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DNA finger printing of *Bacillus thuringiensis* based on repetitive DNA sequences using ERIC-PCR

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ABSTRACT

To assess the distribution and evolutionary conservation of distinct prokaryotic repetitive elements, consensus oligonucleotides were processed in polymerase chain reaction (PCR) amplification with genomic DNA from different *Bacillus thuringiensis* (*Bt*) strains from different parts of Mizoram, India. Oligonucleotides matching enterobacterial repetitive intergenic consensus (ERIC) sequences were synthesized and tested as opposing PCR primers and produced clearly resolvable bands by gel electrophoresis, which provided unambiguous DNA fingerprints of the different *Bt* strains. After analysing with NTYSYS, DARwin and POWERMARKER, a dendogram was constructed, which revealed that the *Bt* strains were divided into three main clusters. Widespread distribution of the repetitive DNA elements enabled rapid identification of these *Bt* strains.

Key words: *Bacillus thuringiensis*; ERIC-PCR; DNA; polymorphism.

INTRODUCTION

Bacteria are the most successful group of insect pathogens from a practical and commercial point of view, but the number of useful species is surprisingly small. Three species of spore forming *Bacillus*, *B. thuringiensis* (*Bt*), *B. sphearicus* and *B. popilliae* are among the most important, with *Bt* being of prime importance.[1] *Bt* has been in use under various formulations since 1938 and is the oldest commercial biopesticide, released in 1959.[2]

Bt is a Gram-positive, soil-dwelling bacterium, and also occurs naturally in the gut of caterpillars of moths and butterflies, as well as on the dark surface of plants.[3] Some *Bt* strains produce insecticidal proteins called δ-endotoxins during sporulation, and are used as biopesticide and more recently genetically modified crops.[4]

The *Bt* strains have a genome size ranging from 2.4-5.7 million bp.[5] Genetic maps of *Bt* and *B. cereus* showed very close similarity between the two genomes near the replication origin, while greater variability was seen in the terminal half of their respective genomes.[6] Chromosomal DNA hybridization,[7] phospholipid and fatty acid analysis,[8] 16S rRNA sequence comparison,[9] amplified fragment length polymorphism analysis[10] and genomic

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restriction digest analysis[5] supported a single-species hypothesis. Analysis of a 16S rRNA variable region remains controversial.[11] The problem arises with the production of the parasporal crystal, the only definitive characteristic of *Bt,* which is too narrow a criterion to taxonomical issue. The large variety of *Bt* strains and toxin diversity could be due to the high degree of genetic plasticity.[12]

The genomic fingerprinting method employed is based on the use of DNA primers corresponding to naturally occurring repetitive elements in bacteria, and the polymerase chain reaction (PCR) reaction (rep-PCR).[13,14] Three families of repetitive sequences have been identified such as the 35-40-bp repetitive extragenic palindromic (REP) sequence, the 124-127-bp enterobacterial repetitive intergenic consensus (ERIC) sequence, the 154-bp BOX element.[15] These sequences appear to be located in distinct, intergenic positions around the genome. The repetitive elements may be present in both orientations, and oligonucleotide primers have been designed to prime DNA synthesis outward from the inverted repeats in REP and ERIC, and from the box A subunit of Box, in PCR.

The use of these primer(s) and PCR leads to the selective amplification of distinct genomic regions located between REP, ERIC or BOX elements. The corresponding protocols are referred to as REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprinting, respectively, and rep-PCR genomic fingerprinting, collectively.[15,16] Hence, in the present study an attempt was made to characterize the variations in the DNA fingerprint patterns among 17 isolates of indigenous *Bt* isolated from soils in different districts of Mizoram.

MATERIALS AND METHODS

Bacterial samples

The study was conducted on a total of 21 *Bt* strains (Table 1). The standard strains *Bt alesti* (ALS), *Bt israelensis* (ISRL), *Bt kurstaki* (KRS) and *Bt tenebrionis* (TENB) were gifted from Bacillus Genetic Stock Culture, Ohio, USA, and were included as controls. DNA extractions were done from lyophilized *Bt* strains following a genomic DNA isolation based procedure.[17]

Table 1. List of *Bt* strains and primers used..

Place	Locations/site	Strain no and abbreviation
Aizawl	University campus; Ramrikawn; Ramrikawn Fish pond; Ramrikawn banana plantation; Sihhmui; Sairang	4 (CAMP), 6 (RK), 6 (RKa), 6b (RKb), 12 (SH),15 (SR)
Champhai	Champhai	16 (CH)
Lawngtlai	Chhimtuipui	5 (CHTP)
Mamit	Chhippui; West Phaileng; Hmunpui; Lengte; Lengpui; Rowpuichhip	2 (CHP), 7 (WP), 9 (HP), 17 (LT), 18 (LPI), 22 (RP)
Saiha	Serkawr Grass; Serkawr Jhum; Serkawr road side	11 (SG), 28 (SK), 29 (SKd)
Serchhip	Thenzawl	24 (TZ)
Myanmar	Rih Dil	3 (RD)
Primer: ERIC 1R 5'- ATGTAAGCTCCTGGGGATTCAC-3' ERIC 5'- AAGTAAGTGACTGGGGTGAGCG-3'		

Isolation of genomic DNA

1.5 ml of *Bt* strains were centrifuged at 8,000 rpm at 28°C for 5 min in separate micro -centrifuge tubes and the pellet was collected by discarding the supernatant. The pellet was resuspended in 500 µl TE buffer (pH 8.0 with HCl) by repeated pipetting. 50 µl of 10% SDS and 4 µl of 100 mg/ml proteinase K was added, and mixed and incubated for 1 hr at 37°C. An equal volume of water-saturated phenol and chloroform (500 µl) was added and mixed well. It was centrifuged at 10,000 rpm for 15 min at room temperature. The upper aqueous phase was transferred to a new tube and an equal volume of phenol and chloroform (500 µl) was added and mixed well. The mixture was centrifuged at 8,000 rpm for 10 min at room temperature. The upper aque-

ous phase was transferred to a new tube and 0.1 volume of the sodium acetate (pH 5.2) was added. 600 µl of isopropanol was added and mixed properly until the DNA precipitates. It was kept in freezer at -20°C for 30 min if no pellet was visible. The mixture was centrifuged at 10,000 rpm for 5 min at 4°C. The pellet was washed with 1 ml of 70% ethanol for 30 sec and again centrifuged at 10,000 rpm for 5 min at 4°C (this step repeated twice). The pellet (DNA) was air dried and dissolved in 30-50 µl TE buffer and stored at 4°C.

Quantification of genomic DNA by agarose gel electrophoresis

0.8 g of agarose was dissolved in 100 ml of 1X TAE buffer (2 ml of 50X TAE and 98 ml of distilled water) from the stock of 50X TAE buffer. 4 µl of 10 mg/ml of ethidium bromide was added. It was allowed to solidify and DNA sample was run at 50 V.

ERIC-PCR fingerprinting

ERIC-PCR genomic fingerprinting[13] was carried out with primer as in Table 1. Briefly, 50-100 ng of purified DNA was used as template in a 10 µl reaction mixture containing 10X PCR buffer (1 µl), 10 pM of ERIC primer (1.5 µl each), 50 mM deoxynucleotide triposphates (0.2 µl each), 2 unit of Taq DNA polymerase, 3 mM $MgCl_2$ and remaining dd H_2O was added to make the mixture up to 10 µl. PCR amplification was performed in the Gradient Eppendorf thermocycler with one initial denaturation (94°C, 7 min), followed by 30 cycles of denaturation (94°C, 45 sec), annealing (52°C, 1 min), and extension (65°C, 8 min), and a single final extension (65°C, 10 min), followed by cooling at 4°C. 10 µl of each PCR reaction were then electrophoresed directly on 1.5% agarose gel containing 1X TAE (Tris-EDTA) buffer. 4 µl ethidium bromide was added along with the low range DNA ruler plus molecular marker. This gel was allowed to run at a constant voltage and was then visualized under UV transilluminator in a gel documentation system.

Data analysis

The amplified fragments were scored manually for their presence (1) or absence (0) for each *Bt* strains using ERIC primer. The amplicons were in the size range of 160-3000 bp. Only distinct and polymorphic bands were recorded and used in the analysis. For generating similarity matrices, only polymorphic bands with PIC values higher than 0.08 were used.[18] The binary matrix was used to estimate genetic similarities using Jaccard's coefficient The similarity matrix was subjected to unweighed pair group method of arithmetic averages clustering in order to construct the phenetic dendrogram. For this, the cophenetic coefficients between the matrix of genetic similarities and the matrix of cophenetic values were computed using appropriate routines of the NTSYS-pc package. The significance of the cophenetic correlation and the correlation coefficient of similarity matrix generated with RAPD data were tested using the Mantel matrix correspondence test.[19] Statistical analysis was performed using NTSYS-pc software version 2.20.[20] The reliability and robustness of the phenograms were tested by bootstrap analysis for 950 bootstraps for computing probabilities in terms of percentage for each node of the tree using the DARWin software.[21]

Genotyping data from RAPD was used for assessing the discriminatory power of the respective assays by evaluating three parameters, namely, (a) polymorphism information content (PIC),[18] (b) marker index (MI), and (c) resolving power (RP). PIC was averaged over the fragments for each primer combination. Resolving power of each primer combination was calculated by $RP=\Sigma Ib$.[22]

RESULTS AND DISCUSSION

Isolated DNA was tested for its quality

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Table 2. Comparative band attributes of different *Bt* strains isolated from Mizoram using ERIC primer.

Primer	Sequence	Total Band	Polymorphic Band	Polymorphism (%)	PIC	RP	EMR	MI
ERIC1	5′ATGTAAGCTCCTGGGGATTCAC-3′	61	34	55.5	0.14	5.8	0.55	0.084
ERIC2	5′AAGTAAGTGACTGGGGTGAGCG-3′							

and quantity on the gel. A single band of genomic DNA was visible (Fig. 1) indicating good quality and there was no shearing of the DNA. The DNA concentration for various samples ranged between 50-100 ng/µl.

21 *Bt* strains were analyzed using ERIC primer. All bands between replicate samples were completely reproducible (Fig. 2) and were able to clearly distinguish all *Bt* strains. A total of 61 bands were detected (Table 2). The number of polymorphic bands detected with ERIC-PCR was 34 (55.5%) and the average heterozygosity obtained was zero. However, all other banding attributes such as EMR, MI, and RP were markedly good. Cluster analysis was done using only polymorphic bands with PIC value above 0.08 to minimize errors due to rare alleles. The total number of such bands was 61. The Jaccard's similarity coefficient ranged from 0.70 to 0.95.

The dendrograms obtained are shown in Figure 3. The overall topology of majority of *Bt* strains was similar in both dendrograms with few exceptions. Two major clusters were formed with both the datasets. In NTSYS, it was deviated into three cluster groups. The first cluster was again divided into a series of sub-clusters, which contained the strains RD, CAMP, RKa, CHTP. The second sub-cluster contained the strains RKb, LT and LPI, the third sub-clusters were SK, SKd, KRS and ALS. RK was placed in the fourth sub-cluster (Fig. 3b). The second sub cluster was also again divided into two sub-clusters. The first sub-cluster contained the strains WP, SH, HP, SR and ISRL. The second sub-cluster contained the strains CH and TENB. The strain TZ was placed in the third cluster. In Darwin also, all the *Bt* strains were placed in three major clusters. The first cluster was again divided into two sub-clusters (i and ii). The sub-cluster (i) was again divided into sub-cluster (a) and (b). In (a) the strains RKa, CAMP and CHTP were present. In (b) strains RD and RK were present together. The sub-cluster (ii) contained the strains SR, HP and SH. The second cluster was also again divided into two sub-clusters (i and ii). The sub cluster (i) was again divide into (a) and (b). In (a), strains RP, LT and LTI were present whereas in (b), strains TENB and CH were present. In sub cluster (ii), strains TZ and RKb were present. In the third cluster, strains KRS, SKd, ALS, WP and ISRL were found to be clustered together (Fig. 3a). Similarity matrix (Table 4) revealed highest (0.971) similarity coefficient between RKa and CAMP, LPI and LT, LPI and RP minimum (0.647) similarity coefficient was observed between SK and LT, TZ and TENB.

Strain specific bands were observed, e.g. a band of 610 bp with CHTP and RK and two bands of 600 and 180 bp lengths with HP and SR were amplified in the strains and these can be considered as marker bands (Table 3). These bands revealed the existence of some conserved regions and suggested the interspecific genetic relatedness, and provided diagnostic profiles for these strains, as reported in three species of white fringed weevils (Coleoptera) and two of *Parnassius* (Lepidoptera: Papilionidae).[23,24] The present results were in accordance with those of Sharma *et al*.[25] in which an interspecific genetic relatedness and polymorphisms were observed. The mean value for the genetic diversity was found to be 0.1525. The mean value for the PIC was found to be 0.1378 (Table 4).

ERIC-PCR, therefore, allows clear distinc-

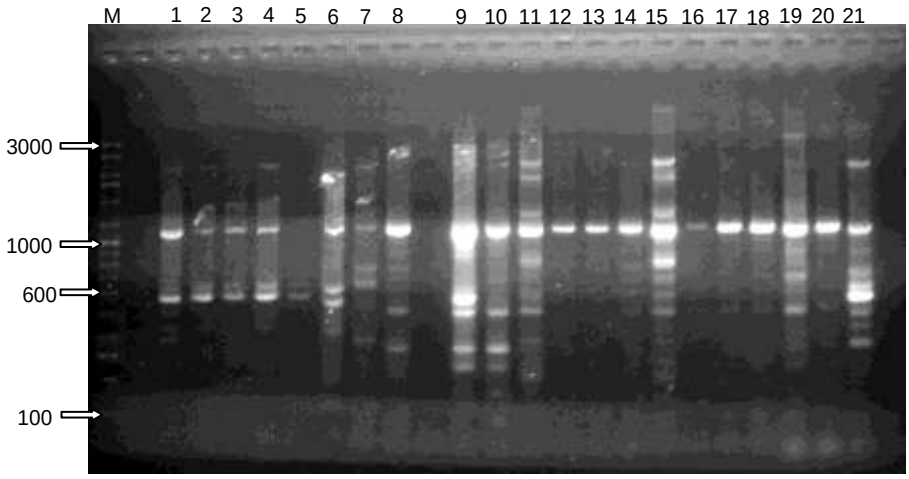

Figure 1. ERIC PCR profile of *Bt* Strains. Lane 1-RD; 2-CAMP; 3-CHTP; 4-RK; 5 -Rka; 6-RK; 7-WP; 8-HP; 9 -SH; 10-S; 11-CH; 12-LT; 13-LPI; 14-RP; 15-TZ; 16-SK; 17-Skd; 18-ALS; 19-ISR; 20-KRS; 21-TENB; M-low range DNA ruler plus used as size markers

Table 3. Similarity matrix of ERIC PCR products obtained on amplification with ERIC primers.

bp	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**	**L8**	**L9**	**L10**	**L11**	**L12**	**L13**	**L14**	**L15**	**L16**	**L17**	**L18**	**L19**	**L20**	**L21**
	RD	CAMP	CHTP	RK	Rka	RKb	WP	HP	SH	SR	CH	LT	LPI	RP	TZ	SK	SKd	ALS	ISRL	KRS	TENB
3700																			3700		
1960													1960		1960						
1940											1940										1940
1820				1820																	
1710	1710																				
1460															1460						
1440											1440										
780											780				780						
650																650					
640																	640			640	
630							630											630	630		
620											620	620	620	620							620
610			610	610																	
600								600		600											
590	590								590												
480																			480		
420											420										
410															410						
380							380														
370																					370
360															360						
350																			350		
330		330					330														
300						300									300						300
290	290	290	290		290																290
280									280												
270						270															
260															260				260		
250								250		250	250										250
240									240												
200							200														
190									190												190
180								180		180											

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Figure 3(a). Dendrogram showing UPGMA clustering of *Bt* strains using ERIC-PCR data (NTSYSpc2.2 software).

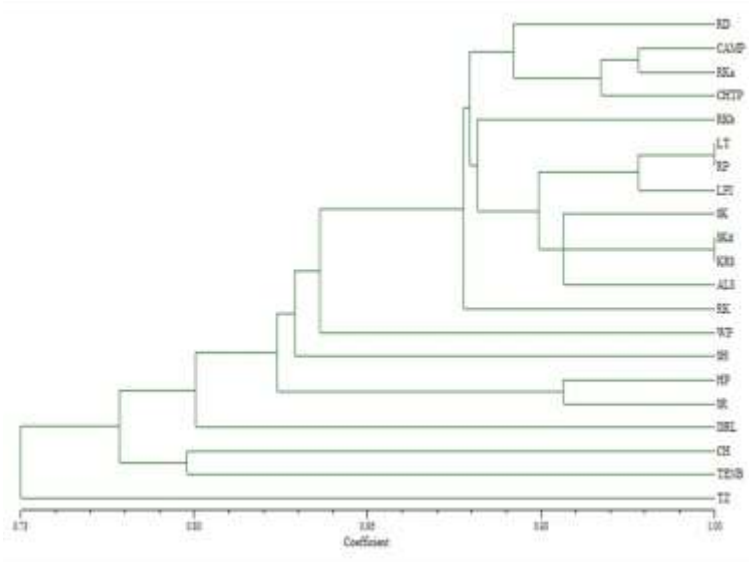
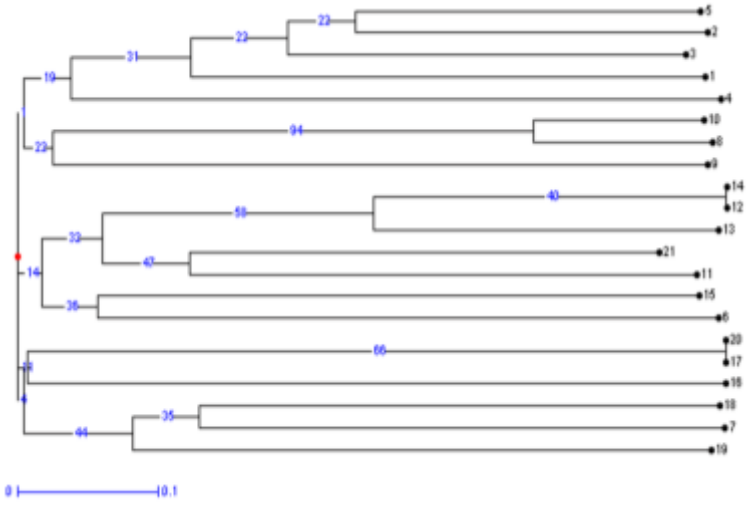


Figure 3(b). Dendogram showing UPGMA clustering of Bt strains ERIC-PCR data. The numbers on nodes indicate % bootstrap values (DARwin software).
1-RD;2-CAMP;3-CHTP;4-RK;5-RKa;6-RKb;7-WP;8-HP;9-SH;10-SR;11-CH;12-LT;13-LPI;14-RP;15-TZ;16-SK;17-SKd;18-ALS;19-ISRL;20-KRS;21-TENB.



tions between different *Bt* strains which contain these repetitive elements. Although the majority of the rep-PCR DNA fingerprinting studies have been performed for epidemiological and strain tracking purposes, this technique also has great versatility and can be used to study microbial ecology and microbial evolution as it has the sensitivity to accurately genotype microbes at the strain or isolates level. Currently, the isolation of the target microorganisms is a prerequisite for performing rep-PCR DNA fingerprinting, due to the complexity of banding patterns in microbial community DNA. However, whole genome amplification (e.g. multiple displacement amplification) can be coupled to the rep-PCR technique to allow for the determination of the genotypes of single cells without cultivation. If the resulting fingerprints are reproducible, it will open a new door for the study of the uncultured microbial world at the strain rather than population or community levels.

Table 4. PIC values of different markers using the Powermarker software.

Marker	PIC	AMOVA	F- statistics
P3700	0.0866	Sum of	Mean- 0.14
P1960	0.1575	square –	SD – 0.028
P1940	0.1575	31.27	
P1820	0.0866		
P1710	0.0866		
P1460	0.0866		
P1440	0.0866		
P780	0.1575		
P650	0.0866		
P640	0.1638		
P630	0.2149		
P620	0.297		
P610	0.1575		
P600	0.1575		
P590	0.1575		
P480	0.0866		
P420	0.0866		
P410	0.0866		
P380	0.0866		
P370	0.0866		
P360	0.0866		
P350	0.0866		
P330	0.1575		
P300	0.2225		
P290	0.3047		
P280	0.0866		
P270	0.0905		
P260	0.1575		
P250	0.2608		
P240	0.0905		
P200	0.0905		
P190	0.1575		
P180	0.1638		
P160	0.1575		
Mean	0.1378		

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