# Cloning and sequencing of a cDNA encoding the coat protein of an Egyptian isolate of *Pepper mild mottle virus*

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A *Tobamovirus* isolated from pepper plants grown in Egypt has been characterized. *Pepper mild mottle Tobamovirus* (PMMoV) was isolated from naturally infected pepper plants grown in Kafr El-sheikh Governorate. RT-PCR using a specific non-degenerate primer pair for the PMMoV coat protein gene (PMM-F and PMM-R) revealed 470 bp amplified product. Dot blot hybridization was used to establish the authenticity and specificity to the RT-PCR amplified products of PMMoV. The coat protein gene of an Egyptian isolate of PMMoV was cloned and sequenced. The sequence contained a full-length ORF coding for the viral CP. It comprises 473 nt and a polypeptide chain of 157 amino acids with a M(r) of 17.27. The comparison of the CP ORF nucleotide sequence of Egyptian isolate (PMMoV-E2) with those of another isolates revealed that it was very close to the Brazilian and Japanese isolates (99 % and 98% respectively). Whereas the CP of PMMoV-E2 isolate was closely related to TMV-CP (99 & 97%) of Japan and Korea isolates, respectively. In the meantime it appeared far from *Tropica Soda* Apple Mosaic Virus-CP (TSAMV-CP) Florida isolate (USA) of *Tobomovirus*

**Key words:** *Pepper mild mottle virus*, RT-PCR, Hybridization, Nucleotide sequencing

## INTRODUCTION

Peppers (*Capsicum* spp) are very important vegetables world wide, they erals.Five species of *Tobamovirus*, *Tobacco mosaic virus* (TMV, P0), *Tomato mosaic virus* (ToMV, P0), *Tobacco mild green mosaic virus* (TMGMV, P0), *Paprika mild mottle virus* (PaMMV, P1), *and Pepper mild mottle virus* (PMMoV) P1,2 or P1,2,3), have been reported as pathogens of *C. annuum* in Japan **(Hamada *et al.*, 2002, 2003; Nagai, 1981; Nagai**

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*et al.*, 1981; Tsuda *et al.*, 1998, respectively). These viruses can cause serious economic losses in both field and greenhouse- grown crops.

*Pepper mild mottle virus* (PMMoV) is one of the major viral pathogens of cultivated peppers; it causes mosaic provide spice and colour to foods, while providing essential vitamins and min Southeast Florida (Lamb *et al.*, 2001). becoming increasingly popular for rapid analysis of gene transcripts, primarily because of its high sensitivity and specificity. The use of reverse transcription-polymerase chain

between the P0 pathotypes (TMV, ToMV, and TMGMV), the P1,2 pathotype of PMMoV, and the Italian type of P1,2,3 PMMoV (Wetter *et al.*,1984 and Berzal-Herranz *et al.*,1995). However, the P1 pathotype of PaMMV could not be distinguished by these methods. In addition to these PMMoVs, two other types of P1,2,3 PMMoV, distinct from the Italian type, have been reported in Japan (Hamada *et al.*, 2002 and Tsuda *et al.*, 1998). Tests based on nucleic acid hybridization can be used for diagnosing

plant viruses (Jones and Torrance, 1986). Until now, radioactive labeled probes have been commonly used for nucleic acid hybridization. However, such techniques have some disadvantages, in concern about the environmental impact, safety and short lifetime and cost using radioactive labels. Therefore, alternative hybridization methods that employ non-radioactive labeled probes have been developed and proving to be much more practical (Leary *et al.*, 1983 and Forster *et al.*, 1985).

Here, we report the sequence of the coat protein gene of an Egyptian PMMoV RNA and compare it with those of other tobamoviruses.

## MATERIALS AND METHODS

### Virus source

*Pepper mild mottle Tobamovirus* (PMMoV) was isolated previously from naturally *Pepper mild mottle Tobamovirus* (PMMoV) was isolated previously from naturally infected pepper plants collected from Kafr El-sheikh Governorate under plastic conditions, and identified on the basis of host range, stability in sap, modes of transmission, inclusion bodies, serological detection and electron microscopy (El-Khewey *et al.*, 2007).

**RNA from purified virus suspension**

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to **Wilcockson and Hull (1974)** using the phenol-SDS method. It was released from the purified virus by using approximately

100µl of purified suspension and extracted in one volumes of 1 x extraction buffer (200 mM Tris base; 100mM NaCl; 10mM EDTA, pH 8.0) and 0.2 volume of 10 % SDS solution followed by vortexing for 2 min. Two volumes of phenol: chloroform: isoamyl alcohol mixture (PCI) were added and shacked vigorously for 10 min. The aqueous phase of each sample was recovered by low-speed centrifugation at 10,000 Xg for 15 min at 4°C (Sigma 2K15 laborzentrifugen). The RNA was precipitated by the addition of 1/10 volume of 3M Na. acetate mixed with 2.2 volume of ice cold absolute ethanol and incubated at -20°C overnight. The RNA pellets were obtained by centrifugation at 10,000Xg for 15 min at 4°C. The RNA pellets were washed with ice cold 75% ethanol and centrifuged as above, dried under vacuum desicator for 15- 20 min, and resuspended in 50µl of

RNase-Free water. The viral RNA was determined by analysis on 1 % agarose gel electrophoresis (**Maniatis *et al.*, 1982**).

**Oligonucleotide primers**

Newly designed specific non-degenerate primers were used in molecular studies for coat protein gene amplification as shown in Table (1). These primers should be amplifying a DNA copy of approx. 470 base pairs of PMMoV virus genome. The virus sense primer (PMM-F) corresponds to nucleotides (nt) positions 5685 to 5704 was designed according to PMMoV complete sequence (**Wang *et al.*, 2006**) while the complementary (antisense) primer (PMM-R) corresponds to (nt) positions 6135 to 6155 of PMMoV coat protein sequence (Table 1). The primers were synthesized at Operon Co., USA.

**cDNA synthesis using random primers**

The PMMoV-cDNA was synthesized and amplified from purified viral RNA using random

**Table 1.** Oligonucleotide sequences of the used primers

| Primer primer | size (mers) | Sequence 5`----------3` | Prod.size |
|---|---|---|---|
| FCP | 30 | ATGTCTTACA GTATCACTAC TCCATCTCAG | 500bp[x] |
| PCP | 18 | TGGGCCCCTACCGGGGGT | 500bp[x] |
| PMM-F | 28 | GGGCTAGA-ATGGCTTACACAGTTTCCAG | 470bp[x] |
| PMM- R | 28 | AGGTGATCCTAAGGAGTTGTAGCCCAGGT | 470bp[x] |

[x] The approximate product size of the primers pair.

primers. A 10-50 ng of viral RNA was used as template for the reverse transcription reaction (RT). The RT reaction was prepared as follows; 0.625 ng of the random primer, 1X first strand cDNA buffer (5X buffer: 250 mM Tris-HCl, pH 8.3, 375mM KCl, 15mM MgCl2, 50 mM DTT) 0.2mM dNTPs, 0.1ul of RNAase inhibitor (40 units/ µl, invitrogen), 0.1 µl of the superscript II reverse transcriptase enzyme (10 µ/µl invitrogen) were added to above mixture, the deionized water was added to final volume 25 µl. Finally, the reaction mixture was incubated at 42 °C for 1hr and the resulting mixture was heated for 5 min at 94 °C and chilled in ice for 2 min.

**PCR amplification using PMMoV-specific primers**

The PCR reaction mixture using specific PMMoV-CP primers was prepared by using 5 µl of the resulting cDNA. The PMMoV cDNA was transferred to a tube containing 45 µl of 1X polymerase chain reaction buffer (10mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% gelatine), 1.5 mM MgCl2, 200µM dNTPs, 10 pmol of PMM-F and PMM-R of forward and reverse primer respectively, 2.5 µ of Taq DNA polymerase (BioLab in rate of 5u /µl). The amplification proceeded in the thermocycler (Uno II Biometra) at 94 °C for 3 min, and through 30 cycles of 92 °C for 30

s and 60°C for 30s and 72 °C for 1min, with a final step at 72 °C for 10 min. Virus detection by nucleic acid hybridization technique

**a. DNA probe labeling**

The Dig-L abeled TMV-cDNA probe was synthesized using PCR-Dig labeling technique according to

Roche, Boehringer Mannheim crop., Indianapolis, IN, USA, protocol. The DNA-PCR product of TMV virus was fractionated in 1% agarose gel and subjected to gene cleaning by Qiaquick gel extraction kit (Qiagen) The purified TMV DNA was used as a template for the polymerase chain reaction using PCR Dig Labeled dNTPs mixture. The PCR reaction was performed using degenerate primers (kindly provided by Dr. Shoman S. Microbiology Dept., Faculty of Science, Ain Shams University) specific for detection of the TMV strains. The virus sense primer (Fcp) and the complementary (antisense) primer (Bcp) were according to Shoman (2004) as shown in Table (1).

The PCR cocktail was performed in 50 µl total volume containing 10mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% (W/V) gelatin, 4mM MgCl2, 200 µM dNTPs, 0.2 µM each primer, the virus sense (FCP) and antisense primer (BCP) and 2.5 units of the thermostable Taq DNA polymerase (Promega). The amplification proceeded in the thermocycler (Uno II Biometra) at 94 °C for 3 min, and through 40 cycles of 92 °C for 30 sec and 60°C for 45 sec and 72 °C for 2 min, with a final step at 72 °C for 10 min

**b. Dot blot hybridization assays**

The preparation of tissue extracts for dot blots was carried out according to **Loebenstein *et al.*(1997)** with some modifications. The leaf tissue (0.5g) was ground in 2.5 ml of denatured solution containing [8X standard saline citrate (SSC) (1X SSC is 150 mM NaCl, 15mM Na. acetate, pH 7.0) plus 10% formaldehyde] and then heated to 60°C for 15 min and kept on ice. 5 µl of the denatured viral RNA and the supernatant of the plant extracts was spotted onto presaturated nitrocellulose membrane (Boehringer Mannheim crop.) with 20X SSC. The membrane was then crosslinked by the U.V irradiation for 2 min, followed by nucleic acid hybridization.

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**Cloning of PMMoV- coat protein gene in Topo-T easy Vector**

The PMMoV-Cp PCR amplified product was directly cloned in the linearized and thymidylated Topo-T-easy plasmid. The construct was transformed into DH5α *E. coli* competent cells according to **Sambrook *et al.* (1989).**

The DNA sequence of PMMoV-isolate was confirmed by direct sequencing the resulted clones briefly. The PMMoV coat protein gene region (470 bp) was sequenced in one direction using the $M_{13}$-21 forward primer. The sequence was carried out using ABI PRISM model 310 versions 3.4 semi-adaptive version 3.2 at gene analysis unit, UCDavis sequences facilities, (California, Davis, USA). Sequence analysis was performed using Bio Edit program version 5.0.6.

## RESULTS

**Virus detection by RT-PCR amplification**

Detection of PMMoV by reverse transcriptase - polymerase Chain Reaction (RT-PCR) was performed on purified viral RNAs extracted using the phenol-SDS method. Fig. (1) show the viral genomic RNA integrity of the TMV (as positive control) and the PMMoV. The PCR using PMMoV specific primers gave a 470 bp amplified product corresponding to the coat protein gene of PMMoV as shown in (Fig. 2 lane 2) whereas no amplification was detected with the healthy pepper plant (Fig. 2 line 3).

Blue/white colonies had screened up to select the bacterial colonies transformed with recombinant Topo plasmids using the PCR technique. Clones from transformed cells were purified using the Wizard minipreps DNA purification system (Promega Corporation MD) and sequenced directly

**Nucleotide sequence analysis**

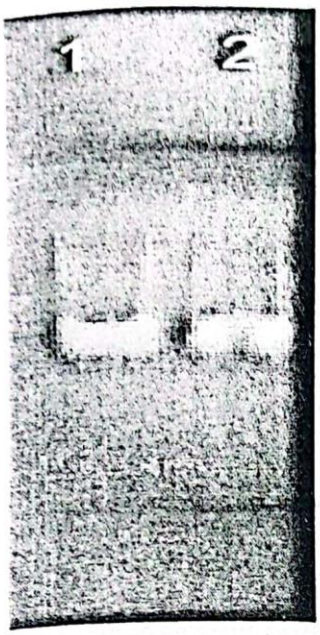


**Fig . 1.** 1% agarose gel electrophoresis for the RNA extraction from purified Viruses. Lane 1: RNA extraction from purified TMV virus, Lane 2: RNA extraction from purified PMMoV

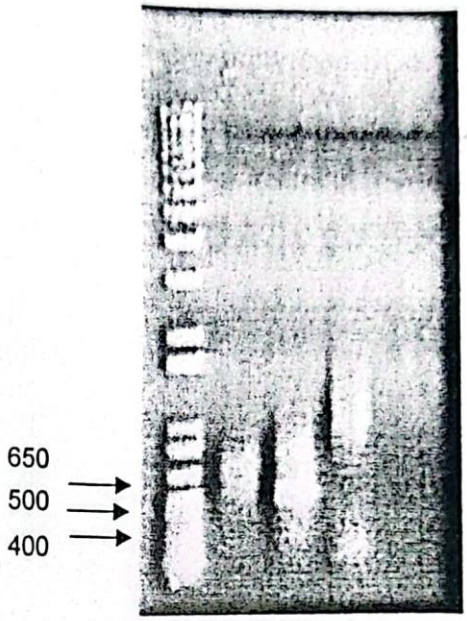


**Fig. 2.** 2 % agarose gel electrophoresis analysis of the amplified product of PMMoV coat protein gene using PMM-1 and PMM-2 specific primers. M:1 Kb ladder, Lane 1: RT-PCR from PMMoV-virus purified from pepper infected plant, Lane 2: RT-PCR amplified product from PMMoV virus purified from *Nicotiana clevelandii* plant, Lane 3: RT-PCR from water PCR negative control.

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**Dot blot hybridization technique**

To detect the authenticity of our amplified product and its correlation to TMV virus previously detected and sequenced; the purified TMV DNA amplified product (TMV control) was used for creation of dig labeled- cDNA probe, specific for the TMV coat protein gene. The non-radioactive-Dig labeled probe was used for dot blot hybridization and clearly hybridized with our PCR products.

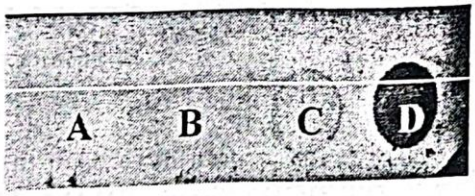


**Fig. 3.** DNA dot blot hybridization on nitrocellulose membrane. The membrane hybridized with specific non- radioactive TMV –DNA –Dig labeled probe. Track A: Uninfected *N. clevelandii* dot blot extract. Track B:denatured PMMoV viral RNA dot blot. Track C: PCR product of PMMoV RNA. Track D: PCR product dot blot from the TMV RNA.

The TMV-cDNA probe hybridized and showed signals with high intensity with amplified product of TMV (positive control) (Fig. 3 track D).

As well as, the cDNA Dig labeled probe showed signals with the denatured PMMoV viral RNA and the amplified PCR product of PMMoV as showed in dot blot tracks B and C, respectively. No signals have been observed with uninfected *N. clevelandii* dot blot extract as shown in track (A). The virus RNA could be detected directly with high sensitivity and specificity from their natural host tissues by dot blot hybridization technique.

**Nucleotide sequence analysis**

One of our research objectives was to clone and sequence the coat protein gene of PMMoV. The 470 bp amplified CP gene product of the isolate PMMoV-E2 was ligated into the Topo-T-easy vector and cloned. Only 6 clones of PMMoV-E2 isolate was sequenced revealing 473 base fragments as expected as shown in Fig. **(4)** with deduced coat protein consisted of 157 amino acids with a M(r) of 17.27. A multiple alignment was done along with sequences previously

obtained which have already been used as reference sequences in other studies. The following sequences were used in the comparisons: Brazil (PMMoV-SaltoBR12 & Itapetininga BR17) with isolate accession No. (AM411434 and AM411438) according to

**Cezar *et al.*, (2006),** PMMoV-Japanese according to **Hamada *et al.*, (2001)** (PMMoV-Ge 1 & Tosa with accession Nos AB062049 &AB062053 respectively), TMV-isolate (CP) Japanese isolate **(Kirita *et al.*, 1997)** with accession No. L35074 and the Korean from the accession AF103778, AB084456 (TMV & PMMoV-P2 isolate) and PMMoV-P91 & PMMoV-CN isolate from Germany and China respectively (Letschert *et al.*, 2002 & Wang *et al.*, 2006) were also included in the comparisons.

The nucleotide sequence alignment Fig. (4) showed 99 %-97% similarity with (PMMoV-SaltoBR12 & PMMoV-Ge 1) isolates belongs to Brazil and Japan respectively. In the meantime the Egyptian isolate (PMMoV-E2 isolate) showed 98 %, 97, 96 % and 94% similarities with Itapetininga BR17, (PMMoV-CN, PMMoV-P2) and (Tosa, PMMoV-Italian and PMMoV-P91 isolate) respectively and correspond to Brazil, China, Korea, Japan, Italy and Germany isolates. However, the Phylogenetic homology tree based on multiple sequence alignments (Fig. 4 & 5) of the Egyptian present isolate (PMMoV-E2 isolate), reveled that the (CP) of the PMMoV-E2 isolate was closely related to TMV-CP (99 & 97%) of Japan and Korea, respectively

These results, revealed that the PMMoV-E2 present isolate appeared far from Tropica Soda Apple Mosaic Virus-CP (TSAMV-CP) Florida isolate (USA) of *Tobomovirus* which showed 81%. Similarity.

The viral sequence obtained in this work was deposited in the National Center for Biotechnology Information Gene Bank with accession numbers EU380719 for PMMoV coat protein gene sequence (CP).

## DISCUSSION

The cDNA of PMMoV was synthesized using random primers and used as a template for PCR amplification of the full length of the viral coat protein gene approximately 470 bp

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amplified product using a specific non-degenerate primer pair for the PMMoV coat protein gene (PMM-F and PMM-R). This size was in agreement with those expected from the nucleotide sequence of the PMMoV-CP gene (**Wang *et al.*, 2006**) .

The RT/PCR technique is highly sensitive, simple and useful in overcoming many difficulties encountered with serological methods, such as low antigen titer, availability of antibodies and cross reactivity of antibodies with heterologous antigens (**Matthews *et al.*, 1997**). It seems also that, RT-PCR is faster than traditional methods, and other methods such as squash blot and dot blot hybridization. It can be concluded from our study that the RT-PCR assay has a great sensitivity that enables the detection of TMV and its strains in infected samples; this result was in agreement with **Tenllado *et al.*, (1994)** who stated that a procedure based on polymerase chain reaction was developed allowing the rapid detection and molecular differentiation of the distinct pathotypes of the tobomoviruses infecting TMV resistance genotypes of pepper in RNA-enriched fractions from infected plants. **Letschert *et al.*, (2002)**, also developed the same technique involving RT- following PCR using single primer pair for detection and differentiation of the five *Tobamovirus* species which are related serologically using the same PCR conditions and primer sets.

This also has intense interest to use this simple technique (RT-PCR) to detect the viral spread earlier. These results in accordance with **Pereda *et al.*, (2000)** who stated that the reverse transcriptase - polymerase chain reaction (RT-PCR) was very sensitive for detecting viral spread earlier and TMV-Cg in Arabidopsis thaliana, but less sensitive for TMV-U1 detection. In addition *Tobacco mosaic virus* (TMV) was used in our study as a positive control, therefore it could be used for addition to. reverse transcription polymerase chain reaction (RT-PCR) assays as an internal control specially incase of the duplex RT-PCR (**Torok and Randles, 2001**). The non-radioactve DIG–labelled TMV cDNA probe, amplified from the full length coat protein gene using Fcp and Bcp degenerate primers, was used for detection of the PMMoV via dot blot hybridization

technique. The TMV-cDNA specific probe and Dot blot hybridization were used to establish the authenticity and specificity of the RT-PCR products. In addition, the virus RNA could be detected directly with high sensitivity and specificity from their natural host tissues by dot blot hybridization technique.

The nucleotide sequence of the coat protein gene of PMMoV was determined. The deduced coat protein consisted of 157 amino acids and this in agreement with Garcia-Luque et al. (1993).The composition of the CP ORF nucleotide sequence of Egyptian isolate (PMMoV-E2) with those of another isolates revealed that it was very close to the Brazilian and Japanese isolates (99 % and 98%).

Whereas the PMMoV-E2 isolate, revealed that the (CP) of the PMMoV-E2 isolate was closely related to TMV-CP of Japan and Korea, (99 & 97%) respectively ( **Park *et al.*, 1994)** in the meantime it appeared far from *Tropica soda apple mosaic virus*-CP (TSAMV-CP) Florida isolate (USA) of *Tobamovirus*.

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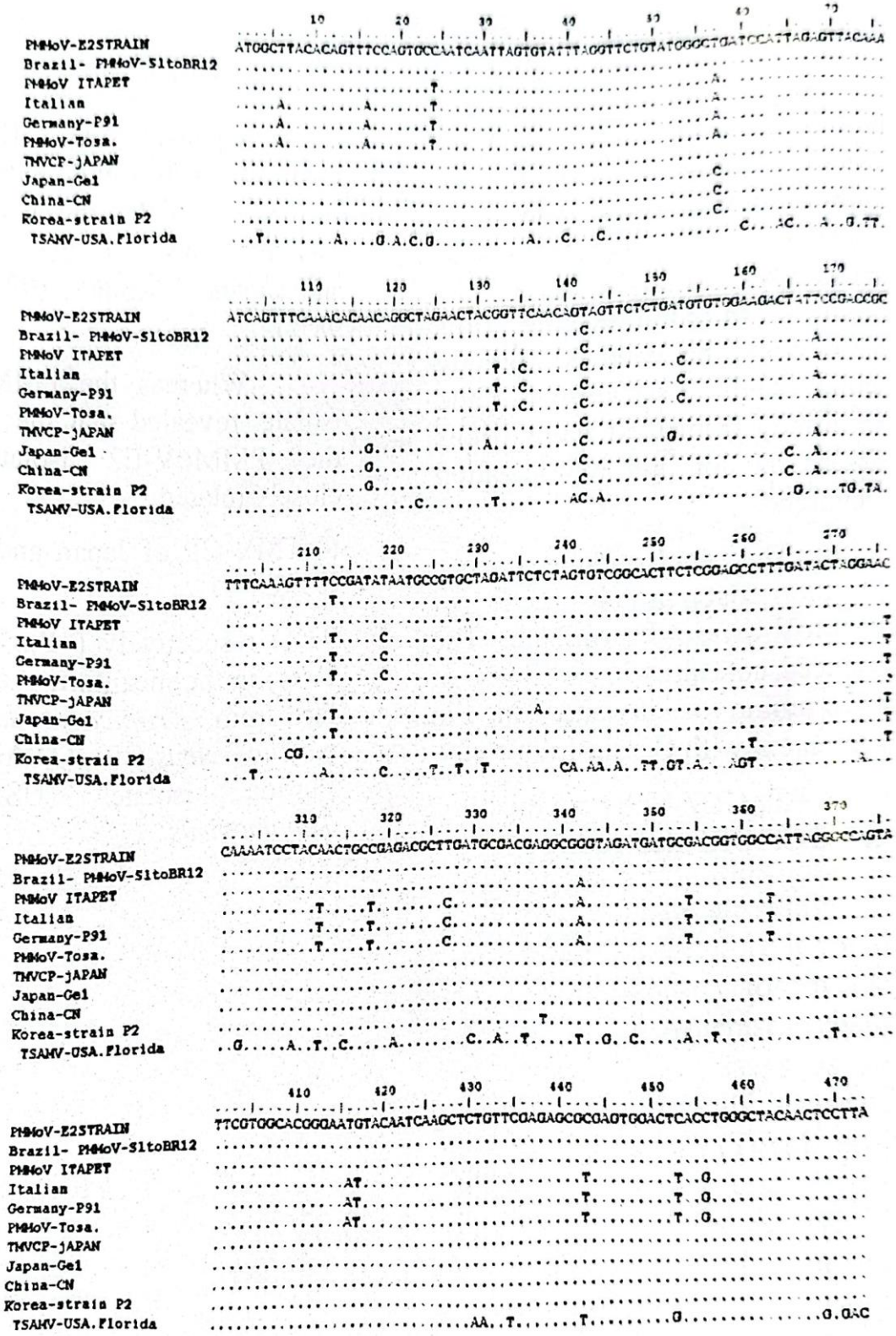
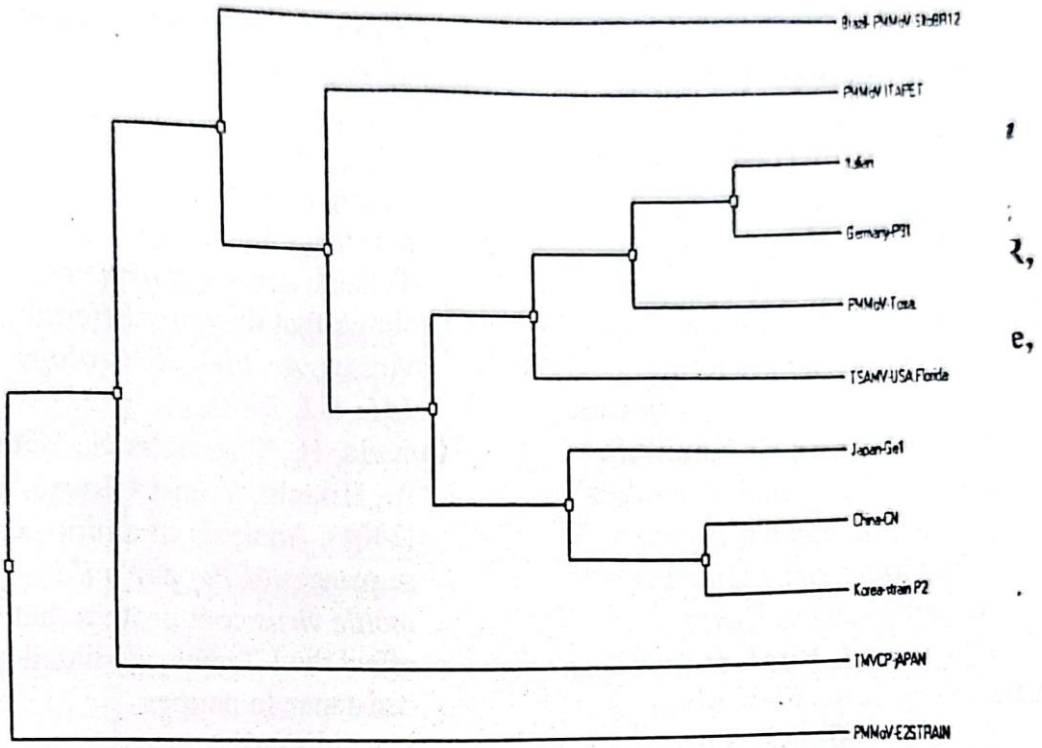



**Fig. 4.** Nucleotide sequence alignment of cloned PMMoV-E2 coat protein gene with r published sequences.




**.5.** The phylogenetic homology tree based on multiple sequence alignments of the PMMoV-E2-Egyptian isolate compared to previously sequenced isolates.

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