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Molecular characterization of Lepidopteran specific *Bacillus thuringiensis* strains isolated from Hilly Zone Soils of Karnataka, India

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Bacillus thuringiensis **(Bt) strains pathogenic to Lepidopteran insects and native to hilly zone soils of Karnataka (India) were explored. 19 strains were isolated from the soils and identified by morphological and microscopic characters. Toxicity level of the Bt isolates was tested by treating third Instar larvae of silkworm (*Bombyx mori L.*) and mulberry leaf roller (*Diaphania pulverulentalis*). Mortality rate of the insect larvae treated with Bt isolates was ranged from 20 to 80%. A few isolates namely, Bt2, Bt6, Bt8, and Bt14 strains were more virulent (caused >50% death) than others. Detection of *cry* genes using *cry* gene specific primers by polymerase chain reaction (PCR) revealed the presence of at least one of the *cry* genes. In one of the isolates (Bt9), the *cry* gene was not detected. The *Cry1Ac* gene was abundant and it was detected in 13 isolates. Cluster analysis for genetic diversity showed two major groups and four sub groups.**

Key words: Molecular characterization, *Bacillus thuringiensis*, bioassay, genetic diversity, *Cry* genes, hilly zone.

INTRODUCTION

Bacillus thuringiensis (Bt) is an endospore forming gram positive bacterium belonging to the family *Bacillaceae*. Bt produces insecticidal delta endotoxin which is also known as crystal protein. This protein crystals can solubilize in alkaline pH in the mid gut lumen of the susceptible insect larvae and release protoxins into the midgut. In the midgut, the protoxin convert to active form by trypsin like proteases. The active toxin binds to specific receptors on the brush border apical membrane of midgut cells leading to formation of pores that disrupt osmotic regulation of the insect epithelium and thereby kills insect larvae (Bravo et al., 1998; Sauka et al., 2007). Quest for novel Bt strains against particular group of insects has resulted in collection of Bt at different habitats. As a result, more than 50,000 strains were isolated from several habitats such as insects, stored products, soil and marine environment (Anderson, 2011). Isolation of Bt strains with novel toxicities is an attractive endeavor for developing successful biopesticide (El-kersh et al., 2012). The hilly zone is one of the 10 agro climatic zones of Karnataka embedded with evergreen forest area where plantation crops are being cultivated. Application of pesticides in this region is minimal due to limited cultivation practices that lead to natural control of the pest and diseases. Therefore, occurrence of novel Bt strains in such soils would be naturally prevalent.

Identification of Bt collections were generally based on morphological, microscopic observations and bioassay tests against specific insect larvae (Keshavarzi, 2008; El-kersh et al., 2012). In addition, polymerase chain reaction (PCR) based technique has also been proposed to be the best method for identifying Bt (Porcar and Perez, 2003).

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Table 1. Specific primers used to amplify endogenous *cry* genes.

Primer pair	Primer sequence (5'--------3')	Crygene	Tm(°C)	Product size (bp)	Reference
Cry1F	CATGATTCATGCGGCAGATAAAC	*Cry1*	48	200	Halima et al., 2006
Cry1R	TTGTGACACTTCTGCTTCCCATT				
Cry3 F	CGTTATCGCAGAGAGATGACATTAAC	*Cry2*	51	700	
Cry3R	CATCTGTTGTTTCTGGAGGCAAT				
Cry 2F	GTTATTCTTAATGCAGATGAATGG	*Cry3*	55	600	Gao et al., 2008
Cry2R	CGGATAAAATAATCTGGGAAATAGT				
Cry4 F	GCATATGATGTAGCGAAACAAGCC	*Cry4*	48	400	
Cry4 R	GCGTGACATACCCATTTCCAGGTCC				
Cry7F	AAGCAGTGAATGCCTTGTTTAC	*Cry7*	52	500	Fatma Ozturk et al., 2008
Cry7R	CTTCTAAACCTTGACTACTT				
Cry1Ac F	TGTAGAAGAGGAAGTCTATCCA	*Cry1Ac*	47	270	
Cry1Ac R	TATCGGTTTCTGGGAAGTA				

At present,over 170 endotoxin encoding genes from Bt have been identified (Glare and O'Callaghan, 2000). PCR amplification of 12 Bt isolates revealed the presence of different *cry* genes which are specific to Lepidoptera, Diptera and Coleoptera (Schnepf et al., 1998; Bravo et al., 1998; Aly, 2007).

Characterization of genetic diversity of Bt strains could provide useful information on the ecological patterns of distribution and opportunities for strain selection to develop novel biopesticides (Bravo et al., 2012). Hundreds of Bt strains isolated from different habitats have showed variations between the strains when analyzed by randomly amplified polymorphic DNA (RAPD) (Malkawi et al., 1999; Yaradoni et al., 2003; Kumar et al., 2010). In this study, we isolated, identified and characterized 19 Bt strains from hilly zone soils of Western ghats of Karnataka.

MATERIALS AND METHODS

Isolation of Bt

Soil samples were collected from 2 to 5" depth at different places of the hilly zone in a sterile plastic bags and stored at 4°C. 19 bacteria were isolated by sodium acetate selection method (Travers et al., 1987). These isolates were identified as Bt by their colony characters and microscopic observations viz., gram reaction, presence of endospore and crystal proteins. Biochemical properties viz., indole, amylase and catalase production were done by following standard procedures (Eswarapriya et al. 2010).

Bioassay of Bt isolates on Lepitopteran insects

Bt isolates were grown in Luria-Bertani (LB) broth at 30°C for 48 h. These isolates having population ~10^7 CFU/ml were individually smeared on fresh succulent mulberry leaves, shade dried and fed to the third Instar larvae of silkworm (*Bombyx mori L, Bobycidae, Lepidoptera*) hybrids (CSR2XCSR4 and PMXCSR2) and the larvae of leaf roller (*Diaphania pulverulentalis* Hampson. Pyralidae, Lepidoptera). Untreated succulent mulberry leaves were used as control. Ten healthy larvae were used in each treatment. The observations for mortality were recorded after 72 h.

PCR detection of *Cry* genes

The *Cry* genes were amplified by PCR using specific primers (Table 1). Plasmid DNA was isolated from Bt strains (Sambrook and Russel, 2001) and PCR was performed in 30 µl reaction mixture composed of 50 ng DNA, 800nM deoxynucleotide triphosphate (dNTPs), 10 nM (each) primer, and 0.3 U of *Taq* DNA polymerase (Genei., Bengaluru, India) in a Thermo cycler (MyCycler^{TM}, Bio-Rad). The PCR conditions were: initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C denaturation for 1 min, annealing as per T_m of the primers (Table 1) for 1 min and extension at 72°C for 1 min and an extra step of extension at 72°C for 10 min. Amplified products were separated on 1% agarose gel stained with ethidium bromide, visualized by transilluminator and documented (Hero lab, USA).

Analysis of genetic diversity by RAPD

The genomic DNA was isolated (Sambrook and Russel, 2001) and amplified using RAPD primers (Table 2). PCR was performed in a 25 µl reaction mixture composed of 2.5 µl buffer, 4 µl (10 mM) dNTP, 3 µl random primer (10mM), 0.5 µl Taq DNA polymerase (3U) (Genei., Bengaluru, India) and 2 µl (40 to 50ng) template DNA. PCR conditions were: initial denaturation at 95°C for 3 min, followed by 40 cycles consisting of denaturation at 94°C for 1 min, annealing for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The amplified products were separated on agarose 1% gel stained with ethidium bromide and photographed. The bands were scored as 1 for the presence and 0 for the absence and then the data was analysed for polymorphism using STATISTICA soft ware.

RESULTS AND DISCUSSION

Bt, a common component of the soil microflora occurs

Table 2. Polymorphism showed genetic variation among Bt isolates.

Primer	Primer sequence	Number of amplified fragment	Number of polymorphic band		Number of monomorphic band
			Shared	Unique	
RAPD-11	GGGTTTAGGG	8	5	2	1
RAPD-13	CACCACCACC	6	4	2	0
RAPD-14	GTCGCTCAGA	7	4	2	1
RAPD-15	CGAAGCTACC	7	4	3	0
RAPD-17	GAATGGGAGG	6	4	1	1
RAPD-18	GCGATCCCCA	8	6	2	0
RAPD-20	AATCGGGCTG	6	3	2	1
RAPD-22	GAAACGGGTG	7	4	2	1
RAPD-23	GTGATCGCAG	5	3	2	0
RAPD-24	CAATCGCCGT	7	3	3	1
RAPD-26	TTCGAGCCAG	8	5	2	1
RAPD-27	GTGAGGCGTC	7	4	3	0
RAPD-29	GAACGGACTC	6	5	1	0
RAPD-31	TGGACCGGTG	5	3	2	0
Total		93	57	29	7
Percentage		100	61.20	31.28	7.52

Table 3. Mortality rate of silkworm hybrids (CSR2XCSR4 and PMXCSR2) and leaf roller larvae treated with Bt *isolates*.

Bt strain	Mortality rate (%)		
	SR2XCSR4	PMXCSR2	Mulberry leaf roller
Bt1	80	30	50
Bt2	60	80	60
Bt3	50	60	60
Bt4	70	50	40
Bt5	40	60	70
Bt6	60	70	80
Bt7	30	30	60
Bt8	70	70	70
Bt9	00	00	00
Bt10	40	30	30
Bt11	60	60	40
Bt12	50	50	50
Bt13	50	60	60
Bt14	60	80	70
Bt15	00	40	40
Bt16	40	40	30
Bt17	50	50	50
Bt18	60	70	20
Bt19	30	20	40
Control	00	00	00

Ten larvae were used in each treatment and percent mortality was assessed by counting the dead larvae after 72 h.

naturally in the environment. Selectively, most of the Bt can be isolated by sodium acetate method (Travers et al., 1987). The major advantage of this method is merely based on heat treatment and acetate usage. Acetate is known to inhibit germination of Bt spores, so other spores germinate and then the growing cells including non-spore forming bacteria are killed by heat treatment (Keshavarzi, 2008). Thus, it facilitates selective isolation of Bt from soil and avoid other contamination. Followed by the selective isolation, Bt can be identified by conventional methods like morphological and microscopic characters (Keshavarzi, 2008). In the present study, 19 Bt strains were isolated from different location of the hilly zone soils and identified by colony characters, microscopic observations (including presence of endospore and crystal protein) and biochemical tests. The isolates formed creamy white colonies on sodium acetate LB agar. The cells were gram positive rods and produced indole, amylase and catalase which are also characteristic feature of Bt. In addition, all the isolates produced protein crystals and endospores (data not shown). The crystal inclusions are the key discriminating factors of Bt to differentiate from other closely related *Bacillus* species such as *B. cereus* and *B. anthracis* which do not produce these inclusions (Glare and Collaghan, 2000; Lima et al., 2008), the 19 isolates have been confirmed as Bt.

Toxicity of Bt isolates to Lepidopteran insect larvae

Consumption of toxin (Bt) treated leaves results in cessation of feeding of Lepidopteran larvae and paralysis of guts. Larvae eating high doses of toxin suffered general paralysis followed by death (Sauka et al., 2007). In this study, 19 Bt strains were tested for their toxicity using third instar larvae of two silk worm hybrids and the larvae of mulberry leaf roller by leaf smear bioassay method (Nethravathi et al., 2010). Results indicate that the mortality rate of insect larvaeranged from 20 to 80% (Table 3

Figure 1. Mortality of larvae of silkworm hybrids (CSR2XCSR4 and PMXCSR2) and leaf roller treated with different *Bt* isolates. A, Untreated healty larvae of silkworm hybrid, CSR2XCSR4; B, larvae of silkworm hybrid, CSR2XCSR4 treated with Bt 1 showing diseased symptom; C, dead larvae of silkworm hybrid, CSR2XCSR4 treated with Bt 1; D, Untreated healty larvae of silkworm hybrid, PMXCSR2; E, larvae of silkworm hybrid, PMXCSR2 treated with Bt 8 showing diseased symptom; F, dead larvae of silkworm hybrid, PMXCSR2 treated with Bt 8;G, bioassay on mulberry leaf roller (*Diaphania pulverulentalis*); H, dead larvae of silkworm hybrid, *Diaphania pulverulentalis*) treated with Bt6.

and Figure 1). The Bt1 strain caused highest mortality (80%) of silk worm hybrid CSR2xCSR4 while Bt2 and Bt8 recorded 80% mortality of another silk worm hybrid, PMxCSR2. This indicated that different Bt strains exhibit varied toxicity to insect larvae even within the hybrids of silkworm. Similarly, 80% mortality of mulberry leaf roller was observed for Bt 6 treatment indicating that the single strain may not be efficient to kill all the Lepidopteran insects though they possess cry1Ac toxin. This could be attributed to the binding of the toxin with low affinity to receptors as interaction of toxin with different receptors depends on the oligomeric structure of the Cry toxin (Bravo et al., 1998). Glare and Collaghan (2000) opined that the individual delta endotoxins and the combination of endotoxins within a single isolate can also interact to increase and occasionally decrease toxicity to a given insect. 70% mortality of the three insect larvae was observed for Bt8 isolate treatment and Bt12 and Bt17 isolates caused 50% mortality of the larvae of the three insects. This variation again is due to the spectra of toxins produced by the Bt isolates (Gao et al., 2008; Liliana et al., 2013). Similarly, El-keresh et al. (2012) reported varied toxicity of Bt isolates against two mosquitoes larvae. However, the Bt9 isolate did not kill any insect larvae even after 72 h of treatment that needs further study.

Screening of *cry* genes from Bt isolates

Cry genes present in 19 Bt isolates were amplified by PCR and sequenced. Results of the present study indicate the presence of more than one *cry* genes in a single isolate. A single Bt strain may contain one or more *cry* genes in their plasmids (Letowski et al., 2005). Amongst the 19 isolates, Bt4, Bt11 and Bt18 possessed 3 *cry* genes whereas others contained two each and a single *cry* gene (*cry7*) was detected in Bt5. Thirteen isolates namely, Bt1, Bt2, Bt3, Bt6, Bt7, BBt10, Bt11, Bt12, Bt13, Bt14, Bt15 and Bt18 possessed *Cry1Ac* gene with an expected size of 275 bp (Table 4, Figure 2). These results are in agreement with those of Aly (2007) who detected 5 *cry* genes in 12 Bt isolates. The native strains of Bt carrying *cry1* genes was reported from the Western ghats of Tamil Nadu by Ramalakshmi and Udaysurian,

Table 4. *Cry* genes profile of nineteen Bt *isolates*.

Bt isolate	*Cry* genes detected					
	Cry1ac	***Cry1***	***Cry2***	***Cry3***	***Cry4***	***Cry7***
Bt 1	+	+	-	-	-	-
Bt 2	+	-	-	-	+	-
Bt 3	+	-	-	+	-	-
Bt 4	-	+	+	+	-	-
Bt 5	-	-	-	-	-	+
Bt 6	+	+	-	-	-	-
Bt 7	+	-	+	-	-	-
Bt 8	+	+	-	-	-	-
Bt 9	-	-	-	-	-	-
Bt 10	+	-	+	-	-	-
Bt 11	+	+	-	+	-	-
Bt 12	+	-	-	+	+	-
Bt 13	+	-	-	+	-	-
Bt 14	+	-	-	-	+	-
Bt 15	+	+	-	-	-	-
Bt 16	-	-	-	+	+	-
Bt 17	-	+	+	-	-	-
Bt 18	+	+	-	-	+	-
Bt 19	-	-	+	+	-	-

+, Presence of the *cry* gene; -, absence of the *cry* gene.

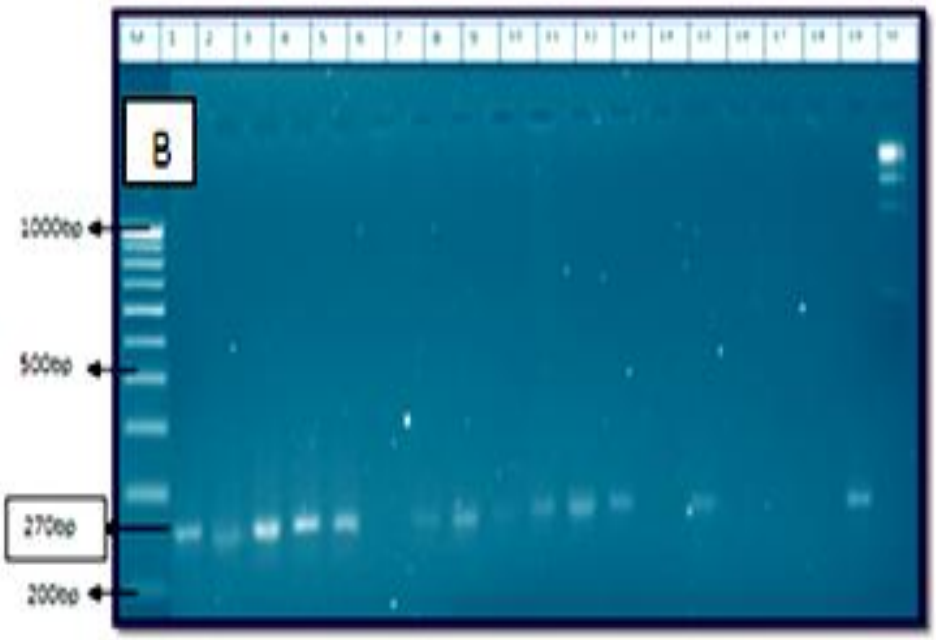

Figure 2. *Cry*1Ac gene detected in different *Bt isolate*. Lane M, 100 bp marker; lane 1, Bt1; lane 2, Bt2; lane 3, Bt3; lane 4, Bt6, lane 5, Bt7, lane 7, Bt8; lane 8, Bt10; lane 9, Bt11; lane 10, Bt 12; lane 11, Bt 13; lane 12, Bt14; lane 14, Bt15; lane 19, Bt 18.

(2009). Saker et al. (2012) reported abun-dance of *cry1* gene in 72.41% of Bt isolates. However, no *cry* gene was amplified for Bt9 isolate which did not kill any insect larvae used. This needs further screening for other *cry* genes to conclude whether or not it is Lepidopteran specific pathogen.

Table 5. Genetic dissimilarity matrix of 19 Bt isolates.

BT1	BT2	BT3	BT4	BT5	BT6	BT7	BT8	BT9	BT10	BT11	BT12	BT13	BT14	BT15	BT16	BT17	BT18	BT19
0																		
5	0																	
5.74	4.9	0																
6.08	6.32	7.21	0															
6.56	7.07	6.63	5.83	0														
6.16	5.39	5.92	6.56	5.74	0													
5.92	5.29	5.66	6.32	6.16	6.4	0												
6	5.74	6.4	5.92	5.74	4.69	5.74	0											
5.83	6.08	6.4	6.86	6.56	6	5.74	5.66	0										
6.63	6.71	6.86	6.71	6.56	6.93	6.4	6.48	4.69	0									
6.24	6.48	6.63	6.93	5.66	6.08	6	5.74	5.39	5.74	0								
6.4	6.16	6.63	7.07	6.63	5.92	6.63	6.56	5.39	5.2	5.83	0							
5.92	5.83	6.63	6.48	6.93	6.71	5.83	6.56	5.57	6.08	6	6	0						
6.08	6.32	6.16	6.32	6.78	6.86	6	6.56	6.4	6.4	6.32	6.63	5.83	0					
6.4	6.16	6.32	6.48	6.16	6.4	5.83	6.08	6.08	6.08	6	6	6.78	6	0				
6.78	6.56	6.24	6.86	5.74	5.83	5.92	5.83	6.48	6.78	6.08	6.4	6.4	5.92	6.08	0			
5.74	5.66	6.16	6.32	5.83	6.08	5.1	6.08	6.24	5.92	6.32	6.32	6.48	5.83	5.1	5.74	0		
5.57	5.66	6.16	5.83	6	6.08	5.83	6.08	6.4	6.08	6	6	6.63	5.29	4.47	5.92	4.47	0	
5.83	5.92	6.56	5.39	6.08	6.32	6.08	6	6.78	6.32	6.86	6.08	6.86	5.2	5.2	6.63	5.2	4.12	0

Genetic diversity of Bt strains

RAPD technology is very useful, fast and informative indifferentiating Bt strains (Gaveria Revera and Priest, 2003) and analysis of genetic diversity is necessary to obtain information on variations between huge numbers of strain within particular group (Sadder et al., 2006). Therefore, to characterize genetic diversity, a total of 21 primers were screened for PCR amplification. Finally, 14 primers were selected for fingerprinting and diversity analysis (Table 2 and Figure 3). Out of 92.48% polymorphic bands obtained, 61.20 formed the polymorphic shared bands and 31.28% formed the polymorphic unique bands, and the remaining 7.53% formed the monomorphic bands. Statistical analysis of RAPD data enabled the classifica-tion of nineteen Bt isolates into two major groups, isolates Bt5, Bt11, Bt16, Bt6, Bt8, Bt9, Bt10, and Bt12 form one group and the isolate Bt17, Bt19, Bt18, Bt15, Bt14, Bt13, Bt4, Bt7, Bt3, Bt2 and Bt1 form the another group. Results of the cluster analysis revealed that all the isola-tes grouped into 2 major clusters. Within these two major clusters, another four subgroups are also present (Figure 4). Ward's dissimilarity index of Bt revealed that the highest dissimilarity obtained was 7.21 and the lowest was 4.12 (Table 5). The highest dissimilarity was present between Bt4 and Bt3 and the minimum dissimilarity was obtained between Bt19 and Bt18. All others were present in between the two ranges of maximum and the minimum values. 70 strains of Bt isolated from

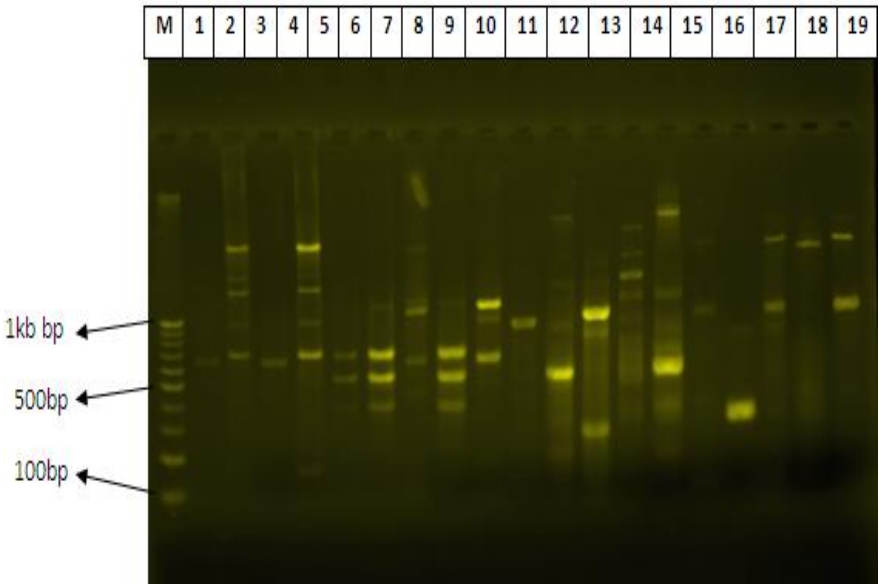

Figure 3. Representative picture showing RAPD in different Bt isolates by RAPD primer 17. Lane M, 100 bp marker; lane 1, Bt1; lane 2, Bt2; lane 3, Bt3; lane 4, Bt4, lane 5, Bt5, lane 6, Bt6; lane 7, Bt7; lane 8, Bt8; lane 9, Bt9; lane 10, Bt 10; lane 11, Bt 11; lane 12, Bt12; lane 13, Bt13; lane 14, Bt14; lane 15, Bt15; lane 16, Bt16; lane 17, Bt17; lane 18, Bt18; lane 19, Bt 19.

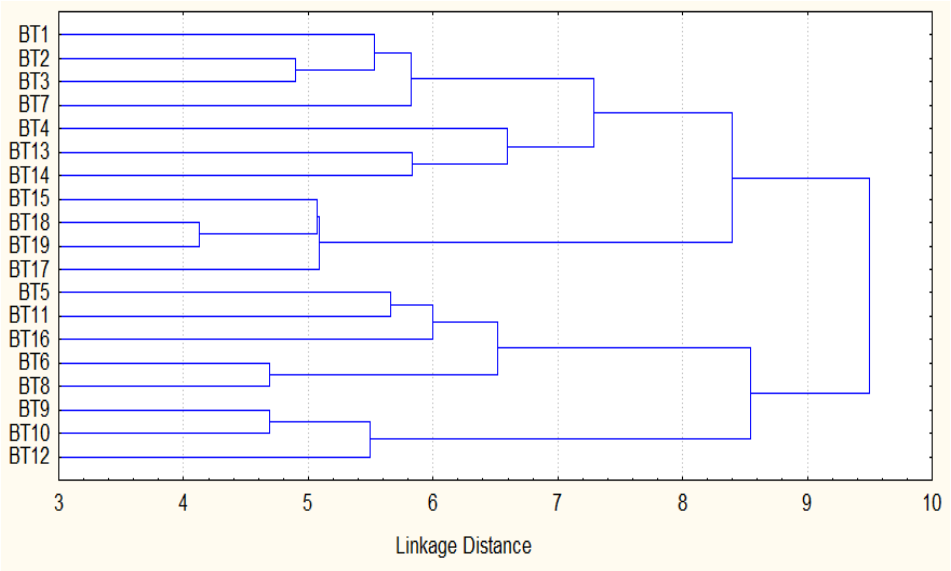

Figure 4. Cluster diagram of 19 Bt isolates.

cotton field soil showed 80% similarity index when genetic diversity was analysed using RAPD (Kumar et al., 2010). Saker et al. (2012) opined that the DNA fingerprinting based RAPD technique has proved to be a reliable method for identification of different Bt strains at DNA level. Similarly, our study has also revealed variations of Lepidopteran specific Bt strains isolated from the hilly zone soils of Karnataka.

REFERENCES

Aly NH (2007). PCR detection of cry Genes in local *Bacillus thuringiensis* Isolates. Australian J. Basic and Appl Sci. 1: 461-466.

Anderson B (2011) Article on *Bacillus thuringiensis*. J. Sci. Technol. Missouri. pp. 21.

Bravo Alejandra, Isabel Gomez, Helena porta, Blanca Ines Garcia-Gomez, Claudia Rodriguez- Almazan, Lillana Pardo and Mario Soberon (2012). Evolution of *Bacillus thuringiensis* cry toxins insecticidal activity. Microb. Biotechnol. 6:17-26.

Bravo Alejandra, Sergio Sarabia, Lorena lopez, Hernesto Ontiveros, Carolina Abarca, Anabel Ortiz, Laura Lina, Francisco J, Villalobos, Guadalupe Pen A, Mari A-Eugenia Nun ez-Valdez, Mario Sobero N, Rodolfo Quintero (1998). Characterization of Cry genes in a Mexican *Bacillus thuringiensis* strain collection. Appl. Environ. Microbiol. 64: 4965-4972.

El-kersh TA, Al-sheikh YA, Al-akeel RA, Alsayed AA (2012). Isolation and characterization of native *Bacillus thuringiensis* isolates from Saudi Arabia. Afr. J. Biotechnol. 11(8): 1924-1938.

Eswarapriya B, Gopalsamy B, Kameswari B, Meera R, Devi P (2010). Insecticidal activity of *Bacillus thuringiensis* IBT- 15 strain against *Plutella xylostella*. Int. J. Pharm. Technol. Res. 2: 2048-2053.

Gao M, Rongsen Li, Shungi8ng Dai, Yan Wu, Dan Yi (2008). Diversity of *Bacillus thuringiensis* strains from soil in chaina and their pesticidal activities. Biol. Control 44: 380-388.

Gaveria Rivera AM, Priest FG (2003). Molecular typing of *Bacillus thuringiensis* Serovars by RAPD-PCR, Syst. Appl. Microbiol. 26: 254-261.

Glare RT, O'Callaghan M (2000) *Bacillus thuringiensis*: Biology, Ecology and Safety. John Wiley & Sons Ltd. Toronto.

Keshavarzi M (2008). Isolation, Identification and Differentiation of local *B. thuringiensis* strains. J. Agric. Sci. Technol. 10: 493-499.

Kumar D, Kamal Chaudhary, Boora KS (2010). Charcterization of native *Bacillus thuringiensis* strains by PCR-RAPD based fingerprinting. Indian J. Microbiol. 50: 27-32.

Letowski J, Bravo A, Brousseau R, Masson L (2005). Assessment of *cry1* gene contents of *B. thuringiensis* strains by use of DNA microassays. Appl. Environ. Microbiol. 71 (9): 5391-5398.

Liliana Pardo-Lopez, Mario Soberon, Alejandra Bravo (2013). Bacillus thuringiensis insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiol. Rev. 37: 3-22.

Lima GMS, Aguiar RWS, Correa RFT, Martins ES, Gomes ACM, Nagata T, Desouza MT, Monnerat DG, Ribeiro BM (2008). Cry 2A toxins from *Bacillus thuringiensis* expressed in insect cells are toxic to two Lepidopteran insects. World J. Microb. Biotechnol. 24: 2941-2948.

Malkawi HI, Al-Momani F, Meqdam MM, Saadoun I, Mohammad MJ (1999). Detection of genetic polymorphisms by RAPD-PCR among isolates of *Bacillus thuringiensis*. New Microbiol. 22: 241–247.

Nethravathi CJ, Hugar PS, Krishnaraj PU, Vastrad AS (2010). Bioefficacy of native *Bacillus thuringiensis* isolates against cabbage leaf webber *Crocidolomia binotalis*. Karnataka. J. Agric. Sci. 23(1): 51-55.

Porcar M, Perez VJ (2003). PCR based identification of *Bacillus thuringiensis* pesticidal crystal genes. FEMS Microbiol. Rev. 26: 419-432.

Ramalakshmi AV, Udaysuriyan V (2009). Diversity of *Bacillus thuringiensis* isolated from Western ghats of Tamil Nadu state. Indian J. Curr. Microbiol. 10: 319-332.

Sadder MT, Khyami H, Horani, Al-Banna L (2006). Application of RAPD Technique to study polymorphism among *Bacillus thuringiensis* isolates from Jordan. World J. Microbiol. Biotechnol. 22: 1307-1312.

Saker M, Salama HS, Ragaei M, El-Ghany NM (2012). Molecular characterization of *Bacillus thuringiensis* isolates from the Egyptian soils. Archives Phytopathol. Plant Prot. 45: 110-125.

Sambrook J, Russel (2001). Molecular Cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, *Cold Spring Harbor* NY pp. 9.31-9.57.

Sauka DH, Sanchez J, Bravo A, Benintende GB (2007). Toxicity of *Bacillus thuringiensis* delta endotoxins against bean shoot borer (*Epinotia aporema* Wals.) larvae, a major soybean pest in Argentina. J. Invertebrate Pathol. 94: 125-129.

Schnepf E, Crickmore N, Van RJ, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62: 775–806.

Travers RS, Martin PAW, Reichelderfer CF (1987). Selective process for the efficient isolation of soil *Bacillus* species. Appl. Environ. Microbiol. 53: 1263-1266.

Yaradoni SN, Krishnaraj PU, Ussuf KK, Kuruvinashetti MS (2003) Molecular characterization of isolates of *Bacillus thuringiensis* from Western Ghats. J. Plant Biol. 30: 7–13.