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Cry gene profiling of PDKV *Bacillus thuringiensis* strains for specificity to *Pectinophora gossypiella* (Saunders)

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Abstract
Bacillus thuringiensis (*Bt*) is a cosmopolitan, antagonistic soil bacterium widely recognized as an eco-friendly alternative to chemical insecticides in pest management. In the present study, ten PDKV *Bt* strains-SGd-1, SY-4, SA-6, SAk-6, SGn-5, SAk-9, SBn-2, SA-18, SGn-4, and SA-20—known for their toxicity against pink bollworm (*Pectinophora gossypiella*), along with the standard *Btk* HD-1 and *Bacillus cereus* strain were screened for the presence of insecticidal *cry* genes using four gene-specific primers: *cry1*, *cry1Ac*, *cry2Ab*, and *cry9* specific to pink bollworm. PCR analysis revealed that the isolates harboured *cry1*, *cry1Ac*, and *cry2Ab* genes either individually or in combination, whereas the *cry9* gene was absent in all strains. Among the screened isolates, *cry1* was the most abundant, detected in 90.91% of the strains (HD-1, SA-6, SA-18, SA-20, SAk-6, SAk-9, SGd-1, SGn-4, SGn-5, SBn-2), followed by *cry1Ac* (54.55%) present in SA-6, SA-20, SAk-6, SAk-9, SGd-1, and SGn-5, and *cry2Ab* (45.45%) found in HD-1, SY-4, SAk-6, SGn-4, and SGn-5.

Keywords: *Bacillus thuringiensis*, *cry gene*, PCR, *Pectinophora gossypiella*

1. Introduction
India is predominantly an agriculture-based country. Despite the availability of advanced technologies in the agricultural sector, the yield and productivity of agricultural and horticultural crops are considerably influenced by both biotic and abiotic stresses. Among the biotic factors, insect pests are a major contributor to crop damage, accounting for an estimated 15.7% loss in yield (Dhaliwal *et al.*, 2015) [6]. Several microorganisms induce toxins that can be used to control the pathogenicity of a wide range of plant pathogens.

Bacillus thuringiensis (*Bt*) is widely regarded as the most effective insect-pathogenic bacterium used in pest control, representing about 2% of the overall insecticide market (Thilagarajan *et al.*, 2023) [12]. It is a Gram-positive, aerobic, spore-forming bacterium that inhabits the soil and is well known for producing insecticidal crystal proteins, termed δ-endotoxins (delta-endotoxins), during sporulation. The taxonomic classification of these toxins placed them in the delta class of insecticidal toxins due to their intracellular localization. They are considered the main factor conferring entomopathogenic properties to *Bt* (Bouslama *et al.*, 2020) [4]. δ-endotoxins of *Bacillus thuringiensis* act by forming pores in the midgut epithelial membranes of insects, leading to cell lysis. These toxins have a three-domain structure: Domain I forms pores in the epithelial cell membranes. Domain II binds to specific receptors, and Domain III stabilizes the toxin-receptor interaction, causing osmotic imbalance and insect death. Therefore, it is evident that the three domains of the toxin protein function in a coordinated manner, with their activities complementing each other (Melo *et al.*, 2016) [8].

To date, over 500 *Bt* toxin genes have been identified and classified into 72 classes based on amino acid sequence similarity and insecticidal specificity (Crickmore *et al.*, 1998) [5]. Among these, only a limited number of Cry toxins are widely utilized for pest control. Notably, Cry1, Cry2, Cry9, and Vip3A proteins have shown toxicity and specificity against various lepidopteran insect pests. However, prolonged use of *Bt* toxins has led to the development of resistance in target insect pests (Hassan *et al.*, 2021) [7]. The continuous exploration of *Bacillus thuringiensis* strains producing diverse toxins is essential for identifying novel and effective isolates.

To determine their potential against insect pests, these isolates must be characterized and screened for the presence of insecticidal genes. Thus, offers promising strategy to overcome resistance to known *Bt* biopesticides.

Polymerase Chain Reaction (PCR) is a highly efficient method for characterizing *Bt* genes. It enables rapid and accurate detection of specific DNA sequences using minimal DNA, allowing large-scale screening of *Bt* isolates to identify and map novel *cry*-type genes. The use of universal primers is a widely adopted method for detecting the presence of *cry* genes (Hassan *et al.*, 2021) [7]. In the present investigation 10 PDKV *Bt* isolates along with standard *Bacillus thuringiensis kurstaki* HD-1 and *B. crereus* have been screened for the presence of *cry* genes which are toxic to *Pectinophora gossypiella* (Saunders).

2. Materials and Methods

2.1 *Bt* culture

A total of 10 PDKV *Bt* isolates (PDKV SA-20, PDKV SY-4, PDKV SGd-1, PDKV SA-6, PDKV SA-18, PDKV SBn-2, PDKV SAk-6, PDKV SAk-9, PDKV SGn-4 and PDKV SGn-5) were obtained from Department of Entomology, Dr. PDKV, Akola, India. Along with standard *Btk* HD-1 and *B. cereus* strain was obtained from NCIM. A single colony of *Bt* cultures was inoculated into 50 ml of Luria broth and incubated at 30 °C overnight at 200 rpm.

2.2 Plasmid DNA isolation

Overnight grown culture of *Bt* isolates was centrifuged at 10,000 rpm for 2 minutes to harvest the cells. Plasmid DNA from the *Bt* isolates was extracted as per the standard protocol of HiPurA plasmid DNA purification kit.

2.3 PCR Screening

2.3.1 PCR primers

The 10 promising PDKV *Bt* isolates along with standard *Btk* HD-1 and *B. cereus* strain were screened for the presence of 4 *cry* genes *cry1*, *cry1Ac*, *cry2Ab* and *cry9* which are toxic to pink bollworm. The details of the sequences, gene recognized, and the predicted amplicon size of the *cry* genes are presented in (Table 1).

Table 1: Details of primers used for screening the *Bt* isolates

Sr. No	Name of the primer		Primer sequence	Predicted size (bp)	Reference
1	*cry*1	F	5'-CATGATTCATGCGGCAGATAAAC-3'	277	Ben-Dov *et al.* (1997) [3]
		R	5'-TTGTGACACTTCTGCTTCCCATT-3'		
2	*cry1Ac*	F	5'-GTATGCTTCTGTAACCCCGATTCACCTC-3'	195	Alberola *et al.* (1999) [1]
		R	5'-CCTGCAGTCCCACTAAAATTTCTAACACCTACTA-3'		
3	*Cry2Ab*	F	5'-GTTATTCTTAATGCAGATGAATGGG-3'	546	Arulslevi *et al.* (2011) [2]
		R	5'-TGGCGTTAATGGGGGGAGAAAT-3'		
4	*cry*9	F	5' CGGTGTTACTATTAGCGAGGGCGG 3'	350	Ben-Dov *et al.* (1997) [3]
		R	5' GTTTGAGCCGCTTCACAGCAATCC 3'		

F-Forward sequence of primer; R-Reverse sequence of primer

2.3.2 Amplification reaction mixture

Isolated plasmid DNA was used as a template DNA for the amplification of *cry1*, *cry1Ac*, *cry2Ab* and *cry9* genes with their specific primers. A total reaction volume of 20 µl consists of 2 µl of template DNA, 1 µl of each primer (forward and reverse), 10 µl of PCR master mix (Make: Takara) consisting of dNTPs, Taq polymerase, and 6 µl of nuclease-free water as given in (Table 2).

Table 2: Reaction constituents for single polymeric chain reaction

Constituents	Quantity
Template DNA (10 ng/µl)	2.0 µl
Master mix vol.	10.0 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
Nuclease free water	6.0 µl
Total reaction volume	20.0 µl

2.3.3 DNA amplification

PCR analysis was performed in thermal cycler (Prima-Trio™) following a temperature cycling program as described by (Temnykh *et al.*, 2000). The temperature profile of the PCR for screening the *Bt* isolates for the presence of *cry1*, *cry1Ac*, *cry2Ab* and *cry9* genes are given (Table 3). The amplification was performed with an initial denaturation at 94 °C for 4 minutes, followed by 30 cycles consisting of denaturation at 94 °C for 1 minute, annealing for 1 minute (at the appropriate annealing temperature), and extension at 72 °C for 1 minute. This was followed by a final extension at 72 °C for 7 minutes and a final hold at 4 °C. The PCR products were resolved by electrophoresis on a 2% agarose gel stained with ethidium bromide (EtBr). The amplified DNA bands were visualized under a UV trans illuminator and documented (Bio-Print, Vilber).

Table 3: Details of PCR conditions for each set of *cry* primers used in the analysis

S. No.	Step	Temperature (°C)	Duration (min.)	No. of cycles
1	Initial denaturation	94 °C	4	1
2	Denaturation	94 °C	1	30
3	Annealing	-	1	
	cry1	53 °C		30
	cry1Ac	61 °C		30
	cry2Ab	66 °C		30
	cry9	60 °C		30
4	Extension	72 °C	1	30
5	Final extension	72 °C	7	1
6	Hold	4 °C		-

3. Results and Discussion

The 10 most potent isolates, based on their entomopathogenic activity against the test insect *Pectinophora gossypiella*, were selected for screening for the presence of different *cry* genes (*cry1*, *cry1Ac*, *cry2Ab and cry9*) along with the standard *Btk* HD-1and *B. cereus* strain. The *cry* gene profile of PDKV *Bt* isolates is present in (Table 4 and 5) depicted in Fig 1. Among the four *cry* gene primers employed in the present study, *cry1* was the most prevalent, present in nine PDKV *Bt* isolates-SA-6, SA-18, SA-20, SAk-6, SAk-9, SGd-1, SGn-4, SGn-5, and SBn-

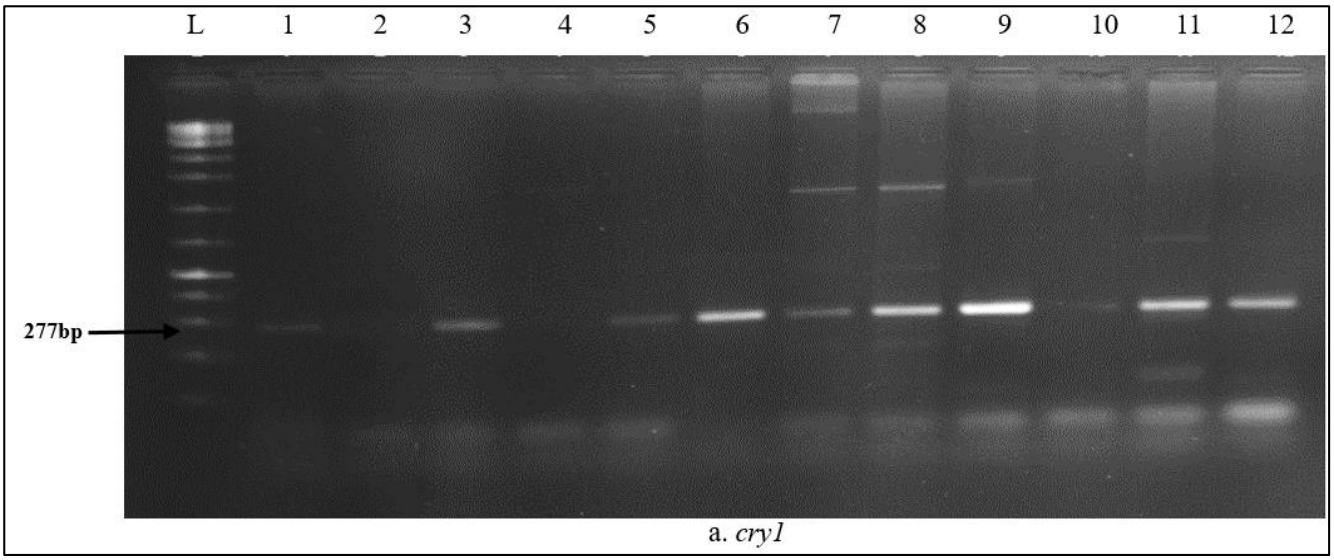
2 as well as in the reference strain HD-1. The *cry1Ac* gene was amplified in six isolates: SA-6, SA-20, SAk-6, SAk-9, SGd-1, and SGn-5. The *cry2Ab* gene was present in five strains: HD-1, SY-4, SAk-6, SGn-4, and SGn-5. In contrast, none of the tested isolates showed amplification for the *cry9* gene, indicating its absence in the strains screened. The present studies are consistent with earlier findings by Naik *et al.* (2018) [9] and Pooja *et al.* (2013) [10].

Table 4: *Cry* genes present in PDKV *Bt* isolates

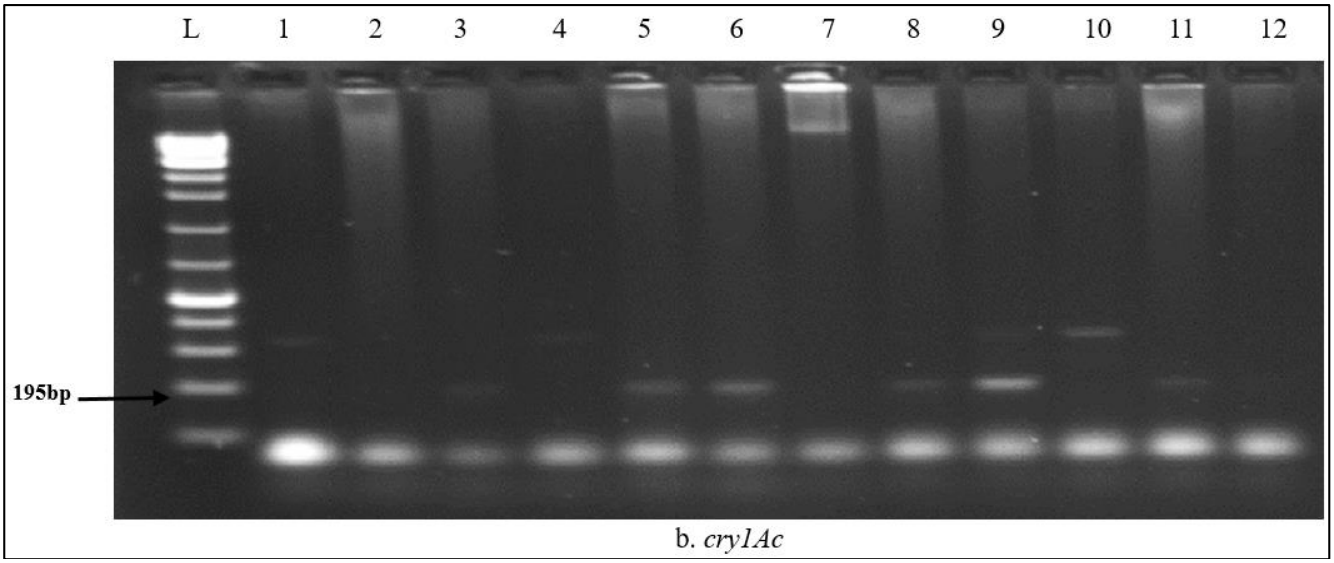
Sr. No	*Cry* gene primer	Size of Amplicon (bp)	Isolates	% occurance
1	*cry1*	277	HD-1, SA-6, SA-18, SA-20, SAk-6, SAk-9, SGd-1, SGn-4, SGn-5, SBn-2	90.91
2	*cry1Ac*	195	SA-6, SA-20, SAk-6, SAk-9, SGd-1, SGn-5	54.55
3	*cry2Ab*	546	HD-1, SY-4, SAk-6, SGn-4, SGn-5	45.45
4	*cry9*	350	-	-

Table 5: Distribution of *cry* genes in PDKV *Bt* isolates

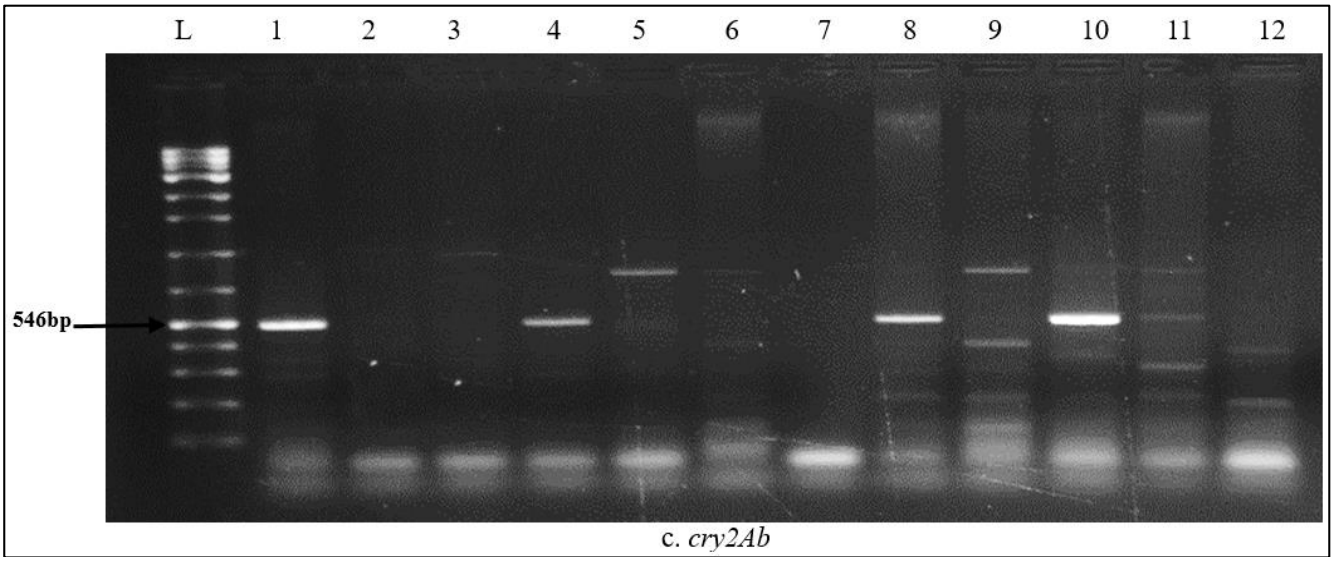
Sr.no	Isolate name	*cry1*	*cry1Ac*	*cry2Ab*	*cry9*
1.	SA-6	+	+	–	–
2.	SA-18	+	–	–	–
3.	SA-20	+	+	–	–
4.	SAk-6	+	+	+	–
5.	SAk-9	+	+	–	–
6.	SGd-1	+	+	–	–
7.	SGn-4	+	–	+	–
8.	SGn-5	+	+	+	–
9.	SBn-2	+	–	–	–
10.	SY-4	–	–	+	–
11.	HD-1	+	–	+	–
12.	*B. cereus*	–	–	–	–



a. *cry1*



b. *cry1Ac*



Lane L: Ladder, 1: *Bt* HD-1, 2: *B. cereus*, 3: SGd-1, 4:SY-4, 5:SA-20, 6:SA-6, 7:SA-18, 8: SAk-6, 9: SAk-9, 10: SGn-4, 11: SGn-5, 12: SBn-2

Fig 1: PCR amplification of *cry* genes

4. Conclusion

The present study highlights the genetic diversity among *Bacillus thuringiensis* isolates, particularly in the distribution of *cry* and *vip* genes, which are directly associated with their insecticidal potential. The variability in *cry*-type genes underscores the importance of molecular characterization in identifying promising *Bt* strains. These findings provide a valuable foundation for selecting potent *Bt* isolates for the development of biopesticides and for the isolation of insecticidal genes that can be utilized in the creation of insect-resistant transgenic crops, contributing to sustainable pest management strategies.

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