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Interspecies diversity of *Bacillus thuringiensis* isolates native from North Western Indian Himalayas

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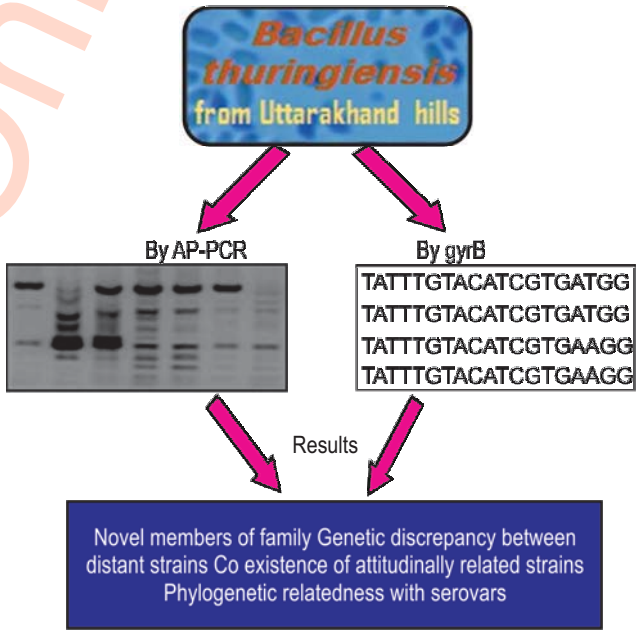
Abstract

Aim : *Bacillus thuringiensis* (Bt) is a ubiquitous pathogenic bacterium exploited worldwide in successful insect pest management programs. The present study aimed at resolving the diversity status and genetic structure of Bt isolates collected from unique and native ecological niche, north-western Indian Himalayas.

Methodology : Forty five *Bacillus thuringiensis* (*Bt*) isolates native to Uttarakhand, India were analyzed for their genetic diversity using Arbitrary Primed-Polymerase Chain Reaction (AP-PCR) patterns (ERIC1, ERIC2, BOX and GTG) and gyrase *B* (*gyrB*) sequences.

Results : Amongst the AP-PCR patterns, ERIC and BOX primers clearly indicated the genetic inconsistency of *Bt* by differentiating low and high hill isolates with unique banding pattern. A dendrogram generated showed fastidious clustering with coexistence of attitudinally related strains, indicating the genetic relatedness with respect to geographical proximity. The *gyrB* (285 bp) dependent phylogeny of seven selected isolates revealed their genetic proximity with different serovars.

Interpretation : The present study demonstrates that ERIC and BOX-PCR can be used in establishment of geographical variation and *gryB* in phylogenetic relatedness with serovars. Genetic relatedness between *Bt* isolates was found to be a function of geographical proximity.



Introduction

Globally rising problems on environmental pollution, resistance development, trade restrictions etc., compels limited use of chemical pesticides against agricultural pests. On the other hand, various pest species belonging to Lepidoptera, Coleoptera and Diptera are continued to be the limiting factors of agricultural production demanding alternative control measures. In this context, use of biopesticides with comparable efficacy to chemical pesticides emerged as a potential tool in successful management of target pests (Chandlera *et al*., 2008). Amongst the bioinsecticides, *Bacillus thuringiensis* (*Bt*) occupies prime position in successful materialization as a commercial insect pathogen. The unique mode of action by the bacterium (Sanahuja *et al*., 2011) offers ecological benefits with no known non-target effects, environmental pollution and bioaccumulation.

Elite strains of *Bt* with toxicity against different non-hosts with putative genes (Kuo and Chak, 1996) having potential in transgenic development has been reported (Kumar *et al*., 2008). So, it is important to look for new *Bt* strains, and great efforts are presently being undertaken at different research centers (Lima *et al*., 2002). Polymerase chain reaction árez-Pérez, 2003; Zara *et al*., 2006), diversity analysis (Awad *et al*., 2007; Katara *et al*., 2012) and target prevision (Carozzi *et al*., 1991) without the need to carry out all the tedious and time-consuming bioassays and biochemical analysis. Regarding the genomic diversity within the *Bt* different molecular approaches have been used, including Restriction Fragment Length Polymorphism (RFLP) (Akhurst *et al*., 1997), Ribotyping (Joung and Cote, 2001), Random Amplified Polymorphic DNA (RAPD) (Malkawi *et al*., 1999), Arbitrary Primed Polymerase Chain Reaction (AP-PCR) (Brousseau *et al*., 1993), Repetitive Extragenic Palindromic sequences (REP-PCR) (Katara *et al*., 2012). However, direct comparison among the results obtained through these methods complicates assessment of genomic variability (Garcia *et al*., 2015) of tested *Bt* collections and were found to be insufficient.

On the other hand, Protein-coding genes exhibit much higher genetic variation, which can be used for classification of closely related taxa (Yamada *et al*., 1999). The desired features of targeted diversity analysis genes would be that they do not transmit horizontally as plasmid DNA does, their molecular evolution rate is higher than that of 16S rRNA and they are distributed universally among bacterial species. Awad *et al*. (2007) reported that variability in a fragment of 352 bp belonging to DNA gyrase subunit B (*gyrB*) protein is an efficient, rapid and easy method to make a distinction between native *Bt collection.* Besides, conservation and understanding diversity in native *Bt* isolates is of prime importance with respect to the evolutionary relatedness and toxicological profiles. Keeping this in view, a collection of 45 *Bt* isolates native to Uttarakhand Himalayas were studied for their genetic diversity and phylogenetic relatedness using a combination of AP-PCR and *gyrB* gene sequences.

Materials and Methods

Bacterial strains : The *Bt* isolates used in this study were collected from different soil samples from all the 13 districts of Uttarakhand state, North Western Indian Himalayas and maintained in pure and viable form in 40 % glycerol stocks at -80 ºC. The threshold height for mid hills was considered as 1000 to 1700 amsl, below and above which were considered as plain/low and high hills, respectively. All the isolates were recovered and retained as single spore colonies in Luria bertani (LB) slants at 4°C for ease in use. The bacterial cultures were obtained by growing in LB broth at 250 rpm at 30°C, unless otherwise specified.

Total DNA extraction : The DNA was isolated by CTAB NaCl procedure as described by Sambrook and Russel (2001), with some modifications. Briefly, the vegetative cells from an overnight grown culture (4 ml) were pelleted by centrifugation (10000 rpm for 2 min at 4°C), washed twice with TE buffer (10 mM tris-HCl, 1 mM EDTA, pH 8.0) and resuspended in 550 µl of same buffer containing 10% SDS and 1 mg ml^{-1} of proteinase K. After incubation for 1 hr at 37°C, the cells were lysed by adding 100 µl of 5 M NaCl and 80 µl of CTAB-NaCl solution (10 mM tris, 20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol in 2% CTAB solution pH 8.0). After incubation at 65°C for 10 min, the suspended proteins were avoided by addition of equal volume of PCI (Phenol, Cholorofarm, Isoamyl alcohol in 25:24:1) followed by centrifugation (10000 rpm for 20 min). The DNA from upper aqueous phase was precipitated by addition of two volumes of ice cold isopropanol preceded with 70% ethanol wash. The air dried pellet was dissolved in 50 µl TE buffer, quantified using spectrophotometer and adjusted to a concentration of 25 ng $µl^{-1}$. The residual RNA was eliminated by RNase treatment at 37 °C for 30 min.

PCR amplification : The details of primers used for amplification of *gyrB,* ERIC I, ERIC II, GTG and BOX-PCR is given in Table 1. The reaction mixture contained 50 ng DNA, 300 µ*M* each dNTP, 1X reaction buffer, 0.2 µ*M* of each primer, 2.5 m*M* $MgCl_2$, 1.25 U Taq DNA polymerase in a total volume of 20 µl. The *gyrB* fragment was amplified in a thermocycler (Biorad) programmed with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of amplification consisting initial denaturation at 94°C for 1 min, annealing at 46ºC for 1 min, extension at 72°C for 1 min. and a final extension step at 74°C for 10 min. Whereas, the annealing temperatures for ERIC, GTG and BOX-PCR were 54ºC, 42ºC and 60ºC, respectively. Each cycle extension time was increased to 8 and 4 min for ERIC and GTG programs for better visualization of amplicons. A negative control without target DNA was also included in each experimental run.

Electrophoretic analysis : The amplicons and patterns were resolved in agarose (1%) gel electrophoresis of 15 µl of PCR product. The gels were run in TAE (40 mM Tris-acetate, 1 mM

Table 1 : Details of the primers used in the study

Primer name and sequence	Amplicon pattern/gene	Annealing temperature (ºC)
BCFW1 - 5'-GTTTCTGGTGGTTTACATGG-3'	*gyrB (*352 bp*)*	46
BCRW1 - 5'-CAACGTATGATTTAATTCCACC-3'		
ERIC I - 5'ATGTAAGCTCCTGGGGATTCAC'3	ERIC I	54
ERIC I - 5'ATGTAAGCTCCTGGGGATTCAC'3	ERIC II	54
ERIC II - 5'AAGTAAGTGACTGGGGTGAGCG'3		
GTG - 5'GTGGTGGTGGTGGTGGTG'3	GTG	42
BOX - 5'GATCGGCAAGGCGACGCTGAC'3	BOX	60

Table 2 : Details of Bt isolates and GeneBank Accession numbers of the *gyrB* sequences

S.No	Isolate	Collection site	Altitude (m)	Accession number
1	UKBt3	Pantnagar	344	KX025078
2	UKBt5	Tanakpur	255	KX025079
3	UKBt10	Ramnagar	345	KX025080
4	UKBt15	Darim	1850	KX025081
5	UKBt28	Ladgav	1518	KX025082
6	UKBt33	Dunagiri	2400	KX025083
7	UKBt36	Gwaldam	1960	KX025084

Table 3 : Patterns of nucleotide substitution (MCL estimate) in *gyrB* fragments of *Bt* isolates native to Uttarakhand Himalayas, India

	A	T	G	C
A	-	*12.26*	*7.05*	**1.18**
T	*18.39*	-	**1.24**	*8.77*
G	*18.39*	**2.16**	-	*8.77*
C	**2.48**	*12.26*	*7.05*	-

Only entries within a row should be compared. Substitutions are shown in bold and those of transversions are shown in italics

EDTA) buffer at 2 V/cm for 5 hrs. After staining with ethidium bromide, bands were visualized using UV illumination and documented with an Alpha image analyzer (Bio-Rad).

Analysis of PCR patterns : Polymorphic bands from each PCR patterns were individually identified by their specific migration rates in the electrophoretic analyses by calculating molecular size of bands against 100 bp DNA ladder (Invitrogen, USA). Once bands were properly and distinctively identified, binary (0/1) matrices were constructed to compare the patterns. Jaccard's similarity coefficients were generated by the SIMQUAL subroutine from the NTSYS- pc 2.02 (Applied Biostatistics, Inc.) package. Cluster analysis along with their corresponding dendrograms were generated by the unweighted-pair group method using average linkages (UPGMA), with the SAHN and TREE subroutines from the NTSYS-pc package.

Sequencing and analysis of *gyrB* fragments : Based on UPGMA dendrogram and REP-PCR patterns seven major clusters were observed, based on which a total of seven *Bt* isolates (UKBt3, UKBt5, UKBt10, UKBt15, UKBt28, UKBt33 and UKBt36) were selected for sequencing of *gyrB* fragments. After amplification, the PCR products of *gyrB* fragments were purified using gel elution columns (Sigma) and sequenced from Scigenome labs, Kochi, India. Nucleotide sequences were aligned with the Clustal Omega (1.2.1) multiple sequence alignment (McWilliam *et al*., 2013) and molecular evolutionary analyses were performed using the software MEGA4 (Molecular Evolutionary Genetic Analysis version 4) (Tamura *et al*., 2007). The phylogenetic tree was constructed by the neighbor-joining method using the distance matrix from the alignment along with other published sequences of different *Bt* serovars (Accession numbers given in tree).

Results and Discussion

The AP-PCR electrophoretic patterns of Uttarakhand *Bt* isolates showing polymorphic bands is presented Fig. 1. They revealed a total of 32 polymorphic DNA fragments. The number of fragments using ERIC 1 primers varied from 1 to 9 per isolate, with a total of 10 electrophoretic patterns with the amplicons size ranging between 182 to 2178 bp (Fig. 1A). ERIC II primers generated 1 to 8 fragments per strain with a total of 12 electrophoretic patterns with fragments ranging from 378 bp and

2983 bp (Fig. 1B). BOX primer generated between 5 and 10 fragments and a total of 16 electrophoretic patterns with fragment sizes between 323 and 2011 bp (Fig. 1C). The GTG primers generated six electrophoretic patterns with fragment sizes ranging between 500 to 3025 bp (Fig. 1D). The ERIC and BOX-PCRs were the most informative. Predominant amplicons in ERIC 1, ERIC 2, BOX and GTG PCRs were found to be 600 bp (71.1%), 572 bp (77.7%), 2000 bp (37.8%) and 3025 bp (91.1%) respectively.

In both the ERIC-PCRs, *Bt* isolates from most of the plain and low to mid hill regions (twenty seven isolates from UKBt1 to UKBt27, accounts to 60%) showed similar amplicon patterns. Overall, most of hill isolates showed highly diverse and unique patterns among them. Amongst the plain and low to mid hill isolates, only two isolates (UKBt5 and UKBt6) showed unique amplicon patterns (7.4%) which were isolated from Tanakpur and Raiwala, respectively. In addition, one isolate, UKBt10 showed

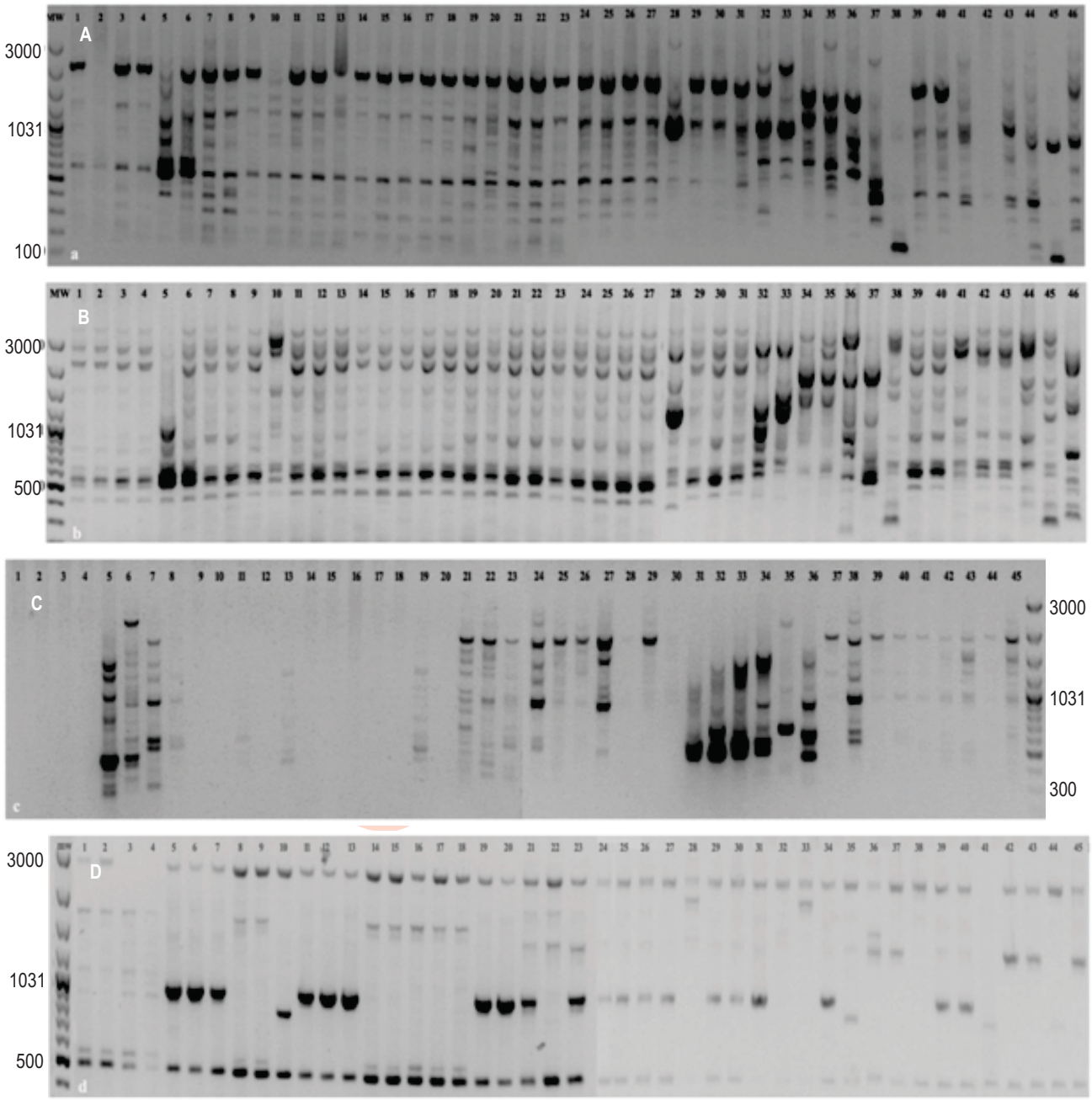


Fig. 1 : AP-PCR patterns of *B. thuringiensis* isolates native to Uttarakhand Himalayas. (a) ERIC1; (b) ERIC2; (c) BOX and (d) GTG. The numbering of each lane indicates the Bt strain isolated from North Western Indian Himalayas

little divergent pattern in ERIC2-PCR which was isolated from Bhowali. The remaining 24 amplicon patterns were similar and can be represented as single genotype. This altitudinal variation indicates the microclimate and ecosystem reliant evolution of the bacterium (Garcia *et al*., 2015; Zara *et al*., 2006; Vilas-Boas and Lemos, 2004). The soaring diversity observed here in the high hill isolates may be due to spatial separation of the strains generated by conquest of hills and mountains.

The BOX-PCR generated no amplicons in 16 isolates. This has been confirmed by permutations of primer and DNA concentrations. However, the isolates with amplicons showed unique patterns except UKBt39, UKBt40, UKBt41, UKBt42, UKBt43, UKBt44 and UKBt45 which showed similarity. All these isolates were collected from altitude range of 1890 to 2200 amsl from Munsyari, Joshimath and Uttarakhasi areas. The same isolates showed little diversity in ERIC and GTG patterns which shows the resolving power of BOX-PCR. de Silva and Valicente (2013) also reported that BOX-PCR is most informative than ERIC. Taken as a whole, the AP-PCR patterns showed a substantial variation in Uttarakhand *Bt* isolates irrespective to the geographical variation indicating heterogeneous distribution with relative low relatedness (Garcia *et al*., 2015). Studies also reported the use of AP-PCR primers in phenotyping of *Bt* serotypes (Katara *et al*., 2012) and wild type collection from India (Katara *et al*., 2012), Russia (Boulygina *et al*., 2009), Mexico (Garcia *et al*., 2015) and *Spodoptera frugiperda* toxic collection from Embrapa (de Silva and Valicente, 2013). The dendrograms generated from all the three amplification patterns (Fig. 2) showed high phenotypic variability among the 45 *Bt* isolates.

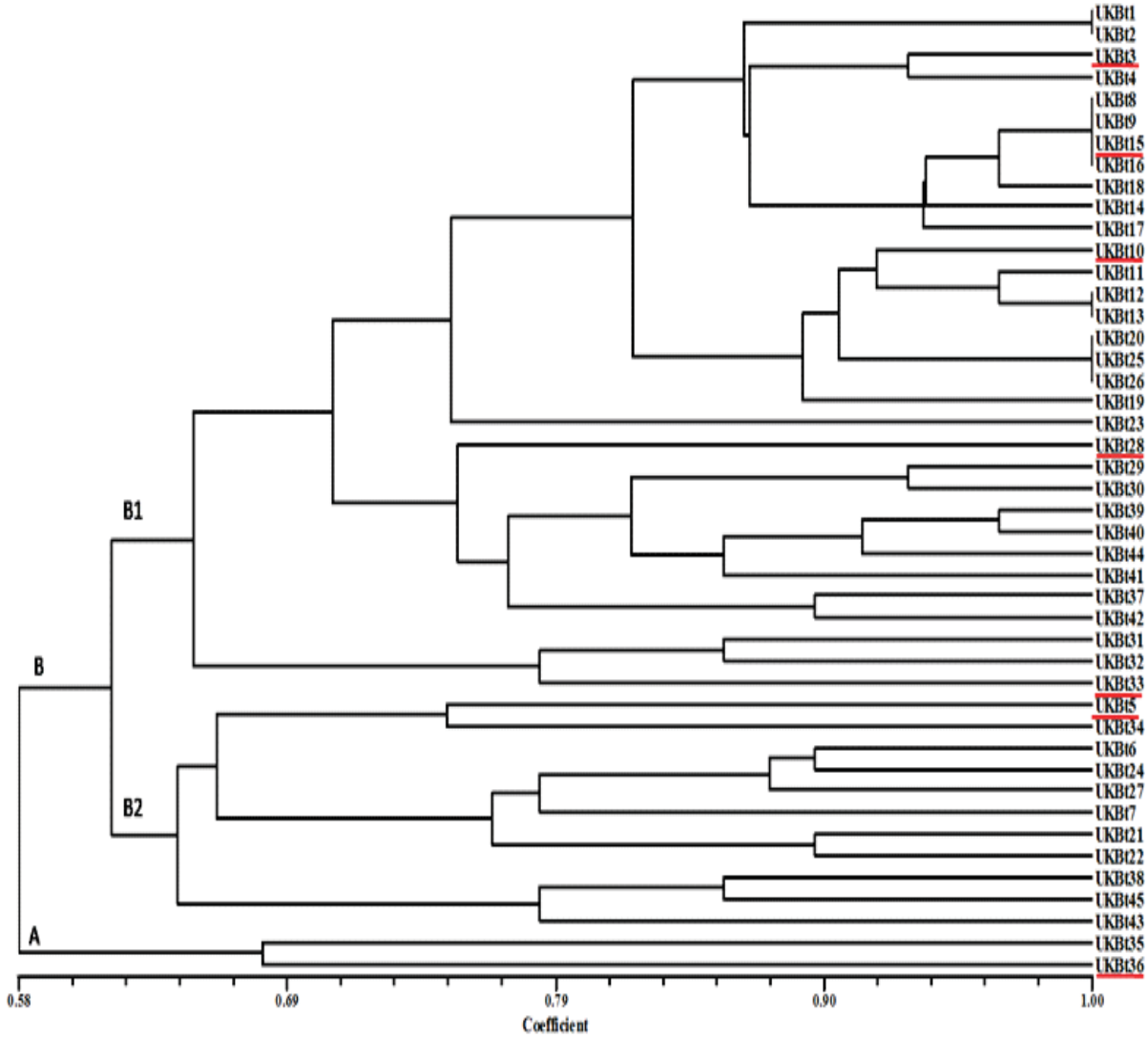


Fig. 2 : Dendrogram generated from AP-PCR patterns of 45 Bt isolates. The coefficient scale close to 1 indicates high similarity values. The isolates sequenced for *gyr*B were underline

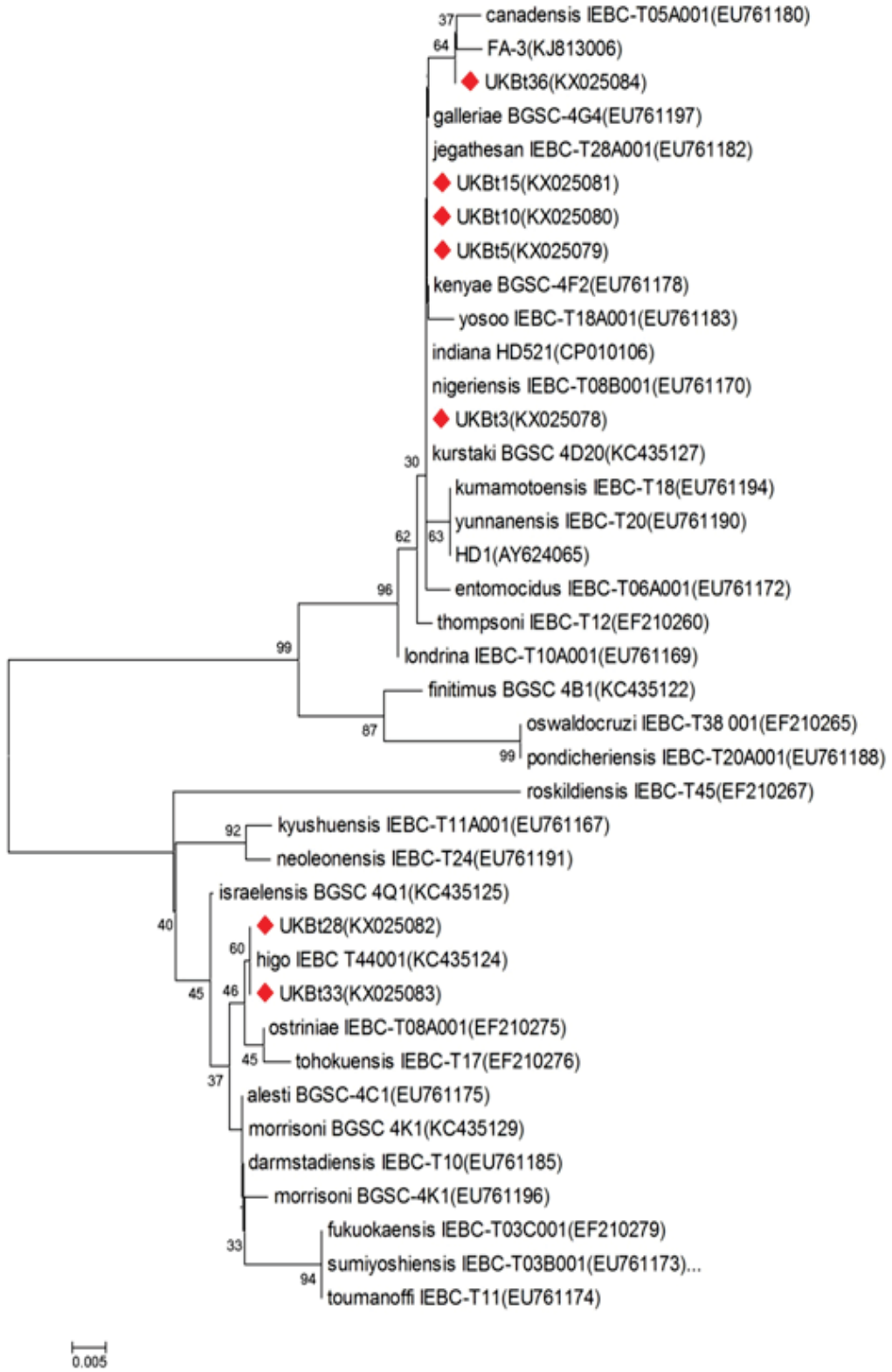


Fig. 3 : Phylogenetic tree based on the *gyrB* sequences of *B. thuringiensis* isolates native to Uttarakhand Himalayas, India in relation to different serovars constructed using neighbor-joining method. The genetic distances were computed by Kimura's model. The numbers at the nodes indicated percentages of occurrence in 1000 bootstrapped trees. Accession numbers were given in parenthesis. Boot strap values higer than 30 were indicated

Even though there are two main clusters, the degree of similarity among strains between and within branches was low, as revealed by the multiple secondary branches and very dissimilar coefficient values. The two major groups, "A" and "B", were formed at a similarity level of 0.58. The largest group "B " consists of 43 isolates covering both hill and plane isolates. However, the group "A" consists of only two isolates, UKBt35 and UKBt36 isolated from around 2000 m elevation. The largest cluster was further divided into two major branches (B1 and B2) at a similarity coefficient of 0.61, which contained mixed elevation isolates. Interestingly, an observation on grouping of the isolates at high similarity values showed coexistence of sequentially collected samples which indicates genetic relatedness with respect to the geographical proximity that is at diminutive levels.

The *gyrB* sequences were submitted to NCBI Gene Bank nucleotide sequence databases (Table 2). Similarity search using the Blast programme (http://www.ncbi.nlm.nih.gov/Blast.cgi) showed close relatedness to *gyrB* sequences of different *Bt* strains and serovars. Moreover, a multiple sequence alignment of 285 bp showed a high similarity level with nucleotide substitutions *viz*., 23 single nucleotide polymorphisms and one pair base substitutions. These substitutions resulted in a neighbor joining phylogenetic tree with two distinct branches, the first one with UKBt28 and UKBt33 (the high hill isolates) with bootstrap consensus of 100 and the second one with remaining plane and mid hill isolates (Fig. 3). In this second clade, the isolate, UKBt36 was found to be distinct among the rest (UKBt5, UKBt3, UKBt10 and UKBt15). The tree also showed phylogenetic proximity of UKBt28 and UKBt33 with *Bt* serovar higo. The high hill isolate, UKBt36 was associated with *Bt* serovar canadensis. In addition, some serovars showed low indexes of statistical confidence indicating the taxonomic relatedness between the serovars. The nucleotide substitution patterns of *gyrB* sequences is presented in Table 3. The analysis showed an overall nucleotide composition of A, T, C and G as 39.6, 26.4, 15.2 and 18.9%, respectively. The transition/transversion rate ratios for purines (k_1) and pyrimidines (k_2) were found to be 0.135 and 0.176, respectively with overall transition/transversion bias of 0.046 (R).

Single-strand conformation polymorphisms of the 16S rDNA amplification products (a frequently used modern bacterial classifications and taxon identifications method) do not allow species discrimination within the *Bacillus anthracis–cereus–thuringiensis* group (Borin *et al*., 1997). In this group, sequence analysis of a house keeping gene, *gyrB* is found to be more highly differential than 16S (La Duc *et al*., 2004; Huang, 1996). Studies have also reported the discriminatory power of this technique in native *Bt* collection (Awad *et al*., 2007; Punina *et al*., 2013), as well as serovars from the same H-serotype and strains from same serovar (Soufiane and Cote, 2009). Awad *et al*. (2007) utilized the nucleotide substitution patterns by *gyrB* PCR-RE digestion using *Sau*3AI to distinguish between *Bt* strains. The originality of this study was the identification followed by confirmation of the exceptionality of native *Bt* strains based on REP-PCR patterns and *gyrB* sequences, respectively. Punina *et al*. (2013) reported an agreement between the AP-PCR and *gyrB* allocation of *Bt* isolates from Ukraine. The nucleotide conservation patterns and resulting phylogeny between the native isolates clearly differentiated isolates from plain or low hill areas (UKBt3, UKBt5, UKBt10 and 15) and high hill regions (UKBt28 and UKBt33) with an exception of UKBt36. This variability also suggests the microclimate dependent evolutionary and ecological relationships between the *Bt* isolates.

In conclusion, the present study showed *Bt* collection from the Uttarakhand Himalayas represent a diverse members of *Bt* family. This uniqueness revealed from the AP-PCR and *gyrB* gene analysis may be the result of prevailing altitudinal variations within the mountain ranges that supported microclimate driven evolutionary discrepancies in Bt. Among the AP-PCR patterns, ERIC and GTG-PCR clearly indicated this ecosystem driven variability among the Bt isolates. Altitudinal dependent difference is clear in ERIC pattern whereas GTG patterns indicated variability between plane isolates. Overall, the study identified putative *Bt* isolates (UKBt3, UKBt35 and UKBt36), which are genetically divergent from the rest of the isolates. In view of this, further studies were planned to identify their uniqueness in bioactivity against insect pests, *cry* gene content, biotechnological applications etc.

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