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Screening and Characterization of Dipteran Specific *cry* Genes from Native *Bacillus thuringiensis* Isolates of Western Ghats, India

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Authors' contributions

This work was carried out in collaboration between both authors. Author DG was responsible for study design, supervision of work and manuscript correction. Author MS was responsible for literature searches, laboratory work, data analysis and manuscript preparation. Both authors read and approved the final manuscript.

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ABSTRACT

Many studies have been done to control lepidopteran by the biological insecticide *Bacillus thuringiensis* crystal protein (Bt protein). But in the current study, we concentrated more on identification and characterization of *cry* genes specific to dipteran insects from twenty native *Bacillus thuringiensis* strains isolated from Western Ghats of Kerala, India. Genomic DNA of the twenty native isolates were subjected to PCR using specific primers for *cry*2, *cry*4, *cry*10, *cry*11, *cry*16, *cry*17, *cry*19 and *cry*21 genes to find out the *cry* genes content. Profiling of *cry* genes showed that *cry*4 was most predominant by 69 per cent in eleven isolates (Bt-23, Bt-133, Bt-242, Bt-245, Bt-250, Bt-326, Bt-411, Bt-420, Bt-447 and reference strain 4Q1). This was followed by *cry*11, which was present in 13 per cent and *cry*10 in 12 per cent. Combinations of c*ry*4 and *cry*11 genes were

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present in Bt-23 and Bt-242 while *cry*21 was present only in isolate Bt-245. None of the isolates carried *cry*2, *cry*16, *cry*17 and *cry*19 genes. Partial *cry*4 gene was cloned around 800 bp from Bt-23 and sequenced from Bt-133, Bt-190 and Bt-242 showing homology with *cry*4 and *cry*4A genes in the NCBI database.

Keywords: Native B. thuringiensis isolates; dipteran specific; primers; cry genes profiling; polymerase chain reaction and cloning.

1. INTRODUCTION

Many insects belong to the Lepidoptera, Diptera and Coleoptera orders cause serious problems to important crops and it's productivity of a variety of important crops. Chemical control based on organochlorated compounds and others have shown little efficiency due to several factors, the rising of resistant organisms; environmental contamination and international restriction of use [1,2,3]. Due to insect resistance, environmental contamination problems and other events, it become a general issue to look for alternative ways of control, such as the use of bioinsecticides. Which act based on specific control mechanisms of the major crop pests, without causing negative impacts similar to those generated by the use of chemical insecticides. Microbial pesticides are becoming an important in crop protection and in insect vector control. These pesticides are natural, disease-causing microorganisms such as viruses, bacteria, fungi, protozoans and nematodes that infect or intoxicate specific pest groups [4,5].

Bacillus thuringiensis (Bt) has been used as a successful microbial insecticide for more than 50-60 years and is a uniquely specific, safe and effective tool for the control of a wide variety of insect pests. Bt is a well known gram positive, spore forming soil bacterium that forms parasporal insecticidal crystal proteins during the stationary phase of its growth cycle. The crystals contain one or more Cry proteins (δ-endotoxins) that are specifically toxic to insect orders such as Lepidoptera, Diptera and Coleoptera and also to some nematodes, mites and protozoa [6,7]. Crystal proteins belonging to Cry1, Cry9, and Cry2 groups are toxic to lepidopteran insects and while Cry3, Cry7, and Cry8 are active against coleopteran insects. Cry proteins toxic to nematodes are Cry5, Cry12, Cry13, and Cry14 whereas Cry2, Cry4, Cry10, Cry11, Cry16, Cry17 and Cry19 are toxic to dipteran insects [8].

Diptera probably have a greater economic impact on both crops and human beings than any other group of insects. There are several pests of economic importance belonging to Diptera, attacking important crops like vegetables, fruits, plantation crops and spices, which some others act as vectors and transmit dreadful diseases to humans and domestic animals. Fruit flies, gall midges and leaf miners etc., are some of the economically important crop pests that belong to order Diptera. Based on the above information the present study mainly concentrated on cloning and characterization of *cry* genes specific to dipteran insect from native isolates of *Bacillus thuringiensis*.

2. MATERIALS AND METHODS

2.1 Bacterial Strains, Growth and Maintenance

Twenty native *Bacillus thuringiensis* isolates were obtained from various locations of Western Ghats soil in Kerala, India. Strain 4Q1 (HD 567) *Bacillus thuringiensis* subsp. *israelensis* obtained from University of Agricultural Sciences, Dharwad, Karnataka was used as a reference strain. All of the Bt strains were maintained at the Laboratory by sub-culturing periodically in Petri plates containing LB Media. After inoculation the Petriplates were incubated at 29℃ for 48 h in BOD incubator and later stored at 4℃.

2.2 Sample Preparation and Total DNA Extraction

One ml of *Bacillus thuringiensis* suspension was inoculated in 25 ml Luria Bertani broth (LB) and incubated overnight at 30℃ with vigorous shaking. The total DNA was isolated from overnight incubated *Bacillus thuringiensis* isolates following the protocol of Sambrook and Russel [9].

2.3 PCR Primers

Native *Bacillus thuringiensis* strains were characterized in terms of dipteran specific *cry* genes *i.e., cry*2, *cry*4, *cry*10, *cry*11, *cry*16, *cry*17, *cry*19 and *cry*21 presence. Eight primers already reported by various scientists were used in this

study. The *cry*2 and *cry*4 primers used were designed by Ben-Dov et al. [10]. Primers used to screen *cry*10, *cry*19 and *cry*21 were described by Ejiofar and Johnson [11]. The primer designed by Bravo et al. [12] used to detect *cry*11gene and the primers used by Barloy et al. [13] were selected for screening of *cry*16 and *cry*17 genes. The details of primers used are given in Table 2.

2.4 PCR Amplification

Polymerase Chain Reaction (PCR) was carried out using *cry* gene specific primers in Eppendorf Master Cycler, Gradient (Eppendorf, Germany) for 30 cycles. Total volume of 25 µl containing 50 ng of template DNA mixed with 10X Taq assay buffer, 10 mM deoxynucleoside triphosphate, 10 pmol forward and reverse primer (each) and 0.3 U of Taq DNA polymerase (Genei). Template DNA was allowed to initial denaturation for 2 min at 94℃ at once, following this denaturation for 45 sec, annealing of primers for 1 min at annealing temperature of respective primers. The extension of PCR products were achieved at 72℃ for 1 min, these steps were repeated for 30 times. An extra step of final extension at 72℃ for 10 min was also carried out once. PCR products were separated using agarose gel electrophoresis in 1% TAE buffer and stained with 0.5 µg/ml ethidium bromide according to Sambrook et al. [14]. PCR products were visualized under UV transilluminator and the sizes of the fragments were estimated based on a DNA ladder of 100 base pairs and λDNA/*Eco*R1+ *Hind*III double digest (Bangalore Genei) molecular weight marker.

Table 1. Details of primers used for amplification of dipteran specific *cry* genes

Primer	Primer sequence	Length (bp)	Annealing temperature (℃)	Expected amplicon size (bp)	Reference
*cry*2	F.P. 5'GTTATTCTTAATGCAGATGAATGGG 3'	25	61	689-701	[10].
	R.P. 5'CGGATAAAATAATCTGGGAAATAGT 3'	25			
*cry*4	F.P. 5' GCATATGATGTAGCGAAACAAGCC 3'	24	53	439, 800	[10].
	R.P. 5' GCGTGACATACCCATTTCCAGGTCC 3'	25			
*cry*10	F.P. 5' ATATGAAATATTCAATGCTC 3'	20	45	404	[11].
	R.P. 5'ATAAATTCAAGTGCCAAGTA 3'	20			
*cry*11	F.P. 5' TTAGAAGATACGCCAGATCAAGC 3'	23	55	305	[12].
	R.P. 5' CATTTGTACTTGAAGTTGTAATCCC 3'	25			
*cry*16	F.P. 5' TCAAAAGGTGTGGCAAG 3'	17	42	1415	[13].
	R.P. 5' ATAAGCCCAATATCATG 3'	17			
*cry*17	F.P. 5' CTGAGGTATTTTGTGGA 3'	17	42	1400	[13].
	R.P. 5' AAGTAAAGATTTCTGGG 3'	17			
*cry*19	F.P. 5' AGGGGAGTCCAGGTTATGAGTTAC 3'	24	55	355	[11].
	R.P. 5' ATTTCCCTAGTTAGTTCGGTTTTT 3'	24			
*cry*21	F.P. 5' ATACAGGGATAGGATTTCAAG 3'	21	53	453	[11].
	R.P. 5' ATCCCCATTTTCTATAAGTGTCT 3'	23			

Table 2. Details of amplicons obtained in *cry* gene profiling

Sl. no.	*cry* gene	Isolates	Expected amplicon size (bp)	Obtained amplicon size (bp)
1	*cry*2	-	689-701	-
2	*cry*4	Bt-23, Bt-133, Bt-190, Bt-242, Bt-245, Bt-250, Bt-277, Bt-326, Bt-411, Bt-420, Bt-447 and 4Q1	439	400, 800
3	*cry*10	Bt-419 and Bt-447	404	400
4	*cry*11	Bt-23, Bt-420 and 4Q1	305	300
5	*cry*16	-	1415	-
6	*cry*17	-	1400	-
7	*cry*19	-	355	-
8	*cry*21	Bt-245	453	450

2.5 Cloning, Sequencing and Database Analysis of PCR Fragments

Competent cells for plasmid transformation were prepared using Genei Competent cell preparation Kit (B) from Bangalore Genei, India following the manufacturer's guideline. *cry*4 gene amplicon size of 800 bp from Bt-23 alone eluted and ligated in pGEMT vector using pGEMT Easy Vector System (Promega Corporation, USA), following the manufacturers protocol. It was used for transformation in competent cells of *Escherichia coli* JM 109 by standard protocols [9]. Cloned product of 800 bp from Bt-23 and eluted product of 400 bp from native isolates Bt-133, Bt-190 and Bt-242 were sequenced.

Recombinant clone with 800 bp insert from the isolates Bt-23 was sequenced at Bangalore Genei using T7 primer to obtain 5'- 3' sequence information of the insert from the forward region, using automated sequencer (ABI–31100 Genetic Analyzer, USA). The eluted products of 400 bp in the isolates Bt-133, Bt-190 and Bt-242 were directly sequenced using the corresponding forward and reverse primers at Chromous Biotech Pvt. Ltd. Blastn was used for DNA sequence analysis to find out the homology of the sequence in the database.

3. RESULTS

3.1 Screening of Dipteran Specific *cry* Genes

The *cry* gene profiling is a method used to find out the genes present in *Bacillus thuringiensis*. All of the twenty native isolates were subjected to PCR using specific primers for *cry*2, *cry*4, *cry*11, *cry*16, *cry*17, *cry*19 and *cry*21 genes to find out the *cry* gene content. *cry* gene profiling revealed *cry*4 gene was dominant in the native Bt isolates. Expected amplicon size for *cry*4 gene was 439 bp. However, two amplicons of 400 bp and 800 bp size were obtained in the isolates Bt-23, Bt-133, Bt-242, Bt-245, Bt-250, Bt-326, Bt-411, Bt-420, Bt-447 and reference strain 4Q1. The isolates Bt-190 and Bt-277 yielded a single amplicon of 400 bp. Two isolates Bt-419 and Bt-447 yielded amplicon of the expected size of 404 bp for *cry*10 gene. *cry*11 gene present in the isolates Bt-23, Bt-420 and standard 4Q1 produced 305 bp amplicon (Fig. 1). *cry*21 gene present in Bt 245, out of twenty isolates this isolates only produced 453 bp amplicons for *cry*21 primers. None of the isolates yielded the expected size amplicons *cry*2, *cry*16, *cry*17 and *cry*19 primers. Details of *cry* genes presence in the native isolates are given in Table 2.

3.2 Cloning and Sequencing of PCR Products

Among native isolates Bt-23 was superior to any other isolates. Amplification of *cry*4 was carried out by PCR with isolate Bt-23, Bt-133, Bt-190 and Bt-242. Two amplicons of 400 and 800 bp were obtained in Bt-23 and remaining three isolates produced 400 bp amplicons only; from Bt-23 only 800 bp amplicon was eluted from the gel. The eluted product from Bt-23 was ligated in pGEMT vector and transferred into competent *E. coli* JM109 cells using the heat shock method. When the transformed *E. coli* cells were grown in LBA ampicillin plates overlaid with X-gal and IPTG, a combination of blue and white colonies were obtained after overnight incubation confirming successful transformation. Colony PCR with plasmids from three white colonies yielded an amplicon of 800 bp, which indicated the presence of insert DNA (Fig. 2.). Sequencing result of Cloned product of 800 bp from Bt-23 and eluted product of 400 bp amplicons from native isolates Bt-133, Bt-190 and Bt-242 were revealed Bt-23*cry*4 sequence contained 780 nucleotides and 243 amino acid residues, Bt-133*cry*4 made of 356 nucleotides and 111 amino acid residues. Bt-190*cry*4 and Bt-242*cry*4 were composed of 364 and 362 with same number of amino acid (116) residues (Table 3).

3.3 Nucleotide Sequence Analysis

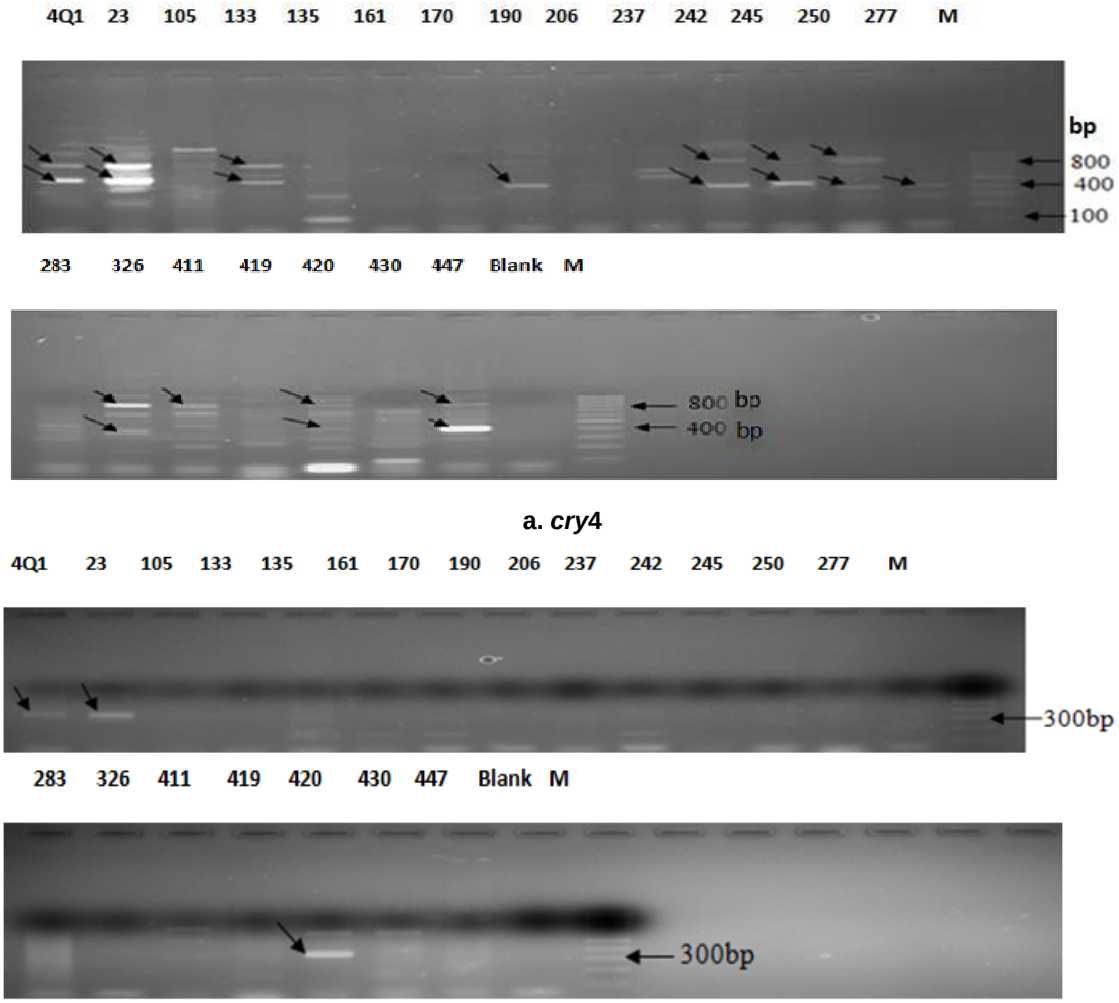
Homology search of nucleotide sequences obtained from isolates Bt-23, Bt-133, Bt-190, and Bt-242 with other reported *cry* gene sequences was carried out in NCBI database. All the four sequences (Bt-23*cry*4, Bt-133*cry*4, Bt-190*cry*4 and Bt-242*cry*4 showed homology with *cry*4A, *cry*4D, *cry*4BLB and other dipteran specific insecticidal genes, truncated pesticidal crystal protein genes and *Bacillus thuringiensis* subsp. *israelensis* 130 kDa pesticidal *cry* gene sequences in NCBI databank.

4. DISCUSSION

4.1 Profiling of Dipteran Specific *cry* Genes

B. thuringiensis has worldwide distribution and individual strains produce potent protein toxins, each of which is specific to a small group of target insects with no effect on higher animals or

on the environment [15]. The need for novel crystal proteins has prompted the development of molecular approaches to quickly and easily characterize toxin genes present in *B. thuringiensis* isolates. PCR is a useful technique for quick and simultaneous screening of Bt strains for classification and prediction of insecticidal activities. In the last few years, several PCR-based methodologies mostly multiplex PCR, which allowed the accurate determination of families of *cry* genes or specific δ-endotoxin genes have been proposed. PCR to detect the *cry* gene content is considered to be the best choice for screening novel organisms for already reported genes because it permits a rapid determination of the presence or absence of a sequence. PCR-based techniques have been used to identify different *cry* genes in *B. thuringiensis* strains [16]. Nemappa et al. [17] used PCR-based techniques for screening *cry1, cry2, cry4, cry10* and *cry11* genes from new *B. thuringiensis* isolates. Carrozi et al. [18] introduced this technique to identify *cry* genes in order to predict the insecticidal activity of Bt isolates. PCR technique was used by Manikandan et al. [6,7] for screening of *cry1Ac* gene and *cry2A* gene from an indigenous isolate of *Bacillus thuringiensis.*

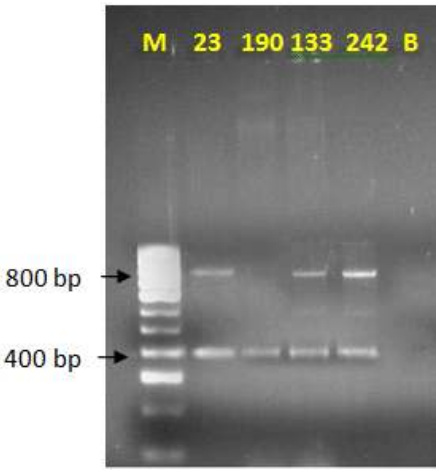


a. *cry*4

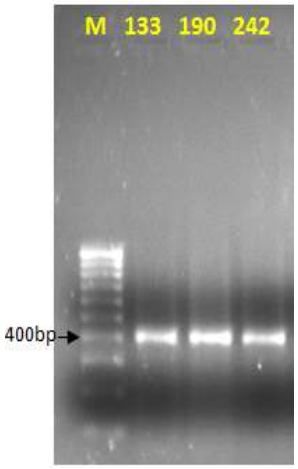
b. *cry*11

Arrows indicate amplicons of the expected size

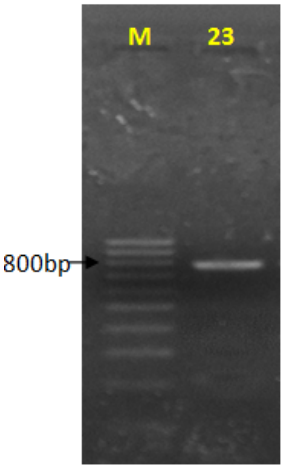
Fig. 1. Profiles of dipteran specific *cry* genes (*cry*4 and *cry*11) in native *B. thuringiensis* isolates



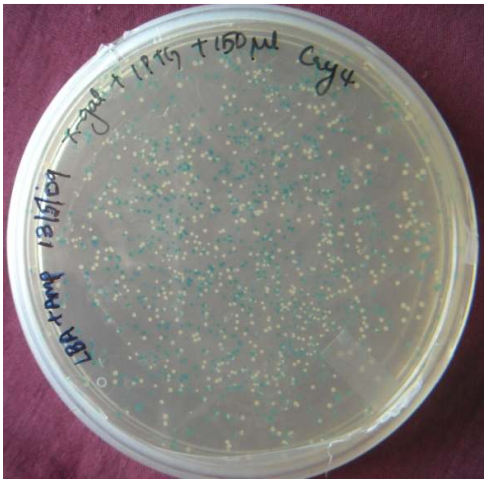
2a. *cry*4 gene amplicons for cloning



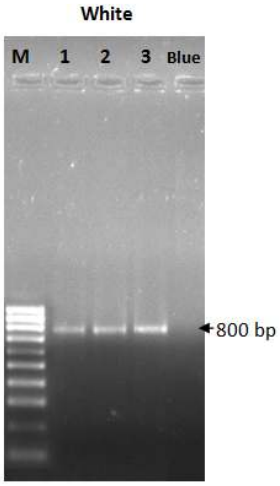
2b. Eluted bands of *cry*4 gene 400 bp fragment



2c. Eluted 800 bp *cry*4 gene fragment



2d. Blue white screening of recombinant *E. coli*



2e. Colony PCR product from blue and white colonies

Fig. 2. Cloning of *cry*4 gene fragment in *E. coli*

In the present study profiling of *cry* genes specific to dipterans in native *Bacillus thuringiensis* by polymerase chain reaction showed that *cry*4 was most predominant by 69 per cent present in eleven isolates. This was followed by *cry*11, which was present in 13 per cent, *cry*10 in 12 per cent and *cry*21 was present in 6 per cent (Fig. 3). None of the isolates yielded amplicon for *cry*2, *cry*16, *cry*17 and *cry*19 genes. The absence of *cry*2 in native isolates correlates with the absence of cuboidal crystal protein, since cuboidal crystals are reported to be encoded by this gene. Of the 20 isolates tested, four contained a combination of two dipteran-specific *cry* genes (Bt-23, Bt-245, Bt-420 and Bt-447). A combination of different genes with specificity to a particular group of insect is considered beneficial, as it may have an additive effect on the target insect. Eight isolates (Bt-133, Bt-190, Bt-242, Bt-250, Bt-277, Bt-326, Bt-411 and Bt-419) harboured only one dipteran specific *cry* gene, while eight other isolates (Bt-105, Bt-135, Bt-161, Bt-170, Bt-206, Bt-237, Bt-283 and Bt-430) lacked any dipteran-specific gene. This

might be due to the reason that they possess other *cry* genes which are not identified by the specific primer used. PCR analysis by Mahalakshmi et al. [19] revealed that 53% of the isolates were positive for the various *cry* and *cyt* genes tested, whereas 47% did not produce any PCR product for the *cry* gene analyzed.

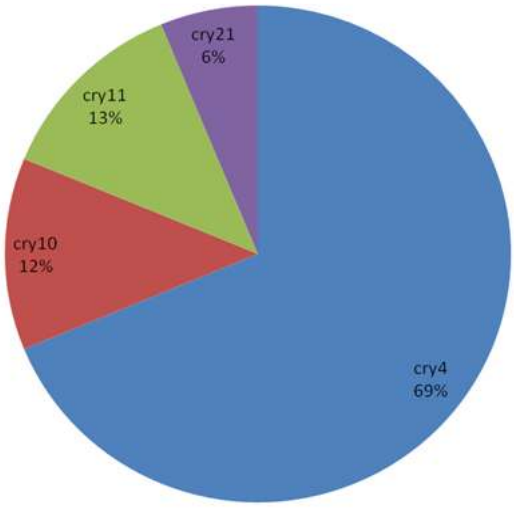


Fig. 3. Distribution of dipteran specific *cry* genes in native *Bacillus thuringiensis* isolates

Table 3. Details of sequenced amplicons from native *B. thuringiensis*

Sl. no.	Sequence name	Nucleotide	Amino acid
1.	Bt-23*cry*4	780	243
2.	Bt-133*cry*4	356	111
3.	Bt-190*cry*4	364	116
4.	Bt- 242*cry*4	362	116

Uribe et al. [20] isolated one hundred and eight different Bt strains from Colombia and carried out gene profiling for *cry*1, *cry*3, *cry*4, *cry*7 and *cry*8 with general and specific primers. Most of the Bt strains (73%) reacted with *cry*1 gene general primers, 27.8 per cent of the Bt isolates reacted with *cry*3, *cry*4, *cry*7 and *cry*8 gene general primers and 17.8 per cent did not react with these two set of primers. Bravo et al. [12] identified that the strains with PCR products of size other than those of predicted ones are candidates for harboring putative novel *cry* genes. Some of the isolates yielded two bands upon amplification by PCR, with *cry* gene primer. This may be owing to the fact that the strains may harbour one or more genes related to *cry* family. Of the twenty isolates under study, four isolates producing numerous crystal proteins and possessing *cry*4 gene were selected for bioassay. These were Bt-23, Bt-133, Bt-190 and Bt-242. Among these Bt-23 harboured two dipteran specific genes viz., *cry*4 and *cry*11.

4.2 Cloning and Sequencing of Partial *cry*4 Genes

PCR was carried out with four selected isolates found positive in *cry*4 gene profiling (Bt-23, Bt-133, Bt-190 and Bt-242), with *cry*4 forward and reverse primer. Two amplicons of size 400, 800 bp were obtained for isolates, Bt-23, Bt-133 and Bt-242. Isolate Bt-190 produced a single amplicon of size 400 bp. Total genomic DNA isolated from new *B. thuringiensis* isolates were subjected to screening of *cry* genes by PCR and predicted possible potential DNA amplicons were cloned and sequenced [17]. Eluted 800 bp DNA fragment of Bt-23 were ligated into pGEMT vector and transformed into *E. coli* JM 109 cells. Balasubramanian et al. [21] amplified 2 kb product through polymerase chain reaction and the amplicon was ligated into pGEMT vector. Beron and Salerno [22] amplified 2.5 kb of *cry*24 gene and it was ligated with pGEMT easy vector and cloned.

In the present study the cloning vector used was pGEMT, specially designed for direct cloning of PCR products. It has a 3' terminal thymidine at both ends and the presence of this at the insertion site greatly improves the efficiency of ligation of PCR product onto the plasmids, by preventing recircularization of the vector and provides a single stranded overhang for PCR products generated by certain thermo-stable DNA polymerases. It contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows the recombinant clones to be directly identified by colour screening of the indicator plate. DNA fragment 450 bp was amplified by *cry*10 gene specific primers from two isolates T109 and T136 were cloned in to T/A cloning vector by Nemappa et al. [17].

Ligated product containing *cry* gene sequence was used to transform the *E. coli* cells, which were later be picked up from the media containing 5-bromo 4-chloro 3-indolyl β-D galactosidase (X-gal) and isopropyl thiogalactoside (IPTG) based on blue white screening. The pGEMT vector contained

polycloning sites inside a β-galactosidase gene. The bacterial cell and vector together provided the complete protein as a result of α complementation [23]. The colonies which have not taken up the plasmid can further utilize the substrate and appear as blue colonies on X-gal chromogenic substrate [24]. All transformed colonies harbouring the recombinant plasmid appeared in white colour due to the disruption of α-complementation.

Presence of amplicons of 800 bp from plasmids obtained from white colonies confirmed the presence of the insert. The Analysis of the sequence data of the four amplified products by Blastn revealed that they were partial *cry*4 genes. Blastn is an algorithm that finds region of local similarity between nucleotide sequences. The homology search revealed that the cloned sequence shared significant homology with *cry*4A, *cry*4D, *cry*4BLB, and *cry* gene sequence of *Bacillus thuringiensis* subsp. *israelensis*. Hundred per cent identity was obtained with that of *cry*4 gene sequence from *B. thuringiensis*, *cry*4A gene sequences from *B. thuringiensis* serovar *israelensis*, delta endotoxin gene of *B. thuringiensis* and other insecticidal toxin *cry* genes reported in NCBI biological databases.

5. CONCLUSION

The *cry* gene profiling of native Bt isolates showed that *cry*4 gene was the most predominant one and isolates Bt-23, Bt-240 possessed two cry genes viz., *cry*4, *cry*11 and three genes (*cry*4, *cry*11and *cry*21) present in Bt-245 isolate. Normally isolates possessed multi-crystal protein genes are more promising then isolates containing single *cry* gene, because each *cry* gene having different mode of action so efficacy will be high. Resistance development of the insect against crystal protein will be delayed for the prolonged time when using isolates with more than one crystal protein gene. So these isolates (Bt-23, Bt-240) can be evaluated further against various dipteran pests, if perform well then these isolates could exploited as a bio-insecticide against dipteran insects since these isolates possess more than one cry genes will be a more suitable choice and therefore better alternative to the chemical insecticides.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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