Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2020; 8(1): 522-530

Received: 16-11-2019
Accepted: 20-12-2019

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Molecular characterization and elucidation of *Bacillus thuringiensis* Cry1I toxin isolated from the insect pest mango leaf webber, *Orthaga exvinacea* (Noctuidae: Lepidoptera)

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Abstract
A new full-length *Bacillus thuringiensis* cry1I type gene was isolated from the mango leaf webber (*Orthaga exvinacea*) that was naturally infested. And its sequence of nucleotides was determined. The new Cry1I protein's deduced amino acid sequence is identical with Cry1b (94%) and Cry1Ia (89%) toxins. NCBI GenBank's accession number was given to the nucleotide sequence of the new cry1I gene: KJ437512 (partial) and KJ437513, showing 93-99 percent homology with Cry1I toxins genes previously published. Bt_MLB_Cry1I 3D toxin homology model was developed, despite low sequence identity with its structural homologues, Cry1I not only resembles the previously reported Cry toxin structures but also shares the typical five conserved amino acid residue blocks and subsequently evaluates the 3D model using bioinformatics tools. Mixtures of spores/inclusions of the *Bt_MLB*_Cry1I were tested for their toxicity against adults of brinjal ash weevil *Myllocerus subfasciatus* (Coleoptera: Curculionidae). In accordance with standard reference strains, adult mortality rates caused by spore and crystalline inclusion are 100 percent. The observed 100% mango leaf webber infestation to Bt in natural condition is a rare report indicating naturally high strain anti-lepidopterone activity. The increase in the number of Bt collections has resulted in an increase in the discovery of new Bt isolates with insecticide activity against a variety of insects or with increased insecticide activity. The current study has extended the potential for the management of Coleopteran pests to collect promising Bt isolates from naturally infected insect pests from different origins.

Keywords: Coleoptera, curculionidae, *cry1I*, Mango leaf webber, *Myllocerus subfasciatus, Orthaga exvinacea, toxicity*

Introduction
Bacillus thuringiensis (Bt) Bacillales: Bacillaceae is a spore-forming, soil-borne, gram-positive bacterium that produces insecticidal crystal proteins known as delta endotoxins or cry proteins that have been widely used over the years as a biopesticide. Cry proteins are oral intoxicants which act on susceptible insects ' midgut cells. To date, based on variations in the amino acid sequence, more than 766 *cry* genes have been characterized and grouped from *cry1* to *cry78* [1]. Most of the cloned toxins consist of Lepidopteran active proteins so far, it is important to look for more Bt strains containing unique Coleopteran genes. The Cry toxins are generally considered less environmentally toxic and can be more easily integrated into biological control-based pest management systems. This is one of the key factors promoting the use of bacterial pathogens control agents. Increasing interest in developing environmentally safe pest control methods has motivated us to look for possible microbial agents for control of agricultural significance in particular Coleoptera pests and insect pests. This is the first research on isolating and characterizing the *Bt* of *Orthaga exvinacea* and assessing the isolates ' insecticidal ability. Bt's ecology and lifestyle has been the target of many questions that have not yet been answered. Some argue that Bt is a soil-dwelling microorganism that gets nutrition from decaying organic matter or roots exudates for its survival and reproduction in nature, reaching the aerial parts of plants when they germinate and emerge from the soil. An opposite view points out that Bt is a versatile pathogen that is then accumulated in the soil and plants by colonizing and destroying its hosts and growing in their corpses, thus becoming natural reservoirs [2].

B. thuringiensis strains from soil, plant surfaces, dead insects and grain samples are identified by extensive screening programs. The analysis of new isolates has resulted in the detection of toxic strains against a wide range of insect orders, including Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, Molophoga, Acari [3], Collembola, Rhabditida and cancer cells [4]. Searching for more toxins is still needed as a significant number of pests remain unregulated with the available Cry proteins. Alternatives to the question of insect resistance are also very interesting, particularly with regard to the expression of *B. thuringiensis* genes that encode insecticidal proteins in transgenic plants [5]. The objective of this study was to isolate and characterize *B. thuringiensis* strains from dead insects and to assess their pathogenicity to Coleopterans. The development of new high-power Cry proteins would provide additional tools for controlling coleopteran *Myllocerus* spp and other insect pests. This is the first study on the isolation and characterization of the mango leaf webber active toxin Coleoptera, *Orthaga exvinacea* (Noctuidae: Lepidoptera) and determining the insecticidal ability of these isolates.

Materials and Methods

Collection of the dead mango leaf larvae

Dead mango leaf larvae *Orthaga exvinacea* (Noctuidae: Lepidoptera) were collected from the farm of the IIHR. Dead larvae samples collected were placed in a plastic container and sealed with a cover. The container containing the sample of the dead larvae was labeled and kept in a box for further processing.

Processing of dead mango leaf larvae

The dead larvae samples were initially homogenized in 1 ml of sterile distilled water in a sterile mortar individually. The homogenate was then transferred to a 6 ml boiling tube and 1 ml of sterile distilled water was applied to the boiling tube. The homogenate was vortexed and was treated with heat shock at 80 °C for 10 minutes in a water bath [6]. The treated specimens were vortex after heat-shock treatment and three-fold serial dilutions were performed. Using the spread plate method, the serially diluted specimens ranging from 10^{-1} to 10^{-3} were mounted on the nutrient agar plate. For 48 h, the inoculated plate of Nutrient Agar was incubated at 30 ° C. The plates were tested for potential growth of *Bacillus* colonies at an interval of 12 hours. The Bacillus was then isolated and grown on new nutrient agar plates using morphological and molecular characterization to further characterize it.

Morphological description of putative colonies of bacillus isolated from dead mango leaf larve

Morphological characterization was performed to identify and isolate the desired microbe on the basis of its morphology and parasporal crystal production ability.

Colony morphology identification and isolation

Colonies in color white to off-white, slightly elevated and with a mat-like appearance with a normal margin were picked and labelled with a marker pen [7]. The colonies selected with the described characteristics may have been colonies of *Bacillus*. Of all the samples, a total for 15 colonies were selected. The colonies selected were sub-cultured and labelled on new nutrient agar plates. In order to obtain pure colonies, further screening was done on the basis of morphological characterization.

Comassie brilliant blue (CBB) staining

The putative Bacillus like isolates was inoculated into a 50 ml sterile conical flask containing nutrient broth. On orbital shaker set at 250 rpm for 4 days, the inoculated nutrient broths were agitated at room temperature. This is done to sporulate the isolated colonies of Bacillus. At two stages of the life cycle of the bacillus, the Bacillus isolates were characterized by a coomassie blue staining process. The first stage is the phase sporulated while the second stage is called the stage of autolysis. The parasporal bodies can be seen clearly and distinguished in the sporulated phase by means of the CBB method [8]. The stage of autolysis was presented at 110 h, where all the bacterial cells would be lysed completely. All the 15 isolates examined showed stained parasporal body and crystals believed to be *B. thuringiensis* because the characteristics shown by the isolates are similar to *B. thuringiensis* characteristics.

Isolation of a *cry1I* full length gene from the *B. thuringiensis* re-isolated from dead mango larvae

To classify *cry1I*-type genes, primers have been designed to amplify complete *cry1I*-type genes (i.e. from start codon to stop codon) based on multiple alignments of all previously described *cry1I* and the consensus sequences of their N- and C-terminal coding regions. PCR was performed using these primers to amplify full cry1I genes (forward primers: 5'- GGA TCC ATA TGA ATA GTG TAT TGA AT- 31, reverse primers 5'- GTA CGG ATC CTC AAA CCT TAA TAA TAA -3 '), 30 amplification cycles at annealing temperature of 630C and Jump Sart TM Accu Taq ™ Taq DNA polymerase (Sigma). Following instructions from the manufacturer, the resulting PCR fragment was excised from the gel and purified using the NucleoSpin ® ExtractII Kit (MN).

Cloning, sequencing and sequence analysis of the *Bt_MLB_Cry1I*

According to Sambrook and Russell [9], the standard recombinant DNA techniques used in cloning were used. The *E. coli DH5α* cells were transformed using the Fermentas DNA ligation kit (# K1214) using a PCR-amplified 2.16-kb *Bt_MLB_cry1I* gene ligated in pTZ57R (T / A cloning vector). Transformed cells (20µl) were distributed to X-gal (270 µg / ml), IPTG (120 µg / ml) and ampicillin (100 µg / ml) containing LB agar plates (Sambrook *et al*., 2001). Then the plates were incubated to screen blue and white colonies at 37°C for 24 hours. The cloning of recombinant plasmid DNA containing the gene Bt_MLB_*cry1I* was confirmed by colony PCR and restriction analysis. The entire 2.16-kb *Bt_MLB_cry1I* gene was sequenced using a series of internal primers designed to determine homology with the known sequences in the NCBI GenBank database and to determine mutations in the cloned genes of Bt isolate.

Nucleotide sequence accession numbers

NCBI GenBank database was provided with the nucleotide sequences of the full-length *Bt_MLB_cry1I* gene obtained from mango leaf webber dead larvae.

Toxicity analysis against *Myllocerus subfasciatus* (Coleoptera: Curculionidae)

At the Indian Institute of Horticultural Research (IIHR) farm, adult weevils of Myllocerus subfasciatus were collected and

reared in the laboratory (Unpublished data). Preliminary leaf dip bioassays were performed with highly concentrated spore – crystal suspensions (around 250 ng of toxin per square centimeter of leaf surface) with adults of brinjal ash weevil / gray weevil. 20 adults were placed on each leaf for each treatment (four replicates per treatment). As negative controls, the solubilization buffer and water were used. Bioassays were conducted with a 16:8 light / dark cycle at 25 °C in 60–70 percent relative humidity. Compared to the parallel control in which leaflets were dipped in sterile distilled water instead of bacterial suspension, the mortality percentage was scored after 4 days. A positive control was used for *B. thuringiensis* subsp *morisoni.*

Results and Discussions

As one of the insect resistance management strategies, the screening of the environment for new and highly potent Bt strains has become inevitable. Several studies on the frequent occurrence of Bt isolates in the natural environment have shown the high potential to isolate a novel strain [10]. Our current knowledge of the ecological distribution of this bacterium in nature has contributed to the worldwide selection creation. The family of *cry1I* (formerly genes of *cryV*) includes genes with rare properties. They are usually silent genes in *B. thuringiensis* strains, for example, but can be expressed as a protoxin form of approximately 81 kDa in Escherichia coli cultures [11], a unique molecular mass in Cry1 toxins. In addition, various Cry1I proteins were identified as active against lepidopteran and coleopteran pests [12-14]. Importantly, currently available data support the idea that Cry1A toxin-resistant insects are not cross-resistant to Cry1I toxins [11, 13]. The Cry1I toxins may be an alternative to combating the issue of insect resistance, making it worthwhile to thoroughly elucidate their potential for insecticides [15].

Morphological characterization

A common colony of Bacillus has a white to off-white colour, a slightly elevated elevation, and a regular margin-like appearance. Colonies with features as a known colony of bacillus are isolated and sub-cultured on new nutrient agar plates.

Coomassie brilliant blue (CBB) staining

Coomassie brilliant blue R-250, binds to virtually all protein in a non-specific way. The coloring of coomassie blue is effective and easy to perform. Once the smear is stained, a destaining solution should be used to wash the stained slide before viewing it under the microscope. CBB staining was exposed to putative Bt MLB isolates in order to have a clear view of the parasporal bodies and spores. Figure 1 shows fully autolysed cells where the Bacillus cells ' rod shape cannot be viewed, the spores are viewed brightly and distinct spherical and amorphous crystal shapes have been observed.

Bt was isolated from various ecological habitats such as soil, plants, animal feces, dead and infected insects, stored food products, aquatic environments, and sludge of waste water. Bt isolate from dead and infected mango leaf webber has been successful in the current investigation. Different authors said that the great diversity and abundance of Bt strains depends on many environmental factors and geographical conditions [16-27]. As Guz *et al*. reported in 2009, insects are a very good source of nutrients for bacterial growth, many Bt strains were expected to be isolated. Nevertheless, the small percentage of insect-based isolated Bt strains was likely caused by limiting factors such as hemocel fluid bactericidal properties, the existence of other gut microorganisms, and their interaction. These factors may also affect the distribution of Bt strains in larvae insect samples and restrict their vegetative development. We were inspired by ongoing research to isolate Bt strains from dead insect pest. We were able to isolate Bt strains from dead insect pest from the result obtained. The common step found in the preparation method is the treatment of heat shock provided to the entire processed samples. This treatment is given to destroy any vegetative type of microorganisms and fungal reproductive spores that remain in the samples collected. In identifying active strains, the strategy to isolate Bt strains from dead insect bodies seems to be highly effective. Only the bacteria that form endospores may withstand the heat shock and when plated, they form colonies. With normal margins and slightly elevated altitude, the selected isolates show white to off white. In 2009, Guz *et al*. reported the occurrence, ecology and toxicity of Bt strains isolated from the *Lymantria dipar* insect pest. During the phase of sporulation and autolysis, the isolated strains were further characterized by coomassie brilliant blue (CBB) staining to have a clear view of the parasporal body. After CBB staining, the isolated strains showed dark blue parasporal body and light unstained endospores. All the isolated colonies have therefore been identified as colonies of Bt.

Cloning and sequence analyses of the *Bt_MLB_cry1I* gene

Using all the known *cry* gene sequences as virtual templates and the software fast PCR, IDT DNA oligo analyzer and primer 6.0, the four primers are designed to be used for each primer. Such primers showed the ability for 100 percent amplification of the *Bt_MLB_cry1I* genes. The main goal of cloning cry gene using recombinant DNA methodologies is to increase the available Bt strains to improve productivity and to improve toxicity. After defining and testing the amplification conditions on known and unknown strains, DNA from the selected strains was amplified using gene-specific primers. Plasmid mobility test, colony PCR, individual restriction digestion analysis (Data not shown) identified the amplification products and specific clones. The cloned amplicons were sequenced and sequences were analyzed using NCBI BLAST programs to detect the known cry gene to which the selected strain gene was the most similar (highest hit) and identity level (Supplementary data 1). A maximum of 12 sequences were analyzed, with identity rates ranging from 81% to 99% with identified cry genes. The gene with the highest hit for each amplicon and its identity rate is shown in Table 1. Rather interestingly, the domain I of the new insect pest mango leaf webber Cry1I toxin, *Orthaga exvinacea* shows 68% and 57% homology to the *cry8* and *cry3* endotoxin genes respectively, and the domain II shows 48% homology to the *cry8* endotoxin genes (Table 1). Such findings demonstrate the toxins of Cry1I play a dual function against insect pests Lepidoptera and Coleopteran. Figure 2 showed the deduced comparisons of amino acids between the published full-length *Bt_MLB_cry1I* genes. They are registered with accession number KJ437512 (partial length gene) and KJ437513 (full length gene) in the NCBI GenBank database.

The similarity of the deduced amino acid sequence ranges from 46-97 percent like sequences of the *Bt_MLB_cry1I* gene and other Bt-like genes. The residues of amino acids ranged from 6.25% (Ala), 8.19% (Thr), 6.11% (Gly) and 0.14%

(Cys). Fig.3 shows the phylogenetic relationship between the *Bt_MLB_cry1I* gene and other cry1 and cry1I genes previously reported from this analysis and other laboratories. Reported from this study, *Bt_MLB_cry1I* is distinctly different from those reported elsewhere. The phylogenetic analysis showed that Bt mango leaf webber Cry1I sequences have almost the same evolutionary range (Fig. 3).

Cry gene content is one of the most important factors influencing toxicity variations. The type of *cry* genes present in a strain typically correlates with its insecticidal activity to some degree. Identifying the gene content in a strain can therefore be used to predict or verify its propensity for insecticides. PCR confirmed the presence of cry1 and cry2 in all isolates tested using multi-author primers [28, 29]. As a reference strain, the *Bt kurstaki HD-1* strain was used because it harbors the genes *cry1Aa*, *cry1Ac*, *cry1Ab*, *cry1Ia*, and *cry2*. Compared to the *Bt kurstaki* HD-1 strain, the protein profile of the crystal protein extract from each local isolate was compared. All profiles were identical and showed 2 bands of about 130 and 65 kDa, supporting the idea that the genes of *cry1* and *cry2* were expressed in all isolates studied.

Historically, polymerase chain reaction (PCR) methods have been used to identify various *cry* genes from Bt strains [30]. Several PCR-based methodologies of universal primers and collections of primers aimed at specific regions of type-specific *cry* genes and these methods make it possible to detect *cry* genes and predict their biological activity. The use of PCR has greatly enhanced the identification of cry genes; however, this technique is mostly restricted to previously described gene family members and requires a large number of primers. Furthermore, universal degenerate primers were designed to amplify all members of various subfamilies of cry genes. While the use of such degenerate oligonucleotides increases the likelihood that novel genes will be expressed, the usefulness is limited to detecting closely related genes in the same population. The ability to detect and classify unknown cry sequences, demonstrated by the characterization of novel cry-related sequences from native Bt strains, was a clear demonstration of the importance of these primers [31]. The present study sets out a gene-specific primary PCR method for the identification of Bt isolate-type *cry1I* genes.

In the NCBI (http:/www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and the Protein Homology / Analogy Recognition Engine (Phyre2) computational tool (http:/www.sbg.bio.ic.ac.uk/phyre2) model, the structure and function of a new *Bt_MLB_Cry1I* toxin is predicted. Such results show that Bt toxin actually belongs to the query protein chain. Three-dimensional (3D) structure is developed with a high confidence match (> 90% confidence) and descriptors of the template's fold and superfamily. Perhaps the model's total fold is right and the model's central core would continue to be accurate. The prediction of consensus scored a confidence value for the predicted secondary structure (0=low confidence, 9=high confidence) whether the regions of the query were structurally ordered (O) or disordered (d). These disordered regions have frequently been found to be involved in protein function and should be considered when examining predicted functional sites. The practical uses of protein structure prediction are numerous, including enhancing crystallographic phasing signals, selecting mutagenesis sites and rational layout of hybrid toxins / domain swapping [32]. A better understanding of Bt_MLB_Cry1I's 3D structure will be useful in designing domain swapping experiments to improve its toxicity to insecticides.

Using NCBI CDD search server, the conserved domains of the cloned *Bt_MLB_cry1I* sequence is analyzed. Bt_MLB_Cry1I's toxic domain ranged from 60 to 644 amino acids and contained endotoxin superfamily N, M and C domains that include most of the N-terminal region that indicates its toxic potential. Bt_MLB_Cry1I alignment with reference strain *B. thuringiensis.* Sub sp. *Kurstaki* (Accession No. X62821) showed changes in position 1 of 75 amino acids.2 (F-L), 13 (S-P), 22 (S-A), 31 (D-V), 35 (Q-K), 37 (I-T), 39(H-I), 42 (C-Y), 44 (K-R), 47 (C-Y), 50 (N-S), 51 (V-I), 52 (E-D), 54 (F-Y)- N-terminal region, 78(F-Y), 82(V-I), 111(I-N), 117(S-L), 126(T-S), 136(A-G), 140(D-E), 147(G-K), 160(S-N), 206(F-Y), 209(S-A), 210(S-P), 228(A-T), 233(Y-D), 237(K-I), 240(S-N), 281(Q-L), 282(M-V), 286(K-I)- Domain I, 289(A-S), 301(G-A), 306(H-N), 308(S-A), 310(T-A), 324(A-V), 330(V-I), 332(N-S), 338(F-Y), 341(Q-I), 364(K-R), 366(F-S), 369(T-P), 373(T-A), 376(I-T), 392(P-Q), 404(L-W), 410(F-Y), 426(K-I), 428(V-P), 430(H-L), 436(F-Y), 440(P-L), 445(I-V), 460(A-T), Domain II- 514(K-I), 519(S-P), 530(F-Y), 555(F-Y), 571(L-V), 596(D-G), 600(K-I), 613(F-L), 614(L-S), Domain III and 655(Y-H), 659(K-E), 670(R-G), 675(D-N), 677(K-T), 678(D-E), 693(D-N), 696(Y-H)-C-terminal region. The study of the deduced Cry1I protein sequence of the T01328 isolate also indicated the existence of an N-terminal sequence acting as a signal peptide, according to the findings of Kostichka *et al*., [33]. Modification of Cry proteins by protein engineering to increase toxicity and insecticide scope is also a promising method, but requires detailed understanding of the structure and function of these proteins and study of toxin-receptor interactions [34, 35].

A preliminary bioassay was conducted on adults of *Myllocerus subfasciatus* (Coleoptera: Curculionidae) with highly concentrated spore – crystal isolate suspensions. The Bt_MLB strains showed different levels of toxicity between 65 and 81%. It was also bioassayed for LC50 estimates with serial dilutions of spore – crystal mixtures (Table 2). *Orthaga exvinacea* was the most toxic with 100% toxicity compared to *B. thuringiensis* subsp *morissoni* as a monitor with 91% toxicity, according to the LC_{50} values and their fiduciary limits, Bt_MLB_Cry1I toxin obtained from insect pest mango leaf webber. The activity of this new toxin is relatively high in its particular activity range may aid in understanding the specificity molecular basis and designing new biopesticides. Most of the cloned toxins consist of lepidopteran active proteins so far, it is important to look for more Bt strains that harbor unique coleopteran genes. The Cry1I toxin may be an alternative to solving the issue of insect resistance and it will therefore be worthwhile to thoroughly elucidate its potential for insecticides. Further studies are underway to determine the nature of the protein and its novelty and toxicity tests are carried out to broaden the understanding of its effects on different insect orders.

Table 1: Mean identity between the protein domains of the new Cry1I protein with the corresponding domains in other members of the Cry1 family and other coleopteran active *Bt* Cry proteins.

	cry1I new KJ437513				
	N-Terminal	**Domain I**	**Domain II**	**Domain III**	**C-Terminal**
cry1I crytal toxin protein	96%	96%	87%	94%	86%
cry1Ia	78%	91%	87%	94%	89%
cry1Ib	89%	95%	97%	94%	89%
cryV	80%	91%	87%	-	89%
cry1I like	89%	-	90%	94%	88%
Delta endotoxin gene	80%	91%	87%	94%	89%
cry8	-	68%	43%	-	-
cry3	-	57%	-	-	-

Table 2: Insecticidal activity of the *B. thuringiensis* isolates against adults of *Myllocerus subfasciatus* (Coleoptera: Curculionidae)

Sl.no.	***Bt* strains**	**Mortality (%)[a]**	**LC_{50}[b] (ng/cm^2)**	**FL (95%)[c]**
1	*cry1I* new KJ437513	100[d]	-	-
2	*Bt* subsp. *morrisoni*. pathovar *tenebrionis*	91	152	141–171

[a] The highest mortality (%) observed in the bioassays (at 250 ng toxin/cm^2).
[b] Results are expressed as nanograms of toxin per square centimeter of surface.
[c] Fl95 min.–max., 95% confidence limit.
[d] LC_{50} value has not been obtained, the stated amount causes 100% mortality.

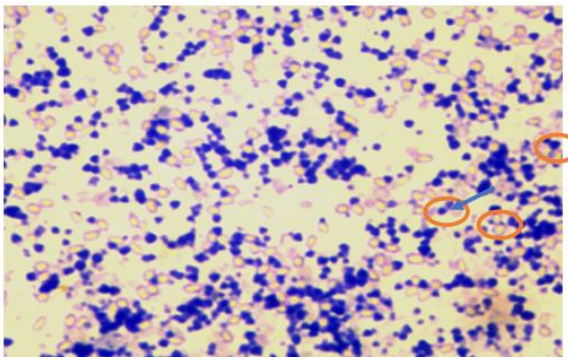

Fig 1: Light microscope photograph of *Bt* obtained from insect pest mango leaf webber, *Orthaga exvinacea* (Noctuidae: Lepidoptera) spores and crystal (magnification x 1000). Circle with arrow indicate the spore, circle indicates the spherical type crystals as well as spore attached with crystal.

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                10        20        30        40        50        60        70        80        90       100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
X62821          MKLKNQDKHQSFSSNAKVDKISTDSLKNETDIELQNINHEDCLKMSEYENVEPFVSASTIQTGIGIAGKILGTLGVPFAGQVASLYSFILGELWPKGKNQ
KJ437513       ...........LP........A........V...K.T.I..Y.R...H.SID.Y.......................Y...I.................S.

               110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
X62821          WEIFMEHVEEIINQKISTYARNKALTDLKGLGDALAVYHDSLESWVGNRNNTRARSVVKSQYIALELMFVQKLPSFAVSGEEVPLLPIYAQAANLHLLLL
KJ437513       ..........N.....L........S..R......G...E......K............N....K.....................................

               210       220       230       240       250       260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
X62201          RDASIFGKEWGLSSSEISTFYNRQVERAGDYSYHCVKWYSTGLNNLRGTNAESWVRYNQFRRDMTLMVLDLVALFPSYDTQMYPIKTTAQLTREVYTDAI
KJ437513       .....Y.......AP.............TR...D...I..N.......................K.....................LV...I..S...........

               310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
X62201          GTVHPHPSFTSTTWYNNNAPSFSAIEAAVVRNPHLLDFLEQVTIYSLLSRWSNTQYMNMWGGHKLEFRTIGGTLNISTQGSTNTSINPVTLPFTSRDVYR
KJ437513       A....NQA.A.............V......I.S.....Y..I.......................R..S.P...A..T................Q........

               410       420       430       440       450       460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
X62201          TESLAGLNLFLTQPVNGVPRVDFHWKFVTHPIASDNFYYPGYAGIGTQLLQDSENELPPEATGQPNYESYSHRLSHIGLISASHVKALVYSWTHRSADRTN
KJ437513       ...W.....Y..............I.P.L......Y..L....V..............T.....................................

               510       520       530       540       550       560       570       580       590       600
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
X62201          TIEPNSITQIPLVKAFNLSSGAAVVRGPGFTGGDILRRTNTGTFGDIRVNINPPFAQRYRVRIRYASTTDLQFHTSINGKAINQGNFSATMNRGEDLDYK
KJ437513       ............I....P..........Y.........................Y.............V.....................G...I

               610       620       630       640       650       660       670       680       690       700
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
X62201          TFRTVGFTTPFSFLDVQSTFTIGAWNFSSGNEVYIDRIEFVPVEVTYEAEYDFEKAQEKVTALFTSTNPRGLKTDVKDYHIDQVSNLVESLSDEFYLDEK
KJ437513       ............LS.....................................H.......E..........G....N.TE..............N..H....

               710
                ....|....|....|....
X62201          RELFEIVKYAKQLHIERNM
KJ437513       ....................
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Fig 2: Amino acid sequence of *cry1I* novel Coleopteran active PCR amplicon from *Bt* mango leaf webber, *Orthaga exvinacea* (Noctuidae: Lepidoptera) strain. Comparison of the amino acid sequence from *Bt* native strain produced with the *cry1I* group primers and that of a known Coleopteran gene, *cryV* (GenBank accession number X62821). A "." is placed at those positions in native *Bt* strain where the amino acid is the same as that in X62821, and a letter denoting the amino acid if it is not. Dashes "-" represent insertions required to align the sequences

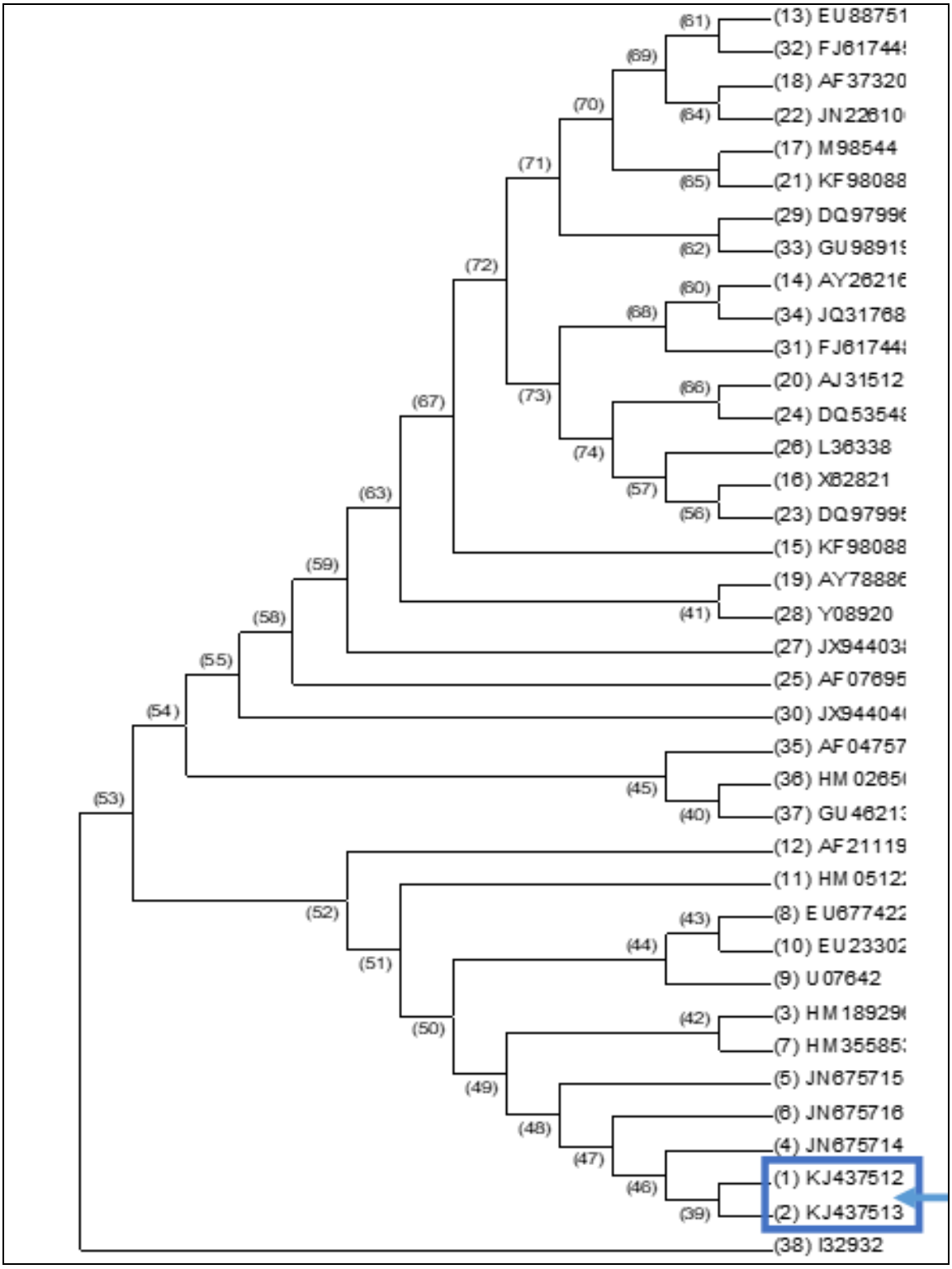


Fig 3: Neighbour-joining tree showing phylogenetic relationship amongst different *Bacillus thuringiensis cry1I* genes based on nucleotide sequences using NCBI GenBank accession number I32932 as an out-group. The NJ tree was constructed using CLUSTAL W with default parameters are indicated at the nodes. Genetic distances between *cry1I* gene sequence profiles were calculated by using Kimura 2-parameter. Bootstrapped data set were generated using phylogeny reconstruction (2000 replicates), and analyzed by using MEGA (version 5.4) software. Sequences were obtained from the databases of the National Center for Biotechnology Information (http:// www.ncbi.nlm.nih.gov). The gene sequences were responsible for geographic separation for divergence within *cry* serotypes, consistent with the evaluation of distinct bacterial population. Despite the geographical distances, *Bt* strains have originated from common ancestors. Some strains have evolved to be quite distinct and others remain as members of closely related groups. Arrow Position of the novel Cry sequence, numbers at nodes percentages of bootstrap resamplings. The values shown at the base of divergent lines are bootstrap values.

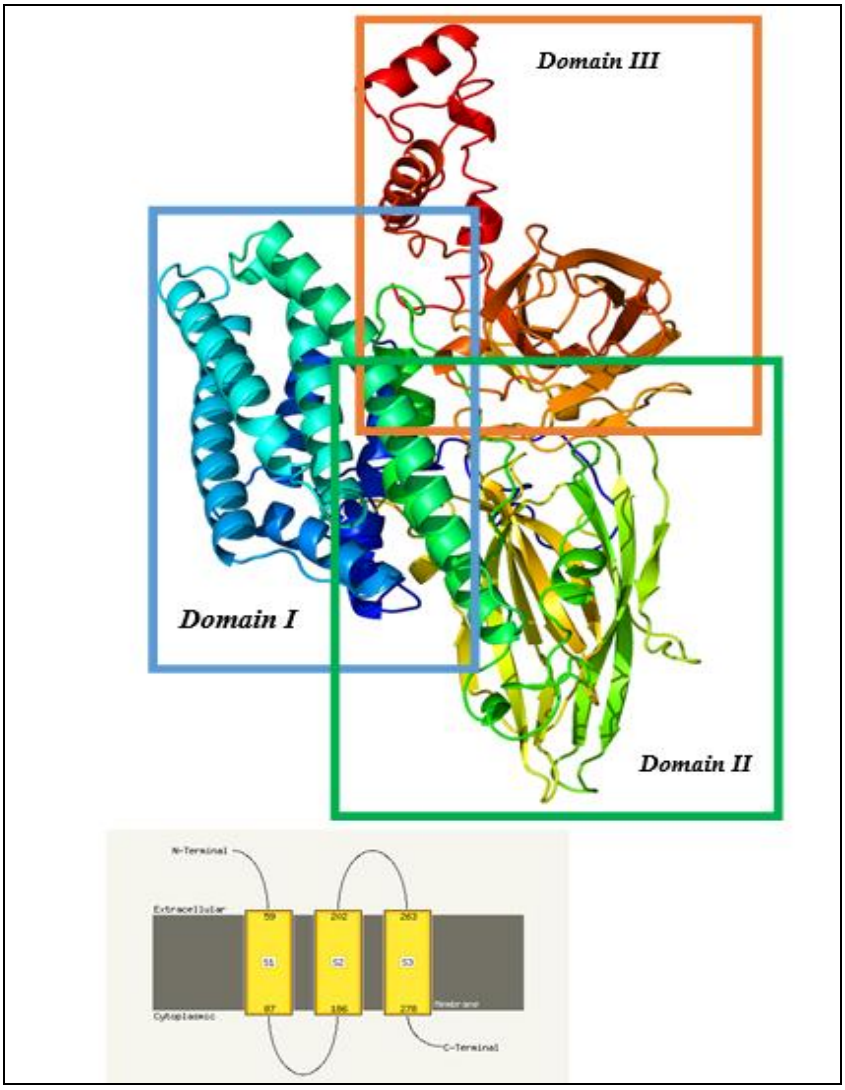
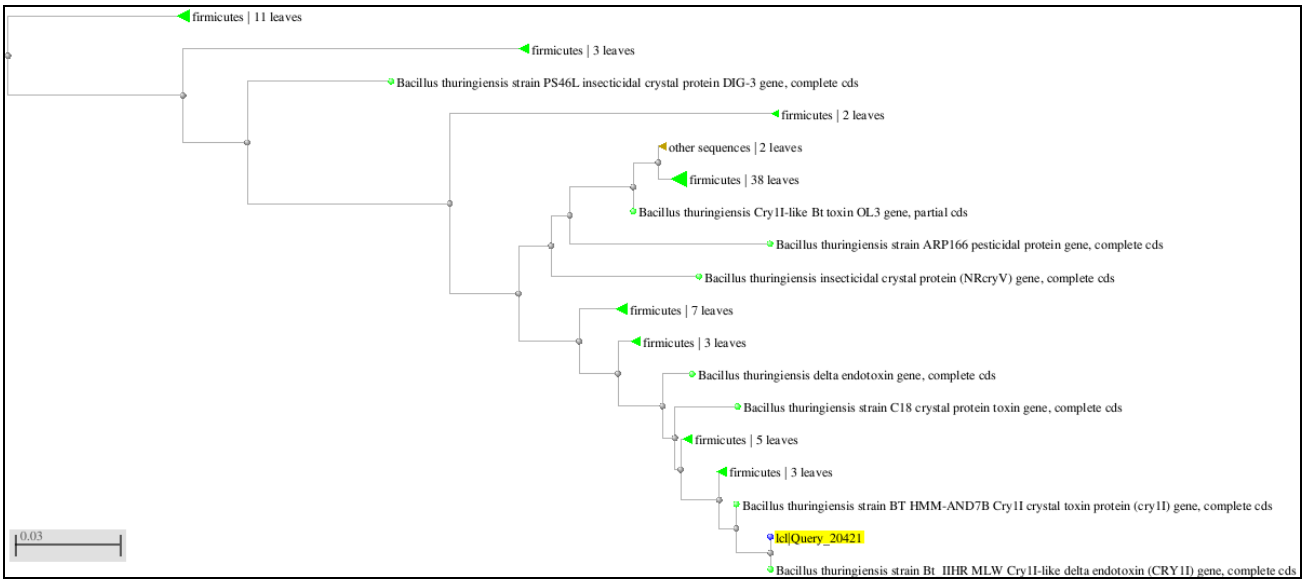
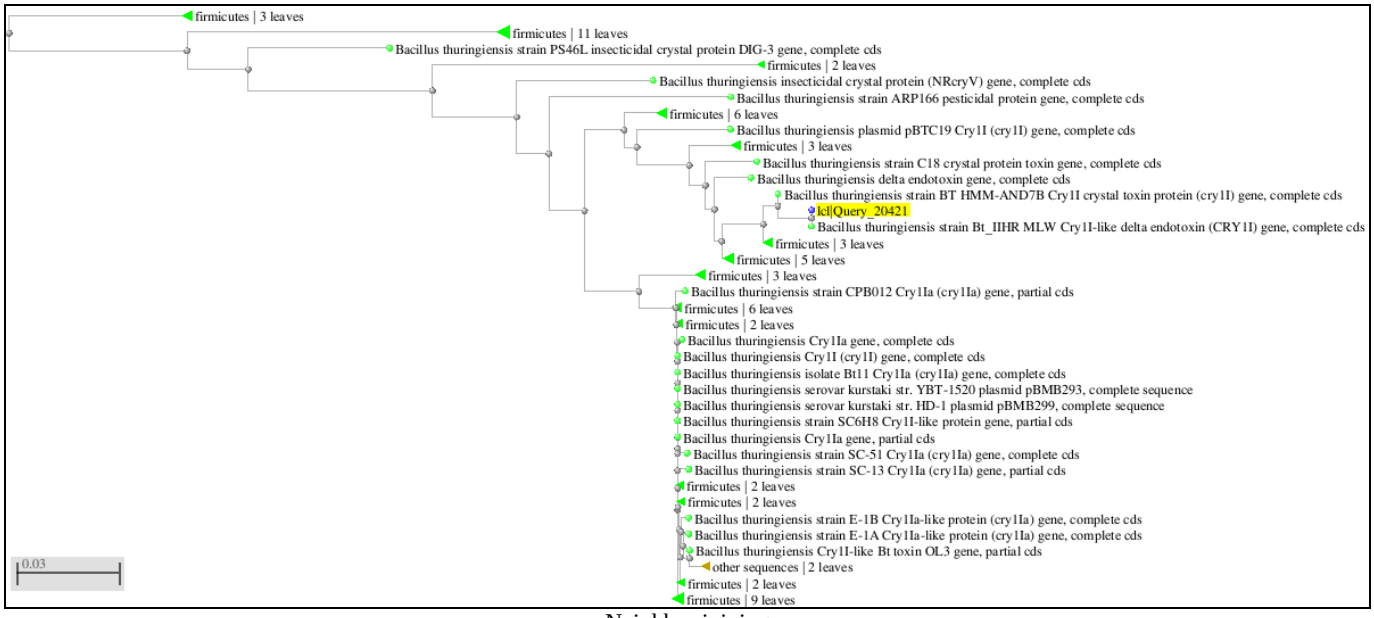


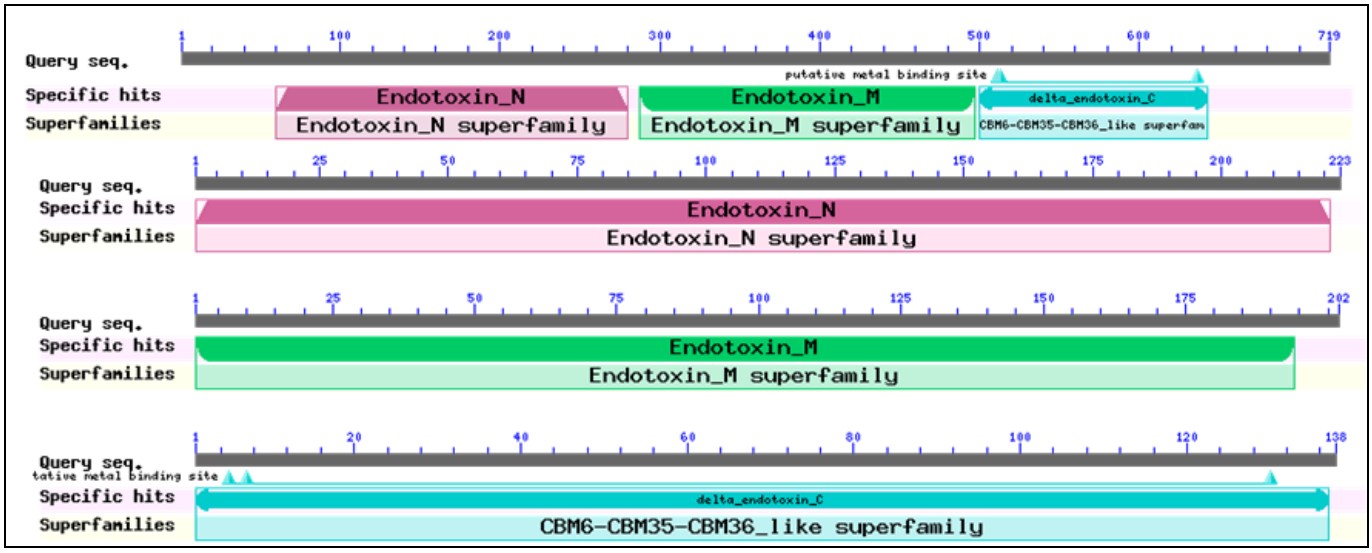
Fig 4: Ribbon representation of the crystal structure of Cry1I (88% of residues modeled). The colored boxes denote the positions of the different domains. The three domains are indicated with domain I (blue), comprised of seven α-helices, designated as the pore forming domain. Domain II (green) is marked as a receptor-binding domain with certain loops being critical for this process. The anti-parallel L-sheet sandwich structure of domain III is indicated in red. This domain function both in binding to the receptor, probably via a lectin binding pocket, and in modulating ion channel activity. Helix α7 is the one closest to domains II and III and may be responsible for the `opening up' of domain I after a second step of toxin-binding to the receptor. B. Topology transmembrane prediction.



Supplementary data 1: Fast minimum evaluation



Neighbor joining



Supplementary data 2: Linear form of three conserved domains of *cry1I* gene. N-terminal domain is involved in membrane insertion; M and C domains are involved in receptor binding. Shown here is the default concise view generated by the CD-Search tool, using pre-calculated alignment information. The view is divided into two panels: a graphical summary and a table detailing the individual matches. The query sequence coordinates are indicated on a gray bar in the top portion of the graphical summary. 'Specific hits' to NCBI-curated domain models are positioned in a separate area below the query sequence, with corresponding balloons rendered in saturated colors. The extent of the best-scoring hit for a region on the query also determines the annotation with the corresponding conserved domain 'Superfamily'. 'Superfamilies' are positioned in the area below the 'Specific hits', and together these are enclosed in boxes to indicate superfamily membership of the NCBI-curated models. Functional sites, as annotated on NCBI-curated domain models, are mapped to the query sequence and depicted as triangles. Sites are mapped from the highest ranked model only, and they are colored according to their source.

Acknowledgements
Authors are grateful to the Director, Indian Institute of Horticultural Research (IIHR) for the encouragement and providing the technical support to carry out the research.

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