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PCR-Based Identification and Detection of *Cry*1 Genes in Some *Bacillus thuringiensis* Isolates

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ABSTRACT

PCR amplification was performed using two universal primers (Un1 and Un2) designed from among the conserved regions of *cry1B* and *cry1Aa* genes. A total of 14 and 5 fragments were displayed among different local *Bacillus thuringiensis* (*Bt*) isolates using Un1 and Un2, respectively. Each isolate differed from the others based on specific amplified patterns of PCR products. Genetic distances calculated from amplified fragments revealed three and two clusters with Un1 and Un2, respectively. Nei's genetic similarity index showed some isolates to be similar while others to be divergent. Furthermore, two pairs of specific primers were designed to identify *cry1B* and *cry1Aa* genes among 14 local *Bt* isolates. Analysis with *cry1B* primer showed that a 1.35-kb fragment was amplified from seven isolates, while with *cry1Aa* primer a 1.30-kb fragment was amplified from five of the seven isolates. SDS-PAGE protein profiles of some local *Bt* isolates, two *Bt* standards and a commercial product revealed a total of 37 protein bands, 21 of which were commonly detected in the seven *Bt* isolates, while the remaining 16 bands showed high variability among the isolates. Protein analysis of the seven isolates showed that both the total and the variable bands were higher in the five isolates containing the two genes (*cry1B* and *cry1Aa*) than the other two isolates containing the *cry1B* gene only. Some unique bands with different density and intensity were detected among the 14 isolates which discriminated them with different characteristic protein profiles, even those isolates with no known or identified δ-endotoxin genes.

Keywords: PCR, universal and specific primers, local *Bt* isolates, cry1 genes, SDS-PAGE

INTRODUCTION

Bacillus thuringiensis (*Bt*) is a spore-forming bacterium producing, upon sporulation, parasporal crystal δ-endotoxin proteins toxic to some invertebrates, mostly insects and nematodes (Crickmore *et al.* 1998). The need for novel environmentally friendly crystal proteins has prompted the development of molecular approaches to quickly and easily characterize toxin genes present in *Bt* isolates. The polymerase chain reaction (PCR) is a molecular tool widely used to characterize *Bt*. It has proven to be a rapid and reliable method and has largely substituted bioassays in preliminary classification of *Bt* collections (Porcar and Juárez-Pérez 2003). An extended multiplex PCR method was established to rapidly identify and classify *Bt* strains containing *cry* genes toxic to species of Lepidoptera, Coleoptera, and Diptera (Ben-Dov *et al.* 1997). A two-step strategy based on PCR has been developed which allows the ability to detect and further clone and sequence genes for which no specific primers are available and in which a variable region exists between two conserved regions (Juárez-Pérez *et al.* 1997). Forty three *Bt* isolates from Brazil and 3 from Argentina were screened using PCR for various Coleoptera-specific *cry* genes (Márquez *et al.* 2000). Moreover, the analysis of 496 *Bt* strains collected from Mexico was based on multiplex PCR with novel general and specific primers to detect different *cry* genes (Bravo *et al.* 1998). Six *Bt* isolates obtained from soil in Nigeria, confirmed to be toxic to mosquito larvae, were differentiated using a PCR-based technique (Ogunjimi *et al.* 2000). A pair of highly degenerated primers was used by Shevelev *et al.* (1998) to carry out a single step PCR-detection of any known and probably unknown *cry* genes of *cry1*, *cry4* and *cry9* encoding a 130-kD protein δ-endotoxin in natural *Bt* strains. Thirty different PCR profiles were found in Colombia *Bt* native strains with *cry1* genes when they were analyzed with specific primers from *cry1A* to *cry1F* (Uribe *et al.* 2003). To verify the δ-endotoxin gene types of K1, Li *et al.* (2002) performed PCR analysis with specific *cry* gene primers. They proved that K1 contained a new *cry* gene in addition to *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1E* and *cry2*. With PCR products as probes, Kuo *et al.* (2000) cloned two new *cry*-type genes from *B. thuringiensis* subsp. *wuhanensis*. These two clones were named *cry1Gb1*, toxic to *Plutella xylostella* larvae, and *cry1Bd1*, toxic to *Pieris rapae* larvae. Two genes encoding the 32 and 40 kD polypeptides of *Bt* crystals were cloned and expressed in an acrystalliferous *Bt* strain and sequenced (Kim *et al.* 2003). A new *cry1Ca9* gene encoding a protein of 1189 amino acid residues was cloned and sequenced from a *Bt* isolate native to Taiwan (Kao *et al.* 2003). A total of 445 isolates from a collection of Colombian *Bt* strains were characterized. The parasporal *cry*stal morphology that was most abundant was bipyramidal (60%). Almost 10% of the isolates were toxic to *Spodoptera frugiperda* and 5.6% against *Culex quinquefasciatus* larvae. *cry* gene content determined by PCR indicated that 10.6% of the isolates contained *cry*1 genes and 1.1% contained *cry*2, *cry*4 or *cry*11 genes. Protein content of the parasporal crystal was determined by SDS-PAGE; 25 and 18 different protein profiles were found in isolates active against *S. frugiperda* and *C. quinquefasciatus*, respectively (Armengol *et al.* 2007). Forty six isolates (65.7%) of a native Iranian *Bt* collection contained a minimum of one Coleopteran-active *cry* gene. Based on universal primers, strains containing *cry*18 and *cry*26 genes were the most abundant and represent 27.1 and 24% of the isolates, respectively, whereas *cry*14, *cry*3, *cry*28, *cry*34, *cry*35, *cry*7, *cry*8 genes were less abundant, found in 14.2, 12.5, 10, 7, 7

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and 5.6% of the strains, respectively. Based on specific primers, isolates containing *cry*1I were the most abundant (48.5%). Two strains containing Coleopteran-active *cry* genes showed higher activity against *Xanthogaleruca luteola* Mull. larvae than *B. thuringiensis* subsp. *morrisoni* pathovar *tenebrionis*. Thirty isolates, when assayed for *cry*1C, *cry*5, *cry*6, *cry*8b, *cry*9, *cry*10, *cry*11, *cry*18, *cry*24 and *cry*35 genes, showed unexpected size bands (Nazarian *et al.* 2009). da Costa *et al.* (2010) aimed to select *B. thuringiensis* isolates efficient against *A. aegypti* larvae. DNA was isolated from a bacterial collection containing 1,073 isolates of *Bt*, obtained from different locations of Brazilian territory, and amplified by PCR using specific primers for *cry*4Aa, *cry*4Ba, *cry*11Aa, *cry*11Ba, *cyt*1Aa, *cyt*1Ab, *cyt*2Aa and *chi* genes. Only 45 isolates (4.2%) presented amplicons for the *cry* and *cyt* genes. The *chi* gene sequence was detected in 25 (54.3%) of those isolates. From the 45 isolates submitted to the selective bioassays, 13 caused 100% mortality of *Aedes aegypti* larvae. The identification of *cry*, *cyt* and *chi* genes of *B. thuringiensis* and the toxicity analysis on *A. aegypti* led to the selection of a set of isolates that have the potential to be used in the formulation of new bioinsecticides.

Eight *Bt* isolates were selected from rural soil, 15 from urban soil and 11 from insects. These were evaluated for entomopathogenicity against larvae of *Anticarsia gemmatalis* and *Culex quinquefasciatus* (Gobatto *et al.* 2010). The pathogenicity tests showed that a higher percentage of isolates were active against *A. gemmatalis* (60%) compared to *C. quinquefasciatus* (31%). SDS-PAGE characterization of two isolates showed a 27-kDa protein fraction related to the *Bt* subspecies *israelensis* cytolytic toxin (cyt) gene. One 130-kDa protein, possibly related to the *Bt cry*stal inclusions (*cry*1) gene, was identified in the other two isolates, which were more toxic to Lepidoptera; another isolate presented a 100-kDa protein. Published data on insecticidal activity of crystal proteins from *Bt* are incorporated into the *Bt* toxin specificity relational database. To date, 125 of the 174 known holotype toxins have been tested in approximately 1700 bioassays against 163 test species, 49 toxins have not been tested at all, 59 were tested against 71 Lepidoptera species in 1182 bioassays, 53 toxins were tested against 23 Diptera species in 233 bioassays, and 47 were tested against 39 Coleoptera species in 190 bioassays (Biomedexperts.com 2011). The activity spectra of the tested toxins were summarized for each order. There are an increasing number of toxin families with cross-order activity, as 15 of the 87 families (secondary rank) that are pesticidal and are active against more than one order. Cross-order activity does not threaten environmental safety of *Bt*-based pest control because toxins tend to be much less toxic to taxa outside the family's primary specificity range (van Frankenhuyzen 2009). Baig *et al.* (2010) aimed to search for *Bt* harboring the *cry*1A gene which could effectively control cotton pest, American bollworm, *Helicoverpa armigera*. *Bt* isolates showed the presence of the *cry*1, *cry*2 and *cry* genes profiling 50 *cry*3, *cry*4, *cry*7, *cry*8 and *cry*9 genes. None of the isolates harbored the *cry*1 gene alone; it was always found in combination with *cry*3. There was no positive isolate for the *cry*10 gene. Considering isolates with single *cry* genes, the frequency of *cry*4 was predominant (22%) followed by *cry*2 (6%), *cry*3 (4%) and *cry*8 (2%). Isolates having two *cry* genes in combination had 14% incidence for *cry2*+*cry4*, 12% for *cry3*+*cry4* and 10% for *cry1*+*cry3*. The three most dominant gene linkages were *cry1*+*cry3*+*cry4*. Further profiling of the *cry*1 gene showed that the *cry*1K + *cry*3 + *cry*1 gene was abundantly present in all combinations such as *cry*1A, *cry*1D, *cry*1F and *cry*1I. However, *cry*1C existed independent of other subtypes.

A total of 28 autoagglutinating strains of *Bt* were isolated from different ecologic niches and distinct sites. Twenty-six strains demonstrated toxicity to mosquito larvae of *A. aegypti* and *Culex quinquefasciatus*. The electrophoretic protein profiles of the crystal components were studied. The same larvicidal activity and the same protein profiles as *B. thuringiensis* serovar *israelensis* were shown by 23/28 strains. RAPD analysis was evaluated using six primers, which demonstrated three different patterns for the 28 autoagglutinating strains, allowing correlation of the profiles obtained with the toxicity observed in the bioassays. The RAPD patterns for mosquitocidal strains were identical to the one of serovar *israelensis*. However, to strains of low toxicity, each primer generated distinctive RAPD patterns, which demonstrated that these strains belong to different serovars. Although the antigenic classification the 26 autoagglutinating strains of *Bt* could not be determined by classical flagellar serotyping and RAPD profiles proved these strains to be compatible with *B. thuringiensis* serovar *israelensis* (Chaves *et al.* 2008).

The aim of the present work was to characterize some local Egyptian *Bt* isolates using RAPD-PCR and to detect *cry1*-type genes among the isolates, as well as to predict their insecticidal activities from toxic protein analysis.

MATERIALS AND METHODS

Bacterial strains

Seventeen local *B. thuringiensis* (*Bt*) strains isolated from soil, *B. thuringiensis* subsp. *thuringiensis* HD-24 and *B. thuringiensis* subsp. *Kurstaki* HD-1 (used as standard *Bt* strains) and the commercial product, Dipel 2x were used in this study (**Table 1**).

DNA extraction from Bacillus thuringiensis

The *Bt* culture was incubated overnight at 30°C in Luria bertani (LB) broth medium with shaking. LB medium (5 ml) was inoculated with 0.1 ml of the overnight culture and incubated at 30°C for 3 h with vigorous shaking. Cells were pelleted by centrifugation for 15 min at 10,000 rpm and resuspended in 100 µl of sterile double-distilled water. Cells were disrupted by incubation for 10 min in boiling water bath followed by rapid chilling on ice. Cell debris was removed by centrifugation for 15 min at 10,000 rpm and 5 µl of the supernatant were used as a DNA template for PCR (Carozzi *et al.* 1991).

RAPD-PCR analysis

PCR amplification was performed in 20 µl reaction volume containing 200 µM each of dATP, dCTP, dGTP, dTTP, 1 unit *Taq* polymerase (Gibco), 20 pmole random primer (Operon), 20 ng genomic DNA and appropriate amplification buffer. The mixture

Table 1 Bacterial strains and *Bt* local isolates with their sources.

Serial No.	Bacterial isolates and strains	Source
1	Is-1	*Bt* isolated from Ismailyia
2, 3	Sn-3, Sn-4	" " " Sinai
4, 5	Gh-2, Gh-3	" " " Gharbya
6, 7	Qa-6, Qa-7	" " " Qaluobyia
8	Fa-8	" " " El-Fayioum
9, 10, 11, 12	As-2, As-5, As-11, As-6	" " " Aswan
16, 17	Ts-1, Ts-7	*Bt* isolated from Toshki
13	*B. thuringiensis kurstaki* HD-1	*Bacillus* Genetic Stock Center (BGSC)
14	*B. thuringiensis* HD-24	
15	Dipel 2X	Commercial *Bt* product

Table 2 The nucleotide sequences of two random primers and two specific primer pairs of *cry* genes.

Primers	Sequence (5′-3′)
Universal primers:	
Un1	5′ TGAAAACAGTA 3′
Un2	5′ TAAAATTAGTTCG 3′
Specific primers:	
cry1Aa	5′ TGCAGCTCCACCCGTACTTGTCTCAT 3′
cry1Aa (r)	5′ TTCCGTCAATTGATGTATAATTG 3′
cry1B	5′ GGCTACCAATACTTCTATTA 3′
cry1B (r)	5′ CTCTTCCCCATTTCCAC 3′

was assembled on ice and DNA amplification was carried out using Biometra thermal cycler by a denaturing step for 3 min at 92°C followed by 45 cycles for 30 sec at 92°C, 35°C for 60 sec and 72°C for 2 min.

PCR amplification of *cry* genes

The presence of selected *cry* genes was detected by PCR according to Xu and Côté (2003). The forward and reverse primers for *cry1Aa* and *cry1B* genes were designed according to Kalman *et al*. (1993). The sequences of the two random primers (Un1 and Un2) as well as the forward and reverse primers of *cry1Aa* and *cry1B* are shown in **Table 2**. PCR amplification products were separated using agarose gel electrophoresis in 1.2% TAE buffer and stained with 0.2 µg/ml ethidium bromide according to Sambrook *et al.* (1989). Nucleic acid bands were detected and photographed under UV light.

Data analysis

The gels were scanned using the Gel Doc 2000 System (Bio-Rad Laboratories, USA). The average genetic distance coefficient D was calculated from amplified fragment data using the following equation:

$$D = 1-2N_{xy} / (N_x-N_y)$$

where N_x = the total number of amplified fragments from strain X, N_y = the total number of amplified fragments from strain Y and N_{xy} = the number of amplified fragments shared by both strain X and Y (Nei and Li 1979). A dendrogram was constructed on the basis of the similarity matrix data by the Unweighted Pair Group Method with Average (UPGMA) cluster analysis (Kumar *et al.* 1993).

Preparation and analysis of spore/crystal mixture

Bacteria were grown in suspension following the method of Alberola *et al.* (1999). Nutrient broth (100 ml) was inoculated into 500-ml flasks with one loop of bacteria and shaken for 3 days at 30°C (220 rpm). At the end of this incubation period, the majority of the population was in the form of free spores and crystals (less than 5% vegetative cells). The suspension was centrifuged for 10 min at 10,000 rpm at 4°C; the pellet was washed twice in water and resuspended in 4 ml of water. This suspension was adjusted with water to give an absorbance of 15 at 600 nm. The supernatants of toxic samples were autoclaved (121°C for 10 min). Colonies were resuspended into 1 ml of ice-cold 0.5 M NaCl. The cells were centrifuged at 13,000 rpm for 5 min. The pellet was resuspended in 1% SDS, 0.01% β-mercaptoethanol, boiled for 10 min, and recentrifuged at 13,000 rpm for 10 min. The supernatant was removed and analyzed by 15% SDS–PAGE. The gel was stained with silver staining method (Echt *et al.* 1996).

RESULTS AND DISCUSSION

Genetic characterization of local *Bt* isolates by RAPD-PCR

PCR amplification was performed using two universal primers Un1 and Un2 selected from a region of the *cry* genes highly conserved in the two oligonucleotide sequences. Not all 17 *Bt* isolates were amplified with the two primers. However, the amplified *Bt* samples for Un1 and Un2 primers are presented in **Fig. 1** and **2**, respectively. The analysis of PCR-amplified products with Un1 primer among different local *Bt* isolates are presented in **Fig. 1**. A total of 14 fragments with sizes ranging from 1.740-0.213 kb were displayed among 10 local *Bt* isolates (**Table 3**). The results revealed that most of the *Bt* isolates are different according

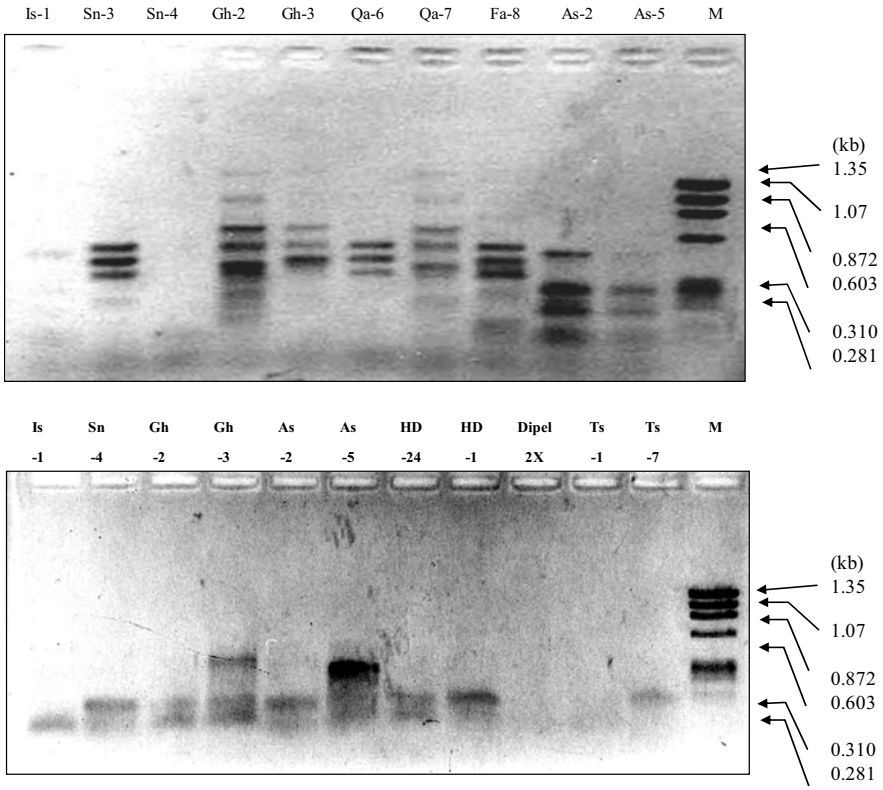

Fig. 1 PCR amplification products generated using two primers Un1 and Un2 of different *Bt* isolates. M = Molecular weight marker (øX174 digested by *Hae*III).

Table 3 PCR amplified products of Un1 primer among ten different *Bt* local isolates.

Band No.	Band size (kb)	*Bt* local isolates									
		Is-1	Sn-3	Sn-4	Gh-2	Gh-3	Qa-6	Qa-7	Fa-8	As-2	As-5
1	1.740				+	+		+			
2	1.256				+	+		+			
3	0.950								+		
4	0.888				+	+		+			
5	0.706		+		+	+	+	+	+		
6	0.650	+								+	+
7	0.632		+			+	+		+		
8	0.564	+			+			+			
9	0.497		+		+		+		+		
10	0.424				+				+	+	+
11	0.320		+					+			
12	0.343	+								+	+
13	0.293				+				+		
14	0.213	+		+						+	+
No of bands		4	4	1	8	5	3	6	6	4	4

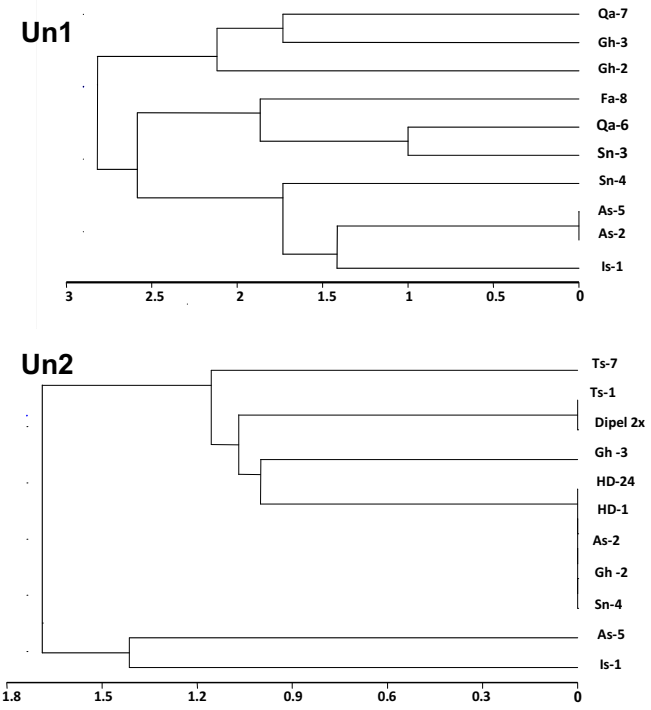

Fig. 2 Genetic distances of different *Bt* isolates using the PCR products of two universal primers Un1 and Un2.

to the specific amplified patterns of the PCR products, except for As-2 and As-5, which showed a similar pattern with four fragments. Three fragments (1.74, 1.256 and 0.95 kb) were uniquely detected and could characterize three isolates: Gh-2, Gh-3 and Qa-7. Two fragments were detected in two different strains only and not in the remaining strains, whereas the first fragment, 0.320 kb in size, existed in Sn-3 and Qa-7 while the second, 0.293 kb in size, existed in Gh-2 and Fa-8; they could both be used as markers for such strains. Another distinct single band ~0.95-kb in size was obtained from fa-8 (**Table 3**). Three isolates (Gh-2, Qa-7 and Fa-8) showed high band numbers (8, 6 and 6, respectively) while Sn-4 and Qa-6 displayed 1 and 3 bands, respectively and could not be precisely characterized.

Genetic distances between the 10 local *Bt* isolates were calculated from the amplified fragment data using UPGMA. A dendrogram was constructed using the coefficient D (**Fig. 1**). From the UPGMA dendrogram, three clusters were divided on the basis of the taxonomic threshold (**Fig. 2**). Genetic similarity between each pair of the 10 isolates revealed that some isolates were closely similar, such as As-2 and As-5 and Qa-6 and Sn-3 while no similarity was observed between Sn-4 and five isolates, and Sn-3 and three isolates (**Table 4**). The analysis of the PCR-amplified products with the universal Un2 primer showed a total of five fragments with sizes ranging from 0.736 to 0.272 kb among eight local *Bt* isolates, two standards *Bt* and a commercial product (**Table 5**). Among the five fragments, band number 5 0.072-kb in size was commonly detected in most isolates, except for As-5 and Ts-7. However, two unique fragments (0.736 and 0.624 kb) were detected in isolates Is-1 and Gh-3, respectively. One fragment with 0.581 kb characterized two isolates Is-1 and As-5 as being unique while the other two fragments (0.361 and 0.272 kb) were detected in two standard *Bt* strains HD-1 and HD-24, as well as in four isolates, but were not present in the commercial *Bt* strain (**Fig. 1; Table 5**). The genetic similarity index between each pair of the eight isolates revealed that some isolates are closely similar, such as (As-2, Gh-2), (Sn-4, Gh-2) and (Ts-1, Ts-7). The two standard *Bt* are mostly similar with each other and also with most isolates, but not with As-5 and Is-1, although no similarity was observed between (Ts-7 and Is-1) and As-5 and most isolates, except for Is-1 (**Table 6**). The UPGMA dendrogram, representing the genetic distances between the eight isolates, revealed two clusters (**Fig. 2**). The results showed a relatively good link between the *Bt* isolates and PCR-amplified patterns. Hence, PCR based on universal primers of *cry* genes could provide a valuable and reliable tool for discriminating local *Bt* isolates. Further, development of this simple molecular approach could be very informative and beneficial, particularly with respect to screening procedures. The results are supported by those of Yu *et al.* (2002), who classified 35 strains of *Bt* using the similarity coefficient and average genetic distance coefficient D, based on differences between major and minor bands of PCR products. Eight *Bt* strains were isolated from soils and identified according to their morphology, the presence of parasporal crystals and a 1400-bp band PCR amplification of the 16S rDNA gene. A bioassay revealed that Ts-5 and As-3 were highly toxic to the 2nd instar larvae of cotton leafworm (*Spodoptera littoralis*) with 100 and 90% mortality, respectively after 4 feeding days. As-4 and Fa-7 showed high mortality (90 and 80%) against northern house mosquito (*Culex pipiens*) larvae after a two-day feeding period. A plasmid profile revealed divergent patterns in the number, molecular size and existence of plasmids, whereas Ts-5 and Sn-2 showed the highest number (7) and Qa-2 the lowest (3). A 3-kb plasmid was found in all isolates and the seven other plasmids varied noticeably among the eight isolates. SDS-PAGE analysis of the spore/crystal mixture individually characterized each of the eight isolates. Protein analysis revealed that each of the eight isolates possessed a unique protein pattern either in their absence, presence or in total numbers. Moreover, protein banding patterns were also quite different between isolates from the same location, such as As-3 and As-4 although they displayed similar total

Table 4 Genetic similarity percentages of ten *Bt* isolates, based on RAPD products of Un1 primer.

	***Bt* local isolates**									
	Is-1	**Sn-3**	**Sn-4**	**Gh-2**	**Gh-3**	**Qa-6**	**Qa-7**	**Fa-8**	**As-2**	**As-5**
Is-1										
Sn-3	00.0									
Sn-4	40.0	00.0								
Gh-2	16.7	33.3	00.0							
Gh-3	00.0	44.4	00.0	61.5						
Qa-6	00.0	85.7	00.0	36.4	50.0					
Qa-7	20.0	40.0	00.0	71.4	72.7	22.2				
Fa-8	20.0	60.0	00.0	42.9	36.4	66.7	16.7			
As-2	75.0	00.0	40.0	16.7	00.0	00.0	00.0	40.0		
As-5	75.0	00.0	40.0	16.7	00.0	00.0	00.0	40.0	100.0	

Table 5 PCR products of Un2 primer among different *Bt* local isolates.

Band No	**Size (kb)**	***Bt* local isolates**								**Standards**		**Dipel**
		Is-1	**Sn-4**	**Gh-2**	**Gh-3**	**As-2**	**As-5**	**Ts-1**	**Ts-7**	**HD-1**	**HD-24**	**2X**
1	0.736	+										
2	0.624				+							
3	0.581	+					+					
4	0.361		+	+	+	+			+	+	+	
5	0.272	+	+	+	+	+		+		+	+	+
No of bands		3	2	2	3	2	1	2	1	1	2	1

Table 6 Genetic similarity of eight Bt isolates, two standards and Dipel 2x, based on RAPD products of Un2 primer.

	***Bt* local isolates**								**Standard *Bt***		**Dipel**
	Is-1	**Sn-4**	**Gh-2**	**Gh-3**	**As-2**	**As-5**	**Ts-1**	**Ts-7**	**HD-1**	**HD-24**	**2X**
Is-1											
Sn-4	40.0										
Gh-2	40.0	100.0									
Gh-3	33.3	80.0	80.0								
As-2	40.0	100.0	100.0	80.0							
As-5	50.0	00.0	00.0	00.0	00.0						
Ts-1	50.0	66.7	66.7	50.0	66.7	00.0					
Ts-7	00.0	66.7	66.7	50.0	66.7	00.0	100.0				
HD-1	40.0	100.0	100.0	80.0	100.0	00.0	66.7	66.7			
HD-24	40.0	100.0	100.0	80.0	100.0	00.0	66.7	66.7	100.0		
Dipel	50.0	66.7	66.7	50.0	66.7	00.0	100.0	00.0	66.7	66.7	

variable 10 bands (Nariman *et al.* 2009). Moreover, Brousseau *et al.* (1993) used arbitrary PCR with a single primer to distinguish and produce discriminating DNA fingerprints for commercial products containing 33 known serovars of *Bt* serovar *kurstaki* (3a3b) and were able to differentiate the closely related *Bacillus cereus* species. The authors stated that the technique proved to be a powerful tool for identification and discrimination of individual *Bt* strains, confirming our conclusions.

Detection of some *cry1* genes in the local *Bt* isolates

PCR analysis was carried out to screen 14 local *Bt* isolates for the presence of *cry1*-type genes. Two pairs (forward and reverse) of specific primers were designed according to Kalman *et al.* (1993) to identify *cry1B* and *cry1Aa* genes. Analysis with *cry1B* primer showed that a fragment of approximately 1.35 kb was amplified from seven local *Bt* isolates: Is-1, Sn-4, Gh-2, Gh-3, Qa-6, Qa-7 and Fa-8. The predicted sizes of the pcr-amplified products and the relative positions of the specific oligonucleotide primer *cry1B* are shown in **Fig. 3A**. Furthermore, PCR analysis with *cry1Aa* revealed one amplified fragment of 1.30 kb, which was amplified from 5/7 isolates: Is-1, Sn-4, Gh-2, Gh-3 and Qa-6 (**Fig. 3B**).

It could be predicted from the results that 7/14 isolates contain the *cry1B* and five of them harbor the two genes, *cry1B* and *cry1Aa*. Similar results were obtained by Wang *et al.* (2003) who used PCR to study the distribution and diversity of *cry* genes and to detect the presence of novel *cry* genes in *Bt* isolates. They found that *Bt* isolates containing *cry1*-type genes were the most abundant and represent 76.5% of the *Bt* isolates in China. Moreover, the *cry1B* gene identified in the six isolates by PCR amplification method is in agreement with Espinasse *et al.* (2002). Moreover, they examined 640 natural isolates of *Bt* and showed that the 58 strains (9%) whose supernatants were toxic to *Anthonomus grandis* (Coleoptera: Curculionidae) produced between 10 and 175 µg of β-exotoxin I/ml. The authors also found that 55 (46%) samples of 118 strains whose culture supernatants were not toxic to *A. grandis* nevertheless produced between 2 and 5 µg/ml. Secretion of large amounts of β-exotoxin I was strongly associated with the presence of *cry1B* and *vip2* genes in the 640 natural *B. thuringiensis* isolates studied.

However, some of other non-specific amplification fragments were observed when using *cry1B*. This could be attributed to nonspecificity annealing with other *cry* genes which could be prevented by increasing temperature. This is in agreement with Bravo *et al.* (1998) who used an expanded set of general and specific primers for detecting three genes of the *cry*9 group. They reported that the specific primer *spe-cry9C*, corresponding to bases 1853 to 1868, is predicted to also nonspecifically anneal to bases 1961 to 1976 in *cry*9Ca; it may thus interfere with amplification of the 306-bp fragment of *cry9Ca*. In addition, it includes a set of *spe-cry9C* predicted to anneal nonspecifically both directly and in the reverse direction to *cry9Ca* and *cry9Aa*, thus giving rise to further nonspecific amplifications (Ben-Dov *et al.* 1999).

SDS-PAGE analysis of total protein mixture from the identified-gene isolates

The SDS-PAGE protein profiles of 17 samples, comprising

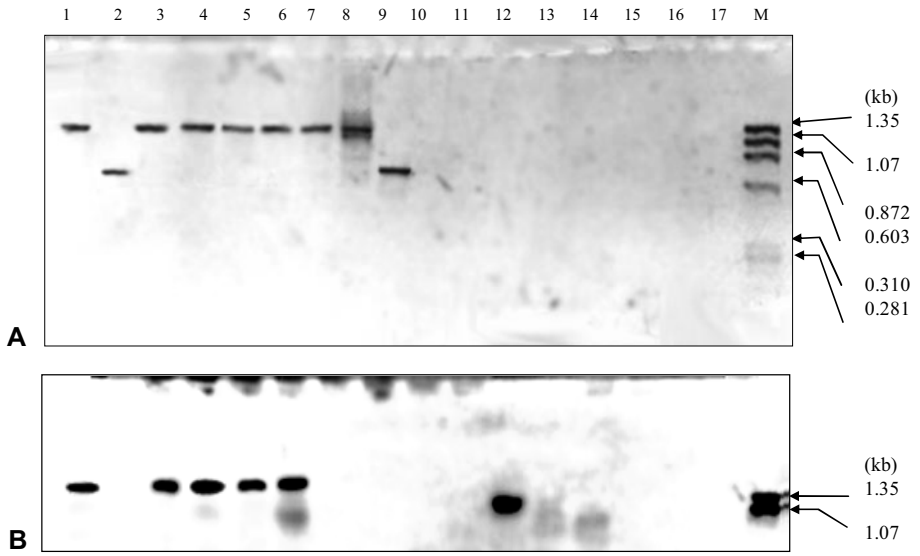

Fig. 3 Agarose gel electrophoresis of PCR products obtained with specific primers for *cry1* genes. Lanes from 1-17 are DNA of local *Bt* collected isolates. M = Molecular weight marker (øX174 digested by *Hae*III). (**A**) *cry1B* gene and (**B**) *cry1Aa* gene.

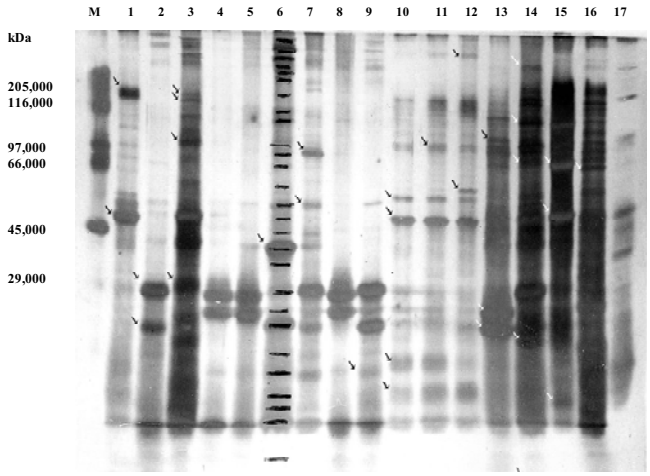

Fig. 4 SDS-PAGE protein band profiles of 17 different *Bt* isolates of spore/crystal mixture proteins. Arrows indicate unique bands with different densities and intensities.

14 local *Bt* isolates, two *Bt* standards and a commercial product are presented in **Fig. 4**. Among the 14 isolates, seven displayed the *cry1B* gene while five of them showed the *cry1Aa* gene. Accordingly, attention has been given to analyze the proteins in the seven isolates. The analysis revealed a total of 37 protein bands with different molecular weights ranging from 240 to 3 kDa (**Table 7**). Among the bands, 21 are commonly detected in the seven *Bt* isolates, while the remainder (16 bands) showed high variability among the isolates. Genetic discrimination of the seven isolates was based upon the absence or presence of unique band(s), as shown in **Table 7**. Protein analysis of the seven isolates showed that both the total and the variable bands are higher in the five isolates containing the two genes *cry1B* and *cry1Aa*, compared with the other two isolates Qa-7 and Fa-8 that contain the *cry1B* gene only. Meanwhile, some unique bands with different density, intensity and appearance were detected among the 17 isolates. These bands could discriminate the isolates with different characteristic protein profiles, even the isolates with no known or identified δ-endotoxin genes, as shown in **Fig. 4**. It is of interest to note that two protein bands 8 and 9 with molecular weights 141 and 132 kDa were detected in the seven isolates, except for Qa-7, which was free of the 132-kD band. Such results are in accordance with Thomas and Ellar (1983), who reported that the majority of crystal proteins

Table 7 Protein analysis of seven *Bt* isolates harboring the two genes *cry1B* and *cry1Aa*.

Band No.	**MW (kDa)**	**Identified - *cry1B* and *cry1Aa* isolates**					**Identified-*cry1B* isolates**	
		Is-1	**Sn-4**	**Gh-2**	**Gh-3**	**Qa-6**	**Qa-7**	**Fa-8**
1	240	+	+	+	+	+		
2	234	+	+	+	+		+	
3	222	+	+	+	+	+	+	+
4	209		+	+	+	+	+	+
5	188	+	+	+	+	+	+	+
6	173	+	+	+	+	+		
7	162		+	+	+	+	+	+
8	141	+	+	+	+	+	+	+
9	132	+	+	+	+	+		+
10	116		+	+		+		
11	110	+	+	+	+	+	+	+
12	105			+	+	+		
13	101	+	+	+	+	+	+	+
14	98	+	+			+	+	+
15	90		+	+	+	+	+	+
16	85	+	+	+	+	+	+	+
17	76	+	+	+	+	+	+	+
18	70				+	+	+	+
19	68	+	+	+	+	+	+	+
20	61	+	+	+	+	+	+	+
21	53	+	+	+	+	+	+	+
22	42	+	+	+	+	+	+	+
23	36	+	+	+	+			+
24	33	+	+	+	+	+	+	+
25	28	+	+	+	+	+	+	+
26	24	+	+	+	+	+	+	+
27	21	+	+	+	+	+	+	+
28	20	+	+	+	+	+	+	+
29	17	+	+	+	+	+	+	+
30	12	+				+	+	+
31	10	+	+	+	+	+	+	+
32	9	+		+	+			
33	8	+	+	+	+	+	+	
34	6	+		+	+	+	+	+
35	5	+	+	+	+	+	+	+
36	4	+	+	+	+	+	+	+
37	3	+	+	+	+	+	+	+
Total numbers		31	32	34	34	34	30	30
Total variable bands		10	11	13	13	13	9	9

* The normal numbers represents the monomorphic bands, while the bold represents the variable bands in the seven isolates.

and all class I lepidopteran-specific crystal proteins are synthesized as 130-140 kDa protoxins, which are then proteolytically cleaved in the insect midgut to 65-70 kDa active toxins. Also, Zhang *et al.* (2000) reported that a *cry1* type gene expressed 130-150 kD proteins in 31 isolates of *Bt*, 16 of which contained the *cry1I* gene encoding an 80-kD protein and was larvicidal to both Lepidopteran and Coleopteran species. The authors added that the identification of this *cry*-type gene and its analysis by SDS-PAGE protein could predict insecticidal activity of *Bt* isolates. Furthermore, the only known *cry* toxin that produces a protoxin band of about 140 kDa on SDS-PAGE gels is *cry1B* (**Fig. 4**), also reported by Espinasse *et al.* (2002). Using PCR with primers specific for *cry1B*, they detected the *cry1B* gene in all 39 strains that produced more than 10 µg of δ-exotoxin/ml, while δ-exotoxin was toxic at a concentration of 5 µg/ml. Moreover, strains carrying *cry1B* and *vip2* genes also possess genetic determinants necessary to promote high levels of production of *δ*-exotoxin.

Hence, PCR could be used to screen many *Bt* samples, to identify and classify *cry* genes, and subsequently predict their insecticidal activities.

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