnosis to have a complete and clear image for viral etiology.

Key words: CMV, RT-PCR, diversity

INTRODUCTION

Cucumoviruses infect over 1000 plant species and cause considerable harm to agriculture worldwide, therefore understanding the molecular mechanism of the virus spread is of utmost importance. *Cucumber mosaic virus* (CMV), *Tomato aspermy virus* (TAV) and *Peanut stunt virus* (PSV) form the *Cucumo virus* genus in the family Bromoviridae. Their genomes consist of three single-stranded, positive-sense RNA molecules. The RNA 3 encodes two proteins, the Movement Protein (MP) and the Coat Protein (CP). The main function of the CPs is to encapsulate the viral RNAs. The virus particle is composed of 180 CPs with a T = 3 truncated icosahedral symmetry (Smith *et al*., 2000). In addition, the CP plays significant role in the infection process. The capsid protein is indispensable for cell-to-cell and long distance movement of the virus within plants and also between plants since it contains determinants for aphid transmission (Palukaitis and Garcýa-Arenal, 2004). Besides the movement protein the cucumoviruses also require the CP for cell-to-cell movement (Suzuki *et al*., 1991), but the assembly of the virus particle is not required (Kaplan *et al*., 1998). If the C-terminal 33 amino acids (aa) were deleted from the MP, the CP was not necessary for the cell-to-cell movement of CMV which will be our main focus here (Nagano *et al*., 2001).

The aim of this study was to characterize the CMV coat protein in the Gladiolus isolate from Egypt and compare it with the known corresponding sequences registered in GenBank.

MATERIALS AND METHODS

Virus isolates and plant materials: The *Cucumber mosaic virus* isolate Gera-EG (Sofy and Soliman, 2011) was kindly donated by Dr. Ahmed Sofy and this isolate was registered in GenBank under accession no. JQ013954. Also, Banana mosaic virus Egyptian isolate was kindly donated by Dr. Khalid El-Dougdoug which was registered in GenBank under accession No. EU851875.

On the other hand, naturally infected Gladiolus plants exhibited virus like symptoms were collected from open fields and protective plastic houses of different regions in Egypt. The virus agent was transferred into Cucumber seedlings by sap inoculation under greenhouse conditions. The symptoms were developed and recorded. The plants and were tested for CMV infection by DAS-ELISA using specific IgG CMV according to (Clark and Adams, 1977). Also, the two isolates of *Cucumber mosaic virus* from Geranium and Banana were confirmed by DAS-ELISA using specific IgG CMV.

Differential hosts: Sap from inoculated plants of each host (Banana, Geranium and Gladiolus) were inoculated on differential hosts under greenhouse conditions (Table 1), the results were confirmed by DAS-ELISA.

Aphid transmission: It was carried out by using virus free aphids (Plant protection Dept., Fac. Agric. Ain Shams Univ.); these aphids were divided into three groups feeding on three individual infected Cucumber plants groups in screened cages under greenhouse conditions for 24 h (acquisition access period). These aphids were transferred on ten healthy Cucumber plants for each CMV isolates in screened cages under greenhouse conditions for 4 access days, then aphids were killed by malathion (2%). These plants were tested for CMV infection by external symptoms and confirmed by DAS-ELISA.

Virus stability *in vitro* as thermal inactivation point and dilution end point were determined according to (Noordam, 1973) using *Chenopodium amaranticolor* as local lesion assay.

The epidermal strips were removed with forceps from lower surface of infected Cucumber leaves with 4 CMV isolates and were scanned using light microscope. The strips were treated with triton×-100 for 5 min according to Noordam (1973).

Molecular virology characters: Total RNA was extracted from healthy and infected Cucumber plants with CMV isolates using high pure RNA tissue kit (Roche Molecular Biochemicals, Cat.No. 2033674). The yield and purity of RNA were estimated spectrophotometrically and electrophoretically on 1-5% agarose gel.

RT-PCR protocol as described by Chung *et al.* (2007) using two primers sets (CMV1 reverse 3'-ttggatcctcagactgggag-5'and forward CMV 2 5'-aaccatggacaaatcagaa-3') for the coat protein gene. Complementary DNA (c-DNA) synthesis was accomplished by as described by Chung *et al.* (2007).

Amplification of c-DNA included the following cycles; initial melting at 94°C for 3 min (1 cycle), denaturation at 94°C for 1 min, primer annealing at 54°C for 2 min and extension at 72°C for 1-5 min for 35 cycles with a final extension at 72°C for 7 min (1 cycle). The PCR products were

analyzed by electrophoresis onto 7-5% agarose gel. The size of DNA fragments (cp gene) was determined in accordance with molecular weight DNA ladder. The PCR products were visualized on a UV transilluminator pharmacia.

DNA sequencing for the partially amplified coat protein gene of CMV isolate from Gladiolus was performed using ABI prism 3100, genetic analyzer by using dye-primer and dye-terminator method at gene link DNA sequencing service, New York USA. The resulting sequence of cp gene was aligned (clustal W software) with the two CMV isolates from Geranium and Banana which registered in GenBank under accession No. JQ013954 and EU851875, respectively for deduction of nucleotide and amino acid sequence comparative analysis.

RESULTS

The results were appeared that diversity of CMV isolates in Egypt based on biological properties and molecular characters.

Symptomatology: Naturally infected plants exhibited symptoms according to the response of plants. Cucumber plants appeared mosaic and stunting varying in severity (Table 1). Banana plants showed mosaic streak on main vein of leaf; Geranium plants exhibiting net yellow mosaic and blisters as well as Gladiolus plants exhibiting symptoms on flower color breaking, leaf mosaic and blotched leaf.

Differential hosts: According to the response of the tested host plants (Table1), host reactions were appeared different symptoms.

Serological relationships: All three CMV isolates infected Cucumber plants were gave serological precipitation reaction with polyclonal antibody specific CMV by Ouchtorlony test. The serological relationship between four isolates was visible as precipitation band with different density degrees (Table 2).

Table 1: Differential host reactions of CMV isolates

CMV gladiolus isolate	CMV geranium isolate	CMV banana isolate	CMV isolates hosts
Small necrotic local lesion and chlorotic centers	Chlorotic local lesion without halo	Large necrotic local lesion	*Chenopodium amaranticolar*
Mild strick mosaic	Sever mosaic	No symptom	*Capsicum annuum*
Mosaic	No symptoms	Mosaic	*Datura stramonium*
Net yellow mosaic	Sever mosaic	Chlorotic spots	*Cucumber sativus* cv. beith alpha
Mosaic leaf narrow plant erect	Mosaic	Vein necrosis necrotic spots	*Datura stramonium*
Sever mosaic	Sever mosaic	Sever mosaic stunting	*Cucumber sativus* cv. beith alpha
Sever mosaic, blasters	Mosaic	No symptoms	*Lycopersicon esculantum cv.Casstlerock*
Sever mosaic	No symptoms	Mild mosaic	*Nicotiana glutinosa*
Sever mosaic	Mosaic	No symptoms	*Datura metel*
Mild mosaic	Sever mosaic	No symptoms	*Petunia hybrida*
No symptoms	Mosaic	Chlorotic spot	*Nicotiana tabacum* cv. white burley *Nicotiana glauca* *Nicotiana rustica*

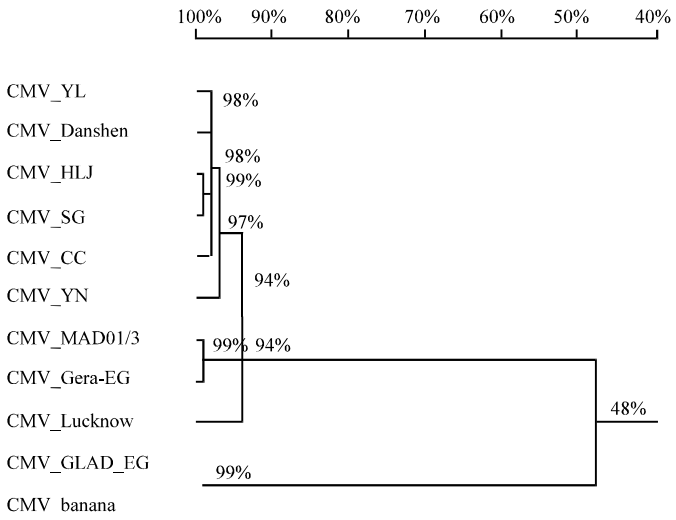

Fig. 1: Phylogenetic tree showing relationships between three Egyptians CMV isolates from Banana, gladiolus and geranium with some reference strains from genbank

Table 2: Biological and serological properties between CMV isolates

CMV gladiolus isolate	CMV geranium isolate	CMV banana isolate	CMV isolate name character
++	++	+++	Serological relationships (density of band precipitation)
			Biological properties
10^{-5}, 0%	10^{-4}, 30.5%	10^{-5}, 3.4%	DEP
70°C, 26.5%	65°C, 0%	75°C, 0%	TIP
4.5 day, 25%	3.0 day, 25.2%	2.5 day	LIV
648	657	585	CP gene size (bp)

Virus stability: All CMV isolates showed marked difference in their DEP, TIP and LIV pattern in such a way that CMV isolate from Banana and Gladiolus showed (10-5, 10-5 75, 70, 2.5 days, 4.5 days), while that from Geranium (10-4, 65 and 3 days) for DEP, TIP and LIV, respectively as shown in Table 2.

Molecular diversity: The total DNA was around 4.2 mg g^{-1} fresh weight of Cucumber leaves infected with four CMV isolates. The total DNA lysates which had been used in PCR reactions were separated and visualized by 1.5% agarose gel. CMV was detected in 3 Cucumber plants infected with CMV isolates. One subgenomic fragment of CMV isolates were amplified using two oligonucleotide primers specific CMV. The expected size 657, 585 and 648 bp for four CMV isolated from Geranium, Banana and Gladiolus plants, respectively. The CMV isolate from Gladiolus showed 99% identity with reference strains of Egyptian CMV isolates (Banana and Geranium) published on genbank with accession numbers EU851875 and JQ013954, respectively, as in Fig. 1. Based on MSA analysis (Fig. 2), the phylogenetic tree (Fig. 3) was performed and shows two clusters in which CMV isolates from Banana and Gladiolus were found to be highly homologous with percentage (99%) while that from Geranium showed distant homology (47%), so it was represented as a separate cluster as in Fig. 3.

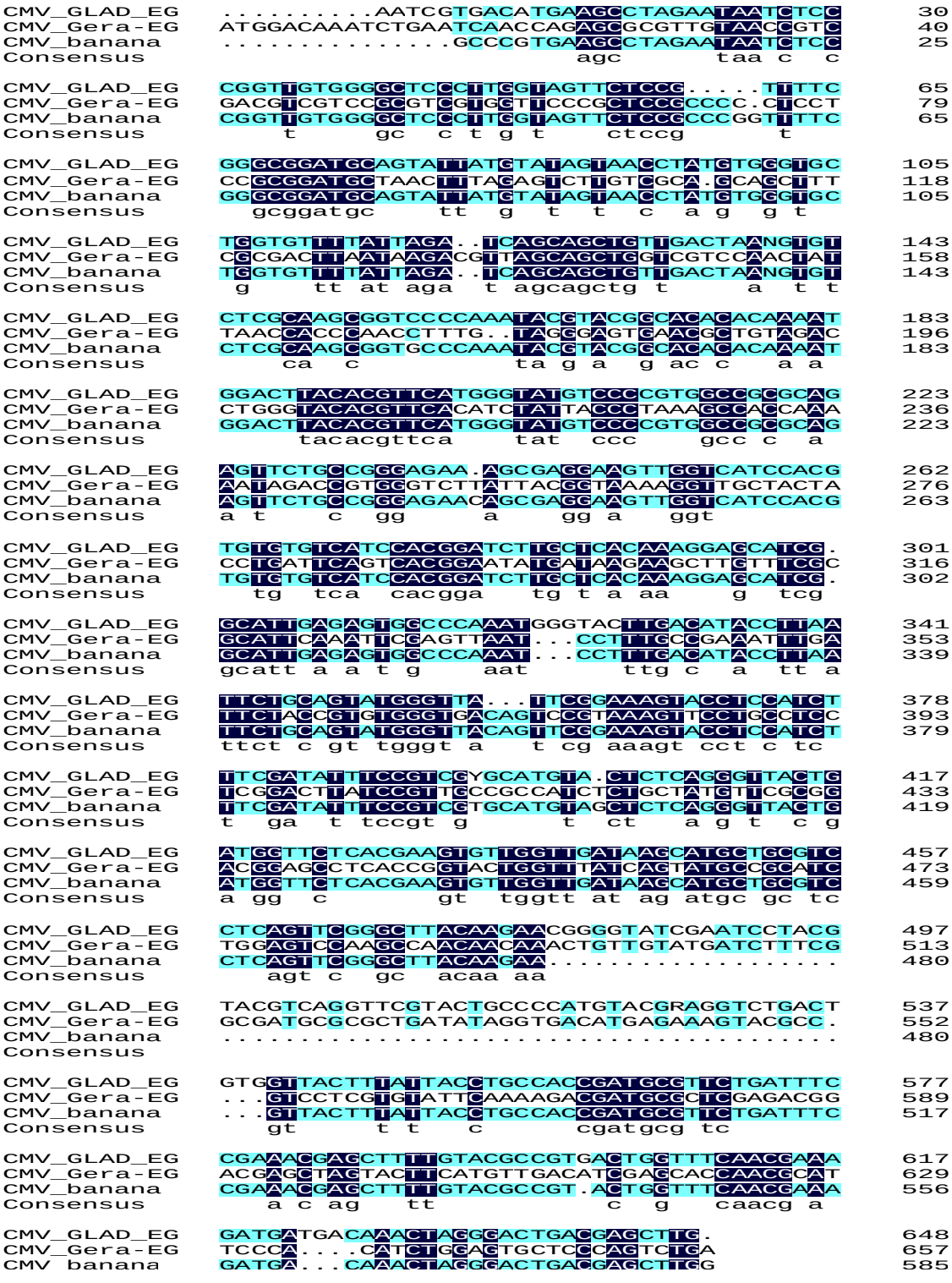

Fig. 2: Multiple sequence alignment for the three CMV isolates based on nucleotide sequence

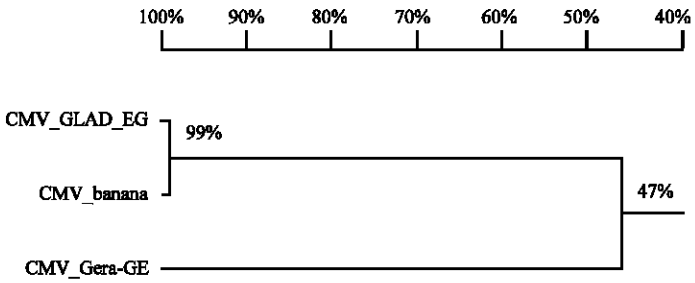

Fig. 3: Phylogenetic tree representing the relationship between the three isolates based nucleotide sequence homology

Table 3: Results from tajima's neutrality test

m	S	p_s	Θ	π	D
3	282	0.500000	0.333333	0.333333	0.000000

m: No. of sequences, S: No. of segregating sites, ps: S/m, Θ: The population genetics parameter, π: Nucleotide diversity and D is the Tajima test statistic

Upon translation of the three CMV nucleotide sequence into the primary amino acids sequence to demonstrate the variability and predict the phylogram relating them based on amino acid sequence homology as in Fig. 4. CMV from Gladiolus and Banana showed moderate similarity (50%) whilst kept much lower similarity to CMV_Gera as shown in Fig. 5.

Tajima's neutrality test: The evolution of living organisms is the consequence of two processes. First, evolution depends on the genetic variability generated by mutations which continuously arise within populations. Second, it also relies on changes in the frequency of alleles within populations over time. That is why the Tajima's test was performed for inspection of whether occurrence of neutrality between such two processes performed the forcing mechanisms for evolutionary change or it would be the role of natural selection by mutation.

There was equivalence (confirmed by nearly equivalence between **Θ** (0.333333) and π (0.333333) between genetic drift and mutation (Table 3). The equivalence between such two values and the zero value of D considered the null hypothesis to be held.

DISCUSSION

CMV appears to be the most important virus of some annual crops in Argentina, eastern China, Croatia, France, Egypt, Greece, Israel, Italy, Japan, Poland, Portugal, Spain, Sweden and in the north east of US. In other countries, CMV ranks second or third in importance (Tomlinson, 1987). To date, a number of reports indicate that CMV populations are well established in Mediterranean areas where they are frequently found in mixed infections with other viruses that have either a wide (e.g., *Alfalfa mosaic virus* and *Tomato spotted wilt virus*) or restricted host range (e.g., potyviruses infecting cucurbits).

The existence of many CMV strains that have a very broad host range suggests that the multi-component nature of the virus does not constrain its ecological success or epidemiological competence (Fulton, 1980). Rather, the consensus is that random reassortment of intact genomic segments from multi-component viruses is a parasexual process generating new variants to

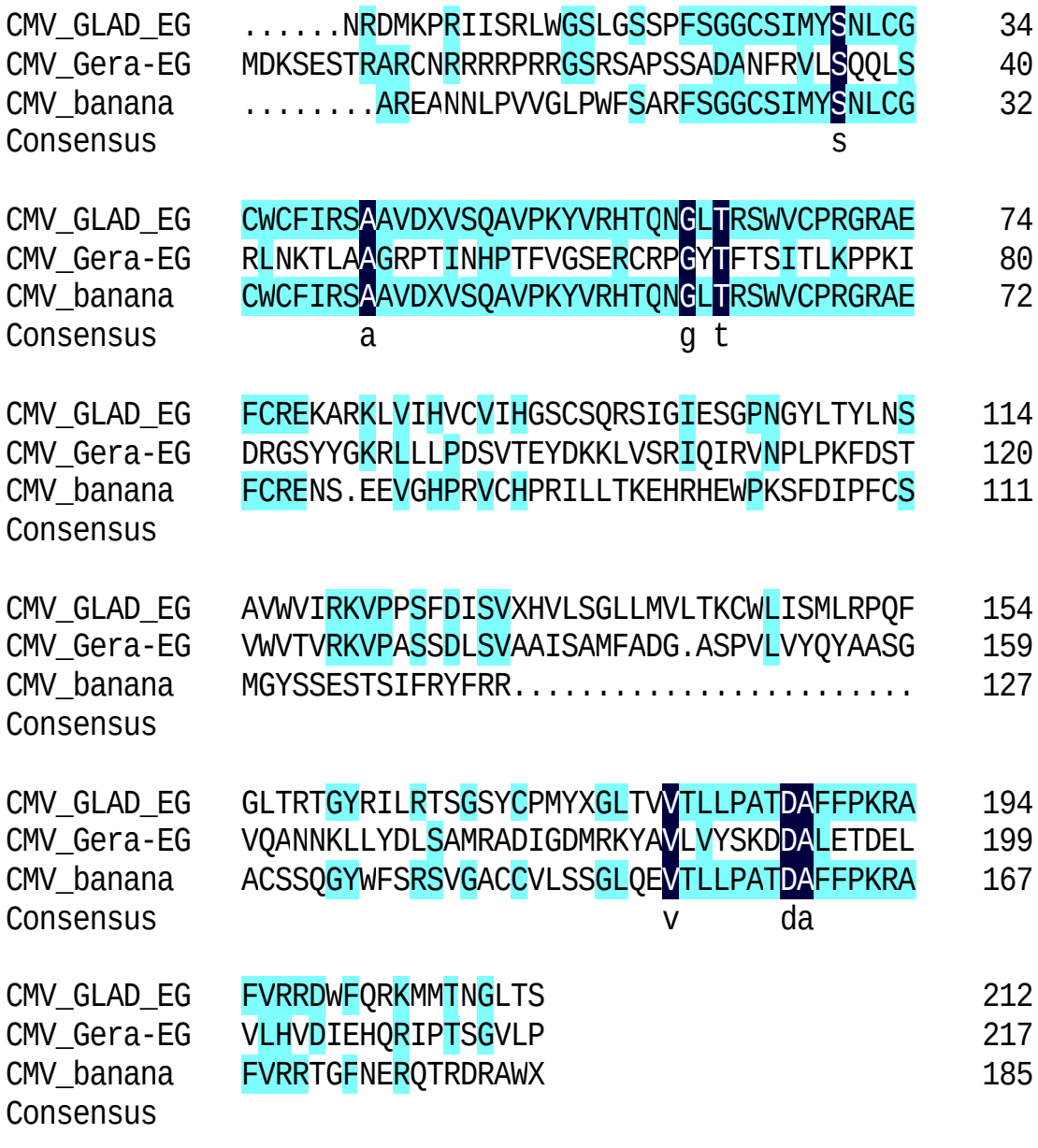

Fig. 4: Multiple sequence alignment for the three CMV isolates based on amino acid sequences

counterbalance losses in fitness due to the to accumulation of deleterious mutations through successive host passages. It has been postulated that in a small asexual population deleterious mutations accumulate through an irreversible, ratchet-like mechanism, known as Muller's ratchet. Genetic exchange by reassortment and by recombination may help in rescuing viable virus fitness from a population that has been ratcheted-down (Roossinck, 1997).

Viable reassortants have been prepared *in vitro* and used to assign specific functions to individual segments of the genome (Carrere *et al.*, 1999). True recombination (RNA-RNA

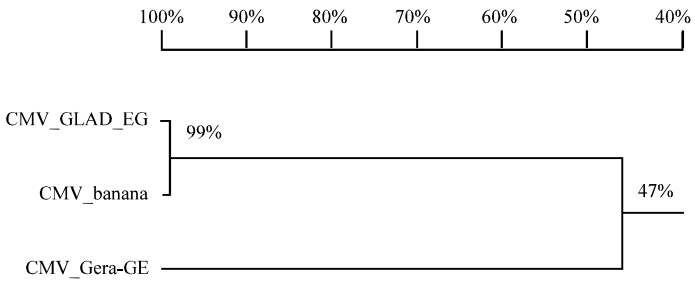

Fig. 5: Phylogenetic tree representing the relationship between the three isolates based on amino acids sequence homology

recombination) and the production of mixed subunit capsids from two different cucumoviruses that may lead to radical recognition changes in insect-mediated transmission were reported also for CMV.

Although postulated several years ago, the natural occurrence of reassortants in the genus *Cucumovirus* was demonstrated only recently for *Bean distortion mosaic virus* (BDiMV), found in Chile (White *et al*., 1995). After a recent survey of CMV in Spain (Fraile *et al*., 1997), it was suggested that reassortment or recombination are rare in natural populations of this virus and that the reassortant genomes that have been found do not correspond to a simple random association of the three genomic segments. Thus, CMV strains with reassortant or recombinant genomes seem not to be favored under natural conditions. Although the nature of this competitive disadvantage is largely unknown, one hypothesis suggests that, in CMV, reassortment is selected against aphid transmission (Fraile *et al*., 1997; Gallitelli *et al*., 1997). Another explanation may rely on the scarcity of natural mixed infections of different CMV strains which are required for genetic exchange to occur in plants. The Tajima's D test is a widely used test of neutrality in population genetics. This statistic illustrates the allele frequency distribution of nucleotide sequence and is based on the difference between two estimators of (the population mutation rate): (1) Tajima's estimator, which is based on the average number of pairwise difference between sequences and (2) Watterson's estimator, which is based on the number of segregating sites in the sample. Tajimas estimator takes into account allele frequency when comparing pairwise differences between sequences whereas Watterson's estimator does not. A segregating site counts as any point where there are differing nucleotides between sequences in the data set, independent of the actual number of differences and hence independent of allele frequency. The difference between these two estimators of is scaled by the standard deviation of their difference. A positive value of D as in our study indicates an excess of intermediate frequency (polymorphic) alleles, while a negative value indicates an excess of rare alleles. The Tajima's D test in which D value was zero ensured that the evolutionary process for Egyptian CMV isolates accomplished by combination of both; mutation and genetic drift (the change in allele frequency for a specific gene within a population) (Tajima, 1989).

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