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Genetic characterization of native *Bacillus thuringiensis* strains isolated from Tamil Nadu, India

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ABSTRACT

B. thuringiensis is a crystalliferous bacteria used as a biocontrol agent against lepidopteran, dipteran, and coleopteran pests. Seventy eight *Bacillus thuringiensis* strains were isolated from 108 soil samples collected from Tamil Nadu, India. Phylogenetic relationship of *B. thuringiensis* isolates were evaluated based on PCR amplified fragment polymorphisms of flagellin genes (PCR-AFPF). The isolated *B. thuringiensis* strains comprised of 51.3% of known biochemical types and 48.7% of undescribed *B. thuringiensis* types. PCR-AFPF UPGMA dendrogram generated using Jaccard coefficient values showed two phylogenetic groups, group A and B comprised of I-XIII and XIV-XV clusters respectively. The present study concluded that *B. thuringiensis* isolates from Tamil Nadu have a high degree of genetic diversity and high rate of genetic exchange.

KEY WORDS: *BACILLUS THURINGIENSIS*, PCR-AFPF, GENETIC DIVERSITY, UPGMA DENDROGRAM

INTRODUCTION

Chemical insecticides that are currently used to control insect pests are extremely toxic to non-target organisms and many insects have developed resistance to different chemical pesticides, resulting in ineffective insect control programs. They are deleterious to the health of humans and animals, lead to cancer and immune system disorders. In addition, chemical insecticides are recalcitrant, it accumulates in the environment and result in soil and water pollution (Devine and Furlong, 2007). The use of microbial insecticides is an alternative to chemical pesticides for insect control. Biological insecticides are mainly based on entomatopathogenic bacteria, *Bacillus thuringiensis.*

B. thuringiensis is a member of a group of crystal-liferous spore-forming gram-positive bacteria of the family Bacillaceae (Schnepf et al., 1998). This bacterium is able to produce proteinaceous parasporal crystals that exhibit specific insecticidal and nematicidal activities (Khyami-Horani et al., 1996; Carneiro et al., 1998; Al-Banna and Khyami-Horani, 2004). *B. thuringiensis* is commonly used as an organic biopesticide against lepidopteran, dipteran, and coleopteran insect pests (Schnepf et al., 1998). *B. thuringiensis* was first isolated from diseased silkworm (*Bombyx mori*) larvae (Ishiwata, 1901). Over the last five decades, *B. thuringiensis* has been developed as a microbial agent against lepidopteran pests (Carlton, 1990). In 1977, Goldberg and Margalit (1977) discovered a novel *B. thuringiensis* strain that expressed specific insecticidal properties against Diptera and another *B. thuringiensis* strain that targeted against Coleoptera was explored by Krieg et al. (1983). The success of various *B. thuringiensis* strains in controlling various insect pests has driven the establishment of several screening programmes for more novel *B. thuringiensis* strains. As a result, it is estimated that today, more than 50,000 *B. thuringiensis* strains are kept in various private and public bacterial collection centres (Sanchis et al. 1996).

There are many methods have been proposed to classify *B. thuringiensis* into sub-species level. The main classification of *B. thuringiensis* isolates was developed on the basis of H-flagellar antigens by de Barjac and Bonnefoi (1962). *B. thuringiensis* strains are classified into more than 82 serovars using H-antigen method. However, there are two limitations with the H-classification for strains lacking parasporal inclusions and auto-agglutinated strains. Some *B. cereus* strains have antigens that cross-react with sera specific for *B. thuringiensis* H-serotypes (14) and such isolates may originate from older *B. thuringiensis* strains that have lost plasmid encoded crystals (Lecadet et al., 1999). These auto-agglutinated strains make up almost 3% of the *B. thuringiensis* in the International Entomopathogenic *Bacillus* Center (IEBC) collection (Burges et al., 1982) at the Institute of Pasteur, Paris, France, but the H-classification is completely useless on these strains. In addition, a few *B. thuringiensis* strains, called non-motile strains such as *B. thuringiensis* var. *wuhanensis*, also escape H-serotyping (de Barjac and Frachon, 1990).

With the development of molecular biology, the classification and identification of bacteria has changed from traditional phenotypic to genotypic methods in recent decades. Pulsed field gel electrophoresis (PFGE), used for genotyping different bacterial strains of a specific species as in the case of *B. thuringiensis* strains (Gaviria and Priest, 2003). However, as PFGE requires special equipment and chemicals, it is not easy to perform in many laboratories. Currently, molecular typing methods including Arbitrary Primer PCR technology (Brousseau et al., 1993), DNA reassociation measurements (Nakamura, 1994), ribosomal RNA gene restriction fragment length polymorphism (Priest et al., 1994; Akhurst et al., 1997), ribosomal RNA gene intergenic spacer sequences comparison (Bourque et al., 1995), DNA-colony hybridization and random amplified polymorphic DNA (RAPD) analysis (Hansen et al., 1998), have also been applied to a limited numbers of *B. thuringiensis* strains. To date, molecular techniques such as RAPD, RFLP, 16S rRNA probe, specific DNA probe, and ISR methods have not provided any great improvement over the H-classification method. Hence, in the present research, a faster, convenient and accurate method was followed to classify all subspecies of *B. thuringiensis* using PCR amplified fragment polymorphism of flagellin genes (PCR-AFPF).

MATERIALS AND METHODS

Soil collection and isolation of *B. thuringiensis*: Soil samples were collected from 108 locations in Tamil Nadu, India, that are very diverse in nature. The samples were collected from agricultural fields, high-altitude mountains, forests, grasslands and sewage. Soil samples were collected by scraping off surface material with a sterile spatula and then obtaining a 10g sample from 1 or 2 cm below the surface. These samples were stored in sterile plastic bags at ambient temperature. One gram of soil was added to 10ml of Luria Broth, which was buffered with 0.25M sodium acetate (Travers et al., 1987). The mixture was shaken for 4h at 250 rpm in 30°C, after incubation 1.5ml of sample mixture was taken and heat shocked at 80°C for 3min. 100µl of the suspension was plated on HiCrome™ Bacillus Agar supplemented with 10µg/ml of polymyxin B. Colonies formed after overnight growth at 30°C were selected based on colour and colony morphology and the colony was transferred onto TCHA medium supplemented with 0.3% glucose. Cultures were allowed to grow and sporulate for 40h at 30°C and the sporulated cultures were then checked for the presence of crystals, which was the criterion used to confirm the isolates as *B. thuringiensis* (Braun, 2000).

Biochemical identification

Fourteen biochemical tests such as acid production from glucose, arabinose, xylose, mannitol, mannose, salicin and sucrose; utilization of citrate and esculin; and production of protease, amylase, phospholipase C or lecithinase, and hemolysin were performed as described by Parry et al. (1983) to identify *B. thuringiensis* strains. For this study, only the results of the following four (the

most relevant) biochemical tests are presented: esculin utilization, acid formation from salicin and sucrose, and lecithinase production (Martin and Travers, 1989). Based on these four biochemical tests the *B. thuringiensis* isolates classified into 16 biochemical types. The classification of *B. thuringiensis* strains in these groups was corresponding to the distribution obtained by additional tests.

PCR–AFPF

DNA C1000 thermal cycler (Bio-Rad) was used to carry out PCR amplification. Cells of different *B. thuringiensis* strains were inoculated on Luria-Bertani (LB) agar plates and incubated at 30°C for 12 h. A loopful of cells was suspended in 100 µl of nuclease free water in a 1.5-ml Eppendorf tube. The cell suspension was frozen at -70ºC for 20 min and then boiled in water bath for 10 min. The resultant lysate was centrifuged at 10,000 rpm for 10 min and five microliters of the supernatant was used as a source of DNA template. The AFPF primers used in this study was previously described by Yu et al. (2002) and are listed (Table 1). Primer was obtained from Eurofins MWG Operon, Germany. Each 50 µl of PCR mixture contained 200 µM deoxynucleotide triphosphates, 2 µM MgCl$_2$, 12.5 pmol per primer, and 2 U of DreamTaq™ DNA polymerase (Fermentas). Amplification was performed using a single denaturation of 3 min at 94°C followed by a 35 cycle program, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 2 min; the final extension step was 72°C for 10 min. The PCR amplified products were detected by 1% agarose gel electrophoresis. The gels were scanned using AlphaImager gel documentation system.

Data analysis

Cluster analysis was used to examine genotypic relationships among the environmental *B. thuringiensis* isolates and it was performed using the AlphaView software, version 4.2 (Proteinsimple, CA). For data analysis, the profiles were converted into binary matrix. The computer cluster analysis was performed on the basis of calculation of the Jaccard Coefficient using unweighted pair group method with arithmetic mean (UPGMA) (Kumar et al., 1993).

RESULTS AND DISCUSSION

Isolation of *B. thuringiensis* from soil: Totally 108 soil samples were collected from different locations of 26 districts in Tamil Nadu, India. Soil samples were collected from cultivated fields (rice, sugarcane, coffee, tea, mango, papaya, cabbage, onion, tomato, coconut, and potato), natural vegetation (pine forests, shola forest,

Table 1. Nucleotide sequences of primers used for PCR-AFPF and multiplex PCR	
Primer Name	*Sequence*
Fla5	F – GGCGTCGACATGAGAATTAATACAAACATT
Fla3	R – CGCCTGCAGTTATTGTAATAATTTAGAAGCC

reserved forest, tropical evergreen forest, and grasslands) and sewage soils. The elevations of the places from which the samples collected were highly variable, ranging from sea level to 7,200 m above sea level. Among 82 soil samples, twenty six samples were collected from hilly regions (Ooty, Yercaud, Kolli hills, Palani, Kodaikanal, Sirumalai).

After acetate and polymyxin B selection, 97 of 108 soil sample yield colonies. Of these 97 samples, 70 (72%) contained at least one crystal protein forming *B. thuringiensis* strains. From the 70 soil samples, 78 *B. thuringiensis* strains were isolated and separated from 173 other spore forming organisms. Overall, this suggests that Tamil Nadu soils were enriched with *B. thuringiensis*. With the advent of a very selective procedure for separating *B. thuringiensis* spores from the spores of other soil microbes, 78 *B. thuringiensis* strains have been isolated through acetate and polymyxin B selection. The distribution of *B. thuringiensis* in Tamil Nadu, is summarized (Table 2). In Tamil Nadu, the soil samples collected from districts such as, Vellore, Thanjavur and Theni were extremely rich in *B. thuringiensis*. Hundred percent of the soil sample contained *B. thuringiensis*. The soil sample collected from Krishnagiri, Nilgiris, Coimbatore, Trichy, Tirunelvelli and Kanyakumari had 75 – 80% of *B. thuringiensis* strains. On the other hand, soil samples collected from Ariyalur, Nagapatinam, Madurai, Virudhunagar and Tuticorin, only 25 – 33% of the soil samples contained *B. thuringiensis* strains.

Biochemical typing: The *B. thuringiensis* strains isolated from Tamil Nadu were identified and classified based on Martin and Travers (1989). In order to discriminate the isolated *B. thuringiensis* strains in to 16 biochemical types, four biochemical tests such as esculin utilization, acid formation from salicin and sucrose and lecithinase production were carried out and these were the most variable among *B. thuringiensis* isolates. *B. thuringiensis* isolates were given the biochemical type number based on the Martin and Travers (1989) and their occurrence in Tamil Nadu is also recorded (Table 3). Eventhough some of these types were differed by a single biochemical tests, these difference were more important. In this study *B. thuringiensis* subsp. *kurstaki* is widely available in the environment differed from leastly available *B. thuringiensis* subsp. *galleriae* by a single biochemical test, lecithinase production. Among

Table 2. Distribution of *B. thuringiensis* in soil			
Districts	*No. of samples examined*	*% of samples with B. thuringiensis isolate (no. of samples)*	*B. thuringiensis index*a *(no. of B. thuringiensis isolates)*
Chennai	4	50.0 (2)	0.22 (2)
Kanchipuram	4	50.0 (2)	0.33 (2)
Vellore	4	100.0 (4)	0.37 (3)
Krishnagiri	4	75.0 (3)	0.28 (4)
Villupuram	4	50.0 (2)	0.18 (2)
Nilgiris	4	75.0 (3)	0.40 (4)
Erode	4	50.0 (2)	0.22 (2)
Salem	4	75.0 (3)	0.38 (5)
Ariyalur	4	25.0 (1)	0.16 (1)
Perambulur	4	50.0 (2)	0.25 (2)
Namakkal	4	75.0 (3)	0.46 (6)
Coimbatore	4	75.0 (3)	0.36 (4)
Tiruppur	4	50.0 (2)	0.28 (2)
Karur	4	75.0 (3)	0.25 (2)
Trichy	4	75.0 (3)	0.27 (3)
Nagapattinam	4	33.3 (1)	0.16 (1)
Tiruvarur	4	66.6 (2)	0.40 (2)
Thanjavur	4	100.0 (4)	0.30 (4)
Pudukottai	4	75.0 (3)	0.33 (2)
Dindigul	4	100.0 (4)	0.30 (4)
Theni	4	100.0 (4)	0.33 (4)
Madurai	4	75.0 (2)	0.44 (4)
Virudhunagar	4	50.0 (2)	0.12 (1)
Tuticorin	4	50.0 (2)	0.28 (2)
Tirunelveli	4	75.0 (3)	0.36 (4)
Kanyakumari	4	75.0 (3)	0.60 (3)
Ramanathapuram	4	50.0(2)	0.28 (2)
Total	108	64.8 (70)	0.31 (78)

a*B. thuringiensis* index was calculated as a number of *B. thuringiensis* strains isolated divided by the number of colonies of all bacteria examined.

78 *B. thuringiensis* isolates, a maximum of 12 (15.4%) *B. thuringiensis* subsp. *kurstaki* strains were isolated from different soil samples. *B. thuringiensis* subsp. *israelensis* was not present in any of the samples. The biochemical types 9, 10, 11, 13, 15 and 16 made up a cluster of strains which accounted for 48.7% of all environmental isolates. The biochemical type of each isolates is recorded (Table 3). The results are similar to the study reported by Martin and Travers (1989), but they have got more isolates of six undescribed *B. thuringiensis* biochemical types (52%) than the known biochemical types (48%).

The widely accepted and well established typing method for *B. thuringiensis* strains is serotyping. But the current serotyping system is not suitable for auto agglutinated strains, non-motile strains and strains lacking a parasporal inclusion body. From the point of bacterial systematic classification, serotyping is a phenotypic system which cannot reveal phylogenetic relationships among the strains (Joung and Côté, 2001).

Analysis of AFPF-PCR amplified products: PCR was performed using cell lysate as DNA template with Fla5 and Fla3 primers. AFPF-PCR yielded multiple distinct DNA products of sizes ranging from approximately 100 to 2000bp of more than 300 fragments from 78 *B. thuringiensis* strains (Fig. 1). All *B. thuringiensis* isolates differed from one another in the specific amplified patterns of the PCR products, which correspond to the presence of flagellin gene sequence. In these isolates the major number of bands was observed at the size of 100, 225 and 425bp. Most of the amplified products were observed

Table 3. Biochemical types of *B. thuringiensis* and its occurrence in Tamil Nadu, India[a]

Biochemical Type	Biochemical test result[b]				*B. thuringiensis* isolates in Tamil Nadu, India (%)
	Esculin	Salicin	Lecithinase	Sucrose	
1 (*thuringiensis*)	+	+	+	+	4 (5.1)
2 (*kurstaki*)	+	+	+	-	12 (15.4)
3 (*indiana*)	+	+	-	+	3 (3.8)
4 (*galleriae*)	+	+	-	-	1 (1.3)
5 (*sotto*)	+	-	+	+	3 (3.9)
6 (*dendrolimus*)	+	-	+	-	11 (14.1)
7 (*morrisoni*)	+	-	-	+	3 (3.8)
8 (*darmstadiensis*)	+	-	-	-	2 (2.6)
9 (Biochemical type 9)	-	+	+	+	4 (5.1)
10 (Biochemical type 10)	-	+	+	-	6 (7.7)
11 (Biochemical type 11)	-	+	-	+	1 (1.3)
12 (*ostriniae*)	-	+	-	-	1 (1.3)
13 (Biochemical type 13)	-	-	+	+	9 (11.5)
14 (*israelensis*)	-	-	+	-	0 (0)
15 (Biochemical type 15)	-	-	-	+	10 (12.8)
16 (Biochemical type 16)	-	-	-	-	8 (10.3)
Total					78

[a]Martin and Travers, 1989
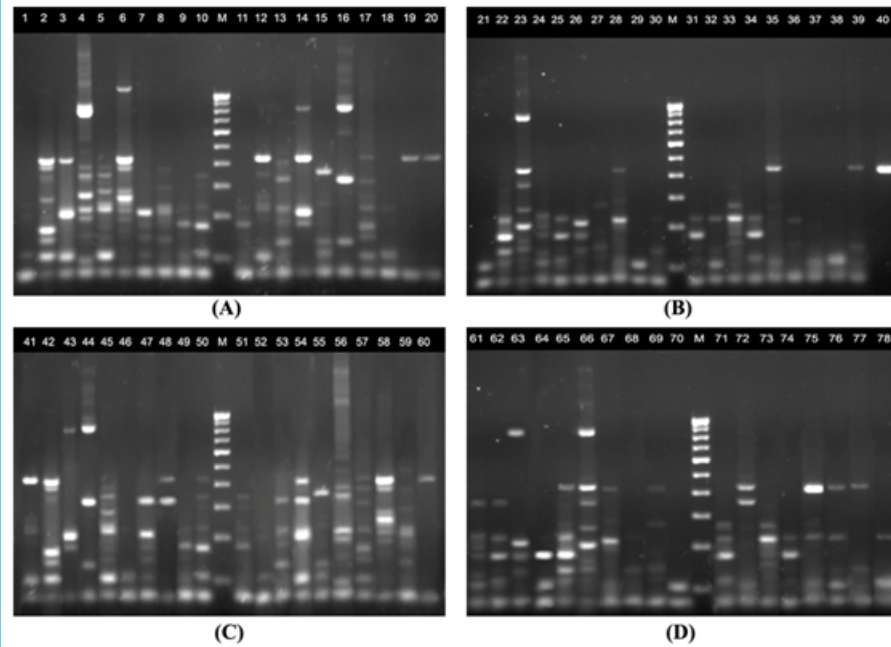[b]The + sign indicates a positive reaction; – sign indicates a negative reaction

FIGURE 1. PCR-AFPR fingerprint patterns of environmental isolates of *Bacillus thuringiensis*. (A) *Bacillus thuringiensis* Tamil Nadu (BTTN) isolates BTTN01 – BTTN20; (B) BTTN20 – BTTN40; (C) BTTN41 – BTTN60; (D) BTTN61 – BTTN78. Lane M, 100bp ladder.
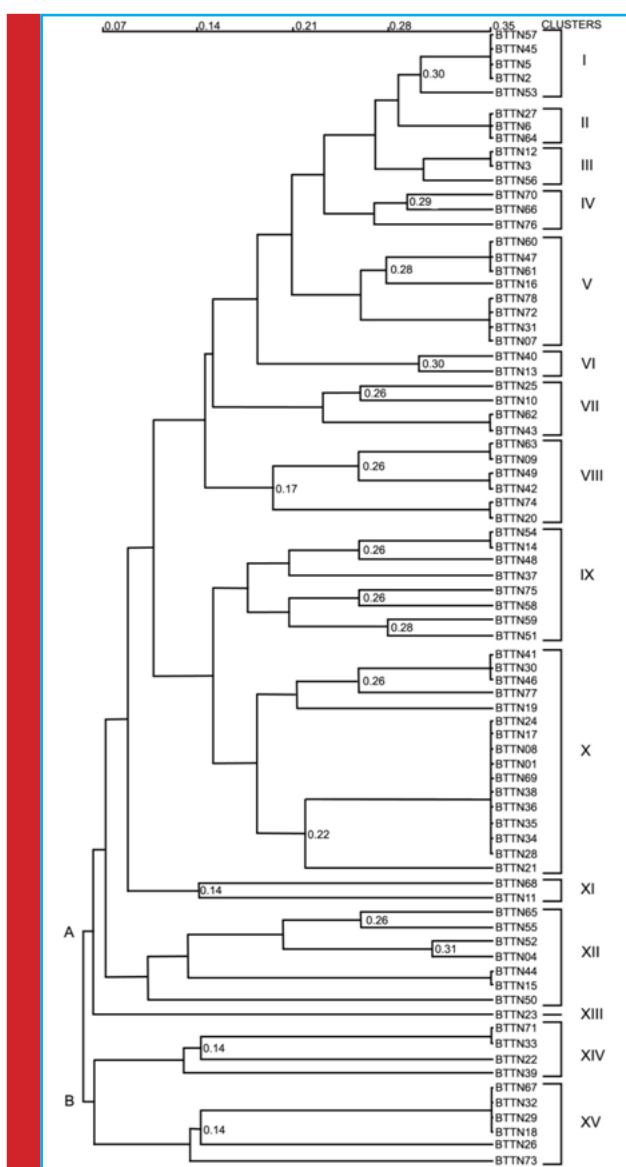
FIGURE 2. UPGMA dendrogram derived from similarity coefficients calculated by the Jaccard method based on the amplified bands obtained during PCR-AFPF analysis. The dendrogram shows the relationships among environmental isolates of *B. thunringiensis.*

between 100 to 1000bp and only few of the isolates (BTTN4, 6, 16, 23, 44, 56 and 66) contained fragments more than 1000bp. Only a distinct single product with a size of about 100 or 425bp was obtained in the strains such as, BTTN19, 20, 21, 29, 38, 40, 60 and 70. The maximum of 12 to 14 bands ranged from sizes 100 – 2000bp were observed in BTTN4, 23, 56 and 66.

PCR-AFPF is a preliminary attempt of Yu et al. (2002) to classify the subspecies of *B. thuringiensis* obtained from the Institute of Pasteur, France and in the present study this tool is used to discriminate between the *B.*

thuringiensis serovars isolated from the environment. The chromosome of all *B. thuringiensis* strains contain flagellin gene and it was present even in their closely related species *B. cereus* and *B. anthracis.* Its PCR amplification pattern analysis is not subjected to any limitation associated with the *B. thuringiensis* serotyping system. The phyolgenetic analysis of *B. thuringiensis* using PCR-AFPF would be more accurate, than previously described molecular classification methods such as RAPD, RFLP, 16s rRNA probe, specific DNA probe, and ISR (Yu et al., 2002). The AFPF primers recognises differences in the prevalence and positions of annealing sites in the genome producing sets of fragments that are considered to reflect the genomic composition of the strain, therefore it gives a good opportunity to detect biodiversity of a group of isolates.

Phylogenetic analysis using UPGMA method: The genetic distance, Jaccard coefficient values were calculated from amplified bands and the values were ranged from 0.07 to 0.035 (Fig. 2). An unweighed pair group method with average (UPGMA) dendrogram was constructed using the Jaccard coefficient. In UPGMA dendrogram, the *B. thuringiensis* isolates were divided into 2 groups, group A and B. Group A was further classified into 13 clusters (I - XIII), which comprised of 68 *B. thuringiensis* isolates and remaining 10 strains, BTTN71, 33, 22, 39, 67, 32, 29, 18, 26 and 73 were categorized in cluster XIV and XV and these two clusters were grouped under group B. Among I to XV clusters, cluster I, X, XII and XIV contains the mixture of different *B. thuringiensis* biochemical types and rest of the 11 clusters contains any one of the biochemical types. The maximum of 20.5% of *B. thuringiensis* isolates were classified under cluster X and it comprised 6.25% of biochemical type 9, 25% of *B. thuringiensis* subsp. kurstaki and 68.75% of *B. thuringiensis* subsp. dendrolimus. At the least of cluster XIII contained only one biochemical type 9 strain and this type showed more degree of variations in their band pattern among their biochemical type and these four biochemical type 9 strains were characterized under 3 clusters (X, XI and XIII). This confirmation of variation or intermixing of biochemical type 9 within the clusters suggests that the specific phenotypes were acquired after the ancestors to each of the clusters were formed. The study supports the idea that horizontal gene transfer of plasmid is an important factor in defining the phenotypes of biochemical type 9 isolates evolved along perceptible large evolutionary distances to give rise to different clusters.

The study of Katara et al. (2012) demonstrated that molecular typing and diversity analysis of *B. thuringiensis* has enormous importance for discrimination of strains isolated from different sources. They distinguished 113 native *B. thuringiensis* strains isolated from various

locations in India using REP-PCR and ERIC-PCR. They explored that the *B. thuringiensis* isolates collected from diverse habitats in India had a high degree of genetic diversity. Similar to them, the *B. thuringiensis* strains which were isolated from Tamil Nadu showed diverse range of patterns and high level of genetic diversity.

CONCLUSION

Through the present study, it is suggested that the association between *B. thuringiensis* and insects is not obligative. *B. thuringiensis* was omnipresent when compared to other bacterial strains and based on the nutrient requirements the growth turnover is possible. There is no need that the population of *B. thuringiensis* has to be more in the soil samples with high levels of insect activity because several soil samples had been collected from the mosquito breeding regions but no *B. thuringiensis* isolates had shown any degree of mosquito larvicidal activity. Finally, the genetic heterogeneity were analysed in the *B. thuringiensis* strains isolated from Tamil Nadu using AFPF-PCR. This technique could be used for the separation of novel *B. thuringiensis* isolates from the environment.

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