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Optimization of RAPD-PCR Conditions for the Study of Genetic Diversity in Nepalese Isolates of *Bacillus thuringiensis Berliner*

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Abstract

Random amplified polymorphic DNA (RAPD) is a simple and reliable method to detect DNA polymorphism and has been used extensively for genetic diversity studies. In the present investigation the RAPD reaction and cycling conditions were optimized for generating RAPD fingerprints of ten Nepalese strains of *Bacillus thuringiensis Berliner (Bt)* isolated from an altitudinal range of 70 meter above sea level (masl) to 5050 masl. To determine the optimum conditions, different concentrations of $MgCl_2$, template DNA, Taq DNA polymerase, primer, dNTPs as well as different cycling programs were analyzed. Reproducible amplification patterns were obtained using 0.4 µM of primer, 2.5 mM of $MgCl_2$, 125 ng of template DNA, 0.2mM of dNTPs and 1U Taq DNA polymerase in 25 µl of the reaction volume. Cycling programs were also optimized. Out of 100 arbitrary primers screened, amplification performed with 24 primers generated the best RAPD fingerprints. The optimized RAPD-PCR conditions and the selected primers are suitable for further work on genetic diversity analysis of Nepalese isolates of *Bt.*

Key words: DNA fingerprint, primer, Taq DNA polymerase, template DNA

Introduction

Bacillus thuringiensis Berliner (Bt) is a spore forming, crystalliferous Gram positive bacteria of the family *Bacillaceae* (Schnepf *et al.*1998). During sporulation, it produces intracellular insecticidal crystal protein (*cry* protein) that is toxic to insect larvae in the orders Lepidoptera, Diptera, Coleoptera etc. The *cry* protein from *Bt* has been developed as a successful biological agent to control insect pests.

Morphological and physiological characteristics have traditionally provided a wealth of Bacillus systematic information for establishing *Bacillus* classification system (Quingming *et al.*1997). The development of molecular techniques such as DNA-DNA hybridization and DNA sequencing in bacterial taxonomy have permitted objective determination of inter and intra relatedness of species. However, all these techniques are time consuming, expensive and complex while dealing with a large number of strains.

More recently, arbitrarily primed PCR-base techniques (variously named as RAPDs, AP-PCR and DAF) have been introduced allowing rapid screening of a large number of genotypes for the study of genetic diversity and systematic relationships (Williams *et al*. 1990, Welsh & McClelland 1990, Caetano-Annoles *et al*.1991a, b). These techniques use short (4-10 bp) synthetic deoxyribonucleotides of arbitrary sequences as primers and do not require specific sequence information for the design of PCR primers. Because the number of potential primers that can be used is very large, numerous polymorphisms can be detected even between closely related organisms.

Of these, RAPD has been a very popular employed technique to generate genus-specific, species specific, or strain-specific diagnostics DNA fragments or fingerprints, identifying genes linked to traits of interest; undertaking genetic diversity studies and gene mapping for development of diagnostics, etc. (Abad *et al*. 1998, Ransom *et al*. 1998, Bazzicalup & Fani 1996).

Nepal is rich in biological diversity due to its unique topography, geography and climatic gradients.

Ten local isolates of *B. thuringiensis* were isolated from different geographical regions of Nepal varying in altitudes ranging from75 masl-5050 masl. (Rana *et al*. 2002 a,b). Present investigation has been undertaken to optimize RAPD-PCR reaction and cycling conditions for the generation of RAPD profiles for the study of genetic diversity and relationship in Nepalese isolates of *B. thuringiensis* species.

Materials and Methods

Ten local strains (Table 1) isolated from various geographical location of Nepal and one commercial strain has been used in the present study.

Table 1. *B. thuringiensis* strains used for optimization

S.N	Area	Code No.	Altitude (masl)
1	Nepalgunj	Nep1	144
2	Nepalgunj	Nep2	144
3	Pokhara	Bag1C	827
4	Pokhara	Bag2A	827
5	Chitwan	Chit-5	400
6	Biratnagar	Birat-6	72
7	Biratnagar	Bir2	72
8	Lobuche	Lob6	5000
9	Lobuche	Lob9	5050
10	Lobuche	Lob10	5050
11	Btk (ringer)	Btk	-

DNA extraction

Four different DNA extraction techniques (Bravo *et al*. 1998, Juarez-Perez *et al*. 1997, Ceron *et.al.* 1995 and Hansen *et.al.* 2001) were assessed for generating DNA fingerprinting.

DNA estimation

DNA quantification as well as quality assessment was carried out spectrophotometrically using Biophotometer (Eppendorf – AG 22331, Germany).

Gel electrophoresis

The quality of extracted DNA was assessed using 1.5 % agarose gel electrophoresis (in EMBI TEC Santiago, CA gel tank) in TAE buffer (Tris / Acetic Acid / EDTA) at 25 V (4.2 V/cm) for half an hour. PCR products were electrophoresed on 1.5% (w/v) agarose gel in 1x TAE buffer at 25 V (4.2 V/cm) for 1.5 h. The gels were stained with ethidium bromide (10mg/ml solution) for 45 minutes, de-stained for 15 minutes in water prior to visualization and photography using UV trans-illuminator (UVITEC, Japan) and Polaroid Gelcam (UK).

PCR optimization

Best RAPD-PCR cycling conditions for *Bt* was selected from four randomly selected PCR cycles *viz*.,Yu and Pauls (1994), Giovana *et al* .(2001), Arango *et al*. (2002), Araujo *et al*. (2004) and were used for DNA fingerprinting.

PCR reactions were performed in 25 µl reaction volume. The optimum RAPD-PCR reaction conditions were selected by varying several parameters *viz*. DNA concentration (50, 75, 100, 125, 150, 175, 200, 225, 250 and 275 ng); $MgCl_2$ concentration (1.5, 2.5, 3.5 and 4.5.mM); Primer concentration (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 µM) Taq DNA polymerase concentration (0.5, 1.0, 1.5 and 2.0 U) and dNTP concentration (100, 200, 300 and 400 mM).

Primer screening

Using optimized reaction and cycling conditions for *Bt*, 100 UBC (University of British Columbia,Vancouver, Canada) random primers were screened and best primers producing multiple crispy bands were selected for subsequent profiling experiments**.**

Results and Discussion

Selection of DNA extraction protocol for *Bt*

Among the four protocols considered for the DNA extraction technique for *Bt*, modified method of Hansen *et al.* (2001) proved to be efficacious in yielding strong and reliable amplification products as compared to

other methods. The quality of the template DNA has a great effect on the generation and resolution of amplified products. Because the amplification process requires such small amounts of template DNA, extraction procedures that emphasize purity rather than quantity are usually most appropriate for RAPD research (Weeden *et al*. 1992).

Optimization of RAPD –PCR reaction and cycling parameters

For the generation of DNA fingerprints for RAPD–based genetic diversity studies, a number of parameters starting from DNA isolation procedure, template DNA concentration, $MgCl_2$ concentration, Taq DNA polymerase concentration, primer concentration and dNTPs concentration need to be optimized as it is highly sensitive to reaction and cycling parameters resulting into lack of reproducibility (Weeden *et.al.* 1992). Furthermore, change of thermal cycle could also induce non-reproducibility. Therefore, this technique demands strict maintenance of all the parameters following its final optimization. Optimized RAPD-PCR reaction and cycling parameters are shown in Table 2.

Table 2. Optimization of the RAPD-PCR reaction parameters for Nepalese *Bacillus thuringiensis* isolates

S. N.	PCR parameters	Tested range (Concentration / RAPD cycles)	Optimum conditions
1.	DNA concentration (ng)	50, 75, 100, 125, 150, 175, 200, 225, 250 and 275	125 ng
2.	$MgCl_2$ concentration (mM)	1.5, 2.5, 3.5 and 4.5	2.5 mM
3.	dNTPs concentration (mM)	100, 200, 300 and 400	0.2 mM
4.	Primer concentration (µM)	0.1, 0.2, 0.3, 0.4, 0.5 and 0.6	0.4 µM
5.	Taq polymerase concentration (U)	0.5, 1.0, 1.5 ,2.0 and 2.5	1.0 U
6.	PCR programs	a) 4 min of initial denaturation at 95^0C, 45 cycles of denaturation at 94^0C for 1 min, annealing at 36^0C for 1 min and extension at 72^0C. (Giovana *et al*. 2001) b) 3 min of initial denaturation at 95^0C, 40 cycles of denaturation at 95^0C for 1 min, annealing at 34^0C for 2 min and extension at 72^0C. Final extension at 72^0C for 5 min. (Arango *et. al*.2002). c) 4 min of initial denaturation at 92^0C, 40 cycles of denaturation at 92^0C for 1 min, annealing at 37^0C for 2 min and extension at 72^0C for 3 mins. Final extension at 72^0C for 3 min.(Araujo *et. al*. 2004) (d) 1 min of initial denaturation at 94^0C, 35 cycles of denaturation at 94^0C for 10 s, annealing at 38^0C for 30 s and extension at 72^0C for 60s. Final extension at 72^0C for 5 min. (Yu & Pauls 1994)	a) Giovana *el al* 2001

The ratio of DNA template to primer is one of the most critical factors to consider when optimizing the PCR. Therefore, a range of DNA concentrations should be tried against a fixed primer concentration for each DNA extraction protocol to obtain the ideal conditions (Tyler *et al*.1997). It was observed that the use of 125 ng of DNA in the PCR mix provided the best results (Fig. 1). The concentration of Taq DNA polymerase had an influence on the amplification. 1U of Taq DNA polymerase produced the best result.

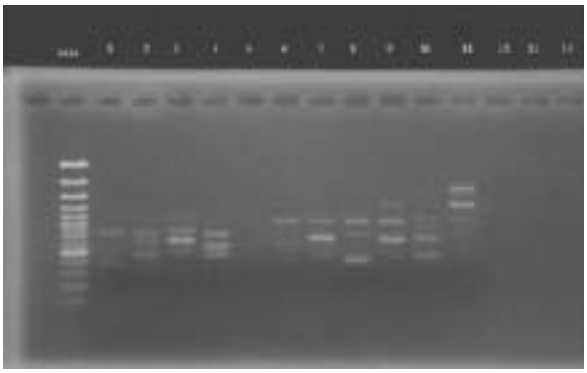

Fig. 1. RAPD-PCR assays for the selection of DNA concentration using primer UBC 1 and DNA of strain Lob 9. Lane marked MM are 100 base pair molecular weight marker; Lanes 1-4 and 6-11 having 50, 75, 100, 125, 150, 175, 200, 225, 250 and 275ng of Bt DNA respectively.

The variation in $MgCl_2$ concentration modified the RAPD profile. The best banding patterns were observed at 2.5mM concentration of $MgCl_2$ (Fig 2). Lower concentration and higher concentrations of $MgCl_2$ had significant effects upon the RAPD profile produced, revealing fewer and non-specific bands. Since the $MgCl_2$ is co-factor of the Taq DNA polymerase enzyme, it influences the DNA amplification process. Lower quantities than the necessary ones lead to the amplification failure or deficient amplification whilst excess Mg^{2+} results in non-specific amplifications as a result of reduced enzyme fidelity (Saiki 1989).

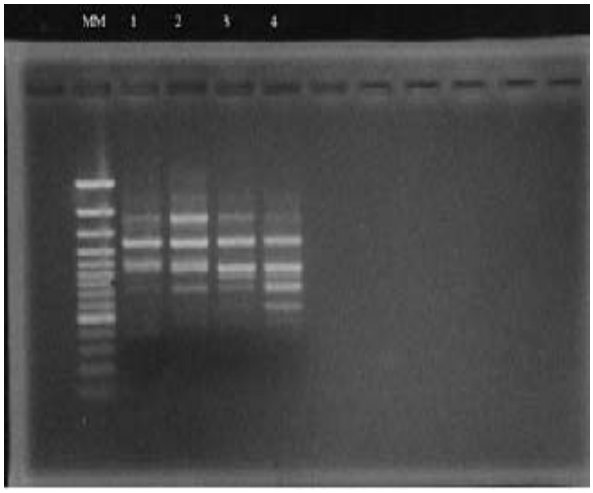

Fig. 2. RAPD – PCR of Bt for selection of $MgCl_2$ concentration using primer UBC1 and DNA of Lob 9 strain. Lane marked MM is 100 base pair molecular weight marker. Lanes 1-4 RAPD fingerprint in of varying concentration of $MgCl_2$ 1.5, 2.5, 3.5, 4.5 mM respectively.

After optimization of all the reagents, RAPD amplification was performed in a total volume of 25 ul reaction mixture. Optimized RAPD-PCR reaction volume of 25 µl contained 125 ng of template DNA, 2.5mM of $MgCl_2$, 0.2mM each of dATP, dTTP, dGTP and dCTP, 2.5 µl PCR buffer, 0.4 µM primer and 1.0 U of Taq DNA polymerase. The method of Giovana *et al.* (2001) produced the best result (Table 2).

Primer Screening

A series of 100 decamer primers from codes 1-100 (obtained from UBC) were tested. Amplification performed with 24 primers generated the best RAPD profiles and were found to amplify genomic DNA fragments with reproducible polymorphisms (Fig. 3 & 4). The GC content of these primers ranged from 60-80%. Amplified fragments ranged from 500 bp to 1300 bp (Table 3).

Table 3. Selected 10 bp random primers for Nepalese *Bt* isolates

S.N	Primer code	Nucleotide sequence 5' to 3'	GC %
1	UBC 1	CCTGGGCTTC	70
2	UBC 2	CCTGGGCTTG	70
3	UBC 3	CCTGGGCTTA	60
4	UBC 4	CCTGGGCTGG	80
5	UBC 6	CCTGGGCCTA	70
6	UBC 16	GGTGGCGGGA	80
7	UBC 17	CCTGGGCCTC	80
8	UBC 43	AAAACCGGGC	60
9	UBC 51	CTACCCGTGC	70
10	UBC 54	GTCCCAGAGC	70
11	UBC 55	TCCCTCGTGC	70
12	UBC 65	AGGGGCGGGA	80
13	UBC 66	GAGGGCGTGA	70
14	UBC 67	GAGGGCGAGC	80
15	UBC 71	GAGGGCGAGG	80
16	UBC 73	GGGCACGCGA	80
17	UBC 74	GAGCACCTGA	60
18	UBC 76	GAGCACCAGT	60
19	UBC 85	GTGCTCGTGC	70
20	UBC 86	GGGGGGAAGG	80
21	UBC 88	CGGGGGATGG	80
22	UBC 92	CCTGGGCTTT	60
23	UBC 96	GGCGGCATGG	80
24	UBC 97	ATCTGCGAGC	60

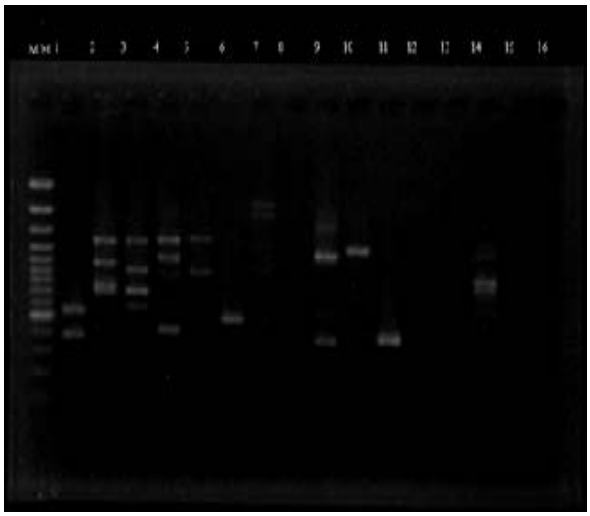

Fig. 3. RAPD – PCR for the primer screening experiment using DNA of Lob 9 strain. Lanes marked MM are 100 base pair molecular weight marker. Lanes 1-14, results of primer screening involving UBC primer 15-28

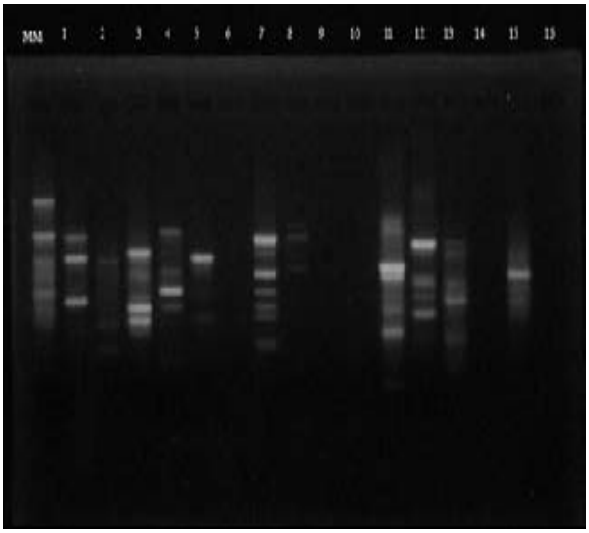

Fig. 4. RAPD – PCR of Bt for the primer screening using DNA of Lob 9 strain ane 1-16 results of primer screening involving UBC primer 85-100

Almost all the tested parameters for RAPD-PCR like the concentration of template DNA, primer, $MgCl_2$, Taq polymerase, dNTPs, temperature and PCR program were optimized which produced clear, multiple scorable amplified products suitable for RAPD amplification. The optimized reaction and cycling parameters thus obtained from present investigation will be used in subsequent experiment on RAPD profiling for genetic diversity study of Nepalese *Bt*. isolates.

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