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Full Length Research Paper

Detection, cloning and bioinformatics analysis of vip1/vip2 genes from local strains of *Bacillus thuringiensis*

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Bio-insecticides based on the spore forming bacterium, *Bacillus thuringiensis* (Bt) have been used for commercial scale for the past 40 years. Bt is a Gram-positive soil bacterium that forms insecticidal crystal proteins (ICPs) during sporulation; it has been characterized as an insect pathogen. Vegetative insecticidal protein (VIP) is a newly discovered family of toxin protein isolated from Bt. A hundred strains of local *Bacillus thuringiensis* were isolated from soil and dead larvae, identified by 16S rRNA and screened for the presence of vip1 and vip2 genes by polymerase chain reaction (PCR) amplification, with only four strains producing the desired bands of Vip1 and Vip2. The amplified fragments were cloned in pGEM-vector, sequenced and analyzed. The nucleotide sequences of vip1 (2.3 kb) and vip2 (1.3 kb) were given Gene-bank accession numbers: JN008908 and JN035904, respectively. Vip1 and Vip2 showed 99% homology with the previously isolated genes.

Key words: *Bacillus thuringiensis*, vegetative insecticidal proteins (vip1 and vip2), 16S rRNA, cloning.

INTRODUCTION

The Gram-positive bacterium *Bacillus thuringiensis* (Bt) is a common soil micro-organism that has been used as a biological pesticide for several decades. Bt insecticidal activity has mainly contributed to parasporal crystalline (δ-endotoxin inclusions) formed during sporulation (Schnepf et al., 1998), and to some extent to the vegetative insecticidal proteins (VIPs) (Warren, 1997; Han et al., 1999). The detailed molecular mechanisms mediating the insecticidal activity of *Bacillus*-produced δ-endotoxins have been described as a multistep process, which initiates upon ingestion of the protein crystals. The insecticidal δ-endotoxins are solubilized in the insect midgut and subsequently undergo site-specific proteolysis from their N- and C- terminus. These activated polypeptides bind to receptors in the midgut epithelium and form ion channels, inducing osmotic lysis of the epithelium cells and subsequent death of the larvae (Lorence et al., 1995). To date, more than 300 insecticidal crystal proteins (ICP) genes have been cloned, sequenced and have been classified into 67 groups of cyt genes, based on the amino acid homology of the corresponding proteins (Crickmore et al., 2004; Noguera and Ibarra, 2010). Moreover, many strains also produce an assortment of various other virulence factors (De Maagd et al., 2003) including the Cry1I toxin (Kostichka et al., 1996), β-exotoxin I (Ohba et al., 1980) and the vegetative insecticidal proteins Vip1, Vip2 and Vip3.

Vip toxins, so-called because of their production during vegetative growth phase, extend their insecticidal properties especially against the western corn rootworm (*Diabrotica virgifera*) and the European corn borer (*Ostrinianubilalis*), both widespread corn pests, less affected by the δ-endotoxins (Warren, 1997). There are more than 90 different vegetative insecticidal genes that have been identified to date. These identified Vip genes are 11 vip1, 15 Vip2 and 70 Vip3. Among them, vip1/vip2 has a priority in future screening of new larvicidal proteins because of their activity against western corn rootworm (WCR) and northern corn rootworm (NCR) (Han et al., 1999). The Vip1-Vip2 toxin is representative of a class of

binary toxins distinct from the classical A-B toxin, such as choleratoxin, that must assemble into a complex composed of two functionally different subunits or domains for activity (Madshus and Stenmark, 1992). Each polypeptide in the Vip1-Vip2 class of binary toxin evidently functions separately, with the membrane binding 100 kDa Vip1 providing a pathway for the 52 kDa Vip2 ADP-ribosylase to enter the cytoplasm of target WCR-define cells (Warren et al., 1996). The NAD-dependent ADP-ribosyltransferase Vip2 likely modifies monomeric actin at Arg177 to block polymerization, leading to loss of the actin cytoskeleton, and eventual cell death due to the rapid subunit exchange within actin filaments *in vivo* (Carlier, 1990). Vip2 also shares significant sequence similarity with enzymatic components of other binary toxins like *Clostridium botulinum* C2 toxin (Aktories et al., 1986), *C. perfringens* iota toxin (Stiles and Wilkens, 1986), *C. spiroforme* toxin (Simpson et al., 1989) and *C. difficile* toxin (Popoff et al., 1988). Though symptoms produced by Vips are similar to those caused by Cry proteins, the symptoms developed over a period of 48 to 72 h after ingestion whereas, it takes only 16 to 24 h for the symptoms to appear in the case of Cry proteins (Estruch et al., 1997). Here, our experimental approaches focused on bacterial stages before sporulation, and has led to identification of non-delta endotoxins. In this work, the distribution of Vip1/Vip2 genes among local *B. thuringiensis* strains was surveyed by PCR; Vip1 and Vip2 genes were cloned, sequenced and analyzed.

MATERIALS AND METHODS

Isolation of *Bacillus thuringiensis* from soil

A total of 75 soil samples were collected from different geographical locations in Egypt; Al-Ayat (29850' N and 31820' E) at Giza area, 40 km south of Cairo, Asyut80 City (27811' N and 31804' E), 350 km south of Cairo and from Tanta city (31800' N and 31800' E), 150 km north of Cairo. Samples were collected from 2 to 5 cm below the surface with a shovel. Each soil sample was placed in a plastic bag at ambient temperature. *Bacillus thuringiensis* strains were isolated from soil samples following the method described by Travers et al. (1987). Briefly, 0.5 g of soil was added to 10 ml of L broth (per liter: 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl) in a 100 ml flask. The L broth was buffered with 0.25 M of sodium acetate. This mixture was shaken for 48 h at 250 rpm at 30°C. Then, 1.5 ml samples were heat treated in a water bath at 80°C for 3 min. An aliquot of 50 μL was plated on L broth with addition of 15 g/L of bacteriological agar, and grown overnight at 30°C. A random sample of colonies was picked and plated onto T3 agar (per liter: 3 g of tryptone, 2 g of tryptose, 1.5 g of yeast extract, 0.05 M sodium phosphate (pH 6.8), 0.005 g of MnCl and 15 g of bacteriological agar).

Isolation of *Bacillus thuringiensis* from dead larvae

A total of 25 bacterial strains were isolated from dead insects. Dead larvae of Egyptian cotton leaf worm (also locally known as Alfalfa leaf worm) *Spodopteralittoralis* (Lepidoptera: Noctuidae), five in total were collected from Giza area (29850' N and 31820' E). Each dead larva was collected using sterile forceps and placed in a sterile plastic screw-top bottle. Each dead larva of *Spodopteralittoralis* was crushed in sterile crucible, and added to a tube containing 9 ml sterile phosphate buffered saline (PBS). The suspension was heated at 80°C for 3 min then spun for 5 min at 8000 rpm. An aliquot of 50 μL of the supernatant were spread on LB agar plates and incubated overnight at 30°C. Plates from soil or dead insect samples were incubated for 72 h at 30°C to allow the bacteria to sporulate. Isolates were preserved at Microbial Molecular Biology Department, AGERI-Egypt.

Total DNA Isolation

Total DNA was prepared from overnight culture in LB medium by the method of Ferrandis et al. (1999). Cells were recovered by centrifugation at 13000 × *g* for 3 min. Cell pellet was resuspended in 1 ml of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 2% (w/v) SDS, and 400 μg/ml proteinase K (Takara, Shiga, Japan) (20 mg/ml) and incubated for 30 min at 55°C. Total DNA was recovered by sequential extractions with phenol chloroform. The aqueous upper layer was transferred into a fresh tube and same volume of isopropanol was added. DNA was precipitated by centrifugation at 13000 × *g* for 20 min at 4°C followed by washing with 70% (v/v) ethanol, drying under vacuum, and re-suspension in 50 μl sterile distilled water.

Polymerase chain reaction (PCR) amplification of 16S rRNA

Bacillus thuringiensis like colonies were used for PCR of 16S rRNA genes, followed by sequencing as molecular confirmatory test. 16S rRNA primer (16S rRNA F and 16 rRNA R), specific for *Bacillus*, were synthesized according to Yoon et al. (2001) (Table 1). Amplified fragments were cloned into T-easy vector (from Promega, Madison, USA) and the DNA sequences were determined. The PCR reaction mixture (50 μL total volume) contained 200 μM of each dNTP, 0.5 μM primers, 10 mM tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 2.5 U Taq polymerase (ABgene, Surry, UK) and 100 ng of template DNA. Amplification of DNA, using primers 16S rRNA (F) and 16S rRNA (R) was performed at the following temperature cycle: denaturation at 94°C for 3 min, 30 cycles at 94°C for 60 s, 45°C for 60 s, and 72°C for 60 s, and final extension at 72°C for 7 min. The full-length PCR product 16SrRNA genes (1.3 kb) were investigated using electrophoresis in a 1% agarose gel.

PCR screening of vip1/vip2 gene

The Vip2 (F) and Vip2(R) primers were used to check for the presence of the Vip2 and to isolate the 1.3 kb full-length Vip2 coding region from local *B. thuringiensis* strains (Table 1). The Vip1 (F) and Vip1 (R) primers were used to check for the presence of Vip1 gene and to isolate the full-length of Vip1 coding region (2.5 kb) from local *B. thuringiensis* strains (Table 1). However, one internal nested primer Vip1 (int R) synthized at AGERI as shown in Table 1 was used only in sequence reaction in order to get the the rest of nucleotide sequences. Primers were synthesized at AGERI according to Warren et al. (1996). A loop of *B. thuringiensis* cells grown in Luria-Bertani (LB) plates at 30°C and the overnight culture, was suspended in 200 μl sterile distilled water, and then boiled vigorously for 5 min. 5 μl of the suspension was used as template DNA for PCR, using the following conditions: 5 min initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for

Table 1. The sequence of the primer for the isolation of 16S rRNA, vip1, and vip2 genes and the nested primer for the DNA sequencing.

Primer	Sequence	Amplified product
16S rRNA(F)	'5-CAGGCCTAACACATGCAAGTC-3'	1.3Kb
16S rRNA (R)	'5-GGGCGGTGTGTACAAGGC-3'	
vip1 (F)	'5-CGCGGATCCATGAAAAATATGAAGAAAAAGTTAG-3'	2.3Kb
vip1 (R)	'5-CGCGCGGCCGCTTATCTAGATTTGTTAGGTCC-3'	
vip2 (F)	'5-GGATCCATGAAAAGAATGGAGGGAAAATTG-3'	1.3Kb
vip2 (R)	'5-CTCGAGTTAATTTGTTAATAATGTTGCATCCA-3'	
vip1 (int R)	'5-ATACTAGGAAAAGCTGCTACTAATGG-3'	Nested primer for DNA sequencing
M13F	M13F '5-GTAAAACGACGGCCAG-3'	
M13R	M13R '5-CAGGAAACAGCTATGAC-3'	

2.5, while for Vip1, it was 2.5 min. Final extension was carried out for 10 min at 72°C (Sambrook and Russell, 2001). The PCR products of Vip1 and Vip2 genes were examined by electrophoresis in 1% agarose gel.

Cloning of the vip1/vip2 genes

The amplified fragment was eluted using the DNA purification kit (from Ferments) and ligated with T4 ligase enzyme and cloned in pGEM-T vector (from Promega). *E. coli* DH5α cells were used for transformation, and selected on LB plates containing ampicillin (100 μg/ml), X-Gal (20 mg/ml) and IPTG (200 mg/ml). White recombinant colonies grown on fresh plates were screened and verified by PCR and restriction enzyme analysis, respectively. All DNA manipulations, including ligation, transformation and restriction digestion were carried out as described by Sambrook and Russell (2001). The Vip1 and Vip2 genes were analyzed and compared with the updated Gen-bank data by using BLAST program (http://www.ncbi.nlm.nih.gov/blast).

Sequencing of vip1 and vip2 genes

The nucleotide sequence of either cloned 16S rRNA fragments or Vip1 (2.3 kb) and Vip2 (1.3 kb) genes were performed at AGERI according to Sanger et al. (1977). DNA sequencing reactions were performed using ABI PRISM BigDye terminator cycle sequencing ready reaction kit (PE applied Biosystems, USA), in conjunction with ABI PRISM (310 Genetic Analyzer) Cycle system 9700 instrument, and the reaction was conducted in a total volume of 20 μl, containing 8 μl of terminator ready reaction mix, 100 to 500 ng of PCR product, and 2 pmol of m13 forward and or m13 reverse, and or primers for sequencing reactions (Vip1 int1R). The sequences were then assembled into a single non-overlapping contiguous sequence using the Fragment assembly program of genetics computer.

RESULTS AND DISCUSSION

Many *Bacillus thuringiensis* strains secrete non δ-endotoxins vegetative insecticidal proteins (Vip1, Vip2 and Vip3) during vegetative growth (Warren, 1997). Vips represent a new type of insecticidal proteins because they are secreted as soluble proteins, rather than forming crystal inclusions inside the *B. thuringiensis* mother cell, and because Vips do not share sequence homology with *B. thuringiensis* crystal proteins. Two classes of Vip toxins have been described; the first consists of a binary system composed of two proteins, Vip1 and Vip2, which are 100 and 52 kDa in size, respectively. These proteins are highly toxic to certain coleopteran species (Warren, 1997; Warren et al., 1996). The second class consists of a 88.5 kDa protein, Vip3, which is active against a wide spectrum of lepidopteran insects and displays acute bioactivity towards the black cutworm (BCW; *Agrotisipsilon*), fall armyworm (FAW; *Spodopterafrugiperda*), beet armyworm (*Spodopteraexigua*), tobacco budworm (*Heliothisvirescens*) and corn earworm (*Helicoverpazea*) (Estruch et al., 1996). These two classes of protein do not display sequence homology with Cry protein. The main objectives of this study were identification, characterization, cloning, sequencing and analysis of Vip1 and Vip2 genes expressed during vegetative growth from local *Bacillus thuringiensis* isolates.

Identification of isolates by sequence of 16S rRNA

A (1.3 k) kb 16S rRNA PCR fragment was amplified using the primer pair described in Table 1. The amplified fragment was sequenced, and a total of 1.3 kb nucleotide sequences were determined. BLAST program was used for the analysis of the sequence and the homology search showed that it is like *Bacillus thuringiensis* serovar Entomocidus strain IEBC-T06 00116S ribosomal RNA gene partial sequence (Figure 1).

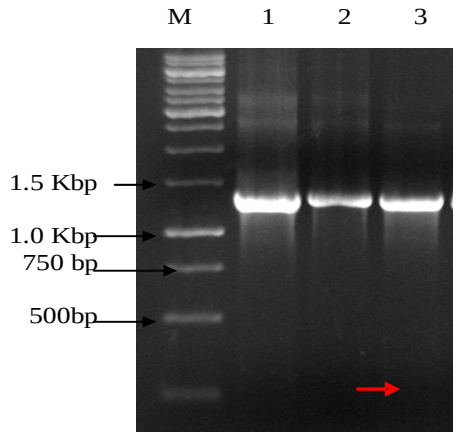


Figure 1. Ethidium bromide stained agarose gel that resolved the PCR amplification fragment of 1.3 kb of 16S rRNA gene from local *Bacillus thuringiensis* isolates. Lanes 1, 2 and 3 shows 16S rRNA fragments. M, DNA marker (1 kbp).

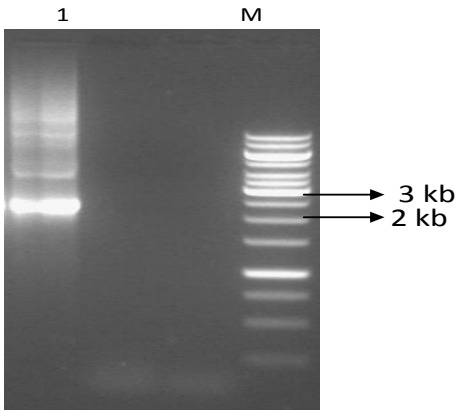


Figure 2. Ethidium bromide stained agarose gel resolution. Lane 1, the PCR amplification fragments of the vip1 gene "2.3 kb" from local *Bacillus thuringiensis* isolate using specific primers; M, DNA marker (1 kbp).

PCR screening for vip1/vip2 genes

Out of 100 *B. thuringiensis* identified by 16S rRNA sequence, four strains amplified PCR product 2.3 kbp for Vip1 (Figure 2) and 1.3 kbp for Vip2 (Figure 3).

Cloning of the vip1 and vip2 genes and sequence analysis

The amplified fragments were cloned into pGEM-T vector; cloning was confirmed by PCR and restriction digestion (Figures 4 and 5) and sequenced. Nucleotide sequence analysis showed a 99% homology to the known Vip1 and Vip2 genes (on-line Blast software)

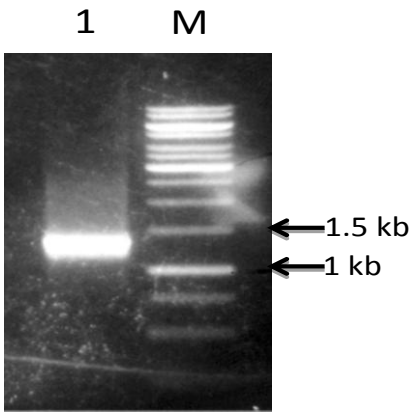


Figure 3. Ethidium bromide stained agarose gel resolution. Lane 1, the PCR amplification fragment of the vip2 gene "1.3 kb" from local *Bacillus thuringiensis* isolates; M, DNA marker (1 kbp).

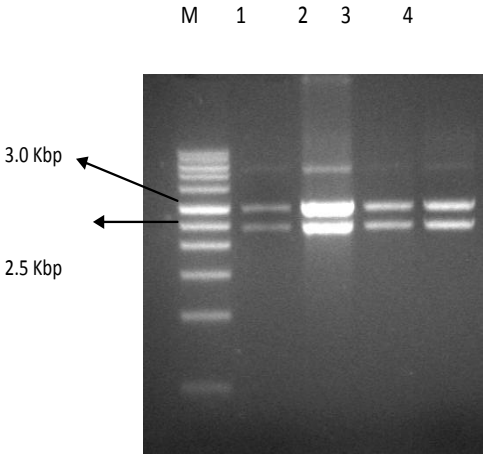


Figure 4. Screening for the presence of cloned vip1 fragment (2.3 kbp), using EcoR 1 cut in pGEM-T vector. Lanes 1, 2, 3, and 4, positive clones; M, is 1 kb DNA marker.

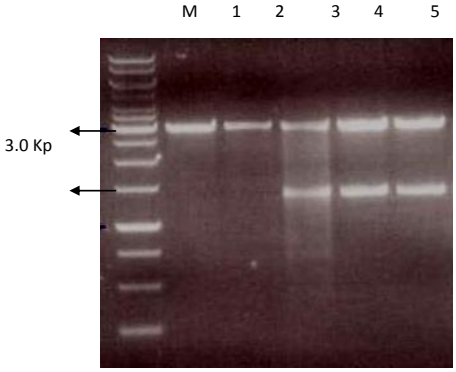
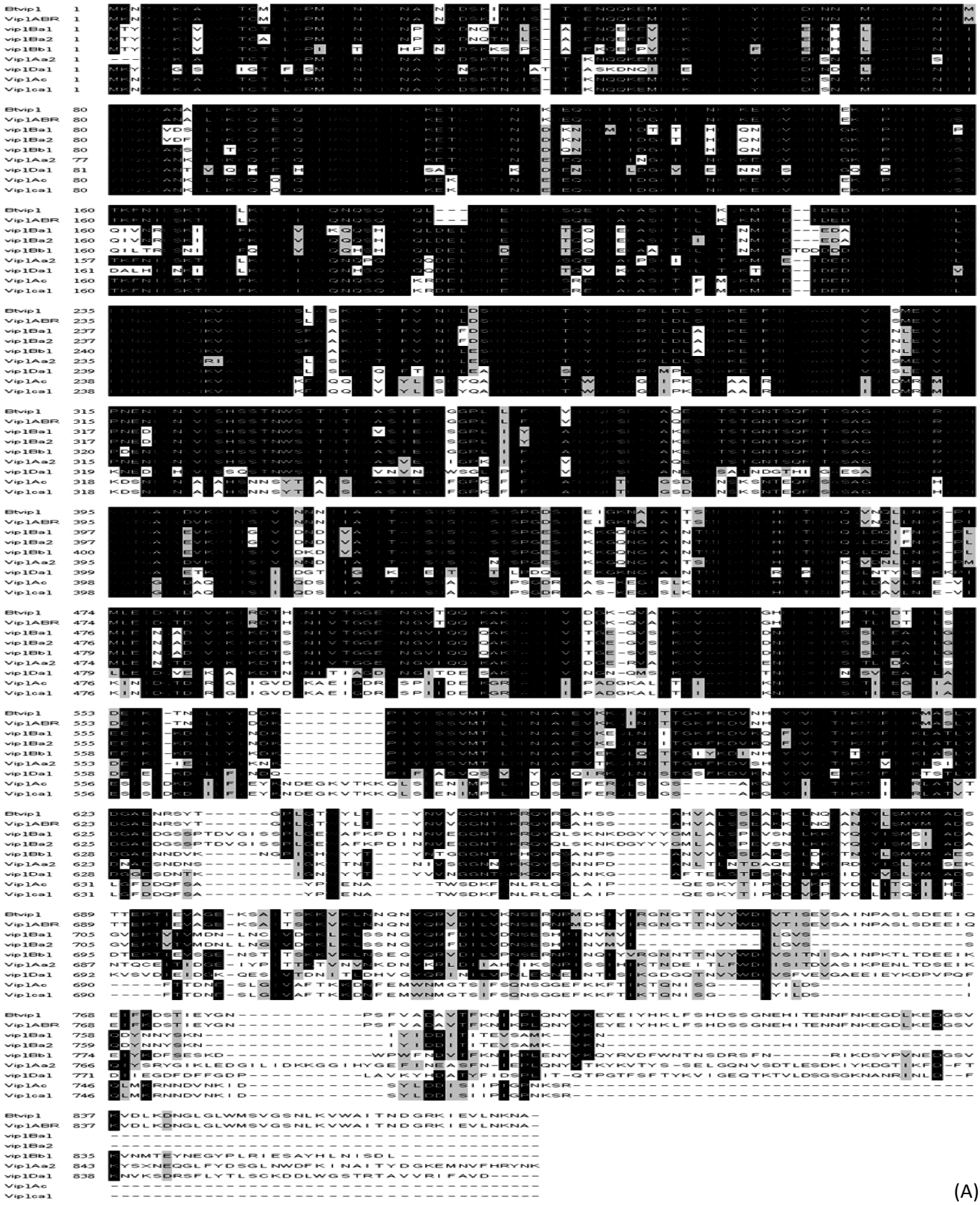


Figure 5. Screening for the presence of cloned vip2 fragment, using EcoR 1 cut in pGEM-T vector. Lanes1 and 2, negative clones; lanes 3, 4, and 5, positive clones; lane M, 1 kb DNA marker.



(A)

Figure 6A. Homologous comparison of Vip1, Vip2, proteins. Black, deep and french gray blocks represents 100, 80 and 60% identity, respectively.

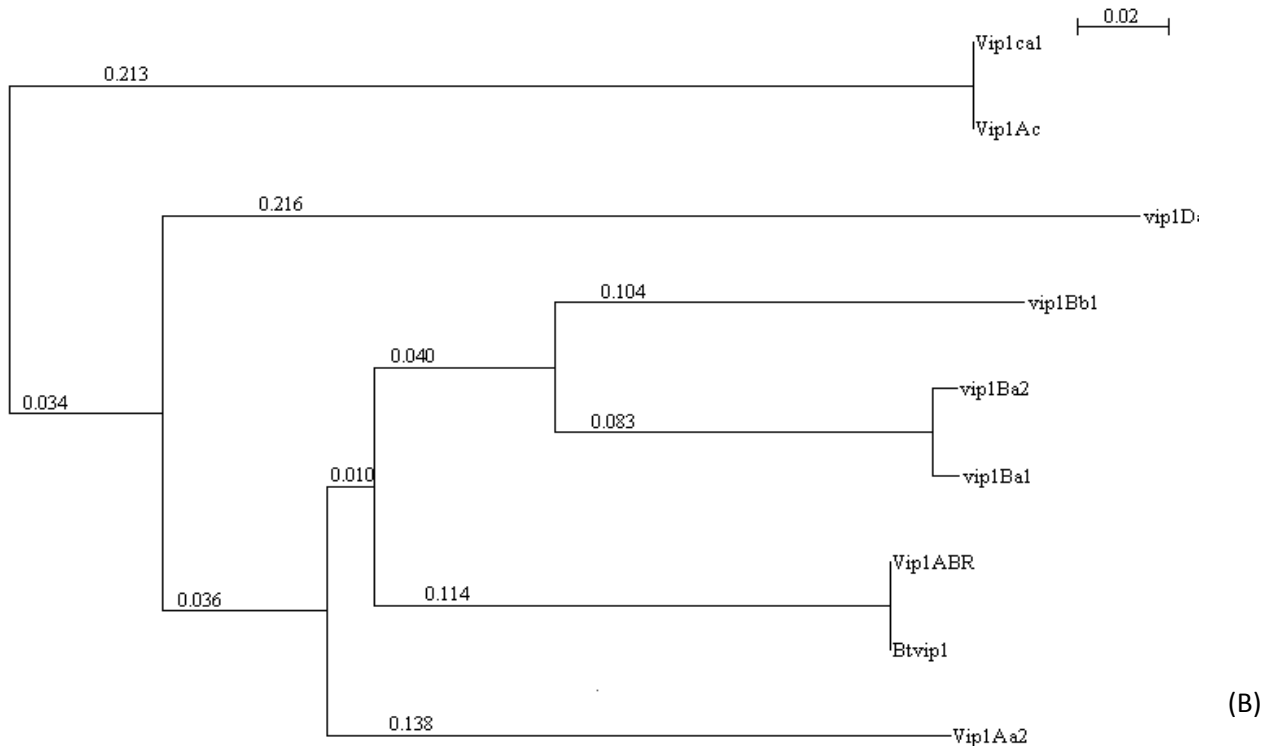


Figure 6B. Homologous comparison and phylogenetic analysis for Btvip1 protein. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events.

sequence. The nucleotide sequence data reported in this paper was filed in EMBL/Gene-bank database under accession number: JN008908 and JN035904 for Vip1 and Vip2 genes, respectively.

Sequence analysis of vip1/vip2 genes

The presence of Vip1 and Vip2 within our local Bt isolates was confirmed by PCR using specific primers for 100 Bt local isolates. Only four isolates showed the presence of Vip1 and Vip2. A careful analysis of the results reveal that Vip1 (2.3 kb) molecular weight was 86980.15 Daltons and contained 778 amino acids and its isolectric point was at pH 5.790. Sequence analysis of Vip2 (1.3 kb) showed that the molecular weight was 51112.35 Daltons and contained 448 amino acids. Also, no convincing similarity was found among Vip1/Vip2 proteins, and any other previously described *B. thuringiensis.* Cry, Cyt or Vip3 proteins could be observed but a significant match to other enzymatic components of other binary toxins such as *Bacillus cereus* Vip2Aa toxin, *Clostridium botulinum* C2 toxin, *C. perfringens* iota toxin, *C. spiroforme* toxin and *C. difficle* toxin was not noticed. An alignment of BtVip1 peptide sequence with other Vip1 toxins revealed 77 to 99% (Figure 6a).

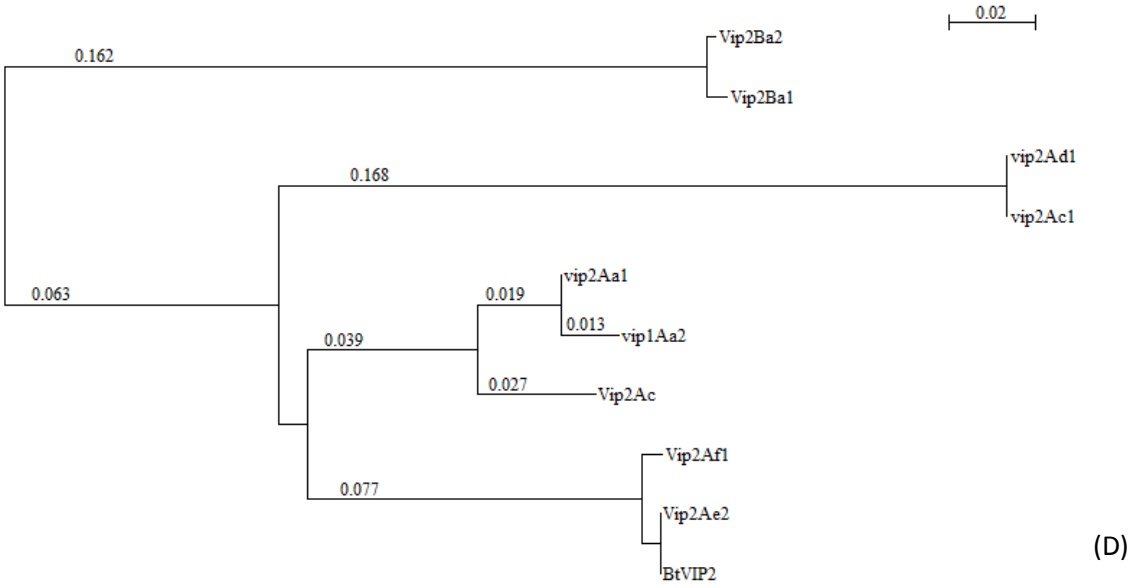
The phylogenetic tree generated by ClustalW showed that Vip1A (BR) and BtVip1 (our isolate) were the same branch as Vip1Ba1, Vip1Ba2 and Vip1Bb (Figure 6b). Vip1Ca1 and Vip1Ac peptide sequences had low identity to other Vip1 proteins and they were located on a separate branch from other Vip1 proteins (Figure 6b). An alignment of BtVip2 peptide sequence with other Vip2A toxins, Vip2B toxins and other binary toxins revealed 77 to 99% (Figure 6c).

The phylogenetic tree generated by ClustalW showed that BtVip2 was the same branch as Vip2Aa1, Vip2Aa2 and Vip2Ac (Figure 6d). BtVip2 peptide sequence had low identity to Vip2Ba1 and vip2Ba2 proteins and they were located on a separate branch from other Vip2 proteins (Figure 6d). An 11.9% distribution of Vip1 and Vip2 genes among 463 Bacillus strains belonging to *B. thuringiensis* and *B. cereus* were indicated by previous researchers (Warren et al., 1996).

However, there are some discrepancies between their results and ours. Only four strains produced the expected PCR products showing a 4% existence of Vip1/Vip 2 genes in *B. thuringiensis*. It was perhaps due to the fact that these two genes have a wider distribution in *B. cereus* than in *B. thuringiensis* that most of our strains belong to (Bt). At present, protein expression and purification of Vip1 and Vip2 proteins, and its insecticidal activity against different insect pests are under investigation.


BtVIP2 1 MKRMEGKLFMVSKKLQLVTKALLFSTVLSIPLLNNEEVKAEHLNLNSQSKYPSFQNQKITDNAEDFKEDKEKAKEWGEVK
Vip2Ae2 1 MKRMEGKLFMVSKKLQLVTKALLFSTVLSIPLLNNEEVKAEHLNLNSQSKYPSFQNQKITDNAEDFKEDKEKAKEWGEVK
Vip2Af1 1 MKRMEGKLFMVSRKLQLVTKALLFSTVLSIPLLNNEEVKAEHLNLNSQSKYPSFQNQKITDNAEDFKEDKEKAKEWGEVK
vip1Aa2 1 -------MFMVSKKLQVVTKTVLLSTVFSISLLNNEVIKAEQLNINSQSKYTNLQNLKITDKVEDFKEDKEKAKEWGKEK
vip2Aa1 1 MKRMEGKLFMVSKKLQVVTKTVLLSTVFSISLLNNEVIKAEQLNINSQSKYTNLQNLKITDKVEDFKEDKEKAKEWGKEK
Vip2Ac 1 MKRMEGKLFMVSTKLQAVTKAVLLSTVLSISLLNNEVIKAEQLNMNSQNKYTNFENLKITDKVEDFKEDKEKAKEWGKEK
vip2Ac1 1 ---MIVIIFTNVKGGNELKKNFYKNLICMSALLLAMPISSNVTYAYGSEKVDYL--VKTTNNTEDFKEDKEKAKEWGKEK
vip2Ad1 1 ---MIVIIFTNVKGGNELKKNFYKNLICMSALLLAMPISSNVTYAYGSEKVDYL--VKTTNNTEDFKEDKEKAKEWGKEK
Vip2Ba1 1 ---------MVSKKLQLITKTLVFSTVLSIPLLNNSEIKAEQLNMNSQIKYPNFQNINIADKPVDFKEDKEKAREWGKEK
Vip2Ba2 1 MKRMEERLFMVSKKLQLITKTLVFSTVLSIPLLNNSEIKAEQLNMNSQIKYPNFQNINIADKPVDFKEDKEKAREWGKEK
BtVIP2 81 EKEWKLTATEKRKINDFLNDTNKIKTNYKEITFSMAGSFEDELKDLKEIDKMFDKANLSSSIITYKNVEPATIGFNKSLT
Vip2Ae2 81 EKEWKLTATEKRKINDFLNDTNKIKTNYKEITFSMAGSFEDELKDLKEIDKMFDKANLSSSIITYKNVEPATIGFNKSLT
Vip2Af1 81 EKEWKLTATEKRKINDFLNDTNKIKTNYKEITFSMAGSFEDELKDLKEIDKMFDKANLSSSIITYKNVEPATIGFNKSLT
vip1Aa2 74 EKEWKLTATEKGKMNNFLDNKNDIKTNYKEITFSMAGSFEDEIKDLKEIDKMFDKTNLSNSIITYKNVEPTTIGFNKSLT
vip2Aa1 81 EKEWKLTATEKGKMNNFLDNKNDIKTNYKEITFSMAGSFEDEIKDLKEIDKMFDKTNLSNSIITYKNVEPTTIGFNKSLT
Vip2Ac 81 EKEWKLTATEKGKMNNFLDNKNDIKTNYKEITFSMAGSFEDEIKDLKEIDKIFDKANLSSPIITYKNVEPATIGFNKSLT
vip2Ac1 76 EKEWKLTVTEKTRMNNFLDNKNDIKKNYKEITFSMAGSFEDEIKDLKEIDKMFDKANLSSSIVTYKNVEPSTIGFNKPLT
vip2Ad1 76 EKEWKLTVTEKTRMNNFLDNKNDIKKNYKEITFSMAGSFEDEIKDLKEIDKMFDKANLSSSIVTYKNVEPSTIGFNKPLT
Vip2Ba1 72 EKEWKLTVTEKGKINDFLDDKDGLKTKYKEINFSKNFEYETELKELEKINTMLDKANLTNSIVTYKNVEPTTIGFNQSLI
Vip2Ba2 81 EKEWKLTATEKGKINDFLDDKDGLKTKYKEINFSKNFEYETELKELEKINTMLDKANLTNSIVTYKNVEPTTIGFNQSLI
BtVIP2 161 EGNTINSDVMAQFKEQFLGKDIKFDSYLDTHLTVQQVSSKERVILKVTVPSGKGSTTPTKAGVILDGNEHKMLIDNGYVL
Vip2Ae2 161 EGNTINSDVMAQFKEQFLGKDIKFDSYLDTHLTVQQVSSKERVILKVTVPSGKGSTTPTKAGVILDGNEHKMLIDNGYVL
Vip2Af1 161 EGNTINSDVMAQFKEQFLGKDIKFDSYLDTHLTVQQVSSKERVILKVTVPSGKGSTNPTKAGVILDGNEPKMLIDNGYVL
vip1Aa2 154 EGNTINSDAMAQFKEQFLDRDIKFDSYLDTHLTAQQVSSKERVILKVTVPSGKGSTTPTKAGVILNNSEYKMLIDNGYMV
vip2Aa1 161 EGNTINSDAMAQFKEQFLDRDIKFDSYLDTHLTAQQVSSKERVILKVTVPSGKGSTTPTKAGVILNNSEYKMLIDNGYMV
Vip2Ac 161 EGNTINSDAMAQFKEQFLDRDIKFDSYLDTHLTVQQVSSKERVILKVKVPSGKGSTTPTKAGIILNNSEYKMLIDNGYMV
vip2Ac1 156 EGNTINTDVQAQFKEQFLGKDIKFDSYLDTHLTAQNVSSKERIILQVTVPSGKGSTIPTKAGVILNNNEYKMLIDNGYVL
vip2Ad1 156 EGNTINTDVQAQFKEQFLGKDIKFDSYLDTHLTAQNVSSKERIILQVTVPSGKGSTIPTKAGVILNNNEYKMLIDNGYVL
Vip2Ba1 152 EGNQINAEAQQKFKEQFLGQDIKFDSYLDMHLTEQNVSSKERVILKVTVPSGKGST-PTKAGVVLNNNEYKMLIDNGYVL
Vip2Ba2 161 EGNQINAEAQQKFKEQFLGQDIKFDSYLDMHLTEQNVSSKERVILKVTVPSGKGST-PTKAGVVLNNNEYKMLIDNGYVL
BtVIP2 241 HVDKVSKVVKRGLECLQVEGTLKKSLDFKNDISAKAHSWGMKNYEEWAANLTDSQRKALDGYARQDYKKINDYLRNQGGS
Vip2Ae2 241 HVDKVSKVVKRGLECLQVEGTLKKSLDFKNDISAKAHSWGMKNYEEWAANLTDSQRKALDGYARQDYKKINDYLRNQGGS
Vip2Af1 241 HVDKVSKVVKKGLECLQVEGTLKKSLDFKNDISAKAHSWGMKNYEEWAANLTDSQRKALDGYARQDYKKINDYLRNQGGS
vip1Aa2 234 HVDKVSKVVKKGVECLQIEGTLKKSLDFKNDINAEAHSWGMKNYEEWAKDLTDSQREALDGYARQDYKEINNYLRNQGGS
vip2Aa1 241 HVDKVSKVVKKGVECLQIEGTLKKSLDFKNDINAEAHSWGMKNYEEWAKDLTDSQREALDGYARQDYKEINNYLRNQGGS
Vip2Ac 241 HVDKVSKVVKKGVECLQVEGTLKKSLDFKNDINAGAHSWGMKNYEEWAKDLTDLQREALDGYARQDYKEINNYLRNQGGN
vip2Ac1 236 HVDNISKVVKKGYECLQIQGTLKKSLDFKNDINAEAHRWGMKNYEGWAKNLTDPQREALDGYARQDYKQINDYLRNQGGS
vip2Ad1 236 HVDNISKVVKKGYECLQIQGTLKKSLDFKNDINAEAHRWGMKNYEGWAKNLTDPQREALDGYARQDYKQINDYLRNQGGS
Vip2Ba1 231 HVENITKVVKKGQECLQVEGTLKKSLDFKNDSDGKGDSWGKKNYKEWSDTLTTDQRKDLNDYGVRGYTEINKYLR-EGDT
Vip2Ba2 240 HVENITKVVKKGQECLQVEGTLKKSLDFKNDSDGKGDSWGKKNYKEWSDTLTTDQRKDLNDYGARGYTEINKYLR-EGGT
BtVIP2 321 GNEQLDAQIKNISETLNNKPIPENITVYRWCGMPEFGYQISEPLPALKDFEWEFLNTIKEDKGYISTSLSSERLAAFGSR
Vip2Ae2 321 GNEQLDAQIKNISETLNNKPIPENITVYRWCGMPEFGYQISEPLPALKDFEWEFLNTIKEDKGYISTSLSSERLAAFGSR
Vip2Af1 321 GNEQLDAQIKNISETLNNKPIPENITVYRWCGMPEFGYQISEPLPALKDFEWEFLNTIKEDKGYISTSLSSERLAAFGSR
vip1Aa2 314 GNEKLDAQIKNISDALGKKPIPENITVYRWCGMPEFGYQISDPLPSLKDFEEQFLNTIKEDKGYMSTSLSSERLAAFGSR
vip2Aa1 321 GNEKLDAQIKNISDALGKKPIPENITVYRWCGMPEFGYQISDPLPSLKDFEEQFLNTIKEDKGYMSTSLSSERLAAFGSR
Vip2Ac 321 GNEKLDAQIKNISDALGKKPIPENITVYRWCGMPEFGYQISDPLPSLKDFEEQFLNTIKEDKGYMSTSLSSERLAAFGSR
vip2Ac1 316 GNEKLDTQIKNISEALEKQPIPENITVYRWCGMAEFGYQISDPLPSLKEMEEKFLNTMKEDKGYMSTSLSSERLSAFGSR
vip2Ad1 316 GNEKLDTQIKNISEALEKQPIPENITVYRWCGMAEFGYQISDPLPSLKEMEEKFLNTMKEDKGYMSTSLSSERLSAFGSR
Vip2Ba1 310 GNTELEEKIKNISDALEKNPIPENITVYRYCGMAEFGYPIKPEAPSVQDFEERFLDTIKEEKGYMSTSLSSD-ATSFGAR
Vip2Ba2 319 GNTELEEKIKNISDALEKNPIPENITVYRYCGMAEFGYPIKPEAPSVQDFEERFLDTIKEEKGYMSTSLSSD-ATSFGAR
BtVIP2 401 KIILRLQIPKGSKGAYLSAIGGFA-NEKEILLDKDSKYHINKITEVVIKGIKRYVVDATLLTN
Vip2Ae2 401 KIILRLQIPKGSKGAYLSAIGGFA-NEKEILLDKDSKYHINKITEVVIKGIKRYVVDATLLTN
Vip2Af1 401 KIILRLQIPKGSKGAYLSAIGGFA-NEKEILLDKDSKYHINKITEVVIKGIKRYVVDATLLTN
vip1Aa2 394 KIILRLQVPKGSTGAYLSAIGGFA-SEKEILLDKDSKYHIDKVTEVIIKVLSDM---------
vip2Aa1 401 KIILRLQVPKGSTGAYLSAIGGFA-SEKEILLDKDSKYHIDKVTEVIIKGVKRYVVDATLLTN
Vip2Ac 401 KIILRLQVPKGSTGAYLSAIGGFA-NEKEILLDKDSKYHIDKVTEVIIKGVKRYVVDATLLTN
vip2Ac1 396 KFILRLQVPKGSTGAYLSAIGGFA-SEKEILIDKDSNYHIDKITEVVIKGVKRYVVDATLLTK
vip2Ad1 396 KFILRLQVPKGSTGAYLSAIGGFA-SEKEILIDKDSNYHIDKITEVVIKGVKRYVVDATLLTK
Vip2Ba1 389 KIILRLQVPKGSSGAYVAGLDGFKPAEKEILIDKGSKYRIDKVTEVVVKGTRKLVVDATLLTK
Vip2Ba2 398 KIILRLQVPKGSSGAYVAGLDGFKPAEKEILIDKGSKYRIDKVTEVVVKGTRKLVVDATLLTK

(c)



(D)

Figure 6(C) and (D). Btvip2 protein. Black, deep and french gray blocks represents 100, 80 and 60% identity, respectively. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events.

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