MOLECULAR CLONING OF *cry* GENES SPECIFIC TO DIPTERANS FROM NATIVE *Bacillus thuringiensis* BERLINER

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THESIS

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Centre for Plant Biotechnology and Molecular Biology

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2010

DECLARATION

I, hereby declare that this thesis entitled **“Molecular cloning of *cry* genes specific to dipterans from native *Bacillus thuringiensis* Berliner”** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara

M. Sivaji
(2007-11-110)

CERTIFICATE

Certified that this thesis entitled **"Molecular cloning of *cry* genes specific to dipterans from native *Bacillus thuringiensis* Berliner"** is a bonafide record of research work done independently by **Mr. M. Sivaji** under my guidance and supervision and that it has not formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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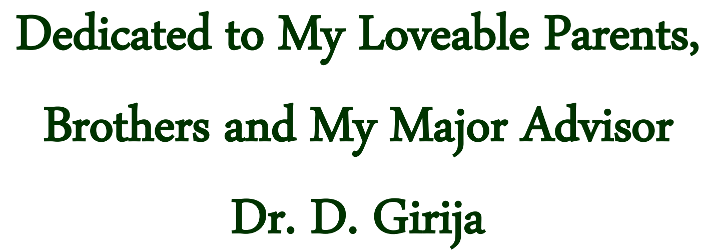
ABBREVIATION

A	Adenine
bp	Base pair
Bt	*Bacillus thuringiensis*
BLAST	Basic local alignment search tool
C	Cytosine
^{o}C	degree Celsius
cm	Centimeter
CPBMB	Centre for Plant Biotechnology and Molecular Biology
DMFO	Dimethyl formamide
DNA	Deoxyribo Nucleic Acid
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
E- PCR	Exclusive PCR
G	Guanine
g	Gram
ICP	Insecticidal crystal protein
IPTG	Isopropyl thio galactoside
IPM	Integrated pet management
KAU	Kerala Agricultural University
kb	kilo base
kDa	kilo Dalton
LB	Luria Bertani
LC	Lethal concentration
M	Mole
MDa	Mega Dalton
mg	Milligram
min	minute
ml	Millilitre
mM	Millimole

µg	Microgram
µl	Microlitre
µM	Micromole
NCB1	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometer
OD	Optical Density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulfonyl flouride
pH	Hydrogen ion concentration
%	Percentage
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
sec	Second
SET	Sucrose, EDTA, Tris HCl
T	Thymine
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UV	Ultra violet
V	Volts
vip	Vegetative insecticidal protein
v/v	Volume by volume
w/v	Weight by volume
X-gal	5- bromo 4 chloro 3- indolyl β-D galactosidase


Dedicated to My Loveable Parents,
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Dr. D. Girija

Introduction

Introduction

Agriculture has been a means of livelihood and a source of food since the dawn of human civilization. About one third of the total crop yield is lost due to insect pests, pathogens and weeds (Huang *et al*., 2002). Control of agricultural pest populations is achieved mainly by the application of chemical insecticides. The continuous use of synthetic pesticides leads to serious problems like environmental degradation and development of resistance in insect pests (Shelton *et al*., 2002). Recently, there has been a renewed interest in the development of biological alternatives to chemical pesticides.

The preference of using biopesticides over synthetic chemicals has been widely accepted in different parts of the world for several reasons. Biological pesticides are more safe, because they are degradable and have a high level of safety for non-target organisms (human beings, animals and fish) in addition to their host specificity (Cartlon, 1988; McGaughy and Whalon, 1992). Another important advantage of biopesticides is their lower resistance in the target pest populations. 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 and protozoans that infect or intoxicate specific pest groups (Carlton, 1988; Spear, 1987).

Different *Bacillus* species (bacteria) have been isolated and are commonly recognized as a definite insect pathogen. The most widely accepted species are *B. thuringiensis*, *B. lentimorbus, B. larvae, B. popilliae* and certain strains of *B. sphaericus* (Spear, 1987). Among these, *Bacillus thuringiensis* Berliner is considered as one of the most versatile microbial insecticides. It is a gram-positive spore-forming soil bacterium. The entomopathogenic activity of this bacterium is principally due to the presence of proteinaceous inclusions that can be distinguished as distinctively shaped crystals

under phase contrast microscope. These inclusions are comprised of proteins known as insecticidal crystal proteins (Cry proteins) or δ-endotoxins (Hofte and Whiteley, 1989).

Cry proteins have been used as biopesticides on a significant scale for more than 30 years, and their safety has been demonstrated. Cry toxins constitute a family of related proteins that can kill insects belonging to Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Homoptera as well as other invertebrates (Schnepf *et al*., 1998 and Feitelson *et al.,* 1999). The greatest success stories in microbial pesticides have come from usage of *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *kurstaki* which are toxic against Diptera and Lepidoptera respectively (Spear,1987).

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.

About 70 species of fruit flies are considered as pests of important agricultural and horticultural crops. Most of the pest species of Tephritidae (fruit flies) attack fruits, the great majority of them belong to the genera *Anastrepha*, *Ceratitis*, *Bactrocera*, *Dacus* and *Rhagoletis*. The hosts of these flies belong to a wide variety of families of plants and include many major commercial crops. The genus *Bactrocera* is the most economically significant genus, with about 40 species considered to be important pests. Orchid fly (*B. dorsalis*), melon fly (*B. cucurbitae*), olive fruit fly (*B. oleae*), Queensland fruit fly (*B. tryoni*) and peach fruit fly (*B. zonata*) are among the most important species (White and Elson-Harris, 1992).

Gall midges induce formation of plant galls and reduce economic yield of crops. Gall insects of melon vegetables, mango and rice are some examples. The family Agromyzidae is comprised exclusively of phytophagous flies whose larvae develop inside host-plant organs, as strictly endophagous herbivores. Agromyzids are frequently called “leaf miner flies” because 80 per cent of known agromyzid species feed within the leaf lamina in their larval stage (Spencer 1990). Leaf-mining larvae reduce the vigour of their host plants by both consuming photosynthetically active leaf tissue and inducing plant response. Heavy infestation not only reduces the yield but in some cases also causes high plant mortality. Some stem miners damage the vascular bundles of stem and root, thereby affecting the transportation of nutrients.

Blood-sucking insects such as mosquitoes and blackflies are vectors of many diseases affecting human and animals. Most important species is *Aedes aegyptii*, the primary carrier for viruses that cause dengue fever and yellow fever. *Culex pipiens* and *Anopheles* are carriers of filariasis and malaria respectively. House flies transmit dreadful diseases such as dysentery and cholera, through protozoa and bacteria carried their feet and mouthparts (Dai, 1992; Georghiou and Wirth, 1997). 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 (Crickmore *et al*, 1998).

Based on the above information, the present study entitled “Molecular cloning of *cry* genes specific to dipterans from native *Bacillus thuringiensis* Berliner” was taken up with the objective of molecular cloning and characterization of *cry* genes specific to dipteran larvae from native isolates of *Bacillus thuringiensis*.

Review of literature

2. REVIEW OF LITERATURE

The damage caused by pests and diseases in various crops in the field and in stored grains in India is estimated to be 30-90 per cent, which accounts for an annual loss of around Rs.10, 000 crores. Several dipteran insects like fruit flies, gall insects and leaf miners are pests of many economically important agricultural and horticultural crops and cause heavy losses. Some dipteran insects like mosquitoes, house flies, sand flies, eye flies and horn flies transmit various fatal and chronic diseases in both human beings and animals. While chemical pesticides have played an important role in increasing food production in India, their indiscriminate use has led to several environmental problems such as pesticide residues in food products, environmental contamination and development of resistance in target organisms (van Rie *et al*., 1989; Ferre *et al*., 1991).

Therefore it has become a general issue to look for alternative ways to control insects and pests, such as the use of bioinsectcides, which act more specifically on major crop pests, without causing biological impacts similar to those generated by the use of chemical insecticides. Bioinsectcides like *Bacillus thuringiensis* are less harmful to non target organisms and the environment than chemical insecticides.

During the past few years, the advancement in plant genetic engineering tools for crop improvement has led to the development of crop plants resistant to insect attack. Several crystal toxin genes of *Bacillus thuringiensis* have been cloned and expressed in bacteria like *Pseudomonas*, baculoviruses like Nucleopolyhedrosis viruses and plants like tobacco. Recent advances in molecular biology and genetic engineering have enabled scientists to modify the genetic constitution of biocontrol organisms with the view of achieving better or desirable traits.

In this study, attempts have been made to characterize and clone dipteran specific *cry* genes from *B. thuringiensis* isolated from the Western Ghats of Kerala. A comprehensive review of the previous research studies related to the topic was carried out in accordance with the objectives of the present study.

2.1 History of *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) is a gram positive, aerobic, endospore-forming bacterium. It is recognized by its parasporal body (known as crystal) that is proteinaceous in nature and possesses insecticidal properties. Those insecticidal proteins, synthesized during sporulation are tightly packed by hydrophobic bonds and disulphide bridges (Jung *et al*., 1995; Srinivas *et al*., 1997).

The first record on Bt goes back to 1901, when Ishiwata discovered a bacterium from diseased silkworm larvae that he named *Bacillus sotto* (Ishiwata, 1901). Between 1902 and 1910, Berliner working at a research station for grain processing in Berlin, investigated an infectious disease of the Mediterranean flour moth (*Ephestia kuehniella*). The infected insects were originally obtained from a mill in the district of Thuringen. In a detailed report, Berliner (1915) described spore-forming bacterium as the causative agent and designated it as *B. thuringiensis.*

The first practical application of Bt was reported by Husz (1928) who isolated a Bt strain from *Ephestia* and tested it on European corn borer. The work eventually led to the first commercial product, sporeine, which was produced in France in 1938 (Luthy *et al*., 1982). There are many subspecies and serotypes of Bt with a range of well characterized insecticidal proteins or Bt toxins. Bt toxins kill insects among the Lepidoptera, Coleoptera, Diptera and nematodes (Hofte and Whiteley, 1989; Feitelson *et al*., 1992). By the virtue of the of lack of toxicity toward other species of animals, human beings, and plants, there is tremendous

potential for exploiting Bt as a biological control agent (Bradley *et al*., 1995; Salama *et al*., 1995).

2.2 Insecticidal crystal proteins

Angus (1956) proved that the insecticidal activity was caused by parasporal inclusions, by separating the toxin from spores. These are known as insecticidal crystal proteins (ICPs) or delta-endotoxins.

Many *B. thuringiensis* strains with different insect host spectra have been identified (Burges, 1981). The crystalline inclusions produced during sporulation consist of one or more insecticidal proteins exhibiting a highly specific insecticidal activity (Aronson *et al.,* 1986; Whiteley and Schnepf, 1986). They are classified into different serotypes or subspecies based on their flagellar antigens. Most strains are active against larvae of certain members of Lepidoptera. But some show toxicity against dipteran (Federici *et al.,* 1990) or coleopteran species (Krieg *et al.,* 1983). Fietelson *et al*. (1992) reported *Bacillus thuringiensis* isolates active against other insect orders like Hymenoptera, Homoptera, Orthoptera, Mallophaga and also against nematodes, mites and protozoa.

B. thuringiensis crystalline inclusions dissolve in the larval midgut, releasing one or more insecticidal crystal proteins of 27 to 140 kilodaltons (kDa). Most crystal proteins are protoxins that are proteolytically converted into smaller toxic polypeptides in the insect midgut. The activated toxin interacts with the midgut epithelial cells of susceptible insects. The genes that encode ICPs reside mostly on plasmids. A subspecies can synthesize more than one type of insecticidal crystal protein (Kronstad *et al.,* 1983). They have various forms such as spherical, bipyramidal, cuboidal, flat rhomboidal or composite with two or more crystal types (Chilcott and Wigley, 1994).

More than 150 insects belonging to the orders Lepidoptera, Diptera and Coleoptera are known to be susceptible to *B. thuringiensis* (Beegle and Yamamoto, 1992). Insecticidal crystal proteins are extremely potent and toxic to target insect larvae at pico mole concentrations. Different domains of insecticidal crystal proteins are responsible for host susceptibility and toxicity.

2.2.1 Classification of *B. thuringiensis* crystal proteins

Bt strains have been classified on the basis of different aspects. Bt strains have been classified on a subspecies level on the basis of flagellar H-antigen typing, biochemical tests of esterases, lectins, enzymes and antibody production (Yamamoto and Powell, 1993). Eighty two serovars of Bt have been recognized on the basis of flagellar H antigen (Lecadet *et al*., 1999). Indirect immunofluorescence (Chitra *et al*., 1998) and Fourier transform infrared spectroscopy (Beattie *et al*., 1998) have also been used for identification of Bt strains.

Bt strains have also been classified on the basis of toxicity towards target insects. Bt strains active against a range of invertebrates including Hymenoptera, Homoptera, Mallophaga, Orthoptera, nematodes and mites have been reported (Marroquin *et al*., 2000). Insect bioassays, however, being time-consuming and exhaustive, pose a limitation for rapid preliminary screening of large Bt collections. Similarly, biochemical testing and flagellar antigen typing, though useful for determination of subspecies do not directly reflect the specific *cry* genes present in a particular Bt strain. *cry* gene profiling could be used for classification of Bt strains (Wang *et al*., 2003).

Many *cry* genes have shown the presence of five conserved blocks of homology, which are of use in their identification. Earlier, *cry* genes were classified into four major classes: *cry*I (Lepidoptera specific), *cry*II (Lepidoptera and Diptera specific), *cry*III (Coleoptera specific) and *cry*IV (Diptera specific), on

the basis of sequence homology and spectrum of insecticidal activity (Hofte and Whiteley, 1989; Feitelson *et al.,* 1992). New genes encoding a diverse set of proteins without a common insecticidal activity each received the name *cry*V*,* based on the next available Roman numeral (Shin *et al.,* 1995).

However, the nomenclature of Hofte and Whiteley (1989) failed to accommodate toxins that belonged to same class but with a different insecticidal spectrum. Hence, Crickmore *et al.* (1998) introduced a system of classification based on amino acid homology, where each protoxin acquired a name consisting of the Cry (or Cyt) and four hierarchical ranks consisting of Arabic numbers, capital letters, lower case letters and Arabic numbers (e.g. Cry22Aa1). Thus, proteins of less than 45 per cent homology differ in primary rank (Cry1, Cry2 etc.), and 78 per cent and 95 per cent identity constitute the border for secondary (Cry1A, Cry1B) and tertiary ranks (Cry1Aa, Cry1Ba), respectively. Quaternary ranks are given to those proteins, which are more than 95 per cent similar in amino acid sequence (Cry1Aa1 and Cry1Aa2).

2.2.2 Crystal morphology and solubility

Crystal morphology in Bt is highly complex and it shows different forms like bipyramidal, cuboidal, spherical, squares and irregular (Chilcott and Wigley, 1994). Bipyramidal crystals are active against lepidopteran larvae (Attathom *et al*., 1995) and cuboidal crystals are active against lepidopteran and dipteran larvae or lepidopteran larvae alone (Yamamoto and Mclanghlin, 1981). Spherical and irregular crystals are mostly mosquitocidal, often active against certain coleopteran species (Krieg *et al*., 1983). Irregular crystals also include those with very little or no identified toxicity (Zelanzy *et al*., 1994). The crystal toxin is insoluble in water or inorganic solvents, but soluble in alkaline solvents. Cry1 proteins are soluble at pH 9.5, while the Cry2 proteins are soluble at a pH of about 12. Similarly, Cry4A, Cry5B and Cyt toxins are soluble at pH 9.5, while the Cry4D toxin requires a pH of 12.

The Cry3A toxin on the other hand, dissolves at pH below 4 and above 9.5 (Koller *et al.,* 1992).

2.3 Structure and mechanism of Bt crystal protein

2.3.1 Structure of crystal protein

The N-terminal domain (domain I) contains seven α-helical domains that are arranged in three pairs around a central helix. It is involved in membrane insertion. Domain II consists of three symmetrically folded β–sheets and plays a role in receptor recognition and binding. The C-terminal (domain III) consists of two β-sheets in a jellyroll conformation and is involved in binding and recognition as well as pore formation and channel specificity. The three domains form an upside-down ”L” shape with domain III stacked on domain II and domain I hanging off the side (Schnepf *et al.,* 1998; de Maagd *et al.,* 2001).

2.3.2 Insecticidal mechanism of crystal protein

Insecticidal crystal proteins, endospores or beta-exotoxins dominate bioactivity of *Bacillus thuringiensis.* To be effective as an insecticide, the insecticidal crystals must first be ingested by *B. thuringiensis* sensitive insects. Phytophagous insects require an extremely alkaline midgut. The pH in insect midgut is normally found to be in the range of 10 to 11. Goblet cells in the midgut epithelium play a critical role in the maintenance of pH by secreting potassium carbonate into the lumen of the midgut.

The Cry proteins solubilized from crystal inclusions of the Bt spore are inactive in their pro-toxin form (Choma *et al.,* 1990). Before toxicity can occur, the pro-toxin must be proteolytically processed. This requires the high pH found in the midgut as well as digestive enzymes from the insect. Activation involves the removal of both carboxyl and amino terminals of the protein (Gringorten,

2001). Once activated, the Cry toxin diffuses from the lumen through the periplasmic membrane into the endoperiplasmic space. The fully processed and active Cry toxin now has access to the surface of the columnar epithelial cells (Hill and Pinnock, 1998).

At the cell surface, the Cry protein binds to its receptor (Ferre and van Rie, 2002). Amino peptidases (Luo *et al.,* 1997) are involved in digestion and cell adhesion and are similar to cadherins (Vadlamudi *et al.*, 1995). These molecules function as receptors for Cry proteins. Cry proteins are composed of three distinct domains. After binding, domain I goes through a rearrangement similar to that of opening an umbrella. The three pairs of α-helices in domain I open and insert into the membrane, placing domain III at the membrane surface over the inserted helices. Insertion of the Cry protein appears to be irreversible (Li *et al.,* 2001). Next, aggregation of inserted Cry protein occurs, resulting in the formation of pores. The pores are most likely tetramers and form a K^+ selective ion channel (Gringorten, 2001).

The formation of the channel immediately leads to two very significant and detrimental physiological changes in the insect. First, the K^+ gradient on the epithelial cells is disrupted, which leads to an increase in haemolymph K^+ concentrations. Next, the pH gradient is disrupted leading to a decrease in the pH of the midgut lumen and an increase in the hemolymph pH. Ultimately, the affected cells are destroyed by the high pH of the midgut and osmotic lysis. As a result of the lysis of cells in the midgut epithelium, the spore is allowed to germinate in a nearly neutral environment, bathed in the nutrients from ruptured cells. Most insects are not killed directly by the effects of the toxin but die as a result of rapidly induced gut paralysis and feeding inhibition, and subsequent starvation or septicemia (Gringorten, 2001).

2.4 Ecology of *Bacillus thuringiensis*

Many *Bacillus thuringiensis* are isolated from numerous sources, including; soil, grain dust, diseased insect larvae and sericulture environments and phylloplane. Soil has been the principal source of novel *B. thuringiensis* (Dulmage and Aizawa, 1982; Smith and Couche, 1991). It also has been found extensively in the phylloplane. Numerous subspecies have been isolated from coniferous trees, deciduous trees and vegetables. These have also been recovered from soil samples and stored products and aquatic habitats (Schnepf *et al*., 1998; Iriarte *et al*., 2000). Spores persist in soil and vegetative growth occurs when nutrients are available.

2.5 Use of Bt in pest management

The application of microorganisms for control of insects was proposed by notable early pioneers in invertebrate pathology such as Louis Pasteur and Elie Metchnikoff (Steinhaus, 1975). *B. thuringiensis* is the most versatile biopesticide for use in pest management. It is used in agricultural crops, harvested product in storage, ornamentals and water bodies to control various groups of insects, depending on the type of toxin produced by the specific isolate of *B. thuringiensis*. Today a number of isolates of the bacterium are commercially produced with activity against Lepidoptera, Coleoptera, and Diptera (Shah and Goettel, 1999).

Sears *et al.* (1983) reported the successful use of *B. thuringiensis* in an IPM programme against cabbageworm (*Pieris rapae*). Goldberg and Margalit (1977) discovered *B. thuringiensis* var. *israelensis* active against mosquito (*Aedes aegypti*) and blackfly larvae (*Simulium* sp). This was the first report of bacterial strain killing non-lepidopteran target and this doubled the number of known susceptible insect orders. The range of *B. thuringiensis* toxicity was once again

expanded by the discovery of bacterial strains active on Colorado potato beetle (*Leptinotarsa decemlineata*).

The first large scale Bt based product was released in 1957, which was a *Bacillus thuringiensis* var. *kurstaki* strain (Feitelson, 1993). As of 1998 about 200 *B. thuringiensis* based products were registered in the United States alone (Schnepf *et al.,* 1998). *B thuringiensis* subsp. *israelensis* is used exclusively or in combination with other interventions for the control of larvae of dozens of species of medically important and pestiferous blackflies and mosquitoes around the world (Lacey and Undeen, 1986; Skovmand *et al.,* 2000). *B. thuringiensis* subsp. *jegathesan* is native to Malaysia, and produces a very complex parasporal body containing at least seven major poly peptides, including Cry11B. This toxin showed higher toxic activity against mosquito (Delecluse *et al.,* 1995).

B. thuringiensis J112 was toxic to third instar larvae of the fruit fly *Drosophila melanogaster* and to second juvenile stage of the nematodes *Meloidogyne incognita* and *M. javanica* (Abu-Hazeem, 2002). In United States *B. thuringiensis* has become the major pesticide used against the gypsy moth (Machesky, 1989). Preparations of *B. thuringiensis* are extensively utilized as a safe and effective pesticide in horticulture and forestry (Kellar and Langenfruch, 1993; Teakle, 1994) and in the control of mosquitoes and blackflies (Ritchie, 1993). Intact parasporal crystals produced by *B. thuringiensis* subsp. *israelensis* are toxic to larvae of several dipteran insects, including mosquitoes, black flies and horn flies (Temeyer, 1984; Margalit and Dean, 1985) and solubilized preparations of the crystal also have been shown to kill adult dipteran insects, such as mosquitoes (Klowden and Bulla, 1984), blackflies (Klowden *et al.,* 1985), and stable flies (Wilton and Klowden, 1985).

Bacillus thuringiensis subsp. *tenebrionis* active on coleopteran pests has been shown to control Colorado potato beetle under field conditions and is now being used commercially (Ferro and Gelernter, 1989). *B. thuringiensis* had been

used operationally for controlling forest caterpillar pests for many years (Cunningham, 1988). Bowen (1991) reported the potential of the bacterium in IPM for arboriculture. It also has the potential to be used as a bioinsecticide against peach twig borer. Edwards and Ford (1992) cited the use of the bacterium as a crop protectant in corn for European corn borer (*Ostrinia nubilalis)*; in soybeans for green clover worm (*Hypena scabra*) and soyabean looper (*Pseudoplusia includens*).

Bacillus thuringiensis based microbial pesticides are used extensively in integrated pest management programmes in vegetables, either due to the concern about residue on harvested products or to decrease the selection pressure from the use of conventional pesticides for control of insects like diamond back moth (*Plutella xylostella*) and cabbage looper (*Trichoplusia ni*) in cole crops, lettuce and tomato (Zalom *et al.,* 1992). It is used against leaf roller in tree fruit crops. New genetically engineered *B. thuringiensis* products may provide more opportunities and choices for growers using IPM programmes. The most successful products will be the ones that provide efficacy and consistency so that these can be competitive with traditional chemicals.

2.6 Insect resistance to *Bacillus thuringiensis*

Development of resistance in insects to pesticides has long been a major concern within the agricultural community. Over 500 species of insects have become resistant to one or multiple synthetic chemical insecticides in the absence of integrated pest management (Georghiou and Tajeda, 1991). In the past, it was hoped that insects would not develop resistance to *B. thuringiensis* toxins, since *B. thuringiensis* and insects have co-evolved. But a number of insect species were found capable of developing resistance to *B. thuringiensis* toxins in the laboratory (McGaughey and Beeman, 1988). Resistance to *Bacillus thuringiensis* insecticides can be initiated by alteration of the target of insect-toxin interaction (Gould *et al.,* 1995; Maclintosh *et al.,* 1991). Resistance to the bacterium was first reported in

Indian mealmoth (*Ploidia interpunctella)* larvae collected from grain storage facilities that had been treated with *B. thuringiensis* (Gelernter, 1997).

Development of resistance to the bacterium due to reduced binding of Bt toxin to the brush border membrane of the midgut epithelium in insect was reported from diamond back moth, *Plutella xylostella* (Tabashnik, 1990; Ferre *et al.,* 1991; Shelton *et al.,* 1993). Substantial resistance to crystal proteins was found in insect populations inhabiting water stress fields treated with *B. thuringiensis* insecticides (Tabashnik, 1994). Resistance was due to the mutation of one locus affecting the ability of a variety of toxins as in the *H. virescens* colony with broad resistance (Gould *et al.,* 1995).

Laboratory selection resulted in *B. thuringiensis* resistance in the tobacco budworm (*Heliothis virescens)* and Colorado potato beetle (Whalon *et al.,* 1993). Kranthi *et al.* (2000) reported seventy six fold resistance in an Indian population of Cotton bollworm (*Helicoverpa armigera*) against *cry*1Ac after ten generations of selection. Indian populations of *Helicoverpa armigera* were significantly less susceptible to *cry*IIAa than *cry*I (Chakrabarthi *et al*., 1998; Babu *et al.,* 2002). A laboratory selected strain of *Heliothis virescens* that is over 10,000 times more resistant to *cry*1Ac than wild type strains was reported by Gould *et al.* (1995).

2.6.1 Resistance management

Various strategies were suggested to tackle the problem of resistance development and these have been summarized by Whalon and McGaughey (1993). These include rotation or alteration of toxins, mixture or sequence of toxins, provision of refuges, ultra high dose of toxin and temporal and spatial expression of Bt toxin genes in transgenic plants. Studies indicating considerable instability of resistance to Bt in *P. xylostella* (Hama *et al.,* 1992), *H. virescens* (Sims and Stone, 1991) and one case of negative cross resistance in *P. interpunctella* (van Rie *et al.,* 1990a,b), suggest that rotations might slowdown

resistance development in certain situations. Trumble (1985) demonstrated the benefit of use of *B. thuringiensis* subsp. *kurstaki* in a pesticide rotation to decrease the development of resistance in target insects.

Denholm and Rowland (1992) advocated a high-dose strategy in conjunction with untreated refuges as a potential means of managing resistance development in transgenic plants. This approach maintains that constitutive and continuous expression of Bt toxins in transgenic plants may be sufficient to kill all of the heterozygotes in a population (McGaughey and Whalon, 1992). Rajamohan *et al.* (1995) constructed several mutant toxins that increased toxicity, especially to gypsy moth about 7-10 times more potent than the parental toxin.

Hybrid wide spectrum toxins, by switching the toxicity determining regions of different Cry toxins, may improve toxicity and yield a toxin with multiple insect specificity through protein engineering. Since continuous exposure of insect pests to a single kind of bacterial toxin had led to rapid development of resistance in insects, it is essential to focus on identification of more potential genes, for the success of *B. thuringiensis* transgenic crops in India.

2.7 Maintenance of *Bacillus thuringiensis*

After isolation of *B. thuringiensis*, it is essential to maintain the organism for further studies. To maintain bacterial cultures there are several methods such as preservation at -40^{0}C in glycerol, freeze drying method (lyophilization), storage at -196^{0}C (liquid nitrogen method) (Aneja, 2003).

2.8 Morphololological and biochemical characterization of *B. thuringiensis*

Many phenotypic and genotypic methods are presently being employed for microbial identification and classification (Louws *et al.,* 1996). Each of these

methods permits a certain level of phylogenetic classification from the genus, species, subspecies, and biovar to the strain specific level.

B. thuringiensis isolates were characterized based on the growth habits on culture medium. Colony morphological features like shape, size, colour, elevation etc., were used as criteria for cultural characterization. *B. thuringiensis* isolates were usually characterized and grouped based on biochemical tests. Martin and Travers (1989) identified *B. thuringiensis* strains by using biochemical tests such as starch hydrolysis, urease production, mannose, sucrose and salicin fermentation, esculin utilization and lecithinase production.

Benhard *et al.* (1997) studied the crystal characteristics of the bacterial strains isolated from different countries. The parasporal inclusions were examined to study the crystal morphology, crystal size relative to the spore, the number of crystals in each spore and whether each cell produced a crystal. Crystal morphology was classified as bipyramidal, spherical, rectangular or cuboidal, irregularly spherical or irregularly pointed.

Porcar and Caballero (2000) compared the morphological characteristics of new *B. thuringiensis* strains isolated from dead larvae of lepidopteran, Cosmopiltan (*Mythemna loreyi)* with standard strains of *Bacillus thuringiensis* var. *kurstaki and Bacillus thuringiensis* var. *aizawai*. The isolated strains showed similar morphological and biochemical characteristics to the standard strains. Kaelin and Gadani (2000) analyzed a total of 133 tobacco samples of different types and origins. Nine percent of samples showed the presence of *B. thuringiensis* and majority of the isolates produced bipyramidal crystals, and 75 per cent of them showed cuboidal or heterogeneous crystals. Only some isolates showed rhomboidal crystal morphology that is characteristic of anti-coleopteran *B. thuringiensis* subsp. *tenebrionis*.

Jung *et al.* (2003) studied seven *B. thuringiensis* strains upon their *cry*I type genes, crystal shapes, crystal protein patterns and insecticidal activities. Silva *et al.* (2004) analyzed Brazilian strains of the bacterium using scanning electron microscopy for their crystal protein morphology. Strains S701 and S764 showed the presence of bipyramidal, cuboidal and round crystals, like in strain HD1 and bipyramidal and round crystals as in strain S1265. Leithy *et al.* (2004) analysed a total of 36 soil samples collected from different locations and specifications for total spore counts and for the occurrence and incidence of *B. thuringiensis*. These isolates were classified using biochemical tests. Obeidat *et al.* (2004) assessed the parasporal crystal morphology of 26 *B. thuringiensis* strains isolated from different habitats of Jordhan, which revealed the presence of bipyramidal, spherical and cuboidal crystal inclusions.

2.9 Bioactivity of *Bacillus thuringiensis* against dipteran and various other insect species

Bacillus thuringiensis (Bt) is a crystalliferous, spore forming bacterium. The insecticidal activity of Bt is the most important feature of this organism. The crystals consist of one or more proteins known as Cry or delta endotoxins (Hofte and Whiteley, 1989). The toxins are in the inclusions as inactive protoxins that are solubilized in the alkaline environment of the insect gut and activated by proteases (Drobniewski and Ellar, 1989). Crystal proteins exhibit a variety of biological actions including cytolytic, haemolytic in addition to entomocidal activities (Orduz *et al*., 1998).

During the last decades, scientific interest was concentrated on the crystals, since cry proteins of many Bt strains showed specific insecticidal activity against insect species of different orders, such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga, in additioin to Nematodes, Mites and Protozoa (Feitelson *et al*., 1992; Schnepf *et al*., 1998).

Table.1 Insecticidal activity of *B. thuringiensis* crystal proteins

***cry* genes**	**Insect specificity**
*cry*1, *cry*2 and *cry*9	Lepidoptera
*cry*2, *cry*4, *cry*10, *cry*11, *cry*16, *cry*17, *cry*19, *cry*20, *cry*21 and *cry*24	Diptera
*cry*3, *cry*7 and *cry*8	Coleoptera
*cry*5, *cry*12, *cry*13 and *cry*14	Nematodes

Source: Crickmore *et al.* (1998); Schnepf *et al.* (1998); Bravo *et al.* (1998)

Chilcott and Wigley (1994) tested the activity of *Bacillus thuringiensis* against eight, first instar larvae of *Planotortrix octa* and *Tenebrio molitor*. Kawalek *et al*. (1995) conducted bioassays using parasporal inclusions isolated from the new mosquitocidal strain, *B. thuringiensis* subsp. *jegasethan* against southern house mosquito (*Culex quinquefasciatus*), yellow fever mosquito (*Aedes aegypti*), Asian tiger mosquito (*Aedes albopictus*), and anopheles mosquito (*Anopheles maculatus*). The LC_{50} values of crystal inclusions for each species indicated that the parasporal inclusions from the new subspecies possessed mosquitocidal toxicity comparable to *B. thuringiensis* subsp. *israelensis.* *B. thuringiensis* isolates were found to be toxic to *D. melanogaster* larvae and adults with 75% of these toxic isolates produced spherical *cry*stals and 25% produced both cuboidal and bipyramidal crystals (Obeidat, 2008).

According to Sivopoulou *et al.* (2000), the crystals of the soil isolated *B. thuringiensis* strain A4 consisted of two polypeptides with molecular mass of 140kDa and 32kDa that exhibited insecticidal activity against adult flies of *B. oleae*. Cavados *et al*. (2004) investigated the effect of *B. thuringiensis* subspecies *israelensis* endotoxins on larvae of the *S. pertinax* (Diptera: Simuliidae), a common blackfly in Brazil, using several concentrations. The most characteristic effects were midgut columnar cell vacuolization, microvilli damages, epithelium cell contents passing into the midgut lumen and finally the cell death.

B. thuringiensis subsp. *israelensis*, which produces Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa toxin is presently used for the control of mosquitoes and blackflies. The expressed Cry20Aa protein showed larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus* (Lee and Gill, 1997). Johnson *et al.* (1996) conducted bioassays with the toxins in *B. thuringiensis* subspecies *tenebrionis* and recombinant *E. coli* against rice weevil. Peyronnet *et al.* (1997) performed bioassays with second instar larvae of the European gypsy moth (*Lymantria dispar*) and Chinese silk worm (*Bombyx mori*) to detect the ability of *B. thuringiensis* toxins to form pores in the midgut epithelial cell membrane of susceptible insects. Mosquito larvicidal activity of recombinant *E. coli* with combination of *cry*IVA and *cry*IVD was seven fold higher than *cry*IVA alone, probably because of the synergism between their activities (Ben-Dov *et al.,* 1995).

The Cry32Aa protein bioassay showed no toxicity against several moths and mosquitoes. However, it exhibited weak toxicity against larvae of the diamondback moth (Balasubramanian *et al*., 2002). Pinto *et al.* (2002) conducted bioassay using non-characterized crystal protein against Hymenoptera (*Acromyrmex lundi*), this assay showed a mortality rate higher than 50 per cent. Cry44Aa, crystals were highly toxic to second instar *Culex pipiens pallens* and *Aedes aegypti* (Takeshi *et al.,* 2006). The Cry1Id protein had toxicity comparable to that of cry1Ia *Plutella xylostella* but it was significantly less active against *Bombyx mori* (Choi *et al.,* 2000).

Kim *et al.* (1998) studied the toxicity effects of 58 strains of *B. thuringiensis* isolated from the soils of various regions in Korea republic. In toxicity tests, 35 per cent of all the isolates were toxic to Lepidoptera, 20 per cent were toxic to Diptera, and 9 per cent were non toxic isolates. A particularly large number of lepidopteran/dipteran active isolates were found. Forty lepidopteran active isolates produced typical rhomboidal inclusions and the remainder belonging to the dipteran active and non-toxic isolates was spherical in shape. Two isolates named

S-333 and S-225 possessed *cry*IC gene, but the S-333 isolate which produced rhomboidal inclusion was toxic to both diamond back moth and mosquito whereas the isolate S-225 showed toxicity only to mosquito and produced spherical inclusion.

Cry4Aa toxin has low activity against all three major genera of mosquitoes, *Aedes, Anopheles,* and *Culex,* while Cry4Ba has higher toxicity against *Aedes* and *Anopheles* but no activity against *Culex*. Cry11Aa has higher toxicity than Cry4Aa against all three genera of mosquitoes (Schnepf *et al*., 1998). Cry19Aa was toxic to *Anopheles stephensi* and *Culex pipiens* but had no measurable activity against *Aedes aegypti* (Rosso and Delecluse, 1997). There is less cross resistance to Cry19Aa in *Culex* resistance to Cry4Aa, Cry4Ba and Cry11Aa toxins (Wirth *et al.,* 2001).

Jyoti and Brewer (1999) conducted bioassay experiments with banded sunflower moth larvae to *B. thuringiensis* to determine the median lethal concentration and its efficacy. Dobrzhanskaya *et al.* (2002) identified a new strain of *B. thuringiensis* 2-7 and found that it was toxic to Coleoptera. Balasubramanian *et al.* (2002) carried out bioassay of a novel strain of *B. thuringiensis* subspecies *yunnanensis,* and it exhibited weak toxicity against larvae of diamond back moth. Bioassays showed no toxicity against several moths and mosquitoes. Asano *et al.* (2003) reported that a strain of *B. thuringiensis* subspecies *galleriae* isolated from Japan was highly toxic to the cupreous chafer (*Anomala cuprea*).

Lee *et al.* (2003) studied the mode of action of vegetative insecticidal protein (Vip), the 88kDa Vip3A full length toxin (Vip3A-F) was proteolytically activated to an approximately 62kDa toxin either by trypsin on lepidopteran gut juice extracts. Biotinylated Vip3A-G demonstrated competitive binding to lepidopteran midgut brush border membrane receptors. In voltage clamping assays with dissected midgut from susceptible insect tobacco hornworm

(*Manduca sexta)*, Vip3A-G clearly formed pores whereas Vip3A-F was incapable of pore formation.

Bhalla *et al.* (2005) reported toxicity of vegetative insecticidal protein (vip) from 24 serovars of *B. thuringiensis* on larvae of *Spodoptera litura* and *Plutella xylostella*. Rajesh *et al.* (2006) studied the diversity of *B. thuringiensis* isolates of the Western Ghats of Kerala and Karnataka and their bioefficacy against *Spodoptera litura* and *Helicoverpa armigera.* Neema (2007) assessed the toxicity of Bt isolates from Western Ghats of Kerala against pumpkin caterpillar (*Diaphania indica*). Cabrera *et al.* (2006) performed bioassays with a laboratory strain of *Sesamia nonagrioides,* the Mediterranean corn borer to determine the mode of action of CryIAb protein and to search for other cry proteins that could be used to control the pest.

2.10 Methods for identification of novel insecticidal genes

DNA based method has recently emerged as a reliable, simple and inexpensive way to identify and classify microorganisms. Traditionally, insect bioassays have been conducted in order to identify novel *cry* genes but these techniques are laborious and time-consuming. The detection of new Bt isolates that are highly toxic against a target insect and cloning of the gene thereof suffers from the limitation that *cry* genes may be detected that are already known (Theunis *et al*., 1998). Hence, molecular techniques to speed up the screening, followed by the ultimately necessary insect bioassays are more useful. Southern hybridization has been used for identification of homologous *cry* genes (Kronstad and Whiteley, 1986).

The sequence homology among different *cry* gene families ranges from 45 to 70 per cent, while among *cry* genes of a particular family the homology can be as high as 95 per cent. Because of this homology, specific probes can be designed for identification of homologous genes. However, with the increasing number of

cry genes being discovered, this technique is deemed cumbersome and slow for large scale screening of Bt isolates, since homologous probes for all the known *cry* genes would have to be used for detection. To improve the ease and efficiency of screening of Bt strains by Southern hybridization, a mixture of various *cry* gene sequences has been used as probe for detection of diverse *cry* genes in a single step (Beard *et al*., 2001).

2.10.1 Screening of Bt *cry* genes by Polymerase Chain Reaction

PCR-based methods have been developed for identification of Bt isolates having novel gene profiles (Porcar and Juarez-Perez, 2003). PCR being a highly sensitive and relatively fast technique is especially suitable for rapid, large scale, first-tier screening of Bt isolates. Bt strains harbouring novel *cry* genes and also the less frequently observed *cry* genes have been identified by PCR using specially designed primers corresponding to the highly conserved regions (Bravo *et al.,* 1998; Kim *et al*., 1998; Porcar and Juarez- Perez 2003; Tounsi *et al*., 2003 ; Wang *et al*., 2003).

A rapid analysis of *Bacillus thuringiensis* strains predictive of insecticidal avticity was established by using PCR. Prediction of insecticidal activity were made on the basis of the electrophoretic patterns of the PCR products, included in the screen were PCR primers specific for *cry*4*, cry*3 genes, which are insecticidal for dipterans and coleopterans, respectively. Known *B. thuringiensis* strains as well as unidentified strains isolated from soil and insect cadavers were analyzed by PCR. Insecticidal activity by PCR screen was found to correspond with the insecticidal activity of insect bioassays (Carozzi *et al*., 1991). Different PCR-based approaches have been devised for identification and characterization of *cry* genes using *cry* gene-specific primers (Ceron *et al*., 1995; Kuo and Chak 1996). For rapid screening and prediction of insecticidal activities of Bt isolates on the basis of presence or absence of the PCR product, a set of 12 primers corresponding to three major classes of *cry* genes - *cry*1*,*

*cry*3 and *cry*4, active against lepidopteran, coleopteran and dipteran insects respectively, was used by Carozzi *et al*. (1991).

2.10.2 Screening of Bt *cry* genes by multiplex PCR

Multiplex PCR, where more than two sets of primers designed from regions of high homology within *cry*1Aa, *cry*1Ab and *cry*1Ac genes are used in the same reaction mixture, has been employed to generate the characteristic PCR product profile of Bt strains (Bourque *et al*., 1993). Multiplex PCR using five sets of primers corresponding to the highly conserved regions of six *cry* gene families *viz*., *cry*1, *cry*2, *cry*3, *cry*4, *cry*7 and *cry*8 gene-families to detect the presence of *cry* genes in Bt isolates from Israel, Kazakhstan and Uzbekistan has also been performed (Ben-Dov *et al*., 1997).

A set of four oligonucleotide primers from highly conserved regions between *cry*1 and *cry*3 genes was used to identify strains carrying these genes (Ceron *et al*., 1995). Strains with unique PCR product profiles can be further characterized by additional set of primers designed from the variable regions between the *cry*1 and *cry*3 genes. Primers that yield products of unexpected size may represent novel genes. The analysis of the strains was based on multiplex PCR with novel general and specific primers that could detect the *cry*1, *cry*3, *cry*5, *cry*7, *cry*8, *cry*9, *cry*11, *cry*12, *cry*13, *cry*14, *cry*21, and *cyt* genes (Bravo *et al*., 1998). However, the limitation of this method is that a new gene cannot be detected if it does not have homology to any of the primer sequences used.

2.10.3 Screening of Bt *cry* genes by exclusive PCR (E-PCR)

The exclusive PCR (E-PCR) has been devised for detection of novel genes, in which the amplification of already known *cry*1 genes is followed by a second conditional amplification using three primers, which will occur only if a new putative *cry*1 gene(s) is present in the strains (Juarez-Perez *et al*., 1997). This

method uses in a multiplex PCR, several specific primers designed to recognize only one type of *cry* gene and 'universal' primers designed to detect *cry* gene families. A single universal primer is combined with many specific oligonucleotide primers that recognize individual *cry* genes. The universal primers are designed to have degeneracy of sequence in order to increase the probability of amplification of sequences having low homology within the gene family.

A 'PCR walking' strategy was devised for identification of variants of specific *cry* genes, by employing a series of primers designed to anneal throughout the length of *cry* gene sequence in a single multiplex PCR reaction (Kalman *et al*., 1993). A modification of the expected PCR profile would indicate the presence of a new *cry* gene. This method was used for detection of the *cry*1Cb1 gene. However, this method has only limited application as it is restricted to closely related genes within the same group and a relatively large number of primers is required to analyze each group of genes. A novel *cry*1Ab18 gene was cloned from a Bt isolate by a PCR-based strategy using a set of three highly specific oligonucleotide primers designed to amplify full-length open reading frame (ORF) of all the known *cry*1Aa, *cry*1Ab and *cry*1Ac genes (Stobdan *et al*., 2004).

2.10.4 Screening of Bt *cry* genes by PCR- RFLP method

An elegant method based on combination of PCR and restriction fragment length polymorphism (RFLP) strategies was designed to detect novel *cry* genes (Kuo and Chak, 1996). This is a two-step approach in which PCR amplification with specific primers is followed by restriction analysis of the PCR products. A particular restriction pattern is expected for each gene, as also for a given combination of genes. A different restriction profile of the PCR products would denote the absence or presence of a given restriction site.

The corresponding fragment(s) can be cloned, sequenced and used as a probe for the cloning of the full length *cry* gene. However, when more than four *cry* genes are present in a strain, the restriction profile may become difficult to analyze. Also, a high degree of similarity among the *cry* genes of a subfamily may make it difficult to detect differences among these genes. A second PCR, using alternative primers, which recognize other regions of the genes, followed by restriction analysis and a long electrophoresis run in order to achieve a better resolution of restriction fragments can overcome this problem. Unexpected restriction products may denote the presence of a new type of gene.

A novel *cry*1A-type gene was detected in a Bt isolate from China by using a combination of PCR and restriction analysis of the amplified products (Wang *et al*., 2003). A PCR-AFLP method for identification of *cry*2 genes from *Bacillus thuringiensis* strains from different sources of Argentina was employed to study the distribution of *cry* 2 genes (Diego *et al*., 2005). Recently Diego *et al*. (2007b) reported PCR-RFLP based method for detection and identification of *cry*1I gene from Bt. Liu *et al*., (2007) reported polymerase chain reaction (PCR)-RFLP system is a powerful tool to find novel Vip3A proteins from large-scale Bt strains.

2.10.5 Screening of Bt *cry* genes by nucleic acid hybridization method

Hybridization with a mixture of *cry* gene sequences is an efficient means of detecting clones containing a diverse range of *cry* genes in a single step. The method uses a cocktail of *cry* gene sequences as a hybridization probe to screen Bt strains and gene libraries prepared from them. Under the hybridization and washing conditions used, cross hybridization between genes from different *cry* families was not observed. Probes containing either partial or complete *cry* gene sequences produced similar patterns when hybridized to genomic DNA of several Bt strains although the pattern produced by the probe composed of entire gene coding regions was somewhat more complex (Beard *et al*., 2001).

Byung *et al*. (1995) conducted DNA dot blot hybridization with *cry*4 and *cry*1 specific probes to screen 24 Bt strains for their gene content. Most of the *cry*4 positive strains also hybridize to the *cry*1 specific probe, indicating that *cry*4 gene are closely related to *cry*1 genes. Oreshkova, (1999) analyzed the genomic DNA of Bt by genomic fingerprinting technique. The biotin labeled single stranded DNA of the phage M13 was used as the marker of hyper variable sequence and a procedure for analyzing the differentiation among various strains was developed.

2.10.6 Screening of *B. thuringiensis cry* genes by protein based technique

Analysis by Western blotting with polyclonal antisera raised against toxins purified from Bt subsp. *israelensis* reveals that proteins in parasporal inclusions of *B. thuringiensis* subsp. *jegathesan* are distinct, because little cross-reactivity was shown (Kawalek *et al.,* 1995). A strain of Bt, STB-1, isolated from soil sample in Korea, which was toxic against *Spodoptera exigua* was analyzed for plasmid and protein profile with the reference strains Bt subspecies *kurstaki* and *kenya* to verify the gene type. PCR analysis was performed with Spodoptera specific *cry* gene primers. The strain STB-1 had *cry*1Aa, *cry*1Ab and *cry*1E suggesting it as a unique strain with respect to gene type (Chang *et al.,* 1999). Werneck *et al.* (1999) analyzed a Brazilian strain Bt subspecies *kurstaki* S93, regarding its *cry* gene and protein content. The spore- crystal mixture analyzed by SDS-PAGE showed two major polypeptides of 130 and 65KDa, corresponding to *Cry*1 and *Cry*2 toxins, respectively. Western blot analysis showed that these proteins were immunologically related to Cry1A protein from *Bacillus thuringiensis* subspecies *kurstaki* HD73. The PCR analysis using total DNA from S93 and specific primers showed the presence of *cry*1Aa*, cry*1Ab*, cry*1Ac*, cry*1A type gene was localized in a plasmid of about 44 MDa.

Bt strains toxic to beet army worm were characterized for crystal protein profile, plasmid profile, plasmid restriction patterns, *cry* gene composition, qualitative determination of beta exotoxin production, RAPD and serotyping. All

strain contained *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1B, *cry*1C and *cry*1D genes (Arango *et al*., 2002). Western blot analysis revealed a low level expression of the cloned *cry*2Aa and *cry*2Ab genes in the recombinant Bt strains (Kumar and Udayasuriyan, 2004).

2.11 Plasmid profiling

Bacillus thuringiensis is an insect pathogen used worldwide as a bioinsecticide. Its great biotechnological success resides in the production of highly specific insecticidal proteins (Cry proteins) simultaneously with the sporulation phase. Plasmids from this group of organisms have been implicated in pathogenicity to various insect pests as they carry the genes responsible for pathogenicity. In general, all plasmid patterns are unique to each strain. These Cry proteins are coded by genes (*cry* genes) harbored in megaplasmids (Gonzalez *et al*., 1982; Hofte and Whiteley, 1989; Kronstad *et al*., 1983; Ward and Ellar, 1983). Plasmids have also been associated with the production of a different toxin called β-exotoxin (Levinson *et al*., 1990). The relevance of plasmids in *B*. *thuringiensis* strains is assumed by the regular presence of a set of plasmids, which can vary in number from 1 to 17 and in size from 2 to 80 MDa (Aptosoglou *et al*., 1997; Gonzalez and Carlton, 1980).

Dipteran specific *B*. *thuringiensis* subsp. *israelensis* harbors several plasmids ranging in size from 3 to 135 MDa (Gonzalez and Carlton, 1984). A 75 MDa plasmid is responsible for the mosquitocidal activity. It includes the genes *cry*IVA, *cry*IVB, *cry*IVC, *cry*IVD and *cyt*A, encoding δ-endotoxin proteins 134, 128, 78, 72, and 27 kDa in size, respectively, which are highly expressed during sporulation (de Barjac and Sutherland, 1990; Delecluse *et al*., 1991; 1995; Porter *et al*., 1993).

The set of plasmids harboured in a strain are normally visualized by agarose gel electrophoresis, where they form an electrophoretic pattern of bands,

according to their differential migration in the gel. *B. thuringiensis* plasmids have been studied either to locate *cry* genes or to transfer them between different strains and species (Aronson and Beckman, 1987; Battisti *et al*., 1985; Hu *et al*., 2004; Reddy *et al*., 1987). Plasmid patterns have frequently been used to characterize strains especially compared to those of standard strains (Porcar *et al*., 1999; Vilas-Boas and Lemos, 2004; Ibarra and Federici, 1987, Ibarra *et al*., 2003; Padua *et al*., 1984).

Several studies revealed correlations between the production of δ-endotoxins and the presence of plasmids (Bulla *et al*., 1980; Carlton and Gonzalez, 1985). It is well known that plasmids play a crucial role in bacterial evolution and adaptation by mediating horizontal exchange of genetic material and providing advantageous functions to their carrier (Andrup *et al*., 2003). The number of plasmids, their sizes and copy numbers can vary considerably among bacterial strains.

In a plasmid pattern, two different groups of plasmids can be recognized, those that are 30 MDa and larger than that called megaplasmids. For practical purposes, each group is divided by the so-called chromosomal band in the agarose gel. Small plasmids are below that band, and megaplasmids are above it. For gram-positive bacteria, small plasmids generally use the rolling-circle replication mechanism, with single-stranded DNA intermediates, while megaplasmids normally use the "theta" replication mechanism. In addition, small plasmids are generally present in high copy numbers, while megaplasmids are present in low copy numbers. No specific function has been found for the small plasmids, which is the reason why they are called "cryptic" (Lopez-Meza *et al*., 2003). As for the megaplasmids, their main recognized function is harbouring *cry* genes, although the sequencing of some of these plasmids indicates the occurrence of other important genes (Berry *et al*., 2002; Chao *et al*., 2007; Jensen *et al*., 1995).

Reyes-Ramirez and Ibarra (2008) were able to determine the diversity of plasmid patterns from 130 type strains and also within six serotypes. The

information obtained from this comparison showed the importance of this tool as a strain characterization procedure and indicates the complexity and uniqueness of this feature. Comparisons between the plasmid profiles of the *B. thuringiensis* isolate INTA-FR7-4 and the reference strain *B. thuringiensis subsp. kurstaki* HD1 showed that the Argentine isolate has a simpler array of plasmids (Amadio *et al*., 2009). The plasmid profile of S76 was compared to that of HD-1strain S76 displays a similar number and size of extra chromosomal elements to that of HD-1. Probably, strain S76 harbours the *cry* genes located in a similar manner (Gitahy *et al*., 2007).

Frederiksen *et al.* (2006) intended to find out occurrence of the natural *Bacillus thuringiensis* contaminants and residues of *Bacillus thuringiensis* based insecticides on fresh fruits and vegetables by performing RAPD analysis and plasmid DNA profiling on the 28 isolates harbouring the same crystal protein and enterotoxin genes as the *B. thuringiensis* strains present in Biobit, Dipel, Foray, and Turex revealed that 23 strains were indistinguishable from the active organisms in these products.

2.12 Cloning of *cry* genes by PCR

The main objective of cloning of *cry* genes using recombinant DNA methodologies is to provide better options for crop production. Recombinant DNA technology has great scope in Cry protein based insect control. It may be used to enhance the levels of expression of the toxin genes with stronger resident promoters and ribosome binding sites (RBSs) and appropriately placed enhancers and terminator sequences (Ben-Dov *et al.,* 1995). The first ever report on cloning of Bt crystal protein gene came in 1981. Ben-Dov *et al.* (1995) cloned *cry*IVA*, cry*IVD genes in seven combinations, encoding 134 and 72 kDa proteins respectively, and the gene for a regulatory 20-kDa polypeptide of *Bacillus thuringiensis* subsp. *israelensis* (serovar H14) in *E. coli* expression vectors pT7 and pUHE. The four combination containing *cry*IVA (*cry*IVA alone, with

*cry*IVD, with the 20-kDa protein gene, and with both) displayed high levels of mosquito larvicidal activity in pUHE. Neema (2007) screened native *Bacillus thuringiensis* isolates for *cry*1 and *cry*4 genes through PCR by using universal primers. The amplicon size obtained was 439bp for *cry*4 and 277bp for *cry*1 genes respectively. These amplified samples were eluted then eluted samples were ligated into pGEMT vector and transformed into *E. coli* JM 109 cells.

Schnepf and Whiteley (1985) cloned a crystal protein gene from Bt subsp. *kurstaki* and expressed in *E. coli*. The recombinant strains of *E. coli* synthesized a 130 kDa protein that showed positive reaction with antibody raised against a peptide of the same size from Bt crystals. A new *cry*1I-type gene, *cry*1Id1, was cloned from a Bt isolate, and its nucleotide sequence was determined (Choi *et al*., 2000). Balasubramanian *et al*. (2002) cloned a novel *cry*32Aa gene of Bt subspecies *yunnanesis*. Misra *et al*. (2002) amplified a 1.9 kb *cry* gene by PCR and the amplified fragment cloned in pBluescript and then sub-cloned in pET3 and pET28a for expression in *E. coli.*

Tzeng *et al*. (2002) amplified a full length DNA *cry*1Ac gene of 3.6kb encoding insecticidal crystal protein from a locally isolated Bt strain by PCR. This *cry*1Ac gene was constructed in to *E. coli* plasmid vector pERTMII. A new *cry*1Ab17 type gene was cloned from *Bacillus thuringiensis* WB9 by PCR (Huang *et al*., 2004). Stobdan *et al*. (2004) cloned a *cry*1Ab10 gene from a new isolate of Bt by PCR. Nucleotide sequence and homology search revealed that the toxin showed 95 per cent homology with known Cry1Ab toxin. Two insecticidal crystal proteins genes of Bt, *cry*2Aa and *cry*2Ab were cloned from new isolates of Bt (Kumar and Udayasuriyan, 2004). Santoso *et al*. (2004) reported cloning of *cry* gene from genomic DNA of Bt by direct cloning of PCR products in pGEMT. Rai *et al*. (2006) constructed a plasmid DNA library on pUC18 vector of a native toxic Bt strain S58.

Two clones designated as *cry*1Ab and *cry*1Ac encoding crystal proteins were identified by screening the plasmid library using 732bp *EcoR*I fragment which is a conserved sequence of across the species. Takeshi *et al*. (2006) cloned two novel crystal protein genes, *cry*30Ba and *cry*44Aa from *Bacillus thuringiensis* subsp. *entomocidus* INA288 and expressed in an acrystalliferous strain. Escudero *et al*. (2006) detected the presence of a novel *cry*2 type gene in Bt by PCR. A general primer pair recognizing both *cry*2Aa and *cry*2Ab genes were used in combination with oligonucleotides specifically recognizing the *cry*2A genes primers. Amplified products were cloned into pGEMT vector; the cloned amplicons were sequenced and analyzed.

Beron and Salerno (2007) cloned and sequenced the encoding gene of one of the crystal proteins consisting of an open reading frame (ORF) of 2061bp that encodes a protein of 687amino acid residues. The deduced amino acid sequence has a predicted relative molecular mass of 78 kDa and is 52% and 45% identical to those of the reported Cry24Aa and Cry24Ba sequences, respectively. Diego *et al*. (2007a) reported cloning of novel *cry*1A genes. Nucleotide and amino acid sequence similarity revealed that *cry*1Aa and *cry*1Ac showing several differences with the other known *cry*1A subclasses. Recently the *cry*8C-type designated *cry*8Ca2 was cloned and sequenced from a Bt isolate HBF-1 in China. The sequence analysis showed that the Cry8Ca2 protoxin of 130.5 KDa had 99.9 per cent sequence homology with the previously reported Cry8Ca1 protein (Shu *et al*., 2007).

Materials and methods

3. MATERIALS AND METHODS

The study entitled "Molecular cloning of *cry* genes specific to dipterans from native *Bacillus thuringiensis* Berliner" was carried out in the Molecular Biology Laboratory of the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2007-2009. The materials used and the methodologies adopted in this study are described below.

3.1 Materials

3.1.1 Chemicals, glassware and plastic ware

The chemicals used for the study were of good quality (AR grade) from various agencies including MERCK, SRL and HIMEDIA. Molecular Biology Grade enzymes and buffers were supplied by Banglore Genei Ltd. All the plastic ware used was obtained from Axygen and Tarson India Ltd.

3.1.2 Equipment and machinery

The equipment items available at Centre for Plant Biotechnology and Molecular Biology were used for present study. Incubation of cultures was done in Incubator Shaker, DK- S1010 (Dai Ki Scientific Co. Korea). Centrifugation was done in KUBOTA centrifuge (Kubota, Japan). A compound binocular microscope (CETI) was used for viewing the slides of bacterial cultures. DNA amplification was carried out in Eppendorf Master Cycler, Gradient (Eppendorf, Germany). Vision works LS software was used to visualize the gel and UVP GelDoc-IT^{TM} imaging system (USA) was used for imaging the gel.

3.2 Bacterial isolates

Twenty isolates of *Bacillus thuringiensis* already obtained from the Western Ghats of Kerala under the DBT funded project entitled "Exploration of

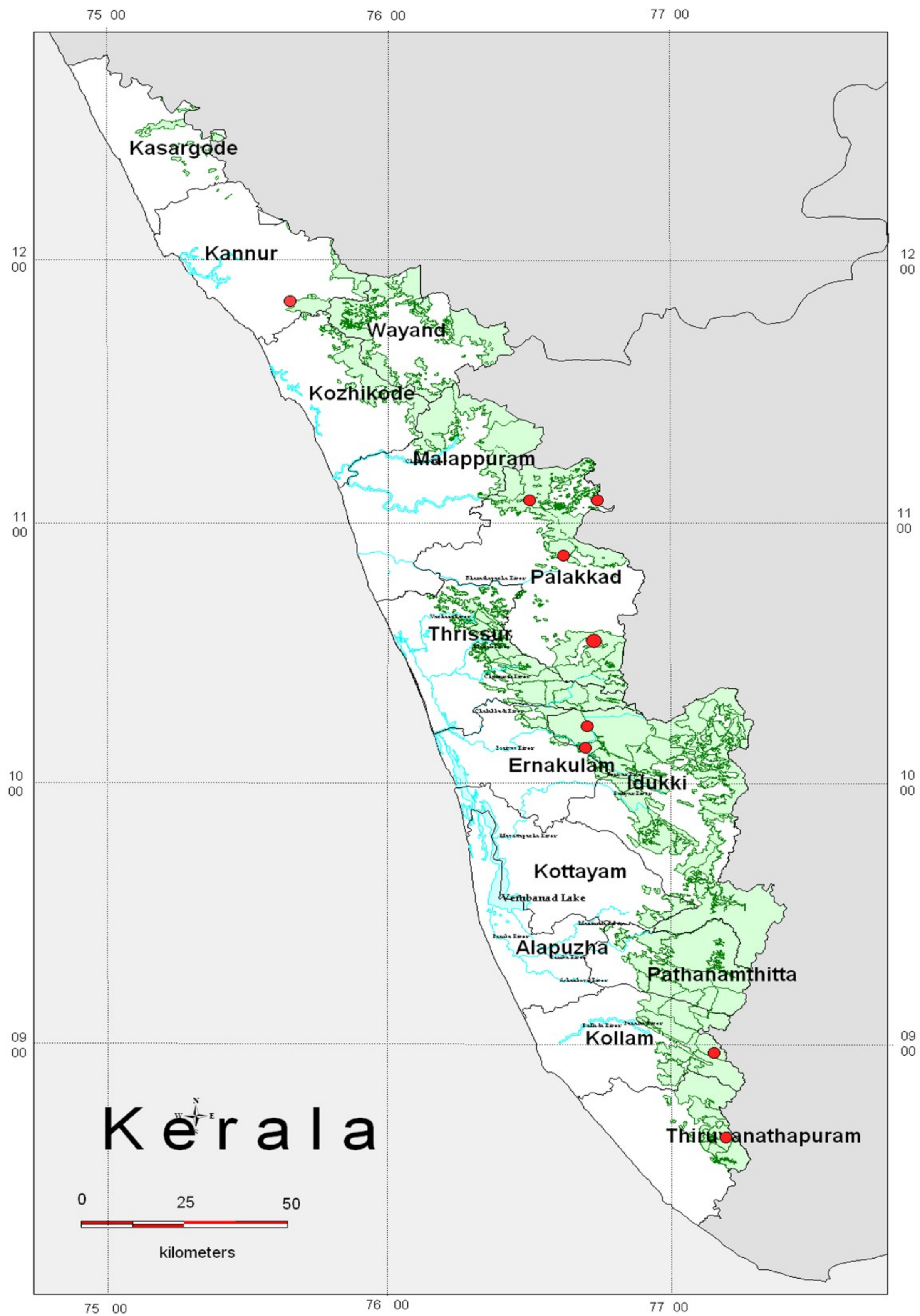


Plate 1. Western Ghats of Kerala indicating locations from where Bt isolates were obtained

Table 2. Details of locations from where Bacillus *thuringiensis* were isolated

Sl. No.	Isolates	Location		GPS reading
		District	Place	
1	Bt-23	Ernakulam	Edamalayar	N: 10^0 12.556 E: 76^0 42.318 Elevation: 989 feet
2	Bt-105	Kollam	Kaduvapara	N: 8^0 57.194 E: 77^0 9.223 Elevation: 1055 feet
3	Bt-133	Palakkad	Dhoni	N: 10^0 51.775 E: 76^0 37.238 Elevation: 1698 feet
4	Bt-135	”	”	N: 10^0 51.775 E: 76^0 37.238 Elevation: 1698 feet
5	Bt-161	”	Minnumpara Nelliyampathy	N: 10^0 32.101 E: 76^0 43.692 Elevation: 5222 feet
6	Bt-170	”	Nelliyampathy	N: 10^0 31.288 E: 76^0 39.933 Elevation: 3079 feet
7	Bt-190	”	”	N: 10^0 32.138 E: 76^0 40.080 Elevation: 2744 feet
8	Bt-206	”	”	N: 10^0 31.485 E: 76^0 38.878 Elevation: 2108 feet
9	Bt-237	”	Pothundy	N: 10^0 31.312 E: 76^0 38.204 Elevation: 1119 feet
10	Bt-242	”	”	N: 10^0 31.312 E: 76^0 38.204 Elevation: 1119 feet
11	Bt-245	Ernakulam	Thattekkad	N: 10^0 7.449 E: 76^0 41.875 Elevation: 517 feet
12	Bt-250	Ernakulam	Thattekkad	N: 10^0 7.550 E: 76^0 41.781 Elevation: 817 feet
13	Bt-277	Thiruvananthapuram	Agasthyavanam	N: 8^0 37.713 E: 77^0 11.816 Elevation: 886 feet
14	Bt-283	”	”	N: 8^0 34.839 E: 77^0 10.917 Elevation: 634 feet
15	Bt-326	”	”	N: 8^0 35.185 E: 77^0 11.734 Elevation: 532 feet
16	Bt-411	Palakkad	Silent valley Pothanthode	N: 11^0 04.605 E: 76^0 29.958 Elevation: 2689 feet
17	Bt-419	”	Silent valley	N: 11^0 08.513 E: 76^0 44.365 Elevation: 3193 feet
18	Bt-420	”	”	N:11^0 09.773 E: 76^0 45.747 Elevation: 3982 feet
19	Bt-430	”	”	N: 11^0 09.142 E: 76^0 45.127 Elevation: 2975 feet
20	Bt-447	Kannur	Kannavam	N: 11^0 84.003 E: 75^0 65.308 Elevation: 653 feet
21	4Q1	*Bacillus thuringiensis* subsp. *israelensis* (Reference strain)		

*N – North, E - East

the molecular diversity and insecticidal spectrum of *Bacillus thuringiensis* isolates of the Western Ghats of India and cloning insecticidal genes from the native isolates" were used for investigation (Plate 1). Strain 4Q1 (*Bacillus thuringiensis* subsp. *israelensis*) obtained from University of Agricultural Sciences, Dharwad was used as a reference strain in all the experiments. The details of the isolates are given in Table 2. These isolates were maintained by sub-culturing periodically in Petriplates containing LB Media (Composition in Appendix I). After inoculation the Petriplates were incubated at 29°C for 48 h in BOD incubator and later stored at 4°C.

3.3 Identification and characterization of *Bacillus thuringiensis* isolates

3.3.1 Morphological characterization

The bacterial isolates were characterized using staining reactions like Gram and crystal protein staining. Chemical compositions of the reagents used for staining reaction are given in Appendix II.

3.3.1.1 Gram staining

The isolates were subjected to Gram staining reaction following the procedure of Hucker and Conn (1923).

1. A smear of the culture was prepared on a clean slide, air dried and heat fixed.
2. One to two drops of crystal violet stain was added and the smear washed after one minute.
3. Gram's iodine solution was added for one minute and then washed.
4. Decolourized with ethyl alcohol for 30 seconds.
5. The smear was treated with the counter stain safranin for one minute.
6. Finally the slide was washed under running tap water, air dried and observed under 100X objective of a compound binocular microscope.

3.3.1.2 Crystal protein staining

The cultures were examined for the presence of crystal protein inclusions by staining with Coomassie brilliant blue as described by Sharif and Alaeddinoglu (1983).

1. Smears of the cultures grown on T3 agar medium for 48 hours were heat fixed.
2. The slides were stained with Coomassie brilliant blue (0.25%) for 3 minutes.
3. Slides were then washed under running tap water and air dried.
4. The smears were observed for the presence of crystal proteins under the 100X objective of a compound binocular microscope.

3.3.2 Cultural characterization

Cultural characteristics of the isolates were studied by streaking it on LB agar medium in Petriplates. Colony characters like pigmentation, size, form, margin, elevation, optical characteristics and diameter were observed after 24 hours of streaking, following Cappuccino and Sherman (1992).

3.3.3 Biochemical characterization

Bacillus thuringiensis isolates were characterized using seven different biochemical tests (Cappuccino and Sherman, 1992). Chemical composition of media used has been provided in Appendix I.

3.3.3.1 Esculinase test

The isolates were tested for their ability to produce the enzyme esculinase by streaking them on esculin agar. After incubation at 30°C for 24

hours, the cultures were examined for blackening around the growth which indicated hydrolysis of esculin.

3.3.3.2 Gelatin hydrolysis

The isolates were analysed for their ability to produce a proteolytic extra cellular enzyme, gelatinase which hydrolyzes the protein gelatin to amino acids. This was carried out by stab inoculating nutrient gelatin deep tubes with the bacterial isolates. The tubes were incubated for 24-48 hours at 30°C and these transferred to a refrigerator at 4°C for 30 minutes. Gelatinase positive cultures remained liquified while in others the medium remained solid.

3.3.3.3 Lecithinase test

Lecithinase test was done by streaking the bacterial cultures on nutrient agar medium supplemented with egg yolk and incubated for 24-48 hours at 30°C. Presence of a clear zone surrounding the growth indicated positive reaction.

3.3.3.4 Solubility in three per cent KOH

At the centre of a sterile glass slide, two drops of three per cent KOH solution were was placed and a loopful of bacterial growth was rapidly agitated on it in a circular motion. After five to eight seconds, the loop was gently raised and lowered just above the slide surface. Presence or absence of viscous strands between the loop and slide surface was observed.

3.3.3.5 Starch hydrolysis

Starch hydrolysis was carried out in order to determine the ability of isolates to excrete hydrolytic extra cellular enzymes capable of degrading the polysaccharide, starch. Bacterial isolates were streaked on starch agar medium.

The plates were incubated for 24-48 hours 30°C. Plates were flooded with Lugol's Iodine solution which produced blue colour on the plate. A clear zone of hydrolysis surrounding the growth of organism when flooded with Lugol's iodine indicated starch hydrolysis.

3.3.3.6 Urease test

Bacillus thuringiensis isolates were tested for their ability to degrade urea by means of an enzyme, urease. The presence of urease was detected by inoculating deep tubes containing urea agar with bacterial cultures. The deep tubes containing inoculated cultures were incubated for 24-48 hours at 30°C and observed for development of deep pink colour, which was the positive reaction for urease test.

3.3.3.7 Voges-Proskauer (VP) Test

This test was carried out to determine whether the isolates were capable of producing non-acidic/neutral end products such as acetyl methyl carbinol from organic acids that result from glucose metabolism. In VP test, the bacterial strains were inoculated into Voges-Proskauer medium and incubated for 48 hours at 30°C. After incubation, 10 drops of Barritt's reagent A was added followed by the addition of Barritt's reagent B (Appendix II). The cultures were examined for the development of pink colour 15 minutes after addition of Barritt's reagent, which indicated a positive reaction.

3.4 Profiling and cloning of crystal protein gene

3.4.1 Isolation of total DNA from *Bacillus thuringiensis*

The total DNA was isolated from *Bacillus thuringiensis* isolates following the protocol of Sambrook and Russel (2001).

(Reagents and their Chemical composition for DNA isolation are given in Appendix III)

1. 25ml of overnight grown culture of *Bacillus thuringiensis* in LB broth was centrifuged at 10,000 rpm for 10 minutes at 4°C.
2. The pellet was resuspended in 10mM Tris HCl and 100mM NaCl.
3. Centrifugation was carried out at 10,000 rpm for 10 minutes at 4°C.
4. The pellet was resuspended in 2.5ml TE and 500µl of lyzozyme from a stock of 50mg/ml and incubated at 37°C for 20 minutes.
5. 25µl of RNase A was added from a stock of 10mg/ml.
6. Incubated at room temperature for 10 minutes.
7. 2.5ml of SDS was added and incubated at 50°C for 45 minutes.
8. 50µl of proteinase K was added from a stock of 20mg/ml and incubated at 50 to 55°C for 10 minutes.
9. An equal volume of phenol was added, mixed gently and centrifuged at 10,000 rpm for 10 minutes at 4°C.
10. The aqueous phase was transferred to a fresh tube and an equal volume of phenol: chloroform (1:1) ratio was added. The mixture was mixed gently.
11. Centrifugation was carried out at 10,000 rpm for 10 minutes at 4°C and the aqueous phase was pipetted into a fresh tube.
12. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently.
13. Centrifugation was carried out at 10,000 rpm for 10 minutes at 4°C and the aqueous phase was separated.
14. $1/10^{th}$ volume of 3M sodium acetate was added and kept in ice for 20 minutes.
15. An equal volume of isopropanol was added and incubated for 5 to 10 minutes.
16. Centrifugation was carried out at 10,000 rpm for 15 minutes at 4°C and the supernatant was decanted.
17. The pellet was washed in 70 per cent alcohol, by giving a centrifugation at 10,000 rpm for 3 minutes. The supernatant was discarded.

18. The pellet was dried and dissolved in 25 to 30µl of TE buffer.

3.4.2 Checking the quality of DNA

3.4.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed based on the method described by Sambrook *et al*. (1989) to check the quality of DNA and also to separate the amplified products.

Materials required for Agarose gel electrophoresis are:

1. Agarose (Bangalore Genei, Low EEO)
2. 50x TAE buffer (pH 8.0)
3. Electrophoresis unit (Biorad, USA), power pack, casting tray and comb
4. 6x Loading/ Tracking dye (Bangalore Genei, Bangalore)
5. Ethidium bromide solution (stock 10mg/ml; working concentration 0.5µg/ml (5µl/100ml gel))
7. Gel documentation and analysis system (UVP GelDoc-IT^{TM} imaging system)

(Chemical composition of the buffer and dyes are given in Appendix IV)

3.4.2.2 Electrophoresis

1. 1X TAE buffer was prepared from 50 X TAE stock solution.
2. Agarose (1.0 per cent (w/v) for genomic DNA and 0.8 per cent (w/v) for PCR) was weighed and added to 1X TAE. It was boiled till the agarose dissolved completely and then cooled to lukewarm temperature.
3. Ethidium bromide was added to a final concentration of 0.5µg/ml as an intercalating dye of DNA and mixed well.
4. The open ends of the gel casting tray were sealed with a cellophane tape and placed on a perfectly horizontal leveled platform.

5. The comb was placed properly and molten agarose was poured into the tray, allowing it to solidify.
6. After the gel was completely set (15 to 20 minutes at room temperature), the comb and cellophane tape were carefully removed.
7. The gel was placed in the electrophoresis tank with the wells near the cathode and submerged in 1X TAE to a depth of 1cm.
8. A piece of cellophane tape was pressed on a solid surface and 1µl 6X loading dye was dispensed in small quantity on the tape. A quantity of 3 to 5µl of DNA was added to each slot (in the case of PCR products, 10.0 to15.0µl) mixed well by pipetting in and out for 2 to 3 times. Then the mixture was loaded in the wells, with the help of micropipette. λDNA/*Eco*R1+ *Hind*III double digest (Bangalore Genei) was used as the molecular weight marker.
9. The cathode and anode were connected to the power pack and the gel was run at a constant voltage of 60 volts.
10. The power was turned off when the tracking dye reached at about 3cm from the anode end.

3.4.2.3 Gel documentation

The DNA bands separated by electrophoresis were viewed and photographed using Vision works LS software and UVP GelDoc-IT™ imaging system.

3.4.3 Purity of DNA

The purity of DNA was further analysed by using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). The absorbance of nucleic acid sample was measured at a wavelength of 260 nm and 280 nm. 260/280 ratio was used to assess the purity of nucleic acids. A ratio of 1.8 to 2 indicated that the DNA preparation was pure and free from protein.

3.5 PCR reaction

3.5.1 Amplification of *cry* gene from native *Bacillus thuringiensis*

The total DNA extracted from *B. thuringiensis* isolates was amplified by PCR. Initially, all the isolates were screened for the presence of dipteran specific *cry* genes *viz*., *cry*2, *cry*4, *cry*10, *cry*11, cry16, *cry*17, *cry*19 and *cry*21 using specific primers. The details of primers are given in Table 3a. Polymerase chain reaction was carried out using *cry* gene specific primers in Eppendorf Master Cycler, Gradient (Eppendorf, Germany).

3.5.2 Composition of the reaction mixture for PCR

1. Genomic DNA (50ng)	-	1.0µl
2. 10X Taq assay buffer	-	2.5µl
3. d NTP mix (10mM)	-	1.0µl
4. Forward primer (10pM)	-	1.0µl
5. Reverse primer (10pM)	-	1.0µl
6. Taq DNA polymerase (0.3 U)	-	2.0µl
7. Autoclaved distilled water	-	16.5µl
		25.0µl

The reaction was set in a 200µl microfuge tube chilled over ice flakes. A momentary spin was given to mix completely all reagents and set in thermal cycler for amplification under suitable programme. Reference strain 4Q1 was used as positive control for amplification of *cry* genes from the bacteria. A negative control was maintained without any template DNA.

3.5.3 Thermal cycler programme

Steps followed in PCR, temperature profile and time duration of each step for amplification of *cry*4 are given in table 4.

Table 3a. Details of primers used for amplification of dipteran specific *cry* genes

Sl. No.	Primer	Primer sequence	Length (bp)	Reference
1	*cry*2	F.P. 5'GTTATTCTTAATGCAGATGAATGGG 3'	25	Bendov *et al.* (1997)
		R.P. 5'CGGATAAAATAATCTGGGAAATAGT 3'	25	
2	*cry*4	F.P. 5' GCATATGATGTAGCGAAACAAGCC 3'	24	
		R.P. 5' GCGTGACATACCCATTTCCAGGTCC 3'	25	
3	*cry*10	F.P. 5' ATATGAAATATTCAATGCTC 3'	20	Ejiofar and Johnson (2002)
		R.P. 5'ATAAATTCAAGTGCCAAGTA 3'	20	
4	*cry*11	F.P. 5' TTAGAAGATACGCCAGATCAAGC 3'	23	Bravo *et al.* (1998)
		R.P. 5' CATTTGTACTTGAAGTTGTAATCCC 3'	25	
5	*cry*16	F.P. 5' TCAAAAGGTGTGGCAAG 3'	17	Barloy *et al.* (1998)
		R.P. 5' ATAAGCCCAATATCATG 3'	17	
6	*cry*17	F.P. 5' CTGAGGTATTTTGTGGA 3'	17	
		R.P. 5' AAGTAAAGATTTCTGGG 3'	17	
7	*cry*19	F.P. 5' AGGGGAGTCCAGGTTATGAGTTAC 3'	24	Ejiofar and Johnson (2002)
		R.P. 5' ATTTCCCTAGTTAGTTCGGTTTTT 3'	24	
8	*cry*21	F.P. 5' ATACAGGGATAGGATTTCAAG 3'	21	
		R.P. 5' ATCCCCATTTTCTATAAGTGTCT 3'	23	

Table 3b. Details of annealing temperature and expected amplicon size for selected dipteran specific primers

Sl. No.	Primer	Annealing Temperature (°C)	Expected amplicon size (bp)
1	*cry*2	61	689-701
2	*cry*4	53	439
3	*cry*10	45	404
4	*cry*11	55	305
5	*cry*16	42	1415
6	*cry*17	42	1400
7	*cry*19	55	355
8	*cry*21	53	453

The thermal cycler programme for other dipteran specific *cry* genes was the same, except annealing temperature, which was different for different cry genes (Table 3b). The PCR product was checked on 0.9 per cent agarose gel and documented.

Table 4. Temperature profile for amplification of *cry*4 gene by PCR

Sl. No	Step	Temperature	Time (min)
1	Initial denaturation	94°C	2.00
2	Denaturation	94°C	0.45
3	Annealing	53°C	1.00
4	Primer extension	72°C	1.00
5	Step 2 to 4	29 cycles	
6	Final extension	72°C	10.00

3.6 Bioassay against vinegar fly (*Drosophila melanogaster* Kent)

Native *B. thuringiensis* isolates were screened for their insecticidal activity against vinegar fly (*Drosophila melanogaster* Kent), a model test insect belonging to order Diptera. The reference strain 4Q1 was used as positive control in assessing the insecticidal activity of native isolates.

3.6.1 Mass rearing of *Drosophila melanogaster* larvae

Large numbers of adult vinegar flies were collected in jam bottles containing banana fruit as an attractant. These flies were released in a netted cage of dimension 45 cm × 45 cm × 43 cm for mating and laying eggs. Inside the cage a Petriplate containing sucrose 2 per cent and agar 1 per cent was kept as a

substrate for egg laying and yeast paste was kept as a food source, which was refreshed thrice a day to maintain uniformity of larvae. Eggs were hatched by incubating at 25±1°C for 24 h. Larvae were collected and transferred into a beaker containing artificial larval diet.

3.6.2 Preparation of artificial diet for larvae

Artificial diet **(**Nakamori and Kakinohana, 1980) was used to feed the larvae. Composition of artificial diet is given in Appendix I. To prepare the larval diet, water was poured initially into the jar of waring blender, followed by adding 3.3 per cent HCl and sodium benzoate. Then pieces of coarse tissue paper were added and mixed thoroughly until the fibre became disentangled and homogenised. The other ingredients *viz*., powdered soybean meal; yeast and raw sugar were added and blended well. Subsequently, wheat bran was added and the contents were mixed thoroughly until it became a homogenous paste.

3.6.3 Harvesting of crystal protein from *Bacillus thuringiensis* isolates

A loopful of culture on T3 agar was inoculated in 5ml T3 broth contained in test tubes. After 24 hours of incubation at 30°C, 2ml of the culture was transferred to 50ml T3 broth and incubated for 72 hours at 30°C with shaking at 160 rpm. Cells were observed under microscope for cell lysis. When completely lysed cells with spores and crystal proteins were observed, crystal protein harvesting was carried out. Chemical composition of the reagents used for harvesting of crystal protein is given in Appendix III.

1. 50ml of T3 grown culture was centrifuged at 10,000 rpm at 4°C for 10 min.
2. The supernatant was discarded and pellet was dissolved in 50ml 10X Tris-EDTA (TE) buffer and 1ml Phenyl Methane Sulfonyl Flouride (PMSF) and centrifuged at 10,000 rpm at 4°C for 10 minutes.
3. Supernatant was discarded and pellet dissolved in 25 ml 0.5M NaCl and centrifuged at 10,000 rpm at 4°C for 10 minutes.

4. Pellet was again dissolved in 50ml 10X TE and 0.5ml PMSF and centrifuged at 10,000 rpm at 4°C for 10 minutes.
5. The pellet was again dissolved in 50ml 10X TE and 0.5ml PMSF, centrifuged at 10,000 rpm at 4°C for 10 minutes.
6. The final pellet obtained was centrifuged to remove water if any present.
7. The pellets were dissolved in 1ml of 50:1 PMSF and stored at -20°C, in Eppendorf tubes.

3.6.4 Enumeration of Bt spores in broth

Before harvesting of crystal protein the bacterial spore count was taken by the help of haemocytometer in order to adjust spore concentration uniformly at the rate of 1.1×10^{10} spores per ml. Spore concentration was calculated from spore count obtained from haemocytometer by using the following formula (Lomer and Lomer, 1996) indicated below:

$$\text{Number of spores per ml} = \frac{X \times 400 \times 10 \times 1000 \times D}{Y}$$

Where,

X = Number of spores counted from 16 small squares

Y = Number of small squares checked

10 = Depth factor

1000 = Conversion factor from mm^3 to cm^3

D = Dilution factor

3.6.5 Standardization of Bt toxin dosage

Toxin dosage for bioassay was standardized by using seven different spore concentrations i.e., 1.1×10^8, 3.3×10^8, 5.5×10^8, 8.8×10^8, 11×10^8, 13.2×10^8 and 16.5×10^8 spores per ml with standard strain 4Q1. These seven spore concentration was prepared from original concentration of 1.1×10^{10} spores per ml by diluting with PMSF solution

3.6.6 Bioassay on *Drosophila melanogaster*

The Cry toxins were tested for their toxicity against third instar larvae of *Drosophila melanogaster* by diet contamination method, as described by Obeidat (2008) with some modification. Crude extract of Bt containing spores and Cry toxin was mixed with artificial diet and used as feed for the larvae. For each isolate, five different concentrations i.e., 3.3×10^8, 5.5×10^8, 8.8×10^8, 11×10^8 and 13.2×10^8 spores per ml were used. The required quantity of spores with toxin was mixed with 2 gram of artificial diet. Three replications were maintained for each isolate and thirty larvae each were introduced in each replication. Bioassay was done in small sterilized and autoclaved glass vials (Plate 2).

Larvae were introduced into the vials which were closed with clean cotton cloth to prevent the escape of the larvae. A vial with 2 gram of diet mixed with PMSF alone was used as a control. These vials were incubated for 24 h at 25°C to find out the toxicity of crystal protein. The per cent larval mortality was recorded after 24 h. The natural mortality was corrected from the original mortality by using the Abbot's formula, as follows:

$$\text{Per cent mortality} = \frac{X - Y}{X} \times 100$$

Where,

X= % live insects in control

Y= % live insects in treatment

Probit analysis was used to find out median lethal concentration (LC_{50}) value i.e., the toxin dosage to kill 50 per cent of the test insect (Finny, 1971).

Adult flies

Rearing netted cage

Vinegar fly eggs

First instar larvae

Third instar larvae

Glass vials for bioassay

Plate 2. Rearing of vinegar fly (*Drosophila melanogaster*) for bioassay

3.7 Plasmid profiling

3.7.1 Isolation of Plasmid DNA

Plasmid was isolated from *Bacillus thuringiensis* culture using Protoplast alkaline lysis (PAL) miniprep procedure (Voskuil and Chambliss, 1993). The details of reagents used for plasmid DNA isolation has been given in Appendix III.

1. One loopful of fresh culture was inoculated in 10ml LB broth and incubated overnight at 30°C with 180 rpm on a shaker.
2. The culture was centrifuged at 10,000 rpm for 5 min and supernatant removed by aspiration.
3. Pellets were suspended by vortexing in 200µl of SET (solution I) with lysozyme, transferred to a microcentrifuge tube and incubated for 10 min at 37°C.
4. 350µl volume of a fresh NaOH sodium dodecyl sulfate solution (SDS) (solution II) was added, and the microcentrifuge tube was repeatedly inverted until the suspension cleared.
5. 350µl of cold potassium acetate solution was added. This was vortexed for 10 sec at 5,000 rpm.
6. The suspension was centrifuged for 5 min at 10,000 rpm in a microcentrifuge and 750µl of the supernatant was transferred to a new microcentrifuge tube.
7. This supernatant fluid was extracted with 650µl of cold phenol-chloroform-isoamylalcohol (25:24:1) by vortexing for 1 min.
8. The mixture was centrifuged for 5 min, and 620µl of the aqueous phase was transferred to a new microcentrifuge tube, where it was extracted with 620µl of cold chloroform-isoamyl alcohol (24:1) by vortexing for 30 sec.
9. The mixture was centrifuged for 3 min, and 550µl of the aqueous phase was transferred to a new microcentrifuge tube.

10. The plasmid DNA was precipitated by adding an equal volume of cold (-20°C) isopropanol and inverting it several times. The suspension was centrifuged for 5 min and isopropanol was removed by aspiration.
11. The pellet was washed with 1 ml of 70 per cent ethanol and centrifuged for 2 min. The ethanol was completely removed by aspiration.
12. The pellet was dried under vacuum for 5 min and suspended in 50µl of deionized H_2O containing DNase-free pancreatic RNase (20 pg/ml).
13. Plasmid DNA was loaded on 0.7 per cent agarose gel and electrophoresis was performed. Then plasmid DNA was stored -20°C.

3.8 Gel elution of PCR amplified fragments

Products obtained in PCR reactions were loaded separately on 0.9 per cent (w/v) agarose gel and the desired band in each case was eluted using DNA Gel Extraction Kit (Chromous Biotech Pvt. Ltd) following the procedure as per the manufacturer's guidelines).

1. The DNA fragment of interest was excised from the gel using a sterile, sharp scalpel while avoiding much exposure to UV on a transilluminator.
2. Gel slice was weighed in a colourless 2ml micro centrifuge tube.
3. Three volumes of gel extraction buffer (w/v) added to one volume of gel.
4. The tube was incubated at 55°C for 5-10 min (or until the gel piece completely dissolved). The sample was mixed by inserting the tube every 2 to 3 min during the incubation to solublise agarose completely.
5. After the gel piece has dissolved completely, 1 gel volume of isopropanol was added to the tube and mixed well.
6. The spin column was placed in to a 2ml collection tube.
7. The gel extracted solution mixed with isopropanol was loaded on to the spin column (600µl each tube).

8. The spin column was spun at 13,000g for 1 min at room temperature. The content of the collection tube was discarded and was placed the spin column back in the same collection tube.
9. 500µl of wash buffer was added to the spin column and spun at 13,000g for 1 min at room temperature. The content of the collection tube was discarded and the spin column was placed back in the same collection tube.
10. The above step was repeated once.
11. The empty column was spun with the collection tube at 13,000g for 3 min at room temperature.
12. The spin column was placed in a fresh 1.5ml micro centrifuge tube.
13. 15µl of elution buffer was added to the spin column.
14. The vial was kept along with spin column at room temperature for 2 min and spun at 13,000g for 1 min at room temperature.
15. Again 15µl of elution buffer was added to the spin column.
16. The vial was kept along with the spin column at room temperature for 2 min and spun at 13,000g for 1 min at room temperature.
17. Purified DNA collected in the tube was checked the presence of DNA on 0.9 per cent agarose gel

3.9 Cloning

3.9.1 Preparation of competent cells

Competent cells for plasmid transformation were prepared using Genei Competent cell preparation Kit (B) from Bangalore Genei, following the manufacturer's guideline. Reagent used for competent cell preparation is given in Appendix III.

Day 1

1. *Escherichia coli* JM 109 was streaked on LB agar plate from stab and incubated at 37°C for 16 to 18 hours.

Day 2

1. 100ml SOC broth in 1000 ml conical flask was inoculated with 10 to 12 moderately sized colonies from SOC plates.
2. Overnight incubation was given at 37°C in a shaker at 200 rpm. When the OD_{600} reached 0.3 (3 to 3.5 h only), growth was arrested by chilling. The flask was chilled for 20 minutes.
3. The entire culture was transferred into a 50ml centrifuge tube and centrifuged at 3500 rpm for 15 minutes at 4°C.
4. The supernatant was discarded. Keeping the tubes on ice, resuspended the bacterial pellet very gently in 33.3ml ice cold solution A.
5. The tubes were kept on ice for 20 minutes and then centrifuged at 3500 rpm for 15 minutes at 4°C.
6. Supernatant was discarded and pellet chilled on ice. The pellet was resuspended in 5 to 6ml of ice cold solution A.
7. The suspension was kept on ice for 10 minutes and aliquots of 100µl were dispensed in chilled 1.5ml Eppendorf tubes.
8. The tubes were frozen on ice for few minutes before storing at -70°C.

   The competence of the cells prepared was confirmed by transformation using pUC18. The cells were plated on a plate containing LBA + 50mg/l ampicillin.

3.9.2 Ligation

The eluted product was ligated in pGEMT vector using pGEMT Easy Vector System (Promega Corporation, USA), following the manufacturer's protocol.

1. Reaction mixture was prepared as described below:

2X rapid ligation buffer	-	5.0µl
pGEMT Easy Vector (50ng)	-	1.0µl
PCR product	-	1.0µl
T4 DNA ligase (3 units/µl)	-	1.0µl
Deionised water	-	2.0µl
		10.0µl

2. The reaction mixture was incubated for one hour at room temperature. Then it was kept at 4^0C overnight. Next day it was used for transformation in competent cells of *E. coli.*

3.9.3 Transformation of ligated DNA into competent cells

1. The vial containing competent cells was thawed on ice.
2. The ligated product was added to the competent cells, contents mixed gently and kept on ice for 40 minutes.
3. The tube was taken from ice; heat shock was given at 42°C exactly for 90 seconds without shaking and placed back on ice for 5 minutes.
4. 250µl of LB broth was added and the tube was inverted twice
5. The tube was incubated at 37°C for one hour with shaking.
6. 100µl and 250µl aliquots of the transformed cells were plated on LB/ampicillin (50mg/l) plates layered with IPTG (6µl) and X-gal (60µl). (Stock: Ampicillin-5mg/ml in water, IPTG-200mg/ml in water, X-gal-20 mg/ml in DMSO) and incubated overnight at 37°C.
7. The recombinant clones were selected based on blue-white screening.

3.9.4 Confirmation of the presence of DNA insert by colony PCR

Colony PCR was carried out with recombinant clones to confirm the presence of inserted DNA. Recombinant bacterial colony was taken by

inoculation loop, mixed with 25µl sterile water and kept at 94°C for 2 min. After a brief centrifugation to sediment the bacterial cell constituents, 2µl of supernatant was taken and used as a template DNA for amplification of specific DNA insert.

3.9.4.1 Composition of the reaction mixture for colony PCR

a) Template DNA	-	2.0µl
b) 10X Taq assay buffer	-	2.5µl
c) d NTP mix (10mM)	-	1.0µl
d) Forward primer	-	1.0µl
e) Reverse primer	-	1.0µl
f) Taq DNA polymerase (0.3 U)	-	2.0µl
g) Autoclaved distilled water	-	15.5µl
		25.0µl

3.10 Sequencing of DNA clones

*Cry*4 amplicons obtained from the isolates Bt-23, Bt-133, Bt-190 and Bt-242 were sequenced. Recombinant clone with 800bp 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 400bp 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.

3.11 Theoretical analysis of sequence

3.11.1 Nucleic acid sequence analysis

The Blastn programme (http://www.ncbi.nm.nih.gov/blast/) was used to find out the homology of nucleotide sequences (Altschul *et al*., 1997). To find the

open reading frame of the nucleotide sequence, the programme ORF finder of NCBI was used (www.ncbi.nlm.gov/gorf/gorf). Nucleotide composition of the given sequence was determined by nucleotide statistics (NASTATS) tool offered by Biology Workbench (http://seqtool.sdsc.edu/). Phylogenetic analysis was done by using Clustal W tool.

3.11.2 Amino acid analysis

Nucleotide sequences were converted to amino acid sequences by using Expasy translate tool (http://www.expasy.ch/tools/dna.html). Physical and chemical properties of the given protein from the deduced amino acid were determined by amino acid statistics (AASTATS) tool offered by Biology Workbench.

3.12 Cloning full length ORF of *cry*4A gene

3.12.1 Primer designing for full length *cry*4A gene

Nucleotide sequence information about *cry*4A gene was collected from NCBI nucleotide database. The following steps were concerned to design primers.

1. Five complete nucleotide sequences (sequences accession numbers are D00248.1, EF424470.1, EF208904.1, Y00423.1, EF424470.1) encoding *cry*4A gene reported by various research workers from *B. thuringiensis* were downloaded from NCBI Genbank (http://www.ncbi.nlm.nih.gov) and copied in FASTA format into a notepad.
2. To assess the homology of the downloaded nucleotide sequences multiple sequence alignment was done using Clustal W (www. ebi.ac.uk/clustalw/) (Thompson *et al.*, 1994).
3. Based upon the homology of nucleotide sequences 18 to 24 bases highly similar regions were selected.

4. The forward and reverse primers were selected from highly similar sequence regions in such a way that
    a) The conserved boxes selected should have GC content not less than 50 per cent
    b) Melting temperature (Tm = 4 GC + 2 AT) ranged between 60°C and 65°C.
    c) It is preferable to have GC content at 3' end.
    d) There should not be any complementarily between forward and reverse primers.
    f) Repeats of single base should not appear within the primer sequence.
    h) Each primer should be 18 to 24bp long
5. For designing forward primer the sequence was taken as such and for the reverse primer, the reverse complementary sequence was taken.
6. Based on above condition forward and reverse primers were designed for full ORF of *cry*4A. These primer details were given in Table 5.

Table 5. Details of *cry*4A gene full length primer

Primer	sequence	Size (bp)	Annealing Temperature (Ta)°C	Expected amplicon size(kb)
Forward	5' ATGGGAGGAACAAATATGAATCC 3'	23	59	3.5
Reverse	5' CACTCGTTCATGCAAATTAATTC 3'	23		

2.12.2 PCR reaction

By using these forward and reverse primers, polymerase chain reaction was done. DNA isolated from Bt isolates Bt-23, Bt-133, Bt-190 and Bt-242 which yielded amplicons for *cry*4A gene was used as a template. The steps followed in PCR already have been mentioned in this chapter, earlier. The PCR products were separated on 0.9 per cent agarose gel.

Results

4. RESULTS

The results of the study on "Molecular cloning of *cry* genes specific to dipterans from native *Bacillus thuringiensis* Berliner" undertaken during the period 2007-2009 at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara are presented in this chapter under different subheads.

4.1 Characterization of *Bacillus thuringiensis* isolates

4.1.1 Cultural characterization

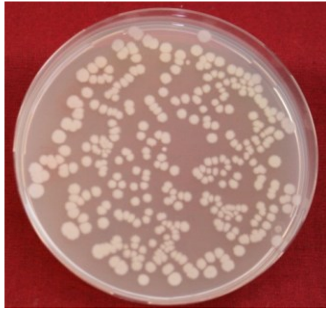
The colony characteristics of *B. thuringiensis* isolate from various places on LB agar medium showed slight variations in cultural characteristics. All the isolates produced creamy white, puffy colonies 24 hours after inoculation on LB agar medium. Isolates Bt-23, Bt-161, Bt-237, Bt-242, Bt-245, and Bt-326 produced large sized colonies, Bt-105, Bt-133, Bt-190, Bt-206, Bt-250, Bt-277, Bt-283, Bt-447 formed medium sized colonies and the other isolates i.e., Bt-135, Bt-170, Bt-411 produced small sized colonies (Table 6). The colonies were circular or irregular and flat. Margins were either entire or undulate (Plate 3).

4.1.2 Staining Reactions

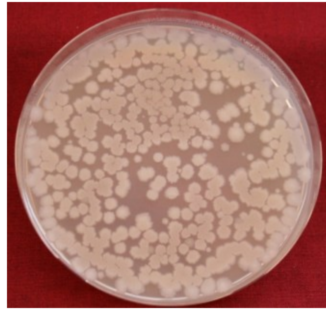
All isolates stained blue upon Gram staining, indicating Gram positive nature. The cells were rod-shaped and arranged in a chain. Staining of the isolates with Coomassie brilliant blue revealed the presence of dark bluish crystal proteins. The shape of crystal proteins varied from irregular, spherical and a composite of spherical and irregular. Isolates Bt-105, Bt-170, Bt-206, Bt-245, Bt-326 and Bt-411 produced a

Table 6. Colony characteristics of native isolates of *Bacillus thuringiensis*

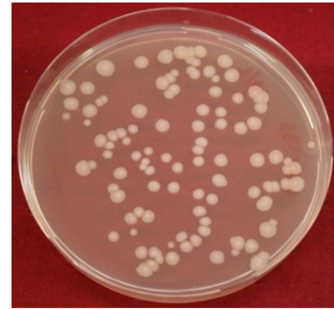
Sl. No.	Isolate	Pigmentation	Size	Form	Margin	Elev-ation	Optical charact-eristics	Diameter (cm)
1	Bt-23	Creamy white	Large	Irregular	Undulate	Flat	Opaque	0.60
2	Bt-105	Creamy white	Large	Circular	Entire	Flat	Opaque	0.60
3	Bt-133	Creamy white	Medium	Circular	Entire	Flat	Opaque	0.40
4	Bt-135	Creamy white	Small	Circular	Entire	Flat	Opaque	0.30
5	Bt-161	Creamy white	Large	Circular	Entire	Flat	Opaque	0.50
6	Bt-170	Creamy white	Small	Irregular	Undulate	Flat	Opaque	0.30
7	Bt-190	Creamy white	Large	Circular	Entire	Flat	Opaque	0.50
8	Bt-206	Creamy white	Small	Circular	Entire	Flat	Opaque	0.30
9	Bt-237	Creamy white	Large	Irregular	Entire	Flat	Opaque	0.50
10	Bt-242	Creamy white	Large	Irregular	Entire	Flat	Opaque	0.60
11	Bt-245	Creamy white	Large	Circular	Entire	Flat	Opaque	0.50
12	Bt-250	Creamy white	Large	Circular	Entire	Flat	Opaque	0.60
13	Bt-277	Creamy white	Large	Circular	Undulate	Flat	Opaque	0.60
14	Bt-283	Creamy white	Small	Circular	Entire	Flat	Opaque	0.30
15	Bt-326	Creamy white	Medium	Circular	Undulate	Flat	Opaque	0.40
16	Bt-411	Creamy white	Small	Circular	Entire	Flat	Opaque	0.25
17	Bt-419	Creamy white	Medium	Circular	Undulate	Flat	Opaque	0.40
18	Bt-420	Creamy white	Medium	Irregular	Entire	Flat	Opaque	0.35
19	Bt-430	Creamy white	Medium	Circular	Undulate	Flat	Opaque	0.35
20	Bt-447	Creamy white	Large	Circular	Undulate	Flat	Opaque	0.60
21	Standard 4Q1	Creamy white	Large	Circular	Entire	Flat	Opaque	0.70



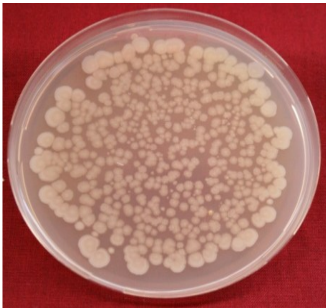
Bt-23



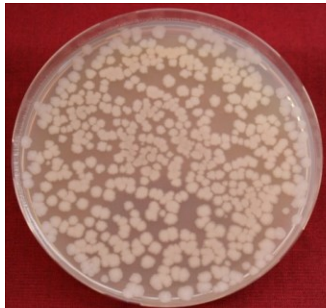
Bt-105



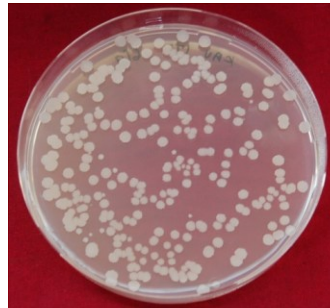
Bt-133



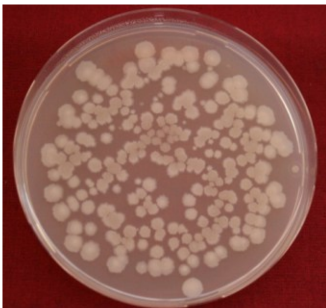
Bt-135



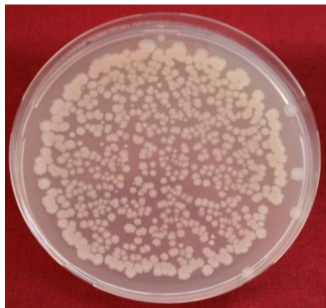
Bt-161



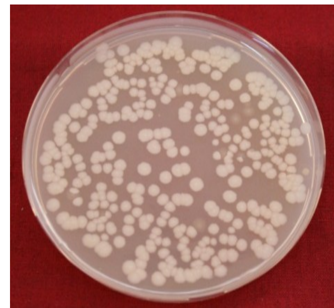
Bt-170



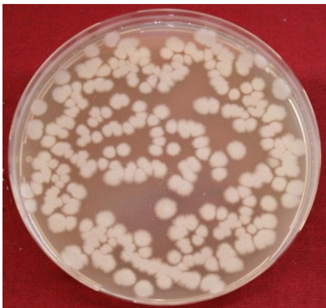
Bt-190



Bt-206

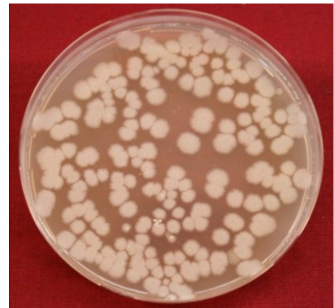


Bt-237



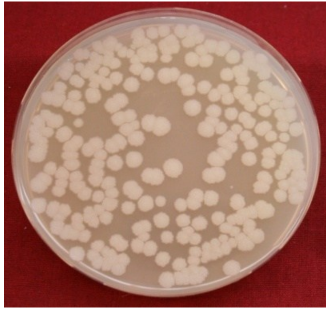
Bt-242

Bt-245

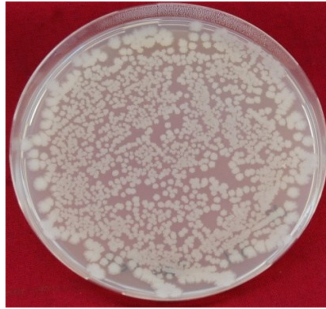


Bt-250

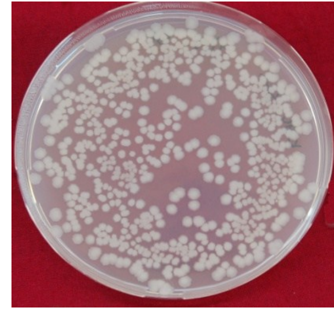
Plate 3. Colony morphology of *Bacillus thuringiensis* isolates



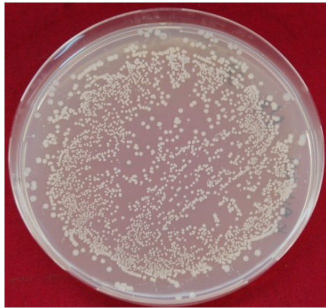
Bt-277



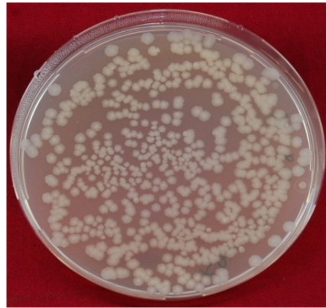
Bt-283



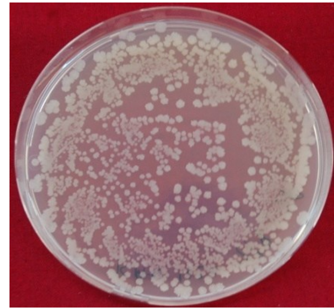
Bt-326



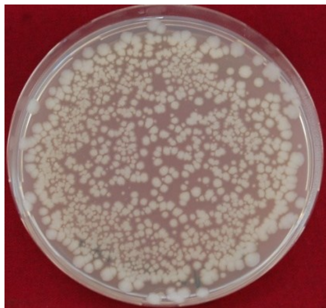
Bt-411



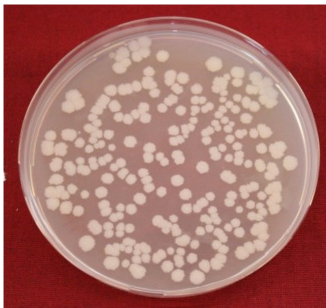
Bt-419



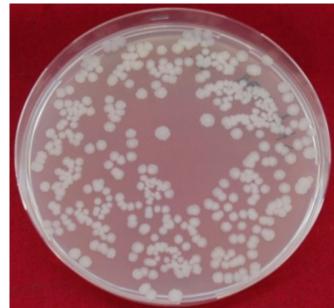
Bt-420



Bt-430



Bt-447



Standard 4Q1

Plate 3. Colony morphology of *Bacillus thuringiensis* isolates

composite of spherical and irregular crystal proteins. The other isolates Bt-23, Bt-133, Bt-161, Bt-190, Bt-237, Bt-242, Bt-277, Bt-283, Bt-419, Bt-420, Bt-430 and Bt-447 produced spherical crystals whereas Bt-135 and Bt-250 produced irregular crystal proteins. Of the total 20 isolates analyzed, 12 isolates produced spherical crystal proteins (Table 7). The morphology of bacterial cell, spore and crystal protein is shown in Plate 4.

4.1.3 Biochemical characterization

The isolates were characterized by seven biochemical tests (Table 8).

4.1.3.1 Esculinase test

All twenty isolates and the reference strain 4Q1 produced browning around the growth in esculin agar media, indicating esculinase activity (Plate 5a).

4.1.3.2 Gelatin hydrolysis

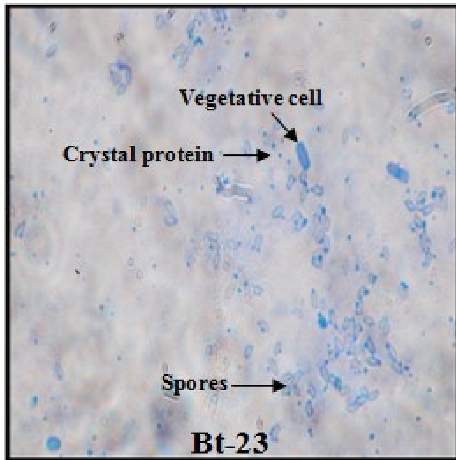
None of the isolates restored the gel characteristics of the gelatin agar medium, which indicated a negative reaction for gelatin hydrolysis.

4.1.3.3 Lecithinase test

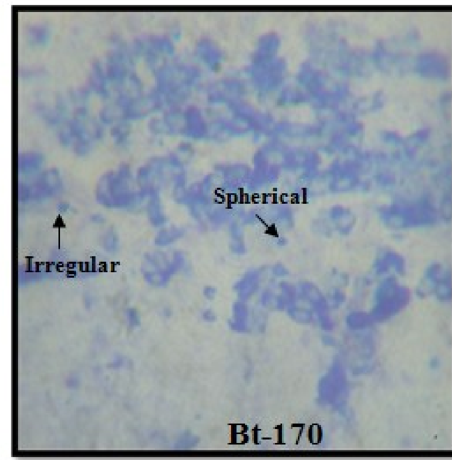
All the isolates including reference strain 4Q1 produced lecithinase. Zones of opalescence around the growth indicated a positive reaction for lecithinase, confirming the ability of isolates to hydrolyze lecithin present in egg yolk medium (Plate 5b).

Table 7. Crystal protein morphology of native isolates of *Bacillus thuringiensis* and the reference strain 4Q1

Sl. No.	Isolate	Shape of crystal protein	Abundance of crystal protein
1	Bt-23	Spherical	Numerous
2	Bt-105	Spherical & Irregular	Few
3	Bt-133	Rhomboidal	Numerous
4	Bt-135	Irregular	Few
5	Bt-161	Spherical	Few
6	Bt-170	Spherical & Irregular	Numerous
7	Bt-190	Spherical	Numerous
8	Bt-206	Spherical & Irregular	Moderate
9	Bt-237	Spherical	Moderate
10	Bt-242	Spherical	Numerous
11	Bt-245	Spherical & Irregular	Moderate
12	Bt-250	Irregular	Few
13	Bt-277	Spherical	Few
14	Bt-283	Spherical	Few
15	Bt-326	Spherical & Irregular	Moderate
16	Bt-411	Spherical & Irregular	Few
17	Bt-419	Spherical	Moderate
18	Bt-420	Spherical	Few
19	Bt-430	Spherical	Few
20	Bt-447	Spherical	Few
21	4Q1	Spherical	Numerous



a. Spherical



b. Irregular & Spherical

Plate 4. Crystal proteins stained with Coomassie brilliant blue in *B. thuringiensis* isolates

4.1.3.4 Solubility in three per cent KOH

None of the isolates formed viscous strands with three per cent KOH, confirming Gram positive nature.

4.1.3.5 Starch hydrolysis

A clear zone of hydrolysis surrounding the growth of bacteria when flooded with Lugol's iodine was observed in all twenty isolates and the reference strain indicating positive reaction (Plate 5c).

4.1.3.6 Urease activity

Ability of Bt isolates to degrade urea was detected by using urea broth with phenol red as a indicator. Colour change of the broth from yellow to deep pink indicated urea hydrolysis. Twelve cultures namely Bt-105, Bt-161, Bt-170, Bt-190, Bt-206, Bt-242, Bt-245, Bt-250, Bt-277, Bt-283, Bt-419 and Bt-447 developed deep pink colour. Bt-23, Bt-133, Bt-135, Bt-237, Bt-326, Bt-411, Bt-420, Bt-430 and standard 4Q1 did not produce any colour change, indicating the absence of urease enzyme (Plate 5d).

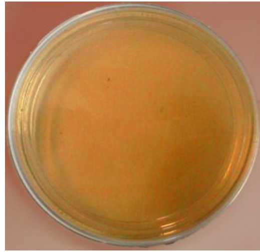
4.1.3.7 Voges Proskauer(VP) test

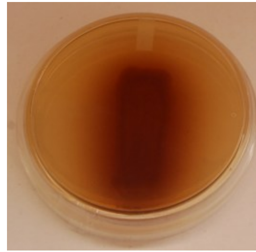
All the isolates were negative for Voges Proskauer test indicating the absence of non-acidic/neutral end products such as acetyl methyl carbinol from glucose metabolism.

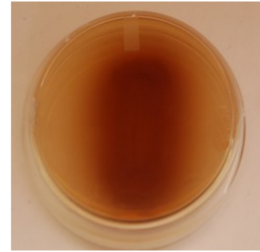
Table 8. Biochemical characteristics of *Bacillus thuringiensis* isolates

Sl. No.	Isolate	BIOCHEMICAL TESTS						
		Esculinase	Gelatin hydrolysis	Lecithinase	Solubility in 3% KOH	Starch hydrolysis	Urease	VP
1	Bt-23	+	_	+	_	+	_	_
2	Bt-105	+	_	+	_	+	+	_
3	Bt-133	+	_	+	_	+	_	_
4	Bt-135	+	_	+	_	+	_	_
5	Bt-161	+	_	+	_	+	+	_
6	Bt-170	+	_	+	_	+	+	_
7	Bt-190	+	_	+	_	+	+	_
8	Bt-206	+	_	+	_	+	+	_
9	Bt-237	+	_	+	_	+	_	_
10	Bt-242	+	_	+	_	+	+	_
11	Bt-245	+	_	+	_	+	+	_
12	Bt-250	+	_	+	_	+	+	_
13	Bt-277	+	_	+	_	+	+	_
14	Bt-283	+	_	+	_	+	+	_
15	Bt-326	+	_	+	_	+	_	_
16	Bt-411	+	_	+	_	+	_	_
17	Bt-419	+	_	+	_	+	_	_
18	Bt-420	+	_	+	_	+	+	_
19	Bt-430	+	_	+	_	+	_	_
20	Bt-447	+	_	+	_	+	+	_
21	4Q1	+	_	+	_	+	_	_

\+ Positive - Negative

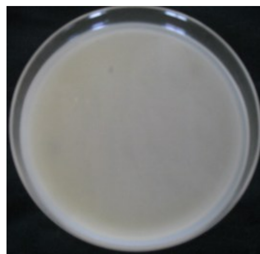


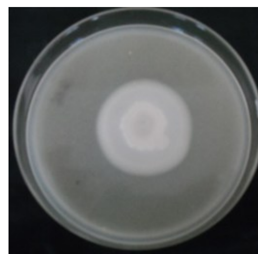


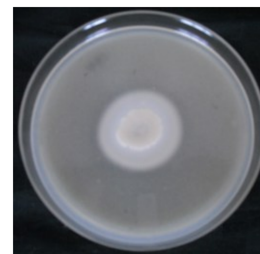


Control **Standard 4Q1** **Bt-23**

a. Esculinase

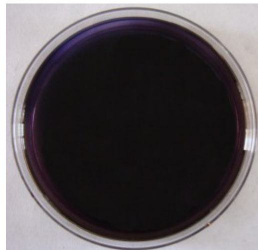


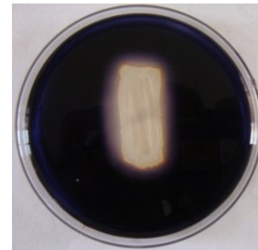




Control **Standard 4Q1** **Bt-133**

b. Lecithinase





Control **Standard 4Q1** **Bt-190**

c. Starch hydrolysis

1. Control
2. Bt-23
3. Bt-190
4. Bt-242

d. Urease

Plate 5. Response of nativeBt isolates to biochemical tests

4.2 Total DNA isolation from *Bacillus thuringiensis* isolates

Total DNA isolated from all the Bt isolates including standard; as per the procedure mentioned earlier was run on 0.7 per cent agarose gel to check whether DNA pure or any protein and RNA contamination. Since RNA and protein will affect the PCR amplification DNA should be free from these contaminants. No RNA contamination was observed (Plate 6a).

4.2.1 Quantification of DNA

The quantity of DNA as assessed by spectrophotometry using Nanodrop varied from 391 ng/µl to 1974 ng/µl of bacterial culture. The OD_{260}/OD_{280} ranged between 1.84 and 2.05 indicating that there was no protein contamination (Table 9).

4.3 Identification of dipteran specific *cry* genes in native *Bacillus thuringiensis* by *cry* gene profiling

cry gene profiling is a method used to find out the genes present in *Bacillus thuringiensis*. All 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* gene content.

4.3.1 *cry*2

The expected size of amplicons by using *cry*2 specific primer was the range in between 681bp to 701bp. None of the isolates produced the expected size amplicon indicating that *cry*2 gene was absent in these (Plate 6b).

Table 9. Quantity of total DNA extracted from native *Bacillus thuringiensis* isolates

Sl. No	Bt isolates	Optical density ratio (260/280)	Quantity of ds DNA(ng/µl)
1	Bt-23	1.92	461.7
2	Bt-105	1.95	1234.2
3	Bt-133	1.99	1029.3
4	Bt-135	2.03	1838.3
5	Bt-161	1.93	1034.9
6	Bt-170	1.90	391.0
7	Bt-190	1.95	947.9
8	Bt-206	1.97	1514.6
9	Bt-237	1.89	1343.6
10	Bt-242	1.83	450.6
11	Bt-245	1.91	1356.3
12	Bt-250	2.01	1974.9
13	Bt-277	1.88	1129.3
14	Bt-283	1.86	883.2
15	Bt-326	1.85	730.6
16	Bt-411	1.84	491.5
17	Bt-419	2.05	1929.4
18	Bt-420	1.96	1255.8
19	Bt-430	2.04	560.1
20	Bt-447	1.90	483.3
21	4Q1	1.98	1833.1

4.3.2 *cry*4

Expected amplicon size for cry4 gene was 439bp. However, two amplicons of 400bp and 800bp 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 400bp (Plate 6c).

4.3.3 *cry*10

Two isolates Bt-419 and Bt-447 yielded amplicon of the expected size of 404bp (Plate 7a).

4.3.4 *cry*11

The isolates Bt-23, Bt-420 and standard 4Q1 produced 305bp amplicon (Plate 7b).

4.3.5 *cry*16 and *cry*17

None of the isolates yielded the expected amplicon of 1415bp for *cry*16 (Plate 7c). No amplification was observed for *cry*17 also the expected size being 1400bp (Plate 8a).

4.3.6 *cry*19 and *cry*21

355bp and 453bp size amplicons were expected for *cry*19 and *cry*21 genes respectively. Out of the twenty isolates only Bt-245 gave expected amplicon for

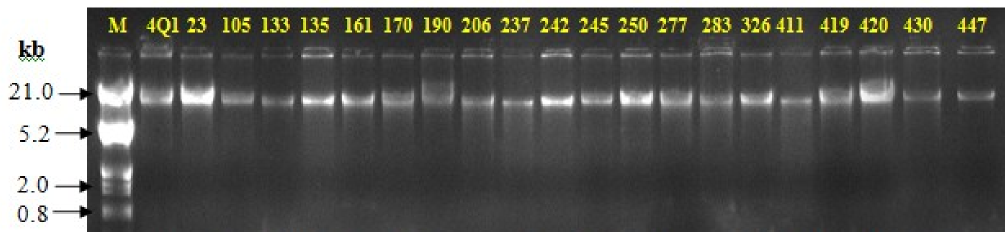
Table10a. *cry* gene content in native isolates of Bt

Sl. No	Isolates	*cry*2	*cry*4	*cry*10	*cry*11	*cry*16	*cry*17	*cry*19	*cry*21
1	Bt-23	-	+	-	+	-	-	-	-
2	Bt-105	-	-	-	-	-	-	-	-
3	Bt-133	-	+	-	-	-	-	-	-
4	Bt-135	-	-	-	-	-	-	-	-
5	Bt-161	-	-	-	-	-	-	-	-
6	Bt-170	-	-	-	-	-	-	-	-
7	Bt-190	-	+	-	-	-	-	-	-
8	Bt-206	-	-	-	-	-	-	-	-
9	Bt-237	-	-	-	-	-	-	-	-
10	Bt-242	-	+	-	-	-	-	-	-
11	Bt- 245	-	+	-	-	-	-	-	+
12	Bt- 250	-	+	-	-	-	-	-	-
13	Bt-277	-	+	-	-	-	-	-	-
14	Bt-283	-	-	-	-	-	-	-	-
15	Bt-326	-	+	-	-	-	-	-	-
16	Bt-411	-	+	-	-	-	-	-	-
17	Bt-419	-	-	+	-	-	-	-	-
18	Bt-420	-	+	-	+	-	-	-	-
19	Bt-430	-	-	-	-	-	-	-	-
20	Bt-447	-	+	+	-	-	-	-	-
21	4Q1	-	+	-	+	-	-	-	-

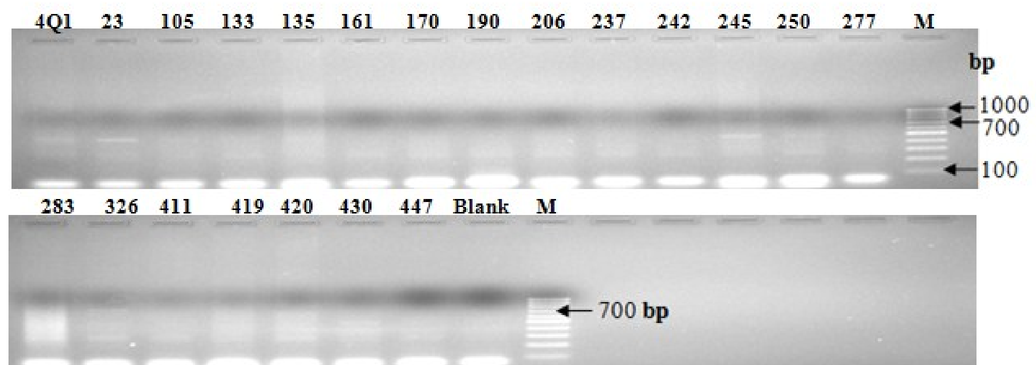
\+ Present - Absent

Table 10b. Details of amplicons obtained in *cry* gene profiling

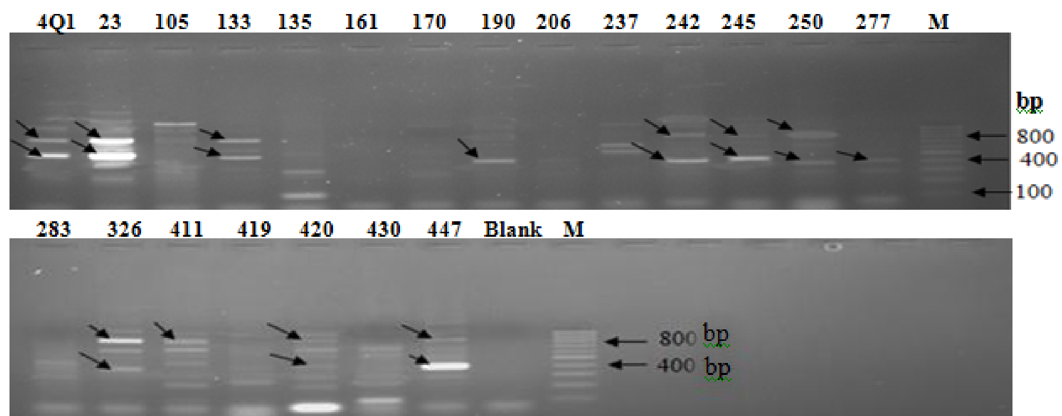
Sl. No	*cry* gene	Isolates	Expected amplicon size (bp)	Obtained amplicon size (bp)
1	*cry*2	-	689-701	-
2	*cry*4	Bt-23, Bt-133, Bt-190, Bt-242, Bt-245, Bt-250, Bt-277, Bt-326, Bt-411, Bt-420, Bt-447 and 4Q1	439	400, 800
3	*cry*10	Bt-419 and Bt-447	404	400
4	*cry*11	Bt-23, Bt-420 and 4Q1	305	300
5	*cry*16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



a. Total DNA isolated from selected *Bacillus thuringiensis* isolates



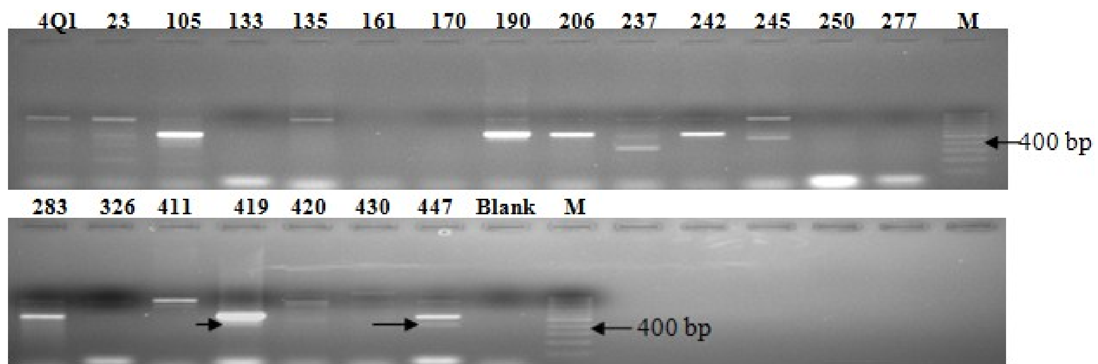
b. *cry*2



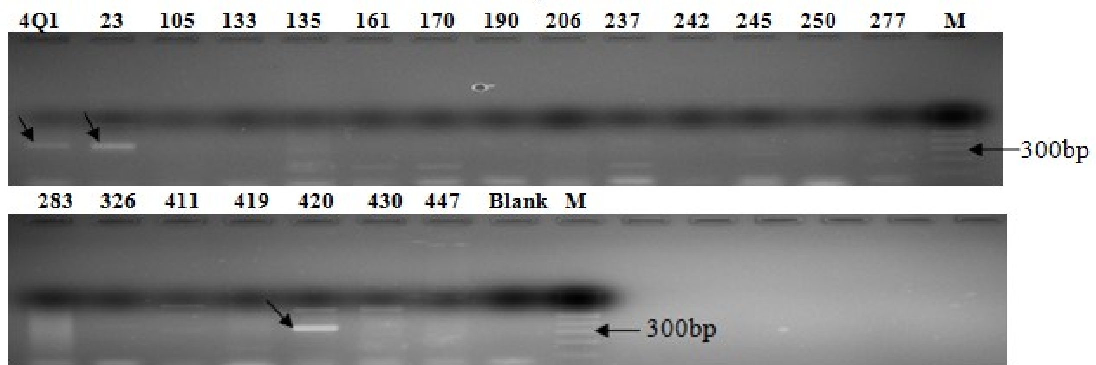
c. *cry*4

Arrows indicate amplicons of the expected size

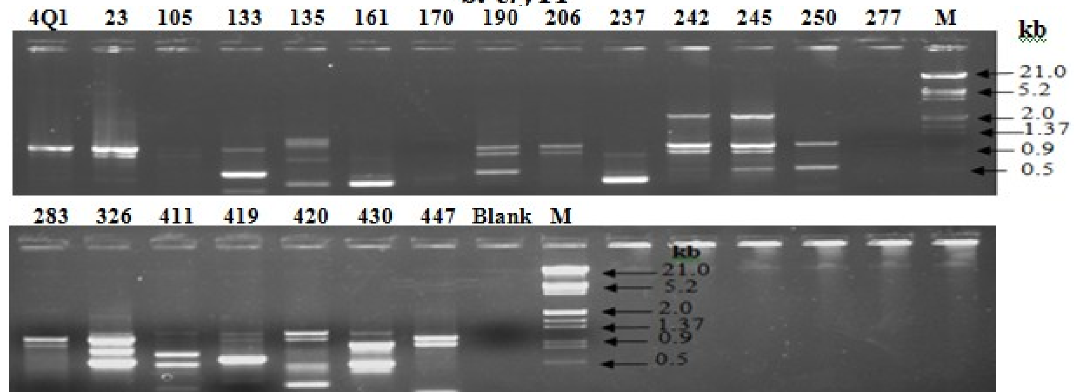
Plate 6. Profiles of dipteran specific *cry* genes (*cry*2 and *cry*4) in native *B. thuringiensis* isolates



a. *cry*10

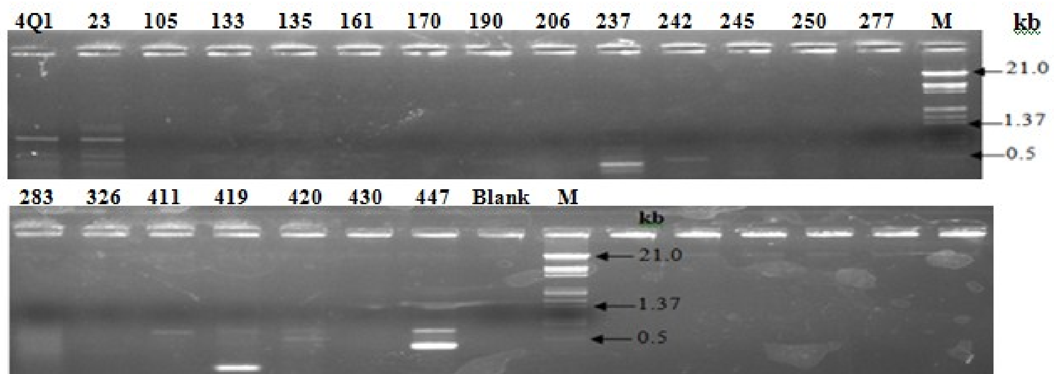


b. *cry*11

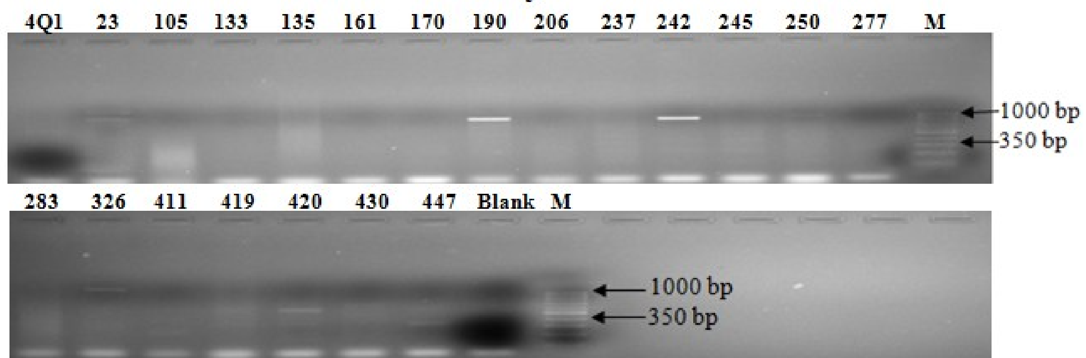


c. *cry*16

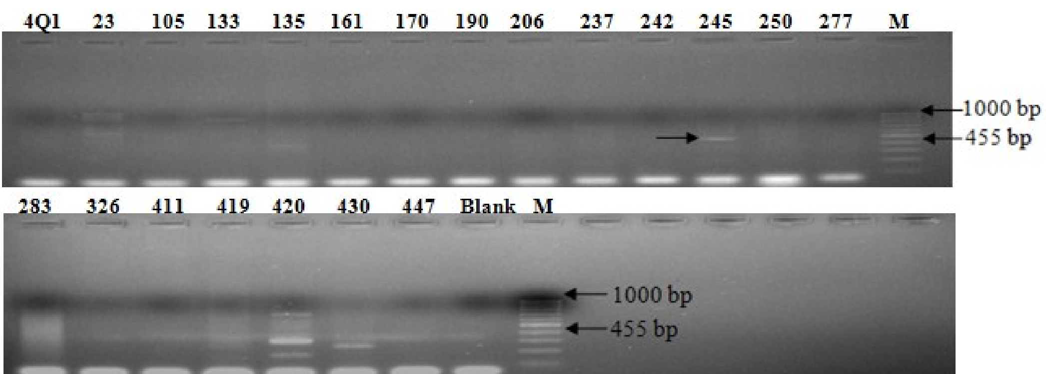
Plate 7. Profiles of dipteran specific *cry* genes (*cry*10, *cry*11 and *cry*16) in native *B. thuringiensis* isolates



a. *cry*17



b. *cry*19



c. *cry*21

Plate 8. Profiles of dipteran specific *cry* genes (*cry*17, *cry*19 and *cry*21) in native *B. thuringiensis* isolates

*cry*21. Except for this isolate, both the genes were absent in all the other isolates (Plates 8b and 8c). Details of *cry* gene profiling are given in Table 10a and 10b.

4.4 Bioassay of native *B. thuringiensis* isolates with *Drosophila melanogaster*

The activity of native *Bacillus thuringiensis* isolates against vinegar fly was assessed by diet contamination method.

4.4.1 Selection of Bt isolates for bioassay

Of the twenty native isolates, four isolates, Bt-23, Bt-133, Bt-190 and Bt-242 were selected for bioassay based on their *cry* gene profiling and the abundance of crystal protein. Standard strain 4Q1 was used in bioassay for standardizing the toxin dosage and comparing with toxicity of native isolates (Table 11).

4.4.2 Standardization of cry toxin dosage

Standardization of dosage of *cry* toxin was carried out by using 4Q1. Seven different concentrations of toxin-spore mixture were used. The dosage of 13.2×10^8 spores per ml of strain 4Q1 itself gave maximum (100 per cent) mortality. So this dosage was fixed as the maximum dose for other native isolates. Since 1.1×10^8 spores per ml gave very low mortality, 3.3×10^8 spores per ml was fixed as the minimum dosage (Table 12).

4.4.3 Symptoms of toxicity

The larva exhibiting the cessation of feeding was identified as the first symptom after feeding on the toxin. Larvae stopped moving and death occurred in 13

Table 11. ***cry*** **gene content and abundance of crystal protein in Bt isolates selected for bioassay**

Sl. No	Isolates	*cry* gene content	Abundance of crystal protein
1	Bt-23	*cry*4, *cry*11	Numerous
2	Bt-133	*cry*4	Numerous
3	Bt-190	*cry*4	Numerous
4	Bt-242	*cry*4	Numerous
5	4Q1	*cry*4, *cry*11	Numerous

Table 12. Mortality of ***Drosophila*** **larvae at different spore concentrations of 4Q1**

Sl. No.	Concentration ($\times 10^8$ spores per ml)	Insect mortality (%)
1	1.1	6.7
2	3.3	24.4
3	5.5	45.6
4	8.8	73.4
5	11.0	88.0
6	13.2	100.0
7	16.5	100.0

to 16 hours. Colour of the larvae changed to dark brownish black and the larval body got putrefied after death (Plate 9). Finally it turned into a black coloured mass and found oozing with of brownish fluid from the body mass.

4.4.4 Larval mortality

The four isolates along with strain 4Q1 were used for bioassay on *Drosophila melanogaster* (vinegar fly) larvae with five different spore concentrations. Moribund larvae were also considered as dead. The per cent mortality was calculated using the number of dead larvae after 24 h (Table 13).

The reference strain, 4Q1 caused 100 per cent morality at a concentration of 13.2 × 10^8 spores per ml after 24 hours. The mortality rate increased with an increasing spore concentration. The lowest concentration of 3.3 × 10^8 spores per ml caused the lowest per cent mortality. Among the native isolates, Bt-23 was found to cause highest mortality (88.8 per cent at the highest concentration of 13.2 × 10^8 spores per ml). The lowest per cent mortality was observed in the isolate Bt-242.

Concentration of spores showed a positive effect on the mortality of larvae in the case of all five *Bacillus thuringiensis* isolates. The per cent mortality was directly proportional to the concentration of spores.

4.4.5. Lethal concentration (LC_{50}) of *B. thuringiensis* isolates

The lethal concentration to kill 50 per cent of test population (LC_{50}) was determined by Probit analysis (Finney, 1971) from the per cent mortality at five different concentrations (Table 14). LC_{50} value was the lowest with the reference strain 4Q1 (6.05 × 10^8 spores per ml), indicating high level of toxicity. Among the

4Q1

4Q1

Bt-23

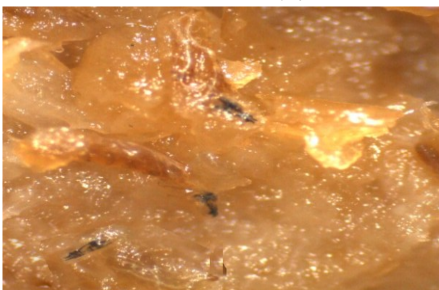
Bt-23

Bt-133

Bt-133

Bt-190

Bt-190


Bt-242

Bt-242

Plate 9. Symptoms of toxicity in *Drosophila* larvae due to Bt crystal protein

Table 13. Effect of crystal proteins from *B. thuringiensis* isolates on larvae of *Drosophila melanogaster*

Sl. No.	Concentration ($\times 10^8$ spores per ml)	Insect mortality of isolates (%)				
		4Q1	Bt-23	Bt-133	Bt-190	Bt-242
1	3.3	24.4	19.0	19.8	21.0	15.8
2	5.5	45.6	35.6	31.0	32.3	28.3
3	8.8	73.4	64.6	57.0	61.1	54.1
4	11.0	88.0	75.5	71.1	73.3	65.0
5	13.2	100	88.8	83.3	85.6	79.3

Table 14. Lethal concentration (LC_{50}) of *B. thuringiensis*

Sl. No.	Isolates	LC_{50} ($\times 10^8$ spores per ml)
1	4Q1	6.05
2	Bt-23	7.39
3	Bt-133	7.98
4	Bt-190	7.62
5	Bt-242	8.64

native isolates, Bt-23 recorded the least value of LC_{50} (7.39×10^8 spores per ml). Highest LC_{50} value of 8.64×10^8 spores per ml was recorded by Bt-242.

4.5 Plasmid profiling

Plasmid profiling was carried out to detect the plasmids present in the native *Bacillus thuringiensis* isolates. 4Q1 was used as a reference strain. In all the isolates one band was observed at 21kb which might be genomic DNA. Four bands of 5kb, 2.5kb, 2kb and 0.9kb size were observed in standard strain 4Q1. Three bands of 5kb, 2kb and 0.9kb were observed in Bt-23. In Bt-133 two plasmids of 3.5kb and 0.9kb were present. The isolate Bt-190 yielded a single band of 0.9kb and Bt-242 yielded two bands of 3.2kb and 1.2kb (Plate 10).

4.6 Amplification of *cry*4 gene from native isolates of selected *B. thuringiensis* isolates

Amplification of *cry*4 was carried out by PCR in selected native isolates viz., Bt-23, Bt-133, Bt-190 and Bt-242. Two amplicons of 400 and 800bp were obtained for three isolates namely, Bt-23, Bt-133 and Bt-242. Isolate Bt-190 yielded a single amplicon of size 400bp (Plate 11a).

4.7 Gel elution of PCR products

The *cry*4 amplicons of 400bp from isolates Bt-133, Bt-190, Bt-242 and 800bp amplicon from isolate Bt-23 were eluted from the gel the products showed good concentration on agarose gel suggesting that recovery of the PCR product was good (Plate 11b and 11c). The eluted band sizes 800bp from Bt-23 was ligated in pGEMT vector and remaining three 400bp amplicons were directly sequenced.

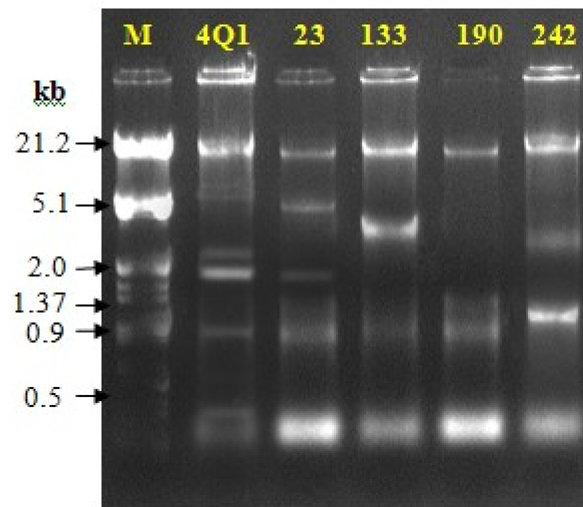


Plate 10. Plasmid profile of selected native *Bacillus thuringiensis* isolates

4.8 Transformation and confirmation of transformation

4.8.1 Transformation

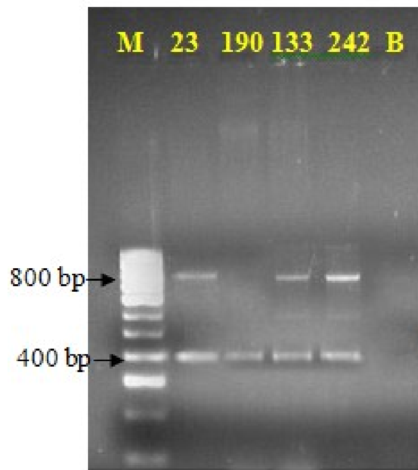
The ligated product was transferred into competent *E. coli* JM109 cells using the heat shock method. When the transformed *E. coli* cells were grown in LBA ampicillin plates overlaid with X-gal and IPTG, a combination of blue and white colonies were obtained after overnight incubation confirming successful transformation (Plate11d).

4.8.2 Confirmation of recombination by colony PCR

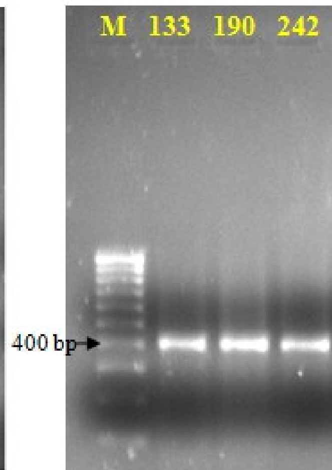
Colony PCR with plasmids from three white colonies yielded an amplicon of 800bp, which indicated the presence of insert DNA. No amplification was obtained from blue colony (Plate 11e).

4.9 Sequencing of *cry*4 amplicons

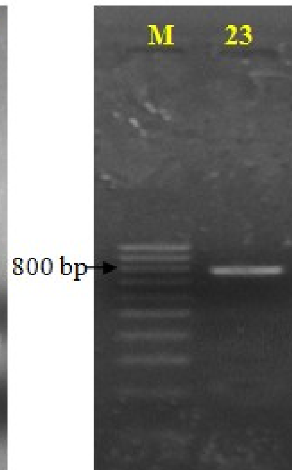
Cloned product of 800bp from Bt-23 and eluted productof400bp from native isolates Bt-23, Bt-133 and Bt-242 were sequenced. The graphical outputs and nucleotide sequences of each isolates are presented in Plates 12, 14, 16 and 18. Number of nucleotides and amino acids of each sequence are presented in Table 15.



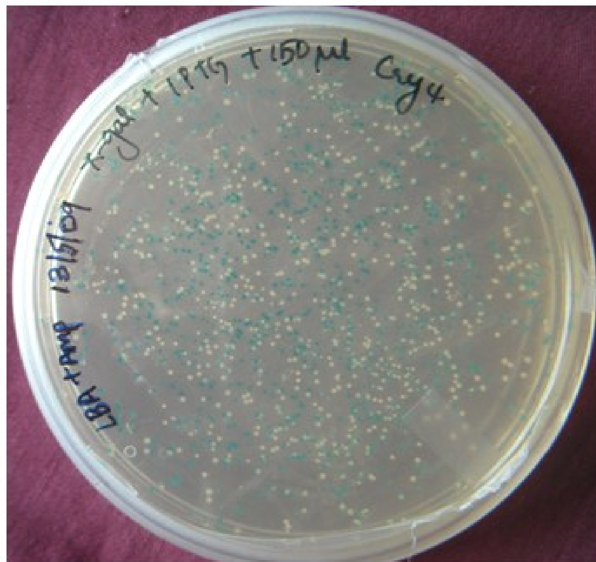
a. ***cry*4 gene amplicons for cloning**



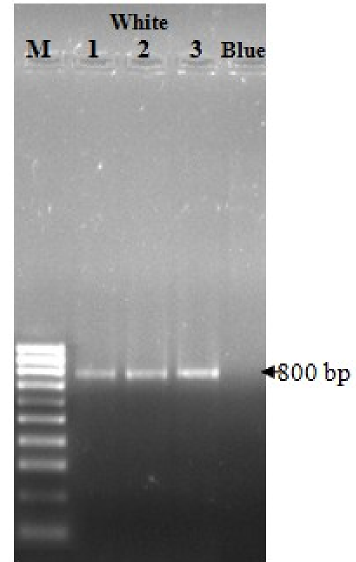
b. Eluted bands of *cry*4 gene 400bp fragment



c. Eluted 800 bp *cry*4 gene fragment



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



e. Colony PCR product from blue and white colonies

Plate11. Cloning of *cry*4 gene fragment in *E.coli*

4.10 Theoretical sequence analysis

Different computer algorithms were used to analyze the nucleotide sequences and amino acid sequences obtained from the isolates Bt-23, Bt-133, Bt-190 and Bt-242.

4.10.1 Nucleotide sequence analysis

4.10.1.1 Nucleotide Blast

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. 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* 130kDa pesticidal *cry* gene sequences in NCBI databank. However, Bt-23*cry*4 also showed homology with *B. cereus* genome. Details of accessions showing homology with the amplicons under the study are provided in plates 13a, 15a, 17a, and 19a.

4.10.1.2 Open reading frame

The sequences were translated in all six opening reading frames (http://www.ncbi.nlm.nih.gov.ORF finder). There were five open reading frames in Bt-23*cry*4, with the longest one located on +3 strand starting from base 279 to 683, having a length of 405 bases, second one on +2 strand starting from the base 581 to 781, with a length of 201 bases, the remaining ORFs were starting from 227 to 346, 2 to 118 and 110 to 211 with 120 bases, 117 bases, 102 bases length on -2, -2 and +2 strands respectively (Plate 13b).

Bt-133*cry*4 had two open reading frames, with the longer one on -3 strand with a length of 237 bases, starting from base 40 to 276 and the other on +3 strand with a length of 102 bases starting from base 93 to 194 (Plate 15b). Bt-190*cry*4 possessed two open reading frames (Plate 17b) with the longest one located on -1 strand with a length of 285bp, starting from base 2 to 286. The second one was encoded on the +1 strand, starting from base 103 to 216, with a length of 114bp. There were four ORFs in Bt-242*cry*4, with the largest one located on -3 strand, having a length of 288bp starting from base 1 to 288. The others were located on the +1, +3 and -1 strands, with a length of 186bp, 183bp and 180bp respectively (Plate 19b). The location and length of ORFs are specified in Table 16.

4.10.1.3 Composition of nucleotide

Nucleotide composition of the above sequences was determined using Biology Work Bench (http://seqtool.sds.edu/). The A+T and G+C base pair composition was 60.6 per cent and 39.4 per cent in Bt-23*cry*4, while Bt-133*cry*4 A+T was 61 per cent and G+C was 38.8 per cent. Bt-190*cry*4 was comparatively rich in A+T (64.3%) among all sequences with very low G+C (35.7%). In contrast, Bt-242*cry*4 was comparatively low in A+T content (57.2%) than the other gene sequences. The details of nucleotide composition of sequences are given in Table 17.

4.10.2 Amino acid sequence analysis

The nucleotide sequences were converted to their respective amino acid by using Expasy translate tool (13c, 15c, 17c and 19c). These amino acid sequences were subjected to various computer algorithms in order to characterize them.

Table 15. Details of amplicons from native *B. thuringiensis* isolates used for sequencing

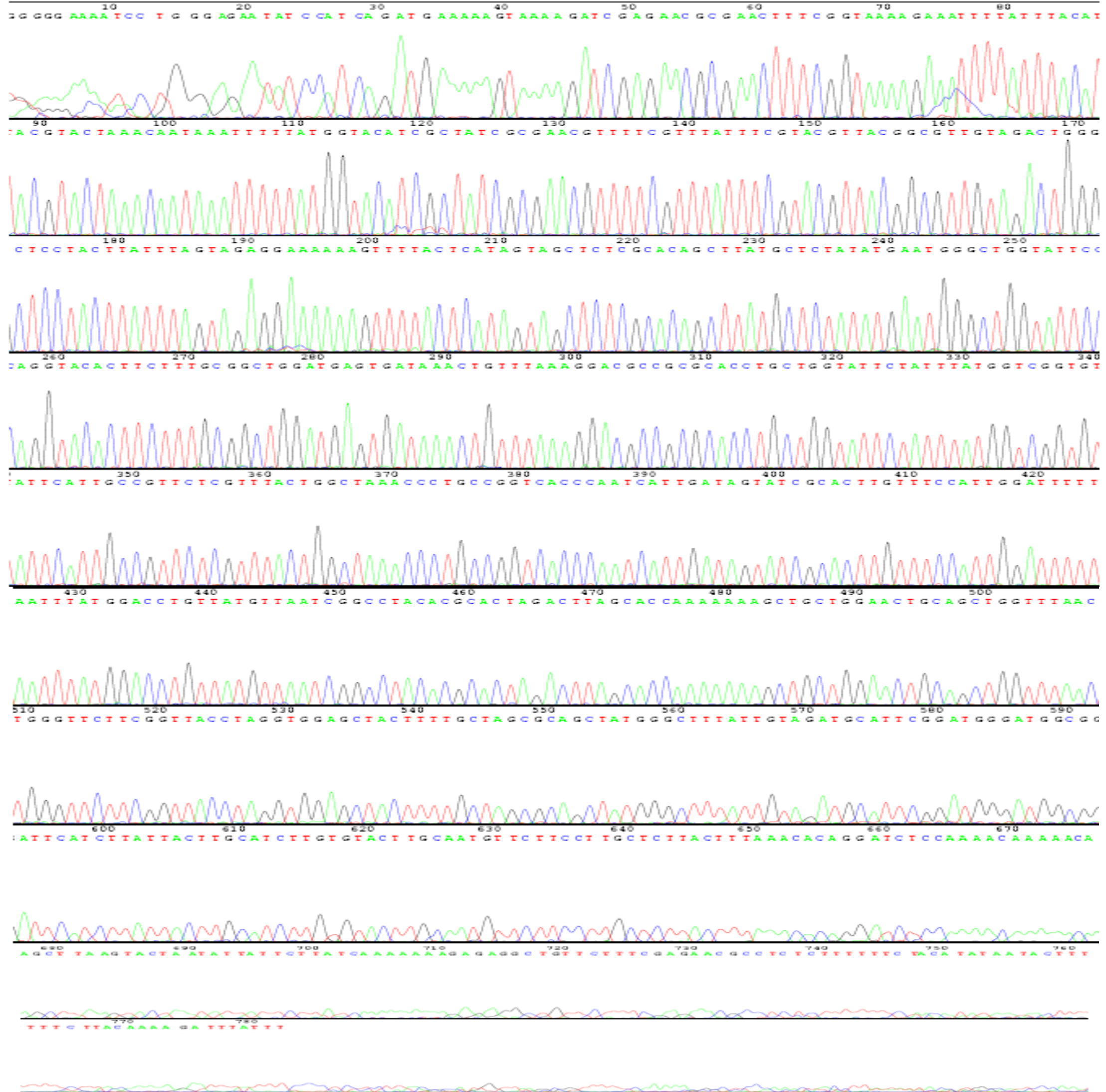
Sl. No.	Sequence name	Nucleotide	Amino acid
1	Bt-23*cry*4	780	243
2	Bt-133*cry*4	356	111
3	Bt-190*cry*4	364	116
4	Bt- 242*cry*4	362	116

Table 16. Open reading frames of *cry* gene fragments from native isolates

Sl. No.	Gene	No. of ORFs	Location	Length (bp)	Reading frame
1	Bt-23*cry*4	5	279-683	405	+3
			581-781	201	+2
			227-346	120	-2
			2-118	117	-2
			110-211	102	+2
2	Bt-133*cry*4	2	40-276	237	-3
			93-194	102	+3
3	Bt-190*cry*4	2	2-286	285	-1
			103-216	114	+1
4	Bt-242*cry*4	4	1-288	288	-3
			1-186	186	+1
			180-361	183	+3
			51-230	180	-1

Table 17. Nucleotide statistics of *cry* gene fragments from native *B. thuringiensis* isolates

Sl. No.	Gene sequence	Nitrogen base percentage (%)					
		A	T	G	C	A and T	G and C
1	Bt-23*cry*4	27.0	33.6	20.1	19.3	60.6	39.4
2	Bt-133*cry*4	30.3	30.9	14.9	23.9	61.2	38.8
3	Bt-190*cry*4	32.1	32.1	13.2	22.5	64.3	35.7
4	Bt-242*cry*4	24.9	32.3	18.0	24.9	57.2	42.8



a. Graphical output

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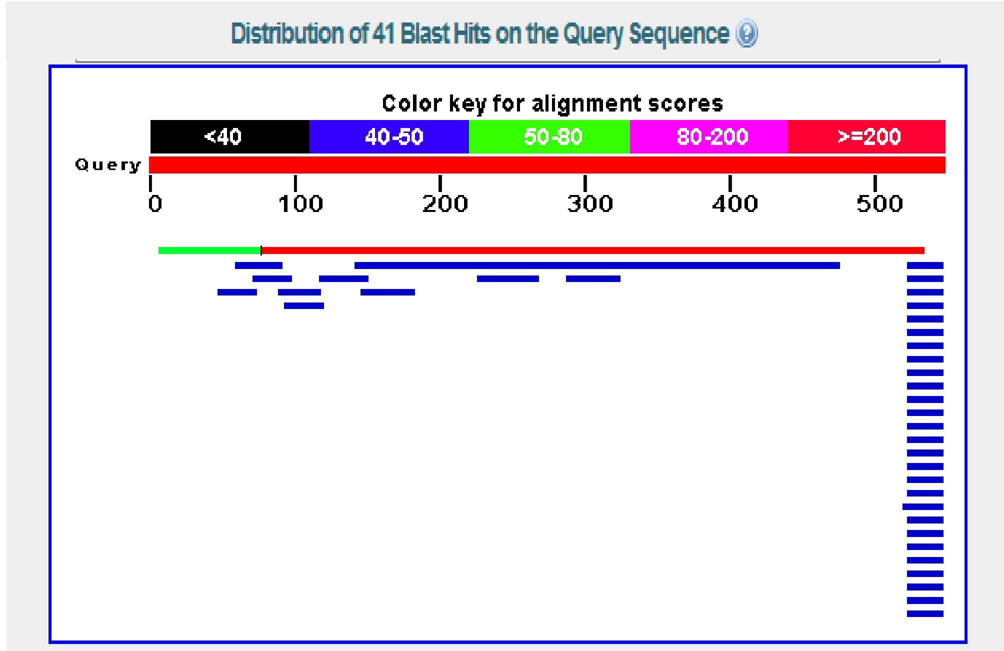
TTTCGGTAAAAGAAATTTTATTTACATACGTACTAAACAATAAATTTTTATGGTACATCG
CTATCGCGAACGTTTTCGTTTATTTCGTACGTTACGGCGTTGTAGACTGGGCTCCTACTT
ATTTAGTAGAGGAAAAAAGTTTTACTCATAGTAGCTCTCGCACAGCTTATGCTCTATATG
AATGGGCTGGTATTCCAGGTACACTTCTTTGCGGCTGGATGAGTGATAAACTGTTTAAAG
GACGCCGCGCACCTGCTGGTATTCTATTTATGGTCGGTGTATTCATTGCCGTTCTCGTTT
ACTGGCTAAACCCTGCCGGTCACCCAATCATTGATAGTATCGCACTTGTTTCCATTGGAT
TTTTAATTTATGGACCTGTTATGTTAATCGGCCTACACGCACTAGACTTAGCACCAAAAA
AAGCTGCTGGAACTGCAGCTGGTTTAACTGGGTTCTTCGGTTACCTAGGTGGAGCTACTT
TTGCTAGCGCAGCTATGGGCTTTATTGTAGATGCATTCGGATGGGATGGCGGATTCATCT
TATTACTTGCATCTTGTGTACTTGCAATGTTCTTCCTTGCTCTTACTTTAAACACAGGAT
CTCCAAAACAAAAACAAGCTTAAGTACTAATATTATTCTTATCAAAAAAAGAGAGGCTGT
TCTTTCGAGAACGCCTCTCTTTTTTCTACATATAATACTTTTTTCTTACAAAAGATTTAT
TT

b. Nucleotide sequence

Plate 12. Details of Bt-23*cry*4 gene fragment

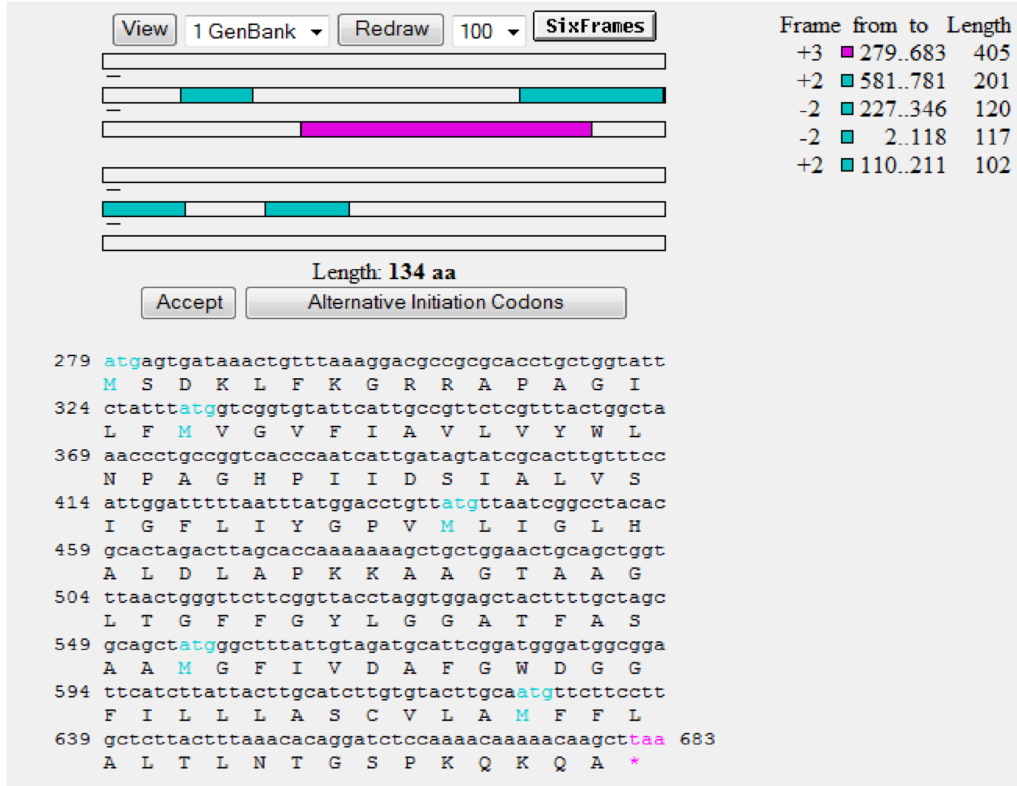


Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
CP001176.1	Bacillus cereus B4264, complete genome	499	552	96%	9e-138	84%	
CP000001.1	Bacillus cereus E33L, complete genome	48.2	48.2	61%	0.047	63%	
GQ202006.1	Bacillus thuringiensis strain AR-56 dipterans toxin crystal protein (cr	46.4	46.4	4%	0.16	100%	
DQ788672.1	Bacillus thuringiensis isolate DAB BT6 Cry4 (cry4) gene, partial cds	46.4	46.4	4%	0.16	100%	
DQ788671.1	Bacillus thuringiensis isolate DAB BT5 Cry4 (cry4) gene, partial cds	46.4	46.4	4%	0.16	100%	
DQ788670.1	Bacillus thuringiensis isolate DAB BT4 Cry4 (cry4) gene, partial cds	46.4	46.4	4%	0.16	100%	
DQ788669.1	Bacillus thuringiensis isolate DAB BT3 Cry4 (cry4) gene, partial cds	46.4	46.4	4%	0.16	100%	
DQ788667.1	Bacillus thuringiensis isolate DAB BT1 Cry4 (cry4) gene, partial cds	46.4	46.4	4%	0.16	100%	
EF468630.1	Bacillus thuringiensis isolate DAB-Bt6 pesticidal crystal protein gene	46.4	46.4	4%	0.16	100%	
EF468628.1	Bacillus thuringiensis isolate DAB-Bt4 pesticidal crystal protein gene	46.4	46.4	4%	0.16	100%	
EF468626.1	Bacillus thuringiensis isolate DAB-Bt2 truncated pesticidal crystal pr	46.4	46.4	4%	0.16	100%	
EF468625.1	Bacillus thuringiensis isolate DAB-Bt1 pesticidal crystal protein gene	46.4	46.4	4%	0.16	100%	
EF424472.1	Bacillus thuringiensis isolate DAB-BT4 Cry4A gene, complete cds	46.4	46.4	4%	0.16	100%	
EF424471.1	Bacillus thuringiensis isolate DAB-BT2 Cry4A gene, complete cds	46.4	46.4	4%	0.16	100%	
EF424470.1	Bacillus thuringiensis isolate DAB-BT6 Cry4A gene, complete cds	46.4	46.4	4%	0.16	100%	
EF424469.1	Bacillus thuringiensis isolate DAB-BT5 Cry4A gene, complete cds	46.4	46.4	4%	0.16	100%	
EF424468.1	Bacillus thuringiensis isolate DAB-BT3 Cry4A gene, complete cds	46.4	46.4	4%	0.16	100%	
EF208904.1	Bacillus thuringiensis Cry4A (cry4A) gene, complete cds	46.4	46.4	4%	0.16	100%	
AY729887.1	Bacillus thuringiensis serovar israelensis delta-endotoxin (cry4BLB)	46.4	46.4	4%	0.16	100%	
AY847707.1	Bacillus thuringiensis strain PBT602 130 kDa crystal protein (cry) ge	46.4	46.4	4%	0.16	100%	
AL731825.1	Bacillus thuringiensis subsp. israelensis plasmid pBtoxis	46.4	135	5%	0.16	100%	
X07423.1	Bacillus thuringiensis israelensis bt8 gene for 130 kDa crystal protei	46.4	46.4	4%	0.16	100%	
Y00423.1	Bacillus thuringiensis gene for 130 kDa delta-endotoxin	46.4	46.4	4%	0.16	100%	
X07082.1	Bacillus thuringiensis gene for 130 kDa delta-endotoxin	46.4	46.4	4%	0.16	100%	
D00248.1	Bacillus thuringiensis israelensis plasmid gene for 130 kDa insecticic	46.4	46.4	4%	0.16	100%	G
D00247.1	Bacillus thuringiensis israelensis plasmid gene for 130 kDa insecticic	46.4	46.4	4%	0.16	100%	
M20242.1	B.thuringiensis mosquitocidal protein (CryD2) gene, complete cds	46.4	46.4	4%	0.16	100%	
BN001301.1	TPA: TPA reasm: Aspergillus nidulans FGSC A4 chromosome I	42.8	42.8	7%	2.0	81%	
BX119919.5	Human DNA sequence from clone RP11-987D21 on chromosome X,	42.8	42.8	6%	2.0	87%	
AL807753.10	Mouse DNA sequence from clone RP23-359L15 on chromosome X C	42.8	42.8	6%	2.0	90%	
EF468629.1	Bacillus thuringiensis isolate DAB-Bt5 pesticidal crystal protein gene	41.0	41.0	4%	6.9	96%	
CR936503.1	Lactobacillus sakei strain 23K complete genome	41.0	41.0	4%	6.9	92%	
AE000657.1	Aquifex aeolicus VF5, complete genome	41.0	41.0	4%	6.9	92%	

a. Blastn output

Plate 13. Sequence analysis of Bt-23*cry*4 gene fragment



b. Open reading frame

```
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 G   G   K   S   W   E   N   I   H   Q   M   K   K   -   K   I   E   N   A   N
tttcggtaaaagaaattttatttacatacgtactaaacaataaatttttatggtacatcg
 F   R   -   K   K   F   Y   L   H   T   Y   -   T   I   N   F   Y   G   T   S
ctatcgcgaacgttttcgtttatttcgtacgttacggcgttgtagactgggctcctactt
 L   S   R   T   F   S   F   I   S   Y   V   T   A   L   -   T   G   L   L   L
atttagtagaggaaaaaagttttactcatagtagctctcgcacagcttatgctctatatg
 I   -   -   R   K   K   V   L   L   I   V   A   L   A   Q   L   M   L   Y   M
aatgggctggtattccaggtacacttctttgcggctggatgagtgataaactgtttaaag
 N   G   L   V   F   Q   V   H   F   F   A   A   G   -   V   I   N   C   L   K
gacgccgcgcacctgctggtattctatttatggtcggtgtattcattgccgttctcgttt
 D   A   A   H   L   L   V   F   Y   L   W   S   V   Y   S   L   P   F   S   F
actggctaaaccctgccggtcacccaatcattgatagtatcgcacttgtttccattggat
 T   G   -   T   L   P   V   T   Q   S   L   I   V   S   H   L   F   P   L   D
ttttaatttatggacctgttatgttaatcggcctacacgcactagacttagcaccaaaaa
 F   -   F   M   D   L   L   C   -   S   A   Y   T   H   -   T   -   H   Q   K
aagctgctggaactgcagctggtttaactgggttcttcggttacctaggtggagctactt
 K   L   L   E   L   Q   L   V   -   L   G   S   S   V   T   -   V   E   L   L
ttgctagcgcagctatgggctttattgtagatgcattcggatgggatggcggattcatct
 L   L   A   Q   L   W   A   L   L   -   M   H   S   D   G   M   A   D   S   S
tattacttgcatcttgtgtacttgcaatgttcttccttgctcttactttaaacacaggat
 Y   Y   L   H   L   V   Y   L   Q   C   S   S   L   L   L   L   -   T   Q   D
ctccaaaacaaaaacaagcttaagtactaatattattcttatcaaaaaaagagaggctgt
 L   Q   N   K   N   K   L   K   Y   -   Y   Y   S   Y   Q   K   K   R   G   C
tctttcgagaacgcctctctttttttctacatataatactttttttcttacaaaagatttat
 S   F   E   N   A   S   L   F   S   T   Y   N   T   F   F   L   Q   K   I   Y
tt
```
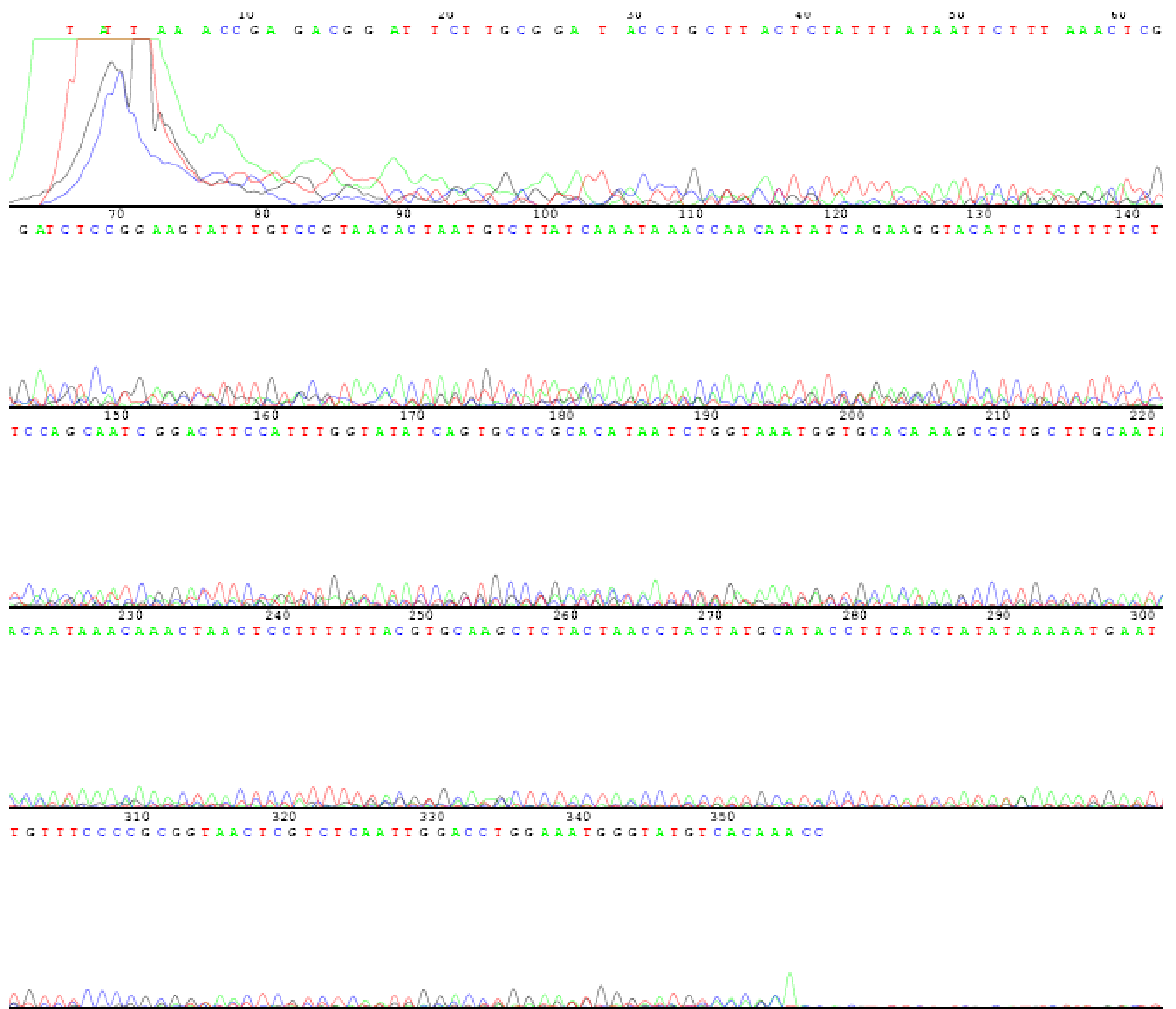

c. Deduced amino acid sequence

Plate 13. Sequence analysis of Bt-23*cry*4 gene fragment



a. Graphical output

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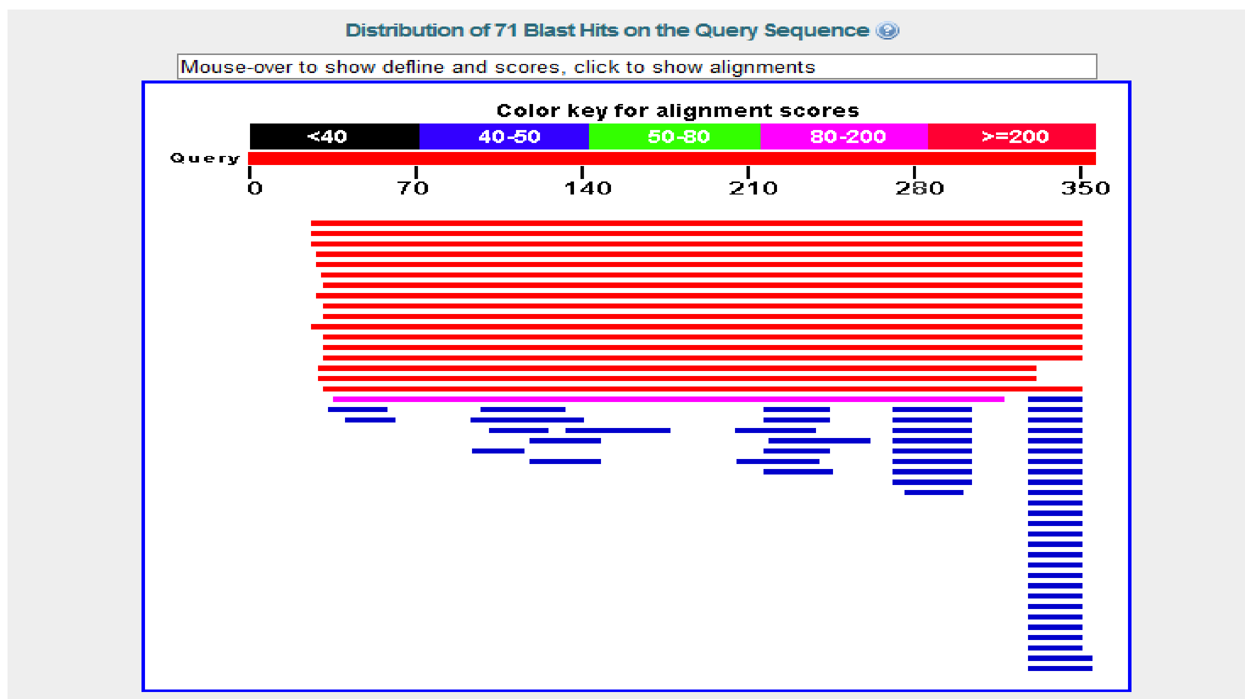
CGGATCTCCGGAAGTATTTGTCCGTAACACTAATGTCTTATCAAATAAACCAACAATATC
AGAAGGTACATCTTCTTTTCTTCCAGCAATCGGACTTCCATTTGGTATATCAGTGCCCGC
ACATAATCTGGTAAATGGTGCACAAAGCCCTGCTTGCAATACAATAAACAAACTAACTCC
TTTTTTACGTGCAAGCTCTACTAACCTACTATGCATACCTTCATCTATATAAAAATGAAT
TGTTTCCCCGCGGTAACTCGTCTCAATTGGACCTGGAAATGGGTATGTCACAAACC

b. Nucleotide sequences

Plate 14. Details of Bt-133*cry*4 gene fragment



Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
GQ202006.1	Bacillus thuringiensis strain AR-56 dipterans toxin crystal protein (cr	42.8	42.8	6%	1.2	100%	
DQ788672.1	Bacillus thuringiensis isolate DAB BT6 Cry4 (cry4) gene, partial cds	42.8	42.8	6%	1.2	100%	
DQ788671.1	Bacillus thuringiensis isolate DAB BT5 Cry4 (cry4) gene, partial cds	42.8	42.8	6%	1.2	100%	
DQ788670.1	Bacillus thuringiensis isolate DAB BT4 Cry4 (cry4) gene, partial cds	42.8	42.8	6%	1.2	100%	
DQ788669.1	Bacillus thuringiensis isolate DAB BT3 Cry4 (cry4) gene, partial cds	42.8	42.8	6%	1.2	100%	
DQ788667.1	Bacillus thuringiensis isolate DAB BT1 Cry4 (cry4) gene, partial cds	42.8	42.8	6%	1.2	100%	
EF468630.1	Bacillus thuringiensis isolate DAB-Bt6 pesticidal crystal protein gene	42.8	42.8	6%	1.2	100%	
EF468628.1	Bacillus thuringiensis isolate DAB-Bt4 pesticidal crystal protein gene	42.8	42.8	6%	1.2	100%	
EF468626.1	Bacillus thuringiensis isolate DAB-Bt2 truncated pesticidal crystal pr	42.8	42.8	6%	1.2	100%	
EF468625.1	Bacillus thuringiensis isolate DAB-Bt1 pesticidal crystal protein gene	42.8	42.8	6%	1.2	100%	
EF424472.1	Bacillus thuringiensis isolate DAB-BT4 Cry4A gene, complete cds	42.8	42.8	6%	1.2	100%	
EF424471.1	Bacillus thuringiensis isolate DAB-BT2 Cry4A gene, complete cds	42.8	42.8	6%	1.2	100%	
EF424470.1	Bacillus thuringiensis isolate DAB-BT6 Cry4A gene, complete cds	42.8	42.8	6%	1.2	100%	
EF424469.1	Bacillus thuringiensis isolate DAB-BT5 Cry4A gene, complete cds	42.8	42.8	6%	1.2	100%	
EF424468.1	Bacillus thuringiensis isolate DAB-BT3 Cry4A gene, complete cds	42.8	42.8	6%	1.2	100%	
CR388145.18	Zebrafish DNA sequence from clone DKEY-264F17, complete seque	42.8	42.8	7%	1.2	96%	
EF208904.1	Bacillus thuringiensis Cry4A (cry4A) gene, complete cds	42.8	42.8	6%	1.2	100%	
AM263198.1	Listeria welshimeri serovar 6b str. SLCC5334 complete genome	42.8	42.8	10%	1.2	86%	
CP000411.1	Oenococcus oeni PSU-1, complete genome	42.8	42.8	13%	1.2	81%	
AY729887.1	Bacillus thuringiensis serovar israelensis delta-endotoxin (cry4BLB)	42.8	42.8	6%	1.2	100%	
AY847707.1	Bacillus thuringiensis strain PBT602 130 kDa crystal protein (cry) ge	42.8	42.8	6%	1.2	100%	
CR759843.7	Zebrafish DNA sequence from clone DKEYP-82B4 in linkage group 4	42.8	42.8	7%	1.2	96%	
BX041693.1	Single read from an extremity of a full-length cDNA clone made fro	42.8	42.8	9%	1.2	88%	U
CR769766.10	Zebrafish DNA sequence from clone DKEY-207J12 in linkage group	42.8	42.8	12%	1.2	83%	
BX855613.7	Zebrafish DNA sequence from clone CH211-208F21 in linkage grour	42.8	42.8	7%	1.2	96%	
AL731825.1	Bacillus thuringiensis subsp. israelensis plasmid pBtoxis	42.8	85.5	6%	1.2	100%	
X07423.1	Bacillus thuringiensis israelensis bt8 gene for 130 kDa crystal protei	42.8	42.8	6%	1.2	100%	
Y00423.1	Bacillus thuringiensis gene for 130 kDa delta-endotoxin	42.8	42.8	6%	1.2	100%	
X07082.1	Bacillus thuringiensis gene for 130 kDa delta-endotoxin	42.8	42.8	6%	1.2	100%	
D00248.1	Bacillus thuringiensis israelensis plasmid gene for 130 kDa insecticic	42.8	42.8	6%	1.2	100%	G
D00247.1	Bacillus thuringiensis israelensis plasmid gene for 130 kDa insecticic	42.8	42.8	6%	1.2	100%	
M20242.1	B.thuringiensis mosquitocidal protein (CryD2) gene, complete cds	42.8	42.8	6%	1.2	100%	
AP001212.3	Homo sapiens genomic DNA, chromosome 2p11.2, clone:cos130	41.0	41.0	9%	4.4	85%	

a. Blastn output

Plate 15. Sequence analysis of Bt-133*cry*4 gene fragment

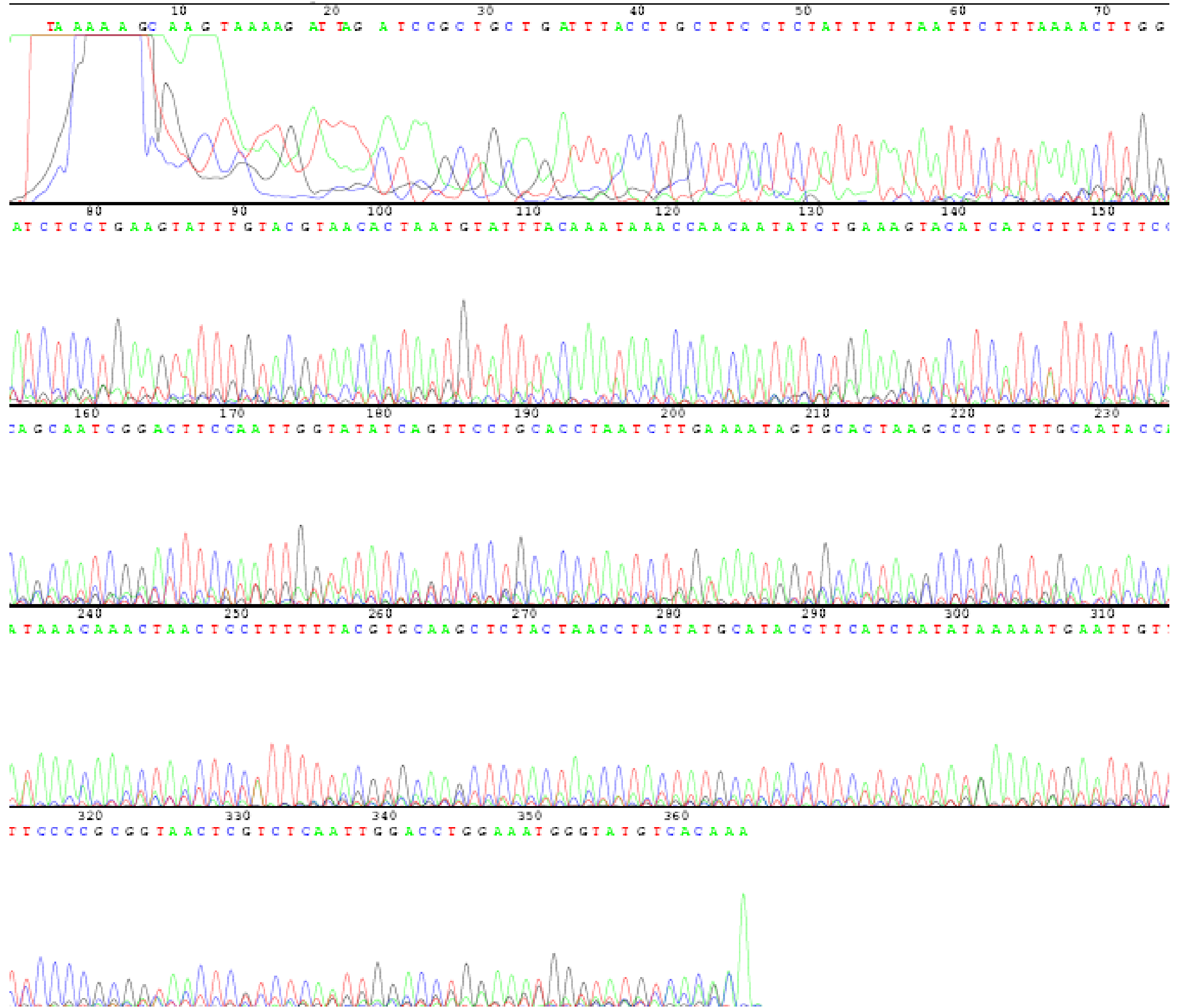
```
View   1 GenBank   Redraw   100   SixFrames

Frame  from  to    Length
 -3         40..276  237
 +3         93..194  102

Length: 78 aa
Accept   Alternative Initiation Codons

276 atgcatagtaggttagtagagcttgcacgtaaaaaaggagttagt
    M  H  S  R  L  V  E  L  A  R  K  K  G  V  S
231 ttgtttattgtattgcaagcagggctttgtgcaccatttaccaga
    L  F  I  V  L  Q  A  G  L  C  A  P  F  T  R
186 ttatgtgcgggcactgatataccaaatggaagtccgattgctgga
    L  C  A  G  T  D  I  P  N  G  S  P  I  A  G
141 agaaaagaagatgtaccttctgatattgttggtttatttgataag
    R  K  E  D  V  P  S  D  I  V  G  L  F  D  K
 96 acattagtgttacggacaaatacttccggagatccgagtttaaag
    T  L  V  L  R  T  N  T  S  G  D  P  S  L  K
 51 aattataaatag 40
    N  Y  K  *
```

b. Open reading frame

```
tattaaaccgagacggattcttgcggatacctgcttactctatttataattctttaaact
 Y  -  T  E  T  D  S  C  G  Y  L  L  T  L  F  I  I  L  -  T
cggatctccggaagtatttgtccgtaacactaatgtcttatcaaataaaccaacaatatc
 R  I  S  G  S  I  C  P  -  H  -  C  L  I  K  -  T  N  N  I
agaaggtacatcttcttttcttccagcaatcggacttccatttggtatatcagtgcccgc
 R  R  Y  I  F  F  S  S  S  N  R  T  S  I  W  Y  I  S  A  R
acataatctggtaaatggtgcacaaagccctgcttgcaatacaataaacaaactaactcc
 T  -  S  G  K  W  C  T  K  P  C  L  Q  Y  N  K  Q  T  N  S
tttttacgtgcaagctctactaacctactatgcataccttcatctatataaaaatgaat
 F  F  T  C  K  L  Y  -  P  T  M  H  T  F  I  Y  I  K  M  N
tgtttccccgcggtaactcgtctcaattggacctggaaatgggtatgtcacaaacc
 C  F  P  A  V  T  R  L  N  W  T  W  K  W  V  C  H  K
```

c. Deduced amino acid sequence

Plate 15. Sequence analysis of Bt-133*cry*4 gene fragment



a. Graphical output

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CAACAATATCTGAAAGTACATCATCTTTTCTTCCAGCAATCGGACTTCCAATTGGTATAT
CAGTTCCTGCACCTAATCTTGAAAATAGTGCACTAAGCCCTGCTTGCAATACCATAAACA
AACTAACTCCTTTTTTACGTGCAAGCTCTACTAACCTACTATGCATACCTTCATCTATAT
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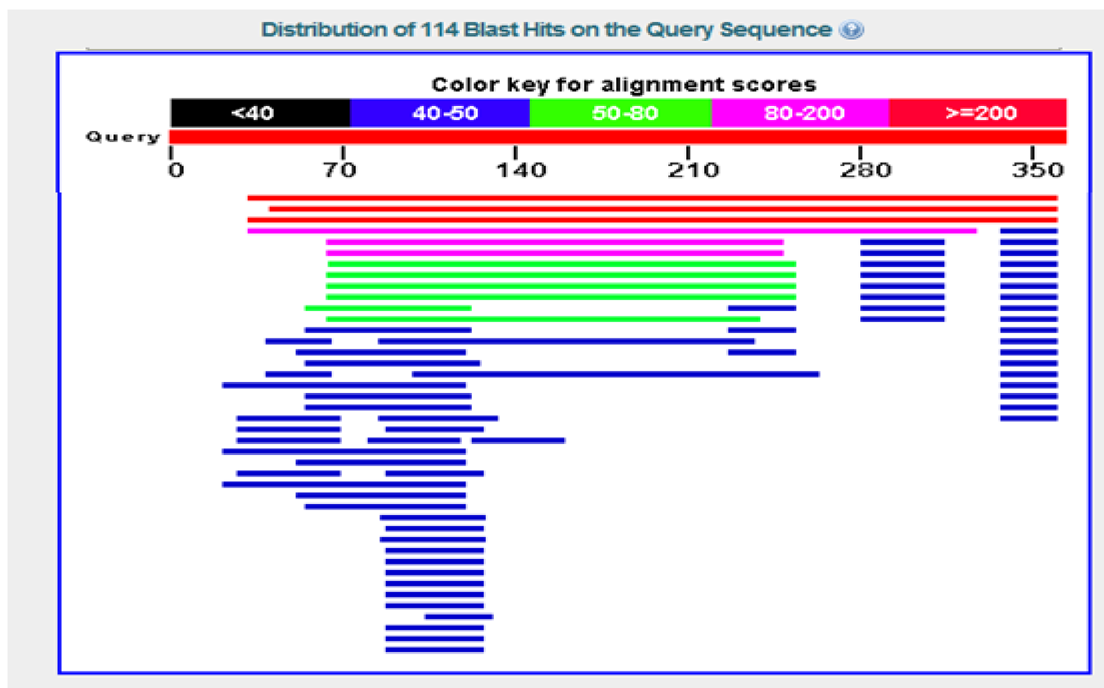
CAAA

b. Nucleotide sequences

Plate 16. Details of Bt-190*cry*4 gene fragment



Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
GQ202006.1	Bacillus thuringiensis strain AR-56 dipterans toxin crystal protein (cr	42.8	42.8	6%	1.3	100%	
XM_002303257.1	Populus trichocarpa predicted protein, mRNA	42.8	42.8	10%	1.3	84%	G
AB481215.1	Microcystis aeruginosa K-139 mipH, mipA, mipB, mipC, mipD, orf1,	42.8	42.8	27%	1.3	69%	
DQ788672.1	Bacillus thuringiensis isolate DAB BT6 Cry4 (cry4) gene, partial cds	42.8	42.8	6%	1.3	100%	
DQ788671.1	Bacillus thuringiensis isolate DAB BT5 Cry4 (cry4) gene, partial cds	42.8	42.8	6%	1.3	100%	
DQ788670.1	Bacillus thuringiensis isolate DAB BT4 Cry4 (cry4) gene, partial cds	42.8	42.8	6%	1.3	100%	
DQ788669.1	Bacillus thuringiensis isolate DAB BT3 Cry4 (cry4) gene, partial cds	42.8	42.8	6%	1.3	100%	
DQ788667.1	Bacillus thuringiensis isolate DAB BT1 Cry4 (cry4) gene, partial cds	42.8	42.8	6%	1.3	100%	
EU109504.1	Planktothrix agardhii NIES-205 ABC transporter (ociD) gene, compl	42.8	85.5	18%	1.3	74%	
EF468630.1	Bacillus thuringiensis isolate DAB-Bt6 pesticidal crystal protein gene	42.8	42.8	6%	1.3	100%	
EF468628.1	Bacillus thuringiensis isolate DAB-Bt4 pesticidal crystal protein gene	42.8	42.8	6%	1.3	100%	
EF468626.1	Bacillus thuringiensis isolate DAB-Bt2 truncated pesticidal crystal pr	42.8	42.8	6%	1.3	100%	
EF468625.1	Bacillus thuringiensis isolate DAB-Bt1 pesticidal crystal protein gene	42.8	42.8	6%	1.3	100%	
EF424472.1	Bacillus thuringiensis isolate DAB-BT4 Cry4A gene, complete cds	42.8	42.8	6%	1.3	100%	
EF424471.1	Bacillus thuringiensis isolate DAB-BT2 Cry4A gene, complete cds	42.8	42.8	6%	1.3	100%	
EF424470.1	Bacillus thuringiensis isolate DAB-BT6 Cry4A gene, complete cds	42.8	42.8	6%	1.3	100%	
EF424469.1	Bacillus thuringiensis isolate DAB-BT5 Cry4A gene, complete cds	42.8	42.8	6%	1.3	100%	
EF424468.1	Bacillus thuringiensis isolate DAB-BT3 Cry4A gene, complete cds	42.8	42.8	6%	1.3	100%	
AY681965.1	Rattus norvegicus SIAH mRNA, complete cds	42.8	42.8	10%	1.3	85%	UG
AM778942.1	Microcystis aeruginosa PCC 7806 genome sequencing data, contig (	42.8	42.8	27%	1.3	69%	
DQ837301.1	Planktothrix agardhii NIVA-CYA 116 cyanopeptin oci gene cluster, c	42.8	85.5	18%	1.3	74%	
DQ075244.1	Microcystis sp. NIVA-CYA 172/5 cyanopeptolin synthetase gene clus	42.8	42.8	18%	1.3	74%	
CR388145.18	Zebrafish DNA sequence from clone DKEY-264F17, complete seque	42.8	42.8	7%	1.3	96%	
CU104769.5	Zebrafish DNA sequence from clone CH73-7P15 in linkage group 24	42.8	42.8	11%	1.3	83%	
EF208904.1	Bacillus thuringiensis Cry4A (cry4A) gene, complete cds	42.8	42.8	6%	1.3	100%	
BC046317.1	Mus musculus seven in absentia 1A, mRNA (cDNA clone MGC:5476	42.8	42.8	10%	1.3	85%	UEG
BC035562.1	Homo sapiens seven in absentia homolog 1 (Drosophila), mRNA (cD	42.8	42.8	10%	1.3	85%	UG

a. Blastn output

Plate 17. Sequence analysis of Bt-190*cry*4 gene fragment

View | 1 GenBank | Redraw | 100 | SixFrames

Frame	from to	Length
-1	2..286	285
+1	103..216	114

Length: **94 aa**

Accept | Alternative Initiation Codons

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241 ttgtttatggtattgcaagcagggcttagtgcactattttcaaga
    L  F  M  V  L  Q  A  G  L  S  A  L  F  S  R
196 ttaggtgcaggaactgatataccaattggaagtccgattgctgga
    L  G  A  G  T  D  I  P  I  G  S  P  I  A  G
151 agaaaagatgatgtactttcagatattgttggtttatttgtaaat
    R  K  D  D  V  L  S  D  I  V  G  L  F  V  N
106 acattagtgttacgtacaaatacttcaggagatccaagttttaaa
    T  L  V  L  R  T  N  T  S  G  D  P  S  F  K
 61 gaattaaaaatagaggaagcaggtaaatcagcagcggatctaatc
    E  L  K  I  E  E  A  G  K  S  A  A  D  L  I
 16 ttttacttgcttttt 2
    F  Y  L  L  F
```

b. Open reading frame

```
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ctttaaaacttggatctcctgaagtatttgtacgtaacactaatgtatttacaaataaac
 L  -  N  L  D  L  L  K  Y  L  Y  V  T  L  M  Y  L  Q  I  N
caacaatatctgaaagtacatcatcttttcttccagcaatcggacttccaattggtatat
 Q  Q  Y  L  K  V  H  H  L  F  F  Q  Q  S  D  F  Q  L  V  Y
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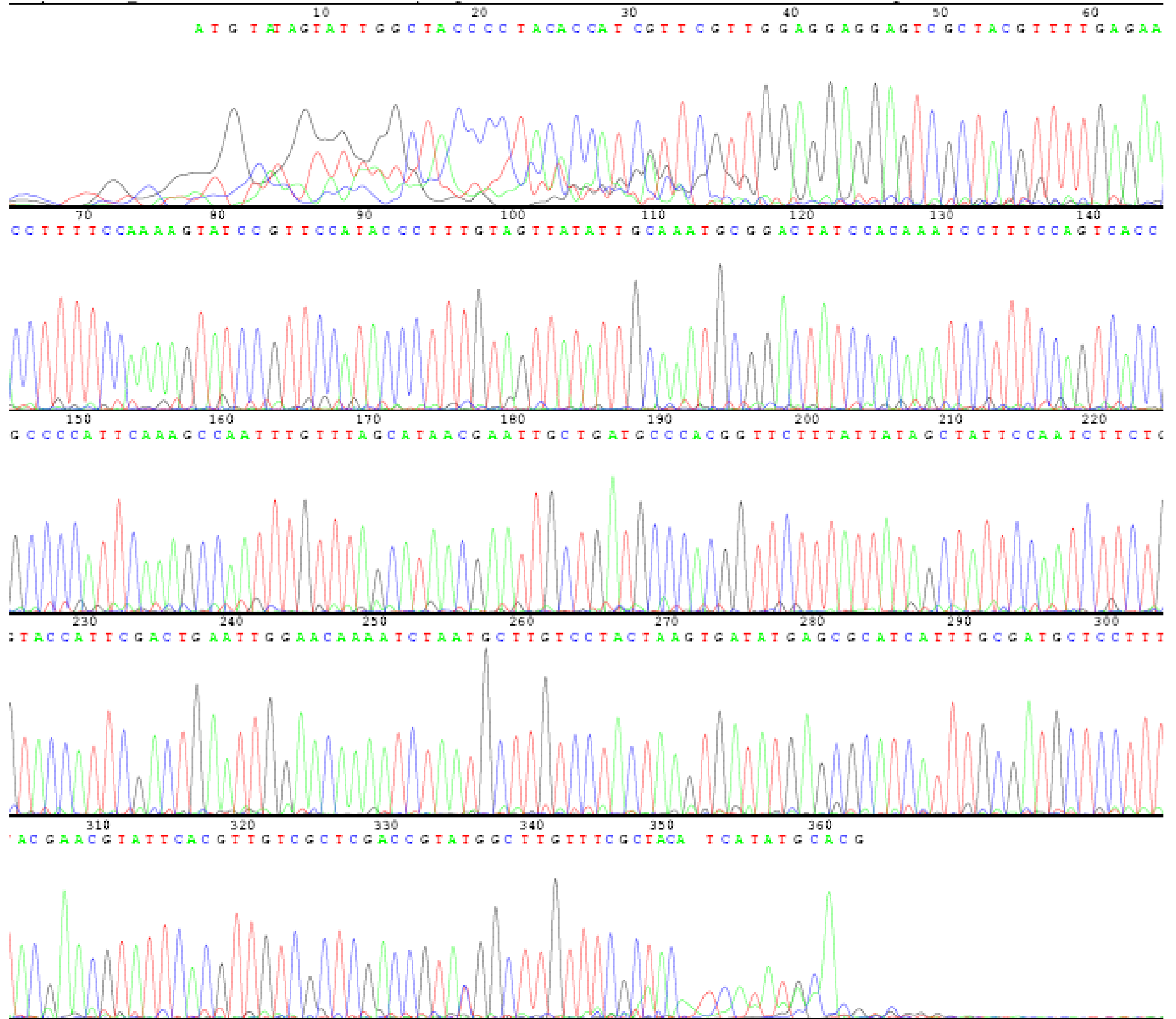
 Q  F  L  H  L  I  L  K  I  V  H  -  A  L  L  A  I  P  -  T
aactaactccttttttacgtgcaagctctactaacctactatgcataccttcatctatat
 N  -  L  L  F  Y  V  Q  A  L  L  T  Y  Y  A  Y  L  H  L  Y
aaaaatgaattgtttccccgcggtaactcgtctcaattggacctggaaatgggtatgtca
 K  N  E  L  F  P  R  G  N  S  S  Q  L  D  L  E  M  G  M  S
caaa
 Q
```

c. Deduced amino acid sequence

Plate 17. Sequence analysis of Bt-190*cry*4 gene fragment



a. Graphical output

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ATCCACAAATCCTTTCCAGTCACCGCCCCATTCAAAGCCAATTTGTTTAGCATAACGAAT
TGCTGATGCCCACGGTTCTTTATTATAGCTATTCCAATCTTCTGTACCATTCGACTGAAT
TGGAACAAAATCTAATGCTTGTCCTACTAAGTGATATGAGCGCATCATTTGCGATGCTCC
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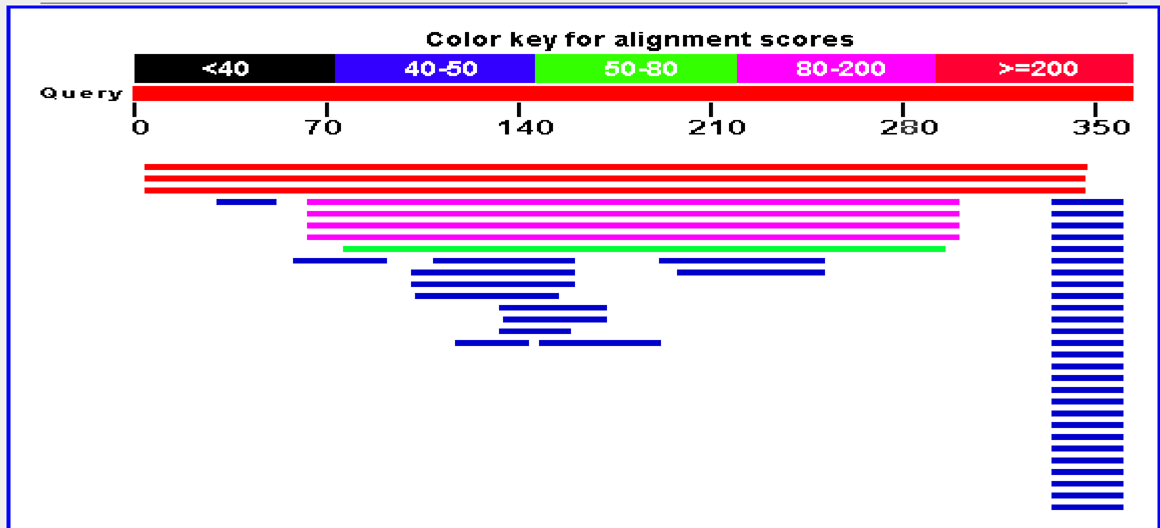
CG

b. Nucleotide sequences

Plate 18. Details of Bt-242*cry*4 gene fragment

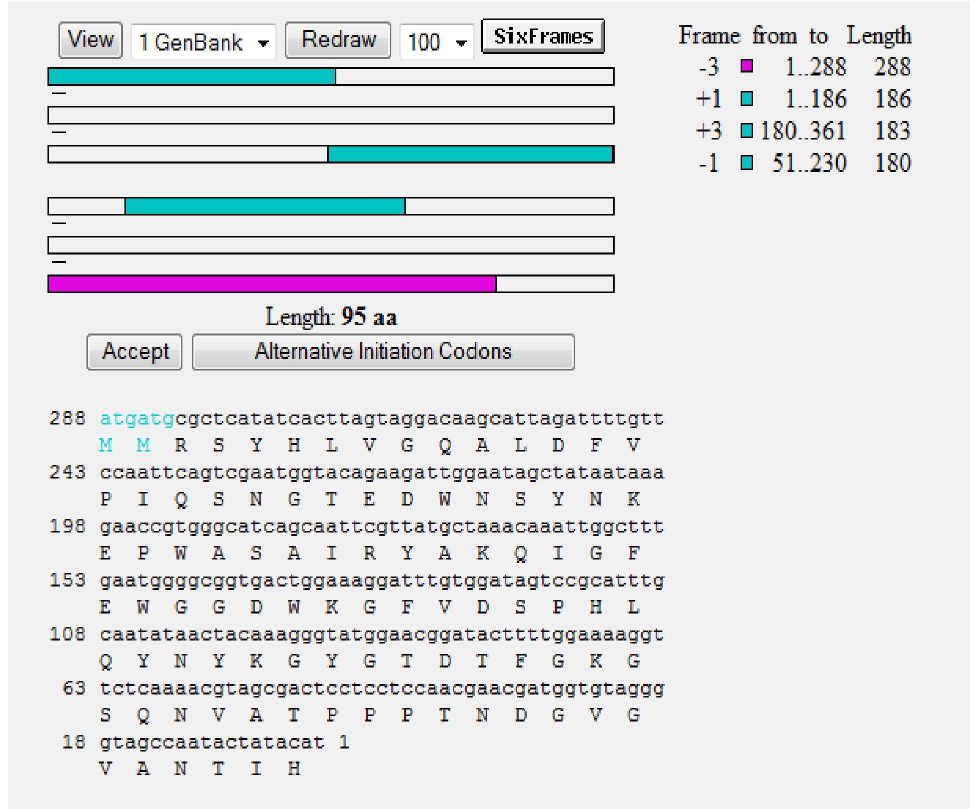


Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
GQ202006.1	Bacillus thuringiensis strain AR-56 dipterans toxin crystal protein (cr	48.2	48.2	7%	0.029	100%	
CP000117.1	Anabaena variabilis ATCC 29413, complete genome	48.2	48.2	19%	0.029	76%	
DQ788672.1	Bacillus thuringiensis isolate DAB BT6 Cry4 (cry4) gene, partial cds	46.4	46.4	7%	0.10	100%	
DQ788671.1	Bacillus thuringiensis isolate DAB BT5 Cry4 (cry4) gene, partial cds	46.4	46.4	7%	0.10	100%	
DQ788670.1	Bacillus thuringiensis isolate DAB BT4 Cry4 (cry4) gene, partial cds	46.4	46.4	7%	0.10	100%	
DQ788669.1	Bacillus thuringiensis isolate DAB BT3 Cry4 (cry4) gene, partial cds	46.4	46.4	7%	0.10	100%	
DQ788667.1	Bacillus thuringiensis isolate DAB BT1 Cry4 (cry4) gene, partial cds	46.4	46.4	7%	0.10	100%	
CP001056.1	Clostridium botulinum B str. Eklund 17B, complete genome	46.4	46.4	12%	0.10	83%	
EF468630.1	Bacillus thuringiensis isolate DAB-Bt6 pesticidal crystal protein gene	46.4	46.4	7%	0.10	100%	
EF468628.1	Bacillus thuringiensis isolate DAB-Bt4 pesticidal crystal protein gene	46.4	46.4	7%	0.10	100%	
EF468626.1	Bacillus thuringiensis isolate DAB-Bt2 truncated pesticidal crystal pr	46.4	46.4	7%	0.10	100%	
EF468625.1	Bacillus thuringiensis isolate DAB-Bt1 pesticidal crystal protein gene	46.4	46.4	7%	0.10	100%	
EF424472.1	Bacillus thuringiensis isolate DAB-BT4 Cry4A gene, complete cds	46.4	46.4	7%	0.10	100%	
EF424471.1	Bacillus thuringiensis isolate DAB-BT2 Cry4A gene, complete cds	46.4	46.4	7%	0.10	100%	
EF424470.1	Bacillus thuringiensis isolate DAB-BT6 Cry4A gene, complete cds	46.4	46.4	7%	0.10	100%	
EF424469.1	Bacillus thuringiensis isolate DAB-BT5 Cry4A gene, complete cds	46.4	46.4	7%	0.10	100%	
EF424468.1	Bacillus thuringiensis isolate DAB-BT3 Cry4A gene, complete cds	46.4	46.4	7%	0.10	100%	
EF208904.1	Bacillus thuringiensis Cry4A (cry4A) gene, complete cds	46.4	46.4	7%	0.10	100%	
AY729887.1	Bacillus thuringiensis serovar israelensis delta-endotoxin (cry4BLB)	46.4	46.4	7%	0.10	100%	
AY847707.1	Bacillus thuringiensis strain PBT602 130 kDa crystal protein (cry) ge	46.4	46.4	7%	0.10	100%	
BA000028.3	Oceanobacillus iheyensis HTE831 DNA, complete genome	46.4	46.4	43%	0.10	67%	
AL731825.1	Bacillus thuringiensis subsp. israelensis plasmid pBtoxis	46.4	133	7%	0.10	100%	
X07423.1	Bacillus thuringiensis israelensis bt8 gene for 130 kDa crystal protei	46.4	46.4	7%	0.10	100%	
Y00423.1	Bacillus thuringiensis gene for 130 kDa delta-endotoxin	46.4	46.4	7%	0.10	100%	
X07082.1	Bacillus thuringiensis gene for 130 kDa delta-endotoxin	46.4	46.4	7%	0.10	100%	
AC006568.7	Homo sapiens chromosome 4 clone C0548P18 map 4p16, complete	46.4	46.4	14%	0.10	82%	
D00248.1	Bacillus thuringiensis israelensis plasmid gene for 130 kDa insecticic	46.4	46.4	7%	0.10	100%	G
D00247.1	Bacillus thuringiensis israelensis plasmid gene for 130 kDa insecticic	46.4	46.4	7%	0.10	100%	
M20242.1	B.thuringiensis mosquitocidal protein (CryD2) gene, complete cds	46.4	46.4	7%	0.10	100%	
CP001804.1	Haliangium ochraceum DSM 14365, complete genome	41.0	41.0	9%	4.3	87%	
GQ425065.1	Hemiberlesia lataniae isolate D0766B cytochrome oxidase subunit I	41.0	41.0	10%	4.3	86%	

a. Blastn output

Plate 19. Sequence analysis of Bt-242*cry*4 gene fragment



b. Open reading frame

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R T F S K S I R S I P F V V I L Q M R T
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I H K S F P V T A P F K A N L F S I T N
tgctgatgcccacggttctttattatagctattccaatcttctgtaccattcgactgaat
C - C P R F F I I A I P I F C T I R L N
tggaacaaaatctaatgcttgtcctactaagtgatatgagcgcatcatttgcgatgctcc
W N K I - C L S Y - V I - A H H L R C S
tttacgaacgtattcacgttgtcgctcgaccgtatggcttgtttcgctacatcatatgca
F T N V F T L S L D R M A C F A T S Y A
cg

c. Deduced amino acid sequence

Plate 19. Sequence analysis of Bt-242*cry*4 gene fragment

The proportion of each amino acid in cloned sequences was calculated by using 'AASTATS' tool (http://seqtool.sdsc.edu/). Bt-23*cry*4 was rich in the amino acid leucine (molar per cent of 18.93) and next to this were serine and phenylalanine, (9.05 and 7.41 per cent respectively). Bt-133*cry*4 was rich in Threonine (12.61%) and isoleucine (9.91%), whereas Bt-190*cry*4 was rich in leucine (26.72%). Cysteine and tryptophan residues were absent in Bt-190*cry*4, while glutamine was absent in Bt-242*cry*4. The details of amino acid composition of the *cry*4 gene fragments are presented in table 18.

4.10.3 Phylogenic analysis of native Bt isolate sequences

Multiple sequence analysis was done with all four *cry*4 gene fragments by using ClustalW tool to find out the relationship among the isolates. This analysis revealed some conserved regions between the four sequences. Phylogenic tree showed that all the four isolates shared a common ancestor and isolates Bt-23*cry*4 and Bt-242*cry*4 formed a sub-cluster showing very close relationship with each other. Bt-190*cry*4 and Bt-133*cry*4 did not form a cluster, showing less similarity between them (plate 20).

4.11 Amplification of full length *cry*4A gene

Two oligonucleotide primers were designed from the conserved boxes obtained from multiple sequence alignment of five *cry*4A gene full length sequences from NCBI database (Plate 21). PCR with these primers, using total DNA of Bt-23, Bt-133, Bt-190 and Bt-242 did not yield any amplification.

Table 18. Amino acid composition of deduced *cry* protein sequences

Polarity		Amino acid (%)	Bt-23*cry*4	Bt-133*cry*4	Bt-190*cry*4	Bt-242*cry*4
Non-polar		Gly	4.12	2.70	1.72	0.86
		Ala	5.35	1.80	4.31	6.31
		Val	5.76	1.80	4.31	6.03
		Leu	18.3	7.21	26.72	6.90
		Ile	3.7	9.91	5.17	12.07
		Met	2.7	1.80	2.59	2.59
		Pro	1.23	3.6	3.45	5.17
		Phe	7.41	6.31	6.03	9.48
		Trp	1.23	4.5	0.00	1.72
Polar	Uncharged	Ser	9.05	9.01	4.31	9.48
		Thr	6.17	12.61	2.59	7.76
		Cys	1.65	7.21	0.00	5.17
		Tyr	7.0	6.31	9.48	5.17
		Asn	4.12	6.31	4.31	4.31
		Gln	4.94	1.8	8.62	0.86
	Basic	Lys	7.0	7.21	5.17	3.45
		Arg	1.65	5.4	1.72	9.48
		His	3.70	2.70	4.31	2.59
	Acidic	Asp	2.47	0.9	3.45	0.86
		Glu	2.06	0.9	1.72	0.00

```
Bt-190CRY4      ------------------------------------------------------TAAAA 5
Bt-133CRY4      ----------------------------------------------------------T 1
Bt-23CRY4       ATTTAGTAGAGGAAAAAAGTTTTACTCATATTTAGCTCTCGCACAGCTTATGCTCTATAG 240
Bt-242CRY4      ----------------------------------------------------ATGTATAG 8

Bt-190CRY4      AGCAAGTAAAAGAT-TAGATCCGCTGCTGATTTACCTGCTTCCTCTATTTTTAATTCTTT 64
Bt-133CRY4      ATTAAACCGAGA---CGGATTC-TTGCGGAT--ACCTGCTTACTCTATTTATAATTCTTT 55
Bt-23CRY4       AATGGGCTGGTATTCCACGTACAGTACTGCAT-GTATAGTTCGGCTACTCATACACCATC 299
Bt-242CRY4      TATTGGCTA------CCCCTACACCATCGTTC-GT-TGGAGGAGGAGTCGCTACGTT-TT 59
                                   * *      *       *              **     *

Bt-190CRY4      AAAACTTGGATCTCCTGAAGTATTTGTACGTAACACTAATGTATTTACAAATAAACCAAC 124
Bt-133CRY4      AAA-CTCGGATCTCCGGAAGTATTTGTCCGTAACACTAATGTCTTATCAAATAAACCAAC 114
Bt-23CRY4       AAGATCC--TTTTCCAAAAGTATCCGTTCCATACCCTACGTAGTTATATTGCAAATGCGG 357
Bt-242CRY4      GAGAACC--TTTTCCAAAAGTATCCGTTCCATACCCTTTGTAGTTATATTGCAAATGCGG 117
                 *        * ***  ******  ** *   ** **      **       ***

Bt-190CRY4      AATATCTGAAAGTACATCATCTTTTCT--TCCAGCAATCGGACTTCCAATTGGTATATCA 182
Bt-133CRY4      AATATCAGAAGGTACATCTTCTTTTCT--TCCAGCAATCGGACTTCCATTTGGTATATCA 172
Bt-23CRY4       ACTATCCACAAATCCTTTCCAGTCACCGCCCCATTCAAAGAAAATTTGTTTAGCATAACG 417
Bt-242CRY4      ACTATCCACAAATCCTTTCCAGTCACCGCCCCATTCAAAGCCAATTTGTTTAGCATAACG 177
                * ****   *  * * *     *  *    ***   *  *    *    ** * *** *

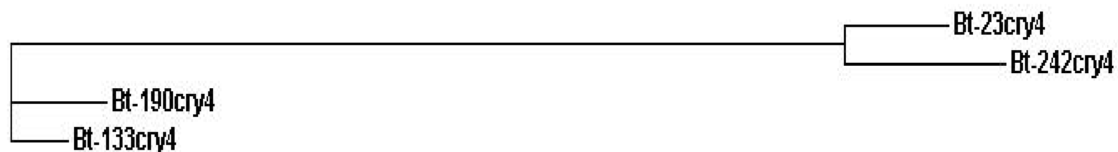
Bt-190CRY4      GTTCCTGCACCTAATCTTGAAAATAGTGCACTAAGCCCTGCTTGCAATACCAT--AAACA 240
Bt-133CRY4      GTGCCCGCACATAATCTGGTAAATGGTGCACAAAGCCCTGCTTGCAATACAAT--AAACA 230
Bt-23CRY4       ATTTGCTGATGCCCACGGTTCTTTATTATAGCTATTCCAATCTTCTGTACCATTCGACTG 477
Bt-242CRY4      AATTGCTGATGCCCACGGTTCTTTATTATAGCTATTCCAATCTTCTGTACCATTCGACTG 237
                        *      *       *  *  *   *  **    * *  *** **   *

Bt-190CRY4      AACTAACTCCTTTTTTACGTGCAAGCTCTACTAACCTAC-TATGCATACCTTCATCTATA 299
Bt-133CRY4      AACTAACTCCTTTTTTACGTGCAAGCTCTACTAACCTAC-TATGCATACCTTCATCTATA 289
Bt-23CRY4       AATTGGAACAAAATCTT-GAAAGTATCCTACTAAGGGATATGAGCGCATCAGCTGCGATG 536
Bt-242CRY4      AATTGGAACAAAATCTA-ATGCTTGTCCTACTAAGTGATATGAGCGCATCATTTGCGATG 296
                ** *    *    * *           *******   *  *  ** * *     * **

Bt-190CRY4      TAAAAATG-AATTGTTTCCCCGCGGTAACTCGTCTCAATTGGACCTGGAAATGGGTATGT 358
Bt-133CRY4      TAAAAATG-AATTGTTTCCCCGCGGTAACTCGTCTCAATTGGACCTGGAAATGGGTATGT 348
Bt-23CRY4       CACCTTTACAAACGTATTCAATTTGTCGCTCGAAGTTAT--GGCTTAT--ACCGCTACAT 592
Bt-242CRY4      CTCCTTTACGAACGTATTCACGTTGTCGCTCGACCGTAT--GGCTTGT--TTCGCTACAT 352
                      *   *  ** * *     **  ****     **   * * *       * **  *

Bt-190CRY4      CACAAA------------------------------------------------------ 364
Bt-133CRY4      CACAAACC---------------------------------------------------- 356
Bt-23CRY4       CATATGCACGTTACTTGCATCTTGTGTACTTGCAACATTTACCCTTGCTCTTACTTTAAA 652
Bt-242CRY4      CATATGCACG-------------------------------------------------- 362
                ** *
```

a. Multiple sequence alignment of the native Bt isolate sequences



b. Phylogenic tree

Plate 20. Phylogenic analysis of the native *B. thuringiensis* sequences

Alignment

Hide Colors | View Alignment File

```
CLUSTAL 2.0.12 multiple sequence alignment

gi|148787932|gb|EF424470.1|          ------------------------------------------ATGAATCC 8
gi|148787936|gb|EF424472.1|          ------------------------------------------ATGAATCC 8
gi|124263654|gb|EF208904.1|          ------------------------------------------ATGAATCC 8
gi|216289|dbj|D00248.1|BACISRH       CAGAAAAAGAGTGTATCTAAATTGAGTATGGGAGGAACAAATATGAATCC 400
gi|40351|emb|Y00423.1|               ------------------------------------------ATGAATCC 8
                                                                               ********
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Forward primer

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gi|124263654|gb|EF208904.1|          TTATCAAAATAAAAATGAATATGAAACATTAAATGCTTCACAAAAAAAAT 58
gi|216289|dbj|D00248.1|BACISRH       TTATCAAAATAAAAATGAATATGAAACATTAAATGCTTCACAAAAAAAAT 450
gi|40351|emb|Y00423.1|               TTATCAAAATAAAAATGAATATGAAACATTAAATGCTTCACAAAAAAAAT 58
                                     **************************************************

gi|148787932|gb|EF424470.1|          TAAATATATCTAATAATTATACAAGATATCCAATAGAAAATAGTCCAAAA 108
gi|148787936|gb|EF424472.1|          TAAATATATCTAATAATTATACAAGATATCCAATAGAAAATAGTCCAAAA 108
gi|124263654|gb|EF208904.1|          TAAATATATCTAATAATTATACAAGATATCCAATAGAAAATAGTCCAAAA 108
gi|216289|dbj|D00248.1|BACISRH       TAAATATATCTAATAATTATACAAGATATCCAATAGAAAATAGTCCAAAA 500
gi|40351|emb|Y00423.1|               TAAATATATCTAATAATTATACAAGATATCCAATAGAAAATAGTCCAAAA 108
                                     **************************************************

gi|148787932|gb|EF424470.1|          CAATTATTACAAAGTACAAATTATAAAGATTGGCTCAATATGTGTCAACA 158
gi|148787936|gb|EF424472.1|          CAATTATTACAAAGTACAAATTATAAAGATTGGCTCAATATGTGTCAACA 158
gi|124263654|gb|EF208904.1|          CAATTATTACAAAGTACAAATTATAAAGATTGGCTCAATATGTGTCAACA 158
gi|216289|dbj|D00248.1|BACISRH       CAATTATTACAAAGTACAAATTATAAAGATTGGCTCAATATGTGTCAACA 550
gi|40351|emb|Y00423.1|               CAATTATTACAAAGTACAAATTATAAAGATTGGCTCAATATGTGTCAACA 158
                                     **************************************************

gi|148787932|gb|EF424470.1|          GAATCAGCAGTATGGTGGAGATTTTGAAACTTTTATTGATAGTGGTGAAC 208
gi|148787936|gb|EF424472.1|          GAATCAGCAGTATGGTGGAGATTTTGAAACTTTTATTGATAGTGGTGAAC 208
gi|124263654|gb|EF208904.1|          GAATCAGCAGTATGGTGGAGATTTTGAAACTTTTATTGATAGTGGTGAAC 208
gi|216289|dbj|D00248.1|BACISRH       GAATCAGCAGTATGGTGGAGATTTTGAAACTTTTATTGATAGTGGTGAAC 600
gi|40351|emb|Y00423.1|               GAATCAGCAGTATGGTGGAGATTTTGAAACTTTTATTGATAGTGGTGAAC 208
                                     **************************************************

gi|148787932|gb|EF424470.1|          TCTGTGCCTATACTATTGTAGTTGGGACCGTACTGACTGGTTTCGGGTTC 258
gi|148787936|gb|EF424472.1|          TCAGTGCCTATACTATTGTAGTTGGGACCGTACTGACTGGTTTCGGGTTC 258
gi|124263654|gb|EF208904.1|          TCAGTGCCTATACTATTGTAGTTGGGACCGTACTGACTGGTTTCGGGTTC 258
gi|216289|dbj|D00248.1|BACISRH       TCAGTGCCTATACTATTGTAGTTGGGACCGTACTGACTGGTTTCGGGTTC 650
gi|40351|emb|Y00423.1|               TCAGTGCCTATACTATTGTAGTTGGGACCGTACTGACTGGTTTCGGGTTC 258
                                     ** ***********************************************

gi|148787932|gb|EF424470.1|          ACAACACCCTTAGGACTTGCTTTAATAGGTTTTGGTACATTAATACCAGT 308
gi|148787936|gb|EF424472.1|          ACAACACCCTTAGGGCTTGCTTTAATAGGTTTTGGTACATTAATACCAGT 308
gi|124263654|gb|EF208904.1|          ACAACACCCTTAGGACTTGCTTTAATAGGTTTTGGTACATTAATACCAGT 308
gi|216289|dbj|D00248.1|BACISRH       ACAACACCCTTAGGACTTGCTTTAATAGGTTTTGGTACATTAATACCAGT 700
gi|40351|emb|Y00423.1|               ACAACACCCTTAGGACTTGCTTTAATAGGTTTTGGTACATTAATACCAGT 308
                                     ************** ***********************************

gi|148787932|gb|EF424470.1|          TCTTTTTCCAGCCCAAGACCAATCTAACACATGGAGTGACTTTATAACAC 358
gi|148787936|gb|EF424472.1|          TCTTTTTCCAGCCCAAGACCCATCTAACACATGGAGTGACTTTATAACAC 358
gi|124263654|gb|EF208904.1|          TCTTTTTCCAGCCCAAGACCAATCTAACACATGGAGTGACTTTATAACAC 358
gi|216289|dbj|D00248.1|BACISRH       TCTTTTTCCAGCCCAAGACCAATCTAACACATGGAGTGACTTTATAACAC 750
gi|40351|emb|Y00423.1|               TCTTTTTCCAGCCCAAGACCAATCTAACACATGGAGTGACTTTATAACAC 358
                                     ******************** *****************************

gi|148787932|gb|EF424470.1|          AAACTAAAAATATTATAAAAAAAAGAAATAGCATCAACGTATATAAGTAAT 408
gi|148787936|gb|EF424472.1|          AAACTAAAAATATTATAAAAAAAAGAAATAGCATCAACGTATATAAGTAAT 408
gi|124263654|gb|EF208904.1|          AAACTAAAAATATTATAAAAAAAAGAAATAGCATCAACGTATATAAGTAAT 408
gi|216289|dbj|D00248.1|BACISRH       AAACTAAAAATATTATAAAAAAAAGAAATAGCATCAACATATATAAGTAAT 800
gi|40351|emb|Y00423.1|               AAACTAAAAATATTATAAAAAAAAGAAATAGCATCAACATATATAAGTAAT 408
                                     ************************************** ***********

gi|148787932|gb|EF424470.1|          GCTAATAAAATTTTAAACAGGTCGTTTAATGTTATCAGCACTTATCATAA 458
gi|148787936|gb|EF424472.1|          GCTAATAAAATTTTAAACAGGTCGTTTAATGTTATCAGCACTTATCATAA 458
gi|124263654|gb|EF208904.1|          GCTAATAAAATTTTAAACAGGTCGTTTAATGTTATCAGCACTTATCATAA 458
gi|216289|dbj|D00248.1|BACISRH       GCTAATAAAATTTTAAACAGGTCGTTTAATGTTATCAGCACTTATCATAA 850
gi|40351|emb|Y00423.1|               GCTAATAAAATTTTAAACAGGTCGTTTAATGTTATCAGCACTTATCATAA 458
                                     **************************************************

gi|148787932|gb|EF424470.1|          TCACCTTAAAACATGGGAGAATAATCCAAACCCACAAAATACTCAGGATG 508
gi|148787936|gb|EF424472.1|          TCACCTTAAAACATGGGAGAATAATCCAAACCCACAAAATACTCAGGGTG 508
gi|124263654|gb|EF208904.1|          TCACCTTAAAACATGGGAGAATAATCCAAACCCACAAAATACTCAGGATG 508
gi|216289|dbj|D00248.1|BACISRH       TCACCTTAAAACATGGGAGAATAATCCAAACCCACAAAATACTCAGGATG 900
gi|40351|emb|Y00423.1|               TCACCTTAAAACATGGGAGAATAATCCAAACCCACAAAATACTCAGGATG 508
                                     *********************************************** **
gi|148787932|gb|EF424470.1|          TAAGGACACAAATCCAGCTAGTTCATTACCATTTTCAAAATGTCATTCCA 558
gi|148787936|gb|EF424472.1|          TAAGGACACAAATCCAGCTAGTTCATTACCATTTTCAAAATGTCATTCCA 558
gi|124263654|gb|EF208904.1|          TAAGGACACAAATCCAGCTAGTTCATTACCATTTTCAAAATGTCATTCCA 558
gi|216289|dbj|D00248.1|BACISRH       TAAGGACACAAATCCAGCTAGTTCATTACCATTTTCAAAATGTCATTCCA 950
gi|40351|emb|Y00423.1|               TAAGGACACAAATCCAGCTAGTTCATTACCATTTTCAAAATGTCATTCCA 558
                                     **************************************************

gi|148787932|gb|EF424470.1|          GAGCTTGTAAACTCTTGTCCTCCTAATCCTAGTGATTGCGATTACTATAA 608
gi|148787936|gb|EF424472.1|          GAGCTTGTAAACTCTTGTCCTCCTAATCCTAGTGATTGCGATTACTATAA 608
gi|124263654|gb|EF208904.1|          GAGCTTGTAAACTCTTGTCCTCCTAATCCTAGTGATTGCGATTACTATAA 608
gi|216289|dbj|D00248.1|BACISRH       GAGCTTGTAAACTCTTGTCCTCCTAATCCTAGTGATTGCGATTACTATAA 1000
gi|40351|emb|Y00423.1|               GAGCTTGTAAACTCTTGTCCTCCTAATCCTAGTGATTGCGATTACTATAA 608
                                     **************************************************

gi|148787932|gb|EF424470.1|          CATACTAGTATTATCTAGTTATGCACACGCAGCAAACTTACATCTGACTG 658
gi|148787936|gb|EF424472.1|          CATACTAGTATTATCTAGTTATGCACACGCAGCAAACTTACATCTGACTG 658
gi|124263654|gb|EF208904.1|          CATACTAGTATTATCTAGTTATGCACACGCAGCAAACTTACATCTGACTG 658
gi|216289|dbj|D00248.1|BACISRH       CATACTAGTATTATCTAGTTATGCACAAGCAGCAAACTTACATCTGACTG 1050
gi|40351|emb|Y00423.1|               CATACTAGTATTATCTAGTTATGCACAAGCAGCAAACTTACATCTGACTG 658
                                     **************************** *********************

gi|148787932|gb|EF424470.1|          TATTAAATCAAGCCGTCAAATTTGAAGCGTATTTAAAAAACAATCGACAA 708
gi|148787936|gb|EF424472.1|          TATTAAATCAAGCCGTCAATTTTGAAGCGTATTTAAAAAACAATCGACAA 708
gi|124263654|gb|EF208904.1|          TATTAAATCAAGCCGTCAAATTTGAAGCGTATTTAAAAAACAATCGACAA 708
gi|216289|dbj|D00248.1|BACISRH       TATTAAATCAAGCCGTCAAATTTGAAGCGTATTTAAAAAACAATCGACAA 1100
gi|40351|emb|Y00423.1|               TATTAAATCAAGCCGTCAAATTTGAAGCGTATTTAAAAAACAATCGACAA 708
                                     ******************* ******************************

gi|148787932|gb|EF424470.1|          TTCGATTATTTAGAGCCTTTGCCAACAGCAATTGATTATTATCCAGTATT 758
gi|148787936|gb|EF424472.1|          TTCGATTATTTAGAGCCTTTGCCAACAGCAATTGATTATTATCCAGTATT 758
gi|124263654|gb|EF208904.1|          TTCGATTATTTAGAGCCTTTGCCAACAGCAATTGATTATTATCCAGTATT 758
gi|216289|dbj|D00248.1|BACISRH       TTCGATTATTTAGAGCCTTTGCCAACAGCAATTGATTATTATCCAGTATT 1150
gi|40351|emb|Y00423.1|               TTCGATTATTTAGAGCCTTTGCCAACAGCAATTGATTATTATCCAGTATT 758
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Plate 21. Multiple sequence alignment with full length *cry*4A gene

(Box indicates forward primer)

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gi|148787932|gb|EF424470.1|          GACTAAAGCTATAGAAGATTACACTAATTATTGTGTAACAACTTATAAAA 808
gi|148787936|gb|EF424472.1|          GACTAAAGCTATAGAAGATTACACTAATTATTGTGTAACAACTTATAAAA 808
gi|124263654|gb|EF208904.1|          GACTAAAGCTATAGAAGATTACACTAATTATTGTGTAACAACTTATAAAA 808
gi|216289|dbj|D00248.1|BACISRH       GACTAAAGCTATAGAAGATTACACTAATTATTGTGTAACAACTTATAAAA 1200
gi|40351|emb|Y00423.1|               GACTAAAGCTATAGAAGATTACACTAATTATTGTGTAACAACTTATAAAA 808
                                     **************************************************

gi|148787932|gb|EF424470.1|          AAGGATTAAATTTAATTAAAACGACGCCTGATAGTAATCTTGATGGAAAT 858
gi|148787936|gb|EF424472.1|          AAGGATTAAATTTAATTAAAACGACGCCTGATAGTAATCTTGATGGAAAT 858
gi|124263654|gb|EF208904.1|          AAGGATTAAATTTAATTAAAACGACGCCTGATAGTAATCTTGATGGAAAT 858
gi|216289|dbj|D00248.1|BACISRH       AAGGATTAAATTTAATTAAAACGACGCCTGATAGTAATCTTGATGGAAAT 1250
gi|40351|emb|Y00423.1|               AAGGATTAAATTTAATTAAAACGACGCCTGATAGTAATCTTGATGGAAAT 858
                                     **************************************************

gi|148787932|gb|EF424470.1|          ATAAACTGGAACACATACAATACGTATCGAACAAAAATGACTACTGCTGT 908
gi|148787936|gb|EF424472.1|          ATAAACTGGAACACATACAATACGTATCGAACAAAAATGACTACTGCTGT 908
gi|124263654|gb|EF208904.1|          ATAAACTGGAACACATACAATACGTATCGAACAAAAATGACTACTGCTGT 908
gi|216289|dbj|D00248.1|BACISRH       ATAAACTGGAACACATACAATACGTATCGAACAAAAATGACTACTGCTGT 1300
gi|40351|emb|Y00423.1|               ATAAACTGGAACACATACAATACGTATCGAACAAAAATGACTACTGCTGT 908
                                     **************************************************

gi|148787932|gb|EF424470.1|          ATTAGATGTTGTTGCACTCTTTCCTATTTATGATGTAGGTAAATATCCAA 958
gi|148787936|gb|EF424472.1|          ATTAGATGTTGTTGCACTCTTTCCTATTTATGATGTAGGTAAATATCCAA 958
gi|124263654|gb|EF208904.1|          ATTAGATGTTGTTGCACTCTTTCCTATTTATGATGTAGGTAAATATCCAA 958
gi|216289|dbj|D00248.1|BACISRH       ATTAGATCTTGTTGCACTCTTTCCTAATTATGATGTAGGTAAATATCCAA 1350
gi|40351|emb|Y00423.1|               ATTAGATGTTGTTGCACTCTTTCCTAATTATGATGTAGGTAAATATCCAA 958
                                     ******* ****************** ***********************

gi|148787932|gb|EF424470.1|          TAGGTGTCCAATCTGAACTTACTCGAGAAATTTATCAGGTACTTAACTTC 1008
gi|148787936|gb|EF424472.1|          TAGGTGTCCAATCTGAACTTACTCGAGAAATTTATCAGGTACTTAACTTC 1008
gi|124263654|gb|EF208904.1|          TAGGTGTCCAATCTGAACTTACTCGAGAAATTTATCAGGTACTTAACTTC 1008
gi|216289|dbj|D00248.1|BACISRH       TAGGTGTCCAATCTGAACTTACTCGAGAAATTTATCAGGTACTTAACTTC 1400
gi|40351|emb|Y00423.1|               TAGGTGTCCAATCTGAACTTACTCGAGAAATTTATCAGGTACTTAACTTC 1008
                                     **************************************************

gi|148787932|gb|EF424470.1|          GAAGAAAGCCCCTATAAATATTATGACTTTCAATATCAAGAGGATTCACT 1058
gi|148787936|gb|EF424472.1|          GAAGAAAGCCCCTATAAATATTATGACTTTCAATATCAAGAGGATTCACT 1058
gi|124263654|gb|EF208904.1|          GAAGAAAGCCCCTATAAATATTATGACTTTCAATATCAAGAGGATTCACT 1058
gi|216289|dbj|D00248.1|BACISRH       GAAGAAAGCCCCTATAAATATTATGACTTTCAATATCAAGAGGATTCACT 1450
gi|40351|emb|Y00423.1|               GAAGAAAGCCCCTATAAATATTATGACTTTCAATATCAAGAGGATTCACT 1058
                                     **************************************************

gi|148787932|gb|EF424470.1|          TACACGTAGACCGCATTTATTTACTTGGCTTGATTCTTTGAATTTTTATG 1108
gi|148787936|gb|EF424472.1|          TACACGTAGACCGCATTTATTTACTTGGCTTGATTCTTTAAATTTTTATG 1108
gi|124263654|gb|EF208904.1|          TACACGTAGACCGCATTTATTTACTTGGCTTGATTCTTTGAATTTTTATG 1108
gi|216289|dbj|D00248.1|BACISRH       TACACGTAGACCGCATTTATTTACTTGGCTTGATTCTTTGAATTTTTATG 1500
gi|40351|emb|Y00423.1|               TACACGTAGACCGCATTTATTTACTTGGCTTGATTCTTTGAATTTTTATG 1108
                                     *************************************** **********

gi|148787932|gb|EF424470.1|          AAAAAGCGCAAACTACTCCTAATAATTTTTTCACCAGCCATTATAATATG 1158
gi|148787936|gb|EF424472.1|          AAAAAGCGCAAACTACTCCTAATAATTTTTTCACCAGCCATTATAATATG 1158
gi|124263654|gb|EF208904.1|          AAAAAGCGCAAACTACTCCTAATAATTTTTTCACCAGCCATTATAATATG 1158
gi|216289|dbj|D00248.1|BACISRH       AAAAAGCGCAAACTACTCCTAATAATTTTTTCACCAGCCATTATAATATG 1550
gi|40351|emb|Y00423.1|               AAAAAGCGCAAACTACTCCTAATAATTTTTTCACCAGCCATTATAATATG 1158
                                     **************************************************

gi|148787932|gb|EF424470.1|          TTTCTTTACACACTTGATAATATATCCCAAAAATCTAGTGTTTTTGGAAA 1208
gi|148787936|gb|EF424472.1|          TTTCATTACACACTTGATAATATATCCCAAAAATCTAGTGTTTTTGGAAA 1208
gi|124263654|gb|EF208904.1|          TTTCATTACACACTTGATAATATATCCCAAAAATCTAGTGTTTTTGGAAA 1208
gi|216289|dbj|D00248.1|BACISRH       TTTCATTACACACTTGATAATATATCCCAAAAATCTAGTGTTTTTGGAAA 1600
gi|40351|emb|Y00423.1|               TTTCATTACACACTTGATAATATATCCCAAAAATCTAGTGTTTTTGGAAA 1208
                                     **** *********************************************

gi|148787932|gb|EF424470.1|          TCACAATGTAACTGATAAATTAAAAGCTCTTGGTTTGGCAACAAATATTT 1258
gi|148787936|gb|EF424472.1|          TCACAATGAAACTGATAAATTAAAAGCTCTTGGTTTGGCAACAAATATTT 1258
gi|124263654|gb|EF208904.1|          TCACAATGTAACTGATAAATTAAAAGCTCTTGGTTTGGCAACAAATATTT 1258
gi|216289|dbj|D00248.1|BACISRH       TCACAATGTAACTGATAAATTAAAATCTCTTGGTTTGGCAACAAATATTT 1650
gi|40351|emb|Y00423.1|               TCACAATGTAACTGATAAATTAAAATCTCTTGGTTTGGCAACAAATATTT 1258
                                     ******** **************** ************************

gi|148787932|gb|EF424470.1|          ATATTTTTTTATTAAATGTCATAAGCTTAGATAATAAATATCTAAATGAT 1308
gi|148787936|gb|EF424472.1|          ATATTTTTTTATTAAATGTCATAAGCTTAGATAATAAATATCTAAATGAT 1308
gi|124263654|gb|EF208904.1|          ATATTTTTTTATTAAATGTCATAAGCTTAGATAATAAATATCTAAATGAT 1308
gi|216289|dbj|D00248.1|BACISRH       ATATTTTTTTATTAAATGTCATAAGCTTAGATAATAAATATCTAAATGAT 1700
gi|40351|emb|Y00423.1|               ATATTTTTTTATTAAATGTCATAAGCTTAGATAATAAATATCTAAATGAT 1308
                                     **************************************************

gi|148787932|gb|EF424470.1|          TATAATAATATTAGTAAAATGGATTTTTTTTATAACTAATGGTACTAGACT 1358
gi|148787936|gb|EF424472.1|          TATAATAATATTAGTAAAATGGATTTTTTTTATAACTAATGGTACTAGACT 1358
gi|124263654|gb|EF208904.1|          TATAATAATATTAGTAAAATGGATTTTTTTTATAACTAATGGTACTAGACT 1358
gi|216289|dbj|D00248.1|BACISRH       TATAATAATATTAGTAAAATGGATTTTTTTTATAACTAATGGTACTAGACT 1750
gi|40351|emb|Y00423.1|               TATAATAATATTAGTAAAATGGATTTTTTTTATAACTAATGGTACTAGACT 1358
                                     **************************************************

gi|148787932|gb|EF424470.1|          TTTGGAGAAAGAACTTACAGCAGGATCTGGGCAAATAACTTATGATGTAA 1408
gi|148787936|gb|EF424472.1|          TTTGGAGAAAGAACTTACAGCAGGATCTGGGCAAATAACTTATGATGTAA 1408
gi|124263654|gb|EF208904.1|          TTTGGAGAAAGAACTTACAGCAGGATCTGGGCAAATAACTTATGATGTAA 1408
gi|216289|dbj|D00248.1|BACISRH       TTTGGAGAAAGAACTTACAGCAGGATCTGGGCAAATAACTTATGATGTAA 1800
gi|40351|emb|Y00423.1|               TTTGGAGAAAGAACTTACAGCAGGATCTGGGCAAATAACTTATGATGTAA 1408
                                     **************************************************

gi|148787932|gb|EF424470.1|          ATAAAAAATATTTTCGGGTTACCAATTCTTAAACGAAGAGAGAATCAAGGA 1458
gi|148787936|gb|EF424472.1|          ATAAAAAATATTTTCGGGTTACCAATTCTTAAACGAAGAGAGAATCAAGGA 1458
gi|124263654|gb|EF208904.1|          ATAAAAAATATTTTCGGGTTACCAATTCTTAAACGAAGAGAGAATCAAGGA 1458
gi|216289|dbj|D00248.1|BACISRH       ATAAAAAATATTTTCGGGTTACCAATTCTTAAACGAAGAGAGAATCAAGGA 1850
gi|40351|emb|Y00423.1|               ATAAAAAATATTTTCGGGTTACCAATTCTTAAACGAAGAGAGAATCAAGGA 1458
                                     **************************************************

gi|148787932|gb|EF424470.1|          AACCCTACCCTTTTTTCCAACATATGATAACTATAGTCATATTTTTATCATT 1508
gi|148787936|gb|EF424472.1|          AACCCTACCCTTTTTTCCAACATATGATAACTATAGTCATATTTTTATCATT 1508
gi|124263654|gb|EF208904.1|          AACCCTACCCTTTTTTCCAACATATGATAACTATAGTCATATTTTTATCATT 1508
gi|216289|dbj|D00248.1|BACISRH       AACCCTACCCTTTTTTCCAACATATGATAACTATAGTCATATTTTTATCATT 1900
gi|40351|emb|Y00423.1|               AACCCTACCCTTTTTTCCAACATATGATAACTATAGTCATATTTTTATCATT 1508
                                     **************************************************

gi|148787932|gb|EF424470.1|          TATTAAAAGTCTTAGTATCCCTGAAACATATAAAACTCAAGTGTATACGT 1558
gi|148787936|gb|EF424472.1|          TATTAAAAGTCTTAGTATCCCTGCAACATATAAAACTCAAGTGTATACGT 1558
gi|124263654|gb|EF208904.1|          TATTAAAAGTCTTAGTATCCCTGCAACATATAAAACTCAAGTGTATACGT 1558
gi|216289|dbj|D00248.1|BACISRH       TATTAAAAGTCTTAGTATCCCTGCAACATATAAAACTCAAGTGTATACGT 1950
gi|40351|emb|Y00423.1|               TATTAAAAGTCTTAGTATCCCTGCAACATATAAAACTCAAGTGTATACGT 1558
                                     *********************** **************************

gi|148787932|gb|EF424470.1|          TTGCTTGGACACACTCTAGTGTTGATCCTAAAAAATACAATTTATACACAT 1608
gi|148787936|gb|EF424472.1|          TTGCTTGGACACACTCTAGTGTTGATCCTAAAAAATACAATTTATACACAT 1608
gi|124263654|gb|EF208904.1|          TTGCTTGGACACACTCTAGTGTTGATCCTAAAAAATACAATTTATACACAT 1608
gi|216289|dbj|D00248.1|BACISRH       TTGCTTGGACACACTCTAGTGTTGATCCTAAAAAATACAATTTATACACAT 2000
gi|40351|emb|Y00423.1|               TTGCTTGGACACACTCTAGTGTTGATCCTAAAAAATACAATTTATACACAT 1608
                                     **************************************************

gi|148787932|gb|EF424470.1|          TTAACTACCCAAATTCCAGCTGTAAAAGCGAATTCACTTGGGACTGCTTC 1658
gi|148787936|gb|EF424472.1|          TTAACTACCCAAATTCCAGCTGTAAAAGCGAATTCACTTGGGACTGCTTC 1658
gi|124263654|gb|EF208904.1|          TTAACTACCCAAATTCCAGCTGTAAAAGCGAATTCACTTGGGACTGCTTC 1658
gi|216289|dbj|D00248.1|BACISRH       TTAACTACCCAAATTCCAGCTGTAAAAGCGAATTCACTTGGGACTGCTTC 2050
gi|40351|emb|Y00423.1|               TTAACTACCCAAATTCCAGCTGTAAAAGCGAATTCACTTGGGACTGCTTC 1658
                                     **************************************************

gi|148787932|gb|EF424470.1|          TAAGGTTGTTCAAGGACCTGGTCATACAGGAGGGGATTTAATTGATTTCA 1708
gi|148787936|gb|EF424472.1|          TAAGGTTGTTCAAGGACCTGGTCATACAGGAGGGGATTTAATTGATTTCA 1708
gi|124263654|gb|EF208904.1|          TAAGGTTGTTCAAGGACCTGGTCATACAGGAGGGGATTTAATTGATTTCA 1708
gi|216289|dbj|D00248.1|BACISRH       TAAGGTTGTTCAAGGACCTGGTCATACAGGAGGGGATTTAATTGATTTCA 2100
gi|40351|emb|Y00423.1|               TAAGGTTGTTCAAGGACCTGGTCATACAGGAGGGGATTTAATTGATTTCA 1708
                                     **************************************************

gi|148787932|gb|EF424470.1|          AAGATCATTTCAAAATTACATGTCAACACTCAAATTTTCAACAATCGTAT 1758
gi|148787936|gb|EF424472.1|          AAGATCATTTCAAAATTACATGTCAACACTCAAATTTTCAACAATCGTAT 1758
gi|124263654|gb|EF208904.1|          AAGATCATTTCAAAATTACATGTCAACACTCAAATTTTCAACAATCGTAT 1758
gi|216289|dbj|D00248.1|BACISRH       AAGATCATTTCAAAATTACATGTCAACACTCAAATTTTCAACAATCGTAT 2150
gi|40351|emb|Y00423.1|               AAGATCATTTCAAAATTACATGTCAACACTCAAATTTTCAACAATCGTAT 1758
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Plate 21. Multiple sequence alignment with full length *cry*4A gene

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gi|148787932|gb|EF424470.1|           TTTATAAGAATTCGTTTTGCTTCAAATGGAAGCGCAAATACTCGAGCTGT 1808
gi|148787936|gb|EF424472.1|           TTTATAAGAATTCGTTTTGCTTCAAATGGAAGCGCAAATACTCGAGCTGT 1808
gi|124263654|gb|EF208904.1|           TTTATAAGAATTCGTTTTGCTTCAAATGGAAGCGCAAATACTCGAGCTGT 1808
gi|216289|dbj|D00248.1|BACISRH       TTTATAAGAATTCGTTATGCTTCAAATGGAAGCGCAAATACTCGAGCTGT 2200
gi|40351|emb|Y00423.1|                TTTATAAGAATTCGTTATGCTTCAAATGGAAGCGCAAATACTCGAGCTGT 1808
                                      **************** *********************************

gi|148787932|gb|EF424470.1|           TATAAATCTTAGTATCCCAAGGGTAGCAGAACTGGGTATGGCACTCAACC 1858
gi|148787936|gb|EF424472.1|           TATAAATCTTAGTATCCCAGGGGTAGCAGAACTGGGTATGGCACTCAACC 1858
gi|124263654|gb|EF208904.1|           TATAAATCTTAGTATCCCAGGGGTAGCAGAACTGGGTATGGCACTCAACC 1858
gi|216289|dbj|D00248.1|BACISRH       TATAAATCTTAGTATCCCAGGGGTAGCAGAACTGGGTATGGCACTCAACC 2250
gi|40351|emb|Y00423.1|                TATAAATCTTAGTATCCCAGGGGTAGCAGAACTGGGTATGGCACTCAACC 1858
                                      ******************* ******************************

gi|148787932|gb|EF424470.1|           CCACTTTTTCTGGTACAGATTATACGAATTTAAAATATAAAGATTTTCAG 1908
gi|148787936|gb|EF424472.1|           CCACTTTTTCTGGTACAGATTATACAAAATTAAAATATAAAGATTTTCAG 1908
gi|124263654|gb|EF208904.1|           CCACTTTTTCTGGTACAGATTATACGAATTTAAAATATAAAGATTTTCAG 1908
gi|216289|dbj|D00248.1|BACISRH       CCACTTTTTCTGGTACAGATTATACGAATTTAAAATATAAAGATTTTCAG 2300
gi|40351|emb|Y00423.1|                CCACTTTTTCTGGTACAGATTATACGAATTTAAAATATAAAGATTTTCAG 1908
                                      ************************* ** *********************

gi|148787932|gb|EF424470.1|           TACTTAGAATTTTCTAACGAGGTGAAATTTGCTCCAAATCAAAACATATC 1958
gi|148787936|gb|EF424472.1|           TACTTAGAATTTTCTAACGAGGTGAAATTTGCTCCAAATCAAAACATATC 1958
gi|124263654|gb|EF208904.1|           TACTTAGAATTTTCTAACGAGGTGAAATTTGCTCCAAATCAAAACATATC 1958
gi|216289|dbj|D00248.1|BACISRH       TACTTAGAATTTTCTAACGAGGTGAAATTTGCTCCAAATCAAAACATATC 2350
gi|40351|emb|Y00423.1|                TACTTAGAATTTTCTAACGAGGTGAAATTTGCTCCAAATCAAAACATATC 1958
                                      **************************************************

gi|148787932|gb|EF424470.1|           TCTTGTGTTTAATCGTTCGGATGTATATACAAACACAACAGTACTTATTG 2008
gi|148787936|gb|EF424472.1|           TCTTGTGTTTAATCGTTCGGATGTATATACAAACACAACAGTACTTATTG 2008
gi|124263654|gb|EF208904.1|           TCTTGTGTTTAATCGTTCGGATGTATATACAAACACAACAGTACTTATTG 2008
gi|216289|dbj|D00248.1|BACISRH       TCTTGTGTTTAATCGTTCGGATGTATATACAAACACAACAGTACTTATTG 2400
gi|40351|emb|Y00423.1|                TCTTGTGTTTAATCGTTCGGATGTATATACAAACACAACAGTACTTATTG 2008
                                      **************************************************

gi|148787932|gb|EF424470.1|           ATAAAATTGAATTTCTGCCAATTACTCGTTCTATAAGAGAGGATAGAGAG 2058
gi|148787936|gb|EF424472.1|           ATAAAATTGAATTTCTGCCAATTACTCGTTCTATAAGAGAGGATAGAGAG 2058
gi|124263654|gb|EF208904.1|           ATAAAATTGAATTTCTGCCAATTACTCGTTCTATAAGAGAGGATAGAGAG 2058
gi|216289|dbj|D00248.1|BACISRH       ATAAAATTGAATTTCTGCCAATTACTCGTTCTATAAGAGAGGATAGAGAG 2450
gi|40351|emb|Y00423.1|                ATAAAATTGAATTTCTGCCAATTACTCGTTCTATAAGAGAGGATAGAGAG 2058
                                      **************************************************

gi|148787932|gb|EF424470.1|           AAACAAAAATTAGAAACAGTACAACAAATAATTAATACATTTTATGCAAA 2108
gi|148787936|gb|EF424472.1|           AAACAAAAATTAGAAACAGTACAACAAATAATTAATACATTTTATGCAAA 2108
gi|124263654|gb|EF208904.1|           AAACAAAAATTAGAAACAGTACAACAAATAATTAATACATTTTATGCAAA 2108
gi|216289|dbj|D00248.1|BACISRH       AAACAAAAATTAGAAACAGTACAACAAATAATTAATACATTTTATGCAAA 2500
gi|40351|emb|Y00423.1|                AAACAAAAATTAGAAACAGTACAACAAATAATTAATACATTTTATGCAAA 2108
                                      **************************************************

gi|148787932|gb|EF424470.1|           TCCTATAAAAAACACTTTACAATCAGAACTTACAGATTATGACATAGATC 2158
gi|148787936|gb|EF424472.1|           TCCTATAAAAAACACTTTACAATCAGAACTTACAGATTATGACATAGATC 2158
gi|124263654|gb|EF208904.1|           TCCTATAAAAAACACTTTACAATCAGAACTTACAGATTATGACATAGATC 2158
gi|216289|dbj|D00248.1|BACISRH       TCCTATAAAAAACACTTTACAATCAGAACTTACAGATTATGACATAGATC 2550
gi|40351|emb|Y00423.1|                TCCTATAAAAAACACTTTACAATCAGAACTTACAGATTATGACATAGATC 2158
                                      **************************************************

gi|148787932|gb|EF424470.1|           AAGCCGCAAATCTTGTGGAATGTATTTCTGAAGAATTATATCCAAAAGAA 2208
gi|148787936|gb|EF424472.1|           AAGCCGCAAATCTTGTGGAATGTATTTCTGAAGAATTATATCCAAAAGAA 2208
gi|124263654|gb|EF208904.1|           AAGCCGCAAATCTTGTGGAATGTATTTCTGAAGAATTATATCCAAAAGAA 2208
gi|216289|dbj|D00248.1|BACISRH       AAGCCGCAAATCTTGTGGAATGTATTTCTGAAGAATTATATCCAAAAGAA 2600
gi|40351|emb|Y00423.1|                AAGCCGCAAATCTTGTGGAATGTATTTCTGAAGAATTATATCCAAAAGAA 2208
                                      **************************************************

gi|148787932|gb|EF424470.1|           AAAATGCTGTTATTAGATGAAGTTAAAAATGCGAAACAACTTAGTAAATC 2258
gi|148787936|gb|EF424472.1|           AAAATGCTGTTATTAGATGAAGTTAAAAATGCGAAACAACTTAGTAAATC 2258
gi|124263654|gb|EF208904.1|           AAAATGCTGTTATTAGATGAAGTTAAAAATGCGAAACAACTTAGTAAATC 2258
gi|216289|dbj|D00248.1|BACISRH       AAAATGCTGTTATTAGATGAAGTTAAAAATGCGAAACAACTTAGTCAATC 2650
gi|40351|emb|Y00423.1|                AAAATGCTGTTATTAGATGAAGTTAAAAATGCGAAACAACTTAGTCAATC 2258
                                      ********************************************* ****

gi|148787932|gb|EF424470.1|           TCGAAATGTACTTCAAAACGGGGATTTTGAATCGGCTACGCTTGGTTGGA 2308
gi|148787936|gb|EF424472.1|           TCGAAATGTACTTCAAAACGGGGATTTTGAACCGGCTACGCTTGGTTGGA 2308
gi|124263654|gb|EF208904.1|           TCGAAATGTACTTCAAAACGGGGATTTTGAATCGGCTACGCTTGGTTGGA 2308
gi|216289|dbj|D00248.1|BACISRH       TCGAAATGTACTTCAAAACGGGGATTTTGAATCGGCTACGCTTGGTTGGA 2700
gi|40351|emb|Y00423.1|                TCGAAATGTACTTCAAAACGGGGATTTTGAATCGGCTACGCTTGGTTGGA 2308
                                      ******************************* ******************

gi|148787932|gb|EF424470.1|           CAACAAGTGATAATATCACAATTCAAGAAGATGATCCTATTTTTAAAGGG 2358
gi|148787936|gb|EF424472.1|           CAACAAGTGATAATATCACAATTCAAGAAGATGATCCTATTTTTAAAGGG 2358
gi|124263654|gb|EF208904.1|           CAACAAGTGATAATATCACAATTCAAGAAGATGATCCTATTTTTAAAGGG 2358
gi|216289|dbj|D00248.1|BACISRH       CAACAAGTGATAATATCACAATTCAAGAAGATGATCCTATTTTTAAAGGG 2750
gi|40351|emb|Y00423.1|                CAACAAGTGATAATATCACAATTCAAGAAGATGATCCTATTTTTAAAGGG 2358
                                      **************************************************

gi|148787932|gb|EF424470.1|           CATTACCTTCATATGTCTGGGGCGAGAGACATTGATGGTACGATATTTCC 2408
gi|148787936|gb|EF424472.1|           CATTACCTTCATATGTCTGGGGCGAGAGACATTGATGGTACGATATTTCC 2408
gi|124263654|gb|EF208904.1|           CATTACCTTCATATGTCTGGGGCGAGAGACATTGATGGTACGATATTTCC 2408
gi|216289|dbj|D00248.1|BACISRH       CATTACCTTCATATGTCTGGGGCGAGAGACATTGATGGTACGATATTTCC 2800
gi|40351|emb|Y00423.1|                CATTACCTTCATATGTCTGGGGCGAGAGACATTGATGGTACGATATTTCC 2408
                                      **************************************************

gi|148787932|gb|EF424470.1|           GACCTATATATTCCAAAAAAATTGATGAATCAAAATTAAAACCGTATACAC 2458
gi|148787936|gb|EF424472.1|           GACCTATATATTCCAAAAAAATTGATGAATCAAAATTAAAACCGTATACAC 2458
gi|124263654|gb|EF208904.1|           GACCTATATATTCCAAAAAAATTGATGAATCAAAATTAAAACCGTATACAC 2458
gi|216289|dbj|D00248.1|BACISRH       GACCTATATATTCCAAAAAAATTGATGAATCAAAATTAAAACCGTATACAC 2850
gi|40351|emb|Y00423.1|                GACCTATATATTCCAAAAAAATTGATGAATCAAAATTAAAACCGTATACAC 2458
                                      **************************************************

gi|148787932|gb|EF424470.1|           GTTACCTAGTAAGGGGATTTGTAGGAAGTAGTAAAGATGTAGAACTAGTG 2508
gi|148787936|gb|EF424472.1|           GTTACCTAGTAAGGGGATTTGTAGGAAGTAGTAAAGATGTAGAACTAGTG 2508
gi|124263654|gb|EF208904.1|           GTTACCTAGTAAGGGGATTTGTAGGAAGTAGTAAAGATGTAGAACTAGTG 2508
gi|216289|dbj|D00248.1|BACISRH       GTTACCTAGTAAGGGGATTTGTAGGAAGTAGTAAAGATGTAGAACTAGTG 2900
gi|40351|emb|Y00423.1|                GTTACCTAGTAAGGGGATTTGTAGGAAGTAGTAAAGATGTAGAACTAGTG 2508
                                      **************************************************

gi|148787932|gb|EF424470.1|           GTTTCACGCTATGGGGAAGAAATTGATGCCATCATGCATGTTCCAGCTGA 2558
gi|148787936|gb|EF424472.1|           GTTTCACGCTATGGGGAAGAAATTGATGCCATCATGCATGTTCCAGCTGA 2558
gi|124263654|gb|EF208904.1|           GTTTCACGCTATGGGGAAGAAATTGATGCCATCATGCATGTTCCAGCTGA 2558
gi|216289|dbj|D00248.1|BACISRH       GTTTCACGCTATGGGGAAGAAATTGATGCCATCATGAATGTTCCAGCTGA 2950
gi|40351|emb|Y00423.1|                GTTTCACGCTATGGGGAAGAAATTGATGCCATCATGAATGTTCCAGCTGA 2558
                                      ************************************ *************

gi|148787932|gb|EF424470.1|           TTTAAACTATCTGTATCCTTCTACCTGTGATTGTGAAGCGTCTAATCGTT 2608
gi|148787936|gb|EF424472.1|           TTTAAACTATCTGTATCCTTCTACCTGTGATTGTGAAGCGTCTAATCGTT 2608
gi|124263654|gb|EF208904.1|           TTTAAACTATCTGTATCCTTCTACCTGTGATTGTGAAGCGTCTAATCGTT 2608
gi|216289|dbj|D00248.1|BACISRH       TTTAAACTATCTGTATCCTTCTACCTTTGATTGTGAAGGGTCTAATCGTT 3000
gi|40351|emb|Y00423.1|                TTTAAACTATCTGTATCCTTCTACCTTTGATTGTGAAGGGTCTAATCGTT 2608
                                      ************************** *********** ***********

gi|148787932|gb|EF424470.1|           GTGAGACGTCCGCTGTGCCGGCTAACATTGGGAACACTTCTGATATGTTG 2658
gi|148787936|gb|EF424472.1|           GTGAGACGTCCGCTGTGCCGGCTAACATTGGGAACACTTCTGATATGTTG 2658
gi|124263654|gb|EF208904.1|           GTGAGACGTCCGCTGTGCCGGCTAACATTGGGAACACTTCTGATATGTTG 2658
gi|216289|dbj|D00248.1|BACISRH       GTGAGACGTCCGCTGTGCCGGCTAACATTGGGAACACTTCTGATATGTTG 3050
gi|40351|emb|Y00423.1|                GTGAGACGTCCGCTGTGCCGGCTAACATTGGGAACACTTCTGATATGTTG 2658
                                      **************************************************

gi|148787932|gb|EF424470.1|           TATTCATGCCAATATGATACAGGGAAAAAGCATGTCGTATGTCAGGATTC 2708
gi|148787936|gb|EF424472.1|           TATTCATGCCAATATGATACAGGGAAAAAGCATGTCGTATGTCAGGATTC 2708
gi|124263654|gb|EF208904.1|           TATTCATGCCAATATGATACAGGGAAAAAGCATGTCGTATGTCAGGATTC 2708
gi|216289|dbj|D00248.1|BACISRH       TATTCATGCCAATATGATACAGGGAAAAAGCATGTCGTATGTCAGGATTC 3100
gi|40351|emb|Y00423.1|                TATTCATGCCAATATGATACAGGGAAAAAGCATGTCGTATGTCAGGATTC 2708
                                      **************************************************

gi|148787932|gb|EF424470.1|           CCATCAATTTAGTTTCACTATTGATACAGGGGCATTAGATACAAATGAAA 2758
gi|148787936|gb|EF424472.1|           CCATCAATTTAGTTTCACTATTGATACAGGGGCATTAGATACAAATGAAA 2758
gi|124263654|gb|EF208904.1|           CCATCAATTTAGTTTCACTATTGATACAGGGGCATTAGATACAAATGAAA 2758
gi|216289|dbj|D00248.1|BACISRH       CCATCAATTTAGTTTCACTATTGATACAGGGGCATTAGATACAAATGAAA 3150
gi|40351|emb|Y00423.1|                CCATCAATTTAGTTTCACTATTGATACAGGGGCATTAGATACAAATGAAA 2758
                                      **************************************************
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Plate 21. Multiple sequence alignment with full length *cry*4A gene

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gi|148787932|gb|EF424470.1|         ATATAGGGGTTTGGGTCATGTTTAAAATATCTTCTCCAGATGGATACGCA 2808
gi|148787936|gb|EF424472.1|         ATATAGGGGTTTGGGTCATGTTTAAAATATCTTCTCCAGATGGATACGCA 2808
gi|124263654|gb|EF208904.1|         ATATAGGGGTTTGGGTCATGTTTAAAATATCTTCTCCAGATGGATACGCA 2808
gi|216289|dbj|D00248.1|BACISRH      ATATAGGGGTTTGGGTCATGTTTAAAATATCTTCTCCAGATGGATACGCA 3200
gi|40351|emb|Y00423.1|              ATATAGGGGTTTGGGTCATGTTTAAAATATCTTCTCCAGATGGATACGCA 2808
                                    **************************************************

gi|148787932|gb|EF424470.1|         TCATTAGATAATTTAGAAGTAATTGAAGAAGGGCCAATAGATGGGGAAGC 2858
gi|148787936|gb|EF424472.1|         TCATTAGATAATTTAGAAGTAATTGAAGAAGGGCCAATAGATGGGGAAGC 2858
gi|124263654|gb|EF208904.1|         TCATTAGATAATTTAGAAGTAATTGAAGAAGGGCCAATAGATGGGGAAGC 2858
gi|216289|dbj|D00248.1|BACISRH      TCATTAGATAATTTAGAAGTAATTGAAGAAGGGCCAATAGATGGGGAAGC 3250
gi|40351|emb|Y00423.1|              TCATTAGATAATTTAGAAGTAATTGAAGAAGGGCCAATAGATGGGGAAGC 2858
                                    **************************************************

gi|148787932|gb|EF424470.1|         ACTGTCACGCGTGAAACACATGGAGAAGAAATGGAACGATCAAATGGAAG 2908
gi|148787936|gb|EF424472.1|         ACTGTCACGCGTGAAACACATGGAGAAGAAATGGAACGATCAAATGGAAG 2908
gi|124263654|gb|EF208904.1|         ACTGTCACGCGTGAAACACATGGAGAAGAAATGGAACGATCAAATGGAAG 2908
gi|216289|dbj|D00248.1|BACISRH      ACTGTCACGCGTGAAACACATGGAGAAGAAATGGAACGATCAAATGGAAG 3300
gi|40351|emb|Y00423.1|              ACTGTCACGCGTGAAACACATGGAGAAGAAATGGAACGATCAAATGGAAG 2908
                                    **************************************************

gi|148787932|gb|EF424470.1|         CAAAACGTTCGGAAACACAACAAGCATATGATGTAGCGAAACAAGCCATT 2958
gi|148787936|gb|EF424472.1|         CAAAACGTTCGGAAACACAACAAGCATATGATGTAGCGAAACAAGCCATT 2958
gi|124263654|gb|EF208904.1|         CAAAACGTTCGGAAACACAACAAGCATATGATGTAGCGAAACAAGCCATT 2958
gi|216289|dbj|D00248.1|BACISRH      CAAAACGTTCGGAAACACAACAAGCATATGATGTAGCGAAACAAGCCATT 3350
gi|40351|emb|Y00423.1|              CAAAACGTTCGGAAACACAACAAGCATATGATGTAGCGAAACAAGCCATT 2958
                                    **************************************************

gi|148787932|gb|EF424470.1|         GATGCTTTATTCACAAATGTACAAGATGAGGCTTTACAGTTTGATACGAC 3008
gi|148787936|gb|EF424472.1|         GATGCTTTATTCACAAATGTACAAGATGAGGCTTTACAGTTTGATACGAC 3008
gi|124263654|gb|EF208904.1|         GATGCTTTATTCACAAATGTACAAGATGAGGCTTTACAGTTTGATACGAC 3008
gi|216289|dbj|D00248.1|BACISRH      GATGCTTTATTCACAAATGTACAAGATGAGGCTTTACAGTTTGATACGAC 3400
gi|40351|emb|Y00423.1|              GATGCTTTATTCACAAATGTACAAGATGAGGCTTTACAGTTTGATACGAC 3008
                                    **************************************************

gi|148787932|gb|EF424470.1|         ACTCGCTCAAATTCAGTACGCTGAGTATTTGGTACAATCGATTCCATATG 3058
gi|148787936|gb|EF424472.1|         ACTCGCTCAAATTCAGTACGCTGAGTATTTGGTACAATCGATTCCATATG 3058
gi|124263654|gb|EF208904.1|         ACTCGCTCAAATTCAGTACGCTGAGTATTTGGTACAATCGATTCCATATG 3058
gi|216289|dbj|D00248.1|BACISRH      ACTCGCTCAAATTCAGTACGCTGAGTATTTGGTACAATCGATTCCATATG 3450
gi|40351|emb|Y00423.1|              ACTCGCTCAAATTCAGTACGCTGAGTATTTGGTACAATCGATTCCATATG 3058
                                    **************************************************

gi|148787932|gb|EF424470.1|         TGTACAATGATTGGTTGTCAGATGTTCCAGGTATGAATTATGATATCTAT 3108
gi|148787936|gb|EF424472.1|         TGTACAATGATTGGTTGTCAGATGTTCCAGGTATGAATTATGATATCTAT 3108
gi|124263654|gb|EF208904.1|         TGTACAATGATTGGTTGTCAGATGTTCCAGGTATGAATTATGATATCTAT 3108
gi|216289|dbj|D00248.1|BACISRH      TGTACAATGATTGGTTGTCAGATGTTCCAGGTATGAATTATGATATCTAT 3500
gi|40351|emb|Y00423.1|              TGTACAATGATTGGTTGTCAGATGTTCCAGGTATGAATTATGATATCTAT 3108
                                    **************************************************

gi|148787932|gb|EF424470.1|         GTAGAGTTGGATGCACGAGTGGCACAAGCGCGTTATTTGTATGATATAAG 3158
gi|148787936|gb|EF424472.1|         GTAGAGTTGGATGCACGAGTGGCACAAGCGCGTTATTTGTATGATATAAG 3158
gi|124263654|gb|EF208904.1|         GTAGAGTTGGATGCACGAGTGGCACAAGCGCGTTATTTGTATGATATAAG 3158
gi|216289|dbj|D00248.1|BACISRH      GTAGAGTTGGATGCACGAGTGGCACAAGCGCGTTATTTGTATGATACAAG 3550
gi|40351|emb|Y00423.1|              GTAGAGTTGGATGCACGAGTGGCACAAGCGCGTTATTTGTATGATATAAG 3158
                                    ********************************************** ***

gi|148787932|gb|EF424470.1|         AAATATTATTAAAAAATGGTGATTTTACACAAGGGGTAATGGGGTGGCATG 3208
gi|148787936|gb|EF424472.1|         AAATATTATTAAAAAATGGTGATTTTACACAAGGGGTAATGGGGTGGCATG 3208
gi|124263654|gb|EF208904.1|         AAATATTATTAAAAAATGGTGATTTTACACAAGGGGTAATGGGGTGGCATG 3208
gi|216289|dbj|D00248.1|BACISRH      AAATATTATTAAAAAATGGTGATTTTACACAAGGGGTAATGGGGTGGCATG 3600
gi|40351|emb|Y00423.1|              AAATATTATTAAAAAATGGTGATTTTACACAAGGGGTAATGGGGTGGCATG 3208
                                    **************************************************

gi|148787932|gb|EF424470.1|         TAACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTGGTT 3258
gi|148787936|gb|EF424472.1|         TAACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTGGTT 3258
gi|124263654|gb|EF208904.1|         TAACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTGGTT 3258
gi|216289|dbj|D00248.1|BACISRH      TAACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTGGTT 3650
gi|40351|emb|Y00423.1|              TAACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTGGTT 3258
                                    **************************************************

gi|148787932|gb|EF424470.1|         CTATTTAATTGGAGTGCTGGCGTATCTCAAAATGTCCATCTCCACCATAA 3308
gi|148787936|gb|EF424472.1|         TTATTTAATTGGAGTGCTGGCGTATCTCAAAATGTCCATCTCCACCAAAA 3308
gi|124263654|gb|EF208904.1|         CTATTTAATTGGAGTGCTGGCGTATCTCAAAATGTCCATCTCCACCATAA 3308
gi|216289|dbj|D00248.1|BACISRH      CTATCTAATTGGAGTGCTGGCGTATCTCAAAATGTCCATCTCCAACATAA 3700
gi|40351|emb|Y00423.1|              CTATCTAATTGGAGTGCTGGCGTATCTCAAAATGTCCATCTCCAACATAA 3308
                                     *** *************************************** ** **

gi|148787932|gb|EF424470.1|         TCATGGGTATGTCTTAGGTGTTATTGCCAAAAAAGAAGGACCTGGAAATG 3358
gi|148787936|gb|EF424472.1|         TCATGGGTATGTCTTAGGTGTTATTGCCAAAAAAGAAGGACCTGGAAATG 3358
gi|124263654|gb|EF208904.1|         TCATGGGTATGTCTTAGGTGTTATTGCCAAAAAAGAAGGACCTGGAAATG 3358
gi|216289|dbj|D00248.1|BACISRH      TCATGGGTATGTCTTACGTGTTATTGCCAAAAAAGAAGGACCTGGAAATG 3750
gi|40351|emb|Y00423.1|              TCATGGGTATGTCTTAGGTGTTATTGCCAAAAAAGAAGGACCTGGAAATG 3358
                                    **************** *********************************

gi|148787932|gb|EF424470.1|         GGTATGTCACGCTTATGGATTGGGAGGAGAATCAAGAAAAATTGACGTTT 3408
gi|148787936|gb|EF424472.1|         GGTATGTCACGCTTATGGATTGGGAGGAGAATCAAGAAAAATTGACGTTT 3408
gi|124263654|gb|EF208904.1|         GGTATGTCACGCTTATGGATTGGGAGGAGAATCAAGAAAAATTGACGTTT 3408
gi|216289|dbj|D00248.1|BACISRH      GGTATGTCACGCTTATGGATTGTGAGGAGAATCAAGAAAAATTGACGTTT 3800
gi|40351|emb|Y00423.1|              GGTATGTCACGCTTATGGATTGGGAGGAGAATCAAGAAAAATTGACGTTT 3408
                                    ********************** ***************************

gi|148787932|gb|EF424470.1|         ACGTCTTGTGAAGAAGGATATATTACGAAGACAGTAGATGTATTCCCAGA 3458
gi|148787936|gb|EF424472.1|         ACGTCTTGTGAAGAAGGATATATTACGAAGACAGTAGATGTATTCCCAGA 3458
gi|124263654|gb|EF208904.1|         ACGTCTTGTGAAGAAGGATATATTACGAAGACAGTAGATGTATTCCCAGA 3458
gi|216289|dbj|D00248.1|BACISRH      ACGTCTTGTGAAGAAGGATATATTACGAAGACAGTAGATGTATTCCCAGA 3850
gi|40351|emb|Y00423.1|              ACGTCTTGTGAAGAAGGATATATTACGAAGACAGTAGATGTATTCCCAGA 3458
                                    **************************************************

gi|148787932|gb|EF424470.1|         TACAGATCGTGTACGAATTGAGATAGGCGAGACCGAAGGTTCGTTTTATA 3508
gi|148787936|gb|EF424472.1|         TACAGATCGTGTACGAATTGAGATAGGCGAGACCGAAGTTTCGTTTTATA 3508
gi|124263654|gb|EF208904.1|         TACAGATCGTGTACGAATTGAGATAGGCGAGACCGAAGGTTCGTTTTATA 3508
gi|216289|dbj|D00248.1|BACISRH      TACAGATCGTGTACGAATTGAGATAGGCGAAACCGAAGGTTCGTTTTATA 3900
gi|40351|emb|Y00423.1|              TACAGATCGTGTACGAATTGAGATAGGCGAAACCGAAGGTTCGTTTTATA 3508
                                    ****************************** ******* ***********
gi|148787932|gb|EF424470.1|         TCGAAAGCATTGATTTAATTTGCATGAACGAGTGA--------------- 3543
gi|148787936|gb|EF424472.1|         TCGAAAGCATTGATTTAATTTGCATGAACGAGTGA--------------- 3543
gi|124263654|gb|EF208904.1|         TCGAAAGCATTGATTTAATTTGCATGAACGAGTGA--------------- 3543
gi|216289|dbj|D00248.1|BACISRH      TCGAAAGCATTGAATTAATTTGCATGAACGAGTGATTAATAAAAAATAAC 3950
gi|40351|emb|Y00423.1|              TCGAAAGCATTGAATTAATTTGCATGAACGAGTGA--------------- 3543
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Reverse primer

Plate 21. Multiple sequence alignment with full length *cry*4A gene

(Box indicates reverse primer)

Discussion

5. Discussion

The use of chemical insecticides is being increasingly discouraged because of several reasons like their toxicity to non-target organisms, environmental persistence leading to serious health hazards and development of resistance by insect populations. Consequently, attention has been directed toward alternative methods of insect control. An attractive alternative involves the use of entomopathogens specific against the pest species. A diverse group of microorganisms pathogenic to insects is available. Among these are viruses, bacteria, and fungi. Each of these subgroups is composed of a spectrum of organisms that vary in their mode of infection, site of replication, and mechanism of pathogenicity. One such organism, *Bacillus thuringiensis* Berliner, is available commercially and was originally thought to affect only lepidopteran insects. However, later reports indicated that, *B. thuringiensis* subsp. *israelensis*, was active against dipteran insects, such as fruit flies, mosquitoes and blackflies (Goldberg and Margalit,1977; Undeen and Nagel, 1978), yet has no adverse effects against most non-target organisms (Colbo and Undeen, 1980; Garcia *et al.,* 1981; Molloy and Jamnback, 1981; Mulla *et al.,* 1982).

Entomocidal properties of *B. thuringiensis* reside in a parasporal crystal protein that is formed during sporulation of the organism. These proteins are deposited as parasporal inclusions and, in some cases, these are also found on the surface of the spore. When the inclusion (or spore) is ingested by susceptible larvae, the protoxin is solubilized in the alkaline, reducing environment of the midgut. The midgut of these larvae also contains proteases necessary to convert the protoxins to toxins and perhaps receptors on the surface of midgut epithelial cells to which the toxins (or protoxins) bind to initiate their action (Fast *et al.,* 1978; Huber and Luthy, 1981; Knowles *et al.,* 1984). For this reason, conventional bioassay procedures

involve feeding test insects a diet containing known concentration of pathogens (Burges and Thomson, 1971; Dulmage *et al.,* 1971).

Intensive screening programmes are going on all over the world, to isolate *B. thuringiensis* strains from soil samples, plant surfaces, dead insects and stored grains. The search for new *B. thuringiensis* strains is a continuous process, since a significant number of pests are not controlled with the available crystal proteins. It is also important to provide alternative means of coping with the problem of insect resistance, especially with regard to the expression of *B. thuringiensis* genes encoding insecticidal proteins in transgenic plants (van Rie, 1989)

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The present study emphasized on cultural, morphological, biochemical and molecular characterization of twenty native isolates from the repository of *Bacillus thuringiensis* available at the Centre for Plant Biotechnology and Molecular Biology. These were originally isolated from soil samples collected from various locations in six districts of Kerala, coming under the Western Ghats region. The insecticidal property of native isolates was also assessed against a test organism, *Drosophila melanogaster* (vinegar fly) belonging to the insect order Diptera. Dipteran specific *cry* genes *cry*2, *cry*4, *cry*10, *cry*11, *cry*16, *cry*17, *cry*19 and *cry*21 profiling was carried out by using general and specific primers of the respective genes. Partial coding sequences of *cry*4 gene were cloned and sequenced from selected potential Bt isolates. Sequence analysis was carried out by using various bioinformatics tools.

5.1 Cultural characterization

Cultural and morphological characters of twenty native *Bacillus thuringiensis* isolates were studied. All the isolates produced creamy white, puffy, and opaque colonies, 24 hours after inoculation on LB agar medium. Colonies were flat, size

ranging from small to large, with circular or irregular form. Margins were either undulate or entire. The colony morphology analysis of 95 isolates from Brazilian territory were made of smooth colonies with an irregular shape (Lima *et al*., 2002). Similar variations in the morphological characteristics of *B. thuringiensis* isolates on nutrient agar medium were recorded by Chatterjee *et al*. (2006). They reported circular, white, flat and undulate or entire colonies of the *B. thuringiensis* isolates obtained from West Bengal.

5.2 Staining reactions

Gram staining of *Bacillus thuringiensis* isolates revealed the presence of blue coloured, rod-shaped cells arranged like a chain.

For the staining of crystal proteins, Comassie brilliant blue was used. This chemical provides a rapid, sensitive and convenient staining procedure and it imparts a dark-blue colour to proteins. Crystal proteins are stained blue and endospores remain unstained. The shape of crystal proteins varied from spherical, irregular, rhomboidal and composite of spherical and irregular for different isolates. Isolates Bt-105, Bt-170, Bt-206, Bt-245, Bt-326 and Bt-411 produced a composite of spherical and irregular crystal proteins. Composite parasporal inclusion bodies have been earlier reported by Obeidat *et al*. (2004).

Of the remaining isolates, Bt-23, Bt-161, Bt-190, Bt-237, Bt-242, Bt-277, Bt-283, Bt-419, Bt-420, Bt-430 and Bt-447 produced spherical crystals. Bt-135 and Bt-250 produced irregular and Bt-133 formed rhomboidal crystal proteins. 55 per cent of native Bt isolates produced spherical crystals, 30, 10 and 5 per cent isolates formed composite, irregular and rhomboidal shape crystal protein respectively (Fig. 1). The study indicated that spherical crystal proteins were most predominant in *B.*

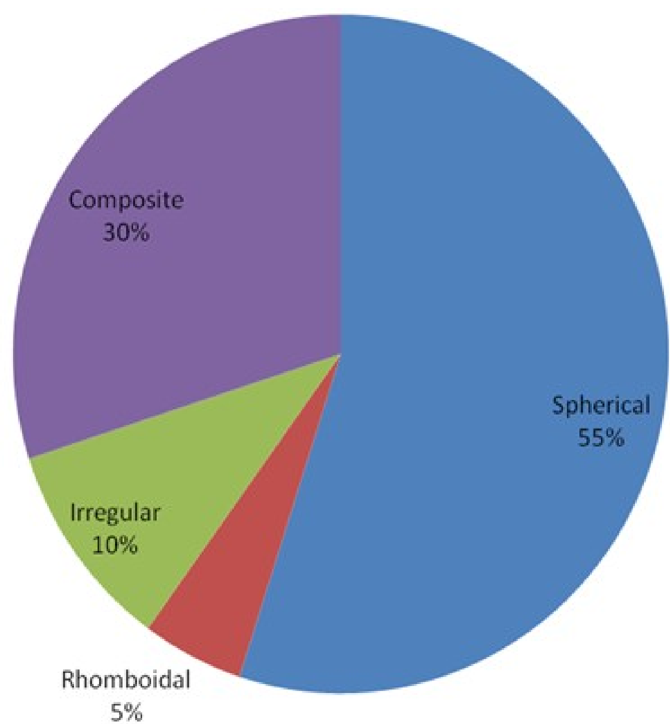

Fig. 1. Morphological type distribution of crystal protein in native *Bacillus thuringiensis* isolates

thuringiensis isolated from the Western Ghats of Kerala. Earlier studies with isoloates from Wonju area showed presence of spherical, irregular and bipyramidal crystal proteins (Yoo *et al*., 1996). In another study, *B. thuringiensis* isolates were found to be toxic to *D. melanogaster* larvae and adults. Majority (75%) of these toxic

isolates produced spherical crystals and 25 per cent produced both cuboidal and bipyramidal crystals (Obeidat, 2008). A correlation between the shape of crystal protein and insect specificity has been reported by earlier workers (Attathom *et al*., 1995; Yamamoto and Mclanghlin, 1981). Spherical crystal protein are reported to be dipteran specific and bipyramidal ones specific to lepidopteran larvae (Lima *et al.,* 2002). Spherical and irregular crystals are mostly mosquitocidal, often active against certain coleopteran species also (Krieg *et al*., 1983).

The parasporal inclusion bodies of *Bacillus thuringiensis* were observed as morphologically heterogeneous (Ohba *et al*., 2001). Crystal protein morphology of *Bacillus thuringiensis* isolates was diverse, ranging from cuboidal, spherical, rhomboidal and irregular (Benhard *et al*., 1997).

The native isolates of *Bacillus thuringiensis* were found to produce crystal proteins in varying numbers. Of the twenty isolates, five produced numerous crystal proteins (Bt-23, Bt-133, Bt-170, Bt-190 and Bt-242). Five isolates produced moderate number and ten, a few crystals. This kind of variations in the number of crystals was earlier reported by Aronson and Fitz-James (1976) and Bechtel and Bulla (1976).

5.3 Biochemical characterization

Seven biochemical tests including production of enzymes like esculinase, gelatinase, lecithinase, urease and amylase, solubility in three per cent KOH and

Voges-Proskauer test were used to characterize *B. thuringiensis* isolates (Cappuccino and Sherman, 1992).

The enzyme esculinase helps the bacterium to hydrolyze the glycoside esculin to form esculetin and dextrin. Esculetin reacts with ferric citrate to produce dark brown or black phenolic iron complex. All the twenty native isolates and the reference strain 4Q1 produced browning around the growth in esculin agar medium, indicating their ability to hydrolyse esculin. Reports on bacterial isolates that differ in their reaction to esculinase were made by various scientists (Gordon *et al.,* 1973; de Lucca *et al.,* 1991; Hernandez *et al.,* 1998).

None of the native Bt isolates were capable of producing proteolytic extra-cellular enzyme gelatinase. In this test after 24 hours incubation, gelatin remained solidified which indicated negative reaction of bacterial isolates to gelatin hydrolysis. This was also reported by earlier workers (Hernandez *et al.*, 1998; Demir *et al.*, 2002).

All the isolates produced lecithinase, a phospholipase enzyme that hydrolyzes lecithin. Presence of a clear zone in the medium around bacterial growth indicated the ability of the bacterium to hydrolyse lecithin present in egg yolk medium. Bacterial isolates were tested for solubility in three per cent KOH, as an effective supplement to Gram staining, proven to be useful for rapid and accurate differentiation of bacteria (Suslow *et al*., 1982). None of the isolates formed viscous thread-like structure with KOH, indicating gram positive nature. KOH test is based on differences in the chemistry of bacterial cell wall. The cell wall of gram-negative bacteria is easily disrupted when exposed to dilute alkali solutions. When the cell walls are disrupted, the suspension in KOH becomes viscous due to the release of relatively unfragmented threads of deoxyribonucleic acid. Weak alkali solutions have

no detectable effect on the cell wall of gram-positive bacteria. Differences in KOH solubility have been used successfully to categorize aerobic and facultative bacteria, including bacteria which display variable Gram staining reactions.

All the isolates were capable of hydrolyzing starch, which was indicated by the presence of a clear zone around the growth, when flooded with Lugol's iodine. The unhydrolyzed starch stained blue. Similar observations were made by Demir *et al*. (2002) who characterized *B. thuringiensis* isolates using starch hydrolysis reaction and all gave positive response.

In the urease test, the bacterial isolates were checked for their ability to degrade urea by means of the enzyme urease. Urease is a hydrolytic enzyme that attacks nitrogen and carbon bonds in amide compounds such as urea and forms the alkaline end product ammonia. Colour change of the medium to deep pink, is due to the reason that, as the substrate urea splits into products, presence of ammonia creates an alkaline environment that causes phenol red to turn deep pink. Twelve cultures namely Bt-105, Bt-161, Bt-170, Bt-190, Bt-206, Bt-242, Bt-245, Bt-250, Bt-277, Bt-283, Bt-419 and Bt-447 developed deep pink colour which was indicative of urea hydrolysis. The isolates Bt-23, Bt-133, Bt-135, Bt-237, Bt-326, Bt-411, Bt-420, Bt-430 and 4Q1 did not produce any colour change, indicating the absence of urease enzyme in these isolates. Different responses of bacterial isolates to hydrolysis of urea had been reported by earlier workers (Hernandez *et al*., 1998; Demir *et al.,* 2002; Chatterjee *et al*., 2006).

Voges-Proskauer (VP) test was carried out to determine the ability of isolates to produce non-acidic or neutral end products such as acetyl methyl carbinol from organic acids that result from glucose metabolism. None of the isolates gave positive reaction. Reports on negative reaction of bacterial isolates to Voges-Proskauer test

were made by various scientists (Hernandez *et al.,* 1988; Demir *et al*., 2002; Lopez and Alippi, 2005).

Among the different biochemical tests used for the characterization of *B. thuringiensis* isolates, differential response was obtained only for urea hydrolysis. The response of bacterial isolates to esculinase test, gelatin hydrolysis, lecithinase test, solubility in three per cent KOH, starch hydrolysis test and Voges-Proskauer test remained uniform. The differential response of bacterial isolates to biochemical tests is indicative of the diversity in locations from which *B. thuringiensis* were isolated.

5.4 DNA isolation

Gram-positive bacteria have a rigid cell wall that can be difficult to lyse, and the methods used for the isolation of genomic DNA from Gram-negative bacteria are generally not successful with Gram-positive bacteria. Molecular epidemiologic studies and many other screening applications may also require preparation of genomic DNA from a large number of samples. Clean DNA is generally required for any *in vitro* amplification procedures, such as PCR. Several procedures are available for preparing genomic DNA from Gram-positive bacteria, but they are time-consuming and are not suitable for application to large sample numbers. Usually, large culture volumes are required to obtain sufficient amounts of genomic DNA for various molecular manipulations. Moreover, they involve a lengthy lysis step, which can result in considerable DNA damage.

In the present study, total DNA was isolated from all the bacterial isolates and reference strain according to the procedure of Sambrook and Russel (2001). Similar procedure was used by several workers for isolating DNA from *Bacillus thuringiensis* (Ben- Dov *et al*., 1997; Beron *et al*., 2004; Rajesh *et al*., 2006; Held *et al*., 1982;

Kawalek *et al*., 1995; Juarez Perez *et al.,* 1997; Ben-Dov *et al*., 1997). The protocol yielded a single sharp band on 0.8 per cent agarose gel. No RNA contamination was observed. The OD_{260}/OD_{280} values of DNA ranged between 1.84 and 2.05 indicating that DNA was good without much protein contamination. Quantity of DNA was found to be highest in the isolate Bt-250 (1974.9 ng/µl) and the lowest in Bt-170 (391.0 ng/µl).

5.4.1 Profiling of dipteran specific *cry* genes in *Bacillus thuringiensis*

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 (Schnepf *et al*., 1998). The extensive variety of bacterial strains and the toxins they produce permit the production of bioinsecticides using the bacteria themselves and also allow use of the toxin genes in the development of transgenic plants (Romeis *et al*., 2006). Specificity of an isolate of *B. thuringiensis* could be determined by bioassay experiments on different groups of target insects. However, this becomes tedious and time consuming with large collections of isolates. 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. 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. Although powerful, PCR approaches are limited to the detection of already-known genes and fail to detect and identify novel *cry* genes even though various strategies have been proposed to increase their efficiency.

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 (Porcar and Juarez-Perez, 2003). Exclusive PCR was used by Juarez-Perez *et al*. (1997) which is a two step procedure which allows both the identification of known *cry* genes present in *B. thuringiensis* isolates and the detection and identification of *cry*1-related sequences unrecognized by specific primers. Carrozi *et al*. (1991) introduced this technique to identify *cry* genes in order to predict the insecticidal activity of Bt isolates. DNA amplification being very sensitive to PCR conditions, various parameters like the concentration of DNA and Taq DNA polymerase, annealing temperature and number of cycles for the thermal cycler programme were standardized for the specific amplification of *cry* gene.

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 (Bt-23, Bt-133, Bt-190, Bt-242, Bt-245, Bt-250, Bt-277, Bt-326, Bt-411, Bt-420 and Bt-447). This was followed by *cry*11, which was present in 13 per cent and *cry*10 in 12 per cent (Bt-419 and Bt-447) (Fig. 2). c*ry*4 and *cry*11 genes were present in Bt-23 and Bt-242. *cry*21 was present only in the isolate Bt-245. 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, 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, Bt-430) lacked any dipteran-specific gene. This might be due to the reason

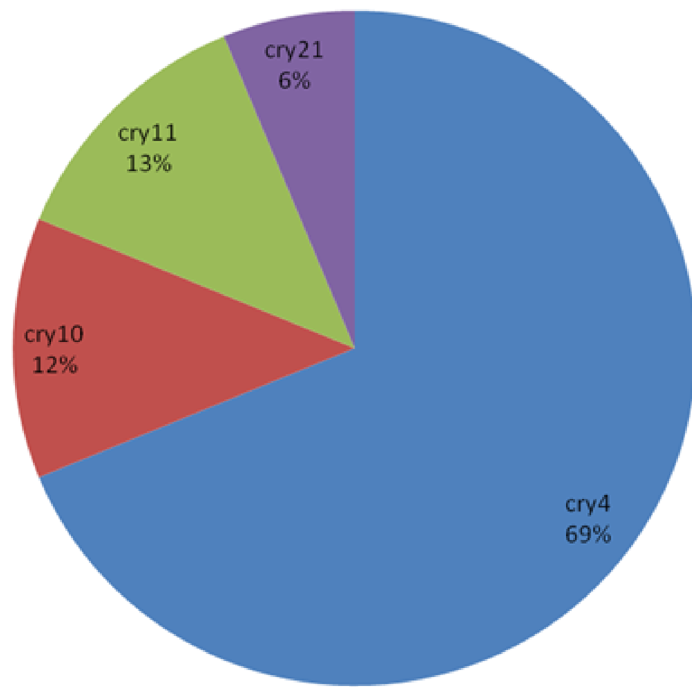

Fig. 2. Distribution of dipteran specific *cry* genes in native *Bacillus thuringiensis* isolates

that they possess other *cry* genes which are not identified by the specific primer used (Table 10b).

Uribe *et al.* (2003) 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.

During *cry* gene profiling for all the eight *cry* genes, some non-specific amplifications were also found. This could be due to novel *cry* genes possessed by the Bt isolates. Bravo *et al*. (1998) 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.

5.5 Insecticidal activity against *Drosophila melanogaster*

Bacillus thuringiensis is a biological control agent that presents several advantages over the use of chemical control agents. Since the parasporal bodies released by such bacteria during their growth are highly specific for some of the major species belonging to insect orders like Lepidoptera, Diptera and Coleoptera, these have no harmful effect on other non-target insects, plants and domestic animals. These entomopathogenic proteins named δ-endotoxins are produced as protoxins that can be solubilized and activated by the action of alkaline proteases (Ferre *et al.,* 1991). The activated toxin molecules are found linked to specific sites located on the apical microvilosites of susceptible intestinal cells of the larvae (van Rie *et al.,* 1990a

and b). After the toxin induction, its molecules are inserted within the plasma membrane and by doing so they punch holes in the cell surface, changing the osmotic equilibrium and resulting in death (van Rie *et al.,* 1989).

The parasporal inclusion body is composed of proteins varying in quantity and type depending on the strain. Each type of crystal protein is characterized by a specific host range. Based upon differences in sequence and specificity, insecticidal crystal δ-endotoxins have been classified into several groups of proteins designated as Cry (Crickmore *et al.,* 1995; Feitelson *et al.,* 1992; Hofte and Whiteley, 1989). The Cry2, Cry4, Cry10, Cry11, Cry16, Cry17, Cry19, and Cyt proteins are toxic to dipteran insects. The characterization of most of the *B. thuringiensis* collections were based on bioassays against different insect larvae without identification of the *cry* genes present in the *B.thuringiensis* strains (Crickmore *et al.,* 1998 and Schnepf *et al.,* 1998).

In the present study, bioassay of *B. thuringiensis* isolates collected from Western Ghats of Kerala against *Drosophila melanogaster* (vinegar fly) was carried out by diet contamination method. Diet contamination method is preferred to surface contamination method, as in the latter, the larvae sometimes bore into the diet instead of feeding on the surface, which results in a low intake of the toxin. In the diet contamination method, crude crystal protein preparation from *B. thuringiensis* isolates were mixed with semi synthetic diet and larvae of *Drosophila melanogaster* were allowed to feed on the diet. Similar method had been used by many workers in their bioassay experiments (Nakamori and Kakinohana, 1980; Obeta and Okafor, 1984; Travers and Martin, 1987; Held *et al*., 1982; Klowden *et al*., 1984; Chak *et al*., 1994; Peyronnet *et al*., 1997; Obeidat, 2008).

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.

5.5.1 Symptoms of Cry toxin infection

Cessation of feeding was observed as the first symptom 10 hours after larvae were introduced into Cry toxin mixed with larval diet. Angus (1956) and Cooksey (1971) reported that the first sign of poisoning following ingestion of crystalline endotoxins was paralysis of gut and mouth parts leading to cessation of feeding. Feeding inhibition has been reported as an immediate effect of the protective action against further feeding on the toxin. Larvae stopped moving and death occurred within 13 to 16 hours after feeding. The cadaver became putrified and changed into black colour. Similar observations were also reported by Brunner and Stevens (1986) and Wilding (1986).

5.5.2 Bioassay of Drosophila larvae with the selected *B. thuringiensis* isolates

The spore concentration of spore-crystal protein mixture for the bioassay experiment was standardized with reference to 4Q1. Of the seven dosages tested, 1.1×10^8 spores per ml gave a very low mortality of 6.7 per cent and this was eliminated for subsequent experiments. Higher concentrations of 13.2×10^8 and 16.5×10^8 spores per ml resulted in a mortality of 100 per cent. Therefore the highest concentration of 16.5×10^8 was also eliminated. Remaining five spore concentrations viz., 3.3×10^8, 5.5×10^8, 8.8×10^8, 11×10^8 and 13.2×10^8 spores per ml were selected for bioassay experiment. Jacob (2008) had used five different spore concentrations of Bt toxin for the bioassay experiment against *Spodoptera litura.*

Beron and salerno (2007) had used six different concentrations of Bt toxin for bioassay with *Aedes aegypti*.

The insecticidal activity of *B. thuringiensis* crystal proteins is traditionally investigated by using crude preparations of spore-crystal mixtures. The results of these studies, however, were difficult to be interpreted. Spore-crystal preparations generally contain other toxic agents also such as the beta-exotoxin and toxic spore components. In some cases it is also not known whether the germination of spores inside the larvae contributes to the observed toxic effects. Hence, studies using spore-protein mixture might not lead to conclusive data that toxicity was due solely to the activity of the crystal proteins. In some recent investigations, gradient-purified crystals have been used in toxicity assays. However, since many *B. thuringiensis* strains simultaneously produce more than one crystal proteins, it is still difficult to accurately determine the toxicity spectrum of individual proteins from these studies.

In the present investigation, insecticidal activity of Bt isolates was carried out with five different concentrations i.e., 3.3×10^8, 5.5×10^8, 8.8×10^8, 11.0×10^8 and 13.2×10^8 spores per ml. The reference strain, 4Q1 caused 100 per cent morality at a concentration of 13.2×10^8 spores per ml after 24 hours. The lowest concentration of 3.3×10^8 spores per ml caused the lowest mortality. Hence 4Q1 was found to be highly effective among all the isolates were tested. In the native isolates, Bt-23 was found to cause the highest mortality of 88.8 per cent at its highest concentration of 13.2×10^8 spores per ml. Lowest mortality (79.3 per cent) was observed with the isolate Bt-242. Isolates Bt-133 and Bt-190 caused larval mortality 83.3 and 85.6 per cent respectively at the concentration of 13.2×10^8 spores per ml. Among the native isolates, Bt-23 was highly effective in its toxicity, since it caused the highest mortality followed by Bt-190, Bt-133 and Bt-242. The larval mortality showed an increase in the mortality per cent with the increase in the concentrations of toxin-

spore suspension (Fig. 3). This was also confirmed by the earlier report by Sareen *et al.* (1983) who described the increase in per cent mortality along with the increase in spore concentration. Variations in the effectiveness among different concentrations of spore or crystal toxins of *B. thuringiensis* were also already reported (Ahmed *et al.,* 1988; Zaz, 1989).

Obeidat (2008) reported that 30 per cent of Jordanian *B. thuringiensis* isolates were toxic to the larvae and adults of *Drosophila melanogaster*. He also indicated that 75 per cent of the 24 isolates which were toxic, produced spherical crystal proteins.

5.5.3 Lethal concentration (LC_{50}) of Bt isolates

Median lethal concentration (LC_{50}) of the five isolates was determined by Probit analysis with SPSS statistical software. Probit analysis was earlier used to find out the LC_{50} value of Bt toxin by Beron and Salerno (2007). The dosage mortality response of *Drosophila* larvae to the different concentration of Bt isolates indicated a slight variability among the different isolates to kill the insects. The lowest LC_{50} value was recorded with Bt-23 which indicated a comparatively high effectiveness of the isolate (Figure 4). This is in agreement with the presence of two dipteran specific genes (*cry*4 and *cry*11) in this isolate, as revealed by *cry* gene profiling. Such difference in the insecticidal activity of different isolates of *Bacillus thuringiensis* might be due to the synergistic effect of two different *cry-cry* gene combinations. Mosquito larvicidal activity of recombinant *E. coli* with combination of CryIVA and CryIVD toxin was seven fold higher than CryIVA toxin alone, probably because of the polypeptides or the synergism between their activities as reported by Ben-Dov *et al.* (1995).

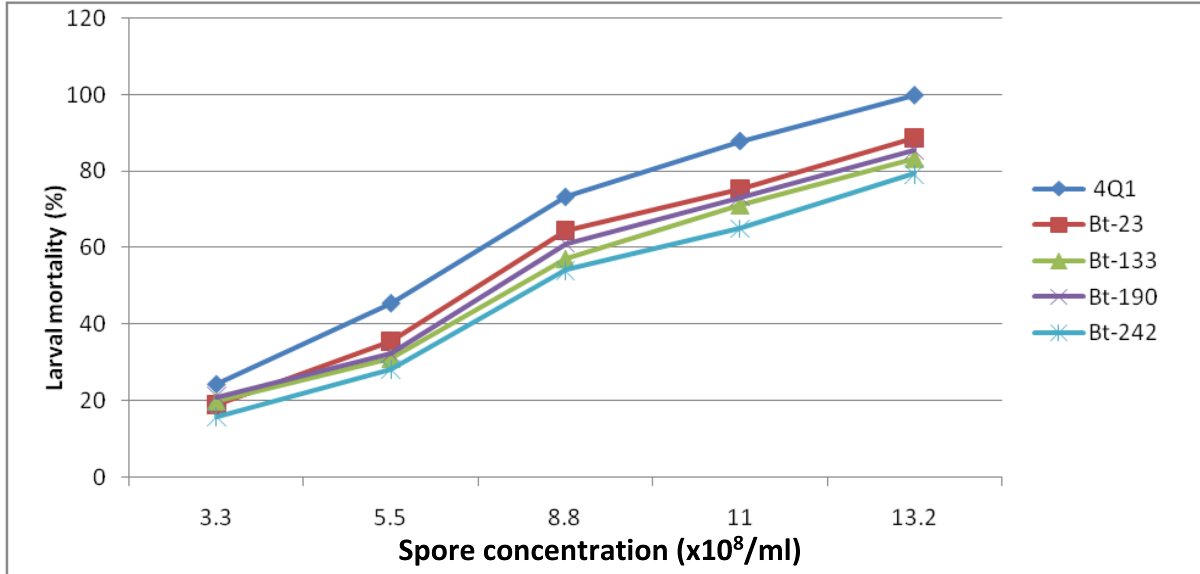

Fig. 3. Dosage – mortality response of Bt spore concentration on larvae of *Drosophila melanogaster*

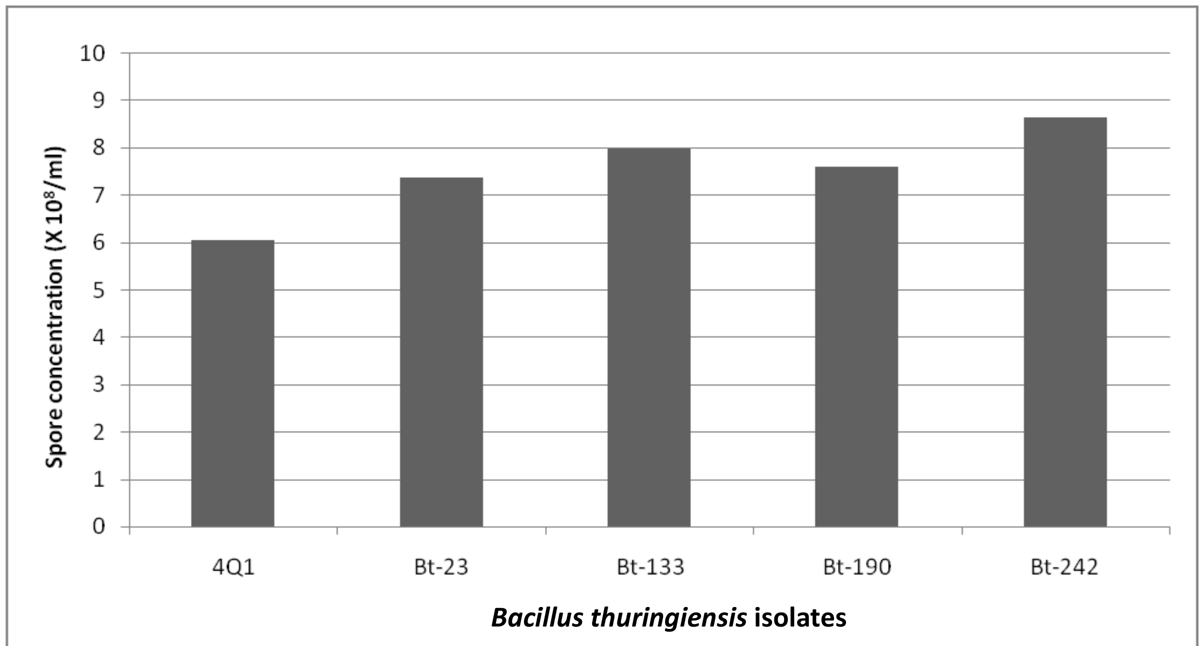

Fig. 4. Relative median lethal concentration values (LC_{50}) of native *Bacillus thuringiensis* isolates on *Drosophila melanogaster* larvae

Another reason for the difference in toxicity of Cry proteins might be the difference in the carbohydrate affinity of the domain II, resulting in variable binding specificity with the receptors at the brush border membrane of the insect (Smedley and Ellar, 1996). Since *Bacillus thuringiensis* strains simultaneously produce more than one type of crystal proteins, bioassay can be greatly influenced by the relative proportion of different proteins within the crystal. Hence it is difficult to accurately determine the spectrum of individual proteins causing toxicity on the test organisms.

5.6 Plasmid profiling

All *Bacillus thuringiensis* strains contain a set of self-replicating, extra-chromosomal DNA molecules or plasmids. Parasporal inclusions, which are composed of insecticidal crystal proteins are the basis for the commercial use of *B. thuringiensis* as a control agent of various insects. *cry* genes encoding crystal proteins are harboured on plasmids of *Bacillus thuringiensis.* The number of plasmids, their sizes and copy numbers can vary considerably among Bt strains.

Plasmids are of great importance in *B. thuringiensis* because the insecticidal protein encoding genes are located on megaplasmids. Plasmids have also been associated with the production of β-exotoxins in these bacteria. *B. thuringiensis* plasmids have been studied either to locate *cry* genes, or to transfer them between different strains or to characterize different strains (Reyes-Ramirez and Ibarra, 2008). The plasmid patterns obtained from gel electrophoresis have been used as a tool to characterize strains, but comparison of the plasmid patterns has been limited in the number and diversity of strains.

In the present investigation, selected isolates were used for plasmid profiling. In all the isolates a band moving on par with 21 kb fragment of molecular weight marker (λ *Hind*III/ *Eco*RI*)* was observed. This could be genomic DNA or mega

plasmids present in *Bacillus thuringiensis.* In *B. thuringiensis*, plasmids are recognized into two groups, those that are ≤30MDa and those that are ≥30MDa (Reyes-Ramirez and Ibarra, 2008). Migration of megaplasmids on agarose gel is often very limited and co-migrating bands are difficult to differentiate. Moreover, megaplasmids are degraded easily during storage. In the present study, no megaplasmids could be located migrating above the genomic DNA band. However, smaller plasmids of 5kb, 2.5kb, 2kb and 0.9kb were observed in 4Q1. Native isolate Bt-23 contained three plasmids size of 5kb, 2kb and 0.9kb. Two plasmids of 3.5kb and 0.9kb were present at in Bt-133. Bt-190 possessed only one plasmid of 0.9kb size and Bt-242 yielded two bands of 3.2kb and 1.2kb.

Reyes-Ramirez and Ibarra (2008) determined the diversity of plasmid patterns from 130 type strains and also from six serotypes. Plasmid patterns of *B. thuringiensis* revealed all the strains contain at least one plasmid, and some strains have a maximum of 13 plasmids. Plasmid patterns from different serotypes were compared, strains within the same serotype showed at least some degree of resemblance. Plasmid profiling was reported by many workers to find out the number of plasmids and their pattern (Frederiksen *et al.,* 2006; Amadio *et al.,* 2009; Gitahy *et al.,* 2007; Voskuil and Chambliss, 1993).

5.7 Amplification of *cry*4 gene from *B. thuringiensis* isolates

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, 800bp were obtained for isolates, Bt-23, Bt-133 and Bt-242. Isolate Bt-190 produced a single amplicon of size 400bp.

5.8 Cloning and sequencing of *cry*4 amplicons

Eluted 800bp DNA fragment of Bt-23 were ligated into pGEMT vector and transformed into *E. coli* JM 109 cells. Balasubramanian *et al*. (2002) amplified 2kb product through polymerase chain reaction and the amplicon was ligated into pGEMT vector. Beron and Salerno (2007) amplified 2.5kb 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.

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 (Ullman *et al*., 1967). The colonies which have not taken up the plasmid can further utilize the substrate and appear as blue colonies on X-gal chromogenic substrate (Horwitz *et al.,* 1964). All transformed colonies harbouring the recombinant plasmid appeared in white colour due to the disruption of α-complementation.

Presence of amplicons of 800bp from plasmids obtained from white colonies confirmed the presence of the insert. Sequence information 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.

5.9 Analysis of the sequence data

The Analysis of the sequence data of the four amplified products by Blastn revealed that they were partial *cry*4 genes. Blastn is a algorithm that finds region of local similarity between nucleotide sequences. The homology search revealed that all the four amplified sequences 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*4A gene sequence from *B. thuringiensis*, *cry*4BLB gene sequences from *B. thuringiensis* serovar *israelensis* delta endotoxin gene of *B. thuringiensis* and other insecticidal toxin *cry* genes reported in NCBI biological databases. However the query coverage was low, ranging from 4 to 6 per cent for different sequences. This could be due to high amount of variability in the *cry*4 gene among different isolates. In the case of Bt-23 *cry*4, the sequence showed homology with *Bacillus cereus* genome also. This could probably be due to the genetic similarity between the two species, which has already been reported (Ash *et al*., 1991)

ORF analysis revealed that out of the five ORF in Bt-23*cry*4 sequence, three of them were located on the plus strands (+3, +2 and +2) and two were located on minus (-2, -2). The longest ORF was on +3 with a length of 405bp. In Bt-133*cry*4 sequence had only two reading frames. The longest one was reading frame -3 with length of 237bp. The longest ORF of Bt-190*cry*4 sequence was located on -1 with

285bp length and Bt-242*cry*4 sequence encoded the longest ORF on -3 reading frame with a length of 288bp.

The analysis for discovering nitrogen base composition revealed that all the *cry*4 sequences were rich in A+T. Johnson *et al.* (1996) made similar observations in which the cloned sequences of *cry* genes were rich in A+T compared to C+G bases. Phylogenetic analysis of the sequences showed all four isolates shared a common ancestor. Bt-23*cry*4 and Bt-242*cry*4 were clustered together, indicating strong phylogenic similarity.

Toxic effect of crystal protein is highly dependent upon the solubility of the crystal protein and membrane receptor-binding sites. The solubility is again based upon the amino acid residues present in the protein. In this study, amino acid analysis revealed that the hydrophobic amino acid leucine was highest (18.3%), followed by hydrophilic amino acid serine with 9.05 per cent in Bt-23*cry*4. Bt-133*cry*4 possessed a high molar percentage of the polar amino acid threonine (12.61). Analysis of Bt-190*cry*4 and Bt-242*cry*4 revealed, those were rich in non-polar amino acids leucine (26.72%) and isoleucine (12.07%).

5.10 Amplification of full length *cry*4A gene

5.10.1 Primer designing

Two oligonucleotide primers (Forward and reverse primers) were designed for full length *cry*4A gene based on already known *cry*4A type genes. Lin *et al*. (2008) designed forward and reverse primers for the full length *cry*2Ab gene from the earlier reported *cry*2Ab genes. The insecticidal crystal protein gene sequences provide the basis for the construction of gene specific primers to screen the *B. thuringiensis* isolates by PCR analysis for the presence of known nucleotide

sequences and characterizing insecticidal crystal proteins from new bacterial isolates (Perfontaine *et al.*, 1987). From this multiple sequence alignment the conserved regions were chosen to design primers. Specific primes were designed by various scientists for amplification of *cry* gene (Ceron *et al.*, 1995; Ben-Dov *et al.*, 1997; Juarez-perez *et al.*, 1997; Pinto and Fiuza, 2003; Beron *et al.*, 2004).

5.10.2 Polymerase chain reaction

An attempt was made to amplify the full length *cry*4 gene present in selected native isolates. However no product was obtained from PCR. Carozzi *et al.* (1991) earlier reported that only *cry*4A full length gene could be amplified using the specific primers from *B. thuringiensis* isolates. For the amplification of *cry*4B, a different set of primers was required. In the present study, since Blastn revealed homology with *cry*4A, *cry*4D and *cry*4BLB, it could be *cry*4B gene present in the test isolates.

In the present study, native isolates isolated from different parts of Western Ghats in Kerala were characterized at morphological, biochemical and molecular levels. *cry* profiling showed *cry*4 gene was the most predominant one in native Bt isolates, and some isolates contained *cry*11, *cry*10 and *cry*21 genes also. Insecticidal activity of the isolates was assessed against the dipteran model insect *Drosophila melanogaster.* Amplicons of *cry*4 genes from native isolates were cloned and sequenced. The sequenced nucleotides showed homology with *cry*4A gene sequences present in the NCBI nucleotide database. Oligonucleotide primers were designed to amplify full length *cry*4A gene but native Bt isolates failed to give full length amplification of *cry*4A gene. Further investigations are required for the amplification full length dipteran specific *cry* genes from native *Bacillus thuringiensis* isolates and their effectiveness against dipteran insects and pests.

Summary

6. Summary

The present investigation entitled ''Molecular cloning of *cry* genes specific to dipterans from native *Bacillus thuringiensis* Berliner'' was undertaken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, Thrissur, during the period 2007-2009. The salient features of the study are summarized below.

Twenty isolates of *B. thuringiensis* which were isolated from the Western Ghats of Kerala were used for the study and *B. thuringiensis* subsp. *israelensis* strain 4Q1 was used as the reference strain.

All the isolates were Gram positive as indicated by blue colour upon Gram staining and by the absence of viscous threads after KOH treatment. The cells were rod-shaped and arranged in chains. Crystal protein staining with Coomassie Brilliant blue revealed the presence of dark blue crystal protein inclusions. The shape of crystal protein in these isolates varied from spherical to irregular. The isolates Bt-105, Bt-170, Bt-206, Bt-245, Bt-326 and Bt-411 produced composite of spherical and irregular crystal proteins. Twelve isolates produced spherical and two isolates produced irregular of crystal proteins.

All the twenty native isolates were characterized by seven biochemical tests. None of the isolates produced neutral-alkaline metabolites in VP test. Twelve Bt isolates produced urease enzyme remaining eight isolates and 4Q1 not able to degrade urea. All the isolates hydrolyzed esculin, lecithin and starch but not gelatin.

Total DNA was isolated from *Bacillus thuringiensis* isolates following the procedure of Sambrook and Russel (2001). The concentration of DNA in the samples

varied from 391.0 ng/µl to 1974.9 ng/µl. The OD_{260}/OD_{280} values of DNA ranged between 1.84 and 2.05, indicating purity of the preparation.

Profiling of eight *cry* genes specific to dipteran insects by polymerase chain reaction indicated *cry*4 was predominant. Eleven native isolates possessed *cry*4 gene and *cry*10 was present in two isolates (Bt-419 and Bt-447). Three isolates contained *cry*11 and *cry*21 was present in only one isolate (Bt-419). *cry*2, *cry*16, *cry*17, *cry*19 genes were absent in native isolates and a combination of two dipteran-specific *cry* genes was present in four isolates (Bt-23, Bt-245, Bt-420, and Bt-447).

The insecticidal activity of selected *Bacillus thuringiensis* isolates was assessed by diet contamination method on *Drosophila melanogaster*, by incorporating crude crystal protein preparations in the diet, at five different concentrations.

The isolate 4Q1 recorded 100 per cent mortality within 24h at a spore concentration of 13.2 x 10^8 per ml. Among the native isolates, Bt-23 produced the highest mortality (88.8 per cent) and the lowest mortality of 79.3 per cent was recorded in Bt-242. The lowest LC_{50} value of 6.05 x 10^8 spores per ml was also recorded by 4Q1. Among the native isolates, Bt-23 recorded an LC_{50} value of 7.39 x 10^8 spores/ ml, indicating that the toxin was more potent.

Plasmid profiles developed for the selected isolates indicated presence of varying number of plasmids.

Polymerase chain reaction with *cry*4 specific primers yielded two amplicons of 400 and 800bp size in the isolates Bt-23, Bt-133, Bt-242 and one amplicon of 400bp in isolate Bt-190. Since Bt-23 was the most efficient in inducing mortality in

larvae of *Drosophila*, the 800p amplicon was clone in plasmid vector pGEMT. Sequence analysis revealed homology of the amplicon with *cry*4A, *cry*4D and *cry*4BLB genes in the NCBI database.

Amino acid statistics of the gene fragments revealed that Bt-23*cry*4 and Bt-133*cry*4 were rich in hydrophilic amino acids. Attempts to amplify the full length ORF of *cry*4 using specifically designed primers did not succeed.

Future research may be concentrated on finding out the efficacy of native *Bacillus thuringiensis* against agricultural pests belonging to the Order Diptera including fruit flies, leaf miners and blood sucking insects like mosquitoes. The full length dipteran specific *cry* genes from native *B. thuringiensis* could be cloned and expressed in *E. coli.* Insecticidal activity and level of gene expression could be assessed in both, before exploiting these for crop improvement through genetic engineering.

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* Originals not seen

Appendices

Appendix I

Composition of different media used in the study

1. Luria Bertani (LB) broth

Tryptone	-	10 g
Yeast Extract	-	5 g
NaCl	-	5 g
pH adjusted to	-	7± 0.2
Distilled water	-	to make up to 1000ml

2. Luria Bertani agar medium

Tryptone	-	10 g
Yeast Extract	-	5 g
NaCl	-	5 g
Agar	-	20g
pH adjusted to	-	7± 0.2
Distilled water	-	make up to 1000ml

3. T3 medium

Tryptone	-	3 g
Tryptose	-	2 g
Yeast extract	-	1.5 g
Sodium phosphate	-	1.2 g
pH adjusted to	-	6.8
Distilled water	-	make up to 1000ml

4. T3 agar medium

Tryptone	-	3 g
Tryptose	-	2 g
Yeast extract	-	1.5 g
Sodium phosphate	-	1.2 g
Agar	-	20 g
pH adjusted to	-	6.8
Distilled water	-	make up to 1000ml

5. Esculin agar medium

Casein enzymic hydrolysate	-	13 g
Sodium chloride	-	5 g
Yeast extract	-	5 g
Beef heart, infusion	-	2 g
Esculin	-	1 g
Ferric citrate	-	0.5 g
Agar	-	15 g
Distilled water	-	1000 ml
pH	-	7.3 - 7.5

6. Nutrient gelatin medium

Peptone	-	5 g
Beef extract	-	3 g
Gelatin	-	120 g
pH adjusted to	-	6.8
Distilled water	-	make up to 1000ml

7. Nutrient agar medium

Peptone	-	5 g
Beef extract	-	3 g
NaCl	-	3 g
Agar	-	15 g
pH adjusted to	-	7
Distilled water	-	make up to 1000ml

(Two egg yolks to be added into the medium under sterile conditions, after autoclaving, when the temperature is around 40 to 50^{0}C).

8. Starch agar medium

Peptone	-	5 g
Beef extract	-	3 g
Soluble starch	-	2 g
Agar	-	15 g
pH adjusted to	-	7
Distilled water	-	make up to 1000ml

9. Urea agar medium

Peptone	-	1 g
Glucose	-	1 g
Sodium chloride	-	5 g
Monopotassium phosphate	-	2 g
Phenol red (1.2%)	-	10 ml
Agar	-	20 g
pH adjusted to	-	6.8
Distilled water	-	make up to 1000ml

(40% urea to a final concentration of 50ml/l after autoclaving).

10. Voges Proskauer medium

Peptone	-	5 g
D- glucose	-	8 g
Disodium phosphate	-	5 g
pH adjusted to	-	6.9
Distilled water	-	make up to 1000ml

11. SOC medium

Tryptone	-	2 g
Yeast extract	-	0.5 g
1M NaCl	-	1 ml
1M KCl	-	0.25 ml
2M Mg^{+2} stock	-	1 ml
2M glucose	-	1 ml
pH adjusted to	-	6.9
Distilled water	-	make up to 1000ml

Both Mg^{+2} stock and glucose should be filter sterilized before adding to the media.

12. Ingredients for *Drosophila melanogaster* larval diet (1000 g of diet):

Wheat bran	-	133 g
Raw sugar	-	37 g
De-fatted soybean meal	-	29 g
Yeast	-	29 g
Coarse tissue paper	-	23 g
Sodium benzoate	-	1 g
HCl (3.3 per cent)	-	20 ml
Water	-	708 ml

Appendix II

I. Reagents used for Gram staining

1. Crystal violet

Solution A

Crystal violet (90%)	-	2 g
Ethyl alcohol (95%)	-	20 ml

Solution B

Ammonium oxalate	-	0.8 g
Distilled water	-	80 ml

Mix solution A & B

2. Gram's iodine

Iodine	-	1 g
Potassium iodide	-	2 g
Distilled water	-	300 ml

3. Ethyl alcohol

Ethyl alcohol (100%)	-	95 ml
Distilled water	-	5 ml

4. Safranine

Safranine O	-	0.25 ml
Ethyl alcohol	-	10 ml
Distilled water	-	100 ml

II. Reagent used for crystal protein staining

Coomassie Brilliant Blue (CBB)

CBB powder	-	0.25 g
Glacial acetic acid	-	7 ml
Absolute alcohol	-	50 ml
Distilled water	-	43 ml

III. Reagent used for VP test

1. Barrit's reagent A

Alpha naphthol	-	5 g
Absolute ethanol	-	95 ml

2. Barrit's reagent B

Potassium hydroxide	-	40 g
Creatine	-	0.3 g
Distilled water	-	100 ml

Appendix III

1. Reagent used for DNA isolation

1. 10mM Tris HCl

1M Tris HCl (pH - 8.0)	-	1ml
Distilled water	-	100 ml

2. 100mM NaCl

1M NaCl	-	10 ml
Distilled water	-	100 ml

3. TE buffer

(Tris HCl - 50mM; EDTA - 20mM)

Tris HCl - 0.05 M (pH - 8.0)	-	0.394 g
0.02M EDTA (pH - 8.0)	-	0.372 g
Distilled water	-	100 ml

4. Lysozyme stock

Lysozyme	-	50 mg
Distilled water	-	1 ml

Stock was prepared by dissolving 50 mg lyzozyme in 1 ml water and was stored under refrigerated conditions.

5. RNase A stock

RNase A	-	10 mg
Distilled water	-	1 ml

Stock was prepared by dissolving 10 mg RNase A in 1 ml water and was stored under refrigerated conditions at -20^0C.

6. 2 % SDS in TE buffer

SDS	-	2 g
TE buffer	-	100 ml

7. Proteinase K

Proteinase K	-	20 mg
Distilled water	-	1 ml

Stock was prepared by dissolving 20 mg Proteinase K in 1 ml water and stored under refrigerated conditions at -20^0C

8. Phenol: chloroform(1:1 v/v)

To 1 part of Tris saturated phenol, 1 part of chloroform was added.

9. Chloroform: isoamylalcohol (24:1 v/v)

To 24 parts of chloroform, 1 part of isoamylalcohol was added and mixed properly. The mixture was stored in refrigerator before use.

10. 3M Sodium acetate

Sodium acetate	-	20.412 g
Distilled water	-	50 ml

11. Chilled isopropanol

12. 70% ethylacohol

To 70 parts of absolute ethanol, 30 parts of double distilled water was added.

II. Reagent used for crystal protein harvesting

1. TE Buffer

10mM Tris HCl (pH 8.0)	-	10 ml
1mM EDTA (pH 8.0)	-	5 ml
Distilled water	-	1000 ml

Autoclaved and stored at room temperature

2. PMSF(Phenyl Methyl Sulfonyl Flouride)

PMSF	-	1.7 g
Propanol	-	100 ml

3. 0.5M NaCl

III. Reagents used for plasmid isolation

1. A. Solution I (SET)

Sucrose	-	25%
Tris HCl	-	50mM
EDTA	-	50mM
pH	-	8.0

B. 5mg of lysozyme per ml of SET

2. Solution II (Lysis buffer)

NaOH	-	0.2 N
SDS	-	1 %

3. Solution III

CH_3COOK	-	3M
pH	-	5.5

IV. Reagent used for competent cell preparation

Solution A

Ice - cold 100mM $CaCl_2$

Appendix IV

Buffers and dyes used in gel electrophoresis

1. 6x Loading/ tracking dye

Bromophenol blue	-	0.25%
Xylene cyanol	-	0.25%
Glycerol	-	30%

The dye was prepared and kept in fridge at 4^0C

2. Ethidium bromide (intercalating dye)

The dye was prepared as a stock solution of 10 mg/ ml in water and was stored at room temperature in a dark bottle.

3. 50x TAE buffer (pH 8.0)

Tris base	-	242.0 g
Glacial acetic acid	-	57.1 ml
0.5M EDTA (pH 8.0)	-	100 ml
Distilled water	-	1000 ml

The solution was prepared and stored at room temperature

Abstract

MOLECULAR CLONING OF *cry* GENES SPECIFIC TO DIPTERANS FROM NATIVE *Bacillus thuringiensis* BERLINER

By

M. SIVAJI

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the

requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

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ABSTRACT

The study entitled "Molecular cloning of *cry* genes specific to dipterans from native *Bacillus thuringiensis* Berliner" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2007- 2009.

The crystal proteins encoded by various *cry* genes present in *B. thuringiensis* exhibit insecticidal activity against members of the orders Lepidoptera, Diptera and Coleoptera. In the present study, an attempt was made to screen and clone dipteran specific *cry* genes from native isolates of *B. thuringiensis*.

Twenty *Bacillus thuringiensis* isolates obtained from different locations of the Western Ghats of Kerala and available in the repository at CPBMB were characterized by routine cultural, morphological and biochemical tests (Cappuccino and Sherman, 1992). Abundance of crystal protein produced during sporulation was assessed by staining. The specificity of Bt strain to a target insect depends on its *cry* gene content and the insecticidal property can be predicted by molecular tools like polymerase chain reaction (PCR). Profiling of eight dipteran-specific *cry* genes (*cry* 2, 4, 10, 11, 16, 17, 19 and 21) was carried out in twenty native Bt isolates using PCR with specific primers. *B. thuringiensis* subsp. *israelensis* strain 4Q1 served as positive control. Eleven native isolates contained *cry*4 gene and four isolates contained two different dipteran-specific genes. Based on the abundance of crystal protein and also the *cry* gene profile, four isolates were selected for further bioassay and molecular studies.

The insecticidal activity of the native Bt isolates was determined by bioassay against the dipteran insect *Drosophila melanogaster* (Vinegar fly). Crystal protein-spore suspension of the selected isolates (Bt-23, Bt-133, Bt-190 and Bt-242) and reference strain 4Q1 at five different spore concentrations were mixed in the semi-synthetic diet for third instar larvae. The percent mortality of larvae was directly proportional to the concentration of spores. 4Q1 was found to be highly toxic, with 100% mortality in 24h. Bt-23 recorded lowest LC_{50} value among the native isolates, indicating high toxicity of crystal protein.

The dipteran-specific *cry*4 gene was amplified by PCR in four selected isolates. The amplified PCR product 800bp of isolate Bt-23 was eluted and ligated into pGEMT vector further cloned in *E. coli* JM 109 strain and sequenced. Remaining three isolates of 400bp amplified fragment were eluted and purified product directly sequenced without cloning.

The gene sequences were analyzed by using various bioinformatics tools like clustalW, Blastn and ORF finder. All the sequenced gene fragments showed 100 per cent homology with *cry*4A gene. Forward and reverse primers were designed for *cry*4A full length gene and PCR amplification was done with the selected Bt isolates. But none of the isolates gave amplification for *cry*4A full length gene primer.

The present study was helpful in predicting the insecticidal activity of twenty native isolates of *B. thuringiensis*. Twelve of these isolates harboured at least one dipteran-specific gene. Bioassay of four selected isolates on *Drosophila melanogaster* revealed that Bt-23 was the best with the lowest LC_{50} value. Sequence analysis of the *cry*4 genes amplified through PCR revealed homology with *cry*4A accessions in the database. The isolate Bt-23 could be further exploited as a bioagent for controlling dipteran pests of crop plants.