

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC STUDIES OF COMPONENT SIX OF BANANA BUNCHY TOP BABUVIRUS (BBTV DNA-6)

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Banana and plantations (*Musa sp.*) are the most fourth most important global food crop after rice, wheat and maize (Piertersen and Thomas, 2001). Banana plantations are subjected to various natural climates but diseases; in particular, viral diseases constitute a major setback to this crop worldwide. Among viral infections, Banana Bunchy Top Virus (BBTV) is the most serious virus affecting banana in Egypt as well as the world (Dale, 1987; Othman *et al.*, 1996; Harish *et al.*, 2008) and is responsible for the massive reduction in banana crop yield. The symptoms of BBTD are yellowing, dark green streaks on midrib, petioles, extending down into pseudostem, dark green dots and dashes along the minor leaf veins, stunted growth with bunchy top appearance (Magee, 1940; Vetten *et al.*, 2005). BBTV belong to genus Babuvirus and family Nanoviridae with isometric particles of 18-20 nm in diameter. It is naturally transmitted, in persistence manner, by banana aphid *Pentalonia nigronervosa* (Hu *et al.*, 1996). BBTV is identi-

fied as a phloem-limited virus used to occur in low concentration in banana-infected plants (Harding *et al.*, 1991 and 2000). The virus has a single coat protein with molecular weight (Mr.) of about 20-21kDa (Dietzgen and Thomas, 1991; Harding *et al.*, 1991; Sadik *et al.*, 1999).

The viron consists of at least six components of circular ssDNA each component of the BBTV genome contains one large (monocistronic) transcriptional active open reading frame (ORF) except BBTV DNA 1 contains two ORFs one master replication protein (Rep) ORF (Hafner *et al.*, 1997) and a small ORF internal to major ORF (Beetham *et al.*, 1997). Additionally, two conserved region: stem-loop common region (CR-SL) and major common region (CR-M), potential TATA box at 3' of the stem-loop and polyadenylation signal (Burns *et al.*, 1995; Beetham *et al.*, 1997 and 1999).

The nucleotide sequence of the component six of Australian isolate BBTV

has 1089 nt. (Burns *et al.*, 1995). The encoded protein of BBTV DNA-6 is a nuclear shuttle protein and is preferentially targeted to the nucleus when expressed alone however in the presence of the movement protein it targeted to the cell periphery (Wanitchakorn *et al.*, 2000a).

The aim of this study is to amplify, clone and sequence the full length of BBTV DNA-6 and compare it with other published sequences of the corresponding sequence.

MATERIALS AND METHODS

Virus source

Infected banana plants were collected from El-Behera Governorate (BBTV-B) in Egypt. The presence of the virus was confirmed serologically by using indirect enzyme-linked immunosorbant assay (I-ELISA) as described by Wu and Su (1990).

Genomic DNA extraction from infected banana plants

The total DNA was extracted from the infected banana midrib by using DNeasy plant mini kit (cat. No. 69104 from QIAGEN).

PCR amplification of the full length of BBTV DNA-6

Two oligonucleotide primers were designed to amplify the full length of BBTV DNA-6, according to the published data of Karan *et al.* (1997) as the following:

Forward primer (FDNA-6)

5'**GACAAGAAGGATCCTATTAGTAA**
CAGCAACA3'

Reverse primer (RDNA-6):

5'ATAAAGCTTCTAACTTCCATGTCT
CT3'

The nucleotides in bold represent *Bam*H1 site in the forward primer and *Hind* III in the reverse primer.

One hundred nanograms of the extracted DNA, from infected banana plants, were used as a template in the PCR reaction. PCR was conducted in a volume of 50 µl containing 1X reaction buffer with MgSO₄, 200 µM each dNTPs, 0.4 pmoles of each primers and 0.025 units of *pfu* DNA polymerase (Promega). PCR amplification was performed in a Perkin-Elmer thermocycler (Gene Amp PCR system 2400) for 35 cycles after initial denaturation for 2 min at 95°C. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min. The primer extension was extended to 7 min at 72°C in the final cycle. The PCR amplified products were detected by agarose gel electrophoresis (1.2%) at 80 volts for 1 hour (hr) (Sambrook *et al.*, 1989).

Cloning the full length of the Egyptian BBTV DNA-6

The PCR fragment was tailed with adenine (A) to be ligated in pJET1.2\blunt vector (2974 bp) (Fermentas) by incubat-

ing 25 µl from the preheated (95°C/20 min) PCR product with 7.5 µl dATP (from a 2mM stock), 1.5 µl MgCl₂ (from a stock 50 mM) and 1 µl (0.5 Unit) of *Taq* DNA polymerase at 70°C for 15 min. The A-tailed PCR fragment was ligated into the pJET1.2/blunt vector to create plasmid pJETH. Competent cells of *E. coli* (Top 10 strain) were prepared as mentioned by Hammond and Hammond (1989) and then transformed with the constructed plasmid. The recombinant plasmid DNA was tested by double digestion with *Bam* HI and *Hind* III (Fermentas) as a confirmatory test.

Sequencing and computer analysis of the full length of BBTV DNA-6

A recombinant clone (pJETH) was sequenced using Genetic Analyzer at AGERI, Giza, Egypt, ARC (ABI prism 310, version 3.4, Semi Adaptive, version 3.2). DNA sequence was translated to protein using EditSeq of DNA star software program. DNA and protein sequences of the BBTV-B (Egy6m) full length were aligned and compared using MegaAlign of DNA star program with eleven isolates.

RESULTS AND DISCUSSION

PCR amplification of the full length of BBTV DNA-6

By using two specific primers for the full length of BBTV DNA-6, 1.1 Kbp fragment was detected in the BBTV-infected banana plants. This fragment was

absent in the healthy plant as a negative control (Fig. 1). Burns *et al.* (1995) and Islam *et al.* (2010) reported that the full length of BBTV DNA-6 was 1089 bp.

Recently, the molecular detection of plant viruses is now available and the PCR was successfully used for detecting several plant viruses (Wetzel *et al.*, 1992; Hadidi *et al.*, 1993) and BBTV (Xie *et al.*, 1994; Harding *et al.*, 2000). Therefore, the early detection of BBTV particular in the symptomless banana plant materials produced *via* tissue culture and the BBTV in banana samples showed the characteristic symptoms of BBTV and also in banana aphid could be detected.

Harding *et al.* (2000) and Abdel-Hamid *et al.* (2003) reported the importance of BBTV detection as early step for virus control, in particular, in the plants produce *via* tissue culture as well as symptomless plants.

Cloning the Egyptian BBTV DNA-6 full length

The purified A-tailed PCR product, which represent the full length of Egyptian BBTV DNA-6 was cloned into pJET1.2/blunt vector to create the plasmid pJETH. After mini-preparation, the purified plasmid DNA was subjected to double digestion with *Bam*HI and *Hind*III enzymes as a confirmatory test. A segment of about 1.1 Kbp was released from all recombinant plasmids (Fig. 2). The success of cloning process was confirmed by using molecular techniques as reported by Wanitchakorn *et al.* (1997).

Sequencing and computer analysis of Egyptian BBTV DNA-6 full length

The sequence analysis of the amplified product revealed that the full length of BBTV DNA-6 (Fig. 3). It contains a sequence of 61 nt sequence which represented the stem loop common region (CR-SL) in which a stem loop structure consists of 31 nt (from position 1-31) as show in Fig. (4), Burns *et al.* (1995) deduced that this sequence was a highly conserved in all BBTV components and contained the pentanucleotide sequence TACCC which has been shown to be the site for initiation of viral strand DNA synthesis.

The second common region, which is located at 5' of the (CR-SL), is called the major common region (CR-M) at the position (801-891) and it consists of 91 nt. In this region, from nts 4 to 19 and 21 to 36, an almost complete 16 nt of direct repeat (ATACAAC/gACg/aCTATa/gA) were observed. Further a 15 nts GC-rich sequence (average of 86.6% G+C) was located from nts 77 to 91, this result came in harmony with Burns *et al.* (1995). The presence of the GC-rich region in the CR-M make it believed to resemble the SP1 binding sites found in promoters of genes in animal cells and viruses (Fenoll *et al.*, 1990). Hence, it was assumed to be the binding site for DNA primers associated with complementary strand synthesis (Hafner *et al.*, 1995; Su *et al.*, 2003). A potential TATA box of nine nucleotide sequence was identified and was located downstream from the stem-loop sequence

in a distance of 177 nt with the nine nucleotide sequence (CTATTAATA). Alberts *et al.* (1994) stated that this box is found in the gene promoters from 20 to 60 bp before the start of transcription, and any removing or altering in this sequence markedly reduces the promotor activity. After this box by 46 bases, one large ORF was identified which started with the translation initiation codon ATG at position 277 to 741 in the virion sense of 465 nts in size. In the Egyptian BBTV DNA-6, two potential polyadenylation signals were observed associated with the 3' end of the major ORF. A GT-rich region contained the trinucleotide TTG was located after this polyadenylation signal. Similarly found by Burns *et al.* (1995) who stated that the combination of a polyadenylation signal and GT-rich region contained the trinucleotide TTG may be required for efficient termination. No other ORFs were identified in the component in either the virion or complementary sense which potentially encoded proteins greater than 10 KDa and had appropriately located potential TATA boxes and polyadenylation signals suggesting that this component encodes a single gene. Burns *et al.* (1995) reported that the full length of BBTV DNA-6 of the Australian isolate had one ORF that was associated with the potential TATA box and a polyadenylation signal and this assumption was confirmed by Beetham *et al.* (1999).

The sequenced full length of Egyptian BBTV DNA-6 was compared with the nucleotide sequences of eleven different geographical isolates of BBTV DNA-

6. From the phylogenetic analysis based on the nucleotide sequences of BBTV DNA-6 full length and the eleven isolates, the tree was divided into two clusters. The first cluster represents Asian group and included the isolate from China and Taiwan. The second cluster represents the South Pacific group including Indian, Australian, Pakistan and Egy 6m (in this study) isolates (Fig. 5).

The comparative analysis revealed the presence of high degree of homology between the studied isolate (Egy 6m) and Pakistan (isolate TJ1) and India isolates (isolates 1, 2 and 3), the percent of identities were 98.4, 97.7, 97.3% and 97.3%, respectively. However, the lowest identity was obtained in comparison with China 3 isolate percent of identity 78.9% as shown in Table (1).

Karan *et al.* (1994 and 1997) and Wanitchakorn *et al.* (2000b) reported that there were two groups of BBTV isolates, Asian and south Pacific groups based on sequence variation in DNA-1, DNA-6 and DNA-3, respectively.

As mentioned before the DNA-6 of the Egyptian isolate of BBTV was found to have one large ORF. The predicted protein of this ORF was consisted of 154 amino acids and had a M.wt of 17.39 KDa.. Burns *et al.* (1995) reported the same results.

The Phylogenetic analysis of BBTV DNA-6 full length, based on the amino acid sequences, of the Egyptian isolate (in this isolate) with the eleven

different isolates resulted in the presence of two clusters. The first group was the Asian group which enclosed the isolates from China and Taiwan and the other group which contained the south pacific group (isolates from Australia, India and Pakistan). The Egyptian isolate was found to be grouped in the south pacific group as shown in Fig. (6).

The comparative analysis (based on the amino acid sequences) showed the highest degree of identity between the Egyptian isolate (Egy 6m) with Australia, India (isolates 4 and 5) and Pakistan TJ1 with a percent of identities 99.4%.

The results confirm the existence of two groups of BBTV isolates (Asian and South pacific groups) based on the nucleotide and amino acid sequences of full length of DNA-6 and the Egyptian isolate belong to the south pacific group.

The two oligonucleotides belonging to the BBTV component-6 which synthesized in this study can use as specific primers in BBTV detection. The PCR technique is play an important role in BBTV detection and could be introduced as an early step for virus control especially in tissue culture stage which the existence of virus used to occur in low concentration.

SUMMARY

Banana bunchy top babuvirus, the causal agent of banana bunchy top disease (BBTD), is the most serious viruses infecting banana all over the world as well as

in Egypt. In this study, component 6 (DNA-6) of Banana bunchy top virus (BBTV) encoding a protein of 17.39 KDa was investigated. The full length of BBTV DNA-6 was amplified *via* polymerase chain reaction (PCR), cloned into the pJET1.2/blunt vector and sequenced. Results showed that the size of the full length of BBTV DNA-6 was 1087 nucleotides (nts) with one open reading frame (ORF) and its molecular length was 465 nt. The full length nucleotide sequences for DNA-6 of the Egyptian isolate (Egy6m) were aligned with eleven overseas isolates (three isolates from China, two from Pakistan, one from Taiwan, four from India and one from Australia). Results showed that the isolates were clustered into two groups, the first cluster called South Pacific group that includes the Egyptian, Indian, Australian and isolates from Pakistan. The second cluster called Asian group that contained isolates from Taiwan and China. The Egyptian DNA-6 (Egy 6m) was found to be grouped in the south pacific group with high similarity with isolate TJ1 from Pakistan based on the level of the nucleotide sequence and the amino acid sequence of the deduced protein.

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Table (1): Sequence distance between the studied isolate of BBTV DNA-1 (A) and the other published eleven sequences based on the nucleotide (nt) and amino acids sequences (aa).

BBTV groups	Overseas BBTV isolates			% Identities	
	Isolates	Accession #	Length	Nt	aa
Asian	China 1	EF470243	1080	79.9	93.5
	China 2 (Hainan 571101)	AY494787	1081	81.9	91.6
	China 3 (Haikou)	FJ463047	1082	78.9	91.6
	Taiwan	DQ826396	1086	79.5	91.7
South Pacific	Pakistan (TJ1)	EF529519	1088	98.4	99.4
	Pakistan 2 (clone CP IM 14)	AM418568	1096	97.1	97.4
	Australia	L41577	1089	95.1	99.4
	India 1	EU190970	1087	97.7	98.7
	India 2(BBTV-LKO)	EU391633	1096	97.3	96.8
	India 3 (Bihar)	FJ609644	1090	97.3	96.1
	India 4 (BT-1)	AY948438	1080	91.1	99.4

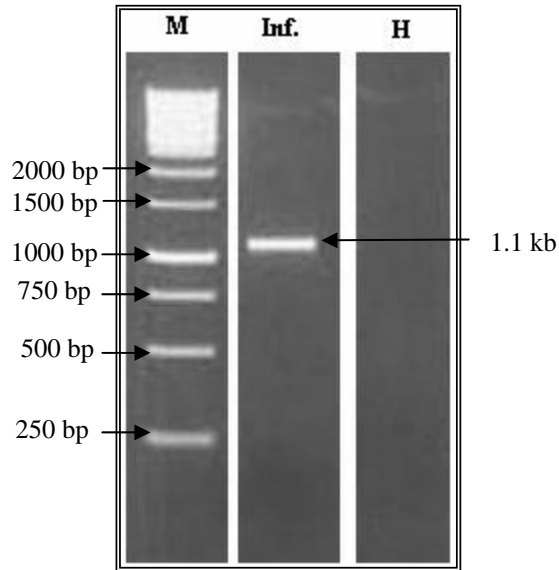


Fig. (1): PCR amplification of the full length of EgyptianS BBTV DNA-6. M: 1Kb DNA marker. Inf: infected banana tissue and H: healthy banana tissue.

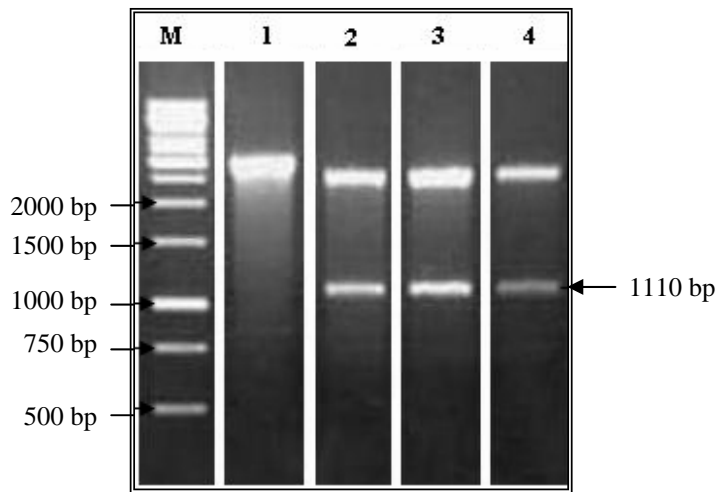


Fig. (2): Digestion of mini-prepared plasmid DNA from recombinant plasmid with *Bam*H1 and *Hind* III. M: 1 kb DNA marker, 1: non-recombinant plasmid. 2, 3 and 4: recombinant plasmids.

<u>AGCACGGGGG</u>	<u>ACTATTATTA</u>	<u>CCCCCGTGC</u>	<u>TCGGGACGGG</u>	40
ACATGACGTC	AGCAAGGATT	ATAATGGGCT	TTTTATTAGC	80
CCATTTATTG	AATTGGGCCG	GGTTTTGTCA	TTTTACAAAA	120
GCCCCGTCCA	GGATAAGTAT	AATGTCACGT	GCCGAATAAA	160
AGGTTGCTTC	GCCTCGAAGA	AACCTAATAT	GAGGTAGCGT	200
ATTCAATACG	CTACCGAATA	<u>TCTATTAATA</u>	TGTGAGTCTC	240
TGCCGAAACA	ATCAGAGCGA	AAGCAAAGCA	GAAGCG ATGG	280
ATTGGGCGGA	ATCACAATTC	AAGACCTGTA	GTCATGGATG	320
CGATTGGAAG	AAGATATCAT	CGGATTCAGC	CGATAATCGA	360
CAATATGTAC	CATGCGTCGA	TTCTGGAGCT	GGAAGAAAGT	400
CGCCTCGCAA	GGTACTTCTT	AGATCTATTG	AAGCTGTGTT	440
TAACGGAAGC	TTCAGCGGAA	ATAATAGGAA	CGTTCGTGGA	480
TTTCTCTACG	TATCGATCAG	AGACGATGAC	GGAGAAATGC	520
GTCCAGTACT	CATAGTACCA	TTCGGAGGAT	ATGGATATCA	560
TAATGATTTT	TATTATTTTCG	AAGGAAAGGG	GAAAGTTGAA	600
TGTGATATAT	CATCAGATTA	TGTTGCGCCA	GGAATAGATT	640
GGAGCAGAGA	CATGGAAGTT	AGTATTAGTA	ACAGCAACAA	680
CTGTAATGAA	TTATGTGATC	TGAAGTGTTA	TGTTGTTTTGT	720
TCTTTAAGAA	TCAAGG<u>AATA</u>	<u>AA</u>AGTTGTGC	TGTAATGTTT	760
ATTAATAAAAA	CGTATATTTG	GGAAATTGAT	AGTTGTATAA	800
AACATACAAC	ACGCTATGAA	ATACAAGACG	CTATGACAAA	840
TGTACGGGTA	TCTGAATGAG	TTTTAGTATC	GCTTAAGGGC	880
CGCAGGCCCG	TTGAAAAATA	ATAATCGAAT	TATAAACGTT	920
AGATAATAAT	CAGAGATAGG	TGATCAGATA	ACCTAAACAT	960
AAACGAAGTA	TATGGCGGTA	CATTAATAAA	ATAAGTTTAA	1000
AAAAATAACC	TAATGAATAC	TAATCTCTGA	TTGGGTCAGA	1040
AGAAAGGCC	ACCAACTAAA	AGGTGGGGAG	<u>AATGTCCCCG</u>	1080
<u>TGACGTA</u>				1087

Fig. (3): The sequence of the Egyptian BBTV DNA-6 full length

- The *CR-SL* is in *italics and underlined* with the stem sequence arrowed (1-31).
- The potential **TATA boxes** are in **bold and double underlined (222-230)**
- The *CR-M* is in **bold and italics (801-891)**.
- The **polyadenylation** sequences were represented in **shaded letters (737,764)**.
- The **ORF** was represented in **bold (277-741)**.
- The start codon was **underlined** at the beginning of the ORF.
- The possible termination signal was **underlined** at the end of the ORF.

Fig. (4): The possible stem-loop structure of BBTV DNA -6 full length of the Egyptian isolate examined in this study. Underlined numbers indicate the nucleotide position on it.

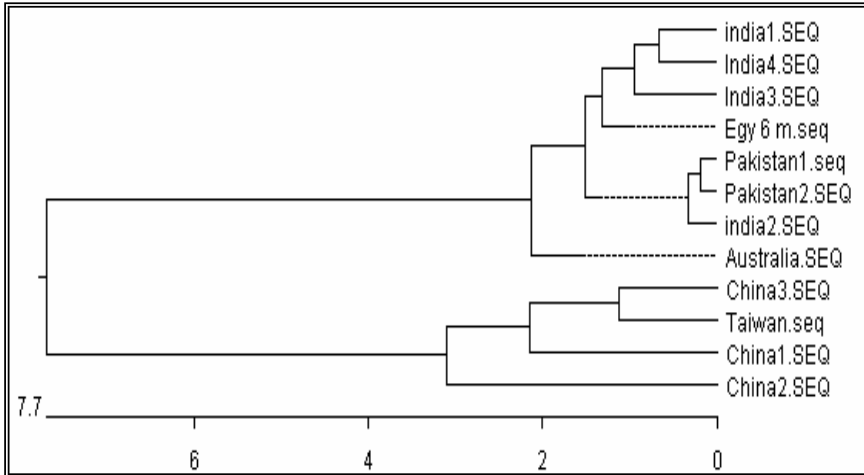
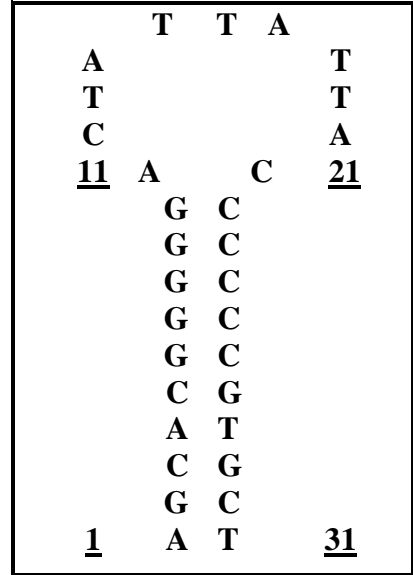


Fig. (5): Phylogenetic analysis of BBTV DNA-6 full length of the Egy 6m with the eleven different isolates based on the nucleotide sequences.

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MDWAESQFKTCSHGCDWKKISSDSADNRQYVPCVDSGAGRKSPR
KVLRLRSIEAVFNFSFSGNNRNVRGFLYVSIRDDDGEMLPVLIVP
FGGYGYHNDFFYFEGKKGKVECDISSDYVAPGIDWSRDMEVSISN
SNNCNELCDLKCYYVVCSLRIKE

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Fig. (6): Amino acid sequences of the translated ORF of Egyptian BBTV DNA-6 of the Egyptian isolate.

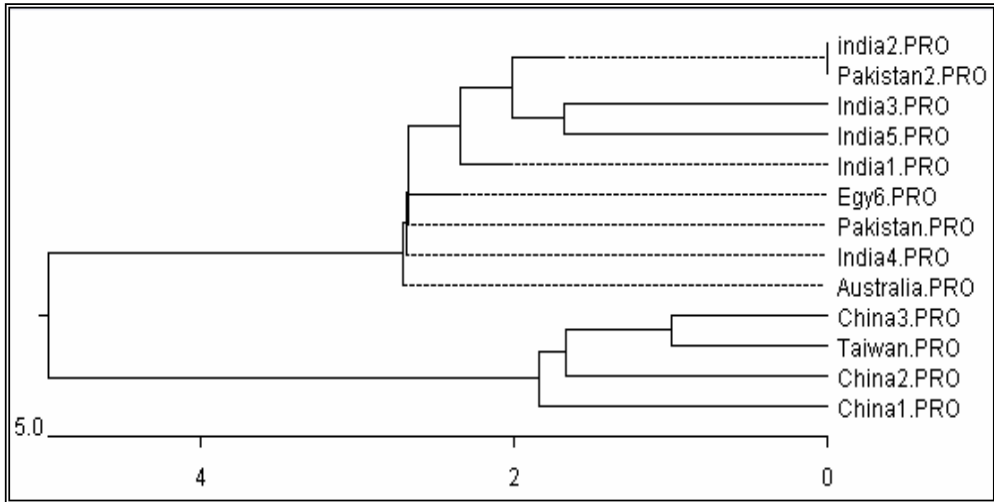


Fig. (7): Phylogenetic analysis of BBTV DNA-6 full length of Egyptian isolate (Egy 6m) with eleven different isolates based on the amino acids sequences.