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RAPD Identification of Local *Bacillus thuringiensis* Isolates Toxic to *Spodoptera littoralis* and *Culex pipiens* using Universal Primers for *cry* Genes

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ABSTRACT

Eight local *Bacillus thuringiensis* (*Bt*) isolates characterized by their toxicity against cotton leafworm (*Spodoptera littoralis*) and *Culex pipiens* mosquito larvae were identified using RAPD analysis. Five universal primers (Un1 to Un5), designed to amplify the conserved regions of five *cry* genes (*cry1Aa*, *cry1Ac*, *cry1I*, *cry2* and *cry3*, after alignment of different accessions obtained from the NCBI GenBank database), were used. A total of 88 RAPD fragments ranging in size from 4.72 to 0.20 kb were amplified, 64 of which were polymorphic while the other were common among the eight *Bt* isolates. The mean percentage polymorphism shown by the five universal primers was 72.7%, specifically decreasing in this order: Un1 (85.7%), Un3 (77.8%), Un4 (76.2%), Un2 (66.7%) and Un5 (58.8%). Among the 64 polymorphic fragments, 20 were *Bt* isolate-specific observed in four isolates, Gh-4, Ts-5, Is-8 and As-3. These isolates varied considerably in their specific fragments: Ts-5 had the highest number (11), Gh-4 with 5, As-3 with 3 and Is-8 with 1. The other four isolates showed no specific amplified fragments. Genetic relationships of the eight local *Bt* isolates provide valuable, reliable information using three UPGMA dendrograms of the three primers (Un1, Un2 and Un3) of *cry1*, Un4-*cry2* and Un5-*cry3* genes.

Keywords: *Bt* isolates, RAPD-PCR

INTRODUCTION

Despite advances in medical science, mosquito-borne diseases including filariasis, dengue and the viral encephalitides remain the most important diseases of humans, with an estimated two billion people worldwide living in endemic areas (WHO 1999). Thus, there is an urgent need for new agents and strategies to control these diseases. Potential strategies include vaccines, new drugs, and transgenic mosquitoes refractory to the causative disease agents, but in the near future control efforts will rely on insecticides and existing drugs (Park *et al.* 2005).

Culex pipiens is the most widely distributed mosquito in the world and it carries a number of diseases, especially arthropod-borne virus. *C. pipiens*, a species that preferentially feeds on birds (Anderson *et al.* 2004), is an important and competent vector of the West Nile virus in both the Old and New Worlds (Hubalek and Halouzka 1999). West Nile virus is a mosquito-borne disease that primarily affects birds but can be spread to humans through the bites of infected mosquitoes. In addition, *C. pipiens* is the main vector in Egypt responsible for transmission of the nematode *Wuchereria bancrofti* that causes lymphatic filariasis (Southgate 1979; Hassan 1991).

Moreover, Rift Valley fever (RVF) virus is one of the most serious transboundary animal diseases. It is a *C. pipiens*-borne viral disease that causes periodic severe epidemics, principally involving ruminant animals and is transmitted to humans causing a potentially fatal disease (Gad *et al.* 1989).

Vector-borne diseases are major public health problems and their prevalence has dramatically increased worldwide (Regis *et al.* 2001). The use of the entomopathogenic bacterium *Bacillus thuringiensis* (*Bt*) as a biolarvicide is a viable alternative in insect control (Baird 2000). *Bt* produces proteinaceous inclusions during sporulation that are toxic towards insect larvae upon ingestion. The parasporal body of *Bt* consists of one or more insecticidal δ-endotoxins (named Cry and Cyt). The revised *cry* gene and their Cry toxin nomenclatures are available at http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/*Bt*/index.html. Vector control products based on *Bt* are designed to control *Culex* mosquito vectors of filariasis and viral diseases. These products have achieved moderate commercial success, but their high cost deters their use in many developing countries.

Bt-based larvicides target larvae in their breeding habitat before they can mature into adult mosquitoes, disperse, have distinct advantages over chemicals because they are nontoxic to humans, and are completely biodegradable, so no residual toxic products accumulate in the environment (Schnepf *et al.* 1998). The search for native strains with activity against dipteran species could have an impact on the control of mosquitoes worldwide (Ibarra *et al.* 2003). New native strains should be dramatically more potent and much faster-acting than any other strain of *Bt* currently used in commercial vector control products. Thereby, the number of known *Bt* strains active on diptera is growing (Yu *et al.* 1991). Novel insecticidal bacteria, with an extended target spectrum are an environmentally safe biocontrol practice and lead to increase food production and postharvest protection (Van Frankenhuyzen 1993). The characterization of *Bt* strain collections may help in the understanding of the role of *Bt* in the environment and the distribution of *cry* genes (Bravo *et al.* 1998).

There has been intense interest in recent years in collecting, analyzing and screening *Bt* strains isolated from environmental samples. Worldwide screening efforts have been based on the possible existence of new strains with new pathogenic spectra or host ranges (Alberola *et al.* 1999). For instance, a screening program developed in Senegal by Kane *et al.* (1998) to isolate new strains of entomopathogenic *Bacillus* led to the isolation of 194 *Bt* strains

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and 9 strains of *Bacillus sphaericus* from various sites and insect samples. these strains were more completely characterized while searching for their toxicity against two major malaria vectors, *Anopheles gambiae* and *A. arabiensis*. Six *Bt* isolates obtained from soil in Nigeria were confirmed to be toxic to mosquito larvae and were differentiated using a PCR-based technique (Ogunjimi *et al.* 2000). Moreover, to establish a method to detect *B. anthracis* in environmental contamination, Kim *et al.* (2007) performed RAPD-PCR with a 10-mer random primer and confirmed the presence of specific PCR bands only in *B. anthracis* species. They reported that the PCR detection method developed in their study is expected to facilitate the monitoring of environmental *B. anthracis* contamination.

Molecular typing of 126 strains of *Bt* representing 57 serovars was performed by Gaviria and Priest (2003) using RAPD-PCR. Serovars *darmstadiensis*, *israelensis*, *Kenya*, *kumamotoensis*, *kurstaki*, *morrisoni*, *Pakistani*, *sotto*, *thuringiensis* and *tolworthi* each encompassed identical or closely related strains. Despite this genomic homogeneity, most of these serovars included at least one variant strain. Of the 57 serovars examined, 31 contained at least one strain with a closely related or identical RAPD pattern to a strain from a different serovar. The authors concluded that while the species is genomically diverse, the homogeneous serovars represent clonal lineages of successful insect pathogens. Pairwise comparisons of RAPD polymorphic products were used by Malkawi *et al.* (1999) to construct a dendrogram applying the cluster analysis of 15 *Bt* isolates recovered from different Jordanian habitats. All isolates were in one major cluster, which was divided into six subclusters. Such analysis showed some regional variation among the isolates, but did not indicate a clearly defined habitat or locational pattern of the DNA polymorphism.

The aim of the present work was to identify the genetic relationship between some local *Bt* isolates characterized by their toxicity against larvae of cotton leafworm (*Spodoptera littoralis*) and *C. pipiens* mosquito using RAPD-PCR with *cry*-universal primers.

MATERIALS AND METHODS

Bacterial strains

Eight local *Bt* strains isolated from the soil of seven governorates; Sinai, Gharbyia, Toshkey, Ismailyia, Qaluobyia, El-Fayioum and Aswan and characterized by their toxicity against larvae of cotton leafworm (*Spodoptera littoralis*) and *Culex pipiens* mosquito were used in this study (**Table 1**).

DNA extraction from *B. thuringiensis* and PCR analysis

Genomic DNA was prepared from 18 h cultures in an exponential phase in Luria-Bertani medium. DNA extraction was performed according to the method of Ben-Dov (1999). Aliquots of 10 ml of bacterial culture were harvested by centrifugation at 12,000 rpm for 15 min at 4°C and washed once in sterile distilled water. the pellets were resuspended in 400 µl of lysis buffer containing 2% glucose, 50 mM Tris-HCl (pH 8.0), 25 mM EDTA, 3 mg/ml lysozyme and 200 mg/ml RNase. The cell suspension was incubated for 1 h at 37°C. Further DNA extraction was performed as described by Sambrook *et al.* (1989).

Table 1 *B. thuringiensis* local isolates and their governorate sources.

Bacterial isolates	Source
Sn-2	Sinai
Gh-4	Gharbyia
Ts-5	Toshkey
Is-8	Ismailyia
Qa-2	Qaluobyia
Fa-7	El-Fayioum
As-3	Aswan
As-4	Aswan

Table 2 Names and sequences of the five universal RAPD primers used for PCR analysis.

Primers	*Cry* genes	Accessions containing the five genes**	Primer sequences (5′-3′)
Un1*	*cry1Aa*	AY31-9967	CCTTGTCGCT
		AY46-6012	
		AY46-6013	
		AY57-0733	
		AY57-0735A	
		Y72-4371	
		AY98-7396	
Un2	*cry1Ac*	AY12-2057	CTCAATGGGA
		AY22-5453	
		U8-9872	
Un3	*cry1I*	AY95-9880	AGCTATGGCC
Un4	*cry2*	AF20-0816	CAAAAGAATG
		AJ48-8143	
		AJ13-2463	
		AJ13-2464	
		AJ13-2465	
Un5	*cry3*	AY57-2010	GGATCCAAGC

* Un = Universal primers
** Obtained from NCBI GenBank database.

PCR amplification was carried out in a DNA thermocycler (Biometra, Germany) for 30 cycles each. The PCR reaction was carried out in a final volume of 25 µl with 1X PCR buffer containing 10 mM Tris-HCl, 25 mM $MgCl_2$, 1 µl of template DNA, 0.2 mM deoxynucleoside triphosphate, 1 to 2 µM (each) primer and 0.5 U of *Taq* DNA polymerase (Promega). PCR conditions consisted of initial denaturation at 95°C for 2 min followed by 95°C for 1 min, annealing to primers at 54°C for 1 min and extension at 72°C for 1 min with a final extension step at 72°C for 5 min. PCR-amplified products were separated using agarose gel electrophoresis in 1% TBE buffer and stained with 0.2 µg/ml ethidium bromide according to Sambrook *et al.* (1989). Amplified fragments were detected and photographed under UV light.

Five random universal primers (Un1, Un2, Un3, Un4 and Un5) were selected from very similar sequences within consensus of the following five *cry* genes (*cry1Aa*, *cry1Ac*, *cry1I*, *cry2* and *cry3*), respectively (after alignment of different accessions obtained from NCBI GenBank database) to detect variation within the five *cry* genes (**Table 2**).

Genetic analysis

RAPD fragments were scored as present (+) or absent. The data was used for similarity-based analysis using the program MVSP (version 3.1b) from www.kovcomp.com. RAPD analyses were analyzed using the Nei genetic similarity index (Nei and Li 1979) based on the equation:

Similarity = 2Nab/(Na+ Nb)

where Nab = number of scored amplified fragments with the same molecular size shared between a and b, Na and Nb = number of scored amplified fragments in a and b, respectively. A dendrogram was constructed based on the similarity matrix data by unweighted pair group method with average (UPGMA) cluster analysis.

RESULTS AND DISCUSSION

Genetic characterization of eight local *Bt* isolates by RAPD analysis

Five universal primers (Un1 to Un5) selected from the highly conserved regions present in five *cry* genes (*cry1Aa*, *cry1Ac*, *crya1I*, *cry2* and *cry3*) were used for RAPD analysis of the eight *Bt* isolates.

Primer Un1 for the *cry1Aa* gene resulted in 14 fragments, 12 of which were polymorphic with sizes ranging from 4.72 to 0.35 kb; two fragments were common among the eight *Bt* isolates (**Fig. 1**; **Table 3**). The total number of amplified fragments of the eight *Bt* isolates varied consider-

Table 3 RAPD analysis of the polymorphic amplified fragments of eight *Bt* local isolates using Un1, Un2 and Un3 primers for *cry1* gene type.

Primers and their genes	Fragment		*Bt* local isolates							
	№	Size (kb)	Sn-2	Gh-4	Ts-5	Is-8	Qa-2	Fa-7	As-3	As-4
Un1 for *cry1Aa* gene	1	4.72			(+)					
	2	3.71			+					+
	3	2.70		(+)						
	4	1.91	+	+	+	+	+			+
	5	1.66		+		+	+			
	6	1.36	+		+			+		+
	8	0.95				+	+	+		
	9	0.74		+						+
	10	0.61	+			+	+	+	+	
	11	0.47	+	+	+					+
	12	0.40			(+)					
	13	0.35	+	+	+					+
	Total № = 12		5	6	7	4	4	3	1	6
Un2 for *cry1Ac* gene	1	3.89		+		+	+			
	2	3.40		+	+	+	+			
	3	3.02		+	+		+			
	4	2.68				(+)				
	5	2.44		(+)						
	6	2.13			(+)					
	9	1.36		+		+				+
	12	0.90	+			+	+			
	13	0.78			(+)					
	15	0.64	+	+	+					
	17	0.48			(+)					
	18	0.37	+	+	+					
	Total № = 12		3	7	7	5	4	0	0	1
Un3 for *cry1I* gene	1	4.06			(+)					
	2	3.24			(+)					
	3	2.41							(+)	
	4	2.17			+					+
	5	1.87							(+)	
	6	1.44		+	+	+	+			
	7	1.26		+	+	+	+			
	8	1.08	+	+						
	9	1.02			+					+
	10	0.91		+		+	+		+	
	11	0.76			(+)					
	14	0.35	+		+					
	16	0.24		(+)						
	17	0.21		+					+	
	Total № = 14		2	6	8	3	3	0	4	2

+ = Presence of amplified fragment
(+)= Presence of specific fragment

ably. For instance, in Ts-5 seven fragments with different molecular sizes were amplified while only one fragment was amplified As-3. RAPD analysis revealed that the amplified PCR products of most of the *Bt* isolates vary, except for Is-8 and Qa-2, which show four fragments with a similar pattern.

Primer Un2 for the *cry1Ac* gene revealed 18 fragments, 12 of which were polymorphic with sizes ranging from 3.89 to 0.37 kb (**Fig. 1**; **Table 3**). The total fragment numbers of the eight *Bt* isolates varied significantly in their amplified fragments: whereas Gh-4 and Ts-5 showed seven, Fa-7 and As-3 revealed none. Five distinct amplified fragments were uniquely displayed in three isolates. For example, Ts-5 displayed three (2.13, 0.78 and 0.48 kb) while Gh-4 and Is-8 displayed two (2.44 and 2.68 kb).

Primer Un3 for the *crya1I* gene revealed 18 fragments, 14 of which were polymorphic with sizes ranging from 4.06 to 0.21 kb (**Fig. 1**; **Table 3**). The variable total fragments numbers of the eight *Bt* isolates varied substantially: whereas Ts-5 showed eight fragments, Fa-7 showed none. Six fragments were uniquely displayed in three isolates: Ts-5 displayed three (4.06, 3.24 and 0.76 kb), As-3 displayed two (2.41 and 1.87 kb) and Gh-4 showed one (0.24 kb). Moreover, five fragments (2.17, 1.08, 1.02, 0.35 and 0.21 kb) were displayed uniquely in two different isolates, except for two (2.17 and 1.02 bp) which appeared in both Ts-5 and As-4.

Primer Un4 for *cry2* gene revealed 21 fragments, 16 of which were polymorphic with sizes ranging from 2.94 to 0.20 kb (**Fig. 1**; **Table 4**). The total number of fragments of the eight isolates varied substantially: for example, Ts-5 and As-4 showed ten, while Gh-4 and As-3 showed two. Ts-5 was characterized by two unique fragments (2.30 and 1.55 kb) that were absent in the other seven isolates. Moreover, each of the five amplified fragments show different sizes; 2.94, 1.31, 0.75, 0.61 and 0.25 kb and displayed in two different isolates among five isolates and Is-8 and As-4 revealed the same two (2.94 and 0.75 kb).

Primer Un5 for *cry3* gene revealed 17 amplified fragments, 10 of which were polymorphic with sizes ranging from 3.61 to 0.32 kb (**Fig. 1**; **Table 4**). A total of three variable fragment numbers were quite similar in five of the eight *Bt* isolates, whereas Gh-4 showed the greatest variability with six fragments, while both of Qa-2 and As-4 showed the least with only two fragments. Four unique fragments (3.61, 3.05, 2.27 and 0.32 kb) were present in all isolates.

A total of 88 DNA fragments ranging in size from 4.72 to 0.20 kb were amplified, 64 of which were polymorphic while the other amplified fragments were common among all eight *Bt* isolates. The mean percentage polymorphism shown by the five universal primers was 72.7%, specifically decreasing in this order: Un1 (85.7%), Un3 (77.8%), Un4 (76.2%), Un2 (66.7%) and Un5 (58.8%) as shown in **Table**

Table 4 RAPD analysis of the polymorphic amplified fragments of eight *Bt* local isolates using Un4 and Un5 primers for *cry2* and *cry3* gene types.

Primers and their gene	Fragment		*Bt* local isolates							
	№	Size (kb)	Sn-2	Gh-4	Ts-5	Is-8	Qa-2	Fa-7	As-3	As-4
Un4 for *cry2* gene	1	2.94				+				+
	2	2.30			(+)					
	4	1.96			+	+				+
	5	1.70		+	+		+			
	6	1.55			(+)					
	7	1.42			+				+	+
	8	1.31					+	+		
	9	1.17		+		+	+	+		+
	11	0.98	+		+	+				+
	13	0.82	+			+				+
	14	0.75				+				+
	15	0.67	+		+	+	+			
	16	0.61				+	+			
	18	0.42			+			+		+
	20	0.25			+					+
	21	0.20			+	+	+		+	+
	Total № = 16		3	2	8	9	6	3	2	10
Un5 for *cry3* gene	1	3.61		(+)						
	2	3.05		(+)						
	3	2.60		+						+
	4	2.27			(+)					
	5	1.95		+	+					
	7	1.42	+			+	+	+	+	+
	8	1.25	+	+	+	+		+		
	11	0.86	+	+			+	+		
	12	0.70				+			+	
	16	0.32							(+)	
	Total № = 10		3	6	3	3	2	3	3	2

+ = Presence of amplified fragment
(+)= Presence of specific fragment

Table 5 RAPD amplified fragments, polymorphism and isolate-specific fragments in four local *Bt* isolates.

Primers	Total fragments	P*	*Bt* isolate-specific fragments				
			Fragments		Local *Bt* isolates		
			№	Size (kb)	Gh-4	Ts-5	Is-8	As-3
Un1 for *cry1Aa* gene	14	12	3	4.72, 0.4		[2]+		
				2.7	+			
		85.7%		21.1%	1	2	0	0
Un2 for *cry1Ac* gene	18	12	5	2.68			+	
				2.44	+			
				2.13, 0.78, 0.48		[3]+		
		66.7%		27.8%	1	3	1	0
Un3 for *cry1I* gene	18	14	6	4.06, 3.24, 0.76		[3]+		
				2.41, 1.87				[2]+
				0.24	+			
		77.8%		33.3%	1	3	0	2
Un4 for *cry2* gene	21	16	2	2.3, 1.55		[2]+		
		76.2%		9.5%	0	2	0	0
Un5 for *cry3* gene	17	10	4	3.61, 3.05	[2]+			
				2.27		+		
				0.32				+
		58.8%		23.5%	2	1	0	1
Total	88	64	20		5	11	1	3
Mean % polymorphism = 72.7%			22.7%					

Total = Total number of amplified fragments.
P* = Number of polymorphic fragments.
+ = Presence of specific fragment.
The polymorphic fragments/total fragments = polymorphic %, thus 12/14 = 85.7%, 12/18 = 66.7% and so on. Mean polymorphic % = 64/88 = 72.7%

5. Among the 64 polymorphic fragments, 20 were *Bt* isolate-specific fragments observed only in four isolates, Gh-4, Ts-5, Is-8 and As-3. These isolates varied considerably in their specific fragments and were genetically variable: Ts-5 had the highest number (11) of specific fragments, Gh-4 with 5, As-3 with 3 and Is-8 with one.

Brousseau *et al.* (1993) used PCR to distinguish and produce discriminating DNA fingerprints for commercial products containing 33 known serovars of *Bt* serovar *kurstaki* (3a3b) and were able to differentiate closely related *Bacillus cereus* species. The authors stated that the technique proved to be a powerful tool for identifying and discriminating individual *Bt* strains. Stephan *et al*. (1994) examined 46 *B. licheniformis* strains isolated from food matrices (37 strains isolated from spoiled blood sausage, 3 strains isolated from pig blood and 6 strains of unknown origin) using RAPD analysis. They observed quite low heterogeneity, grouping the strains into 5 and 10 pattern types depending on the primer used. RAPD analysis was also used to fingerprint 31 mosquito-pathogenic and 14 non-pathogenic strains of *B. sphaericus* using eight primers and the band patterns obtained were analyzed using the Jaccard's coefficient and unweighted pair group with arithmetic average clustering (Woodburn *et al.* 1995). Daffonchio *et al.* (1998) used RAPD

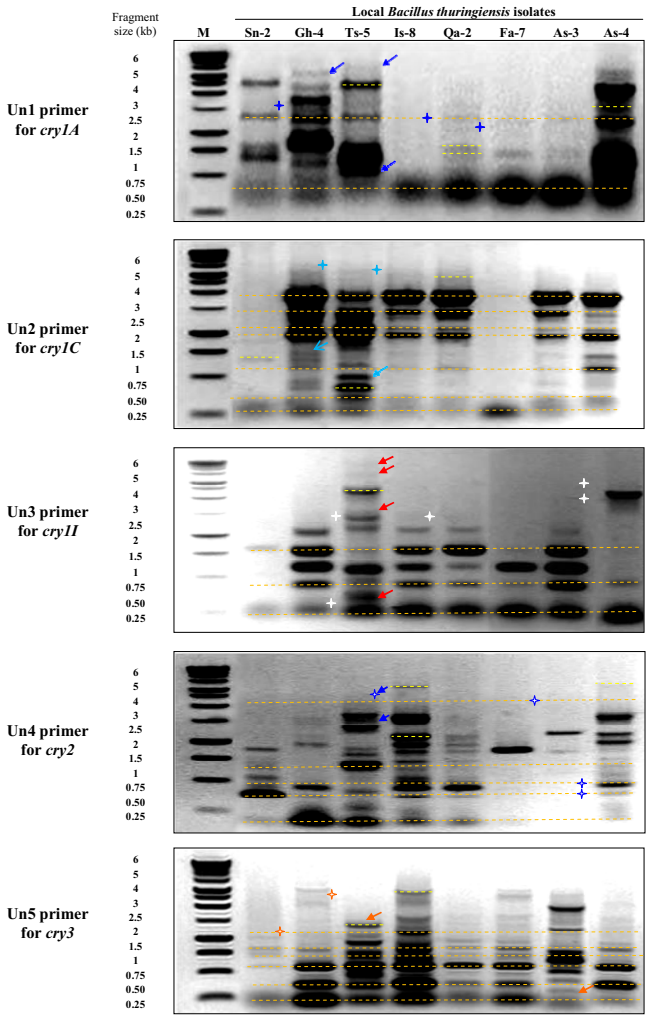

Fig. 1 RAPD amplification profiles of eight local *Bt* isolates using five universal primers (Un1 to Un5). M = 1 kb DNA ladder. In order to minimize the variation between *Bt* isolates, each amplified fragment was identified by eye with the aid of Adobe Photoshop program. The orange dotted lines refer to the common fragments, yellow lines refer to non-common, arrows indicate specific fragments and stars are the visible fragments on gels that did not clearly appear on inverted photos.

analysis to group and type 21 strains of *B. cereus* and another 12 *Bacillus* strains of *B. licheniformis*, *B. subtilis* and *B. circulans*. Moreover, RAPD-based fingerprinting of 21 serovars of *Bt* representing different serotypes was performed using 19 random primers, whereas a total of 172 polymorphic fragments ranging in size from 161 to 2789 bp were amplified from 13 of the 19 primers (Pattanayak *et al.* 2001). Valadares *et al.* (2001) used RAPD analysis, *cry* gene-specific PCR and dot blot DNA hybridization techniques to screen over 11,000 isolates of *Bt* subsp. *kurstaki*. They identified bacteria with genetic patterns consistent with those of *Bt* subsp. *kurstaki* HD1 in 9,102 of 10,659 isolates (85.4%) obtained from air samples, 13 of 440 isolates (2.9%) obtained from water samples, and 131 of 171 isolates (76.6%) obtained from nasal swab samples. Moreover, Chaves *et al.* (2008) used six RAPD primers to demonstrate three different patterns for 28 autoagglutinating strains of *Bt*, allowing correlation of the profiles obtained with the toxicity observed in the bioassays and found that the RAPD patterns for mosquitocidal strains were identical to one serovar, *israelensis*. However, for strains of low toxicity, each primer generated distinctive RAPD patterns, which demonstrated that these strains belong to different serovars.

Genetic similarity of the eight *Bt* isolates using RAPD analysis

Genetic similarity between each two pairs of the eight *Bt* isolates was performed using the Nei similarity index on the basis of RAPD amplified fragments of the three *cry* gene types: *cry1*, *cry2* and *cry3* (**Table 6**). Genetic similarity between the eight local *Bt* isolates was calculated from the amplified fragment data using unweighted pair group method with averages (UPGMA). Three dendrograms were constructed using the three universal primers; Un1, Un2 and Un3 that represented the *cry1* gene type including *cry1Aa*, *cry1Ac* and *cry1I* genes as well as Un4 for *cry2* gene and Un5 for *cry3* gene (**Fig. 2**).

The UPGMA dendrograms of the three primers revealed clearly two main clusters for each primer and were classified the eight local *Bt* isolates into different subgroups with various value percentages of bootstraps as shown in **Fig. 2**. Accordingly, using the three primers; Un1, Un2 and Un3, Fa-7 revealed most high similarity with As-2, Gh-4 and Sn-2 with 92, 82 and 100%, respectively. Qa-2 also showed high similarity with Is-8 (89%) using Un1 and with Sn-2 and Fa-7 (95%) using Un3. In addition, Sn-2 showed a similar manner with As-4 (85%) using primer Un1 as shown in **Table 6**. However, Ts-5 revealed the lowest genetic similarity among isolates with Is-8, Fa-7 and As-3 (64, 66 and 67%, respectively) using Un1 and with Fa-7 (56%) using Un2. As-3 displayed also the lowest similarity with Gh-4 (61%) using Un3. The obtained results were agreed with Pattanayak *et al.* (2001) who used pairwise genetic similarity analysis of 21 *Bt* serovars representing different serotypes and found very low similarity values ranging from 3

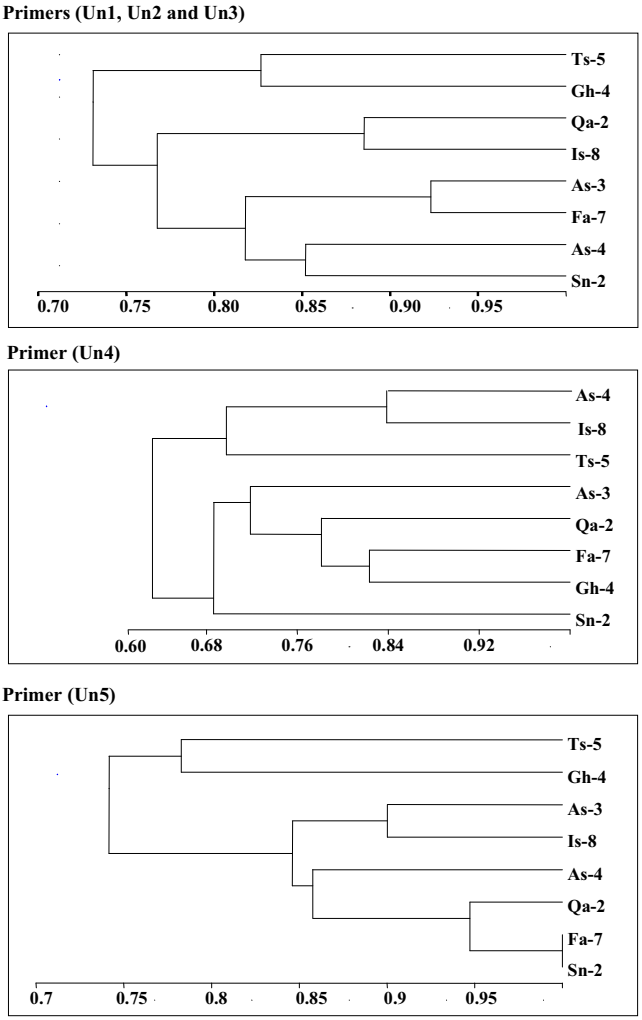

Fig. 2 dendrogram representing the genetic relationships among the eight local *Bt* isolates using UPGMA cluster analysis of Nei's genetic similarity coefficients generated from five RAPD universal primers.

Table 6 Genetic similarity percentages of eight *Bt* isolates based on RAPD products of the primers.

Primers and their genes	Isolates	Sn-2	Gh-4	Ts-5	Is-8	Qa-2	Fa-7	As-3
Un1, Un2 and Un3 for *cry1* gene	Gh-4	80						
	Ts-5	79	83					
	Is-8	75	77	64				
	Qa-2	79	81	74	89			
	Fa-7	79	67	66	78	80		
	As-3	81	68	67	72	78	92	
	As-4	85	83	75	77	75	83	84
Un4 for *cry2* gene	Gh-4	71						
	Ts-5	64	58					
	Is-8	75	61	65				
	Qa-2	67	80	64	74			
	Fa-7	67	82	56	58	76		
	As-3	71	75	67	61	70	71	
	As-4	64	58	75	84	57	64	67
Un5 for *cry3* gene	Gh-4	78						
	Ts-5	80	78					
	Is-8	90	70	80				
	Qa-2	95	73	74	84			
	Fa-7	100	78	80	90	95		
	As-3	80	61	70	90	84	80	
	As-4	84	73	74	84	89	84	84

to 68% among the serovars, indicating high genetic divergence. They also showed that 19 serovars fell into two major clusters while the remaining two formed solitary clusters in the dendrogram. Clustering of *Bt* strains established genetic relatedness between serovars and serotypes. These authors suggested that RAPD analysis can be used for genotypic characterization of *Bt* to complement flagellar serotyping. Yu *et al.* (2002) classified 35 strains of *Bt* using the similarity coefficient and average genetic distance coefficient D, based upon the differences between the major and minor bands of PCR products.

On the other hand, the eight *Bt* isolates revealed high DNA polymorphism using the five RAPD primers, either in the occurrence of amplified fragments or in the variable genetic similarities of each isolate with the others even though they should display the narrowest and lowest variation due to the structure of the designed RAPD primers. This takes into consideration that the five primers were designed from similar consensus sequences within the five types of *cry* genes; Lepidoptera-active (*cry1Aa*, *cry1Ac* and *cry1I*), Lepidoptera/Diptera-active *cry2* gene and Coleoptera-active *cry3* gene. Furthermore, in order to narrow the level of sequence variation among the eight isolates, 7 accessions (for *cry1Aa* gene), 3 (for *cry1Ac*) and five of (*cry3*) obtained from the NCBI GenBank database were performed using multiple sequence alignment (**Table 1**).

Another view-point is that the eight *Bt* isolates under study may harbor different *cry* genes as stated by several reports. For instance, Nariman (2007) found five *Bt* isolates harboring a combination of different *cry* genes, such as Gh-3 harbored *cry1Aa*, *cry1Ac*, *cry2* and *cry3* genes. Sn-4 and Ts-1 isolates harbored *cry1Ac* and *cry2*, while As-2 is containing *cry1Aa* and *cry1Ac*. Gh-5 was harbored *cry1Aa* and *cry1I*. Ben-Dov *et al.* (1997) reported the presence of *cry1* genes and *cry3*, *cry8*, or *cry7* genes in the same *Bt* isolate. Bravo *et al.* (1998) observed *cry1* genes and coleopteran-active *cry3A*, *cry3Ba* and *cry7A* genes in the same isolate.

Eventually, the fluctuation of genetic similarity values of each of the eight isolates with others using the three primers evidently revealed the divergent genetic backgrounds of such isolates with their high DNA polymorphism patterns.

CONCLUSION AND PERSPECTIVES

The identification and discrimination of eight individual *Bt* strains isolated from different soils in seven governorates in Egypt revealed that they are novel strains with different serovars and that each isolate could be characterized by unique RAPD patterns. This demonstrates that the eight *Bt* isolates harbored different types of *cry* genes; *cry1Aa*, *cry1Ac*, *cry1I*, *cry2* and *cry3* that produce parasporal proteins with high toxicity against lepidoptera (*Spodoptera littoralis*) and diptera (*Culex pipiens*) insects. The eight new *Bt* isolates with new pathogenic spectra or host ranges could be registered and used as new commercial insecticidal bacteria.

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