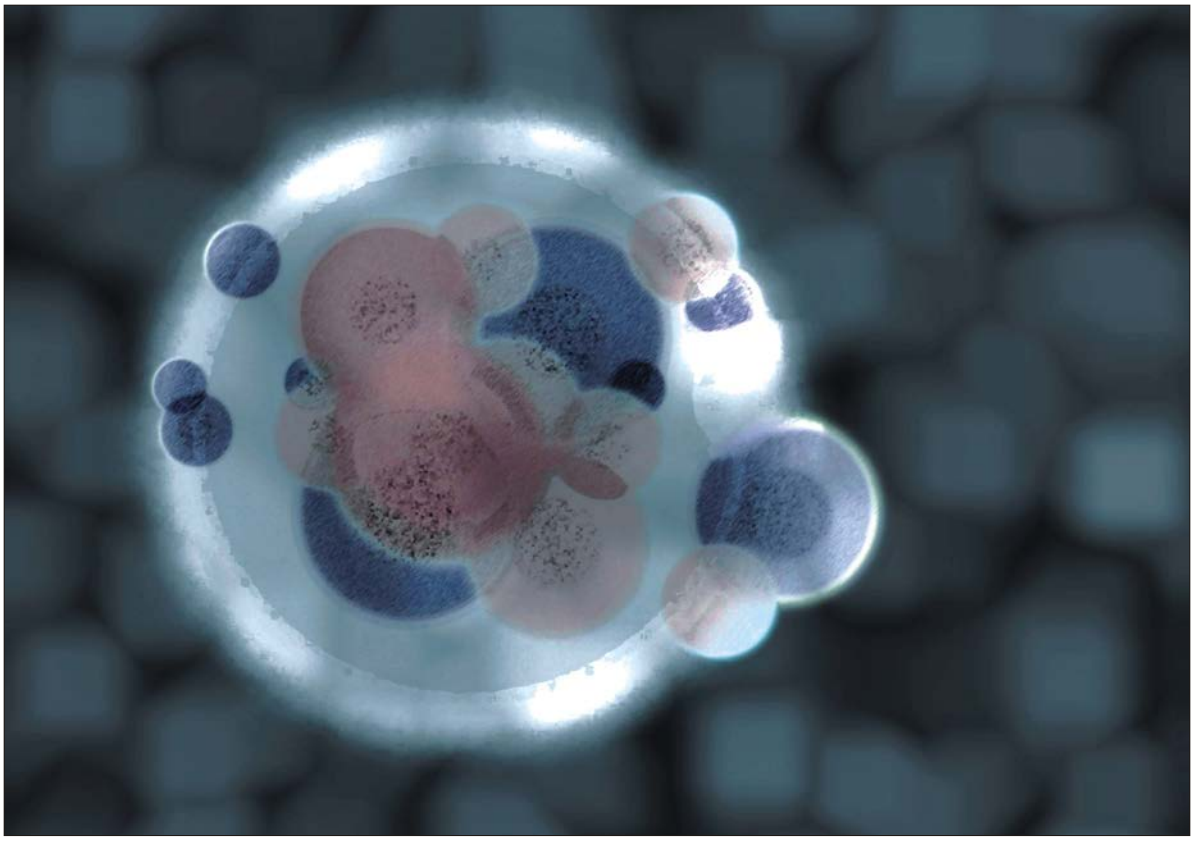

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Genetic Similarity Among Four *Bacillus thuringiensis* subspecies Based on Random Amplified Polymorphic DNA (RAPD)

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Abstract: The aim of this study was to evaluate the genetic similarity and phylogenetic relationship among four *Bacillus thuringiensis* subspecies (new isolate and three reference strains). Random amplified polymorphic DNA (RAPD) technique was used in this study. Based on RAPD pattern obtained with total of seventeen random primers, the similarity index was calculated for each primer separately and the average for all primers was carried out with each comparison. Moreover, the scored band data was subjected to cluster analysis using Statistica/W5.0 Software package. Based on RAPD data obtained with all primers, the average of genetic similarities between the new isolate (66) and ACCC 10061, tt4 and i977 was 42.47; 51.10; and 45.90, respectively. The four strains are grouped into two major clusters A and B. Cluster A consisted of *B. thuringiensis Berliner* (ACCC 10061) strain and cluster B consisted of the three *B. thuringiensis* (66, tt4 and i977) strains. RAPD-PCR could be used extensively to study phylogenetic relationship among *B. thuringiensis* strains from different subspecies as well as for identification new isolate bacterial strains.

Key words: *Bacillus thuringiensis* subspecies, new isolate, genetic similarity, RAPD-PCR, phylogenetic relationship

INTRODUCTION

One commonly used biopesticide is the gram positive bacterium *Bacillus thuringiensis* (*B. thuringiensis*). Pesticidal *B. thuringiensis* strains are known to produce crystal proteins during sporulation, which are specifically toxic to certain orders and species of insects and nematodes

RAPD technology has been particularly used for genetic and molecular studies as it is a simple, powerful and rapid method. This technology is proving to be quite useful in typing strains of bacteria. Genetic typing of bacteria by PCR amplification of variable DNA has been described for a large series of species (Abdul Manaf *et al*., 2006; Salem *et al*., 2006; Tankson *et al*., 2006; Aymerich *et al*., 2006; Naffa *et al*., 2006; Seppola *et al*., 2006; Catzeddu *et al*., 2006; Gomes *et al*., 2005; Naureen *et al*., 2005; Fujii *et al*., 2005; Duarte *et al*., 2005; Yin *et al*., 2005; De la Puente-Redondo *et al*., 2000; Gaviria-Rivera and Priest, 2003; Van Belkum, 1994).

In this study, RAPD-PCR technique was employed to study genetic similarity among four subspecies of *B. thuringiensis* including three reference strains and new isolate.

MATERIALS AND METHODS

Strains: Four *B. thuringiensis* strains were used in this study. The new *B. thuringiensis* isolate (designated 66) was recovered from dead *Biomphalaria alexandrina* snails in Egypt and selected from several isolates on the basis of bioassay of its toxicity against *Biomphalaria alexandrina* snails, in addition to sequence of morphological, biochemical and molecular tests (Salem, 2004). *B. thuringiensis tenebrionis* (tt4), *B. thuringiensis israelensis* (i977) were kindly supplied by Prof. Priest, FG, Heriot University, England. *B. thuringiensis Berliner* (ACCC 10061) was purchased from China Center of Industrial Culture Collection (CICC), Beijing, China.

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Genomic DNA isolation and concentration: Genomic DNA was extracted from the four bacterial strains using DNeasy Mini Kit (Gene Company, Limited, Guangzhou, China). The concentration of DNA and its relative purity was determined using UV spectrophotometer based on absorbance at 260 and 280 nm, respectively.

RAPD-PCR primers: A total of seventeen random sequence primers that were 9-10 bases long were used in this study (Table 1). Eleven primers (R1-R11) were synthesized by Shanghai DNA BioTechnologies Co., Ltd, Shanghai, China. Other primers (R12-R17) are package as components of Ready-To-Go™ RAPD Analysis Kit (Guangzhou Amsure Trade Ltd., China).

RAPD reaction and PCR conditions: DNA amplification was performed using Ready-To-Go™ RAPD Analysis Beads (27-9500-01, Guangzhou Amsure Trade Ltd., China) according to the manufacturer's description. The amplification reactions were performed in 25 μL volumes. From 10-15 ng template DNA and 25 pmol of a single RAPD primer were added to the tube of RAPD analysis Beads. The mixture was heated to 95°C for 5 min followed by 45 cycles consisting of 95°C for 1 min, 36°C for 1 min and 72°C for 2 min and finally 72°C for 3 min in a Peltier Thermal Cycler (PTC-200, MJ Research Inc., Watertown, USA). After amplification, the banding pattern of the randomly amplified DNA was fractionated by electrophoresis on 1.2% agarose gel and run at 60 V in 1X TBE buffer containing 0.5 μg ethidium bromide mL^{-1}. Gels were imaged with the gel documentation system (UVP, USA).

RAPD analysis: The RAPD bands were scored for their presence (1) or absence (0). The similarity index among strains was calculated using the formula: Bab = 2Nab/(Na+Nb), where Nab is the number of common fragments observed in individuals a and b and Na and Nb are the total number of fragments scored in a and b, respectively (Lynch, 1990). The similarity index was calculated for each primer separately and the average for all primers was carried out with each comparison. Also, the scored band data (Presence or absence) was subjected to cluster analysis using Statistica/W5.0 Software package (StatSoft Inc., Tulsa, OK, USA). The Dendrogram was constructed by Ward's method of clustering using minimum variance algorithm. The dissimilarity matrix was developed using Squared Euclidean Distance (SED).

Table 1: Nucleotide sequence of RAPD primers used in this study

Primers	Nucleotides Sequences (5'-3')	GC (%)	Reference
R1	CCG AGT CCA	66.7	Nilsson *et al.* (1998)
R2	ACG CGC CCT	77.8	
R3	CCG GCG GCG	100	
R4	CGG CCA CTG T	70	
R5	ACG TAT CTGC	50	Meunier and Grimont (1993)
R6	CCGAGTCCA	66.7	Gaviria-Rivera and Priest (2003)
R7	CGG CCC CTG T	80	Meunier and Grimont (1993)
R8	GTT TCC GCC C	70	Vettori *et al.* (1996)
R9	CGG CCT CTG C	80	Stephan (1996)
R10	ACA ACT GCT C	50	
R11	ACG TAT CTG C	50	
R12	GGT GCG GGA A	70	Ready-To-Go RAPD Analysis Kit
R13	GTTTCGCTCC	60	(27-9502-01), Amersham
R14	GTA GAC CCG T	60	Biosciences.
R15	AAG AGC CCG T	60	
R16	AAC GCG CAA C	60	
R17	CCC GTC AGC A	70	

RESULTS

The genomic DNA isolated from *B. thuringiensis* strains was analyzing using RAPD with seventeen random decamer oligonucleotide primers. All RAPD primers yielded reproducible DNA profiles. All amplification products were found to be reproducible when reactions were repeated using the same reaction conditions. Among the primers used, some of them were successfully amplified polymorphic bands among the four strains (Fig. 1-3).

A phylogenetic tree was generated from RAPD patterns of the four strains (Fig. 4). The four strains are grouped into two major clusters A and B. Cluster A consisted of *B. thuringiensis Berliner* (ACCC 10061) strain and cluster B consisted of the three *B. thuringiensis* (66, tt4 and i977) strains based on genetic similarity in their RAPD profiles. The maximum linkage distance between the two major clusters (A and B) was 96 units. Cluster B was grouped into two subclusters (B1 and B2) with maximum linkage distance about 94 units.

Table 2: Genetic similarity estimated for each primer between the new isolate and other strains

	Comparisons		
Primers	66 X ACCC 10061	66 X tt4	66 X i977
R1	36.36	22.22	28.57
R2	20.00	72.73	25.00
R3	54.54	66.67	50.00
R4	44.44	15.38	28.57
R5	80.00	60.00	66.67
R6	75.00	66.67	66.67
R7	36.36	28.57	25.00
R8	80.00	80.00	25.00
R9	16.54	60.00	50.00
R10	0.00	16.67	33.33
R11	30.77	18.18	18.18
R12	28.57	28.57	33.33
R13	66.67	60.00	50.00
R14	33.33	57.14	75.00
R15	25.00	50.00	50.00
R16	50.00	75.00	75.00
R17	44.44	90.91	80.00
Average	42.47	51.10	45.90

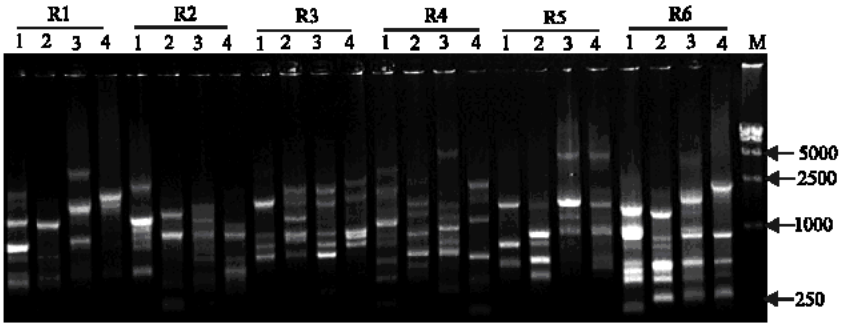

Fig. 1: RAPD fingerprint profiles of four strains obtained with six primers (R1-R6). Lanes 1-4: Strains (ACCC 10061, 66, tt4 and i977 for each primer, respectively). Lane M: DNA molecular marker (DL 15 Kb)

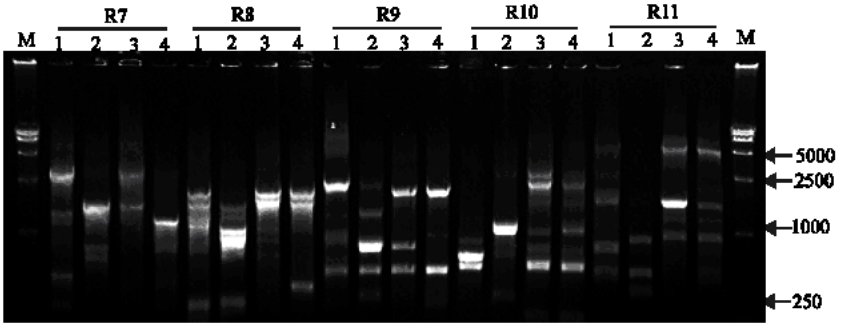

Fig. 2: RAPD fingerprint profiles of four strains obtained with five primers (R7-R11). Lanes 1-4: Strains (ACCC 10061, 66, tt4 and i977 for each primer, respectively). Lanes M: DNA molecular marker (DL 15 Kb)

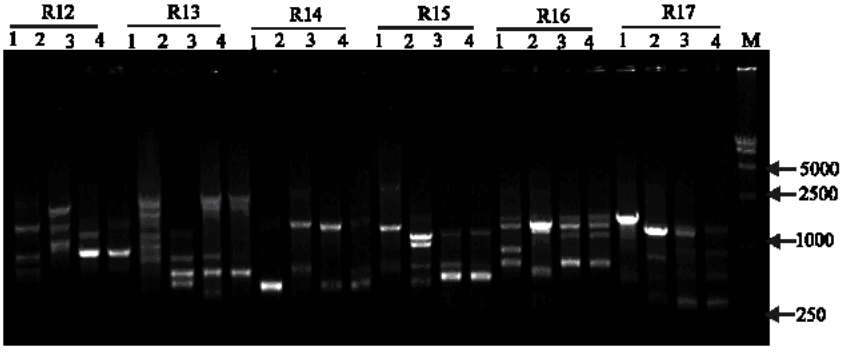

Fig. 3: RAPD fingerprint profiles of four strains obtained with six primers (R12-R17). Lanes 1-4: Strains (ACCC 10061, 66, tt4 and i977 for each primer, respectively). Lane M: DNA molecular marker (DL 15 Kb)

The subcluster B1 consisted of the new isolate strain (66). However, subcluster B2 was grouped into two subclusters (B2.1 and B2.2) consisted of tt4 and i977, which showed less variation than the first cluster.

Genetic similarity estimated as Band Sharing (BS) between the new isolate and the other *B. thuringiensis* strains (ACCC 10061, tt4 and i977) was presented in Table 2. Among primers used in this study, primers (R17) showed the highest similarity (90.91%) between the new isolate (66) and tt4. On the other hand, the highest polymorphism (100.00%) between the isolate 66 and ACCC 10061 was obtained with primers (R10). Based on RAPD data obtained with all primers, the average of genetic similarities between the new isolate (66) and ACCC 10061, tt4 and i977 was 42.47; 51.10 and 45.90, respectively.

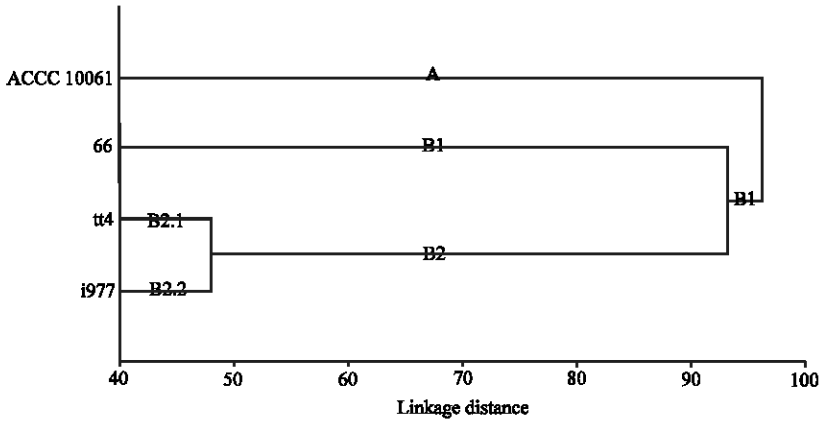

Fig. 4: Phylogenetic tree built on the bases on RAPD profiles of four strains using seventeen primers

DISCUSSION

RAPD is a simple and rapid mean for establishing the polymorphism between biology without DNA sequencing (Chen *et al*., 2001). Moreover, RAPD-PCR has proved to be an informative method suitable for the study of a large number of strains in a short time. RAPD is a well-established easy method used to classify closely related strains (Pinchuk *et al*., 2002; Ghelardi *et al*., 2002; Williams *et al*., 1990). In the present work, RAPD method was used to study genetic similarity and phylogenetic relationship among four *B. thuringiensis* strains.

To ensure that the amplified DNA bands originated from genomic DNA and not primer artifacts, negative control was carried out for each primer/strain combination. No amplification was detected in control reactions. On other hand, the GC% of primers used in this study ranged from 50 to 100% and the results showed that GC content is not essential requirements for generation of polymorphic bands. This result is in agreement with the findings of other researchers (Gomes *et al*., 2005). On the contrary, it has been observed that random primers with high GC content (>60%) resulted in a greater and better reproducible number of strain specific bands in enterotoxigenic *E. coli* (Pacheco *et al*., 1996; Makino *et al*., 1994; Akopyanz *et al*., 1992). In general, RAPD patterns provide a convenient and simple method for bacterial typing with reasonably reproducible results so long as care is taken with the concentrations of template DNA (Davin-Regli *et al*., 1995) and magnesium in the reaction (Park *et al*., 1994). The arbitrary primers recognize differences in the prevalence and positions of annealing sites in genome producing sets of fragments that are considered to reflect the genomic composition of the strain (Gaviria-Rivera and Priest, 2003).

A phylogenetic tree was generated from the diverse four strains RAPD patterns and different primers were highly suggestive of a genetically diverse population (Fig. 4). The placement of the three *B. thuringiensis* (tt4, i977 and 66) in one major cluster and ACCC 10061 in the second major cluster indicated the effectiveness of RAPD-PCR technique as powerful method to differentiate between bacterial strains from different subspecies. Moreover, the placement of tt4 and i977 in on subcluster demonstrated the highest genetic similarity between them. Also, results showed that the closer proximity of the new isolate (66) to tt4 and i977 (51.10 and 45.90%, respectively) and the farthest from ACCC 10061 (42.47%, respectively) as average of similarity index obtained with all seventeen random primers. The RAPD study presented here indicated that it could provide an alternative to Serotyping for *B. thuringiensis*. Serotyping has provided a valuable subspecific classification of *B. thuringiensis* for over four decades but suffers limitations (Gaviria-Rivera and Priest, 2003). Some strains cannot be typed because they lack flagella or autoagglutinate and specialist antisera are needed. Moreover, typing based on whole genome patterns of one kind or another has become the norm for medically important bacteria (Gurtler and Mayall, 2001).

The genetic variation between the new isolate and the other reference strains (tt4, i977 and ACCC 10061) is in agreement with the results of other molecular biology approaches used for identification of the new isolate strain (data not shown). It can be concluded that RAPD-PCR is powerful tool to study genetic similarity and/or polymorphisms among bacterial strains from different subspecies.

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