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Research Article

Diversity of *cry* genes occurring in the North East

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ABSTRACT: The search for new *Bacillus thuringiensis (Bt)* strains is a continuous process and researchers are now focusing on finding toxin proteins that are toxic to pests of insect orders that are not reported. In the present study soil and insect cadaver samples were collected from North East India comprising the states of Assam, Tripura and Mehhalaya and native *Bt* were isolated using standard protocols. At total of 30 *Bt* isolates were purified and characterized. Various types of crystal morphology were encountered that included bipyramidal, cuboidal, square, rhomboid, spherical and irregular. PCR analysis showed that diverse *cry* genes were expressed. The *cry* genes identified were Lepidoptera, Coleoptera and Diptera specific. Detected genes included *cry1Ac, cry2A, cry4A, cry10A, cry16A, cry17A, cry19A, cry30Aa, cry44Aa, cry11A, cry4B, cry12A, cry8A* and *cry7A*. Many of them were positive for Vip3A protein. The coleopteran specific *Bt* were evaluated against *Sitophilus oryzae* and *Callosobruchus chinensis* and NBAIR-AgBt6 was found to be toxic. The isolates are being further evaluated for use as biopesticides.

KEY WORDS: *Bacillus thuringiensis*, bioassay, cry genes, diversity, North East

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INTRODUCTION

The search for new *Bacillus thuringiensis* (*Bt*) strains is a continuous process and researchers are now focusing on finding toxin proteins that are toxic to pests of insect orders that are not reported. Basically search for novel Cry toxins is the norm. However studies on *Bt* diversity are well documented from different countries (Bravo *et al*., 1998; Porcar and Juarez Perez, 2003; Uribe *et al.*, 2003; Quesada Moraga *et al*., 2004; Nazarian *et al*., 2009; Thammasittirong and Attathom, 2008; Baig and Mehnaz, 2009). In India limited work on *Bt* diversity has been done (Ramalakshmi and Udayasuriyan, 2010; Asokan *et al*., 2014) but the studies were restricted to certain Cry types like *cry*3 or *cry*1. Improved PCR based techniques have been designed to detect *cry* genes (Bravo *et al*., 1998; Beron *et al*., 2005). Documentation of *Bt* diversity in northeast India is very limited or not available. Currently there are 240 holotype toxins available (www.lifesci.sussex.ac.uk). Under *cry1* there are 44 types like *cry1Aa, cry1Ab, cry1Ac* etc, similarly under *cry2* there are 9 types like *cry2Aa, cry2Ab* etc. We have toxins listed from *cry1 to cry72* (having 229 holotypes). Hence we undertook a systematic study to analyse the type of *Bt* that occur in northeast India (Tripura, Assam and Shillong) and also to ascertain the *cry* gene diversity that occurs in these isolates. The strategy used was based on multiplex PCR analysis with novel, general and specific primers for identification of Cry toxin genes for lepidopteran, coleopteran and dipteran pests.

MATERIALS AND METHODS

Isolationof *Bacillus thuringiensiss* strains from soil samples and insect cadavers

Soil and insect cadaver samples were collected from the three states of northeast India (Tripura, Assam and Meghalaya). Samples were drawn from forests and crop growing areas. It was ascertained that commercial *Bt* formulation was not used in these areas. The *Bt* isolates were selected as per the methodology of Travers *et al*. (1987) and Santana *et al*. (2008).

Staining and microscope analysis

The *Bt* isolates were screened for production of crystalline inclusions by use of amido black stain. The stain was prepared as A. 1.5g amido black stain 10b dissolved in 1L containing 50% methanol, 40% distilled water, 10%

glacial acetic acid, filtered and stored for 3 days; and B. 0.5% aqueous solution of safranin. Heat fixed smear was stained with A for 2-3 minutes, washed and stained with B for 2-3 minutes, washed, dried and observed under oil immersion using Olympus BX-41. Crystals appeared blue black and spores appeared with pinkish margin. Crystals were also characterized by Transmission Electron Microscopy (TEM) studies by uranyl acetate staining and observation in TEM make HITACHI.

PCR amplification and detection of *cry* genes

The *Bt* isolates that produced protein crystals were purified. The purified cultures were grown overnight in LB broth in rotary shaker (model Orbitek) at 250 rpm. DNA was extracted by using HiPurA™ Bacterial Genomic Purification Kit (MB505) as per the manufacturer's protocol with mini-prep column formation as per Sambrook (2001). Purity of DNA was tested by running in 1.25% agarose gel for 1hr at 100V. Multiple *cry* gene profile in PCR was done using Quantarus (UK) make thermocycler for 30 reaction cycles each. PCR reactions were carried out in 25 µl containing 20-100ng of DNA mixed with 1X Taq reaction buffer, 150 mM - dNTP mix, 0.2-0.4 µM – Primer (forward and reverse), 1U - Taq DNA Polymerase (GeNei), 14 µM - $MgCl_2$. Denaturation of template DNA was done for one minute at 94ºC, annealing at 45-59ºC (Table 1) for one minute and elongation at 72ºC. An extra denaturation and elongation step was provided at 94ºC for 2-5 minutes and at 72ºC for 5-10 minutes respectively (Aly, 2007). PCR amplification of products were separated using agarose gel electrophoresis in 1.2% TAE buffer and stained with 0.2 mg/ml ethidium bromide (Sambrook *et al*., 1989). PCR products were visualized under UV transilluminator and the sizes of the fragments were estimated based on a DNA ladder (GeNei) of 100 to 1500 base pairs. The specific primers used are listed in (Table 1). For Vip3A specific primers were designed by us.

Preparation of *Bacillus thuringiensis* extracts for bioassays

Bacillus thuringiensis 24h overnight culture in single colony of each strain grown in T3 agar medium were inoculated in 10 ml liquid LB broth medium and grown for 48h at 28°C and 200 rpm. An aliquot was taken to verify spore and crystal formation (over 90% sporulation is optimum), and the pre-culture was incubated for 20 min at 70°C to eliminate vegetative cells (synchronization). The main culture (40 ml) was inoculated with 1/1,000 volumes of synchronized pre-culture and grown as mentioned above. Optimal crystal formation was checked by phase-contrast microscopy. The whole culture was centrifuged at 9,000×g for 10 min. An aliquot of the supernatant (1 ml) was kept at -20°C for future bioassays. The pellet was washed once with ice-cold 1 mol/l NaCl, 10 mmol/l EDTA solutions. Finally, the pellet was suspended in 1 ml of 10 mmol/lKCl. OD 590nm was measured and suspensions were stored at -20°C until bioassay. All steps after centrifugation were done on ice to limit proteolysis.

Protein estimation

The pellet obtained from above was, dissolved in 500µl of lysis buffer containing 100mM Tris HCl (pH 7.0), 20mM EDTA, 5mg/ml lysozyme, 2% SDS and it was centrifuged at 8000 rpm for 7 minutes. Pellet was resuspended in 200µl resuspension buffer (0.1% SDS + 10mM EDTA), further diluted with treatment buffer (2.0%SDS +5% â mercaptoethanol+130mM Tris HCl, pH 10.0) and incubated at 90ºC for 7 minutes (Morris *et al*., 1998). Protein concentration was measured as per Lowry *et al.* (1951).

Bioassay

Sitophilus oryzae and *Callosobrochus chinensis* were obtained from the division of insect ecology that were maintained as stored grain pests on chickpea seeds. The beetles were reared in the laboratory at 28-32 ºC and 70-80% of relative humidity on maize and chickpea grains. The spore crystal protein concentrations obtained from the *Bt* that expressed coleopteran specific toxin genes were serially diluted to obtain six different concentrations. Two types of grains were used, for *C. chinensis* chickpea groundnut seeds were used and for *S. oryzae* maize seeds were used. The fully grown seeds (100 numbers) were first disinfested (65°C for 2-3h), cooled and then surface coated with the 2 ml of different protein (spore crystal) concentration with 3 replications for each treatment. The seeds were then transferred into Petri dishes. For each treatment ten healthy laboratory reared beetles were exposed with help of soft brush. For control both dry grains and wet (sterile water treated) grains with three replicas were maintained. Mortality was recorded on daily basis separating dead from alive and average of three replicas were considered as final mortality (Md. Abdur Rashid *et al*., 2012). The results were subjected to probit analysis using SPSS version 10 software and LC_{50} values obtained.

RESULTS AND DISCUSSION

Thirty *Bacillus thuringiensis* bacteria were isolated from a total of 123 samples including soil, and dead insects obtained from North East encompassing three states (Assam,

Table 1. List of primers used for the detection of different *cry* genes from North-East isolates of *Bacillus thuringiensis*

Cry gene	Product size (bp)	Primers sequence	Annealing Temp. (°C)	Reference
Cry 1Ac	3686	P1 5'GTCGACTATGGATAACAATCCG'3 P2 5'-GGCTCCDDAACCTGAGTTTGC-3'	58	Designed in this experiment
Cry 2A	1170	5'-CGATATGTTAGAATTTAGAAC-3' 5'-TACCGTTTATAGTAACTCG-3'	50	Porcar and Perez 2003
Cry 3A	951	5'-CGTTATCGCAGAGAGATGACATTAAC-3' 5'-TGGTGCCCCGTCTAAACTGAGTGT-3'	59	Ben Dov *et al., 1997*
Cry 4A	1529	5'-GGGTATGGCACTCAACCCCACTT-3' 5'-GCGTGACATACCCATTTCCAGGTCC-3'	53	Ben Dov *et al., 1997*
Cry 4B	1925	5'-GGGTATGGCACTCAACCCCACTT-3' 5'-GCGTGACATACCCATTTCCAGGTCC-3'	55.9	Ben Dov *et al., 1997*
Cry 7A	1320	5'-CATCTAGCTTTATTAAGAGATTC-3' 5'-GATAAATTCGATTGAATCTAC-3'	59.2	Ben Dov *et al*., 2001
Cry 8A	342	5'-ATGAGTCCAAATAATCTAAATG-3' 5'-TCTCCCCATATATCTACGCTC-3'	50	Bravo *et al*., 1998
Cry 10A	651	5'-ATAAATTCAAGTGCCAAGTA-3' 5'-CCGAACCTACTATTGCGCCA-3'	45	Porcar *et al*., 1999
Cry 11A	445	5'-CCGAACCTACTATTGCGCCA-3' 5'-CTCCCTGCTAGGATTCCGTC-3'	55	Ben Dov *et al, 1997*
Cry 12A	363	5'-CTCCCCCAACATTCCATCC-3' 5'-AATTACTTACACGTGCCATACCTG-3'	59.3	Ejiofor A.O. and Johnson T. (2002)
Cry 16A	1415	5'-TCAAAAGGTGTGGCAAG-3' 5'-ATAAGCCCAATATCATG-3'	46	Barloy *et al*., 1998
Cry 17A	1400	5'-AAGTAAAGATTTCTGGG-3' 5'-CTGAGGTATTTTGTGGA-3'	48	Barloy *et al*., 1998
Cry 19A	355	5'-AGGGGAGTCCAGGTTATGAGTTAC-3' 5'-ATTTCCCTAGTTAGTTCGGTTTTT-3'	46.9	Ejiofor A.O. and Johnson T. (2002)
Cry30Aa	1600	5'-ACAAATTATAAAGATTGGCT-3' 5'-GAGTAATTGGCAGAAATTC-3'	52	Ito *et al*., 2006
Cry44Aa	1800	5'-ACAAATT ATAAAGATTGGCT-3' 5'-GAGTAATTGGCAGAAATTC-3'	53	Ito *et al*., 2006
Vip3A	2370	F 5' CTC AAT GGG ACG CAT TTC TT 3' R 5 'GTTGTAAGGGCACTGTTC 3'	50	Rangeshwaran *et al*., 2016

Tripura and Shillong). The crystal structures of these isolates were identified using Amido black staining. The isolates expressed varied type of crystals (bypramidal, square, spherical, irregular) as observed through transmission electron microscopy (Fig. 1).

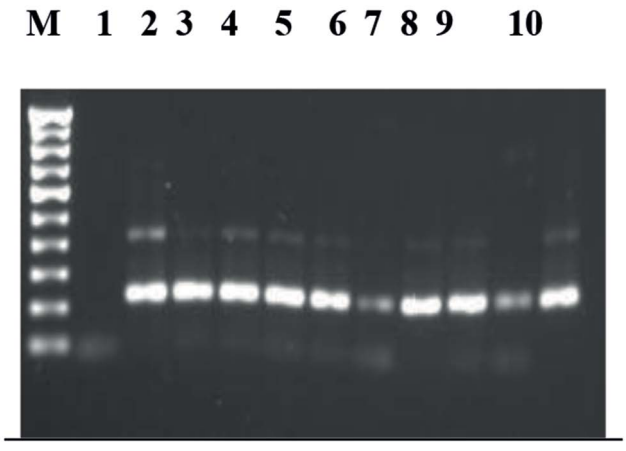
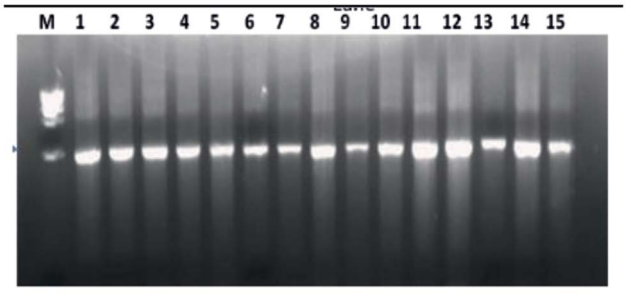
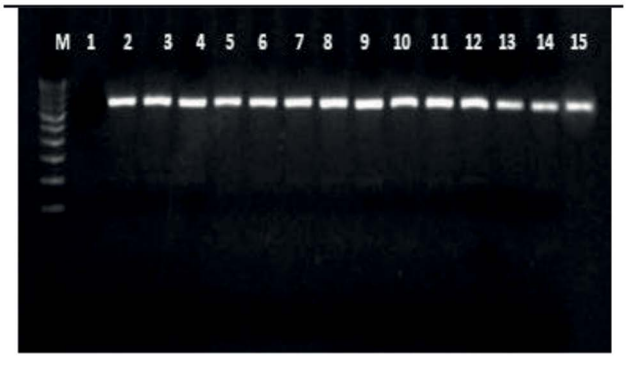
The *cry* gene profiling of the 30 *Bt* isolates from North-east was done by amplification of specific *cry* genes using degenerate primers, PCR amplification and sequence analysis (Table 2). Here 16 types of *cry genes* were detected from the 30 isolates. Some of them were sequenced and submitted to GenBank. The amplified Cry protein genes included *Cry1Ac, Cry2A, Cry3A, Cry4A, Cry4B, Cry7A, Cry8A, Cry10A*, *Cry11A, Cry12A, Cry16A, Cry17A, Cry19A, Cry30Aa, Cry44Aa* and *Vip3A* (Table 2 and 3). All the isolates harboured genes that target pests of more than one insect order. For example ten strains namely TrBt10, TrBt17, AsBT21, AsBT20, AsBT24, TrBt8, TrBt10, AsBt16, TrBt18 and AsBt16 harboured *cry* genes that are Lepidoptera-Diptera-Coleoptera-active. Groups or combination Cry protein genes detected against insect orders were Lepidoptera; Coleoptera, Diptera; Lepidoptera-Diptera; Lepidoptera-Coleoptera; Coleoptera-Dipteraand Lepidoptera-Diptera-Coleoptera. Vip3Aprotein gene amplification was found in 17 of the 30 North-East isolates. *Cry1Ac* and *Cry2A* invariably occurred together in all the isolates (Table 2). Hence Lepidoptera-Diptera active cry genes were abundant. One unique isolate TrBt-18 which showed bipyramidal and spherical crystals harboured *Cry2A, Cry4A, Cry7A, Cry8A, Cry16A*, and Vip3A

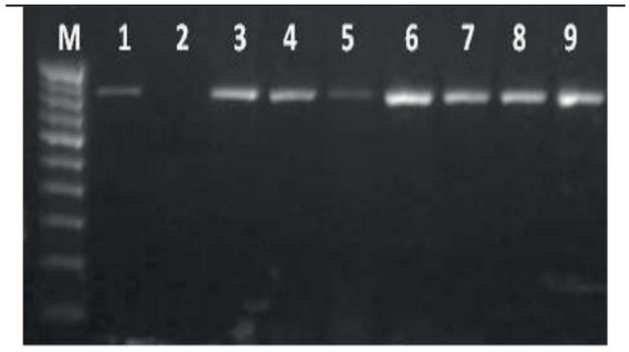
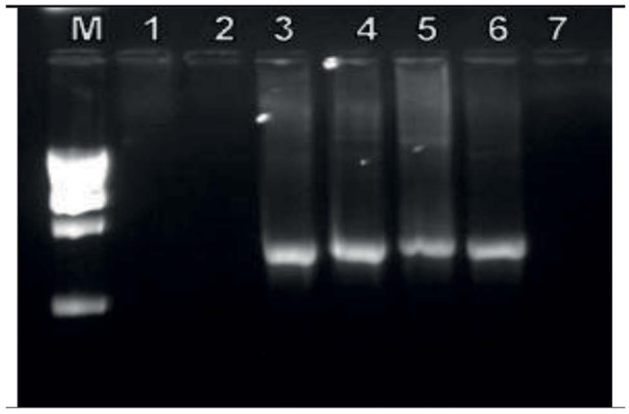
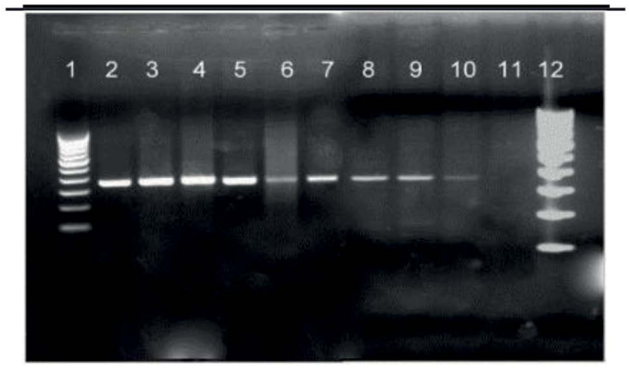
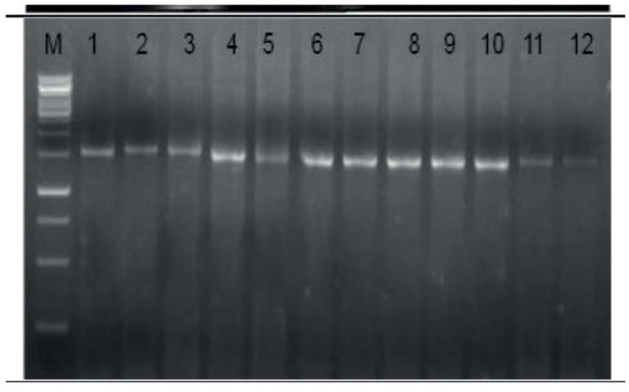
Table 2. *Cry* gene profiles of the *Bacillus thuringiensis* isolates from North East

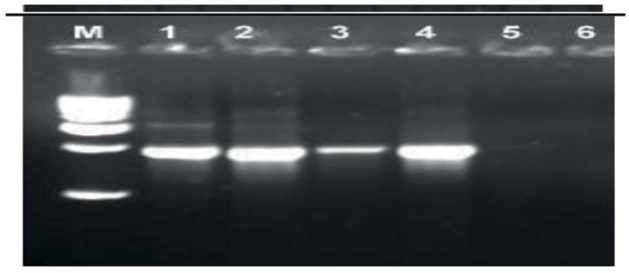
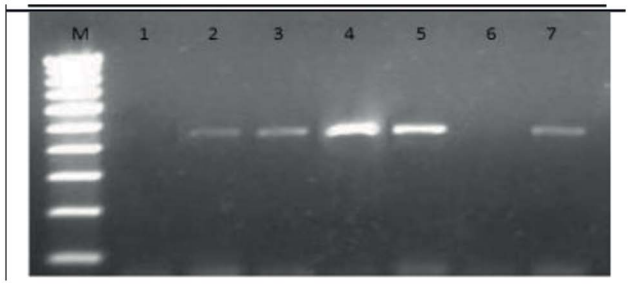
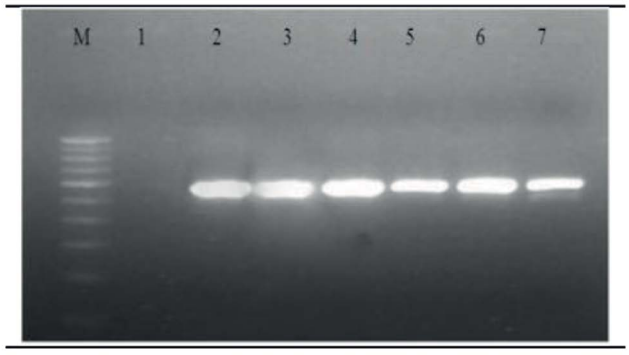
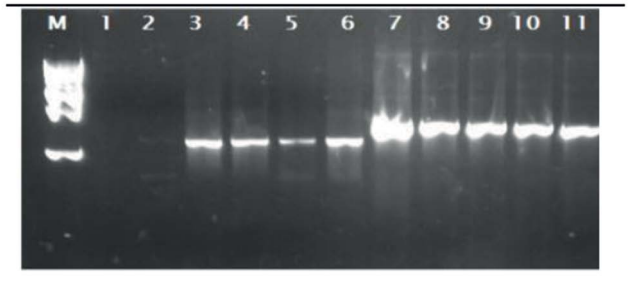
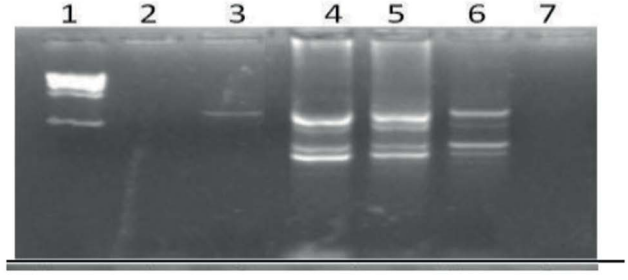
Strain Name	Crystal Morphology	Cry gene profile	Isolation source	GPS data	Accession No	Predicted insecticidal activity
AgBt-1	Bipyramidal+ cuboidal	*Cry1Ac, Cry2A, Cry10A, Cry16A, Cry17A, Cry19A, Cry30Aa, Cry44Aa* and Vip3A	Adult larvae	23°51'N91°16'E		Lepidoptera+Diptera
AgBt-2	Bipyramidal + spherical	*Cry 1Ac*	Pupae	23°51'N91°16'E		Lepidoptera
AgBt-3	Bipyramidal	*Cry1Ac, Cry16A, Cry17A, Cry19A, Cry 30Aa, Cry44Aa* and Vip3A	Leaf	23°51'N91°16'E		Lepidoptera + Diptera
AgBt-4	Bipyramidal + Irregular	*Cry1Ac, Cry11A* and *vip*3A	Dead larvae	23°51'N91°16'E	KC596019	Lepidoptera + Diptera
AgBt-5	Bipyramidal	*Cry1Ac, Cry2A, Cry4A, Cry10A, Cry11A, Cry16A, Cry19A* and Vip3A	Dead larvae	23°51'N91°16'E		Lepidoptera + Diptera
AgBt-6	Bipyramidal + irregular	*Cry1Ac, Cry2A, Cry4A, Cry10A, Cry11A, Cry16A, Cry19A, Cry44Aa* and Vip3A	Dead larvae	24°32'N 92°21'E	KC596018	Lepidoptera + Diptera
AgBt-7	Bipyramidal crystal	*Cry1Ac, Cry16A, Cry19A*	Leaf	24°32'N 92°21'E		Lepidoptera + Diptera
TrBt-8	Bipyramidal crystal	*Cry4A, Cry4B, Cry10A, and Cry11A, Cry 30Aa* and C*ry 44Aa*	Leaf	24°32'N 92°21'E		Lepidoptera + Diptera
TrBt-9	Bipyramidal crystal	*Cry4B, Cry12A* and Vip3A	Leaf	24°32'N 92°21'E		Lepidoptera + Diptera
TrBt-10	Bipyramidal + spherical	*Cry2A,Cry3A, Cry8A, Cry11A* and Vip3A	Dead larvae	24°32'N 92°21'E	KC416619 KC596017	Coleoptera
AsBt-11	Bipyramidal + irregular	*Cry1Ac, Cry16A*, Vip3A	Leaf	24°32'N 92°21'E		Lepidoptera + Diptera
AsBt-12	Bipyramidal crystal	*Cry1Ac,Cry4B, Cry11A, Cry16A* and Vip3A	Leaf	24°32'N 92°21'E	KC596015	Lepidoptera+ Diptera
AsBt-13	Bipyramidal + spherical	*Cry1Ac* and Vip3A	Leaf	26°18'N91°16'E		Lepidoptera
AsBt-14	Bipyramidal + Rhomboidal+ Flat	No amplification	Leaf	26°18'N91°16'E		
AsBt-15	Bipyramidal	*Cry 2A, Cry12A*, and Vip3A	Leaf	25°42'N 88°24'E	KC596008	Lepidoptera + Diptera
AsBt-16	Bipyramidal + Rhomboidal	*Cry2A,Cry4A,Cry7A,Cry8A, Cry10A, Cry12A, Cry19A* and Vip3A	Dead larvae	26°18'N91°16'E		Lepidoptera + Diptera
TrBt-17	Bipyramidal	*Cry2A, Cry3A, Cry7A, Cry8A, Cry10A, Cry12A, Cry16A* and Vip3A	Soil	23°51'N91°16'E	KC416620	Coleoptera
TrBt-18	Bipyramidal + spherical + cuboidal	*Cry2A, Cry 4A,Cry7A, Cry8A ,Cry 16A*, and Vip3A	Leaf	23°51'N91°16'E		Lepidoptera + Diptera+ Coleoptera
TrBt-19	Bipyramidal	*Cry 4A* and Vip3A	Leaf	23°51'N91°16'E	KC596007	Lepidoptera + Diptera
AsBt-20	Sphaerical + cuboidal	*Cry2A, Cry3A, Cry7A, Cry8A, Cry10A, Cry12A, Cry16A* and Vip3A	Leaf	26°18'N91°16'E	KC416622	Coleoptera
AsBt-21	Sphaerical + cuboidal	*Cry2A, Cry3A, Cry7A, Cry 8A, Cry0A, Cry12A* and Vip3A	Leaf	26°18'N91°16'E	KC416621	Coleoptera

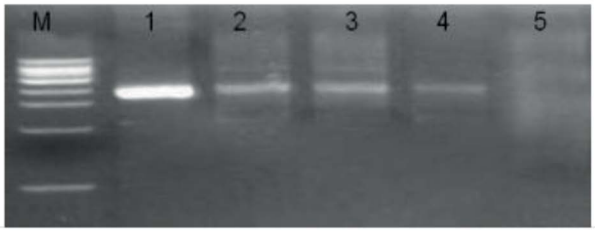
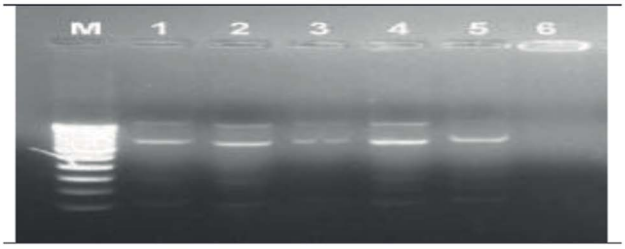
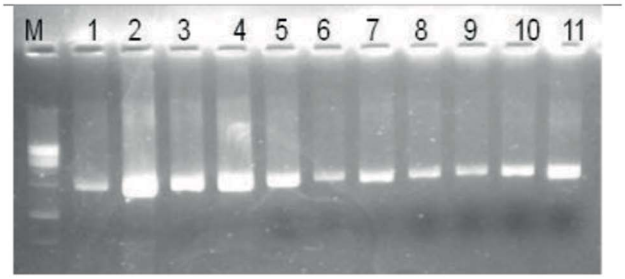
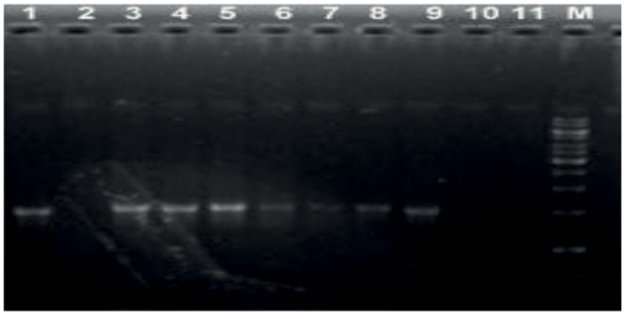
AsBt-22	Bipyramidal	*Cry16A*	Leaf	26°18'N91°76' E		Diptera
AsBt-23	Bipyramidal	*Cry16A*	Soil	22°70'N78°20' E		Diptera
AsBt-24	Spherical + cuboidal	*Cry3A, Cry8A* and *Cry12A*	Soil	24°30'N91°73'E	KC416623	Diptera+ Coleoptera
AsBt-25	Bipyramidal +Rhomboidal	*Cry2A, Cry 4A, Cry16A* and Vip3A	Soil	24°30'N91°73'E	KC596011	Lepidoptera + Diptera
AsBt-26	Bipyramidal	No amplification	Soil	24°30'N91°73'E		
AsBt-27	Bipyramidal	No amplification	Soil	24°30'N91°73'E		
AsBt-28	Bipyramidal	No amplification	Soil	24°30'N91°73'E		
Bt-Assam	Bipyramidal	*Cry 10A* and *Cry17A*	Soil	26°18'N91°16'E		Diptera
NE-60	Bipyramidal	*Cry 4A, Cry30Aa* and Vip3A	Soil	26°18'N91°16'E	KC596010	Lepidoptera + Diptera

Table 3. PCR analysis of *cry* genes occurring in the *Bacillus thuringiensis* isolates from North east

Sl. No.	PCR analysis	*Cry* gene profile
1		*Cry1Ac*, 238bp. M- 1kb Marker, 1- Blank, 2-4HD1 (reference), 3-AgBt1, 4-AgBt2, 5-AgBt3, 6- AgBt4, 7-AgBt5, 8-AgBt6, 9-AgBt7, 10-TRBt9.
2		*Cry2A* gene (1170bp). M-1kb marker, 1- 4HD1 (reference), 2- AgBt1, 3-AgBt5, 4- AgBt6, 5 -TrBt8, 6-TrBt10, 7- AsBt16, 8- TrBt17, 9-AsBt20, 10-AsBt21, 11- AsBt25, 12-AsBt15, 13-TrBt18, 14-BtAN4, 15- BtAN5.
3		Vip3A gene (675bp), M-100bp marker,1-Blank, 2- 4HD1 (reference), 3- AgBt1, 4-AgBt3, 5- AgBt4, 6- AgBt5, 7- AgBt6, 8- TrBt8, 9- TrBt9, 10- AsBt11, 11- AsBt12, 12- AsBt13, 13- AsBt15, 14- AsBt16, 15- AsBt21.

4		*Cry3A* gene 951 bp. M-100bp Marker, 1-4AA1 (reference), 2- Blank, 3- BTAN4, 4-BTAN5, 5- TrBt10, 6-TrBt17, 7- AsBT21, 8-AsBT20, 9- AsBT24.
5		*Cry7A* gene 1320 bp. M-1KB marker 1- Blank, 2-TrBt18, 3- AsBt16, 4-AsBt20, 5- TrBt17, 6-TrBt18, 7-AsBT24,
6		*Cry8A* gene 342 bp. 1- 100 bp marker. 2- HD867 (reference), 3- TRBt10, 4-AsBt16, 5- Asbt20, 6- AsBt21, 7- AsBt24, 8- AsBT21, 9- AsBt16, 10-AsBT20, 11- Blank and 12- M.
7		*Cry4A* gene 1529 bp M-1Kb marker, 1-4Q1 (reference), 2-TrBt8 , 3-TrBt9, 4-TrBt-11, 5- AsBt-12, 6- AsBt16, 7 –TrBt-18, 8-TrBt-19, 9- AsBT25, 10-NE-60, 11-AgBt5, 12-AgBt6.

8		*Cry4B* gene (1925bp). M-1kb marker, 1- 4Q1 (reference), 2- TrBt8, 3-TrBt9, 5- AsBt11, 5-AsBt12
9		*cry10A* gene 615 bp M- 100 bp marker, 1-Blank, 2-4Q1 (reference), 3-AsBt -16, 4- AsBt 19, 5- BtAssam, 6- AgBt5
10		*Cry11A* gene 445 bp M- 100 bp marker, 1- Blank, 2-Bt. 4Q1 (reference), 3-AgBt5, 4-AgBt6, 5-AgBt6, 6- TrBt8, 7-TrBt 10.
11		*Cry16A* gene 1415 bp product. M- 1kb marker, 1, Blank, 2- 4Q1 (reference), 3- AsBt1, 4- AsBt3, 5- AgBt5, 6-AgBt6, 7- AgBt7, 8- AsBt11, 9- AsBt12, 10-AsBt22, 11-AsBt23
12		*Cry17A* gene1400 bp. M- 1Kb marker, 2- Blank, 3- 4Q1 (reference), 4- AgBt1, 5- AgBt3 and 6- Bt Assam, 7- Empty

13		*Cry30Aa* gene. M- 1000bb marker 1- 4Q1 (reference), 2- AgBt1, 3-Agbt3 and 4- TrBt8 and 5-NE-60.
14		*Cry44Aa* gene. M- 1000bp marker 1- 4Q1 (reference), 2- AsBt1, 3- AsBt3, 4- Asbt6 and 5- TrBt8 and 6-Empty
15		*Cry12A* gene 363 bp M- 100 bp marker, 1-4D1(reference), 2-Trbt17, 3-EG1, 4-AgBt20 , 5- AgBt15, 6- AsBt24, 7-TrBt9, 8-Asbt21, 9- BtAN4, 10-BtAN5,11-TrBt16
16		*Cry19A* gene 355 bp 1- 4Q1 (reference), 2- Blank, 3- AsBt1, 4- AsBt7, 5-AsBt6, 6- AsBt3, 8-AsBt5,9-BtAN4, 10-BtAN5,11-TrBt16, M - 1000bp marker.

which could be active against Lepidoptera/Diptera/Coleoptera group of insects. The relative abundance of the cry genes in Northeast was analysed (Fig. 2). Vip3A was encountered in 16% of the samples, *Cry16A* was detected in 11% samples, 10% of specimens showed *Cry1Ac* and *Cry2A, Cry10A* were 10%, *Cry12A* and *Cry30A* was 6%, *Cry11A*, *Cry12A, Cry8A* and *Cry7A* was 4-5% and the other Cry protein genes ranged from 2-3%. The cry toxins that are primarily active against lepidopteran insects belong to the *Cry1, Cry2* and Vip3A groups. *Cry3, Cry7*, and *Cry8* toxins are active against coleopteran insects. The *Cry2A, Cry4A, Cry 4B Cry10A, Cry11A, Cry12A, Cry16A, Cry17A, Cry19A, Cry30Aa*, and *Cry44Aa* proteins are act against insects coming under order Diptera. *Cry* genes were amplified using specific primers and also reference strains. *Cry* gene profiling using specific or degenerate primers have been previously described (Ben Dov *et al*., 1997; 2001, Bravo *et al*., 1998, Ejiofor, 2002; Aly, 2007). *Cry* gene profile based on Lepidoptera encode for insect toxins namely cry1, cry2 and cry 9A whereas cry7 and cry8 encode for Coleopteran toxic proteins (Porcar and Perez, 2003, Ito et al., 2006). Konecka *et al*. (2012) analyzed the *cry* gene profile of 8 isolates and that they expressed diverse crystals and harboured cry genes active against pests of Coleoptera, Diptera and Lepidoptera. They could detect multiple cry genes like *Cry1Aa, Cry1Ab, Cry1Ac, Cry1B*,

Cry1C, Cry1D, Cry1I, Cry2Aa, Cry2Ab, Cry9B, Cry9E, and *Cry15. Cry16A* and *Cry17A* were first amplified in certain species of *Clostridium* (Barloy *et al.,* 1996) and the gene products, *Cry16A* and *Cry17A*, showed a remarkable

Table 4. Bioassay of *Bacillus thuringiensis* samples expressing *cry3a* protein against *Sitophilus oryzae*

Isolate	LC50 value (µg/ml)	95% confidence limits		Std. Error
		Lower	Upper	
4AA1	27.129	15.736	35.792	0.51
AgBT 6	8.049	0.425	17.725	0.49
TrBT 17	13.893	1.894	23.624	0.54
AsBT 20	21.826	8.699	31.286	0.51
TrBT 10	19.122	1.842	31.638	0.48
AsBT 24	36.381	11.619	54.773	0.46

Table 5. Bioassay of *Bacillus thuringiensis* samples expressing *cry3a* protein against *Callosobrochus chinensis*

Isolate	LC50 value (µg/ml)	95% confidence limits		Std. Error
		Lower	Upper	
4AA1 (Standard)	15.963	4.579	24.487	0.56
AgBT 6	8.371	0.042	18.403	0.57
TrBT 17	40.928	21.738	58.298	0.47
AsBT 20	8.431	0.000	21.026	0.55
TrBT 10	15.898	4.219	24.795	0.54
AsBT 24	13.312	2.107	22.366	0.57

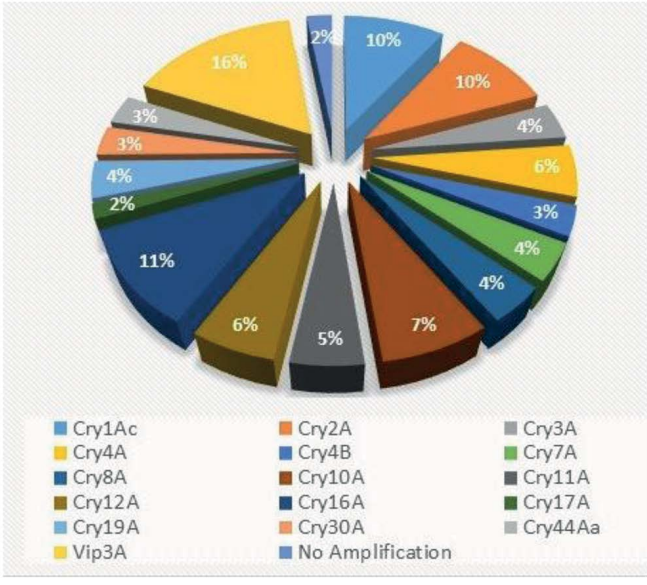


Fig. 1. Diversity of *cry* genes occurring in the North East.

Fig. 2. Crystal morphology imaged through Transmission Electron Microscope (TEM).

mosquitocidal activity, and are the first reported cases of secreted or excreted mosquitocidal toxins derived from an anaerobic bacterium. In our studies we could amplify *Cry17A* in 3 isolates namely Ag-Bt1, Ag-Bt3 and Bt-Assam (Fig. 13) surprisingly *Cry16A* was detected in 10 of the isolates (Fig. 12 and Table 3) based on size 1415 kb and 1400 kb gel picture. Nucleotide sequence BLAST analysis showed 99% similarity with the available database at NCBI. However further work on their cloning, sequence and bioassay needs to be studied. Ito *et al*. (2006), first reported the cloning and expression of two novel crystal protein genes, *Cry44Aa/orf2-44A* and *Cry30Ba/orf2-30B*, from highly mosquitocidal *B. thuringiensis* subsp. *entomocidus* INA288. The *Cry44Aa/orf2-44A* gene is highly toxic to *C. pipiens* and *A. aegypti* and appears to be a promising alternative to *B. thuringiensis* subsp. *israelensis* or may be used in combination with *B. thuringiensis* subsp. *israelensis* toxins. In the present study we detected these two rare *cry* genes in some of the isolates that showed presence of dipteran toxic cry proteins. Wild strains isolated from environmental samples can synthesize crystals that display higher activity against insect pests in comparison to *B. thuringiensis* strains already used in pesticide production (Konecka *et al.,* 2012). The knowledge on coding for genes toxins in crystalline inclusion is useful in predicting potential pathogenicity of *B. thuringiensis* isolates against insects (Baig and Mehnaz, 2010; Nazarian *et al.,* 2009).

Since some of the isolated *Bt* had Coleoptera specific *cry* genes, they were evaluated against *Callosobrochus chinensis* and *S. oryzae*. The results showed that the Agartala isolate NBAIR-AgBt6 was most toxic recording LC_{50} of 8.049 µg/ml against *S. oryza*e and 8.371 µg/ml against *C. chinensis* (Table 4 and 5). The isolate could be safer alternative to chemicals especially for stored grains. Stored grain pests cause heavy loss to granaries all over the world and accounts for 10 to 40 per cent loss (Chaubey, 2011). The pests mostly belong to the order Coleoptera. In India the main stored grain pests include *C. chinensis, Sitophilus* spp. and *Tribolium* spp. Strain HD73 was found to be the most active strain against *C. chinensis* that could be due to the variability of delta-endotoxins of different subspecies of these bacilli (Federiei, 1990). Asokan *et al*. (2013) obtained 36 new *Bt* isolates from Andaman and Nicobar islands and found variations in crystal morphology

and mass of crystal protein(s). Based on the toxicity test, 50 % of isolates were toxic to Ash weevils (coleopteran pest) and PCR analysis unveiled prepotency of *cry1B* and *cry8b* like genes in these isolates.

The results showed that dipteran and lepidopteran specific genes were predominant. In all the studies reference strains *B. thuringiensis* subsp. *kurstaki* (HD-1), *B. thuringiensis* subsp. *israelensis*, (4Q1), *B. thuringiensis* subsp. *tenebrionis* and *B. thuringiensis* subsp. *japonensis* were used for PCR standardization. Presence of *Cry16A* and *Cry17A* is a first report for *B. thuringiensis* isolates from soils of North East.

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