NUCLEOTIDE SEQUENCES OF SEGMENT FIVE OF THE EGYPTIAN BANANA BUNCHY TOP BABUVIRUS

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Banana and plantains (*Musa* sp.) are the fourth most important global food crop after rice, wheat and maize (Piertersen and Thomas, 2001), it's also occupy an important position in the agriculture economies of Australia, Malaysia, Taiwan, Sirilanka and south China. The world banana production is estimated at 64.6 million tons in 2000 (Anonymous, 2001).

Banana production is subjected to many natural climates, but diseases such as fungal, viral, bacterial and nematodes constitute a major problem. Bisheng *et al.* (1997) reported that the viral diseases threaten banana production with a ratio of about 20-30 % and occasionally reach 50-80% worldwide. Banana bunchy top babuvirus (BBTV) is the casual agent of banana bunchy top disease (BBTD) (Dale, 1987) and is classified as a member of nanoviradae based on its molecular characterization (Dugdale *et al.,* 1998; Harding *et al.,* 2000).

BBTV is a single-stranded DNA (ssDNA) virus and has small isometric virions of 18-20 nm, and is persistently transmitted by the banana aphid *Pentalonia nigronervosa* (Harding *et al.,* 1991). Its genome consists of at least six components of circular ssDNA each about 1 Kb in size (Burns *et al.,* 1995). Each of the six DNA components associated with BBTV encoded at least one gene (Beetham *et al.,* 1997; Beetham *et al.,* 1999). The intergenic or non-coding regions of the BBTV DNA components have three regions of homology. The major common region (CR-M) incorporates a 66 - 92 nucleotide (nts) region (Burns *et al.,* 1995), the second region of homology is the stem-loop common region (CR-SL) incorporates a 69 nts region (Burns *et al.,* 1995) and contains a stem loop sequence, while the third region is common to all BBTV DNA components and has a potential TATA box, with consensus nanonucleotide sequence CTATa/ta/tAt/aa (Burns *et al.,* 1995) and is one of a number of transcription elements identified within the intergenic region.

A common strategy of DNA viruses is the creation of an environment favorable for efficient replication of their genome by subverting the cell cycle

control of the host and forcing cells into DNA synthesis or S phase.

Dougdale *et al.* (1998) hypothized that the BBTV DNA-5 gene product is the first expressed in the host after BBTV infection and may function in creating a cellular environment permissive for viral DNA replication and transcription. In addition, the level of self-primed extension products synthesized appeared to be greatest for BBTV DNA-5 that was several fold higher than the products for all the other components (Hafner *et al.*, 1997).

The encoded gene product of the BBTV DNA-5 is called cell cycle link, Clink, protein with a molecular weight of 19 - 20 KDa, this protein may represent a new class of viral cell cycle moderate (Aronson *et al.*, 2000; Gronenborn, 2004).

The aim of this study is to amplify, clone and sequence the full length of BBTV DNA-5 of the Egyptian isolate and compare it with other published sequences of full length of BBTV DNA-5.

MATERIALS AND METHODS

Virus source

Infected banana plants were collected from El-Qalubia Governorate 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).

DNA isolation from infected plants

The infected midrib was ground into a fine powder and the DNA was extracted by using DNeasy plant mini kit (cat. No. 69104) from QIAGEN.

PCR amplification for the full length of BBTV DNA-5

Two oligonucleotide primers for PCR were designed to amplify the full length of BBTV DNA-5 according to the published data of Burns *et al.* (1995). FLO5F represented the forward primer (5\ **GGG** ATC CCC GGA GTG AAG GAT ATG A 3\) and FLO5R represented the reverse primer (5\ GAA GCT TCA AGG GCA TCC TCT **TGA T** 3\). Nucleotides in bold represents *Bam*H1 site in the forward primer and *Hind*III in the reverse primer.

Two hundred nanograms of the extracted DNA were used as a template. PCR was conducted in a volume of 50 µl containing 1 x reaction buffer with $MgSO_4$, 200 µM each dNTPs, 0.4 pmol of each primer, 0.025 U *Pfu* DNA polymerase (Promega) and the 200 ng of extracted DNA as a template.

PCR amplification was performed in a Perkin-Elmer thermocycler (Gen 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 57°C for 1 min and extension at 72°C for 2 min. The primer extension was extended to 7 min at 72°C in the final cycle. The PCR ampli-

fied products were detected by agarose gel electrophoresis (1.2%) at 80 volts for 1hr (Sambrook *et al.,* 1989).

Cloning the full length of BBTV DNA-5

The PCR fragment was tailed with adenine (A) to be ligated in pGEM®-T Easy vector (3018 bp) (Promega) by incubating 25µl from the preheated (95°C/ 20 min) PCR product with 7.5 µl dATP (2 mM), 1.5 µl $MgCl_2$ (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 pGEM–T easy vector to create plasmid pNH5. Competent cells of *E. coli* (strain DH5α) were prepared and then transformed with the constructed plasmid as mentioned by Hammond and Hammond (1989). The recombinant plasmid DNA was tested by the digestion with *Eco* R1 enzyme as a confirmatory test.

Sequencing and computer analysis of the BBTV DNA-5 full length

The PCR product was sequenced using Genetic Analyzer (ABI Prism 310, version 3.4, SemiAdaptive, version 3.2) by forward and reverse primers. The ORF of DNA-5 was determined in the full length. The data base searches were performed using BLAST (basic local alignment search tool at NCBI). The nucleotide sequence of the full-length PCR product and the amino acid of the ORF sequences were aligned and compared using MegAlign of DNA Star Program with corresponding sequences of eight isolates as following: Australian-L41578, indian1-EUO51379.1, Indian2 -AY267898.1, Indian3 -AY845636.1, Hinanian -AY606085.1, Taiwan1-EFO95166.1 and Taiwan2 -DQ826395.1. Multiple comparisons were based on Jotun Hein method obtained in the program.

RESULTS AND DISCUSSION

PCR amplification for the full length of BBTV DNA-5

By using two specific primers for the full length of BBTV DNA-5, 1031 bp fragment was detected in the positive control and the BBTV-infected banana plants. This fragment was absent in the negative control (Fig. 1). Burns *et al.* (1995) reported that the full length of Australian BBTV DNA-5 was 1018.

By using PCR technology, BBTV was successfully detected in the virus-infected samples, therefore, early detection of BBTV particular in the symptomless banana plant materials produced *via* tissue culture could be detected. Harding *et al.* (2000) reported that this technique play an important role in detection of BBTV as well as in BBTV purification steps. (Sadik *et al.,* 1999).

Cloning Egyptian BBTV DNA-5 full length in pGEM-T easy vector

The purified A-tailed PCR product, which represent the full length BBTV DNA-5 was cloned into pGEM-T

easy vector (Fig. 2) and the created plasmid called (pNH5) with Mwt. about 4049 bp.

After minipreparation for the resulted colonies, the purified DNA plasmids were digested with *Eco* R1 enzyme as a confirmatory test. A segment of about 1031 bp was released from the recombinant plasmid; on the other hand, no fragment was detected in the non-recombinant plasmids (Fig. 3). Wanitchakorn *et al.* (1997) was also used the same technique to screen the recombinant plasmids.

- ***Sequencing of the Egyptian full length of BBTV DNA-5***

The DNA sequence of the full length of BBTV DNA-5 revealed 1017 bp (Fig. 4). This sequence was analyzed using DNA Star Program. It was found that, the sequence of the full length of BBTV DNA-5 contain a sequence of up to 69 nts sequence which represented the stem loop common region (CR-SL), in which a loop structure was consisted of 31 nts (from position 1- 31). Burns *et al.* (1995) found the similar result and they reported 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 was located at 5\ of the CR-SL it was called the major common region (CR-M) at the position (821-912) and consisted of 92 nts. In this region from nucleotide 4 to 20 and 21 to 36, an almost complete 16 nucleotides of direct repeat (ATACAACACGCTATAA) were observed. Furthermore, 15 nts with GC-rich sequence (average of 80% G+C) was located from nucleotides 78 to 92. These results were in harmony with the results obtained by Burns *et al.* (1995) with slite differences as the nucleotide in position 15 was found to be A instead of G.

A potential TATA box of nine nucleotides sequence was identified and was located downstream from the stem-loop sequence in a distance of 157 nucleotides. This result was compatible with Burns *et al.* (1995).

One open reading frame (ORF) was identified downstream of TATA box which started with the translation initiation codon ATG at position 239 to 725 in the virion sense with a size 486 nts and code 18.7 kDa. Burns *et al.* (1995) was also found that the full length of BBTV DNA-5 had one ORF associated with the potential TATA box and a polyadenylation region. However, the ORF was started from position 240 to 723 with a size 453 nt and coding 161 aa with molecular weight 18.9 kDa. In addition one potential polyadenylation signal was observed and associated with the 3\ end of the major ORF after 19 nts. A GT-rich region contained the trinucleotide TTG was located after this polyadenylation signal. This region was found to have the trinucleotide sequence TTG. These se-

quences with these criteria were not identified elsewhere in the BBTV DNA-5 full length suggesting that this component encodes a single gene. The similar observation was found by Burns *et al.* (1995).

No other ORFs were identified in the component five in either the virion sense or complementary sense which potentially encoded proteins greater than 10 kDa and had appropriately located potential TATA boxes and polyadenylation signals.

Based on the nucleotide sequences, the Egyptian isolate collected from Qalubia (Q) Governorate was compared with seven different isolates of BBTV as following: Australian, Hainana, Indian1, Indian2, Indian3, Taiwan 1 and Taiwan 2.

The phylogenetic analysis based on the nucleotide sequences of the eight different isolates (Fig. 5) revealed that the presence of two groups of BBTV, the first group represents Asian group (including Hainan (AY606085.1) and two isolates from Taiwan (1: EFO95166.1 and 2: DQ826395.1) and the second group represents South Pacific group (including Indian (1: EU051379.1, 2: AY267898.1 and 3: AY845636.1), Australian (L41578) and Egyptian isolate in this study). Karan *et al.* (1994 and 1997) and Wanitchakorn *et al.* (2000a) also reported that there were two groups of BBTV strains, Asian and South Pacific groups. Nour El-Din *et al.* (2005) reported that there were two groups of BBTV stains and the Egyptian isolate belong to the South Pacific group.

Results of the comparative analysis in Table 1 showed that the Egyptian isolate (Q) revealed high degree of homology to Australian (L41578), Indian2 (AY267898.1), and Taiwan 1 (EFO95166.1) in percentage 94.6, 94.1 and 94.1% respectively.

Based on the amino acid sequence for the ORF of BBTV DNA-5, the predicted protein consisted from 162 amino acids (Fig. 6), two motives were identified, the first motif was the cell cycle link binding motif LXCXE (LYCDE) in the position from 112 to 116 and the second motif was the F-box (MPDDVKREIKEI) in the position from 9 to 20.

In agreement with the obtained results, Wanitchakorn *et al. (*2000b) reported that the predicted gene product of BBTV DNA-5 contained an LXCXE motif in the C-terminal half of the protein (amino acids 111-115).

Amati and Vlach (1999) reported that the importance of the F-box (amino acid) with the cell cycle link, Clink, protein (the encoded protein of BBTV DNA-5). The phylogenetic analysis based on the amino acid sequences of the eight isolates of the full length of BBTV DNA-5 (Fig. 7) showed two clusters, cluster 1 included Hainan, Taiwan 2, Indian1, Taiwan 1, Australian and Indian 2 isolates, while cluster 2 included the Egyptian (Q) and the Indian 2 isolates.

Data in Table No.2 represent the percentage of similarity between the

Egyptian isolate and the seven different BBTV isolates based on the amino acid sequence.

The percentage of identity of the full length of BBTV DNA-5 was as the following: 90.0, 78.8, 83.1, 90.6 and 91.9 and 89.4 and 78.8% with isolates from Australian, Hainan, India 1, 2 and 3, Taiwan 1 and 2, respectively.

The Egyptian isolate, was aligned with the seven different isolates of BBTV (Fig. 8) and showed that the LXCXE and F-box motives were found in all eight isolates of BBTV in the same position.

The *Nanoviradae* group, based on the amino acid sequences, characterized with the presence of LXCXE motif (Katual *et al.*, 1998) and F-box motif (Aronson *et al.,* 2000) and these motives play an important role in stimulating the viral DNA replication and regulate pathway of the cell cycle.

SUMMARY

Banana and plantains (*Musa sp.*) are the most important staple food and source of carbohydrates in different geographical regions mainly in Africa. The productivity of banana crop is attacked by many pests and diseases. Banana bunchy top babuvirus (BBTV) is the most destructive viral disease because it has an enormous negative impact on the productivity of banana in all of banana-cultivated-countries in the world. Accordingly, in this study we are focusing on the molecular characterization of the full length of the Egyptian isolate of BBTV DNA-5. The full length of BBTV DNA-5 was synthesized *via* polymerase chain reaction (PCR) then manipulated and cloned into pGEM-T easy vector and sequenced. The size of the full length of DNA-5 was 1017 nucleotides (nts) which includes one open reading frame (ORF) of 489 nts. The nucleotide sequences of the full length of DNA-5 of the Egyptian isolate was aligned with those of seven overseas isolates (three isolates from India, two from Taiwan and one from Hainan). Results showed that the major outcome of this alignment was restricted in two groups, the first group called Asian group that comprised Taiwan 1, Taiwan 2 and the second group contained the Egyptian, Indian 1, Indian 2, Australian and Hainan isolates that formed the South Pacific group. As a conclusion, one can recommend that a strategy of PCR detection of BBTV could be introduced as an early step for controlling such virus based on the nucleotide sequence of the Egyptian BBTV Q-DNA-5 beside the other published sequences.

ACKNOWLEDGMENT

My special thanks to Dr. Amgad Abd El-Maged Saleh, Researcher in Agricultural Genetic Engineering Research Institute (AGERI) and Prof. Dr. Atef Shoukry Sadik, Professor of Agriculture Virology, Faculty of Agriculture,

Department of microbiology, Ain Shams University, for their input and cooperation in this study.

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Table (1): Sequence distance between the studied isolate of BBTV DNA-5 (Q) and the other published seven sequences on the base of the nucleic acid.

	Percent identity							
	1	2	3	4	5	6	7	8
1- Q full length. SEQ		94.6	84.4	93.2	94.1	89.8	94.1	84.5
2- Australian. SEQ	5.6		87.8	97.4	98.8	97.9	99.3	88.2
3- Hainan. SEQ	17.5	13.4		86.1	86.5	87.7	87.2	93.7
4- Indian 1. SEQ	7.1	2.6	15.4		97.0	94.6	97.1	86.1
5- Indian 2. SEQ	6.1	1.2	14.9	3.1		98.0	98.4	87.8
6- Indian 3. SEQ	11.0	2.4	13.5	5.6	2.0		97.4	88.2
7- Taiwan1. SEQ	6.1	0.7	14.0	4.0	1.6	2.6		88.3
8- Taiwan 2. SEQ	17.4	12.9	6.5	15.3	13.4	12.8	12.7	

Table (2): Sequence distance between the studied isolate of BBTV DNA-5 (Q) and the other published seven sequences on the base of the nucleic acid.

	Percent identity							
	1	2	3	4	5	6	7	8
1- Q PRO		90.0	78.8	83.1	90.6	91.9	89.4	78.8
2- Australian. SEQ	10.8		85.8	91.4	98.1	96.9	98.1	89.4
3- Hainan. SEQ	25.0	15.8		80.9	85.8	84.0	85.2	93.2
4- Indian 1. SEQ	19.2	9.2	22.1		90.7	89.5	91.4	81.5
5- Indian 2. SEQ	10.0	1.9	15.8	9.9		97.5	97.5	86.4
6- Indian 3. SEQ	8.6	3.2	18.1	11.3	2.5		96.3	84.6
7- Taiwan1. SEQ	11.5	1.9	16.6	9.2	2.5	3.8		85.8
8- Taiwan 2. SEQ	25.0	15.0	7.1	21.3	15.0	17.3	15.8	

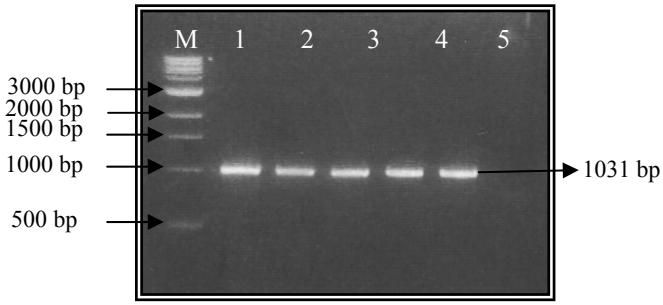


Fig. (1): 1.2% agarose gel in TAE buffer stained with ethidium bromide shows PCR detection of BBTV DNA-5. M: Fermantas 1 kb marker (500, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 8000 and 10,000 bp), 1: positive control, 2-4: BBTV infected samples, 5: negative control (Healthy plant).

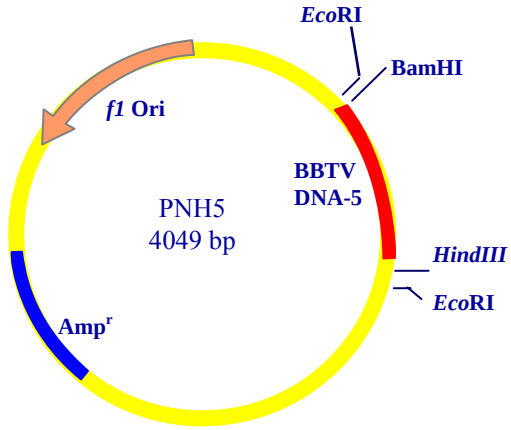
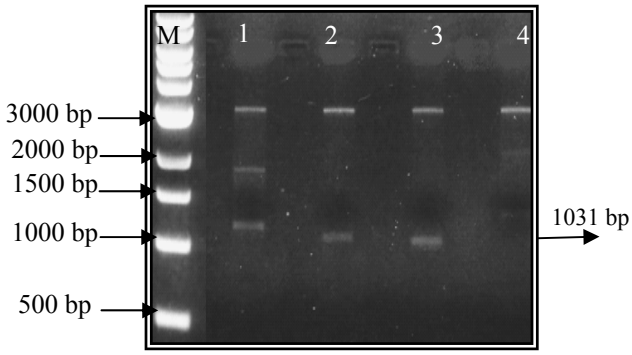


Fig. (2): A diagram showing the recombinant plasmid PNH5 (4049 bp).

Fig. (3): 1.2% agarose gel in TAE buffer stained with ethidium bromide showing digestion of mini-prepared plasmid DNA from recombinant colonies with *Eco*R1. Note: 1017bp fragment was released from the recombinant colonies. M: 1kb marker (500, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 8000 and 10,000 bp), 1: uncut plasmid, 2, 3: recombinant plasmids and 4: non-recombinant plasmid.



AGCGCTGGGG	*ACTATTATTA*	*CCCCCAGCGC*	*TCAGGACGGG*	**40**
*ACAT*CACGTG	CGACTAACAG	ACGCACGTGA	GAATGCAGTA	**80**
GCTTGCAGCG	AAAGATAGAC	GTCAACATCA	ATAAAGAAGA	**120**
AGGAATATTC	TTTGCTTCGG	CACGAAGCAA	AGGGTATAGA	**160**
TATTTGTTCG	AGATGCGAAA	ATGGAGG**CTA**	**TTTAAA**CCTG	**200**
ATGGTTTTGT	GATTTCCGAA	ATCACTCTTC	GGAAGAGAA**A**	**240**
TGGAGTTCTG	**GGAATCGTCT**	**GCCATGCCTG**	**ACGATGTCAA**	**280**
GAGAGAGATT	**AAGGAAATAT**	**ATTGGGAAGA**	**TCGGAAGAAA**	**320**
CTTCTGTTCT	**GTCAGAAGTG**	**AAGAGCTCCG**	**GAGGGAGGGA**	**360**
TTTGTGATGG	**GAACCCTAGG**	**GCCTGCACAA**	**GCTCAAAGAC**	**400**
GGGGGGGAAG	**AAATACTTCT**	**ATCATTCGCT**	**ATAGCGAATA**	**440**
CTGAAGAAAC	**CATGTGTGGT**	**AATGTGTTGT**	**GTTAGCAATA**	**480**
AATCACTTGT	**GTATAGGCTA**	**AACAGCATGG**	**TGTTCTTTTA**	**520**
TCATGAATAC	**CTTGAAGAAC**	**TAGGTGGTGA**	**TTTCTCAGTA**	**560**
TATCAAGATC	**TCTATTATGA**	**TGAGGTTCTC**	**TCTTCTTCAT**	**600**
CGACAGATGA	**AGAAGATGTA**	**GGAGTAATAT**	**ACAGGAATGT**	**640**
TATCATGGCA	**TCGACTCAAG**	**AGAAGTTCTC**	**TTGGAGTGAT**	**680**
TGTCAGAAGA	**TAGTAATATC**	**AGACTATGAT**	**GTAACATTAC**	**720**
TCTAATGTAA	TATCCATTAT	CATCAATAAA	ATAATGGAAT	**760**
GTTGATTATG	TATTTATCAT	AAATACATAA	TGGTATACGT	**800**
ATAGCATAAA	ATACATTACC	***AACATACAAC***	***ACGCTATAAA***	**840**
ATACAACACA	***CTATAACAAA***	***TGTACGGGTA***	***TCTGATTGGG***	**880**
CTATATTAAC	***CCCTTAAGGG***	***CCGAAGGCCC***	***GT***TTAAATAT	**920**
GTGTTGGACG	AAGTCCAAAC	ACAAAAAAGT	ATGCAGAACA	**960**
ACGGAATAAA	ATGAGCTGGC	AACGTAGGGT	CC*ATGTCCCG*	**1000**
AGTTAGTGCG	*CCACGTA*			**1017**

Fig. (4): The sequence of the Egyptian BBTV DNA-5 full length

- The potential **TATA boxes** are in **bold and double underlined**
- The *stem-loop structure* is in *italics and underlined*, with the stem sequence arrowed.
- The *CR-SL* is *underlined*
- The ***CR-M*** is ***in bold and italics***
- The polyadenylation sequence was represented in shaded letters.
- The **ORF** was represented **in bold**.

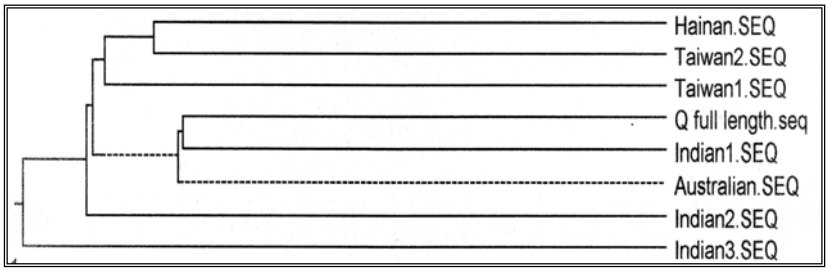


Fig. (5): Phylogenetic analysis of BBTV DNA-5 full length with seven different isolates based on the nucleotide sequences.

MEFWESSAMPDDVKREIKEIYWEDRKKLLFCQKL
KSSVGRVLVFGALGAALARRWGVRSTSIIRYSEYL
KKPCVVICCVSNKSLVYRLNSMVFFYDEYLEEVG
GDFSVYQDLYCDEVLSSSSTDEEDVGVIYRNVIMA
STQEKFSWSDCQKIVISDYDVTLL

Fig. (6): Amino acids sequences of the translated ORF of BBTV DNA-5 of the Egyptian isolate. The underlined sequences represent the F-box and LXCXE motifs.

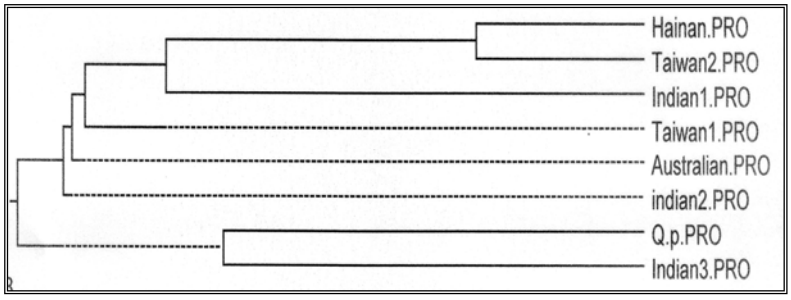


Fig. (7): Phylogenetic analysis of BBTV DNA-5 full length with seven different isolates based on the amino acids sequences.

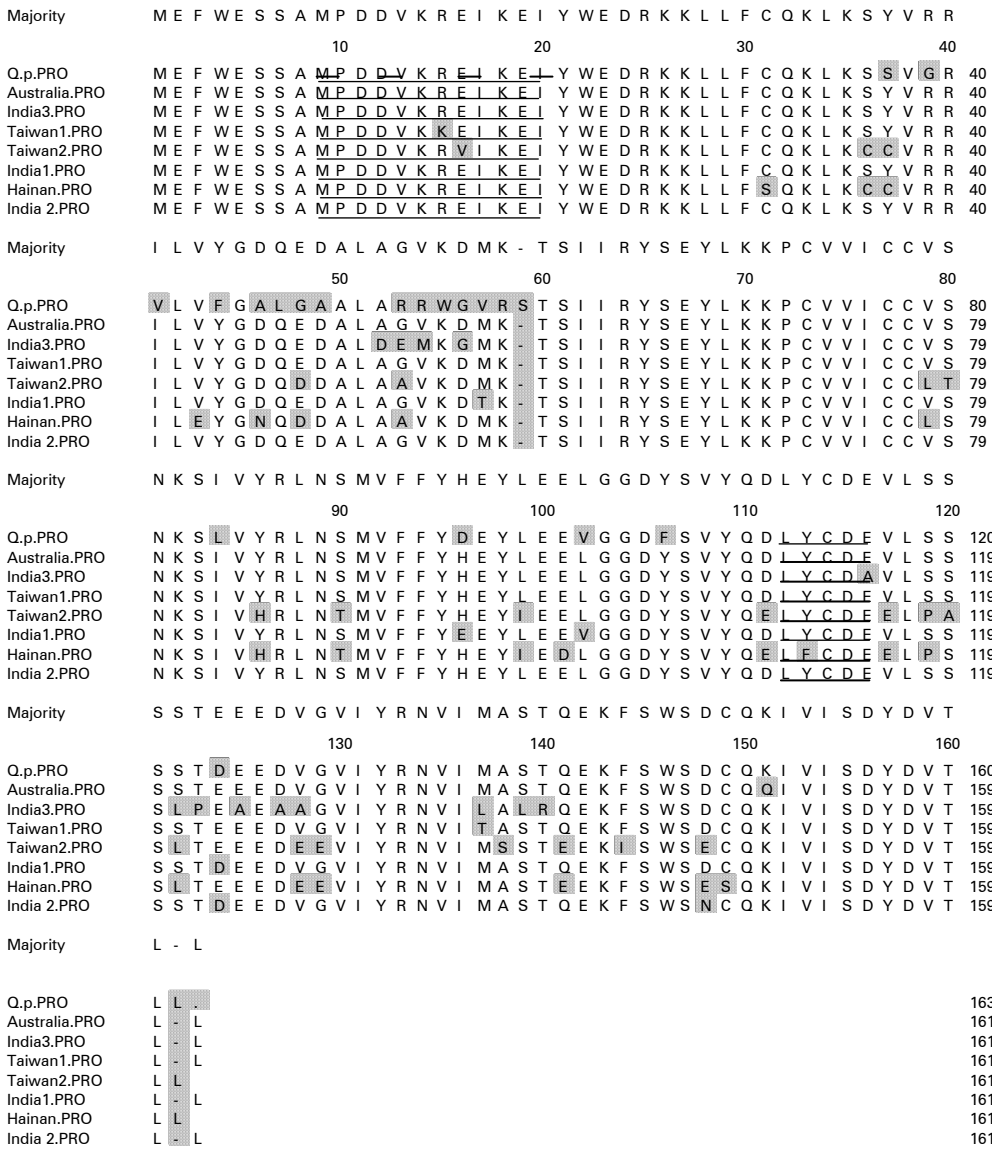


Fig. (8): Alignment of amino acids sequence of BBTV DNA-5 ORF with seven geographical isolates from Australian (L41578), Hainan (AY606085.1), india1 (EUO51379.1), Indian2 (AY267898.1), Indian3 (AY845636.1), Taiwan1 (EFO95166.1) and Taiwan2 (DQ826395.1). Taiwan**1 (EFO95166.1)**). **MPDDVKREI** represents the F-box motif and **LYCDE** represents LXCXE motif.