Curr Microbiol (2011) 63:420–425
DOI 10.1007/s00284-011-9998-x

Cloning, Characterization and Diversity of Insecticidal Crystal Protein Genes of *Bacillus thuringiensis* Native Isolates from Soils of Andaman and Nicobar Islands

H. M. Mahadeva Swamy · R. Asokan · S. N. Nagesha · D. K. Arora · Ajantha Birah · Riaz Mahmood

Received: 25 February 2011 / Accepted: 7 August 2011 / Published online: 21 August 2011

Abstract *Bt* strains were isolated from soils of Andaman and Nicobar Islands and characterized by microscopic and molecular methods. Diversity was observed both in protein and *cry* gene profiles, where majority of the isolates showed presence of 65 kDa protein band on SDS-PAGE while rest of them showed 130, 72, 44, and 29 kDa bands. PCR analysis revealed predominance of *cry1I* and *cry7*, *8* genes in these isolates. The PCR screening strategy presented here led us to identify putative novel *cry* genes which could be active against Coleoptera insects. Variation in the nucleotide sequences of *cry* genes from the isolates suggests that the genetic diversity of Bt isolates results from the influence of different ecological factors and spatial separation between strains generated by the conquest of different habitats in the soils of Andaman and Nicobar islands. The implications of our studies are important from the point of view of identifying novel *cry* genes that could be toxic to insects other than lepidoptera.

This project is funded under ICAR network project on Application of microbes for agriculture and allied sector through National Bureau of Agriculturally Important Micro organisms (NBAIM). This is a part of Doctoral degree research work.

Electronic supplementary material The online version of this article (doi:10.1007/s00284-011-9998-x) contains supplementary material, which is available to authorized users.

H. M. Mahadeva Swamy (✉) · R. Asokan (✉) · S. N. Nagesha
Bio Pesticide Laboratory (BPL), Division of Biotechnology, Indian Institute of Horticultural Research (IIHR), Hessarghatta Lake Post, Bangalore 560089, Karnataka, India
e-mail: clintonbio@gmail.com

R. Asokan
e-mail: asokaniihr@gmail.com

D. K. Arora
National Bureau of Agriculturally Important Micro Organisms (NBAIM), Mau Nath Bhajan 275101, Uttar Pradesh, India

A. Birah
Central Agricultural Research Institute, Port Blair 744101, India

R. Mahmood
Post-Graduate Department of Studies and Research in Biotechnology and Bioinformatics, Kuvempu University, Jnanasahayadri, Shimoga, Shankaraghatta 577451, Karnataka, India

Introduction

Climate driven emergence of new plant pests and diseases is of concern—emerging pests are often plant pests of related species known as "new encounter" pests, which come into contact with new hosts that do not necessarily have an appropriate level of resistance, or are plant pests introduced without their biological control agents (in particular, insect pests, nematodes and weeds). In addition, the use of synthetic insecticides is not recommended because of the long residual action and toxicity to a wide spectrum of organisms, including humans. Consequently, interest has developed in the use of alternative strategies for biological control, such as *Bacillus thuringiensis* [1]. As a result of this, it occupies more than 90% of the world biopesticide market [2]. Along with the use of *cry* genes in transgenic plants recently, it has contributed significantly to reduce environmental pollution by chemical pesticides.

Worldwide isolation of Bt have established it to be a natural inhabitant of soil [3–5]. However, isolation of Bt from diverse environments such as phylloplane, dead insects, stored grains, brackish sediments, aquatic environments, etc., has created an enigma about the role of Bt in nature [6, 7]. There is currently great interest in isolating novel strains of *Bt* with either unique host specificity or

elevated toxicity. Search for novel *Bt* strains may lead to the discovery of additional insecticidal proteins with higher toxicity and/or wider spectrum. Novel toxins are also important for providing alternatives to cope with the emergence of resistant insect populations.

Diversity available in nature has to be collected systematically, classified, analyzed, and harnessed for whatever it is worth. It is a basic resource, and it has to be collected and made available in an easily usable form. The characterization of native *Bt* strains helps in understanding the role of *Bt* in the native environment and distribution of *cry* genes in local condition [6, 7]. The aim of this work was to make an attempt to isolate, characterize and predict insecticidal activity of *B. thuringiensis* isolates and to evaluate their potential usefulness in developing new biopesticides with broader and higher spectrum of toxicity against insect pests belonging to order Coleoptera. We used PCR screening to detect *cry* genes and SDS-PAGE method to estimate the composition of toxins in bacterial crystals, cloning, and sequencing to determine the diversity of the isolates. Hence, this study was carried out to explore potential Bt diversity present in soil samples of Andaman and Nicobar Islands (undisturbed ecosystem) between 6° and 14° north latitude and 92° and 94° east longitude of India.

Materials and Methods

Sample Collection

A total of 120 soil samples were collected randomly for isolating *B. thuringiensis.* One gram of soil sample was collected from 5 cm depth, after gently removing the debris in the top soil (data not shown), using a sterile spatula and placed immediately inside the sterile polythene covers (10 cm × 5 cm). Labels containing the details on date of collection, place of collection, collector's name, description of the place of collection, and the agro climatic zone in which the sampling was carried out, were written using a pencil and placed inside the polythene cover and secured properly. The soil samples were stored at 4°C until further processing. Before collection of soil samples it was ensured that there was no previous history of use of *B. thuringiensis* in all the sampling places.

Isolation of *Bacillus thuringiensis*

Isolation of *B. thuringiensis* strains was carried out according to the method described by Travers et al. [8]. 1.00 g of soil sample was put in 100 ml of conical flask containing 10 ml of L broth, buffered with 0.25 M sodium acetate pH 6.8. This soil suspension was rotated in a rotary shaker at 250 rpm for 4 h at 30°C. Later the soil suspension was heated at 80°C for 3 min, in a water bath, in order to eliminate the vegetative form of most of the gram positive bacteria and gram negative bacteria. After cooling, serial dilutions (10^{-1} to 10^{-4}) were made in sterile double distilled water. Different dilutions (100 µl) were placed on 'Luria-Bertani (LB) agar' and incubated at 30°C overnight in a bacteriological incubator. There were three replications for each dilution. Anywhere from 1 to 30 different colony types were obtained. The cultures were examined for the presence of parasporal crystals by phase-contrast microscopy. Crystal-forming strains were purified by sub culturing on nutrient agar plates until cultures were axenic as indicated by visual and microscopic examinations. Non-crystal-forming colonies were restreaked on nutrient agar and checked for crystal production after 48 h, since many isolates did not produce crystals until the second or third passage following acetate treatment. Crystal producing strains were grown in 300 ml of T3 medium at 37°C and agitated at 200 rpm on a shaker until sporulation completes, as observed by phase-contrast microscopy. All the selected colonies were purified using single colony isolation and spotted on to master plate on nutrient agar at 4°C until further use. Population of the total *Bacillus* isolates and crystalliferous Bt isolates were determined for each sample by taking into account dilution of the sample. The population was expressed as number of colony forming units (cfu)/unit area per gram of soil used for isolation. *Bt* isolation index was worked out by dividing the population of crystalliferous Bt isolates by the total population of *Bacillus* for each sample collected (Supplementary data).

Oligonucleotide Primers and PCR Analysis for the Detection of Coleopteran Genes

Universal and gene specific primers for different Coleoptera *cry* genes detections were selected from the previous works [9] (supplementary data). The Oligonucleotides were synthesized at Sigma Aldrich Pvt. Ltd. USA. Total DNA was extracted and purified following the method described by Ferrandis et al. [10]. PCR was conducted for 250 ng of total *B. thuringiensis* DNA with 2.5 U of Taq DNA polymerase, 200 nM each dNTP, 1 mM each primer 3 mM MgCl2 in a final volume of 50 µl. Amplification was done in a Applied Biosystems thermal cycler under the following conditions: 5 min of denaturation at 94°C followed by 25 cycles of amplification with a 1 min denaturation at 94°C, 45 s of annealing at 45°C, and 2 min of extension at 72°C. An extra extension step of 10 min at 72°C was added after completion of the 25 cycles [11]. PCR products were analyzed by 1% agarose gel electrophoresis.

Molecular Cloning

PCR amplified products were ligated to the TA cloning vector pTZ57R/T, which was used for transforming *Escherichia coli* DH5 α by standard protocols (Sambrook and Russell, 2001) using Fermentas DNA ligation kit. The transformed cells (60 µl) were spread on LB agar plates containing X-gal (270 µg/ml), IPTG (120 µg/ml) and ampicillin (100 µg/ml). The plates were then incubated overnight at 37°C to screen blue and white colonies. Cloning was confirmed by colony PCR and recombinant plasmid comparison with colony without insert. Finally, cloned fragments were sequenced. BLAST X (version 2.2.6) was used for DNA sequence analysis. Each gene was sequenced at least three times, and a consensus sequence was obtained. Known Cry sequences were obtained from the nonredundant protein database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The sequences of more than 50 Cry proteins were used. Sequence alignments were generated with the CLUSTAL X software program (version 1.8). Dendrograms were compiled from 1,000 independent trials of CLUSTAL X.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the partial *cry* genes obtained from Andaman and Nicobar Islands were submitted to NCBI GenBank database.

Characterization of Parasporal Inclusions and Protein Electrophoresis

For each Bt isolate, a single colony was inoculated into 5 ml T3 broth and incubated in a rotatory shaker, maintained at 30°C at 200 rpm for nearly 48–60 h, and the bacterial sporulation was monitored through a phase contrast microscope. When more than 90% of cells had lysed, the sporulated broth culture was transferred to 4°C, at least half-an-hour before harvesting. The T3 broth containing sporulated culture was centrifuged for 10 min at 10,000 rpm at 4°C. The pellet was washed once with 5 ml of ice-cold Tris–EDTA buffer [Tris 10 mM, EDTA 1 mM, pH 8.0 with 1 mM phenyl methyl sulphonyl fluoride (PMSF)], once with 5 ml of ice-cold 0.5 M NaCl followed by two more washes with 5 ml of Tris–EDTA buffer with 0.5 mM PMSF by centrifuging at the same speed and time. Finally, the spore-crystal pellet was suspended in 100 µl of sterile distilled water containing 1 mM PMSF and stored in −20°C. The protein content of spore–crystal mixtures of the strains containing Coleopteran-specific active *cry* genes was determined by SDS-PAGE analysis, as described by Nazarian et al. [9]. Gels were stained with Coomassie brilliant blue R-250 for 40 min, and de-stained in a solution containing 6.75% (v/v), glacial acetic acid and 9.45% (v/v) methanol.

Results

Out of 120 soil samples, Bt isolates were obtained from 54 soil samples (45% frequency). The highest frequency for isolation of Bt (18.75%) was recorded in the soil samples of Middle Andaman. The lowest frequency (6.25%) for isolation of Bt was observed in the soil samples of South Island, Karmalnd, Rrotland Island, Kanaka, and Bananga. For this purpose, a total of 1634 bacillus like colonies observed through bright field microscopy, 16 isolates were identified as Bt based on the presence of crystalline inclusions (Supplementary data). The isolates with crystalline inclusions were further purified as single colonies and stored as glycerol stocks at −80°C for future studies. These colonies produced both spore and crystal protein. The shape of the crystalline inclusions varied among the Bt isolates. Based on crystal morphology, the 16 isolates of Bt fall into the following eight groups: Spherical, Rectangular, Irregular, Bipyramidal, Spindle, Cuboidal, Rhomboidal, amorphous and crystal attached to spore (Supplementary data). Bipyramidal inclusions were predominantly present in 68.17% of the isolates when compare to other shapes (Supplementary data) [2, 9, 12–14].

Observations of different *Bt* isolates in the vegetative and lysis phase clearly showed the different crystal shapes. The vegetative cells were rod shaped was also similar for all the *B. thuringiensis* isolates. The various crystal shapes like bipyramidal, rhomboidal and spherical were distinct [2, 15]. The crystal shapes that were observed in the light microscope were the same in the transmission electron microscopy also (Fig. 1a–e).

The PCR methodology described here utilized 31 primers to detect 19 different Coleopteran-active *cry* genes, including *cry1* (*cry1B*, *cry1I*), *cry3* (*cry3A*, *cry3B*, *cry3C*), *cry7* (*cry7A*), *cry8* (*cry8A*, *cry8B*, *cry8C*), *cry14*, *cry18*, *cry26*, and *cry28*, *cry34 and cry35*, described to codify for proteins active against Coleoptera Fig. 2 shows some PCR products obtained with native strains [9]. Each known *cry* gene should produce a PCR product with a unique molecular weight. PCR results showed that 13 native strains contained at least one Coleopteran-active *cry* gene (81.25%), and 3 isolates (18.75%) did not give any PCR product when assayed with primers of the Coleopteran-active genes (Fig. 3). Three isolates AND7B, AND8B, and BRT2B contains more than one *cry* gene, suggesting that *B. thuringiensis* strains in general have a high frequency of genetic information exchange compare to other isolates. The results with different *B. thuringiensis* isolates harboring cry1I gene was more prevalent (37.5%) followed by

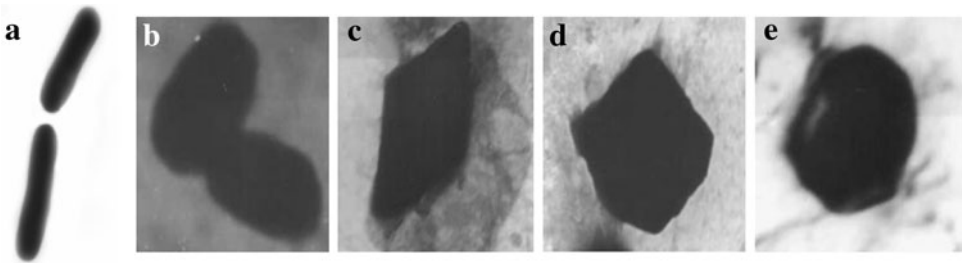

Fig. 1 Transmission electron micrographs of vegetative cell, spore, and crystals. **a** Vegetative cell (×56000), **b** spores (×72000), **c** bipyramidal (×72000), **d** rhomboidal (×72000), **e** spherical (×72000)

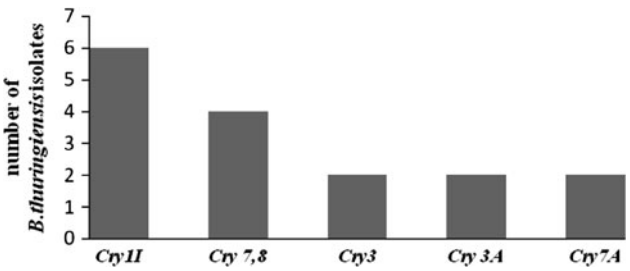

Fig. 2 Distribution of Coleopteran-active *cry*-type genes obtained from 16 fields collected Strains of *B. thuringiensis* identified by PCR analysis with universal and gene specific primers

cry7, 8 genes (25.0%) [9]. The cloned amplicons were subjected to sequencing, and sequences were analyzed with BLAST programs to detect the known *cry* gene to which the gene of the selected strain was the most similar (highest hit) and the level of identity (Fig. 4). A total of 66 sequences were analyzed, and the levels of identity with known *cry* genes ranged from 25 to 99%. All the partial sequences were deposited to NCBI GenBank database HQ018646, HQ018647, HQ018648, HQ018649, HQ018650, HQ018651, HQ018652, HQ018653, HQ018654, HQ018655, HQ018656, HQ018657, HQ018658, HQ018659, HQ018732, and HQ018733.

Bacillus thuringiensis isolates containing *cry* proteins analyzed by SDS-PAGE to estimate the molecular weights of the cry proteins. Isolates presented δ-endotoxins with molecular weights between 20 and 135 kDa [9, 12]. Isolates showed between 1 and 6 different bands (Figs. 5, 6). The SDS-PAGE analysis showed presence of major polypeptides. Cry protein production in isolate AND6, AND8B and SITA was very high and intense. This may be due to higher copy number of plasmids in the cell [16]. One extra band by the isolates AND8B and SITA ~60 kDa might also be responsible for higher activity. Thus, future investigation of this study will include bioassays against resistant insects and various other insects to explore their toxic potential and cloning of novel *cry* genes.

Discussion

As the innovative capacity of the biotechnology fields mostly relies on prokaryotic cells engineering, microbe hunters are in the process of finding more and better strategies to capture and culture brand new strains with potential applications in agriculture [7]. *B. thuringiensis* popularly known as *Bt* is a soil bacterium which is distributed all over the world from diverse habitats, including soil, insects, stored products, dust, deciduous and coniferous leaves, phylloplane, and other miscellaneous habitats [3, 17]. The soil samples of Andaman and Nicobar Islands may yield new isolates of Bt with novel Cry proteins which could be used for control of various pests. In this study, 120 soil samples from Andaman and Nicobar islands were used as source material for isolation of indigenous Bt strains.

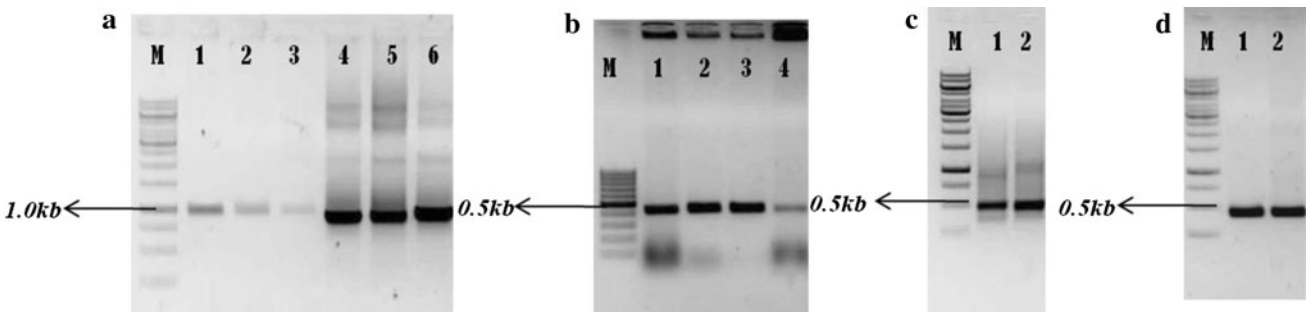

Fig. 3 Agarose (1.0%) gel electrophoresis of PCR products amplified from the selected native *B. thuringiensis* strains of Andaman and Nicobar islands of India. **a** Cry1I specific primer; *M* molecular weight marker (1 kb); *lane 1* AND5, *lane 2* AND7A, *lane 3* AND8A, *lane 4* AND10, *lane* 5 BRT2A, and *lane 6* CON+: positive control standard strain. **b** Cry3A specific primer; *M* molecular weight marker (100 bp); *lane 1* AND7A, *lane 2* AND8B, *lane 3* AND10, and *lane 4* SITA. **c** Cry7, 8 universal primers *M* Molecular weight marker (1 kb); *lane 1* AND7B, and *lane* 2-AND10. **d** Cry7A specific primer; *M* molecular weight marker (1 kb), *lane 1* AND6 and *lane 2* KLP1

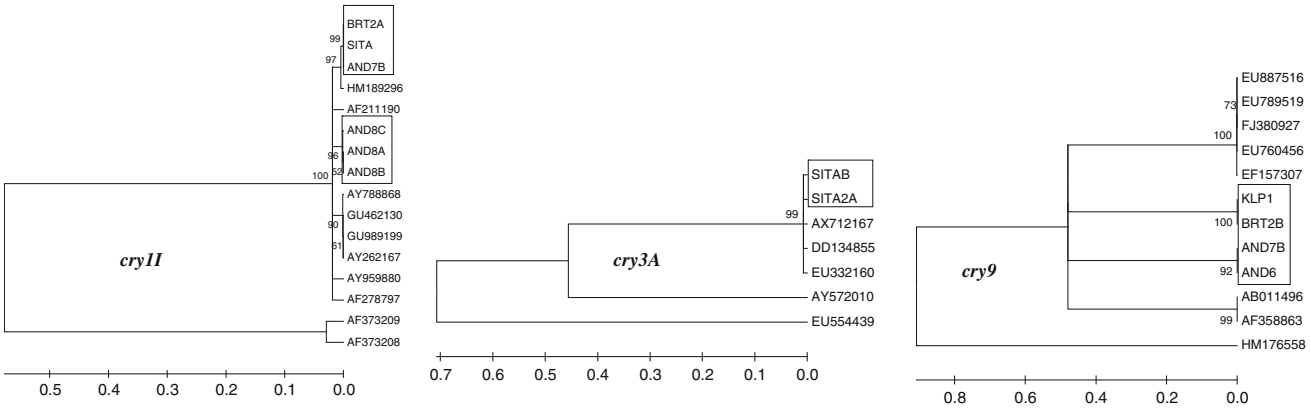

Fig. 4 Rooted neighbor-joining tree showing phylogenetic relationship among different *Bacillus thuringiensis* isolates based on nucleotide region of *cry1I*, *cry3A*, and *cry9* coleopteran-active *cry* genes. The NJ tree was constructed using CLUSTAL W with default parameter. The tree was generated by using TREEVIEW. Sequences were obtained from the databases of the National Center for Biotechnology Information. 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, *cry* strains have originated from common ancestors. Some strains have evolved to be quite distinct and others remain as members of closely related groups

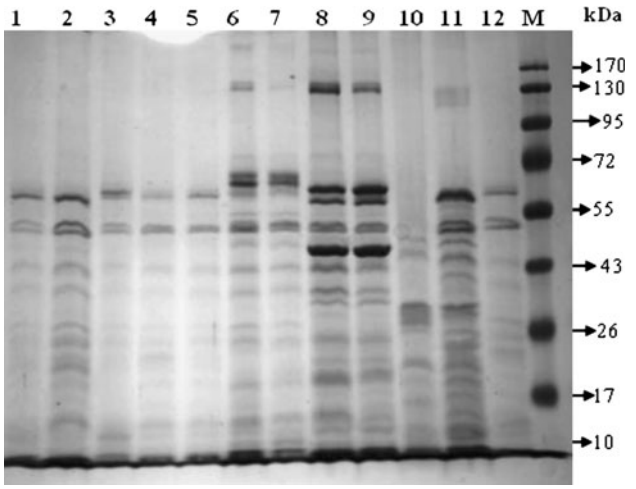

Fig. 5 SDS-PAGE of spore-crystal suspension of selected new isolates of *B. thuringiensis* strains form Andaman and Nicobar islands. *M* protein molecular weight marker, *lane 1* AND7A, *lane 2* AND7B, *lane 3* AND8B, *lane 4* AND8C, *lane 5* AND10, *lane 6* AND6, *lane 7* KLP1, *lane 8* AND8B, *lane 9* SITA, *lane 10* SITAB, *lane 11* SITA2A, and *lane 12* BRT2B

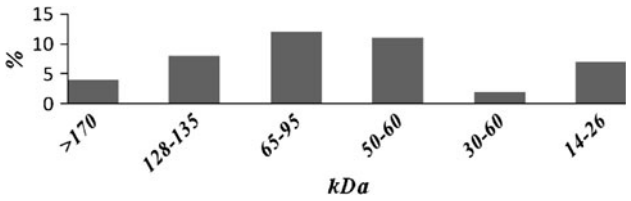

Fig. 6 Protein band patterns in field-collected strains of *B. thuringiensis* identified by SDS-PAGE electrophoresis

Results showed that about 45% of the 120 samples were positive for Bt and yielded 16 isolates. Earlier studies reported varied frequency for isolation of Bt from soil samples ranging from 3–85% [2, 8, 15–17].

Initial identification of Bt is mainly based on the presence of crystalline inclusions. The bright field microscopy is more useful than phase contrast microscopy for high throughput evaluation of bacterial colonies for the presence of crystals and also for identification of small crystals [13]. In this study, 16 of the 1,634 stained bacterial colonies observed through bright field microscopy showed that the presence of crystalline inclusions, and were identified as Bt. Based on the number and shape of the crystalline inclusions, the new isolates of Bt were characterized into eight groups viz., bipyramidal, Cuboidal, bipyramidal with Cuboidal, rectangular, spherical, small spherical, amorphous, and crystals attached to spore. These findings differed from the earlier reports [13]. Rampersad and Ammons [13] reported that 30 and 3.8% of the 79 isolates characterized by them contained dark staining body which appeared as a cap on the spore and small parasporal bodies, respectively. Differences observed in the morphology of crystalline inclusions of Bt suggested presence of diversity in the Bt isolates of Andaman and Nicobar islands [18]. The identification of known *cry* genes in *B. thuringiensis* strains is important, since the specificity of action is known for many of the Cry toxins. This fact allows the possibility of selecting native strains that could be used in control of some targets and of selecting strains with the highest activity. PCR analysis can be considered, with some limitations, a tool to predict the biological activity of a *B. thuringiensis* isolate [6, 8, 17, 19]. This technique was first introduced by Carozzi et al. [7] to identify *cry* genes in order to predict insecticidal activity. Over the last decade, PCR methods for screening *cry* genes present in *B. thuringiensis* collections, including multiplex PCR methods, have been used to identify strains that harbor genes coding for known Cry types [1, 4–6, 11, 17, 18]. Then, these

results were used for prediction of toxicity of the isolates on different pests based on the PCR results.

Cry proteins are very selectively active against certain insect species, so the strains containing several types of *cry* genes encoding highly active insecticidal crystal proteins might be predicted to have a wider pest spectrum or increased activity. This would lead to additional broad spectrum and highly toxic *B. thuringiensis* insecticides [9, 17]. This study was the first research in which *B. thuringiensis* isolates were screened almost for all coleopteran-active *cry* genes (19 *cry* genes). The final aligned sequences were deposited to NCBI and phylogenetic tree was constructed which clearly indicate the nucleotide variation among the Bt isolates containing *Cry1I* gene of Andaman and Nicobar.

Grouping of Bt isolates according to crystal protein(s) profile studied by SDS-PAGE will give a prelude for the presence of diversity in *cry* genes. The diversity of *cry* gene profiles, molecular weights of the δ-endotoxins observed in the SDS-PAGE, and different shapes of crystals, suggest that there are strains expressing different Cry proteins that could be toxic against Coleopterans, Dipterans, and Lepidopterans, as were shown previously [8, 9, 12, 19, 20]. Isolates presented δ-endotoxins with molecular weights between 20 and 135 kDa. Therefore, analysis of crystal proteins(s) profile could be useful to predict the presence of *cry* genes. In this study, 45.45% of the 16 isolates are having 135 and/or 65 kDa proteins suggesting the presence of genes related to Cry families. Other isolates showed that the presence of 95 or 30 or 14 kDa proteins indicating the presence of other novel cry genes also [12]. These results lead us to suggest the presence of diversity in Bt isolates of Andaman and Nicobar isolates. Results of preliminary studies on PCR and partial sequencing of nucleotides of *cry* genes also indicated variations in the Bt isolates of Andaman and Nicobar islands. Further studies on isolation of full length gene, cloning and characterization, recombinant protein production of *cry* genes and toxicity studies on different order of insect pests from these new isolates of Bt will be useful to open new vistas in the area of integrated pest management for sustainable agriculture.

Acknowledgment Authors are grateful to the Director, Indian Institute of Horticultural Research (IIHR) for providing the encouragement and technical support to carry out the research. This project is funded under ICAR network project on Application of microbes for agriculture and allied sector through National Bureau of Agriculturally Important Micro organisms (NBIAM) is being acknowledged.

References

1. Kaur S, Singh A (2000) Distribution of *Bacillus thuringiensis* isolates in different soil types from North India. Indian J Ecol 27:52–60
2. Asokan R, Puttaswamy (2007) Isolation and characterisation of *Bacillus thuringiensis* Berliner from soil, leaf, seed dust and insect cadaver. J Biol control 21(1):83–90
3. Ben-Dov E, Manasherob R, Zaritsky A, Barak Z, Margalith Y (2001) PCR analysis of *cry7* genes in *Bacillus thuringiensis* by the five conserved blocks of toxins. Curr Microbiol 42:96–99
4. Ben-Dov E, Wang Q, Zaritsky A, Manasherob R, Barak Z, Schneider B, Khamraev A, Baizhanov M, Glupov V, Margalith Y (1999) Multiplex PCR screening to detect *cry9* genes in *Bacillus thuringiensis* strains. Appl Environ Microbiol 65:3714–3716
5. Bernhard K, Jarrett P, Meadows M, Butt J, Ellis DJ, Roberts GM, Pauli S, Rodgers P, Burges HG (1997) Natural isolates of *Bacillus thuringiensis*: worldwide distribution, characterization, and activity against insect pests. J Invertebr Pathol 70:59–68
6. Beron CM, Curatti L, Salerno GL (2005) New strategy for identification of novel *cry*-type genes from *Bacillus thuringiensis* strains. Appl Environ Microbiol 71(2):761–765
7. Carozzi NB, Kramer VC, Warren GW, Evola S, Koziel MG (1991) Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl Environ Microbiol 57:3057–3061
8. Travers RS, Martin PAW, Reichelderfer CF (1987) Selective process for efficient isolation of soil *Bacillus* spp. Appl Environ Microbiol 56:1263–1266
9. Nazarian A, Jahangiri R, Jouzani GS, Seifinejad A, Soheilivand S, Bagheri O, Keshavarzi M, Alamisaeid K (2009) Coleopteran-specific and putative novel *cry* genes in Iranian native *Bacillus thuringiensis* collection. J Invertebr Pathol 102:101–109
10. Ferrandis MD, Juárez-Pérez VM, Frutos R, Bel Y, Ferré J (1999) Distribution of *cryI*, *cryII* and *cryV* genes within *Bacillus thuringiensis* isolates from Spain. System Appl Microbiol 22: 179–185
11. Juárez-Pérez VM, Ferrandis MD, Frutos R (1997) PCR-based approach for detection of novel *Bacillus thuringiensis cry* genes. Appl Environ Microbiol 63:2997–3002
12. Ramalakshmi A, Udayasuriyan V (2010) Diversity of *Bacillus thuringiensis* isolated from Western Ghats of Tamil Nadu State. India Curr Microbio 61:13–18
13. Rampersad J, Ammons D (2005) A *Bacillus thuringiensis* isolation method utilizing a novel stain, low selection and high throughput produced atypical results. BMC Microbiol 5:52–63
14. Vidal-Quist JC, Castañera P, González-Cabrera J (2009) Diversity of *Bacillus thuringiensis* strains isolated from citrus orchards in spain and evaluation of their insecticidal activity against *Ceratitis capitata*. J Microbiol Biotechnol 19(8):749–759
15. Whiteley HR, Schnepf (1986) The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. Ann Rev Micobiol 40:549–576
16. Vilas-Boas GT, Lemos MVF (2004) Diversity of *cry* genes and genetic characterization of *Bacillus thuringiensis* isolated from Brazil. Can J Microbiol 50:605–613
17. Uribe D, Martinez W, Ceron J (2003) Distribution and diversity of *cry* genes in native strains of *B. thuringiensis* obtained from different ecosystems from Colombia. J Invertebr Pathol 82: 119–127
18. Martin PAW, Travers RS (1989) Worldwide abundance and distribution of *B. thuringiensis* isolates. Appl Environ Microbiol 55:2437–2442
19. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
20. Jouzani GS, Abad AP, Seifinejad A, Marzban R, Kariman K, Maleki B (2008) Distribution and diversity of dipteran-specific *cry* and *cyt* genes in native *Bacillus thuringiensis* strains obtained from different ecosystems of Iran. J Ind Microbiol Biotechnol 35(2):83–94