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Diversity of *Bacillus thuringiensis cry* genes in soils of Andhra Pradesh, India

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A total of 61 bacterial strains was isolated in soils from Chittoor and Kadapa districts of Andhra Pradesh, India using sodium acetate selection method and preliminarily identified as *Bacillus thuringiensis* based on Gram's staining, endospore staining and crystal protein stud. These strains were tested against the insect, *Spodoptera litura* under laboratory conditions and found 18 strains with ≥ 50% insect mortality. Further, these eighteen strains were analyzed for the insecticidal crystal protein composition with fourteen cry specific primers. The results indicated that, among all 14 *cry* genes, *cry*1I was the observed in 11 strains, followed by *cry*1Aa in 10 strains. At the same time a strain, F493 collected from Talakona area identified as effective against *S. litura* with 100 per cent larval death and which was also identified with 8 different crystal protein encoding genes (*cry*1Aa, *cry*1Ac, *cry*1Fa1, *cry*1I, *cry*2, *cry*2A(a)1, *cry*8, *cry*9Ca1).

Keywords: *Bacillus thuringiensis* strains, *Cry* gene, Southern zone, *Spodoptera litura*, δ-endotoxins PCR

Bacillus thuringiensis, a Gram-positive rod-shaped soil bacteria is capable of producing crystalline inclusions comprised of Crystal and Cytolytic proteins that act as potential biopesticides against many crop insect pests. Biopesticides based on cell, spore-crystal mixtures of *B. thuringiensis* seem to be a good alternative for chemical insecticides. They are eco- friendly, do not have a negative impact on non-target organisms, including vertebrates, and are effective in reducing the insect pest population[1]. Search for novel *B. thuringiensis* isolates with a higher and broader spectrum of activity in various natural habitats is gaining momentum. New strains are cultured from samples collected from soil[2], leaves[3] dead insects[4], and other sources[5-7], and their bioefficacy is being evaluated.

Entomopathogenecity of *B. thuringiensis* is mainly due to its ability to produce insecticidal crystalline proteins (ICPs) 'δ endotoxins' during the stationary phase of its growth with concomitant spore production[8]. The crystal proteins produced by each *B. thuringiensis* strains are different and are encoded by specific *cry* genes. Based on toxin production, the insecticidal spectrum of strain is varied. Therefore, large screening programmes, leading to important collections of *B. thuringiensis* strains have been conducted worldwide from different environments and characterized to evaluate their toxic potential against various pests orders[9,10]. *B. thuringiensis* strains are characterized in a number of ways, such as flagellar serotyping[11] profiling plasmid arrays or proteins, the use of monoclonal antibodies and hybridization, or PCR amplification, based on sequences of known *cry* genes[12] or by molecular fingerprints[13-15]. Among the various techniques, PCR based identification is more reliable and having wider acceptability. Extensive screening of *B. thuringiensis* strains for *cry* genes encoding various crystal proteins has led to the identification of more than 700 *cry* gene sequences. These sequences have been classified according to their amino acid sequence identity in at least 70 different *cry* gene groups (*cry*1 to *cry*70). *Cry* toxicity is mainly due to receptor recognition by cry toxins that is fundamental for insect specificity[16]. In the same way, the present studies were conducted to isolate *Spodoptera litura* effective strains and characterization of those strains for *cry* genes encoding the crystal proteins from the soils of Andhra Pradesh, India.

Materials and Methods

Isolation and Identification of *B. thuringiensis*

The present studies were conducted from 2014-15 to 2016-17 at the Department of Entomology, Institute of Frontier Technology, Regional Agricultural Research Station, Tirupati. A total of 264 soil samples were

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collected from forest ecosystems in Chittoor and Kadapa districts of Andhra Pradesh, India. Collection sites were 0.5 to 1.0 Km distance from the road and were chosen in such a way that the soils were not in cultivation, were never applied with any *B. thuringiensis* formulations. Samples were obtained from a depth of 10-15 cm in sterile polyethylene bags by using a sterilized spatula and brought to the laboratory and were stored at 4°C for further processing.

Sodium acetate selection method given by Travers *et al.*[17], was followed for isolating *B. thuringiensis* soil samples with slight modifications. Half a gram of soil sample was added to 10 mL of Luria Bertani broth (LB) composed of Tryptone, Yeast extract, and NaCl in a conical flask of 100 mL capacity. The Luria Bertani broth was buffered with 0.5 M sodium acetate. The mixture was kept on a shaker at 250 rpm for 4h at 28°C, followed by heat shock at 80°C for 15 min. The samples were serially diluted till 10^{-5} concentration and 100 µL of the sample was spread on Luria Bertani (LB) agar media. Three replications were maintained for each dilution. The plates were incubated at 37°C overnight. Colonies were picked up after comparing with morphological characters (cream- colored and have the appearance of fried egg like colonies on the plate) and were purified by four- way streaking[18] These cultures were subjected to Gram's staining[19] for separation of Gram-positive and negative (bacteria). The Gram-positive isolates (Fig. 1) were streaked onto T3 media plates and allowed to multiply for 72 h and tested for the endospore production[20] (Fig. 2) with malachite green staining. The isolates which were found to be positive in malachite green staining were further screened for crystal production[21] using Coomassie brilliant blue as per standard protocols (Fig. 3). The resultant crystal positive *B. thuringiensis* isolates were further used for bio-efficacy studies and molecular characterization with *cry* gene specific primers belongs to *cry*1, *cry*2, *cry*8, and *cry*9.

Bioassay studies

Sixty- one (61) bacterial cultures identified as *B. thuringiensis* based on various microbial techniques coupled with microscopy were bioassayed to ascertain insecticidal activity against third instar *S. litura*. *B. thuringiensis* (HD1) strain from National Beaureau of Agricultural Insect Resources (NBAIR), Bengaluru was used as a reference strain. Individual *B. thuringiensis* culture was streaked on plain Luria Bertani agar plates and incubated overnight at 37°C. One loop of the overnight culture was inoculated in Luria Bertani broth and kept for sporulation under shaking conditions at 28°C for 72 h. The culture containing spore crystal mixture was pelletized in Eppendorf centrifuge at 10000 rpm for 10 min. The resultant

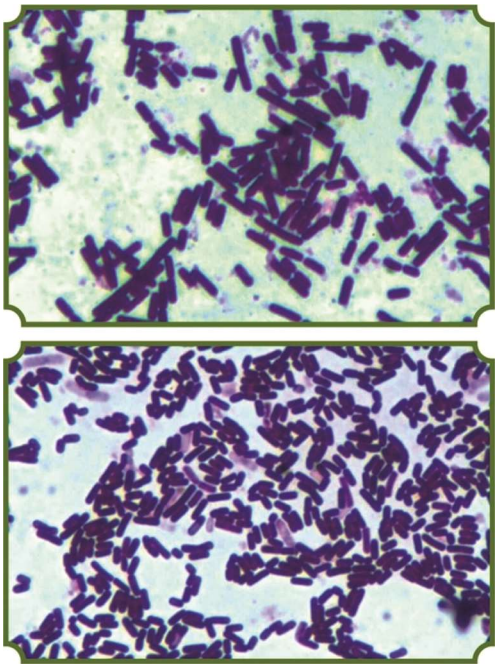


Fig. 1 — Identification *Bacillus* through Gram's staining

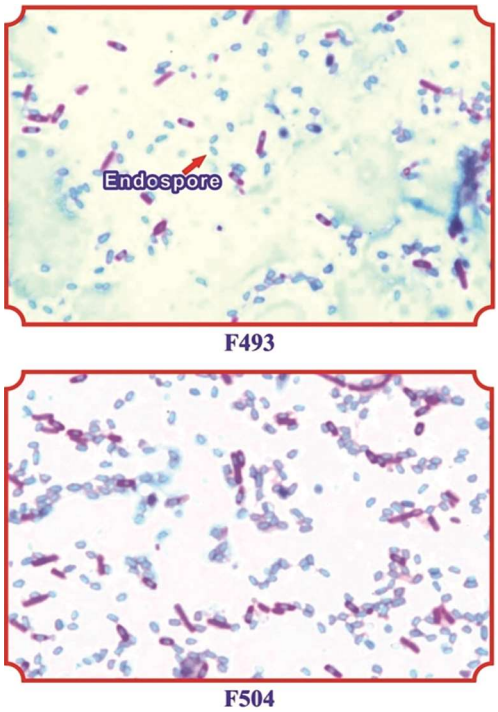


Fig. 2 — Endospore stating of *Bacillus* strains

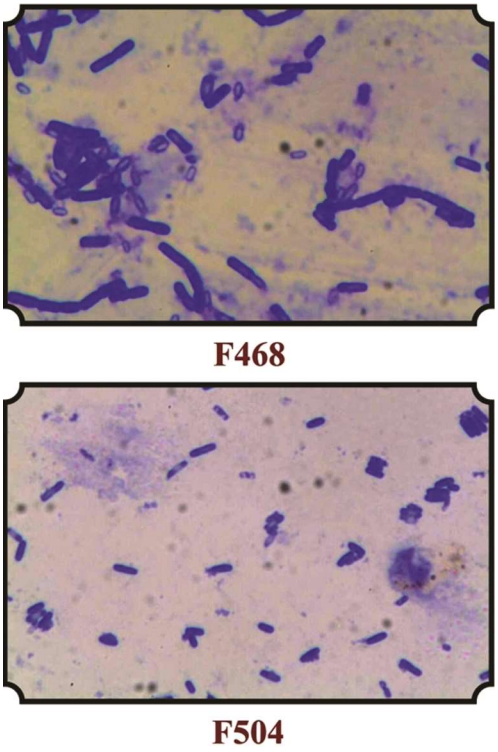


Fig. 3 — Crystal staining of *B. thuringiensis* strains

pellet was diluted in sterile water. The bioassay was followed by leaf dip method developed by Shelton *et al.*[22] A compound leaf of Groundnut was dipped for 10 min into *B. thuringiensis* culture broth (5×10^8 CFU mL^{-1}) containing 0.2% Triton X-100, then kept leaf for air drying till leaf surface free from moisture. After air drying, the petiole of the leaf was swabbed with wet cotton to maintain leaf succulence and turgidity. Two compound leaves were used for one replication, which was placed on a Petri plate. Ten larvae were released per one replication. HD-1 strain of *B. thuringiensis* served as a reference strain. The leaf dipped in distilled water served as control. The larval mortality was assessed after 72 h at regular intervals till pupation. Day wise larval mortality was counted, converted to percentages and subjected to Analysis of Variance (ANOVA) for drawing necessary conclusions.

DNA extraction and PCR amplification

From the results of insect bioassay studies, 18 strains were chosen for screening an abundance of *cry* genes. Total DNA was isolated from 18 *B. thuringiensis* strains with 50% ≥ mortality of III instar *S.litura* along with standard check HD1 by adopting the protocol of Sambrook and Russell[23] with slight modifications as follows. Twenty five mL of an overnight culture grown in Luria broth at 37°C for 16 h was centrifuged at 10000 rpm for 10 min. The pellet was resuspended in 10 mm Tris, 100 mm Sodium chloride solution and centrifuged at 10000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 2.5 mL of TE buffer and 500 µL of Lysozyme (50 mg mL^{-1}). The solution was incubated at 37°C for 20 min and 25 µL of RNAse (10 mg mL^{-1}) was added and incubated at 50°C for 45 min. 50 µL of Proteinase K (20 mg mL^{-1}) was added and incubated at 50 to 55°C for 10 min. Equal volumes of Phenol was later added, mixed gently and centrifuged at 10000 rpm for 10 min at 4°C. The aqueous phase was transferred to a fresh tube and extraction was repeated twice. Equal volume Phenol: Chloroform (1:1) was added, centrifuged and the aqueous phase was separated out. Equal volumes of Chloroform: Isoamyl alcohol (24:1) was added, centrifuged and the aqueous phase was separated out. To the supernatant, $1/10^{th}$ volume of 3 M Sodium acetate (pH 5.5) was added and incubated in ice for 20 min. Two volumes of cold, 2-propanol were added and centrifuged for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 70% Ethanol, dried DNA and pellet dissolved in 25 µL of TE buffer. The presence of DNA and its quality was checked through agarose gel electrophoresis as well as nanodrop spectrophotometer.

A total of 14 cry primers (*cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1C, *cry*1Da1, *cry*1Ea1, *cry*1F, *cry*1Fa1, *cry1I*, *cry*2, *cry*2A(a)1, *cry*8, *cry*9Aa1 and *cry*9Ca1) was used in the study. The primers were synthesized with the known sequences available in the database. The composition of the reaction mixture was Taq assay buffer (10X) 2.5 µL, dNTP's (2 mM) 1.5 µL, 2.5 µL of each forward and reverse primer, $MgCl_2$ (2.0 mM) 1.5 µL, Taq DNA polymerase (3 U μL^{-1}) 0.5 µL, Template DNA (100 ng) 2.5 µL and made to a final volume of 25 µL. The annealing temperature ranged from 46.2°C to 57.2°C for various primer sets (Table 1).

Results and Discussion

Out of 264 soil samples, 71 Gram-positive isolates were identified of which 68 having endospore producing nature and finally 61 isolates were identified in crystal production. These 61 isolates along with standard check HD-1 were tested for bio-efficacy against third instar larvae of *S. litura*. The bioassay has resulted in cumulative mortality of 0.00 to 100% at 168 h after treatment and F493

strain has recorded 100% mortality, followed by F468 (86.67%) which were comparable with standard strain HD-1 for their efficacy. The other strains, F287 (76.67%), F504 (76.67%) were identified as effective treatments next to F493, HD-1, and F468 which were statistically on par with each other. In untreated control, the larval mortality was zero percent (Table 2 & Fig. 4).

Soil is considered as a rich source of microbial fauna, *B. thuringiensis* said to be one such organism which is abundant in soil samples. The diversity of *B. thuringiensis* from various soil samples was reported by several researchers across the countries [24] including Brazil[25], China[26], Egypt[27], India[28], Iran[29], Mexico[30], Sudan[31], Syria[32], and Thailand [33]. The results of present study are comparable with earlier studies of Nariman *et al.*[34] who reported 100 and 90% mortality of second instar *S. littoralis* with two *Bt* isolates Ts-5 and As-3 collected from seven governorates of Egypt. Similarly, Lalitha and MuraliKrishna[35] evaluated 114 Bt strains against I & III instar *S. litura* and found 375 strain recorded a mortality of 90 and 70% which were on par with standard strain HD1. Further, Li *et al.*[36] reported that a Korean *B. thuringiensis* isolate with high insecticidal activity against two lepidopteran pests *viz*., *Plutella*

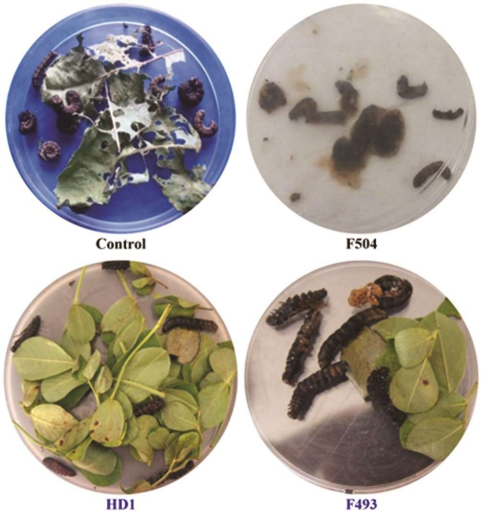


Fig.4 — Efficacy of indigenous *B. thuringiensis* strains against *S. litura* in laboratory bioassay

Table 1 — Details of *cry* primers used in the study

S. No.	Primer name	Direction	Primer sequence	Reference
1.	*cry*1Aa	Direct	ATTATCATATTGATCAAGTTC	Salek, *et al.*, 2012
		Reverse	CATAAGGAACCCGTACCTGG	
2.	*cry*1Ab	Direct	GGACCAGGATTTACAGGAGG	-do-
		Reverse	GTTCTCCTACTAATGGTTTCC	
3.	*cry*1Ac	Direct	CTCAATGGGACGCATTTCTT	-do-
		Reverse	CGGTTGTAAGGGCACTGTTC	
4.	*cry*1C	Direct	AAAGATCTGGAACACCTTT	Ceron *et al.*, 1994
		Reverse	CAAACTCTAAATCCTTTCAC	
5.	*cry*1Da	Direct	GTAGCAGACATTTCATTAGG	Pooja *et al.*, 2013
		Reverse	ACATGAATAAGGCTAGTCAG	
6.	*cry*1Ea1	Direct	ATATAGAAGTAGGGGGACAG	-do-
		Reverse	TAGCCCTAGTTGATTTGTAG	
7.	*cry*1Fa1	Direct	GATTTGCTAATACAGACGAC	-do-
		Reverse	CGTGAACTCACTAAGTGTCC	
8.	*cry*1F	Direct	TGTAGAAGAGGAAGTCTATCCA	Ceron *et al.*, 1994
		Reverse	TATCGGTTTCTGGGAGTA	
9.	*cry*1I	Direct	AGCTATGGCCTAAGGGGAAA	Nariman, 2007
		Reverse	TTCCAACCCAACTTTCAA	
10.	*cry*2	Direct	GTTATTCTTAATGCAGATGAATGGG	Ben-Dov *et al.*, 1997
		Reverse	CGGATAAAATAATCTGGGAAATAGT	
11.	*cry*2A(a)	Direct	AAGGAGGAATTTTATATGAA	Ogunjimi *et al*., 2002
		Reverse	CATTTAGTTCCGTCAATATG	
12.	*cry*8	Direct	ATGAGTCCAAATAATCTAAATG	Bravo *et al.*, 1998
		Reverse	TTTCATTAATGAGTTCTTCCACTCG	
13.	*cry*9Aa1	Direct	ATCGTAGAGAGTGACATTG	Pooja *et al.*, 2013
		Reverse	TGTTGTCCAGAGATTAGTTC	
14.	*cry*9Ca1	Direct	GGATCTAAATGCAAGTGTAG	-do-
		Reverse	ACCATTTACATCGTAGTCAC	

xylostella and *Spodoptera exigua* harbored *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1E, *cry*2 genes and another *cry* gene similar to *cry*1Fa1.

Molecular characterization of indigenous *Bt* strains

Molecular screening of *Bt* strains facilitates the identification of novel *cry* genes with high entomopathogenic activity against various insect pests. Bravo *et al.*[37] reported that the crystal proteins toxic for lepidopteran insects belong to the *cry*1, *cry*9, and *cry*2 groups, toxins active against coleopteran insects are *cry*3, *cry*7, and *cry*8 proteins, *cry*1B, and *cry*1I proteins have dual activity. The *cry*5, *cry*12, *cry*13, and *cry*14 proteins are nematocidal, and the *cry*2, *cry*4, *cry*10, *cry*11, *cry*16, *cry*17, *cry*19, and *cyt* proteins are toxic for dipteran insects. At the optimum annealing temperature, each primer set produced an amplicon of respective target band. The amplicon size for different primer set was as follows, *cry*1Aa (390 bp), *cry*1Ab (1111 bp), *cry*1Ac (238 bp), *cry*1C (130 bp), *cry*1Da1 (503 bp), *cry*1Ea1 (694 bp), *cry*1F (290 bp), *cry*1Fa1 (580 bp), *cry1I* (189 bp), *cry*2 (689 bp), *cry*2A(a)1 (2000 bp), *cry*8 (376 bp), *cry*9Aa1 (376 bp), *cry*9Ca1 (697 bp) (Fig. 5).

Diversity of *cry* genes in indigenous *Bt* strains collected from Andhra Pradesh

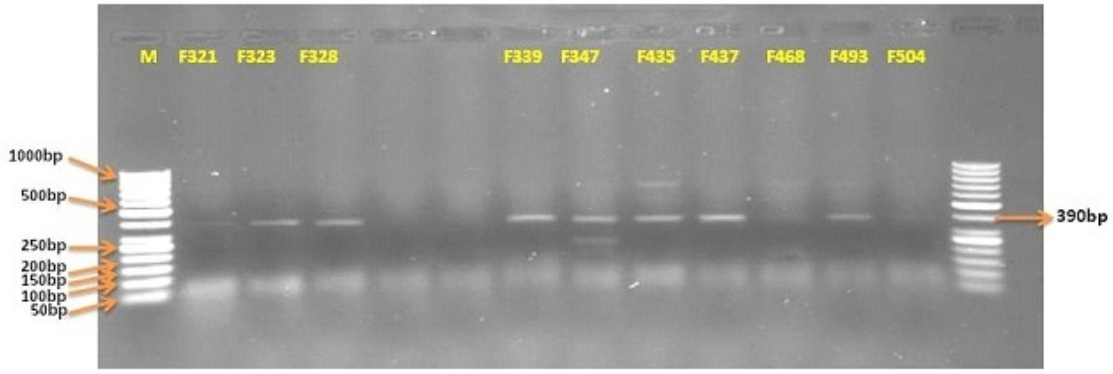
A total of 18 strains were tested for the *cry* gene availability with 14 cry primers. Among the 14 *cry* gene primers used in the present study, *cry*1I was the predominant which was observed in 11 *Bt* strains (F268, F287, F323, F339, F361, F399, F435, F437,

Table 2 — Evaluation of Bt strains against *S. litura* under *in vitro* conditions

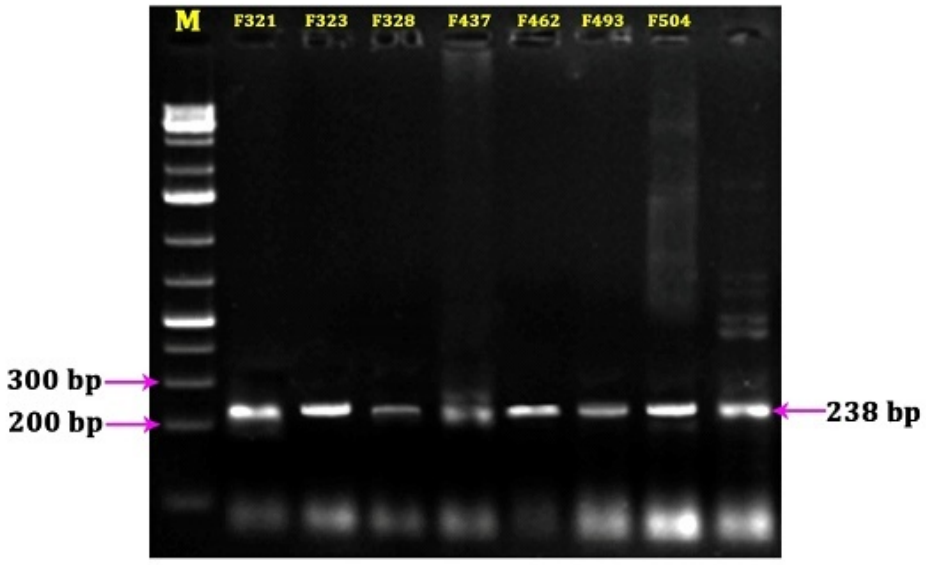
S. No	Strain	Percent mortality	S. No	Strain	Percent mortality
1	F252	0.00 (0.00)^{a}	34	F440	26.67 (31.00)$^{d-i}$
2	F254	26.67 (30.79)$^{d-i}$	35	F441	10.00 (15.00)^{abcd}
3	F256	16.67 (23.36)^{def}	36	F443	26.67 (31.00)$^{d-i}$
4	F258	33.33 (35.22)$^{e-j}$	37	F444	26.67 (30.29)$^{d-i}$
5	F261	43.33 (41.07)^{ghijk}	38	F445	33.33 (35.01)$^{e-j}$
6	F262	46.67 (43.08)^{hijk}	39	F447	13.33 (21.14)^{de}
7	F263	10.00 (18.43)^{de}	40	F455	10.00 (15.00)^{ad}
8	F268	60.00 (50.85)^{jklm}	41	F457	16.67 (23.86)^{defg}
9	F277	0.00 (0.00)^{a}	42	F459	0.00 (0.00)^{a}
10	F281	43.33 (41.15)^{ghijk}	43	F462	63.33 (53.07)^{klm}
11	F284	43.33 (40.78)^{fghijk}	44	F463	26.67 (30.79)$^{d-i}$
12	F287	76.67 (61.22)^{lmn}	45	F468	86.67 (72.78)^{no}
13	F297	50.00 (45.00)^{ijkl}	46	F482	43.33 (41.07)^{ghijk}
14	F307	53.33 (47.01)^{ijkl}	47	F484	10.00 (18.43)^{de}
15	F309	26.67 (31.00)$^{d-i}$	48	F486	33.33 (35.22)$^{e-j}$
16	F316	16.67 (23.86)^{defg}	49	F487	60.00 (50.94)^{jklm}
17	F321	50.00 (45.08)^{ijkl}	50	F490	33.33 (34.93)$^{e-j}$
18	F323	56.67 (49.22)^{jkl}	51	F491	50.00 (45.00)^{ijkl}
19	F328	50.00 (45.00)^{ijkl}	52	F493	100.00 (90.00)^{p}
20	F339	50.00 (45.00)^{ijkl}	53	F498	26.67 (30.79)$^{d-i}$
21	F347	50.00 (44.71)^{ijkl}	54	F500	16.67 (23.86)^{defg}
22	F361	53.33 (47.22)^{ijkl}	55	F503	10.00 (15.00)^{abcd}
23	F396	50.00 (45.00)^{ijkl}	56	F504	76.67 (65.85)^{mn}
24	F399	66.67 (54.99)^{klm}	57	F505	43.33 (41.15)^{ghijk}
25	F429	16.67 (23.86)^{defg}	58	F506	26.67 (26.07)^{defgh}
26	F430	10.00 (15.00)^{abd}	59	F508	0.00 (0.00)^{ab}
27	F432	33.33 (35.01)$^{e-j}$	60	F510	16.67 (23.86)^{defg}
28	F433	50.00 (45.08)^{ijkl}	61	F514	33.33 (35.22)$^{e-j}$
29	F434	10.00 (18.43)^{de}	62	HD1	96.67 (83.86)^{op}
30	F435	50.00 (45.00)^{ijkl}	63	Control	0.00 (0.00)^{abc}
31	F436	43.33 (41.15)^{ghijk}		F.Pr.	<.001
32	F437	60.00 (50.85)^{jklm}		LSD	14.06
33	F438	0.00 (0.00)^{a}			

462, F493, F504), followed by *cry*1Aa in 10 strains (F321, F323, F328, F339, F347, F435, F437, F468, F493, F504). The other *cry* genes such as *cry*1Ac (F321, F323, F328, F437, F462, F493, F504) and *cry*9Ca1 (F323, F328, F347, F361, F399, F493, F504) were observed in 7 strains each. *cry*2A(a)1 was observed in 4 strains (F268, F323, F396, F493), *cry*1C (F361, F435, F504), *cry*2 (F323, F437, F493), *cry*8 (F487, F493, F504), *cry9Aa1* (F297, F328, F347) identified in three strains each. Whereas *cry*Da1 (F321) and *cry*1Fa1 (F437, F493) were observed in one and two strains respectively. Whereas none of the strains positive for *cry*1Ab, *cry*1Ea and *cry*1F genes (Table 3). From the present study, it is evident that, among the various *cry* genes screened, *cry*1 related genes were the most predominant, followed by *cry*9 and *cry*2. Earlier researchers[38-48] reported a higher frequency of *cry*1 gene positive *B. thuringiensis* strains from their studies. In the same way, the present *B. thuringiensis* strain collections had *cry*1 related genes in higher frequency. Added to this, Shishir *et al.*[49] also observed the abundance of insecticidal *cry* genes from the soils of Bangladesh and found *cry*1,*cry*2 and *cry*3 were the most abundant among the *cry* genes tested *viz.*, *cry*1, *cry*2, *cry*3, *cry*4a, *cry*8, *cry*9, *cry*10 and *cry*11. Contrary to the present investigations, Mendoza *et al.*[50] found high frequency of *cry2* genes (71%) followed by *cyt* (40%) and *cry*1 (36%) from the samples of Tijuana-Ensenada region of northwestern Mexico.

Regarding the abundance of *cry* genes in the present *B. thuringiensis* collection, eight *cry* genes (8 *cry* genes) were observed in F493 (*cry*1Aa, *cry*1Ac, *cry*1Fa1, *cry*1I, *cry*2, *cry*2A(a)1, *cry*8, *cry*9Ca1). This strain was collected from the Talakona



B. thuringiensis strains with *cry*1Aa gene



B. thuringiensis strains with *cry*1Ac gene

Fig. 5 — Molecular characterization of *B. thuringiensis* strains with different *cry* gene specific primers

forest area which recorded 100% mortality against

Table 3 — Diversity of *cry* genes in indigenous *Bt* strains collected from Andhra Pradesh

S. No	*Cry* gene	*Bt* strain	No. of strains
1	*cry*1Aa	F321, F323, F328, F339, F347, F435, F437, F468, F493, F504	10
2	*cry*1Ab	-	0
3	*cry*1Ac	F321, F323, F328, F437, F462, F493, F504	7
4	*cry*1C	F361, F435, F504	3
5	*cry*Da1	F268, F287, F297, F321	4
6	*cry*1Ea	-	0
7	*cry*1Fa1	F437, F493	2
8	*cry*1F	-	0
9	*cry*1I	F268, F287, F323, F339, F361, F399, F435, F437, 462, F493, F504	11
10	*cry*2	F323, F437, F493	3
11	*cry*2A(a)1	F268, F323, F396, F493	4
12	*cry*8	F487, F493, F504	3
13	*cry*9Aa1	F297, F328, F347	3
14	*cry*9Ca1	F323, F328, F347, F361, F399, F493, F504	7

Table 4 — List of *Bt* strains identified as effective against *S. litura* indigenous to forest ecosystem in Southern zone of Andhra Pradesh.

S. No.	*Bt* strain	Area of collection	Mortality (%)	*cry* gene(s) observed	No. of *cry* genes
1	F268	Bhakarapet ghat	60.00	*cry*1Da1, *cry*1I, *cry*2A(a)1	3
2	F287	Talakona	76.67	*cry*1Da1, *cry*1I	2
3	F297	Talakona	50.00	*cry*1Da1, *cry*9Aa1	2
4	F321	Talakona	50.00	*cry*1Aa, *cry*1Ac, *cry*1DaI,	3
5	F323	Kalyanidam	56.67	*cry*1Aa, *cry*1Ac, *cry*1I, *cry*2, *cry*2A(a)1, *cry*9Ca1	6
6	F328	Rangampet	50.00	*cry*1Aa, *cry*1Ac, *cry*9Aa1, *cry*9Ca1	4
7	F339	Sugalibidiki	50.00	*cry*1Aa, *cry*1I	2
8	F347	Chinagottigallu	50.00	*cry*1Aa, *cry*9Aa1, *cry*9Ca1	3
9	F361	Rayachoti	53.33	*cry*1C, *cry*1I, *cry*9Ca1	3
10	F396	Mamanduru	50.00	*cry*2A(a)1	1
11	F399	Rayachoti	66.67	*cry*1I, *cry*9Ca1	2
12	F435	Talakona	50.00	*cry*1C, *cry*1Aa, *cry*1I	3
13	F437	Bhakarapet ghat	60.00	*cry*1Aa, *cry*1Ac, *cry*1Fa1, *cry*1I, *cry*2	5
14	F462	Talakona	63.33	*cry*1Ac, *cry*1I	2
15	F468	Bhakarapet ghat	86.67	*cry*1Aa	1
16	F487	Talakona	60.00	*cry*8	1
17	F493	Talakona	100.00	*cry*1Aa, *cry*1Ac, *cry*1Fa1, *cry*1I, *cry*2, *cry*2A(a)1, *cry*8, *cry*9Ca1	8
18	F504	S.V. Zoo park	76.67	*cry*1Aa, *cry*1Ac, *cry*1C, *cry*1I, *cry*8, *cry*9Ca1	6

S. litura in laboratory bioassay studies. Two more strains F504 (*cry*1Aa, *cry*1Ac, *cry*1C, *cry*1I, *cry*8, *cry*9Ca1) and F323 (*cry*1Aa, *cry*1Ac, *cry*1I, *cry*2, *cry*2A(a)1, *cry*9Ca1) identified with six *cry* genes which were collected from S.V. Zoo park area and Kalyanidam. Present *B. thuringiensis* strains showed differed efficacy against test insect, *S. litura* under laboratory bioassay as well as varied types of *cry* genes. This might be due to variation in the expression of genes governing the crystal toxin expression (Tabashnik[51]) which require further investigations related to protein profiling (Table 4).

Further, the present studies revealed that, the coexistence of *cry*9 gene is very common with any of the *cry* genes either *cry*1, *cry*2 or *cry*8. Among these, the frequency of *cry*1 and *cry*9 combination was

more. Similar way, Wang *et al.*[52] stated that among 90.7% strains of *cry*1 gene containing strains also harboured a *cry*2 gene. Baig *et al.*[53] also reported that, out of 50 *Bt* strains, none of the strains harboured *cry*1 gene alone and it was always found in combination with *cry*3. Further, they reported that the combination of *cry*2 + *cry*4 (14%) was high. And also they reported that, the most dominant three gene linkage was *cry*1 + *cry*3 + *cry*4. Further profiling of the *cry*1 gene showed that the *cry*1K gene was abundantly present in all combinations such as *cry*1A, *cry*1D, *cry*1F, and *cry*1I. However, *cry*1C existed independently of other subtypes. Likewise, Shahram *et al.*[54] revealed that, among the forty eight strains from 744 different samples, *cry*1 genes were the most abundant (93.75%).

Added to this, in the present studies, some of the *cry* genes observed at very low frequency in the present *B. thuringiensis* strains, *viz*., *cry*1Ab*, cry*1Ea1 and *cry*1F similar to Valicente *et al.*[55] and Dos Santos *et al.*[56] who also obtained a low frequency of *cry*1F gene in strains of *B. thuringiensis* isolated from soils in China (0.3%) and Mexico (4.8%), respectively. Thammasittirong and Attathom[57] reported a complete absence of *cry*1F gene *B. thuringiensis* isolates collected from Thailand.

Conclusion

The present investigations revealed the diversity of *cry* genes in indigenous *B. thuringiensis* strains collected from undisturbed environments of Andhra Pradesh. Bioassay studies against *S. litura* indicated the effectiveness of indigenous strains in insect control. As the present collection having novel strains with different *cry* genes encoding various crystal proteins, these can also be tested against other lepidopteran insects and also can be used as potential candidates for developing biopesticides.

Conflict of interest

All authors declare no conflict of interest.

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