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ORIGINAL ARTICLE

# Characterization of native *Bacillus thuringiensis* strains by PCR-RAPD based fingerprinting

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**Abstract** Seventy isolates of *Bacillus thuringiensis* were isolated from soil samples collected from cotton fields. These isolates were characterized by randomly amplified poylmorphic DNA (RAPD) markers to determine their genetic diversity pattern based on their source of origin. Different random decamer primers were used for RAPD amplification, which generated a total of 1935 fragments; of these 1865 were polymorphic and 68 monomorphic. The primers OPA03, OPA08, OPD14, OPD19, OPD20, OPE17 and OPD19 produced 100% polymorphic fragments, whereas primers OPC06, OPC20 and OPD17 produced 20, 31 and 17 monomorphic fragments, respectively. When the RAPD banding pattern data was subjected to dendrogram construction, the 70 isolates fell into two separate clusters, cluster I and cluster II, which includes 26 and 44 *B. thuringiensis* isolates, respectively. These two main clusters were further divided into four subclusters at Eucledian distance of 150 and 80% similarity index. All primers showed amplification and indicated the good diversity of *B. thuringiensis* isolates. The RAPD pattern showed 4–10 bands per isolate, with MWt in the range of 0.4–3.5 Kb and an average of 193.5 fragments were produced per primer. The primer OPE17 was found to be the most discriminatory as it produced 286 polymorphic bands.

**Keywords** *Bacillus thuringiensis* · RAPD analysis · Genetic diversity · Dendrogram

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## Introduction

The continuous use of chemical insecticides to control insect pests of agriculture, forestry and horticultural crop plants, leads to deleterious effects on the environment. Some insects have developed resistance to insecticides. An alternative strategy used to control harmful insects is biopesticides based on *Bacillus thuringiensis*. It is a rod-shaped, gram-positive, endospore-forming bacterium, and distinguished from other closely related three *Bacillus* spp. *viz*. *B. cerus*, *B. anthracis* and *B. mycoides*, because of its ability to synthesize delta endotoxins as protein inclusion crystals (or Cry proteins) during sporulation [1]. For more than 50 years, *B. thuringiensis* has been used to control various insects pests due to its ecofriendly nature, safety and target specificity. Delta endotoxin protein in *B. thuringiensis* is ingested by insect larvae. The protoxin in the parasporal bodies are dissolved and activated under alkaline conditions in the midgut of target insects, thereby releasing the active peptides that bind to specific receptors in the insect's midgut epithelial cells and create pores in the epithelial membrane. Soon the insect stops feeding and ultimately dies due to starvation [2]. The different Cry proteins are toxic to a variety of insects that cause serious damage to crop and lead to decrease in crop yield [3, 4].

Native *B. thuringiensis* isolates, retrieved from different locations (Table 1), were subjected to randomly amplified polymorphic DNA (RAPD) marker-based analysis for characterization of their genetic diversity. Recently, various techniques that rely on different nucleic acid pattern and discriminate at genetic level have been developed to gain information about the genetic diversity and genetic relationship between different organisms [5–7]. The RAPD marker-based analysis was found to be an easy, quick and reliable technique to assess the diversity of different types of organisms [8, 9] and this technology was successfully applied to characterize the genetic diversity in various *B. thuringiensis* isolates [10]. It has been found that using RAPD analysis, characteristic fingerprints of different bacterial strains have been generated, and even individual strains within the same serotype can be distinguished [11].

In the present investigation, an attempt was made to characterize the genetic diversity of 70 isolates of *B. thuringiensis* isolated from soil samples collected from cotton fields. The fields were devoid from any chemical spray. There may be a possibility that insects died due to ingestion of *B. thuringiensis* delta endotoxin protein. The native *B. thuringiensis* isolates were further subjected to characterization for their genetic diversity and differentiation using polymerase chain reaction (PCR)-based RAPD analysis.

## Materials and methods

### Isolation of native *B. thuringiensis* isolates

The isolation of *B. thuringiensis* was done according to method of Travers *et al* [12], and Ohba and Aizawa [13]. One gram of each soil sample was suspended in 10 ml of sterilized distilled water and kept at 80°C for 30 min. The *B. thuringiensis* isolates was selected by adding 1 ml of each suspension to 10 ml of Luria Bertani (LB) broth to which 0.25 M sodium acetate, pH 6.8 was added, incubated at 30°C for 4 h and kept at 80°C for 10 min. Further, suspensions were diluted and plated on $T_3$ medium (per liter: 2 g tryptose, 1.5 g yeast extract, 0.05 M sodium phosphate, 0.005 g of $MnCl_2$ and pH 6.8) and these plates were incubated at 30°C for 24 h. The selection method eliminates most of other spore-forming bacteria and non-spore forming organisms in soil sample. Heat shock eliminates the unwanted spore-forming bacteria and only spore-forming *B. thuringiensis* remains. Therefore, this method is specific to *B. thuringiensis*. The *B. thuringiensis* isolates were confirmed on the basis of morphology, gram staining and production of protein crystals. Smears were examined under a light microscope to observe the parasporal crystal protein as inclusion bodies inside the bacterial cell. The crystal-forming colonies were selected and subcultured for further use.

### DNA extraction from *B. thuringiensis* isolates

The *B. thuringiensis* cultures were purified by repeated re-streaking on solid LB medium plates. The fresh *B. thuringiensis* cultures were streaked on LB agar plate and incubated overnight at 30ºC. Then, a loopful of culture was suspended in 100 µl of sterile distilled water or in 1X TE buffer (10 mM Tris-HCl; and 1mM EDTA; pH 8.0) in a microfuge tube. The culture was heat-shocked for 10 min in a water bath set at 80ºC. Then, the heat-shocked bacterial cultures were centrifuged at 10,000 rpm for 10 min. After centrifugation, the supernatant containing DNA was transferred into fresh sterile eppendorf tube and stored at –20°C until further used for the RAPD analysis.

### PCR-RAPD analysis

PCR-RAPD analysis was carried out using decamer oligonucleotide primers, which had minimum 60% G+C content and lacked internal repeats (Operon Technologies, USA). Ten random decamer primers were used for PCR amplification (Table 2). PCR was carried out in a reaction volume of 25 µl containing 1X PCR buffer (10 mM Tris HCl (pH 8.3); 50 mM KCl, 0.1%, Triton X100), 2.5 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphates (dNTPs), 25 pmol primer, 100 ng genomic DNA and 1 U of Taq DNA polymerase (Ampli Taq, Perkin Elmer). A PTC-100 programmable thermal cycler (MJ Research, USA) was used for all amplification reactions. The PCR-RAPD analysis performed with the temperature profile, initial denaturation 94ºC for 3 min, denaturation 94ºC for 1 min, primer annealing 40ºC for 1 min, extension 72ºC for 3 min and final extension 72ºC for 15 min. These steps constitute one PCR cycle and these steps were repeated for subsequent cycles. A total of 35 cycles of amplification were performed and PCR completion reaction mixtures were stored at 4ºC until electrophoresis was performed.

### Data analysis

The RAPD profile (banding patterns) was visualized as pink bands in gel documentation system (Pharmacia Biotech, USA) and the photographs were documented. The bands were scored 1 for the presence and 0 for the absence of band. The data collected were normalized and further subjected to generation of dendrogram for correspondence analysis by using cluster analysis with the criteria of Ward's

**Table 1** Source of soil samples from different locations used for *Bacillus thuringiensis* isolation

| Sr. No. | Source of soil samples | Code of *B. thuringiensis* isolate(s) |
|---|---|---|
| 1. | Behtapur | A1, A2, A3, A4, A5, A6, A7, A8, A9, A10 |
| 2. | Tigawata | B1, B2, B3, B4, B5, B6, B7, B8, B9, B10 |
| 3. | Behni Badshapur | C1, C2, C3, C4, C5, C6, C7, C8, C9, C10 |
| 4. | Daulatpur | D1, D2, D3, D4, D5, D6, D7, D8, D9, D10 |
| 5. | Chamarkhera | E1, E2, E3, E4, E5, E6, E7, E8, E9, E10 |
| 6. | CICR, Sirsa | F1, F2, F3, F4, F5, F6, F7, F8, F9, F10 |
| 7. | Gillonkheri | G1, G2, G3, G4, G5, G6, G7, G8, G9, G10 |

**Table 2** Nucleotide sequence of random primers used for RAPD analysis of *Bacillus thuringiensis* isolates

| Sr. No. | Primer name | Primer sequence |
|---|---|---|
| 1. | OPA03 | AGCTCAGCCA |
| 2. | OPA08 | GTCCACACGG |
| 3. | OPC06 | GAACGGACTC |
| 4. | OPC20 | ACTTCGCCAC |
| 5. | OPD14 | CTTCCCCAAG |
| 6. | OPD17 | ATTCCCACGG |
| 7. | OPD19 | CTGGGGACTT |
| 8. | OPD20 | ACCCGGTCAC |
| 9. | OPE17 | CTACTGCCGT |
| 10. | OPE19 | ACGGCGTATG |

distance, using Windostat software. For maximum accuracy of comparison, all isolates were processed with the same batch of PCR master mix.

## Results and discussion

In the present study, native strains of *B. thuringiensis* were isolated from soil samples. The different *B. thuringiensis* isolates were confirmed on the basis of the method described by Travers et al. [12]—shape, gram staining and the presence of spores and crystals. All isolates were rod-shaped, gram-positive, and spores and crystals were seen inside the bacterium. The diversity of 70 *B. thuringiensis* strains were characterized by RAPD markers their genetic diversity determined, which differentiates the isolates of *B. thuringiensis* isolated from the different places using ten random decamer primers (Tables 1 and 2). The RAPD analysis is considered to be a fast and simple method. Once the primers revealing the polymorphism were identified and PCR conditions optimized, slight differences in primer sequences caused significantly different RAPD patterns and enabled the easy discrimination among the strains [7]. In comparison to other molecular typing methods, RAPD is faster, reproducible and less labor-intensive, and eliminates the need for pure DNA. Only a small amount of template is required for the amplification reaction and shows clear differences among bacterial strains [14].

The RAPD marker has been effectively used to detect the genetic diversity among the 70 *B. thuringiensis* isolates obtained from cotton field soil. The genetic diversity among *B. thuringiensis* strains characterized by employing RAPD marker analysis has been reported earlier and hundreds of *B. thuringiensis* strains were characterized from diverse locations and fall into different groups revealing the strain differences [15–17]. The different random decamer primers used for RAPD-based fingerprinting of 21 serovars of *B. thuringiensis* obtained 172 polymorphic fragments ranging from 161 to 2789 bp in size, 19 serovars of *B. thuringiensis* fall in two major clusters and remaining two formed solitary clusters in the dendrogram. The clustering of *B. thuringiensis* strains established genetic relatedness between serovars and serotypes [18].

Our study indicates that RAPD provides a high degree of discrimination between *B. thuringiensis* isolates. On analysis of the dendrogram (Fig. 2) it was observed that the *B. thuringiensis* isolates clustered according to their geographical location. It was observed that all the

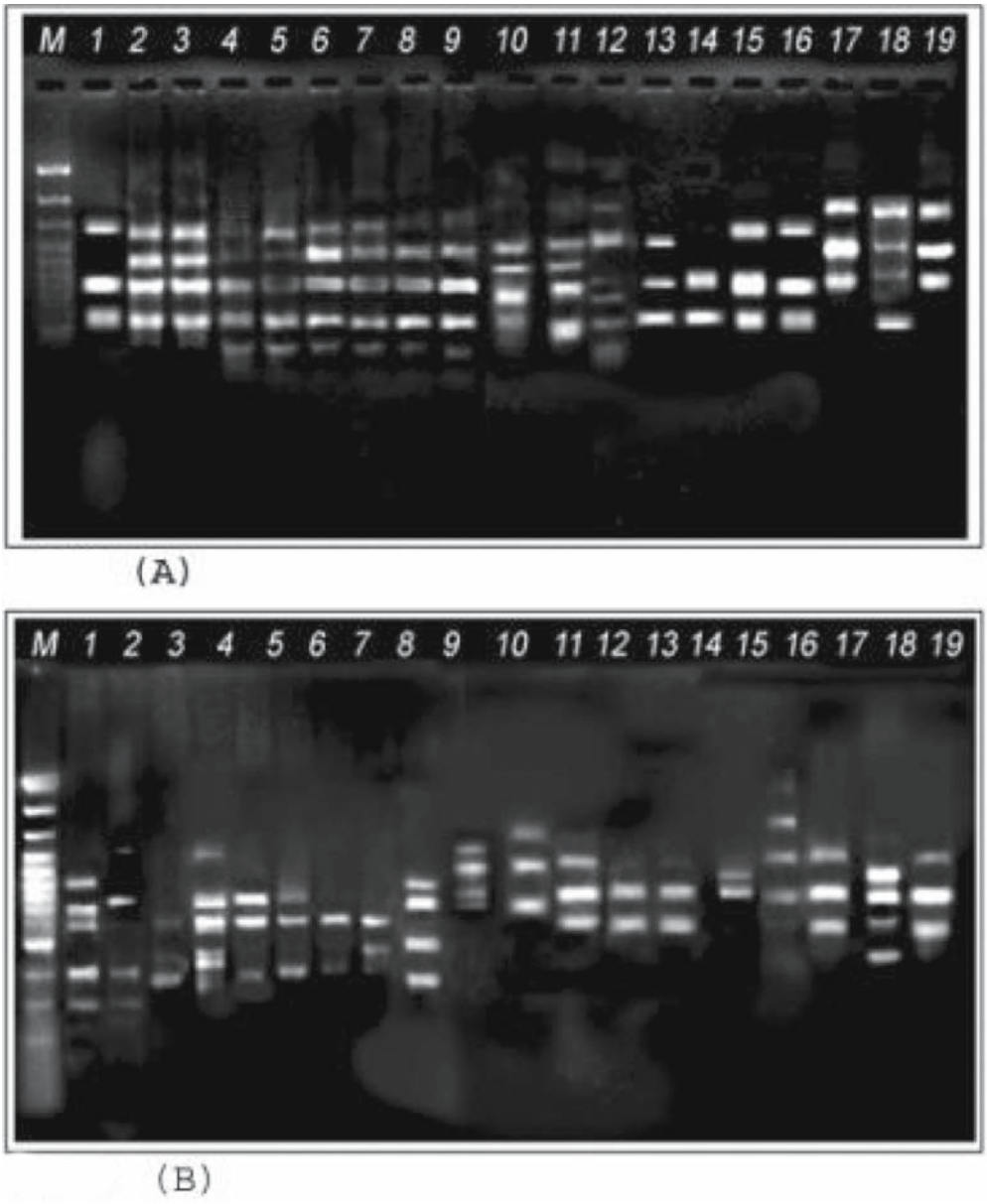


**Fig. 1.** Representative RAPD profiles showing polymorphism among *Bacillus thuringiensis* isolates, electrophoresed on 1% agarose gel and stained with ethidium bromide. The amplification of DNA was carried out using (A) for primer OPD17 and (B) for primer OPC20. Lane 1 molecular weight marker (100 bp) ladder. Lanes 2 to 20 indicate A1 to A10, and B1 to B9 *Bacillus thuringiensis* strains.

primers used for RAPD analysis showed amplification and generated RAPD fingerprint for *B. thuringiensis* isolates (Fig. 1A and 1B). The primers OPA03, OPA08, OPD14, OPD19, OPD20, OPE17 and OPE19 produced 100% polymorphic fragments, whereas primers OPC06, OPC20 and OPD17 produced 20, 31 and 17 monomorphic fragments, respectively; which contributes to 90.1, 89 and 88% of polymorphism (Table 3). An average of 193.5 fragments was produced per primer. The primer OPE17 was found most discriminatory as it produced the highest number of 286 polymorphic fragments. Cluster I contained 26 *B. thuringiensis* isolates, which were isolated from three different locations Behtapur, Gillonkhera and Daulatpur. Cluster II contained 44 *B. thuringiensis* isolates and standard *B.thuringiensis* strain at 70% similarity. Cluster II was further divided into two subclusters; subcluster I contained isolates from the Chamarkhera, Tigawata and Daulatpur and subcluster II had isolates from Tigawata, Behni Badshahpur, Chamarkhera, CICR (Sirsa) and subcluster III contained isolates from Behni Badshahpur, Chamarkhera, CICR (Sirsa). It was observed in this study that isolates from Daulatpur fell into cluster I and cluster II, this

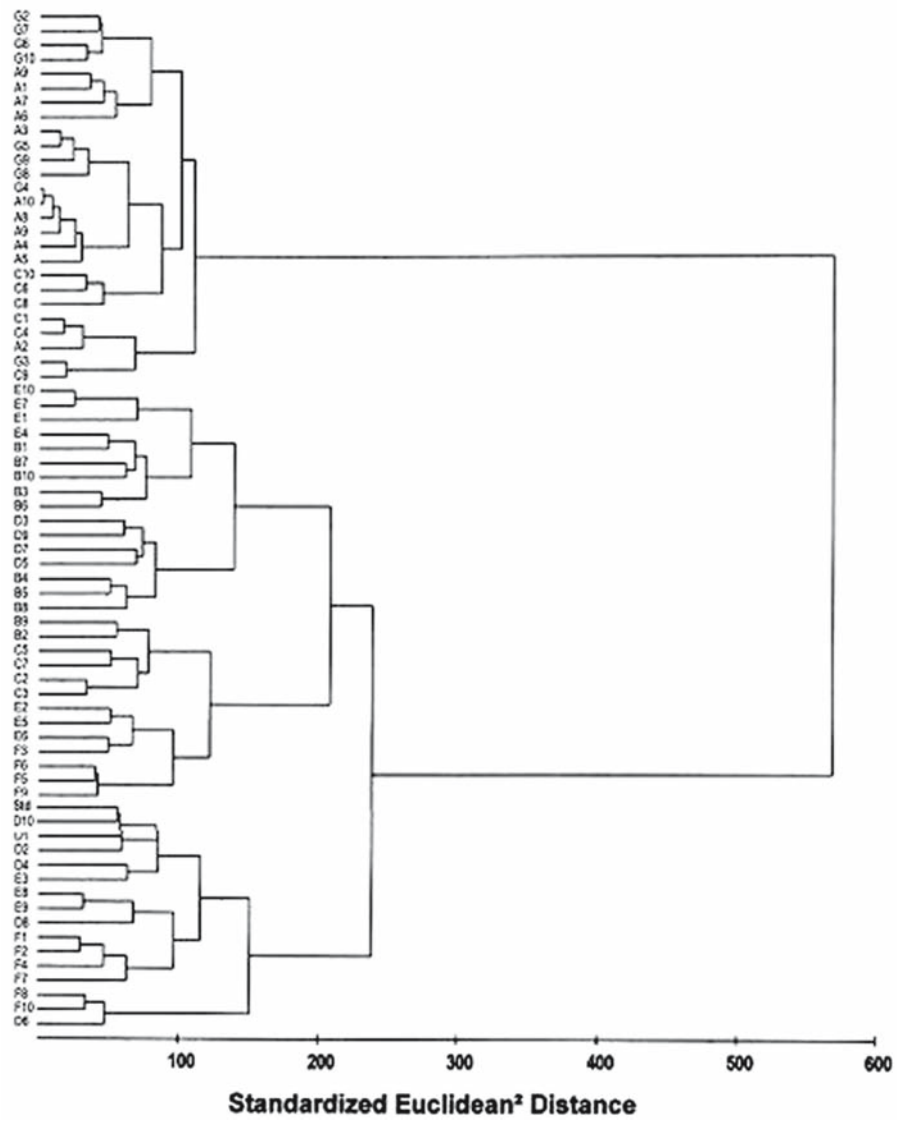


**Fig. 2.** Ward's minimum variance dendrogram of 70 *Bacillus thuringiensis* isolates based on RAPD fingerprinting pattern

**Table 3** Random primers showing polymorphism among native isolates of *Bacillus thurigiensis*

| Sr. No. | Primer | Primer sequence | No. of isolates amplified | Total bands obtained | Polymorphic bands | Monomorphic bands | Polymorphism (%) |
|---|---|---|---|---|---|---|---|
| 1. | OPA03 | AGCTCAGCCA | 70 | 191 | 191 | - | 100 |
| 2. | OPA08 | GTCCACACGG | 70 | 212 | 212 | - | 100 |
| 3. | OPC6 | GAACGGACTC | 70 | 186 | 166 | 20 | 90.1 |
| 4. | OPC20 | ACTTCGCCAC | 70 | 295 | 264 | 31 | 89 |
| 5. | OPD14 | CTTCCCCAAG | 70 | 213 | 213 | - | 100 |
| 6. | OPD17 | ATTCCCACGG | 70 | 153 | 136 | 17 | 88 |
| 7. | OPD19 | CTGGGGACTT | 70 | 158 | 158 | - | 100 |
| 8. | OPD20 | ACCCGGTCAC | 70 | 150 | 150 | - | 100 |
| 9. | OPE17 | CTACTGCCGT | 70 | 286 | 286 | - | 100 |
| 10. | OPE19 | ACGGCGTATG | 70 | 71 | 71 | - | 100 |

indicated that these isolates originated in one region and later diverged from the main lineage and occupied different ecological niche. Cluster analysis of the dendrogram indicated that the *B. thuringiensis* isolates obtained from the same region occupied the same clusters, whereas the different clusters indicated the location difference of the isolates.

The RAPD analysis could effectively distinguish the different native isolates of *B. thuringiensis* isolated from soil samples. RAPD analysis is considered an important molecular biology technique, which is used for the identification of indigenous *B. thuringiensis* isolates. In comparison to other molecular typing methods, RAPD is faster, less labor-intensive and eliminates the need for pure DNA; only a small amount of template DNA is required for amplification reaction [7, 19]. Seventy *B. thuringiensis* strains subjected to genetic diversity determination using RAPD as a marker. Of these 10 random decamer primers were utilized and were further used to detect the genetic diversity and differentiation of these strains according to the source of origin (Tables 1 and 2) [7, 19]. Our results clearly differentiated *B. thuringiensis* isolates based on their location of origin. Further investigations is needed to characterize the *B. thuringiensis* isolates from the other different habitats so that new strains of *B. thuringiensis* can be identified and used as the source of new genes. These strains could possibly have a broad insecticidal spectrum against insects of different orders.

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