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MOLECULAR CHARACTERIZATION AND BIOINFORMATICS ANALYSIS OF VIROID ISOLATE ASSOCIATED WITH CITRUS GUMMY BARK DISEASE IN EGYPT

[32]

Sofy[1]*, A.R.; Soliman[2], A.M.; Mousa[1], A.A. and El-Dougdoug[3], Kh.A.

ABSTRACT

This work reports the characterization of *Hop stunt viroid* which has been isolated previously in Egypt from sweet orange infected with gummy bark disease namely *Citrus viroid II*. The native structure of mature, circular forms of the gummy bark agent was detected by gel electrophoresis. The expected size of amplified cDNA by RT-PCR was approximately 300 bp. A phylogenetic tree of the Egyptian citrus gummy bark agent (Accession no. FJ984562) revealed 100%, 99.7% and 97.8% a moderate degree of similarity to CVd-IIb (USA), CCaVd (Spain) and CCaVd (Egypt) respectively. The minimum free energy of a secondary structure for HSVd-EG-RNA was determined using its primary sequence at 37°C. The sequence appeared to fold into a rod-like structure at -122.1 kcal mol^{-1} while CCaVd-EG at -120.5 kcal mol^{-1}. The five domains of the rod-like structure were determined. The sequence variations between Egyptian citrus gummy bark isolate and Egyptian citrus cachexia isolate in the pathogenic domain (P) tend to influence the pathogenicity of the HSVd-EG. Finally, the genetic diversity and evaluation of entropy power for the Egyptian citrus gummy bark agent and HSVd-citrus populations registered in GenBank, were viewed against the phylogenetic background of known CVd-II variants including the non-cachexia (CVd-IIa) and the causal agents of severe (CVd-IIb, CVd-IIc), more moderate (Ca903) and mild (Ca909).

Keywords: Citrus, Viroids, Cachexia disease, Nucleotide sequence, genetic diversity.

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[1]Botany and Microbiology Department, Faculty of Science, Al-Azhar University, 11884 Nasr City, Cairo, Egypt.
[2]Plant Virus and Phytoplasma Research Section, Plant Pathology Institute, Agriculture Research Center, Giza, Egypt.
[3]Virology Lab., Agric. Microbiology Department, Faculty of Agriculture, Ain Shams University, 11241 Cairo, Egypt.
*ahmd_sofy@yahoo.com

INTRODUCTION

The disorder of trees of sweet orange grafted on sour orange rootstock in Egypt, was described by **Nour-Eldin (1956; 1959)** as phloem discoloration of sweet orange. Further study by **Nour-Eldin (1968)** showed gummy deposits are largely in the outer bark tissues, then the name was changed to gummy bark of sweet orange. When the bark of affected trees is scraped, a line of reddish-brown, gum-impregnated tissue can be seen around the circumference and especially near the bud union. The discoloration and gumming may extend 60 cm or more above the bud union in the bark of the trunk and main branches of the sweet orange. The causal agent of citrus gummy bark disease has been transmitted experimentally by bud inoculation from infected trees to sweet orange; however, symptoms are visible only after 5–10 years incubation period (Nour-Eldin, 1968; Roistacher, 1991; Önelge *et al.,* 1996). Etiology of gummy bark disease is unknown, but viroids have been suggested as the causal agent. sPAGE and RT-PCR results indicated that, CVd-II associated with gummy bark disease as reported by **Önelge *et al.* (2004); Sofy *et al.* (2010).**

Hop stunt viroid (HSVd; synonym: citrus viroid [CVd]-II) is the causal agents of citrus cachexia disease which specific to mandarins and tangelos as reported by **Childs (1952).** Citrus viroid (CVd) group II is comprised of *Hop stunt viroid* (HSVd)-related variants of 295 to 302 nucleotides. This group include; cachexia-inducing agents *Citrus cachexia viroid* (or CVd-IIb), CVd-IIc, Ca-903, and Ca-909 as well as the non-cachexia-inducing variant CVd-IIa (Reanwarakorn and Semancik, 1999; Palacio-Bielsa *et al.,* 2004). *Hop Stunt viroid*-citrus was reported previously in Egypt associated with cachexia disease by El-**Dougdoug *et al.* (1997); Amin (1999).** Symptoms of citrus gummy bark, a disease specific to sweet orange **(Nour-Eldin, 1956; 1959)** bear similarities to cachexia, a disease specific to mandarins and tangelos **(Childs, 1952)** caused by variants of the *Hop stunt viroid* (HSVd)-related CVd-II (Semancik *et al.,* 1988). It could be suggested that a variant of this viroid may also be involved in citrus gummy bark disease expression **(Önelge *et al.* 2004)**. Sequence analyses of the Egyptian gummy bark agent and CVd-IIb (Ca902) strain did not reveal nucleotide change or unusual

mutations that might suggest a relationship to CVd-II with citrus gummy bark disease (**Sofy *et al.*, 2010**).

This work aims at characterization of the viroid isolate associated with gummy bark disease which reported by **Sofy *et al.*, (2010)** in Egypt using RT-PCR, sequence analysis and bioinformatics.

MATERIALS & METHODS

Plant material

The principal gummy bark isolate namely *Citrus viroid II* used in this study was derived from an old line navel orange tree in 2008 from Kalyobiya governorate, Egypt (**Sofy *et al.*, 2010**). This isolate has been maintained in sour orange as rootstock. The viroid isolate was transmitted by blind bud inoculation to Arizona 861-S1 'Etrog' citrons and kept in a greenhouse under controlled temperatures (28-32°C) for at least 5 months.

Dot-blot Hybridization

The fresh leaf tissue (1 g) was ground in 4 ml of grinding buffer (200 mM glycine, 100 mM Na_2HPO_4, 600 mM NaCl, 1% SDS pH 9.5, autoclave for 20 min at 120°C and then added 0.1% DIECA, 0.1 mM DTT) using sterilized pestle and mortar. The extract was clarified by centrifugation for 10 min at 10000 rpm. Aliquots (10 µl) of the partially purified nucleic acids were denatured with formaldehyde denaturation buffer {5X SSC, 25 mM Na_2HPO_4, 5X Denhart (0.1% each of BSA, Ficoll and PVP 360), 50% deionized formamide, 200 µg/ml of denatured calf thymus DNA}. The mixture was incubated for 60 min at 60°C and loaded under vacuum on positively charged nylon membranes with a Bio-Dot-SF apparatus (Biorad Laboratories, Hercules, CA, USA) and immobilized by 5 min of UV treatment. Dot-blot analyses were performed using digoxigenin (Dig)-labeled cDNA probes specific for HSVd and CEVd (kindly supplied by Dr. El-Dougdoug Virology Lab. Fac. of Agric. Ain Shams Univ.). Pre-hybridization and hybridization were performed in 50% formamide and 5X SSC. The membranes were pre-hybridized at 42°C for 2 h and hybridized overnight at 50°C. After hybridization, they washed three times for 5 min each in 2X SSC, 0.1% SDS at room temperature, followed by two times for 15 min each in 0.2X SSC, 0.1% SDS at

50°C. The Dig-labeled hybrids were detected with an anti-Dig-alkaline phosphatase conjugate and visualized with the chemiluminescent substrate CSPD (Roche Diagnostic, Indianapolis, USA) (**Candresse *et al.*, 1990**).

Isolation of low molecular weight RNAs

Five grams of leaf tissues of each citrus tree was triturated in 5 ml of extraction buffer (0.4 M Tris-HCl, pH 8.9; 1% SDS; 5 mM EDTA, pH 7.0; 4% β-mercaptoethanol) and 15 ml of water-saturated phenol neutralized at pH 7.0. The nucleic acids were partitioned in 4 M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in 300 µl TKM buffer (10 mM Tris, 10 mM KCl, 0.1 mM $MgCl_2$, pH 7.4) (**Semancik *et al.*, 1975**).

Polyacrylamide gel electrophoresis.

Aliquots (20 µl) of partially purified RNAs were analyzed by 5% sPAGE. The first gel was polymerized in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2), and subjected to a constant current of 54 mA at 4°C for 3 h. A segment of the gel defined by the xylene-cyanol dye and 1 cm below was excised and placed on the top of a second gel containing 8 M urea and polymerized with TBE buffer (22.25 mM Tris, 22.25 mM boric acid, 0.5 mM EDTA, pH 8.3). The second gel was subjected to a constant current of 15 mA at room temperature for 4 h and stained with silver nitrate (**Igloi, 1983; Rivera-Bustamante *et al.*, 1986**).

RT-PCR

Total RNA was extracted using RNA Minipreps Super Kit (BIO BASIC, Cat. # BS584). The resulting RNA preparations were subjected to RT-PCR amplification. Samples were heated to 70°C for 5 min in the presence of the HSVd-specific oligonucleotide 26-mer HSVd(-) (5`-dAGGGGCTCAAGAGAGGATC CGCGGCA-3`) and immediately cooled on ice. The mixture was then subjected to reverse transcription (RT) with moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI, USA) at 37°C for 60 min in 25 µl volume. One-tenth of the RT product was directly subjected to PCR amplification in a 25 µl volume reaction in the presence of the oligonucleotide HSVd(-) and the 24-mer HSVd(+) (5`-

dCTGGGGAATTCTCGAGTTGC CGCA-3`) (El-Dougdoug *et al.*, 2010). PCR cycling parameters were: denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 2 min, to finish with extension at 72°C for 10 min. PCR-amplified DNA fragments were separated by agarose gel electrophoresis in 1.5 % agarose (Seakem LE, FMC, Bio products, Cat. # 50004) minigels in 0.5X TBE buffer (Tris-borate-EDTA, 90 mM Tris acetate, 90 mM boric acid, 2 mM EDTA, pH 8.0) using 100 bp DNA ladder (Promega, Cat # G2101). The gels were visualized with UV light after staining for 10 min with 10 µg/ml of ethidium bromide [2,7-Diamino-10-ethyl-9-phenylphenanthridinium bromide; homidium bromide. (Sigma, Cat. # E7637)] (**Sambrook *et al.*, 1989**), and visualized with UV illumination using Gel Documentation System (Gel Doc 2000 BIO RAD 1000/115 V ~ 50/60 Hz-150 VA).

Cloning and sequence analysis

The amplified RT-PCR products were purified from the agarose gel using the QIAEX II Agarose Gel Extraction Protocol (Qiagene, Cat. # 20021). The resulting fragments were cloned into the pGEM®-T vector (Promega), and transformed into *E. coli* DH5α. The cDNA clones from all isolates were identified by PCR and restriction analysis. Selected clones were sequenced using an automated DNA sequencer (ABI Prism 3730XL DNA Analyzer) and and analyzed by FinchTV™ version 1.4.0 software. The sequence was reported to GenBank and are archived under the accession number : FJ984562 (**Sofy *et al.*, 2010**). Sequences were aligned with the Egyptian CVd-II sequence that was archived in GenBank database (accession number: FJ984562) using ClustalW (Ver.1.74) program (Thompson *et al.*, 1994). The nucleotide distances were estimated considering alignment gaps by using Jukes and Cantor (1969) method for correction of superimposed substitutions using the Molecular Evolutionary Genetics Analysis (MEGA) software (Ver. 4.0) (**Tamura *et al.*, 2007**). Neighbour Joining (NJ) implemented through MEGA 4.0 software, and bootstrap analysis (1000 replicates) was performed to assess the reliability of the constructed phylogenetic tree.

Prediction of the Secondary Structure of Viroid RNA

Secondary structures were obtained with the MFOLD program (circular version) of the GCG package (**Zuker, 1989**) at 37°C. Circular structures were obtained with CLC RNA Workbench software, Denmark.

RESULTS

A) Primary characterization of gummy bark-causing agent

The principal gummy bark disease agent was collected from an old line navel orange tree in 2008 in Kalyobiya governorate exhibited vein chlorosis leaf, gummy bark symptoms and tree stunted. When the bark was scraped, a line of reddish-brown, gum-impregnated tissue can be seen around the circumference and especially near the bud union. The discoloration and gumming may extend 60 cm or more above the bud union in the bark of the trunk. Also, dark brown streaks of gum-impregnated tissue were seen in both the circumference and in longitudinal sections (**Figure 1**).

Dot blot hybridization was used to confirm the source of gummy bark infected with viroids. The samples gave clear positive with HSVd DIG-labeled probe compared with positive and negative control. Also, the source of gummy bark not infected with *Citrus exocortics viroid* (CEVd) using CEVd Dig-labelled probe compared with positive and negative control (**Figure 2**). HSVd-EG isolate (cv. Navel) was indexed by graft inoculation on Etrog citron plants. It gave petiole wrinkle and midvein browning symptoms appeared after 12-16 weeks.

S-PAGE for gummy bark-causing agent

The RNA of gummy bark-causing agent that was extracted from citrus cv. Navel tree appeared gummy bark and grafted Etrog citron was determined by sPAGE. It was found an identical relative mobility on sPAGE with the electrophoretic mobility of HSVd (**Figure 3**).

B) Molecular characterization of gummy bark-causing agent

1) Total RNA extraction

Total RNA was extracted, from citrus cv. Navel tree appeared gummy bark and grafted Etrog citron, using RNA Minipreps Super Kit (BIO BASIC, Cat. # BS584). The integrity and quantity of the purified RNA were confirmed by gel electrophoresis and UV

spectrophotometer. The concentration of RNA was 45 µg / 0.5 g of infected tissues and the purity of the total RNAs obtained measured by an A260/280 absorbance ratio (1.8) for indicating high yield and purity of the extracted RNAs.

2) Amplification of the gummy bark-causing agent

Gummy bark-causing agent RNA that was prepared previously, was reversed by M-MLV reverse transcriptase. The resulting cDNA was amplified by PCR using primers specific for HSVd. the RT-PCR products were analyzed using 1.5% agarose gel by electrophoresis. The expected size of amplified cDNA was approximately 300 bp. While the healthy leaves have no reaction (**Figure 4-A**).

3) Cloning of gummy bark-causing agent into pGEM®-T Easy Vector

pGEM®-T Easy Vector System I (Promega, Cat # A1360) provides a highly efficient one step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for regulated expression in *E. coli* DH5α.

4) Isolation of recombinant plasmids & Enzyme digestion

Several white colonies resistant to ampicillin were selected to test for recombinant plasmids containing the gummy bark agent (~300 bp). PCR was done to confirm if the transformation of pGEM®-T Easy Vector with gummy bark agent into strain DH5α bacterial strain was carried out or no (Fig. 4-B). Restriction enzyme digestion with *Eco*RI representing insert in the right direction at ~320 bp (**Figure 4-C).**

5) Nucleotide sequence analysis

Nucleotide sequencing of the RT-PCR amplified fragment in the recombinant plasmid for the gummy bark-causing agent was completed to confirm PCR fragment is belonging to *Pospiviroidae* group and to compare the sequence with other sequences of citrus viroids available in GenBank (**Figure 5**).

C) Bioinformatics analysis for characterization of gummy bark-causing agent

1) Statistical analysis of sequence and alignment

For sequence heterogeneity within HSVd-EG isolate, the sequence was aligned using CVd-

IIa, CVd-IIb, CVd-IIc, Ca903 and Ca909 as the original reference sequences for HSVd-citrus. In addition to that, genetic diversity of the HSVd-EG isolate characterized in this work was carried out together with the 17 reported HSVd-citrus sequences from Egypt, Spain, USA, Pakistan, Cuba, Iran, Colombia, Japan, Indian, Tunisia, China and Sudan. All of these sequences were multiple-aligned using the ClustalW program with minor manual adjustments, resulting in 325 positions including the gaps (**Figure 5**). Total of 20 variable sites were found in HSVd and 16 were parsimoniously informative nucleotide sites as well as 4 were singleton sites (**Figure 6**).

The nucleotide sequence of complete genome for HSVd-EG strain (Accession no. FJ984562) revealed highest content for Cytosine (C) 87 (29.1%) followed by Guanine (G) 82 (27.4%), then Thymine (T) 69 (23.1%), and Adenine (A) 61 (20.4%). Data also showed that, the ratio between C+G to A+T was 1.3 (Table 1). Moreover, bases composition data for complete genome sequence of HSVd-EG (gummy bark agent) and 17 isolates in GenBank was tabulated to determine C+G and A+T ratio (**Table 1**).

The nucleotide distances between isolates ranged from 0.000 to 0.042 (Table 2). The lower values were recorded for isolate pairs HSVd-EG with CVd-IIb (1-2), CVd-IIc with Indian (4-5), China with Iran (7-8), CCaVd Egyptian isolate with Ca903 (11-12) and Cuba with Japan (17-18) confirming that these isolates had the same nucleotide sequences (Table 2). The higher nucleotide distance values were recorded for isolate pairs CVd-IIc with Colombia (4-15) and Indian with Colombia (5-15) (Table 2). Genetic diversity value between HSVd populations, representing the average number of nucleotide substitutions per site between pairs of sequences, was 0.021 ± 0.005 (**Table 2**).

Entropy power explains the sequence mutates by the function of entropy plot using BIOEDIT. As shown in Fig. (7), there are several peaks, which contains high variable regions. So entropy power can be used to screen the variable regions (**Table 3**).

A phylogenetic tree was constructed for showing the clustering relationship among HSVd-citrus populations (**Figure 8**). It, generated using the Neighbour-Joining method and

bootstrap analysis of 1000 repetitions. It produced two distinct groups. The first group is Asian strain with USA, Spain and Egypt. Second group is African including Egypt and South American with Japan and USA. HSVd-EG (gummy bark agent) has 100%, 97% and 96.5% homologous with CVd-IIb USA (accession no. aF131249), CCaVd isolate X-704-M Spain (accession no. AF213487) and CCaVd Egypt (accession no. AY513267) respectively.

2- Analysis of HSVd-EG-RNA structure

The primary structure of HSVd-EG-RNA is determined by the sequence. Secondary structure of HSVd-EG-RNA has been characterized by forming intramolecular base pairs among of its bases, which include interior loops, hairpin loop, bulge loop and external loop (**Figures 9 and 10**). On the other hand, the secondary structure of HSVd-EG-RNA differed than secondary structure of CCaVd-EG-RNA specially in pathogenic domain as shown in **Figure (9).** The minimum free energy of a secondary structure for HSVd-EG-RNA is determined as -122.10 kcal mol^{-1} by summing the energy contribution of all base pairs, interior loops, hairpin loop, bulge loops and external loop at 37°C using MFOLD program. It is rod-shaped structure composed of alternating single- and double-stranded regions (**Figure 9**).

Table (4) illustrated $\Delta G°37$ (Gibbs free energy) of stalking pairs and interior, hairpin and bulge loops depend on predicated free energy values (Kcal mol^{-1} at 37°C) for secondary structure of HSVd-EG-RNA. It was observed that base pairs lead to free energies < 0 while bulges and loops lead to free energies > 0. Consequently total free energy of HSVd-EG-isolate was -122.10 kcal mol^{-1}. Partition function of HSVd-EG isolate (Q) sum all free energies of secondary structure. This can be calculated as base pairing probabilities for each pair of bases.

Figure 1. Infected citrus navel orange tree in the field showing different types of gummy bark symptoms as discoloration of phloem tissues and vein chlorosis in leaves.

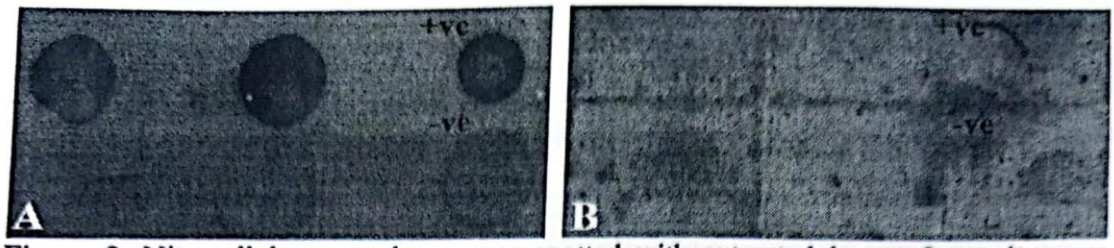

Figure 2. Nitrocellulose membrane was spotted with extracted leaves from citrus cv. Navel tree appeared gummy bark and grafted Etrog citron using A) HSVd Dig-labeled cDNA probe and B) CEVd Dig-labeled cDNA probe by dot-blot hybridization.

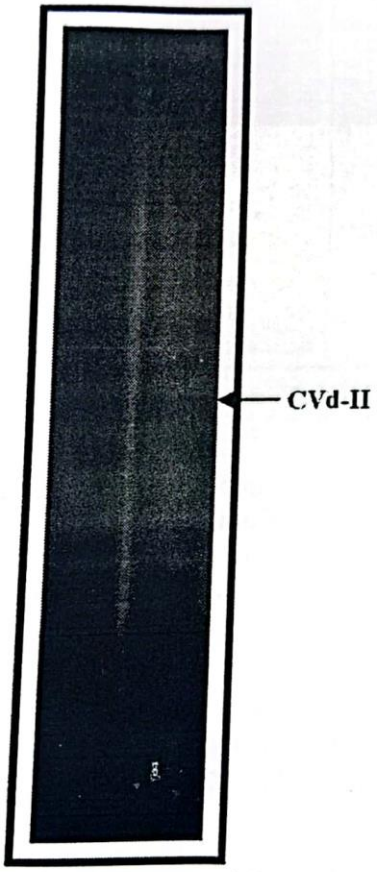

Figure 3. 5% S-PAGE showing RNA bands extracted from citrus cv. Navel tree appeared gummy bark symptoms and grafted Etrog citron stained with silver nitrate.

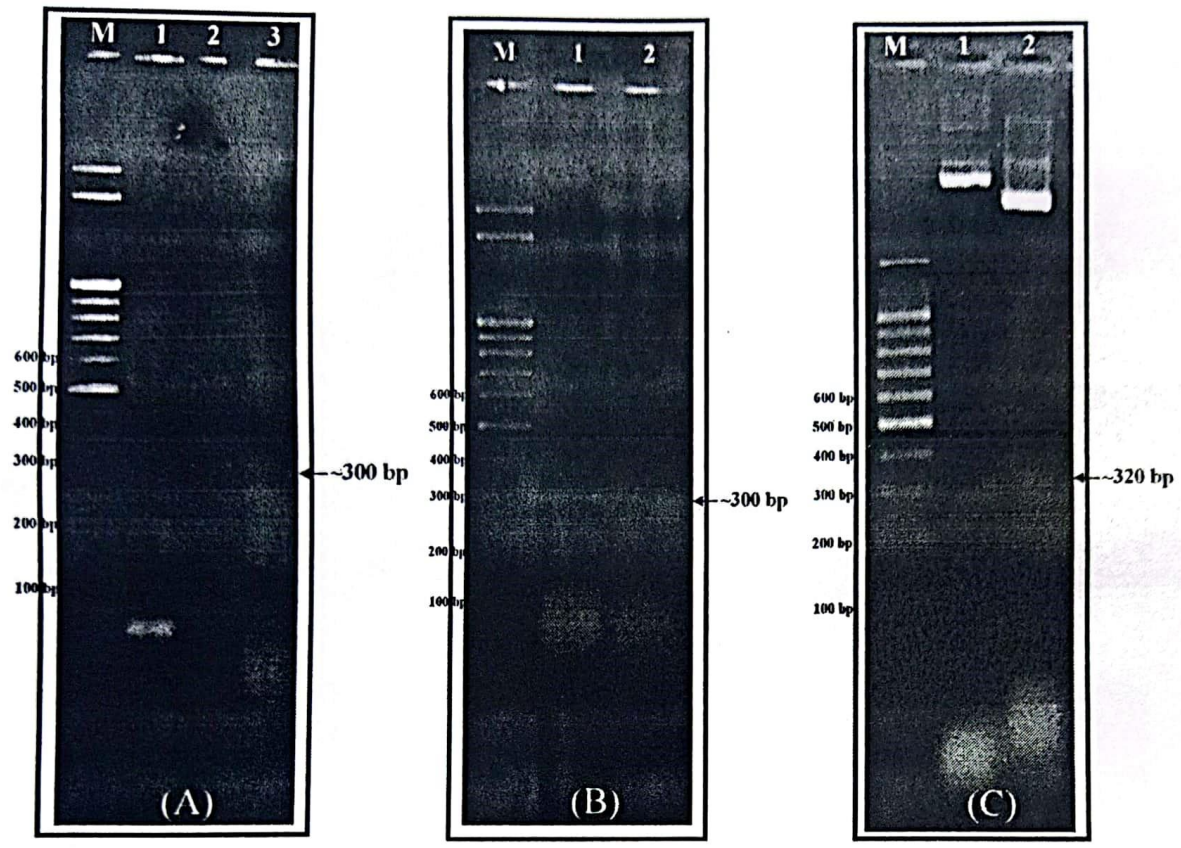

Figure 4. 1.5% agarose gel electrophoresis showing: **A)** RT-PCR products amplified from total RNA extracted from citrus cv. Navel tree appeared gummy bark and grafted Etrog citron infected with Egyptian isolate of HSVd using specific primer Lanes (Lanes 2 & 3) as well as healthy Lane (1). **B)** PCR products of two transformed colonies from the transformation plate (Lanes 1 & 2). **C)** Enzyme digestion of the recombinant plasmids from the cloning of the RT-PCR amplified product where Lane (1): DNA minipreps before digestion and Lane (2): DNA minipreps digested with *Eco*RI. M: DNA molecular weight marker (100 bp ladder) is indicated on the left. Arrow indicates the location of the products.

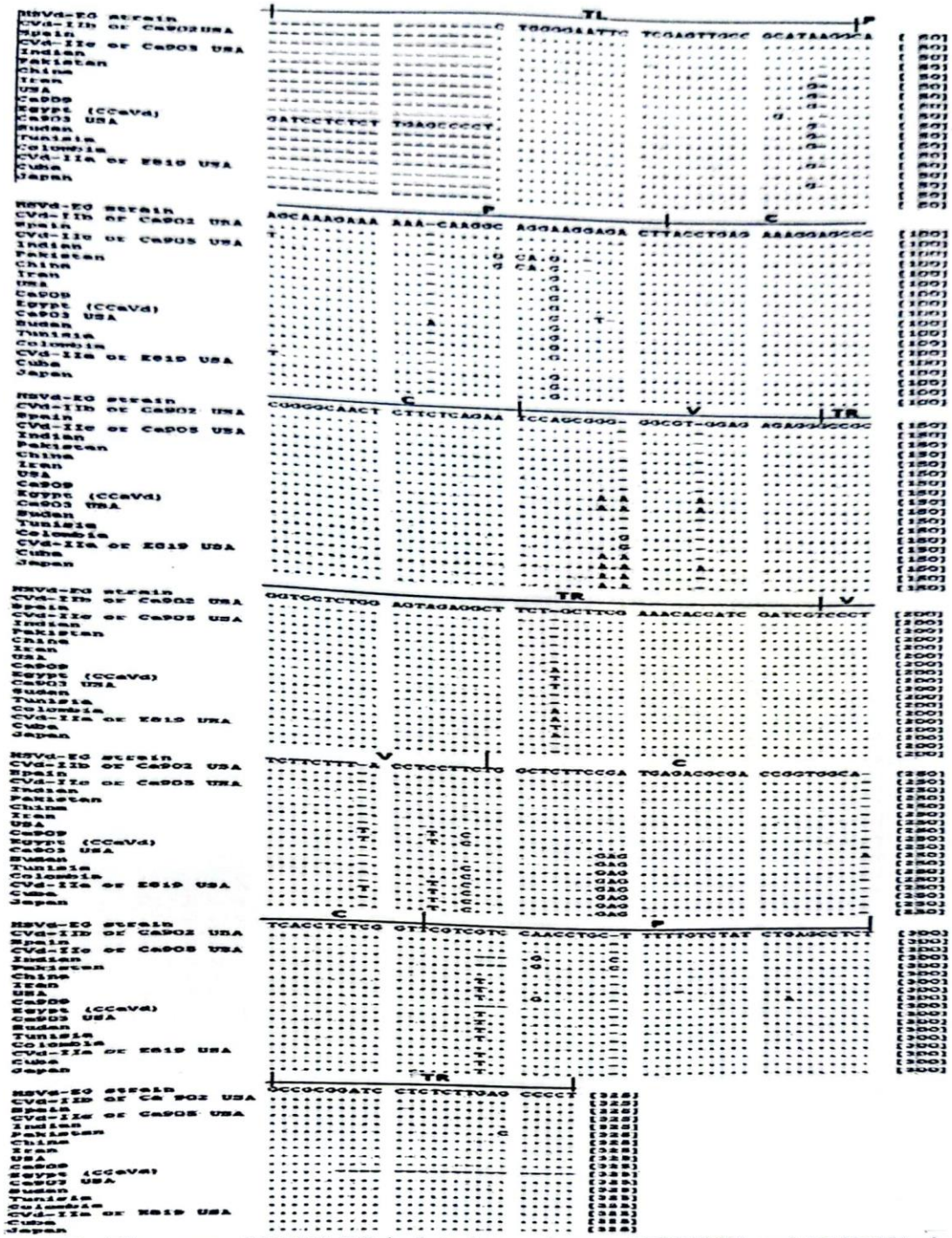

Figure 5. Alignment of HSVd-EG isolate (accession no. FJ984562) and 17 HSVd-citrus sequences using ClustalW program resulting in 325 positions including the gaps. Domains: TL (terminal left), P (pathogenic), C (central), V (variable) and TR (terminal right) (**Keese and Symons, 1985**).

V

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HSVd-EG strain              CAACAGAAG- -CTCGAGAGG  [320]
CVd-IIb or Ca902 USA        .........- -.........  [320]
Spain                       ..T......- -.........  [320]
CVd-IIc or Ca905 USA        ...GCAG..- -......-G..  [320]
Indian                      ...GCAG..- -......-G..  [320]
Pakistan                    .G....G..- -.........C  [320]
China                       .G....G..- -......T...  [320]
Iran                        .G....G..- A.....T...  [320]
USA                         G.....G.AA TTC...TGA.  [320]
Ca909                       .G....GTAA TTC...-...  [320]
Egypt (CCaVd)               .G....G..- -..GAGT..-  [320]
Ca903 USA                   .G....G..- -..GAGT...  [320]
Sudan                       ......G..G A.CGAGT...  [320]
Tunisia                     .G....G..G A.CGAGT...  [320]
Colombia                    ..T.....AA TTCGAG....  [320]
CVd-IIa or E819 USA         .G....G.AA ATCGAGT...  [320]
Cuba                        ......G.AA -TCGAGT...  [320]
Japan                       ......G.AA -TCGAGT...  [320]
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Pi

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HSVd-EG strain              AACAGAG--C TCGAGA [272]
CVd-IIb or Ca902 USA        .......--. ......  [272]
Spain                       .T.....--. ......  [272]
CVd-IIc or Ca905 USA        ..GCAG.--. ....-G  [272]
Indian                      ..GCAG.--. ....-G  [272]
Pakistan                    G....G.--. ......  [272]
China                       G....G.--. ....T.  [272]
Iran                        G....G.-A. ....T.  [272]
USA                         .....GAATT C...TG  [272]
Ca909                       G....GAATT C...-.  [272]
Egypt (CCaVd)               G....G.--. .GAGT.  [272]
Ca903 USA                   G....G.--. .GAGT.  [272]
Sudan                       .....G.GA. CGAGT.  [272]
Tunisia                     G....G.GA. CGAGT.  [272]
Colombia                    .T....AATT CGAG..  [272]
CVd-IIa or E819 USA         G....GAAAT CGAGT.  [272]
Cuba                        .....GAA-T CGAGT.  [272]
Japan                       .....GAA-T CGAGT.  [272]
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S

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HSVd EG strain                    CAGG  [320]
CVd-IIb or Ca902 USA              ....  [320]
Spain                             ....  [320]
CVd-IIc or Ca905 USA              ....  [320]
Indian                            ....  [320]
Pakistan                          ...C  [320]
China                             ....  [320]
Iran                              ....  [320]
USA                               G.A.  [320]
Ca909                             .T..  [320]
Egypt (CCaVd)                     ...-  [320]
Ca903 USA                         ....  [320]
Sudan                             ....  [320]
Tunisia                           ....  [320]
Colombia                          ....  [320]
CVd-IIa or E819 USA               ....  [320]
Cuba                              ....  [320]
Japan                             ....  [320]
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Figure 6. Nucleotide diversity of HSVd-EG strain with 17 HSVd-citrus isolates showing the characteristic changes variable (V), parsimoniously informative nucleotide (Pi) and singleton (S) sites.

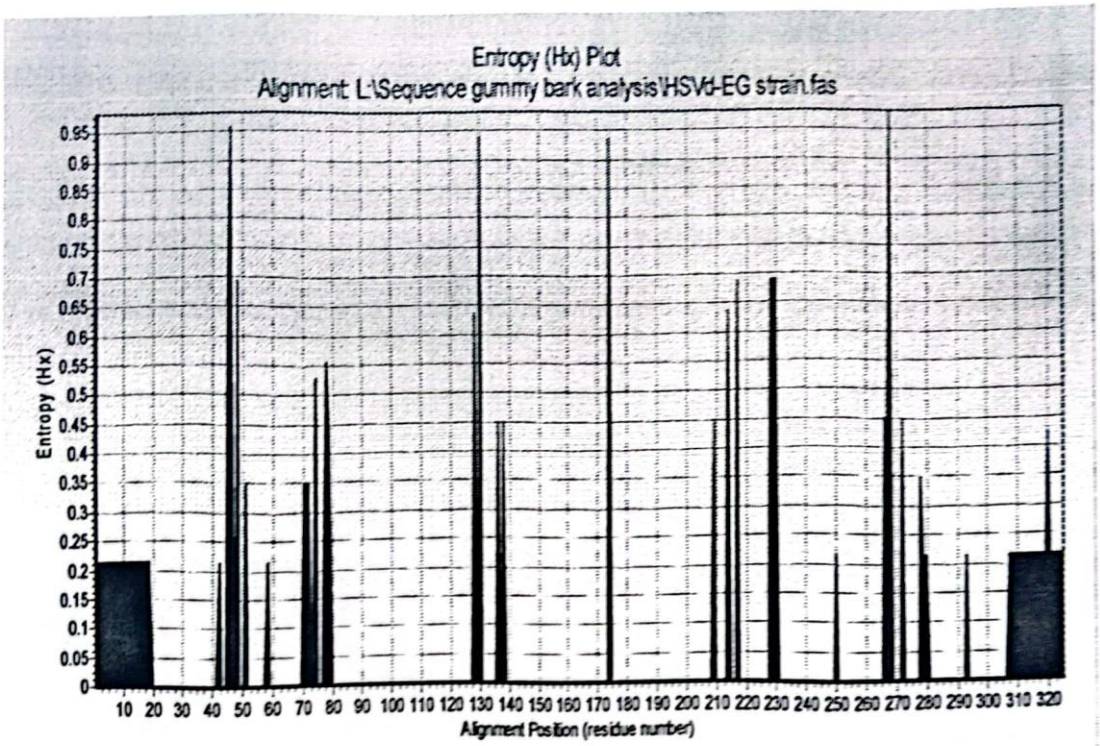

Figure 7. The entropy power result of HSVd-EG and 17 HSVd-citrus isolates by BIOEDIT.

Table 1. Comparison between bases composition of complete genome sequence for HSVd-EG isolate (Accession no. FJ984562) and 17 HSVd- citrus isolates published in GenBank.

Accession No. and isolates	Total (bp.)	Weight (kDa)	Base											
			A		C		G		T		C+G		A+T	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
HSVd-EG strain	299	91.163	61	20.4	87	29.1	82	27.4	69	23.1	169	56.5	130	43.5
CVd-IIb or Ca902	299	91.163	61	20.4	87	29.1	82	27.4	69	23.1	169	56.5	130	43.5
Spain	299	91.138	60	20.1	87	29.1	82	27.4	70	23.4	169	56.5	130	43.5
CVd-IIc or Ca905	295	89.935	58	19.7	87	29.5	82	27.8	68	23.1	169	57.3	126	42.7
Indian	295	89.935	58	19.7	87	29.5	82	27.8	68	23.1	169	57.3	126	42.7
Pakistan	298	90.842	58	19.5	88	29.5	83	27.9	69	23.2	171	57.4	127	42.6
China	298	90.841	58	19.5	87	29.2	83	27.9	70	23.5	170	57	128	43
Iran	298	90.866	59	19.8	87	29.2	83	27.9	69	23.2	170	57	128	43
USA	302	92.051	62	20.5	86	28.5	82	27.2	72	23.8	168	55.6	134	44.4
Ca909	298	90.808	60	20.1	86	28.9	81	27.2	71	23.8	167	56	131	44
Egypt (CCaVd)	300	91.507	60	20	86	28.7	84	28	70	23.3	170	56.7	130	43.3
Ca903	298	90.881	58	19.5	86	28.9	84	28.2	70	23.5	170	57	128	43
Sudan	301	91.821	61	20.3	87	28.9	84	27.9	69	22.9	171	56.8	130	43.2
Tunisia	300	91.524	59	19.7	87	29	85	28.3	69	23	172	57.3	128	42.7
Colombia	301	91.763	62	20.6	86	28.6	82	27.2	71	23.6	168	55.8	133	44.2
CVd-IIa or E819	302	92.092	62	20.5	86	28.5	83	27.5	71	23.5	169	56	133	44
Cuba	300	91.475	62	20.7	86	28.7	82	27.3	70	23.3	168	56	132	44
Japan	300	91.475	62	20.7	86	28.7	82	27.3	70	23.3	168	56	132	44

Table 2. Nucleotide distances and standard error between HSVd-EG strain (gummy bark agent) and 17 HSVd-citrus isolates published in GenBank

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
[1]		*0.000	0.003	0.008	0.008	0.006	0.006	0.006	0.009	0.008	0.009	0.008	0.008	0.009	0.009	0.010	0.009	0.009
[2]	*0.000		0.003	0.008	0.008	0.006	0.006	0.006	0.009	0.008	0.009	0.008	0.008	0.009	0.009	0.010	0.009	0.009
[3]	0.003	0.003		0.008	0.008	0.007	0.007	0.007	0.010	0.009	0.009	0.009	0.009	0.010	0.008	0.011	0.010	0.010
[4]	0.017	0.017	0.021		0.000	0.008	0.007	0.007	0.009	0.010	0.010	0.010	0.010	0.010	0.012	0.012	0.011	0.011
[5]	0.017	0.017	0.021	0.000		0.008	0.007	0.007	0.009	0.010	0.010	0.010	0.010	0.010	0.012	0.012	0.011	0.011
[6]	0.010	0.010	0.014	0.021	0.021		0.005	0.005	0.010	0.007	0.007	0.007	0.009	0.008	0.011	0.009	0.010	0.010
[7]	0.010	0.010	0.014	0.017	0.017	0.007		0.000	0.009	0.007	0.006	0.006	0.008	0.007	0.011	0.008	0.009	0.009
[8]	0.010	0.010	0.014	0.017	0.017	0.007	0.000		0.010	0.008	0.006	0.006	0.008	0.007	0.011	0.008	0.009	0.009
[9]	0.027	0.027	0.031	0.028	0.028	0.031	0.024	0.027		0.007	0.011	0.011	0.010	0.011	0.010	0.009	0.008	0.008
[10]	0.021	0.021	0.024	0.031	0.031	0.017	0.014	0.017	0.017		0.010	0.009	0.010	0.009	0.009	0.007	0.007	0.007
[11]	0.022	0.022	0.026	0.030	0.030	0.014	0.011	0.011	0.037	0.026		0.000	0.005	0.003	0.009	0.006	0.007	0.007
[12]	0.020	0.020	0.024	0.028	0.028	0.017	0.010	0.010	0.034	0.024	0.000		0.005	0.003	0.009	0.006	0.007	0.007
[13]	0.020	0.020	0.024	0.028	0.028	0.024	0.017	0.017	0.034	0.031	0.007	0.007		0.003	0.008	0.007	0.006	0.006
[14]	0.024	0.024	0.027	0.031	0.031	0.020	0.014	0.014	0.038	0.028	0.004	0.003	0.003		0.009	0.006	0.007	0.007
[15]	0.024	0.024	0.020	0.042	0.042	0.034	0.034	0.038	0.031	0.024	0.026	0.024	0.024	0.027		0.007	0.006	0.006
[16]	0.031	0.031	0.034	0.038	0.038	0.027	0.020	0.020	0.027	0.017	0.011	0.010	0.013	0.010	0.017		0.003	0.003
[17]	0.027	0.027	0.031	0.035	0.035	0.031	0.024	0.024	0.020	0.017	0.014	0.014	0.010	0.013	0.010	0.003		0.000
[18]	0.027	0.027	0.031	0.035	0.035	0.031	0.024	0.024	0.020	0.017	0.014	0.014	0.010	0.013	0.010	0.003	0.000	

(*) Distances in lower left and Standard error in upper right (Bootstrap (1000 replicates; seed=17114)).

[1] HSVd-EG_strain, [2] CVd-IIb_or_Ca902_USA, [3] Spain, [4] CVd-IIc_or_Ca905_USA, [5] Indian, [6] Pakistan, [7] China,

[8] Iran, [9] USA, [10] Ca909_USA, [11] Egypt_(CCaVd), [12] Ca903_USA, [13] Sudan, [14] Tunisia, [15] Colombia, [16] CVd-IIa_or_E819_USA, [17] Cuba and [18] Japan.

Table 3. The entropy power data of the nucleotide variable positions of HSVd-EG and 17 HSVd-citrus isolates by BIOEDIT.

Position	Entropy data	Position	Entropy data
1	٠,٢١٤٥٦	174	٠,٩٣٣٨٢
2	٠,٢١٤٥٦	209	٠,٤٥٠٥٦
3	٠,٢١٤٥٦	214	٠,٦٣٦٥١
4	٠,٢١٤٥٦	217	٠,٦٨٦٩٦
5	٠,٢١٤٥٦	228	٠,٦٨٦٩٦
6	٠,٢١٤٥٦	229	٠,٦٨٦٩٦
7	٠,٢١٤٥٦	230	٠,٦٨٦٩٦
8	٠,٢١٤٥٦	250	٠,٢١٤٥٦
9	٠,٢١٤٥٦	266	٠,٤٥٠٥٦
10	٠,٢١٤٥٦	267	٠,٤٥٠٥٦
11	٠,٢١٤٥٦	268	٠,٩٨٠٩٩
12	٠,٢١٤٥٦	272	٠,٤٥٠٥٦
13	٠,٢١٤٥٦	278	٠,٣٤٨٨٣
14	٠,٢١٤٥٦	279	٠,٢١٤٥٦
15	٠,٢١٤٥٦	280	٠,٢١٤٥٦
16	٠,٢١٤٥٦	293	٠,٢١٤٥٦
17	٠,٢١٤٥٦	307	٠,٢١٤٥٦
18	٠,٢١٤٥٦	308	٠,٢١٤٥٦
19	٠,٢١٤٥٦	309	٠,٢١٤٥٦
42	٠,٢١٤٥٦	310	٠,٢١٤٥٦
45	٠,٦٨٦٩٦	311	٠,٢١٤٥٦
46	٠,٩٥٨٠٠	312	٠,٢١٤٥٦
48	٠,٦٩٣١٥	313	٠,٢١٤٥٦
51	٠,٣٤٨٨٣	314	٠,٢١٤٥٦
58	٠,٢١٤٥٦	315	٠,٢١٤٥٦
70	٠,٣٤٨٨٣	316	٠,٢١٤٥٦
71	٠,٣٤٨٨٣	317	٠,٢١٤٥٦
72	٠,٣٤٨٨٣	318	٠,٢١٤٥٦
74	٠,٥٢٩٧١	319	٠,٢١٤٥٦
77	٠,٣٤٨٨٣	320	٠,٤٢٥٨٥
78	٠,٥٥٦٦٥	321	٠,٢١٤٥٦
79	٠,٤٥٠٥٦	322	٠,٢١٤٥٦
128	٠,٦٣٦٥١	323	٠,٢١٤٥٦
130	٠,٩٣٦٨٩	324	٠,٢١٤٥٦
136	٠,٤٥٠٥٦	325	٠,٢١٤٥٦

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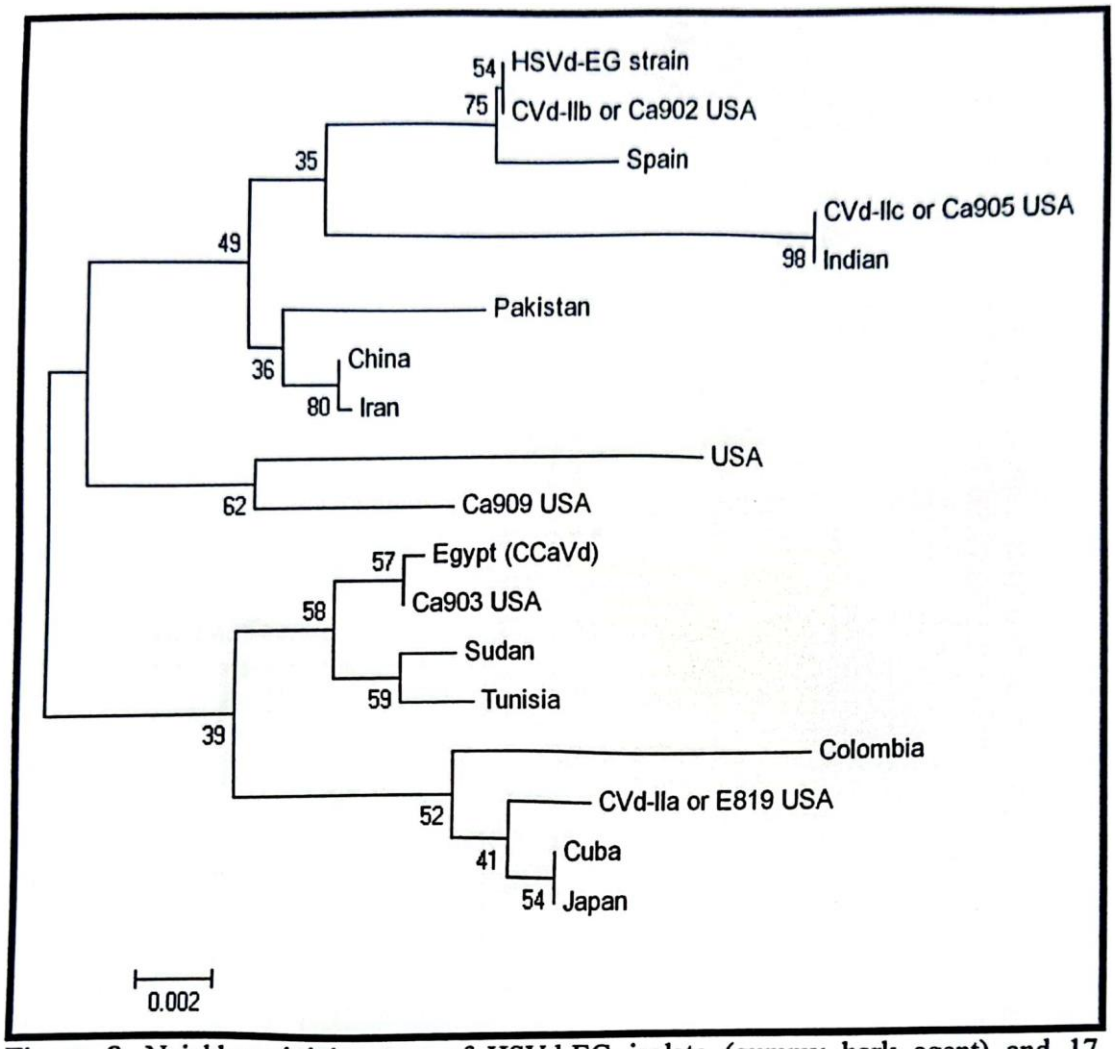

Figure 8. Neighbour-joining tree of HSVd-EG isolate (gummy bark agent) and 17 isolates of HSVd-citrus published in GenBank. Numbers represent bootstrap percentage values based on 1000 replicates.

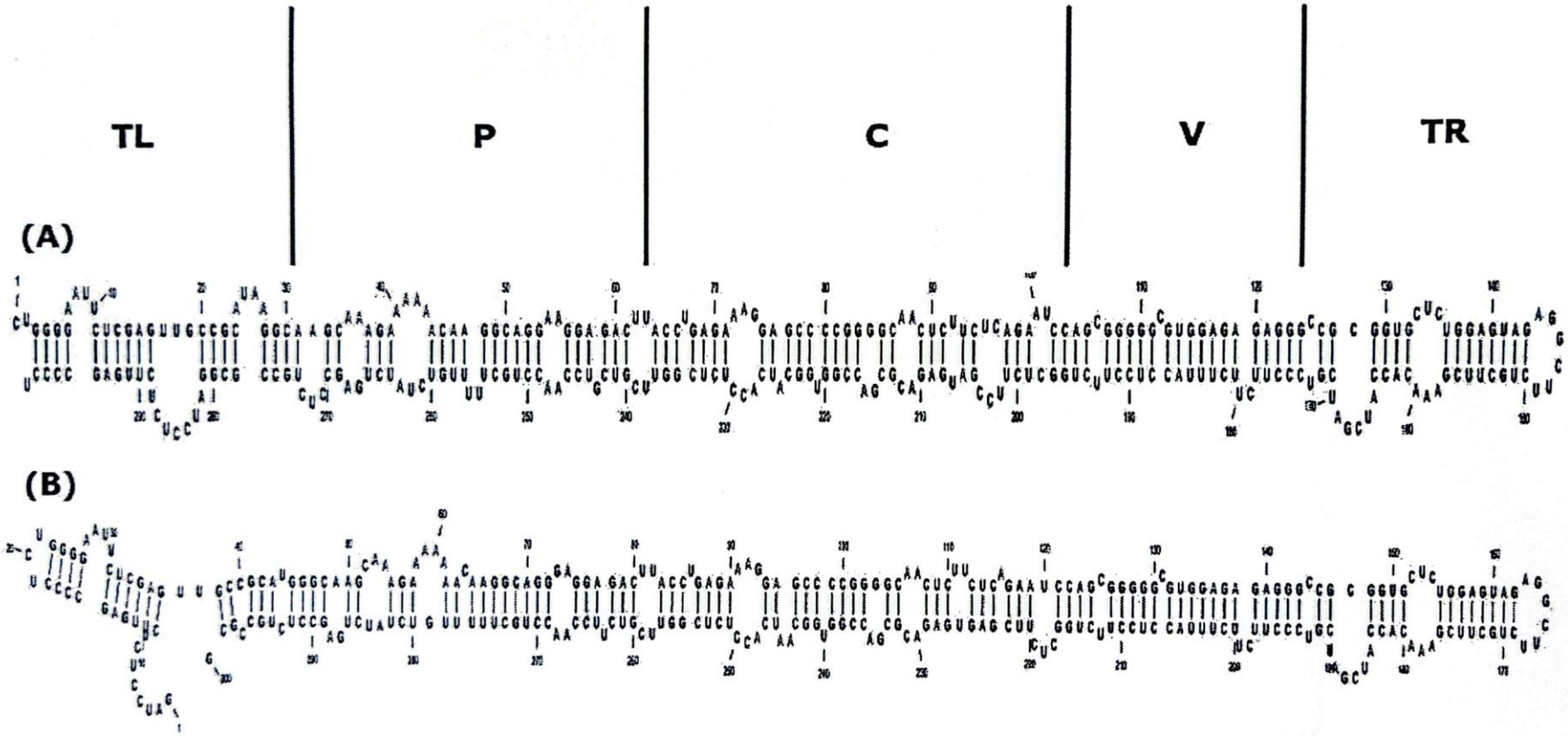

Figure 9. The secondary structure of A) HSVd-EG-RNA (gummy bark agent) and B) CCaVd-EG-RNA (cachexia agent) is rod-shaped structure. Domains: TL (terminal left), P (pathogenic), C (central), V (variable) and TR (terminal right) (Keese and Symons, 1985).

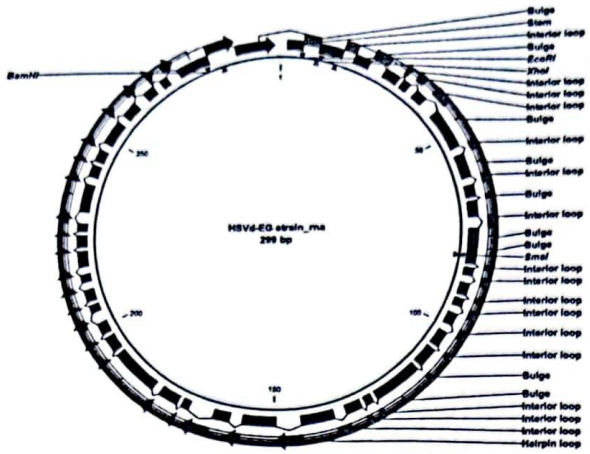

Figure 10. Diagrame illustrated distribution of secondary structure on circular HSVd-EG-RNA.

Table 4: Thermodynamic of a secondary structure for HSVd-EG-RNA.

Structural element		Information	ΔG°37	Structural element		Information	ΔG°37
Stacks	1	G1-C299	-3.30	Stacks	35	G75-C227	-2.40
	2	G4-C297	-3.30		36	G77-C224	-3.40
	3	G6-C296	-3.30		37	C78-G223	-3.30
	4	C11-G294	-2.10		38	C80-G220	-2.10
	5	U12-A293	-2.40		39	C81-G219	-2.40
	6	C17-G291	-1.40		40	G82-C218	-3.40
	7	G18-U290	-1.30		41	G84-C214	-3.40
	8	A19-U289	-2.10		42	C86-G213	-2.10
	9	C20-G288	-3.30		43	U90-A209	-2.40
	10	C21-G287	-2.40		44	U92-A206	-2.40
	11	G23-C285	-3.40		45	A97-U203	-2.10
	12	G25-C277	-3.30		46	G98-C200	-3.40
	13	G26-C276	-3.40		47	C102-G197	-3.30
	14	C30-G275	-2.10		48	C103-G196	-2.10
	15	G31-C270	-3.40		49	A104-U193	-2.10
	16	A37-U268	-2.10		50	G107-U192	-3.30
	17	G38-C267	-3.40		51	G108-C191	-3.30
	18	A44-U260	-2.20		52	C109-C190	-1.50
	19	C45-G259	-2.10		53	G110-U189	-2.10
	20	A46-U258	-0.90		54	G113-C187	-2.20
	21	G47-U257	-2.10		55	U114-A186	-2.10
	22	G48-C233	-3.40		56	G115-U185	-0.50
	23	C50-G231	-2.10		57	G116-U184	-1.30
	24	A51-U230	-2.10		58	A117-U183	-2.10
	25	G53-C280	-3.30		59	G118-C182	-2.40
	26	G55-C246	-3.30		60	G120-U178	-1.30
	27	G57-C245	-3.40		61	A122-U177	-2.10
	28	G58-C244	-3.40		62	G123-C176	-3.30
	29	A60-U241	-2.20		63	G125-C175	-3.40
	30	A64-U237	-2.20		64	C128-G175	-2.40
	31	G65-C236	-3.30		65	G129-C144	-2.30
	32	G68-C234	-2.40		66	G130-C143	-2.30
	33	A69-U233	-2.10		67	U131-A162	-2.10
	34	G70-C232	-2.40		68	U136-G157	-1.40
Stacks	69	G137-C161	-1.50				
	70	G138-C160	-3.40				
	71	A139-U159	-2.10				
	72	G140-C158	-3.40				
	73	C141-G157	-2.10				
	74	A142-U156	-2.10				
Bulge loops	1	G5-C295	3.60				
	2	C31-G276	3.60				
	3	C37-U267	3.80				
	4	A39-U266	3.70				
	5	A52-U255	3.70				
	6	A79-U236	3.60				
	7	C81-G235	3.60				
	8	C111-C188	0.60				
	9	A150-C181	3.80				
Interior loops	1	G15-C289	3.70				
	2	A21-U274	3.40				
	3	C24-G265	1.00				
	4	A28-U262	4.10				
	5	G32-C249	0.10				
	6	C34-G248	3.70				
	7	A35-U247	3.60				
	8	G63-C239	0.80				
	9	U66-G238	0.60				
	10	U91-G208	3.00				
	11	C94-G205	3.00				
	12	A98-U199	3.00				
	13	G100-C198	0.10				
	14	G124-C174	0.10				
	15	G127-C171	4.70				
	16	G131-C161	2.70				
External loop	1	Dangling U at 299, dangling from position 298	-1.20				
Hairpin loop	1	G1-C150	3.30				
Total			-122.10				

DISCUSSION

The etiology of gummy bark disease is unknown, but viroids have been suggested as the causal agent. sPAGE and RT-PCR results indicated that, CVd-II could be associated with gummy bark disease as reported by **Önelge *et al.* (2004).** This study supported evidence that, the viroid associated with this disease was identified through biological and molecular characters as well as bioinformatics analysis.

Source material of this study (HSVd-EG) was isolated and propagated on healthy citrus seedlings Sour orange and Etrog citron by grafting inoculation. It was kept under greenhouse conditions 32-40°C (max. day) / 27-30°C (min. night) as reported by **Roistacher (1991); Duran-Vila *et al.* (1993); El-Dougdoug *et al.* (2010).** These results were confirmed by dot-blot hybridization using specific HSVd Dig-labeled cDNA probe. HSVd-EG isolate that was indexed by graft inoculation on Etrog citron plants gave petiole wrinkle and midvein browning symptoms appeared after 12-16 weeks. On the contrary, **Duran-Vila *et al.* (1988); Semancik *et al.* (1988)** reported that the cachexia-xyloprorosis agents CVd-IIb and CVd-IIc are not symptomless on Etrog citron. On the other hand, **Duran-Vila *et al.* (1993)** reported that CVd-IIb and CVd-IIc induce mild symptoms in 6-9 months as incubation periods and 3 months at 23-32°C or 7 months at 18-25°C are necessary for the detection of all citrus viroids.

The HSVd-EG isolate was transmitted through grafting by eye and blind buds from naturally infected citrus cv. Navel trees to healthy Sour orange and Etrog citron plants where **Nour-Eldin (1968); Roistacher (1991); Önelge *et al.* (1996)** reported that the causal agent of citrus gummy bark disease has been transmitted experimentally by bud inoculation from infected trees to sweet orange; however, symptoms are visible only after an incubation period of 5–10 years.

HSVd-EG-RNA was amplified by PCR after adding two primers oligo (dt) which amplify ~300 base fragment of RNA (Fig. 4-A and 4-C) and this is in harmony with **Reanwarakorn and Semancik (1998); El-Dougdoug *et al.* (2010).** Sequence of HSVd-EG isolate is 299 nucleotides in length as reported by Sofy *et al.* (2010) where submitted to GenBank under accession No.

FJ984562. The nucleotide sequence of complete genome for HSVd-EG strain (Accession no. FJ984562) revealed the highest content for Cytosine (C) 87 (29.1%) followed by Guanine (G) 82 (27.4%), then Thymine (T) 69 (23.1%), and Adenine (A) 61 (20.4%) (Table 1).

Genetic diversity and evaluation of entropy power for the Egyptian citrus gummy bark agent (Accession no. FJ984562) and HSVd-citrus populations registered in GenBank, were viewed against the phylogenetic background of known CVd-II variants including the non-cachexia (CVd-IIa) and the causal agents of severe (CVd-IIb, CVd-IIc), more moderate (Ca903) and mild (Ca909) cachexia (Fig. 7 and 8) (Table 3) as reported by Reanwarakorn and Semancik (1999). The relationships between CVd-II related clone recovered from the citrus gummy bark symptomatic tree and HSVd-citrus sequence registered in GenBank were determined by nucleotide sequence homology (**Figure 5 and 6**). Phylogenetic and molecular evolutionary analyses were conducted using MEGA software version MEGA 4.0 (**Tamura *et al.*, 2007**) (**Figure 8**). The minimum evolution based distance method, Neighbor Joining (NJ), was utilized (**Table 2**) due to limited number of parsimonious informative sites of the relatively small and conserved genomes of CVd-II variants. The Jukes–Cantor estimate the number of nucleotide substitutions per site (d) ranging between $0 \leq d \geq 0.042$. It is supporting the use of p-distance model for the Neighbor Joining (NJ) method. Since the viroid-sequence alignment produces series of distinct insertion/deletion events, the gap sites were included in the phylogenetic analysis (Fig. 8). That could be done using the pairwise-deletion option as well as Bootstrap analysis (1000 replicates) which assess reliability of the constructed phylogenetic tree.

Egyptian gummy bark agent (Accession no. FJ984562; Sofy *et al.*, 2010), is distinctly defined from the non-cachexia variant (CVd-IIa) as well as from the *Citrus cachexia viroid* Egyptian isolate (Accession no. AY513267; **Amin, 1999**). This is supported by the Jukes–Cantor method (**Jukes and Cantor, 1969**) where the genetic distance calculated between Egyptian gummy bark agent (HSVd-EG) and CVd-IIb (d=0), CVd-IIc (d=0.017), Ca903 (d=0.020), Ca909 (d=0.021),

CCaVd-EG (d=0.022) as well as with the non-cachexia CVd-IIa (d=0.031) variant. The relationship between HSVd-EG and CVd-IIb as determined by the estimated genetic difference is identical to the value between the two cachexia variants CCaVd-EG and Ca903-USA (**Table 2 and Figure 8).**

The minimum free energy of a secondary structure for HSVd-EG-RNA was determined from its primary sequence by summing the energy contribution of all base pairs, interior loops, hairpin loop, bulge loops and external loop at 37°C by MFOLD analysis, since this is the temperature at which viroids symptoms are consistently observed by **Roistacher (1991); Duran-Vila *et al.* (1993)**. The HSVd-EG sequence appeared to fold into a rod-like structure with the minimum free energy -122.1 kcal mol^{-1} while CCaVd-EG with the minimum free energy -120.5 kcal mol^{-1}. Serra *et al.* (1995) mentioned that RNA molecule folds into that structure with the minimum free energy (mfe). Free energy models typically assume that the total free energy of a given secondary structure for a molecule is the sum of independent contributions of adjacent, or stacked, base pairs in stems (which tend to stabilize the structure) and of loops (which tend to destabilize the structure).

Symptoms of citrus gummy bark, a disease specific to sweet orange (**Nour-Eldin, 1956; 1959**) bear similarities to cachexia, a disease specific to mandarins and tangelos (**Childs, 1952**) caused by variants of the *Hop stunt viroid* (HSVd) related CVd-II (**Semancik *et al.*, 1988).** Thus, it has been suggested that a variant of this viroid may also be either the causal agent or a factor directly involved in citrus gummy bark disease expression (**Önelge *et al.* 2004).** In addition, sequence analyses of the Egyptian gummy bark agent and CVd-IIb (Ca902) strain did not reveal any common nucleotide change or unusual mutations that might suggest a relationship to CVd-II with citrus gummy bark disease. While, the variant CVd-IIa does not induce any severe disease symptoms, but bark cracking on *Poncirus trifoliata* rootstock has been attributed to CVd-IIa (**Roistacher *et al.*, 1993**). This condition does not result in economic damage to trees or crop but to the contrary sweet orange with CVd-IIa exhibit an enhancement in commercial performance (**Semancik *et al.*,**

2002). So, a case can be made for all CVd-II except CVd-IIa as the most probable agent in support of a viroid etiology for citrus gummy bark disease.

The previous data indicates that the high frequency of detection of Egyptian gummy bark agent as the principal CVd-II-related variant found in symptomatic Navel sweet orange and not found in asymptomatic trees suggests a possible relationship between that variant and citrus gummy bark but no highly distinct cachexia-like variant could be identified as a specific gummy bark disease inducing agent. With the virtual identical genome to CVd-IIb, the gummy bark symptom may constitute an expression of cachexia disease in sweet orange. But the cachexia disease symptoms which reported by **Reichert and Perlberger (1934)**. Moreover, the Egyptian gummy bark agent differ from Egyptian isolate of *Citrus cachexia viroid* infecting mandarin in Egypt (**Amin, 1999**). So, we suggest for this Egyptian strain, the name of *Citrus gummy bark viroid* cachexia strain with taxon identifier 648750 and scientific name *Citrus gummy bark viroid* related to family *Pospiviroidae*, genus *Hostuviroid* and species *Hop stunt viroid* http://www.uniprot.org/taxonomy/648750.

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