Isolation and Identification of Cry protein producing species of *Bacillus* and characterization of their insecticidal properties

Thesis submitted in partial fulfillment
of the requirements for the
degree of Doctor of Philosophy in Biotechnology

by

Zothansanga

Ph.D. Registration No: MZU/Ph.D/222 of 28.11.2008

Under the Supervision of

Dr. N. Senthil Kumar
Professor
Department of Biotechnology

&

Joint- Supervision of

Dr. G. Gurusubramanian
Associate Professor
Department of Zoology

Department of Biotechnology
School of Life Sciences
Mizoram University
Aizawl, Mizoram

DECLARATION

I declare that the thesis entitled "**Isolation and Identification of Cry protein producing species of *Bacillus* and characterization of their insecticidal properties**" submitted to the Mizoram University for the award of degree of Doctor of Philosophy in Biotechnology is a bonafide record of work carried out by me during the period from 2008 to 2012 under the guidance of **Prof. N. Senthil Kumar** (Supervisor), Department of Biotechnology and **Dr. G. Gurusubramanian** (Co-Supervisor), Department of Zoology and has not formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles in this University or other University or institution of higher learning.

Signature of the Candidate

(ZOTHANSANGA)

ACKNOWLEDGEMENT

First of all, I would like to thank God who guides me from the start of my research till the competition. It's because of His blessings, strengthening me and giving me the sense of understanding.

I feel immense pleasure in expressing my thanks and heartfelt gratitude to Professor. N. Senthil Kumar, Head and supervisor, Department of Biotechnology, Mizoram University, Aizawl, Mizoram, for his painstaking guidance, assistance and direction in carrying out the experimental work.

I express my special thanks to my co supervisor Dr.G. Gurusubramanian, Associate Professor, Department of Zoology, Mizoram University for his valuable guidance and sharing his precious treasure of Knowledge for this work.

I earnest wish to express my sincere thanks to the faculties of the Department - Dr. Bhim Pratap Singh, Dr. Jyotirmoy Bhattacharya and Dr. Th. Robert Singh and Mrs. Brindha Senthil Kumar (Bioinformatics Lab) Mizoram University, Aizawl for their guidance which enhance me for learning.

I also extend my heartfelt thanks to my lab mate Rita Zomuanpuii, PC Lalrinfela, Moirangtheim Lakhsmi Pyari, Lalhmachhuani, Lalrotluanga, Lalremsiami hrahsel, Lalthapuii hauhnar, Catherine Vanlalruati, Isacc Laldikbera, Sangzuala sailo, A. Ibeyaima, Liansangmawii chhakchhuak, Ruth Lalfelpuii, Rebecca Laldinmawii hauhnar who spared their valuable time and gave me constant support and necessary help.

I would also like to thank my wife, my son and all of my family members for their constant support, assistance and encouragement.

Last, but not least, I thank UGC, New Delhi (Reference No. F. No. 34-452/2008 (SR) dt. 30 Dec 2008) for supporting research project and for the Meritorius fellowship. I also thank the Department of Biotechnology (DBT), New Delhi for the Bioinformatics Infrastructure Facility, State Biotech Hub and Sophisticated Analytical Intrumentation Facility, NEHU for providing me the necessary facilities for my research.

I am also very much indebted to Dr. Zeigler, Bacillus Genetic Stock Centre (BGSC), Ohio, USA for providing the Standard *Bacillus thuringiensis* strains.

Table of contents

Table of contents

1. INTRODUCTION AND REVIEW OF LITERATURE

Microorganisms being a pioneer colonizer on this planet have come to stay as a cosmopolitan conglomerate of highly compatible organisms. They are abounding in habitats with extremes of temperature, pH, water and salt stress. Bestowed with remarkable inherent physiological and functional diversity, microbes have found application in agriculture, industry, medicine and environment. Till now, efforts were made to tapping of microbial diversity, identification, evaluation, molecular characterization, bioprospecting and conserving them for various applications. So, the potential of microbes needs to be explored and investigated.

The competition for crops between human and insects is as old as agriculture. The use of chemical substances to control pests was started in the mid-1800s. Early insecticides were inorganic chemicals and organic arsenic compounds followed by Organochloride compounds, organophosphates, carbamates, pyrethroids and formamides. Many of these chemicals are also being used today. Certain properties made these chemicals useful, such as long residual action and toxicity to a wide spectrum of organisms. However, chemical pesticide applications have caused many environmental problems including insect resistance, toxicity to humans and to beneficial insects (Glazer and Nikaido, 1995). Like all organisms, insects are susceptible to infection by pathogenic microorganisms. Many of these infectious agents have a narrow host range and, therefore, do not cause uncontrolled destruction of beneficial insects and are not toxic to vertebrates.

Insect pathogenic bacteria, viruses, fungi and nematodes have shown potentials as inundative biocontrol agents. Viruses are highly selective control agents, but as mass production depends on living hosts, few products are available in the market. Fungi can be useful pathogens but their dependency on specific environmental conditions severely limits their applicability. Though bacteria are the most successful group of insect pathogens from a practical and commercial point of view, the number of species so far proven to be useful for biological control is surprisingly small.

The Genus *Bacillus*

Kingdom: Eubacteria
Domain: Bacteria
Phylum: Firmicutes
Class : Bacilli
Order : Bacillales
Family: Bacillaceae
Genus: *Bacillus*
Species: *B. thuringiensis* (Berliner 1915)

Bacillus is a genus of Gram-positive, rod-shaped bacteria and a member of the phylum firmicutes (Cohn, 1872). *Bacillus* species can be obligate aerobes or facultative anaerobes. Ubiquitous in nature, *Bacillus* includes both free-living and pathogenic species. Under stressful environmental conditions, the cells produce oval endospores that can stay dormant for extended periods (Madigan, 2005).

Vegetative cells of certain other organisms, notably of the genera *Bacillus* and *Clostridium,* can undergo a special, unequal type of binary fission where – instead of one cell dividing into two separate cells – one of the cells forms inside the other

("parent") cell. This special kind of cell is called an endospore, and it possesses several resistant outer layers and the internal features of a vegetative cell, except for being almost completely devoid of water. When the "parent cell" lyses, the endospore becomes "free" and can remain viable for extended periods of time. Endospores are metabolically inert and can withstand a wider variety of deleterious conditions (such as radiation, abrasion, extremes of heat and cold, and lack of nutrients and water) than reproductive spores (Sneath,1986). Like a reproductive spore, an endospore will germinate and form a vegetative cell when favorable conditions return for the growth of these cells, and generations of vegetative cells will again thrive as long as the appropriate nutrients and environmental conditions are present. When the nutrients gets depleted, endospores are again produced; this can be seen to happen on artificial media as well as in the normal habitat (Prescott, 2002).

Bacillus species are widely distributed in nature, particularity in soil from where they are spread in dust, in water and on animal or plant materials. The majority of *Bacillus* species are non pathogenic and rarely are associated with disease in humans and lower animals. Exception include *Bacillus anthracis* - the causative agent of anthrax; *Bacillus cereus* - the causative agent of human food poisoning ;*Bacillus popillae* and *Bacillus thuringiensis* - known pathogen of specific insect groups.

Bacteria belonging to the genus *Bacillus* have a long and distinguished history in the realms of Biotechnology. Manufacturer of extra cellular amylase and protease for industrial application began early this century but significant production and use was delayed until after 1950 when advantage of including the alkaline protease (subtilisin

Carlsberg) of *Bacillus licheniformis* in washing detergent was realized. This was followed by the development in the starch processing industry based on the- amylase from *Bacillus licheniformis* particularly the conversion of starch to high fructose corn syrups as sucrose replacement in food and beverages . The *Bacillus* includes very versatile bacteria and the most effective biocontrol agent for various insects pest. *Bacilli* are also good sources of numerous antibiotics, flavor enhancer such as purine nucleosides, surfactants and various other products (Priest, 1988). Strains of *Bacillus* were found to produce mixture of lactic, isovaleric, isobutyric and acetic acids. Other organic acids, such as glycolic, oxalic, malonic and succinic acid have also been identified among phosphate solubilizers. Strains from the genera *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers (Rodriguez *et al*., 1999). Three species of spore forming *Bacillus* - *Bacillus thuringiensis, Bacillus sphearicus* and *Bacillus popilliae* are of practical importance, with *Bacillus thuringiensis (Bt)* being of prime importance due to the production of crystal proteins (cry proteins) encoded by cry genes (Entwistle *et al*., 1993).

Since World War II, insect disease control methods have relied heavily on broad spectrum synthetic chemical insecticides to reduce vector populations. However, synthetic chemical insecticides are being phased out in many countries due to insecticide resistance in mosquito populations. Furthermore, many governments have restricted chemical insecticide use due to their environmental effects on non-target beneficial insects and, especially, on vertebrates through contamination of food and water. To counteract this contamination, attention and efforts were directed to the use of

biological control agents including insect pathogens. As a result, the use of biopesticide, as a component of integrated pest management (IPM), has been gaining acceptance over the world. However, an entomopathogenic organism must fulfill several requisites before being released to the environment as a potential control agent. It should be highly specific and effective against the target pest. The organism should demonstrate the potential to be successfully processed by continuous production technology. The control agent should be available in formulations with a reasonable shelf life, should be stable, and should be harmless to human and non-target flora and fauna (Sansinenea, 2012).

As an entomopathogenic organism, *Bacillus thuringiensis* (*Bt*) fulfills all these requirements. *Bt* has been used as a biopesticide in agriculture, forestry and mosquito control. Its advantages are specific toxicity against target insects, lack of polluting residues and safety to non-target organisms such as mammals, birds, amphibians and reptiles. Although several proteins and other compounds produced by *Bt* contribute to its insecticidal activity, by far the most important components are the proteins that form parasporal crystalline inclusions during sporulation (Sansinenea, 2012). Transgenic crops based on insecticidal crystal proteins of Bt are now an international industry with revenues of several billion dollars per year.

Bt was first discovered in Japan as the causal agent of sotto disease in silkworms (*Bombyx mori*) larvae and was named as Sottokin, which means “sudden death *bacillus*” (Ishiwata, 1901). He also described the pathology and cultural

characteristics in silkworm larvae and it was observed that many of the larvae that did not die, when exposed to the *bacillus,* were very weak and stunted. Further, he stated that "From these experiments the intoxication seems to be caused by some toxine, not only because of the alimentation of *bacillus*, but the death occurs before the multiplication of the *bacillus*…" (Ishiwata, 1905). This showed that from the very beginning it was realized that a toxin was involved in the pathogenicity of *Bt*. The first morphologically valid description was made by the German bacteriologist Ernst Berliner, who isolated the *bacillus* from the Mediterranean flour moth (*Anagasta kuehniella*) (Berliner,1915). He named it *Bacillus thuringiensis* (*Bt*), which is derived from Thuringia, the German town where the moth was found.

Bt was first to be used as an insecticide in the 1950s in the USA. The first commercial name was Thurincide, which was prepared from *B. thuringiensis* subsp. k*urstaki* (Beegle and Yamamoto, 1992). Dulmage discovered more active *B. thuringiensis* var. *kurstaki* (HD1), which was commercialized in the USA as Dipel (Glazer and Nikaido, 1995). The demand of *Bt* based insecticides in agriculture sector declined, in the mid 1970s, because of more effective chemical pesticides. In the 1980s, *B. thuringiensis* research was stimulated by progress in biotechnology. First, Schnepf and Whiteley (1981) cloned a crystal toxin gene from *B. thuringiensis* subsp. k*urstaki* into *E. coli*, since then much research has been performed to improve target spectra and to find out more infectious strains of *B. thuringiensis.*

General Characteristics of *Bacillus thuringiensis*

Bt is a Gram-positive rod which varies 3-5 μm width in size, makes a dormant spore which are resistant to inactivation by heat, desiccation and organic solvents. The spore formation of the organism varies from terminal to subterminal in sporangia that are not swollen, therefore, *Bt* resembles other *Bacillus* species in morphology and shape (Stahly *et al.,* 1991). When nutrients and environmental conditions are sufficient for growth, the spore germinates producing a vegetative cell that grows and reproduces by binary fission. Cells continue to multiply until one or more nutrients, such as sugars, amino acids, or oxygen, become insufficient for continued vegetative growth. Under these conditions, the bacterium sporulates producing a spore and parasporal body, the latter, as noted above, composed primarily of one or more insecticidal proteins in the form of crystalline inclusions (Sansinenea, 2012). This is the most distinguishing feature of *Bt* from closely related bacillus species (e.g. *B. cereus, B. anthracis*) is the presence of a parasporal crystal body that is near to the spore, outside the exosporangium during the endospore formation (Figure 1) (Andrews *et al*., 1987; Bulla *et al*.,1995). These parasporal inclusions contain proteins, called δ-endotoxins (Heimpel, 1967). This observation led to the development of bio-insecticides based on *Bt* for the control of certain insect species among the orders Lepidoptera, Diptera, and Coleoptera (Bernhard *et al*., 1997). There are more recent reports of *Bt* isolates active against other insect orders (Hymenoptera, Homoptera, Orthoptera, and Mallophaga) and against nematodes, mites, and protozoa (Feitelson, 1993).

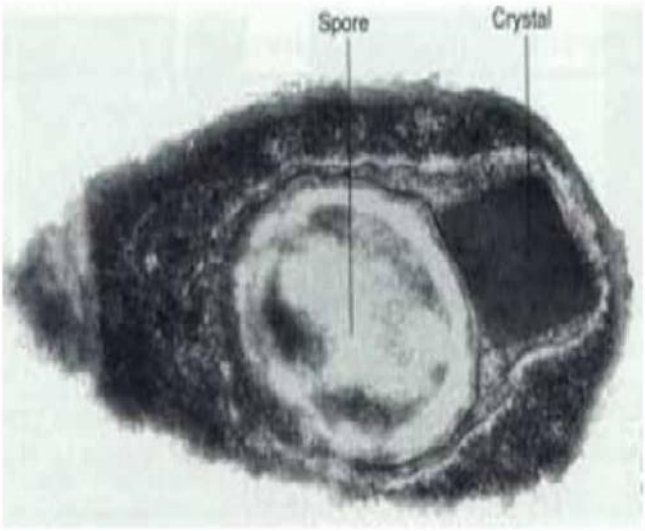


Figure 1. Formation of the toxic parasporal crystal in *B. thuringiensis* (Madigan *et al.*, 2000, Brock Biology of Microorganisms, Chapter 12, pp 509).

The parasporal inclusions are commonly referred as insecticidal crystal proteins (ICP), or δ-endotoxins, which are selectively toxic to different species of several invertebrate phyla. ICPs include the more prevalent Cry (crystal) proteins, as well as the Cyt (cytolytic) proteins produced by some *Bt* strains. A strong correlation between crystal morphology, ICP composition, and bioactivity against target insects has been established (WHO, 1999).

Morphological Properties of *Bacillus thuringiensis*

Colony morphology can help to distinguish *B. thuringiensis* colonies from other *Bacillus* species. The organism forms white, rough colonies, which spread out and can expand over the plate very quickly. *B. thuringiensis* strains have unswollen and ellipsoidal spores that lie in the sub terminal position. The presence of parasporal crystals that are adjacent to the spore in the mother cell is the best criteria to distinguish *Bt* from other closely related *Bacillus* species. The morphology, size, and number of parasporal inclusions may vary among *Bt* strains. However, four distinct crystal morphologies are apparent: the typical bipyramidal crystal, related to Cry 1 proteins (Aronson *et al*., 1976); cuboidal inclusions related to Cry 2 proteins and usually associated with bipyramidal crystals (Ohba and Aizawa, 1986); amorphous and composite crystals related to Cry 4 and Cyt proteins (Federici *et al*., 1990); and flat, square crystals, related to Cry 3 proteins (Herrnstand *et al*., 1986; Lopez-Meza and Ibarra, 1996). Spherical and irregular pointed crystal morphologies can also be observed in *B. thuringiensis* strains. There is a relationship between toxic activity and

crystal shape, so that the observation of crystal morphology by phase contrast microscopy can provide important clues. For instance, Maeda *et al*. (2000) collected 22 isolates of *B. thuringiensis* from marine sediments in Japan. Two isolates of *B. thuringiensis* subsp. *kurstaki,* which are toxic to lepidopteran larvae, formed typically bipyramidal inclusions, whereas isolate *higo*, which is toxic to mosquitoes, formed spherical crystals. The observation of crystal morphology is the first step for establishing *B. thuringiensis* strain collections. Ohba and Aizawa (1986) isolated 189 isolates of *B. thuringiensis* from 136 soil samples from nonagricultural areas of Japan. The classification was based in part on the possession of parasporal bodies. Bernhard *et al*. (1997) isolated 5303 *Bt* from 80 different countries and 2793 of them were classified as bipyramidal shaped crystals (45.9%), spherical (14%) and rectangular (4 %).

Ecology and Prevalence of *B. thuringiensis*

Bt occurs naturally and it can also be added to an ecosystem artificially to achieve insect control. For this reason, the prevalence of *Bt* in nature can be defined as "natural" and "artificial". The habitat is considered as natural, where *Bt* can be isolated, when there is no previous record of application of the organism for insect control. The artificial habitats are areas sprayed with *Bt* based insecticides (usually a mixture of spores and crystals) (Stahly *et al*., 1991).

Bt is indigenous to many environments including soil worldwide **(**Martin and Travers, 1989; Bernhard *et al*., 1997**),** insect cadavers (Carozzi *et al*., 1991; Kaelin *et*

al., 1994; Itaqou-Apoyolo *et al*., 1995; Lopez-Meza and Ibarra, 1996; Cadavos *et al*., 2001), stored product dust (Chambers *et al*., 1991; Meadows *et al*., 1992; Hongyu *et al*., 2000), leaves of plants (Smith and Couche, 1991; Bel *et al*., 1997; Mizuki *et al*., 1999), and aquatic environments (Iriarte *et al*., 2000; Ichimatsu *et al*., 2000). Moreover, *Bt* has recently been isolated from marine sediments (Maeda *et al*., 2000), and also from the soils of Antarctica (Forsty and Logan, 2000). Thus, it is obvious that *Bt* is widespread in nature. *B. thuringiensis* accounts for about 5-8% of *Bacillus* spp. population in the environment (Hastowo *et al.,* 1992).

Toxicity and Mode of Action of Its Insecticidal Proteins

The parasporal crystals of *B. thuringiensis* contain the ICPs in the form of protoxins. After ingestion of parasporal crystals by the susceptible insect, the crystals are dissolved in alkaline conditions (pH 10-12) in the insect mid-gut, generating 130 to 135 kDa protein chains called protoxin. These proteins are then processed to the actual toxic fragments of 60-65 kDa by the gut proteases (Gill *et al*., 1992; Höfte and Whiteley, 1989; Knowles, 1994). Finally, these activated toxins bind to specific receptors present in the larval mid-gut epithelia to the specific receptors on the cell membrane creating ion channels or pores. The pore formation causes osmotic shock. As a result of this process, the cell membrane lyses, paralysis occurs and consequently, the insect stops feeding and dies from starvation (Knowles, 1994).

Individual Cry toxin has a defined spectrum of insecticidal activity, usually restricted to a few species in one particular order of Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes), Coleoptera (beetles and weevils), Hymenoptera (wasps and bees), and nematodes, respectively (de Maagd *et al*., 2001). A few toxins have an activity spectrum that spans two or three insect orders due to the combination of toxins in a given strain. The Cry proteins comprise at least 50 subgroups with more than 200 members (Bravo *et al*., 2007) and the toxins are classified only on the basis of amino acid sequence homology, where each protoxin acquired a name consisting of the mnemonic Cry (or Cyt) and four hierarchical ranks consisting of numbers, depending on its place in a phylogenetic tree (Crickmore *et al*., 2010). The members belong to a three-domain family, and the larger group of Cry proteins is globular molecules with three structural domains connected by single linkers. The protoxins are characteristic of this family and have two different lengths. The C-terminal extension found in the long protoxins is necessary for toxicity and is believed to play a role in the formation of the crystal within the bacterium (de Maagd *et al*., 2001).

Endotoxin crystals must be ingested to have an effect. This is the reason sucking insects and other invertebrates such as spiders and mites are not sensitive to Cry proteins used in *Bt* insecticides or *Bt* crops. Their mode of action involves several events that must be completed several hours after ingestion in order to lead to insect death. Following ingestion, the crystals are solubilized by the alkaline conditions in the insect midgut and are subsequently proteolytically converted into a toxic core fragment (de Maagd *et al*., 2003). Under the highly acidic conditions in stomachs of many

vertebrates, including humans, Cry and Cyt protein crystals may dissolve, but once in solution they are rapidly degraded to non-toxic peptides by gastric juices, typically in less than 2 min. During proteolytic activation, peptides from the N terminus and C terminus are cleaved from the full protein. Activated toxin binds to receptors (glycoprotein or glycolipid) located on the apical microvillus membranes of epithelial midgut cells (Griffiths *et al*., 2005). For some toxins, at least four different binding sites have been described in different lepidopteran insects: a cadherin-like protein (CADR), a glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP) and a 270 kDa glycoconjugate (Lee *et al*., 1996; Jurat-Fuentes and Adang, 2004). After binding, toxin adopts a conformation allowing its insertion into the cell membrane and form a cation-selective channel. Subsequently, oligomerization occurs, and this oligomer forms a pore or ion channel induced by an increase in cationic permeability within the functional receptors contained on the brush borders membranes (Bravo *et al*., 2004). The mode of action of *Bt* can be summarized in the following stages (Schnepf *et al*., 1998) (Figure 2):

(1) Proteins are produced by *Bt* as protoxins encapsulated in crystalline inclusion bodies;

(2) The crystals dissolve in the alkaline environment of the insect midgut and the protoxins are digested by specific proteases leading to activated toxins;

(3) Activated toxins bind specific receptors on the insect midgut;

(4) Receptor-bound toxins undergo conformational changes−in some cases resulting in oligomeric structures that are able to modify the target membrane; and

(5) The protein alters the structural stability of the membrane producing channels or pores that alter the membrane potential leading to cell lysis and insect death.

GENETICS AND MOLECULAR BIOLOGY

Genome

B. thuringiensis strains have a genome size of about 2.4 to 5.7 Mb (Carlson *et al*., 1994). A physical map has been constructed for two *B. thuringiensis* (Carlson and Kolstø, 1993, Carlson *et al*., 1996). Most *B. thuringiensis* isolates have several extrachromosomal elements (plasmids) ranging in size from 2 to >200 kb, some of them circular and others linear. The parasporal crystal proteins are generally encoded by large plasmids. Sequence hybridizing studies with *cry* gene probes have been shown that *cry* genes are also found in the bacterial chromosome (Carlson *et al*., 1994). *B. thuringiensis* also contains large variety of transposable elements which are thought to be involved in the amplification of the *cry* genes in the bacterial cell (Mahillon *et al.,* 1994). Another possible role of these elements could be mediating the transfer of plasmid by a conduction process involving the formations of co-integrate structures between self-conjugative plasmids and chromosomal DNA or non-conjugative plasmids. The last function of these elements may be the horizontal dissemination of genetic material, including *cry* genes, within *B. cereus* and *B. thuringiensis* species (Schnepf *et al*., 1998).

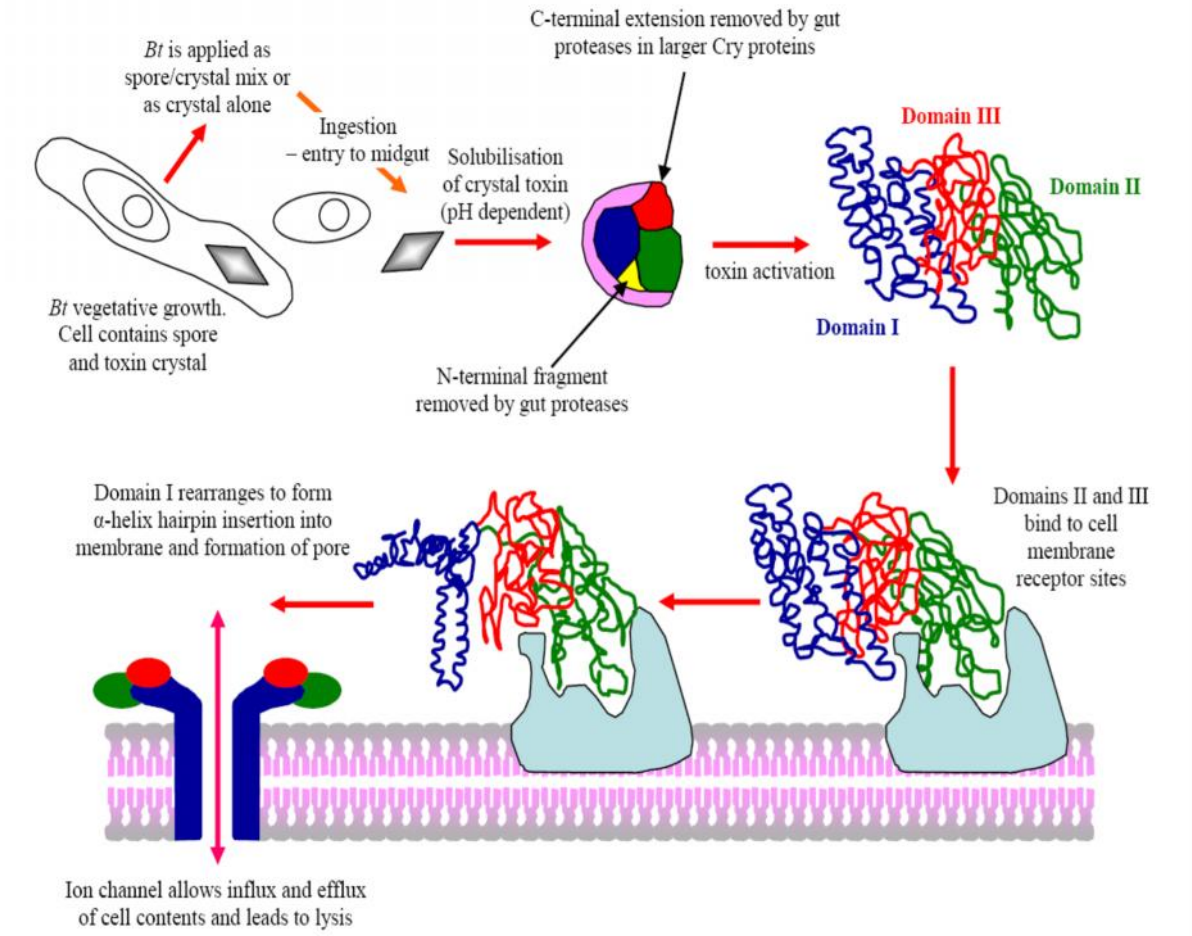


Figure 2. Mode of action of *B. thuringiensis* (de Maard et al., 2001)

cry Genes

The genes coding for the insecticidal crystal proteins are normally associated with plasmid of large molecular mass (Gonzales and Carlton, 1980). Many Cry protein genes have been cloned, sequenced, and named *cry* and *cyt* genes. Each type of crystal protein has a specific host range, and based upon differences in sequence and specificity, Cry toxins are classified by their primary amino acid sequence and more than 500 different cry gene sequences have been classified into 67 groups (Cry1-Cry67) (Crickmore *et al*., 2010). For example, the proteins toxic for lepidopteran insects belong to the Cry 1, Cry 2, and Cry 9 groups. The toxins against coleopteran insects are the Cry 3, Cry 7, and Cry 8 proteins and Cry1Ia1, which is a subgroup of Cry 1 proteins. The Cry 5, Cry12, Cry 13 and Cry 14 proteins are nematocidal, and the Cry 2Aa1, which is a subgroup of Cry 2 proteins, Cry 4, Cry 10, Cry 11, Cry 16, Cry 17, Cry 19, and Cyt proteins are toxic to dipteran insects (Zeigler, 1999).

Each of the *Bt* strains can carry one or more crystal toxin genes, and therefore, strains of the organism may synthesize one or more crystal protein. Transfer of plasmids among *Bt* strains is the main mechanism for generating diversity in toxin genes (Thomas *et al*., 2001). The remarkable diversity of *Bt* strains and toxins is due at least in part to a high degree of genetic plasticity. The genes encoding the crystal proteins are found (often clustered) on transmissible plasmids and flanking transposable elements, explaining how they can spread easily within the species (Schnepf *et al.,* 1998).

cry Gene Expression

The insecticidal crystal proteins are synthesized during the stationary phase of the bacterial life cycle growth. These proteins generally accumulate in the mother cell. The dry weight of the proteins account for up to 25% in sporulated cells of *B. thuringiensis*. The high level of crystal protein synthesis in *B. thuringiensis* is controlled by a variety of mechanisms. These mechanisms may occur at the transcriptional, post-transcriptional and post-translational levels (Agassie and Lereclus, 1995). The sporulation-specific genes controls *cry* gene expression. However, some of *cry* gene expression occurs during the vegetative growth. Thus, the expressions of cry gene mechanisms have been grouped in two groups, sporulation-dependent and sporulation independent. The *cry* 1Aa gene, encoding toxins active against lepidoptera, is a typical example of a sporulation-dependent *cry* gene. This gene is only expressed during the sporulation phase. On the other hand, *cry* 3Aa gene, isolated from the coloepteran-active *B. thuringiensis* var. *tenebrionis*, is expressed during the vegetative growth and also during the stationary phase. In the stationary phase, the expression of this gene has been found to be less than the vegetative phase (Sekar, 1988; De Souza *et al*., 1993).

Taxonomy

The taxonomy of *Bt is* based on morphological and biochemical characteristics. The classification of *Bt* subspecies based on the serological analysis of the flagella (H) antigens was introduced in the early 1960s (de Barjac and Bonnefoi, 1962). This

classification by serotype has been supplemented by morphological and biochemical criteria (de Barjac, 1981). Until 1977, only 13 *Bt* subspecies was described, and at that time all subspecies were toxic to Lepidopteran larvae only. The discovery of other subspecies toxic to Diptera (Goldberg and Margalit, 1977), Coleoptera (Krieg *et al*., 1983) and apparently Nematode (Narva *et al*., 1991) enlarged the host range and markedly increased the number of subspecies. Up to the end of 1998, over 67 subspecies based on flagellar H-serovars had been identified. Updated lists of the serovarieties can be obtained from the reference centre of the Pasteur Institute. Crystal serology has shown that a particular crystal type may be produced by more than one H-serovar (Krywienczyk *et al*., 1978; Smith, 1987).

Molecular Characterization of *B. thuringiensis*

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE describes a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of the length of a polypeptide chain and its charge) and no other physical feature. SDS is an anionic detergent applied to protein sample to linearize proteins and to impart a negative charge to linearized proteins. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Characterization by SDS-PAGE can reveal the protein profile of cry toxin and ultimately the insecticidal activity of the *Bt* can be established by bioassay (Xavier *et al*., 2007).

Determination of the compostion and toxicity of the parasporal crystals, by means of SDS – PAGE analysis and bioassay, is a useful complement for gene identification (Hiren *et al*.,2009). The characterization of native *B. thuringiensis* strains helps in understanding the role of *B. thuringiensis* in the native environment and distribution of *cry* genes in local conditions (Ben-Dov *et al*., 1997; Bernhard, 1997)The intensity of bands were very high in the isolates which showed higher mortality whereas it was less intense in the case of isolates showed lower mortality (Allwyn *et al*.,2007). Protein profile of whole cell can differentiate organisms up to species level, but for *Bacillus* species it can differentiate up to subspecies level (Berber, 2004).

POLYMERASE CHAIN REACTION (PCR)

PCR is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Developed in 1983 by Kary Mullis, PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications (Bartlett and Stirling, 2003). These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases (Saiki *et al*., 1988). An important aspects for establishing *B. thuringiensis* strain collections is to have a method which allows for rapid and exact characterization. Many methods have been described for characterization of *B. thuringiensis* strains, such as bioassay, serotyping (De Barjac and Franchon, 1990),

southern blot analysis for search of known homologous genes (Kronstad and Whiteley, 1986), analysis for reactivity to different monoclonal antibodies (Höfte *et al.,* 1988), and electrophoretic analysis of PCR products using specific primers (Carozzi *et al.,* 1991). Analysis of δ-endotoxin genes by bioassays is an exhaustive and time consuming process, since it is necessary to screen all target insect isolates. Serotyping is also an impractical method; it does not reflect the specific *cry* gene classes of strains. The main disadvantage of the analysis of reactivity to different monoclonal antibodies is cross-reaction. Because of this, Polymerase Chain Reaction (PCR) is the best alternative of such methods. PCR has also been exploited to predict insecticidal activities of *B. thuringiensis* strains, to determine the distribution of *cry* genes, and to detect new genes. Carozzi *et al.* (1991) reported the sequence of twelve PCR primers, which distinguish three major classes of *cry* genes (*cry* I, *cry* III, and *cry* IV). These primers were exploited to predict insecticidal activities of *B. thuringiensis* strains collected from soil samples and insect cadavers.

Each *cry* gene group is divided into different subgroups, which can show toxic effect to different species of an insect order. Multiplex-PCR method has been used to detect subgroups of a cry gene family (Ben-Dov *et al*., 1999). This method is based on two sets of primers. The first set of primers, the universal primer set is applied to detect the related *cry* gene family, and the other set of primer called the specific primer set is used for the detection of the subgroup of the *cry* gene family. The universal primers are chosen from highly conserved regions of a *cry* gene family. The specific primers, on the other hand, are designed from variable regions of these gene family sequences. For

instance, Ceron *et al*. (1994, 1995) exploited this method to detect subgroups of *cry* I and *cry* III genes from soil collected *B. thuringiensis* strains. Detection of novel *cry* genes has also been achieved by this method. For example, Juarez-Perez *et al*. (1997) applied PCR technology to detect new *cry* genes.

RAPD-PCR

Random amplification of polymorphic DNA - Polymerase Chain Reaction (RAPD-PCR) is one of the marker techniques widely used in genome characterization using a single primer which is able to anneal and prime at multiple location throughout the genome producing a spectrum of polymorphic amplification products. The advantage of RAPD is that no prior knowledge of the genome is necessary (Williams *et al*., 1990. Welsh *et al*., 1990). The random amplified polymorphic DNA (RAPD) technique based on polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers (Cocconcelli *et al.,* 1995).

H-serotyping has been used for classification and identification of *Bt* varieties, but the ‘‘auto-agglutinated’’ *Bt* strains and non-motile strains are not type-able by this H-serotyping method (Sadder *et al.,* 2006). As a result, molecular typing methods such as random amplified polymorphic DNA (RAPD) analysis have been applied to differentiate isolates of *Bt* strains (Hansen *et al.*, 1998). Konecka *et al.* (2007) used RAPD analysis to estimate phylogenetic relationships among twelve *Bacillus thuringiensis* strains

isolated from intestinal tracts of *Cydia pomonella* larvae, indicating a tendency of bacterial strains to cluster according to their source.

16s rDNA

The taxonomy of B. thuringiensis has been the subject of studies since the late 1950s. Methods of identifying B. thuringiensis varieties were proposed, based on morphological and biochemical characterization, using conventional microbiological techniques (Heimpel and Angus 1958). H-serotyping, the immunological reaction test to the fagellum antigen (de Barjac and Bonnefoi 1962, 1973), was also developed early and is still widely accepted for classification and identify cation of B. thuringiensis varieties (de Barjac and Frachon 1990; Lecadet *et al.* 1999). Isolates within the serotype, however, are not always the same in terms of their biochemical characteristics, plasmid patterns and host range insecticidal activity. For specifc purposes, other methods, such as plasmid pattern analysis (Lereclus *et al.,* 1982), immunological tests using monoclonal antibody against crystal proteins (Lynch and Baumann, 1985) and identification of the crystal protein gene using specific DNA probes (Visser, 1989), were introduced.

16S ribosomal RNA (or 16S rRNA) is a component of the 30S small subunit of prokaryotic ribosomes. It is approximately 1.5kb (or 1500 nucleotides) in length (greengenes.16sRNA data base). The genes coding for it are referred to as 16S rDNA. Multiple sequences of 16S rRNA can exist within a single bacterium (Case *et al*., 2007).

The 16SrRNA gene is used for phylogenetic studies (Weisburg *et al*., 1991) as it is highly conserved between different species of bacteria and archaea (Coenye *et al*., 2003). PCR primers are used to amplify the 16SrRNA gene providing the phylogenetic information, the most common universal primer pair was devised by Weisburg *et al.* (1991). 16S rRNA gene analysis could be used for novel *B. thuringiensis* strains, allowing them not only to be grouped but also to be positioned on the phylogenetic tree. (Joung and Cote, 2001). 16S rRNA based - molecular genotyping methods, (ribotyping), has proved to be very effective as a molecular taxonomic tool for estimating chromosomal genetic diversity and relationships among various bacterial species and subspecies (Saunders et al. 1988; Grimont and Grimont 1991;).

Scenario of *Bacillus thuringiensis* in Mizoram

Mizoram (21° 57' - 24° 30' North and 92° 15' - 93° 26' East) is a part of the 34 mega-biodiversity hotspots of the world (Indo- Burma global biodiversity hotspot). The entire state is hilly and mountainous and the altitude, temperature and annual rainfall range from 500 to 2157 m, from 7° to 34°C and 2,000 to 4,000 mm, respectively (Myers *et al*., 2000) which gives unique geographical features and abundant biological resources to ecologically study the distribution of this organism in the soil . It is necessary to search for natural strains and toxins in Mizoram (Northeast India), since a significant number of pests are not controlled with the available pest management programs.

The ecological distribution of this bacterium in Mizoram soil remains unexplored and in the present study, *Bt* was isolated from different soils to know the geographical diversity with insecticidal activity and further assess the molecular polymorphism among the isolates. Hence, the present study was carried out to explore diversity of *Bt* present in soil samples of eight districts in Mizoram state, India, in terms of i) the collection, isolation and distribution of *Bt* from soils of different habitats from Mizoram; ii) identification of types of crystal inclusion bodies and biochemical typing; iii) growth curve studies; iv) Larvicidal toxicity; v) Cry protein and gene profiling vi) RAPD-PCR and vi)16s rRNA gene sequencing to identify their phylogenetic relationship.

2. OBJECTIVES OF THE STUDY

i) To study the distribution of *Bacillus sp.* and isolation of *B. thuringiensis (Bt)* from agricultural, forest and urban soils of Mizoram.

ii) To assess the diversity of *Bt* isolates by morphological, physiological and biochemical characterization.

iii) To test the insecticidal activity of *Bt* isolates against Dipterans and Lepidopteran insects.

iv) *Cry* gene and protein profiling of *Bt strains* for identification of different pathotypes.

v) Molecular characterization of pathogenic *Bt* strains using RAPD-PCR.

vi) 16s rRNA gene sequencing of selected *Bt* strains to identify their phylogenetic relationship.

3. MATERIALS AND METHOD

3.1 Soil Sample Collection

A total of 428 soil samples from five different habitats [forest, agriculture land (garden, paddy, banana, teak and mulberry), aquatic (river, pond, lake), fallow, and shifting cultivation (Jhum)] from thirty nine locations covering eight districts (Champhai, Lunglei, Mamit, Kolasib, Aizawl, Serchhip, Saiha, Lawngtlai) – geographic coordinates 21° 57' - 24° 30' North and 92° 15' - 93° 26' East) in Mizoram, Northeast India (Figure 3) were used for isolation of *Bt*. Till date, no commercial *Bt* based product had been used in any of the sampled areas. All the soil samples (200 g) were collected aseptically from top to a depth of 10 cm after scrapping off the surface material with a sterile spatula and placed immediately inside the sterile polythene covers (Travers *et al*., 1987). Labels containing the details on date of collection, place of collection, collector's name, description of the place of collection and the agro climatic zone in which the sampling was carried out, were written using a permanent marker and placed inside the polythene cover and secured properly. Samples were stored at room temperature and processed within one week from the date of collection.

3.2 Isolation of *Bacillus sps* and *B. thuringiensis* from soil samples

One gram of soil sample in 10 ml of sterile de-ionised distilled water were pasteurized at 60°C for 1 h. Plated on nutrient agar (NA). Following incubation at 30°C

for 5 days, flat white matt colonies (characteristic of *Bacillus* spp.) were sub-cultured to NA slopes for identification (Chilcott and Wigley, 1993).

Isolation of *B. thuringiensis* strains was conducted according to the method described by (Travers *et al*., 1987). One gram of soil sample was suspended in 10 ml of Luria's broth buffered with 0.25M sodium acetate (pH: 6.8) and incubated for 4 hours. The acetate delays the germination of *B. thuringiensis* spores. After incubation, the sample was subjected to heat treatment at 80^0C for 3 min. Heat-treat treatment was given at the end of 4 hours incubation to kill other species of *Bacillus* and other bacteria that would germinate ahead of *B. thuringiensis* spores. Serial dilutions (up to 10^{-4}) were made and 20µl of each serial dilute was spread on LB agar and incubated at 37^0C overnight in a bacteriological incubator. The colonies resembling *Bt* (cream-colored and have the appearance of a fried egg on a plate) were selected, gram stained, sub-cultured as ribbon streak (four colonies per plate) on T_3 agar medium (Travers *et al*., 1987). After 72 hrs of incubation, endospore and crystal staining was done as described by Chilcott and Wigley (1988).

3.3 Identification and authentication of *Bacillus thuringiensis* isolates

3.3.1 Gram staining (Gerhardt *et al*., 1981)

Bt isolates were smeared thinly on a slide and heat fixed.The slide was flooded with crystal violet solution for up to one minute and briefly washed off with tap water (not over 5 seconds), drained. Gram's Iodine solution was flooded, and allowed to act (as a mordant) for about one minute and washed off with tap water, drained. Excess water was removed from slide and blot, so that alcohol used for decolorization is not diluted.

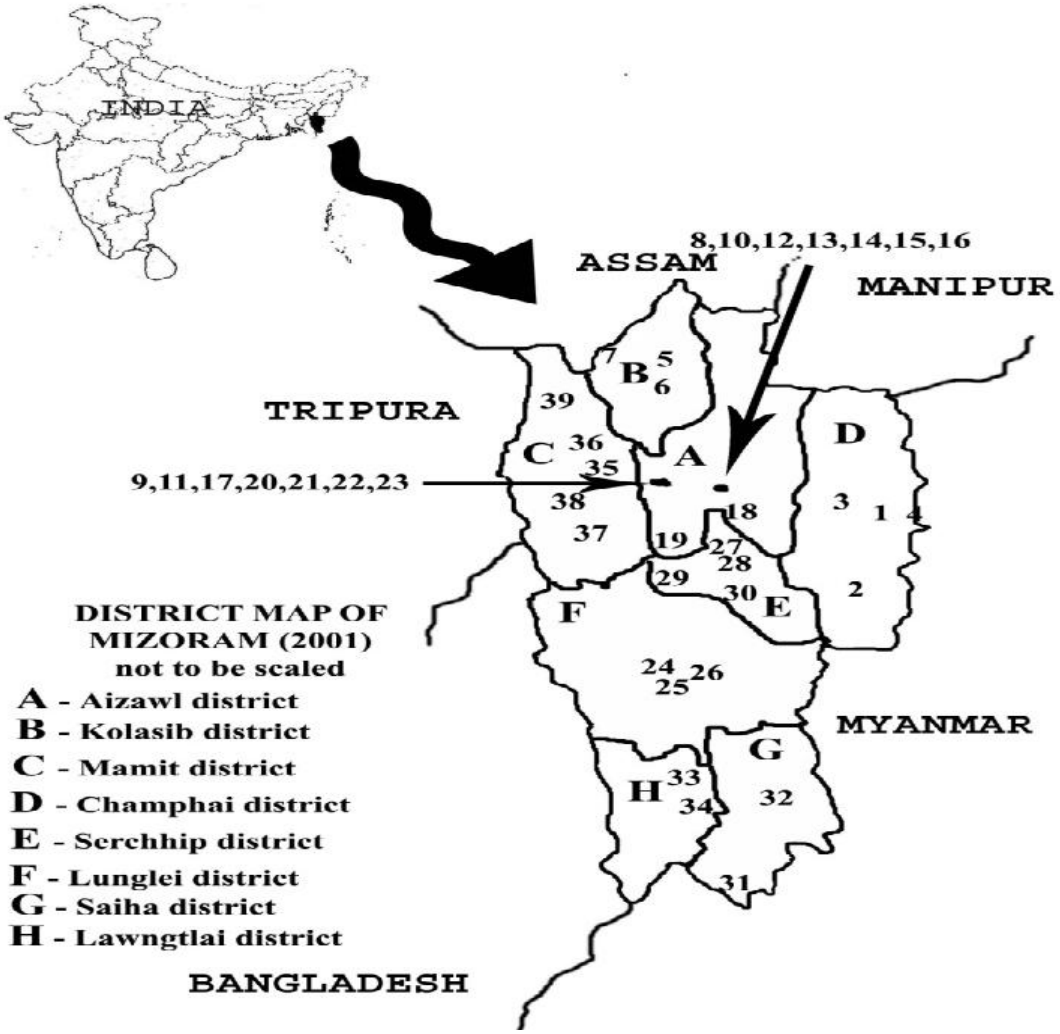


Figure 3. Details of soil sample collection from thirty nine locations covering eight districts in Mizoram state, India for the isolation of *Bacillus thuringiensis*. 1: Champhai; 2: Bungtlang; 3: Khawzawl; 4: Rih Dil; 5: Kolasib; 6: Thingdawl; 7: Bairabi; 8: Dawrpui; 9: Tanhril; 10: Chanmari West ; 11: Chawlhhmun; 12: Zotlang; 13: Chawnpui; 14: Kannan; 15: Vaivakawn; 16: Zarkawt ; 17: Ramrikawn ; 18: Seling ; 19: Sailam ; 20: MZU Campus ; 21: Sairang ; 22 : Sihhmui ; 23 : Tuivamit 24 Serkawn; 25: Lunglei; 26: Zobawk; 27: Chhingchhip; 28: Chhiahtlang; 29: Thenzawl; 30: Serchhip; 31: Khengkhawng ; 32 : Saiha ; 33: Lawngtlai ; 34: Chhimtuipui ; 35: Lengte ; 36: Lengpui ; 37: West Phaileng ; 38: Rawpuichhip; 39: Chhippui.

The slide was flooded with 95% alcohol for 10 seconds and wash off with tap water. (Smears that are excessively thick may require longer decolorization. This is the most sensitive and variable step of the procedure, and requires experience to know just how much to decolorize). The slide was drained and flooded with safranin solution and allowed to counterstain for 30 seconds. Washed off with tap water, drained and blot dry with tissue paper. After drying, the slide can then be viewed under a light microscope with oil immersion.

3.3.2. Endospore staining (Schaeffer–Fulton stain,1930)

Using an aseptic technique, *Bt* isolates were smeared thinly on a slide and heat fixed. The slide was then suspended over a water bath with some sort of porus paper over it, so that the slide is steamed. Malachite green was applied to the slide, which can penetrate the tough walls of the endospores, staining them green. After five minutes, the slide was removed from the steam, and the paper towel was removed. After cooling, the slide was rinsed with water for thirty seconds. The slide was then stained with diluted safranin for two minutes, which stains most other microorganic bodies red or pink. The slide was then rinsed again, and blotted dry. After drying, the slide was viewed under a light microscope with oil immersion.

3.3.3. Presence of spores and crystals (Chilcott and Wigley, 1988)

Thin films of aqueous suspensions of *B. thuringiensis*, grown on agar plates, were placed on a microscope slide, air-dried, and incubated at 100°C for 10 min. The hot slide was placed into naphthalene black 12B solution (1.5 g naphthalene black 12B in

35% v/v glacial acetic acid) for 2 min, washed with tap water, and immersed in Gurr's improved R66 Giemsa stain (BDH) for 1 min. The slides were washed and dried. The bacterial cells and crystals stained black and spores were differentially stained pale to light blue with a dark blue margin.

3.3.4 Scanning electron microscopy (SEM) of spores and crystals (Suzuki, 2002)

SEM of selected cultures were carried out at SAIF, NEHU, Shillong, Meghalaya. Sporulated cultures of *Bt* in NYSM broth were pelleted out. They were then fixed in 2.5% glutaraldehyde in PBS buffer for 45 minutes and washed 15 times with Phosphate buffer saline (pH. 7). Refixed in 1% Osmium tetra oxide (OsO4) in PBS buffer for 1 h. Dried and attached to stub. Sputter coating with gold was done and viewed with SEM.

3.4 Biochemical characterization of the *Bt* Isolates

The colonies that formed on T3 agar was again confirmed by Biochemical characterization based on: Fermentation of sugars, Methyl Red –Voges Proskauer test, starch hydrolysis, Growth on varying % of Nacl, nitrate reduction, Urease , Tryptophan, Catalase, arginine dihydrolase, Growth on D-mannitol, Indole (Holt,1984; Lacey, 1991; Stahly *et al*., 1991) agar medium. All tests were performed with a negative control (without innoculum) and a positive control(Table 1) using standard strains of *Bt* supplied by Dr. Zeigler (Bacillus genetic Stock Center, BGSC, Ohio, USA).

3.4.1 Carbohydrate Fermentation Test

The *Bt* Isolates were inoculated in a peptone broth base containing an indicator (Andrade's peptone water) with the fermentable substance at a concentration of 2.0% level. Two fermentable substances were used and tested for fermentative degradation and they were D-glucose and xylose.

3.4.2 Methyl Red Voges-Proskauer Test (MRVP)

To MR-VP broth, *Bt* Isolates were inoculated and incubated at 37°C for 48hrs. After incubation 0.6ml of alpha-napthol was added and mixed thoroughly. 0.2ml of potassium hydroxide with creatinine was added. The tubes were observed for the color change.

VP Positive : Pink or red color at the surface of the medium (acetoin present).

VP Negative : Yellow or copper color at the surface of the medium (acetoin absent).

3.4.3 Starch Hydrolysis

The *Bt* Isolates were inoculated on starch agar plates by single line inoculation. The plates were incubated at 35°C for 24hrs. After incubation, the plates were flooded with iodine solution. A clear zone around the growth indicates hydrolysis and unchanged starch will give a blue color.

3.4.4 Salt (Sodium chloride) tolerance test

The *Bt* Isolates were inoculated on Nutrient agar plates with varying concentrations of sodium chloride (3%, 5%, 7%) to check the salt tolerance level. Growth of *Bt* was monitored after 24 h.

3.4.5 Nitrate Reduction Test

Nitrate broth was prepared, sterilized at 121°C for 15min at 15lbs / inch inoculated with *Bt* isolates and incubated at 37°C for 18-24hrs. Following incubation, 0.5ml of Reagent A and 0.5ml of Reagent B was added to 5ml of the culture medium. A positive reaction is indicated by the red color before the addition of zinc dust while negative reaction is colorless after the addition of zinc dust.

3.4.6 Urease Fermentation Test

Inoculated slants were incubated at 35ºC. The slant for a change of color was observed at 6 hours, 24 hours, and every day for up to 6 days. Urease production is indicated by a bright pink (fuchsia) color on the slant that may extend into the butt; any degree of pink is considered a positive reaction.

3.4.7 Tryptophan Broth

Tryptophan broth was prepared, sterilized at 121°C for 15minutes at 15lbs/inch inoculated with *Bt* Isolates and incubated at 37°C for 18-24hrs. After incubation 2-3 drops of ferric chloride was added and mixed thoroughly. The tubes were observed for the color change. A positive reaction is indicated by orange brown color.

3.4.8 Catalase Test

Nutrient agar slant were inoculated with *Bt* Isolates and incubated at 37°C for 24hrs. After incubation, 1ml of 3%hydrogen-per-oxide was trickled down the slant. A positive test is indicated by evolution of bubbles.

3.4.9 Arginine-Dihydrolase Test

Arginine dihydrolase broth was prepared, sterilized at 121°C for 15lbs/inch inoculated with *Bt* Isolates and incubated at 37°C for 24 - 48hrs. The color must change from purple to yellow which indicates positive reaction. If it remains purple, it indicates negative reaction.

3.4.10 Growth and acid production from D-mannitol

D-mannitol (filter sterilized using 0.22 μM filter) was added before slants were made to make 1 percent final concentration of pre-sterilized ammonium salts and sugar medium contained in test tubes. 24-h old test culture was inoculated and the tubes were incubated for 15 days at 31°C. No change in color of the medium indicated negative test for the fermentation of D-mannitol.

3.4.11 Indole Production Test

Peptone broth was prepared, sterilized at 121°C for 15 min at 15lbs / inch inoculated with *Bt* Isolates and incubated at 37°C for 24hrs. Following incubation, 0.2ml of Kovac's reagent was added to 5ml of the culture medium. A positive reaction is indicated by the cherry red color in the alchol layer.

3.4.12 Casein Hydrolysis

The *Bt* Isolates was inoculated on skim milk agar plates by single line inoculation. The plates were incubated at 37°C for 24hrs.after incubation; the plates were flooded with trichloro acetic acid solution. A clear zone around the growth which indicates hydrolysis of casein.

Table 1. Reference strain used for comparison in the present study.

Strains	BGSC Code	Original Code	Genotype	*cry* Genes
Bt serovar. *kurstaki*	4D1	HD1	serotype 3a3b	*cry 1,2*
Bt serovar. *aizawai*	4J3	HD133	serotype 7	*cry 1,2,9* *cry 7,8*
Bt serovar. *tenebrionis*	4AA1	*tenebrionis*	serovar *tenebrionis*	*cry 3*
Bt serovar. *israelensis*	4Q2	HD500	serotype 14	*cry 4,11*
Bt serovar. *alesti*	4C1	HD16	Serotype 3a-3c	*cry 1*

3.5 Identification of isolates by Biochemical typing

As described by Martin and Travers (1989) biochemical tests were performed to identify isolates. This system was based on the biochemical tests that have been published for known varieties for which the serotypes have been identified (de Barjac, 1981). The following four biochemical tests were performed: esculin utilization, acid formation from salicin and sucrose, and lecithinase production (Parry *et al*., 1983) which was the most variable among *B. thuringiensis* (Martin and Travers (1989).

3.5.1 Esculin

Esculin agar was prepared, sterilized at 121°C for 15minutes at 15lbs/inch inoculated with *Bt* culture and incubated at 37°C for 18-24 hrs. Following incubation the Esculin reacts with ferric ions to produce black colored complex which indicates positive reaction. Abundant growth on the slant indicates a positive test for growth in the presence of bile. If growth is present, esculin hydrolysis can be observed if the medium has taken on an intense, chocolate brown coloration.

3.5.2 Salicin fermentation test

Salicin solution was prepared as 10% stock solution and filters sterilized. 5 ml of Peptone broth was dispensed in test tubes along with phenol red indicator (0.01%) and sterilized at 121°C for 15 min at 15lbs / inch. To each test tube of peptone water, 0.5 ml of Salicin stock solution was added and inoculated with two drops of individual isolate suspension. The tubes were incubated at 37°C for 7 days. Positive or negative results were observed by change in the color of media or air bubble formation in the tube indicating acid and gas production, respectively. If salicin is fermented to produce acid end products, the p*H* of the medium will drop. A pH indicator in the medium changes color to indicate acid production. A positive test consists of a color change from red to yellow, indicating a pH change to acidic.

3.5.3 Sucrose fermentation test

Sucrose solution was prepared as 10% stock solution and filters sterilized. 5 ml of Peptone broth was dispensed in test tubes along with phenol red indicator (0.01%) and sterilized at 121°C for 15 min at 15lbs / inch. To each test tube of peptone water, 0.5 ml of sucrose stock solution was added and inoculated with two drops of individual isolate suspension. The tubes were incubated at 37°C for 7 days. Positive or negative results were observed by change in the color of media or air bubble formation in the tube indicating acid and gas production, respectively. A positive test consists of a color change from red to yellow, indicating a pH change to acidic.

3.5.4 Lecithinase production test

To check the lecithinase activity, egg yolk powder agar was used. In egg yolk agar, the lipoprotein component Lecithovitellin can also be split by lecithinase into phosphorylcholine and an insoluble diglyceride, which results in the formation of a precipitate in the medium. This precipitate occurs as a white halo, surrounding the colony that produces lecithinase enzyme. The opalescence created is due to the release of free fat. Lecithinase activity is used to characterize several gram positive and gram negative bacteria. Inoculated are incubated at -37^{o}C for 24 hours.

3.6 Protein Profiling

Fresh culture of each strain were grown from stock cultures in LB (Luria-Bertani)-agar medium and incubated at 37°C overnight. To prepare sporulated culture, one loop of each of one night old strains was again inoculated in 50 ml of NYSM medium (Myers and Yousten, 1978) for 72 hrs at 37°C at 200 rpm. The culture were monitored by

staining, after more than 90% of cells had lysed spore-crystal mixture was centrifuged for 10 min at 10,000g at 4^0C and washed with sterile water twice and stored in -80 °C. The spore crystal mixture was used for protein profiling and insecticidal toxicity assay.

3.6.1Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis analysis of Cry proteins (Sambrook *et al.*, 2001; Laemmli, 1970; Moraga *et al.*, 2004)

Reagents required

(a) 10 ml of 10% Separating gel solution:
(4.0 ml of distilled H_2O, 3.3 ml of 30% acrylamide mix, 2.5 ml of 1.5 M Tris buffer (pH 8.8), 0.1 ml of 10% SDS, 0.1 ml of 10% ammonium persulphate, 0.004 ml of TEMED)

(b) 5ml of 5% Stacking gel solution:
(3.4 ml of distilled H_2O, 0.83 ml of 30% acrylamide mix, 0.63 ml of 1.0 M Tris buffer (pH 6.8), 0.05 ml of 10% SDS, 0.05 ml of 10% ammonium persulphate, 0.005 ml of TEMED)

(c) Tris-glycine electrophoresis buffer:
25mM Tris base, 250mM Glycine, 0.1% SDS

(d) Sample loading buffer

(e) Standard molecular-weight protein marker (medium range; PMWM-105979; Bangalore Genei)

8ml of sporulated culture of each isolate was pelleted down at 8000 rpm for 3 min. The supernatant were removed and 100µl sterile distilled water was added. 10 µl of 1N NaOH was added after vortexing, and incubated for 5min. 30 µl of sample buffer was added and boiled for 2 min. The mixture was centrifuged with minicentrifuge. 35 µl of the supernatant was loaded per well of SDS-PAGE. A protein marker was also loaded.

3.6.2 Dendrogram and cluster analysis

Protein markers were scored in a binary form as presence or absence of protein bands (respectively 1 and 0) for each sample. Cry protein profile data was used to construct a dendrogram following the method of NJ. Nei's genetic distances were calculated between each pair of the 27 *Bt* isolates using the Binary data. The genetic distance matrix was used to generate a phylogenetic dendrogram using UPGMA. Consistency of tree was checked by a bootstrap value of 1000 at 95% confidence intervals using NTSYSpc 2.1 software. The genetic similarity matrix of twenty seven *Bt* isolates was estimated using Jaccard's coefficient and was run on SAHN using the NJ clustering algorithm to generate dendrogram. All computations were performed using the NTSYSpc 2.1 (Roholf 1998).

3.7 Insecticidal toxicity assay

3.7.1 Dipteran larvicidal bio-assay

Culex tritaeniorhynchus larvae were collected from fish pond in Lengpui, Aizawl, Mizoram and reared at the Department of Biotechnology, Mizoram University, Aizawl, Mizoram (WHO, 2005). The third instar larvae used in these assays belonged to the 2nd generation and were maintained at 27 ± 2°C with 65 ± 5% relative humidity and 12h photoperiod. The activity of twenty five (25) selected *Bt* isolates and Two (2) standard strains (*Bt. alesti* and *Bt. israelensis*) were screened against third-instar larvae of *C. tritaeniorhynchus* according to WHO procedure (2005). Bioassays were carried out by testing four doses of each isolates. 1ml of sporulated culture from NYSM was taken and serially diluted to 1:10, 1:100, 1:1000, and 1:10000 with tap water. 5ml of each dilution

of each *Bt.* isolate was added to disposable cups (10 x 8 cm) containing 45ml of tap water and 10 larvae of *C. tritaeniorhynchus*. The final volume in each cup was 50 ml. *Bt. israelensis* and *Bt. alesti* which are active against *Diptera* was used as positive control. One cup without *Bt* was used as the negative control. Mortality rate was observed after every 24 hrs. Lethal concentration (LC50 and LC95) and lethal time (LT50 and LT95) was calculated by Probit analysis (Finney, 1971).

3.7.2 Lepidopteran larvicidal bio-assay

The Greater Wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) was used for this study. This moth is pest in beehives, tunneling through the combs, feeding on pollen, wax and honey. Initially the eggs were obtained from Department of Biotechnology, Bharathidasan University, Tamil Nadu and were kept in rearing plastic boxes with artificial diet and the insects was maintained in aerated plastic containers (32.5 x 17.6 x 10 cm) at 25 ± 2°C. The diet was prepared in plastic tray for 10,000 larvae at each experiment with the ingredients, Wheat flour 200 g, Wheat bran 200 g, Milk powder 200 g, Yeast 100 g, Honey 150 ml and Glycerin 150 ml and covered with muslin cloth before and after use. Approximately 200-300 eggs of *G. mellonella* were placed on a piece of artificial diet in cylindrical plastic containers (11cm height x 6cm) and were kept 72-75% relative humidity to avoid fungal contamination. The eggs hatched in 3-4 days later on larvae were given new diet and after 4-5 weeks late instar larvae were collected and used for bioassay study. Further, for insect mass rearing 10-20 larvae were allowed to pupate within the designed box and the emerged adult moths were encouraged for mating and oviposition in the wax coated paper.

Diet incorporation method

In this study, *Bt* formulations were mixed with diet and the larval mortality was assessed. Before going to treatment the late instars larvae were collected and rinsed first for 20 seconds in 60°C water, then for 10 seconds in cold tap water for arresting the silk production and cocoon formation. (WHO procedure, 2005)

3.8 Growth Curve Studies

Ten *Bt* isolates (Thenzawl TZ1, Sailam SL1, Chhippui CHP1, Khengkhawng KK1, Serkawr SK1, Chhimtuipui CHTP1, Sailam SL2, Lengte LT1, Serchhip SC1, Hmunpui HP7) and three reference strains (*Bt. kurstaki* 4D1, *Bt. israelensis* 4Q1, *Bt. alesti* 4C1) were used for growth curve studies . Three µl of each *Bt* culture (24 h old) was transferred into the 10 ml of LB broth and incubated at 37˚C for overnight. After 12 h, the *Bt* LB broth was subjected to turbidometric observations at regular intervals (2 h) and OD was measured at 600 nm. OD readings were taken for all the *Bt* cultures for 48 h. The data was plotted on a graph with OD vs. time, and the vegetative growth phase which is equivalent to the exponential phase was determined (Pelczar *et al*., 1957). Both time (h) and absorbance were plotted on the graph and absorbance units on a logarithmic scale. By connecting the dots, the line was drawn and OD values plots to represent the phases of growth lag, exponential, and the start of the maximum stationary phase. For the growth rate formula two points on the straight line drawn were chosen through the exponential phase and made note of the time interval between them (t). Two points were chosen for which the logs are obtained (Higher CFU/ml = X_t = at

final hours of exponential hours; Lower CFU/ml = X_0 = at initial hours of exponential phase; Time interval (in hours) between the 2 points = t).

Calculation of growth rate constant (μ)

It is the number of generations (doublings) per hour was found as under:

$$\text{Growth rate constant } (\mu) = [\log_{10}N_t - \log_{10}N_0] \times 2.303/[t_f - t_0]$$

Where, N_0 - Initial OD value of exponential phase; N_t – Final OD value of exponential phase; t_f – final time and t_0 – initial time.

Calculation of mean generation time (g)

It is the time it takes for the population to double was calculated by using the following formula:

$$\text{Mean generation time (g)} = [\log_{10}N_t - \log_{10}N_0]/\log_{10}2$$

Where, N_0 - Initial OD value of exponential phase; N_t – Final OD value of exponential phase.

Calculation of growth rate index (C)

Growth rate index, c, is a measure of the number of generations (the number of doublings) that occur per unit of time in an exponentially growing culture.

$$C = \frac{\ln 2}{g}$$

where ln 2 is the natural log of 2 (0.693) and g is the time in hours taken from the population to double during the exponential phase of growth.

3.9 *cry* gene detection by PCR technique

3.9.1 Total DNA isolation

A total of 28 - 45 isolates were selected after identification and biochemical characterization, DNA was extracted according to Bobrowski *et al.* (2001), and was used as a template for PCR. The cultures were incubated overnight at 30°C in LB agar at 37˚C .After 16-20 hrs one loop full of culture was transferred to 300µl of milliQ water and vortexed. It was then kept in -80˚C for 15 minutes. The frozen DNA was immediately transferred to boiling water and kept for 10 minutes. The resulting cell lysate was briefly spun at 6000rpm for 3-4 seconds. The supernatant was used as the DNA template.

3.9.2 PCR conditions

Identification of known lepidopteran, dipteran and coleopteran specific *cry* genes (*cry* 1, 2, 3, 4 and 9) was performed with 250 ng of total *Bt* DNA (3 µl) with reaction buffer (10X Tris, 1µl), 0.5 or 1.0 U of Taq DNA polymerase (Genei, Bangalore), 10 mM each deoxynucleoside triphosphate (0.2 µl), 0.5 mM of each reverse primer and forward primer (specific type primer for each cry gene), and 1.5 mM $MgCl_2$ (0.6 µl) in a final volume of 10 µl . Amplification was done in an Eppendorf thermal cycler under the

following conditions: 3 min of denaturation at 94°C followed by 30 cycles of amplification with a 1 min denaturation at 94°C, 45 sec of annealing at 54 - 60°C, and 30 sec of extension at 72°C. An extra extension step of 5 min at 72°C was added after completion of the 30 cycles. All PCR products were analyzed by 1.5% agarose gel electrophoresis in Tris-borate-EDTA buffer (0.6g of agarose was dissolved in 40 ml of 1X TBE buffer and melted in micro wave oven for 1-2 minutes) and stained with 10 mg/ml ethidium bromide (1 µl) (Sambrook *et al*., 2001). DNA samples were run at 50 volts and PCR products were visualized under UV transilluminator and the sizes of the fragments were estimated based on a DNA ladder of 100 base pairs (Bangalore's genei). The *Bt* isolates were compared with standard strains. The Cry primers used were universal primers as described by Ben-Dov *et al*. (1997) (Table 2).

3.10 Genomic DNA isolation

Isolation of genomic DNA from *Bt* was carried out by following Sambrook and Russell (2001) with slight modifications. The selected strains are streaked on LB agar and incubated overnight at 37°C and then subsequently inoculated in LB broth overnight in a shaker at the same conditions. Genomic DNA was isolated when the cultures were about 16 – 18 hrs. The broth culture of bacteria was centrifuged at 8,000 rpm at 4 °C for 2 minute and the pellet was collected by discarding the supernatant. The pellet was washed by TE buffer pH 8.0(10 mM Tris- HCl pH –8.0) and 1mM EDTA pH 8.0), this process was repeated twice. The cell pellet was resuspended in 0.5 ml SET buffer (75mM NaCl, 25 mM EDTA pH 8.0, 20mM Tris- HCl, pH 8.0). 10 µl (100mg/ ml) lysozyme was added to the above suspension and incubated at 37^0C for 30 - 60

minutes. 2.5μl of RNase was added and incubated for 30 minutes. After incubation, heat inactivation of RNase was done by incubating at 65 ^{0}C for 10 mins. 50μl of 20% SDS and 10 μl of proteinase K (25 mg/ ml) was added to the above suspension and incubated at 55^{0}C for 60 minutes. Tris water saturated phenol: chloroform: isoamyl alcohol (25: 24: 1) was added followed by gentle vortexing, centrifuged at 12000 rpm for 15 minute. To the aqueous phase, 0.1 volume of the sodium acetate (pH 4.8) was added and gently vortexed. Tris water saturated phenol: chloroform: isoamyl alcohol (25: 24: 1) was added followed by gentle vortexing, centrifuged at 10000 rpm for 5 minute. 600 μl of chilled absolute ethanol was added by following gentle extraction and incubated for 30 minute -20^{0}C. The mixture was centrifuged at 10,000 rpm for 5 minute at 4^{0}C. The pellet was washed with 70% ethanol and again centrifuged at 10,000 rpm at 4^{0}C for 5 minute (repeated this step twice). The pellet was air dried and dissolved in T E buffer/water and stored at 4^{0}C.

3.11 Genetic Polymorphism studies through RAPD- PCR

Nine (09) selected isolates harboring different cry genes combination, and two (02) standard strains – *B. israelensis B.aizawai* were used for polymorphism studies (Table 3). The genomic DNA was quantified and diluted to 50ng/μl using Biophotometer Plus (Eppendorf, Germany) and used as a template for RAPD-PCR.

3.11.1 RAPD-PCR conditions

A total of twenty six (26) random primers manufactured by Bangalore's genei were screened (Table 4). Amplification reactions were carried out in 10μl volumes containing 2mM Tris - HCL taq buffer, 1.5 mM of MgCl2, dNTP 2mM, BSA 0.8 %, primer

0.4μM, taq polymerase 1 unit, and 50 ng of template DNA. The PCR program ran as follows - 4 min at 94°C, 35 cycles of 94°C for 1 min, 35°C for 1 min, 72°C for 2 min followed by a final extension for 5 min at 72°C. Amplified DNA fragments were analyzed in 1.5% agarose gel at 50 volt in 1x TAE buffer. Agarose gels are visualize using UVP gel documentation system and analyzed by Doc-ITLS image analysis software (UVP, Cambridge, UK).

3.11.2 Data analysis

Amplified products were scored as either present (1) or absent (0). A data matrix was prepared to determine the genotypes. The data matrix was used to calculate dissimilarity using the Jaccard function supported by Darwin 5 (Perrier X., Jacquemoud-Collet J.P. (2006). DARwin software) by using the formula at a bootstrap value of five thousand (5000)

$d_{ij} = \frac{b+c}{a+(b+c)}$ Where, d_{ij}: dissimilarity between units *i* and *j*

a: number or variables where X_i = presence and X_j = presence

b: number or variables where X_i = presence and X_j = absence

c: or variables where X_i = absence and X_j = absence

Cluster analysis and factorial and co-ordinates analysis of the cluster was done by the same software. Based upon the above method, phylogenetic tree is being created. The reliability and robustness of the phenograms were tested by bootstrap analysis for 5,000

bootstraps for computing probabilities in terms of percentage for each node of the tree using the DARwin software (Perrier and Jacquemoud-Collet, 2006).

The genotyping data from RAPD PCR was further used for assessing the discriminatory power of the primers by evaluating six parameters of the following: - polymorphism percentage, frequency, polymorphism information content (PIC), resolving power (RP), effective multiplex ratio (EMR) and marker index (MI). The PIC of each RAPD marker was computed as $PIC_i = 2f_i\ (1 - f_i)$; where PIC_i is the polymorphic information content of the marker *i*, f_i is the frequency of the amplified allele (band present), and $(1-fi)$ is the frequency of the null allele (Roldan-Ruiz *et al*., 2000). PIC was averaged over the fragments for each primer combination. The MI was calculated using formula, $MI = PIC - EMR$ (Roldan-Ruiz *et al*., 2000), where, effective multiplex ratio (EMR) is the total number of polymorphic loci/fragments per primer. Resolving Power, this is based on the distribution of alleles within the sampled genotypes. Resolving power of each primer combination was calculated using formula, $RP=\Sigma I_b$; where, I_b represents band informativeness expressed as $I_b = 1 - (2 \times I0.5 - pI)$, where, *p* is the fraction of the total accessions in which the band is present (Prevost and Wilkinson, 1999).

Table 2. PCR cocktail mixture, conditions and primers used for *Cry* gene detection.

Components	Working concentration				
	Cry 1	Cry 2	Cry 3	Cry4	Cry9
Buffer	1x				
$MgCl_2$	3 mM				
dNTP	0.2 mM				
Taq. DNA Polymerase	1 U				
DNA Template	3 µl				
Forward Primer	0.5 mM				
Reverse Primer	0.5 mM				
Milli Q Water					
Forward primer – 5'- 3' (Universal)	CATGATTCATGCGGCAGATAAAC	GTTATTCTTAATGCAGATGAATGGG	CGTTATCGCAGAGAGATGACATTAAC	GCATATGATGTAGCGAAACAAGCC	CGGTGTTACTATTAGCGAGGGCGG
Reverse primer – 5'- 3' (Universal)	TTGTGACACTTCTGCTTCCCATT	CGGATAAAATAATCTGGGAAATAGT	CATCTGTTGTTTCTGGAGGCAAT	GCGTGACATACCCATTTCCAGGTCC	GTTTGAGCCGCTTCACAGCAATCC
PCR condition (30 cycles)	94°C for 3 min; 94°C for 1 min; 54-60°C for 45 sec; 72°C for 30 sec (30 cycles); 72°C for 5 min				
Annealing temperature	54°C	60°C	60°C	62°C	60°C
Expected product size (bp)	270 - 320	680 - 720	580 - 620	420 - 450	351 - 354

Table 3. Strains used for RAPD-PCR with site of isolation, vegetation and *cry* genes detected.

No.	Strain ID	Site	Vegetation	Source of Isolation	Cry gene(s) Present
1	*CHP1*	Chhippui	Jhum	Soil	Cry 2,9
2	*CAMP*	Campus	Shrub	Soil	Cry 2,4
3	*CHTP*	Chhimtuipui	River banks	Soil	Cry 2,3,4,9
4	*RRK*	Ramrikawn	Fish pond	Soil	Cry 2,3,9
5	*SK1*	Serkawr	Grass	Soil	Cry 1,2,4
6	*CH1*	Champhai	Grass	Soil	Cry 1,2,9
7	*LL1*	Lunglei	Flower garden	Soil	Cry 1,4,9
8	*CHP5*	Chhippui	Roadside	Soil	Cry 1,9
9	*SK2*	Serkawr	barren	Soil	Cry 4,9
10	*Bt israelensis Q1*				Cry 4, 11,
11	*Bt aizawai 4J3*				Cry 1, 2, 7, 8, 9

Table 3. Primers used for RAPD-PCR analysis

Primer name	Primer Sequence 5'—3'	Primer name	Primer Sequence 5'—3'
BT-1	CAGGCCCTTC	BT-14	CCGGCGGCGC
BT-2	CAATCGCCGT	BT-15	TGCCGAGCTG
BT-3	TCATCGCGCT	BT-16	CAAACGTCGG
BT-4	GCGATCCCCA	BT-17	GAGAGCCAAC
BT-5	CAGCACCCAC	BT-18	ACGGCCGACC
BT-6	GTGAGGCGTC	BT-19	CGCCCCCATT
BT-7	GAACGGACTC	BT-20	TGCAGTCGAA
BT-8	GGTGCGGGAA	BT-21	AGGCCGCTTA
BT-9	GTT TCGCTCC	BT-22	CCGGGCAAGC
BT-10	AAGAGCCCGT	BT-23	AGGATCAAGC
BT-11	AACGCGCAAC	BT-24	CAGGCGCACA
BT-12	CCCGTCAGCA	BT-25	AAACAGCCCG
BT-13	ACGCGCCCTA	BT-26	TGTCAGCGGT

3.12. 16s rRNA gene characterization through PCR and sequence analysis

10 isolates (Mzubt 1,2,4,5,6,11,23,25,26,29) and two standard (*Bti* and *Btk*)were selected for 16s rRNA gene characterization. The genomic DNA harvested was diluted to 150ng/µl which serves as a template. Universal primer (Forward: 5' AGAGTTTGATCCTGGCTCAG 3' Reverse: 5'ACGGCTACCTTCTTCTTACGA 3') for 16s described by Weisburg (1991) was used for characterization. Amplification reactions were carried in 25µl volumes containing 1x taq buffer, 3 mM of Mgcl2, dNTP 0.2mM, primer 0.3µM, taq polymerase 1.5 unit, and 150 ng of template DNA. Amplifications were carried out in a DNA thermal cycler (Eppendorf). The conditions for PCR were as follows: a single denaturation step for 5 min at 95 °C, a step cycle program set for 40 cycles with a cycle of denaturation step for 1 min at 95 °C, annealing for 1 minute at 55 °C with extension for 2 minutes at 72 °C. Finally, an extra extension step for 7 minutes at 72 °C was used. Amplified DNA fragments were analyzed in 1.5% agarose gel at 100 volt in 1x TAE buffer. Two 16s rRNA of local isolates mzubt 6 and mzubt 29 (ramrikawn RRK, Serkawr SK2) were sent for sequencing to GCC biotech, Kolkata.

The sequences of 16s r RNA gene was analyzed by Multiple sequence alignment with other Bt 16s rRNA sequences from NCBI data base alongwith the 16s rRNA gene sequence of *Staphylococcus aureus* as an out group. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Tamura *et al*., 2011). The evolutionary history was inferred by using

the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The boot strap value was given as 5000. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 9 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.,* 2011).

4. RESULTS

Isolation of *Bacillus sp.* and *Bt* from soil samples

A total of 428 soil samples were screened for *Bacillus sp*. from five different habitat soils by from thirty nine locations in eight districts of Mizoram (Figure 3). Out of the 428 soil samples, fifty soil samples showed *Bt* like characteristics. All the colonies formed were found to be *Bacillus*, the procedure for isolation was optimized for Mizoram soil conditions (Table no 4). LB agar was used instead of NA since colonies developed within 24 h instead of 3-5 days with NA. The 50 soil samples were screened using acetate selection process. Acetate inhibited the germination of *Bt* spores allowing other spores to germinate, while the growing cells and other non-spore-forming bacteria were eliminated by heat treatment. *Bt*-like colonies, which are usually described as cream-colored and have the appearance of a fried egg on a plate, were labeled and subcultured (Figure 4). *Bacillus* sp. population ranged between $12x10^2$ - $48x10^7$cfu/g of soil. Population is high in jhum area, paddy field, forest area, fish pond, Marsh area, shrub, teak plantation, river, grass, banana plantation, flower garden low in construction site and barren land area. The population of *Bacillus* sp. is highest in Mamit district and lowest in lunglei district (Table 5). One hundred and seven (107) colonies produced proteinaceous crystals out of 8676 colonies screened for *Bt* and the remaining 8569 colonies were found to be *Bacillus*.

Figure 4: Colony morphology of *B. thuringiensis* isolates

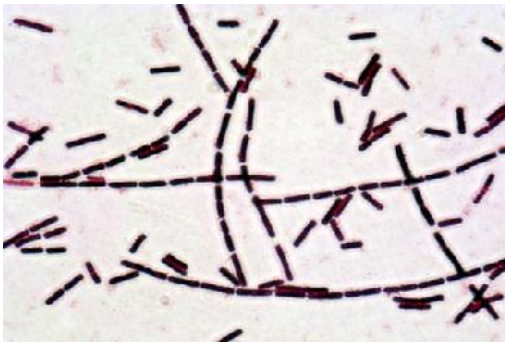


Figure 5a. Gram staining

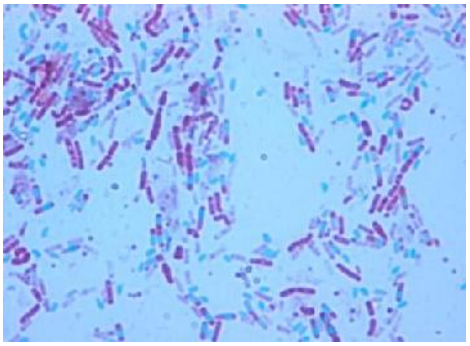


Figure 5b. Endospore staining

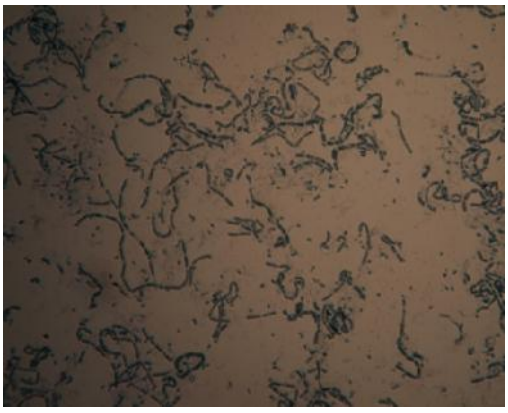


Figure 5c. Spores and crystals staining (Light microscopy)

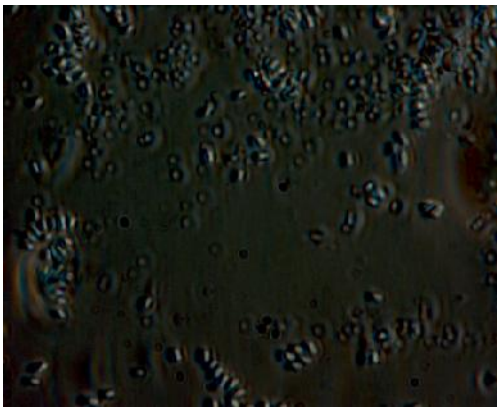


Figure 5d. Spores and crystals Staining (Phase contrast)

(Cells and spores phase light under phase contrast microscopy, crystals appeared phase dark)

Table 4. Optimized Procedure for Mizoram Soil samples

Method	Isolation procedure	Serial dilution	Amount	Plated on
1	1gm of sample in 10 ml of sterile distilled water. Heat treatment at 60°C for 60 minutes *(for Bacillus & Bacillus thuringiensis)*	10^2	20 µl	LB agar
2	1gm of soil sample in 10 ml of Luria broth medium(0.25M sodium acetate buffered) incubate at 37°C for 4 hrs. Heat treatment at 80°C for 3 minutes. Incubate at 37°C, observed colonies after 24 hrs *(for Bacillus thuringiensis only)*	10^3	20 µl	LB agar

Table 5. District wise mean Population of *Bacillus sp.* in the soil.

District	**Cfu/gm of soil**
Mamit	20- 48 $x10^7$
Lawngtlai	25-27 $x10^7$
Saiha	26- 28 $x10^7$
Champhai	2 – 28 $x10^7$
Aizawl	18-38 $x10^7$
Lunglei	12-19 $x10^2$
Serchhip	8-28 $x10^3$
Kolasib	8-28 $x10^3$

cfu – colony forming units

Table 6. Distribution of *Bt* isolates from soil sample collected from different habitats in Mizoram.

S. No	Habitat	Location	Number of samples			Number of colonies		
			Examined	*Bt*	%	Total	With *Bt*	*Bt* index@
1	Forest	MZU Campus, Kolasib, Chanmari West, Chawlhhmun, Khengkhawng, Tanhril, Zotlang, Chawnpui, Kannan, Serkawn, Lunglei, Zobawk, Lawngtlai, Chhingchhip, Chhiahtlang, Bungtlang, Khawzawl, Lengte, Chhimtuipui	19	6	31.57	1731	24	0.014
2	Agriculture	Lunglei, Kolasib, Dawrpui, Thenzawl, Lengpui, Champhai, Sailam, Serchhip, Tuivamit, MZU Campus, Saiha, Lengpui	14	8	57.14	2593	31	0.012
3	Aquatic	Chhimtuipui , Lengpui, Ramrikawn, Kolasib, Thenzawl, Rih Dil	7	5	71.42	1273	19	0.015
4	Fallow	MZU Campus, Lunglei, Vaivakawn, Zarkawt, Ramrikawn	6	2	33.33	573	6	0.010
5	Jhum shifting cultivation	Hmunpui, Bairabi, Seling, Lengpui, West Phaileng, Rawpuichhip, Chhippui, Sairang, Sihhmui	9	8	88.88	2506	27	0.012
Total			55	29	52.72	8676	107	0.012

@The *Bt* index was calculated for each sample as the number of isolates of *Bt*/number of isolates of sporulated bacilli

Table 7. Morphology of vegetative cells, spores & crystals

	Shape
Bacillus sp.	
Vegetative cells	Rod
Spores	Oval
B. thuringiensis	
Crystals	Oval, spherical and bypyramidal.

Table 8. Identification of *Bacillus sp*.

	Bacillus sps	***Bt***
Gram staining	Gram +ve rod	Gram +ve rod
After 72 hrs of incubation,		
Schaeffer-Fulton differential staining of endospore	√	√
Spores and crystals staining	-nil-	The bacterial cells and crystals stained black and spores were differentially stained pale to light blue with a dark blue margin

Table 9. Measurements of vegetative cells, spores & crystals

Bacillus thuringiensis	Length(µm)	Breadth(µm)
Vegetative cells	3.62 ± 1.12	1.02 ± 0.35
Spores	1.25 ± 0.25	0.75 ± 0.25
Crystals	0.75 ± 2.08	0.52 ± 0.10

Measured by using occular micrometer, ERMA company Japan.

Identification and authentication of isolates

Bt isolates were obtained from 29 soil samples (52.72% frequency) (Table 6). The highest frequency of *Bt* (88.88%) was recorded in the shifting cultivation soil followed by aquatic (71.42%) and agriculture (57.14%) soil samples. The lowest frequency (33.33 and 31.57%) of *Bt* was observed in the soil samples of fallow and forest habitats. *Bt* index was calculated as the number of *Bt* colonies divided by the number of colonies examined. The overall *Bt* index of the soil samples from five different habitats in Mizoram was 0.012 (Table 6).

The colony morphology of *Bacillus* and *Bt* observed in the isolates can be summarized given in Table 7. Identification by gram staining, endospore staining after 72 hrs of incubation by Schaeffer-Fulton differential staining of endospore method were carried out for both *Bacillus* and *Bt* (Table 8). All the isolates are gram positive spore forming rods.

Spores and crystals staining was done for *Bt* isolates and observed in light and phase contrast microscope. The bacterial cells and crystals stained black and spores were differentially stained pale to light blue with a dark blue margin in light microscope whereas in phase contrast microscope, cells and spores *Bt* appeared phase light under phase contrast microscopy, while the crystals appeared phase dark. (Figure 5a, 5b, 5c, 5d). Further observation to confirm the presence of crystals in *Bt* isolates was carried out on selected strains using Scanning Electron microscope (Figure 6). The shape of the crystalline inclusions varied among the *Bt* isolates. Based on crystal morphology, the 107 isolates of *Bt* fall into the following three groups: bipyramidal, spherical and

oval. All of the isolates were crystal forming rods. The distribution of *Bacillus sp.* is 98.7 % from soils of Mizoram and out of that *B. thuringiensis* is 1.3 %.

Biochemical characterization

All isolates were rod shaped, gram positive, having both spores and crystals. The length of the spores and crystals were measured using ocular micrometer (ERMA, Japan) and were 1.25 and 0.75 µm, respectively (Table 9). The *Bt* isolates were positive to catalase forming bubbles; urease production indicated by a bright pink (fuchsia) color; Methyl Red Voges Proskauer producing pink or red color at the surface of the medium (acetoin present); and negative to indole without any change in colour; purple with arginine dihydrolase and tryptophan colourless after addition of ferric chloride. All the *Bt* isolates reduced nitrate to nitrite indicated by the red color after addition of reagents; hydrolysed casein forming a clear zone; utilized glucose and xylose for growth; tolerated 3% and 5% Nacl (but not 7%) and did not ferment D- mannitol (*Bt* seldom utilizes D-mannitol) (Figure 7). They were able to produce amylase and protease due to which starch hydrolysis occurred forming a clear zone around the colonies. All tests were performed with a negative control (without innoculum) and a positive control using standard strains (Table1,Table 10).

Biochemical typing of *Bt* isolates

Using the biochemical typing method, all the *Bt* strains isolated were divided into eleven biochemical types (Table 11). In some cases, undescribed combinations of biochemical types (six numbers) were yielded which were referred to by numbers.

Among the biochemical types, *israelensis* subspecies was observed at higher frequency (30.84%) and undescribed biochemical type combinations ranged from 3.73 – 14.0%.

Analysis of crystal protein profile in *Bt* isolates

All the 107 isolates of *Bt* obtained from the different habitats were selected and studied for crystal protein profile(s) by SDS-PAGE. The reference strains, 4C1, 4D1, 4Q1 and 4J3 showed many crystal proteins particularly 135 and 65 kDa (Tables 14). The new isolates of Bt showed that eleven different types of crystal protein profile viz., group I (43 kDa), group II (30 kDa), group III (43 and 30 kDa), group IV (65 kDa), group V (135 kDa), group VI (65 and 135 kDa), group VII (17 and 60 kDa), group VIII (19 kDa), group IX (27 and 95 kDa), group X (14 and 25 kDa) and group XI (22 and 106 kDa) (Table 12, Figure. 8). Out of the 107 Bt isolates analyzed by SDS-PAGE, 60 isolates exhibited three major polypeptide bands with molecular weights in the range of 65 (18.69%), 135 (16.82%) and both 65 and 135 (20.56%) kDa as in the case of reference strains. Whereas, 19 isolates belonging to groups I – III (43, 30 and both 43 and 30 kDa) were observed at a lower frequency (between 3.73 and 8.41 %). Four *Bt* isolates did not show any distinct band of crystal protein(s) (Table 12).

Analysis of the selected 27 isolates of *B. thuringiensis,* along with two standards, revealed very high similarity in the dendrogram obtained through UPGMA clustering method (Figure 9). Out of 33 protein fractions, 14 were common to all the isolates and the similarity indices was more than 66 %. Hence, it is difficult to differentiate these groups and this rules out the incipient speciation among the isolates of *B. thuringiensis*.

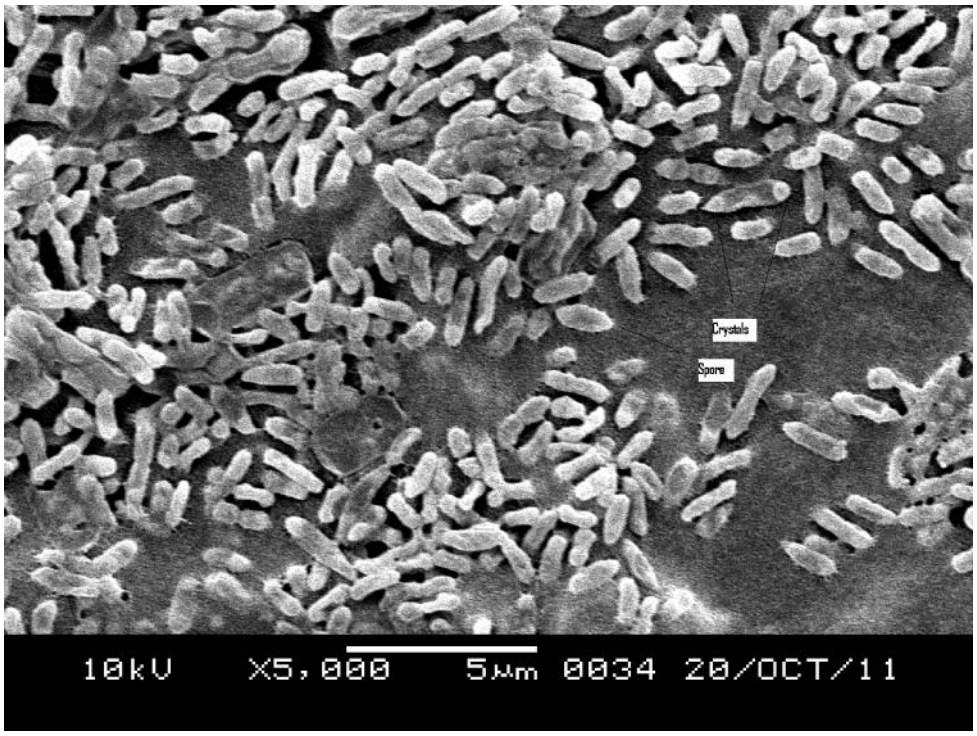


Figure 6. **Scanning Electron micrograph of isolate (SK1).**

Figure: 7. **Biochemical characterization of the *Bt* isolates**

Table 10. Morphology and biochemical tests of *Bt* isolates

S. No	CHARACTER/TEST	*Bt* Isolates	*Bt .alesti* 4C1 and *Bt. kurstaki* (BGSC, USA)
1	Shape	Rods with rounded ends	Rods with rounded ends
2	Spore (Spore staining)	+ Oval	+ Oval
3	Crystal staining	+ varied	+ varied
4	Sporangium	Not swollen	Not swollen
5	Gram stain	+	+
6	Motility	Motile & non motile	Motile & non motile
7	Growth on D-glucose	+	+
8	Growth on xylose	+	+
9	Growth on D-mannitol	-	-
10	Methyl Red	+	+
11	VP	+	+
12	Indole	-	-
13	Tryptophan	-	-
14	Arginine dihydrolase	-	-
15	Starch hydrolysis	+	+
16	Catalase	+	+
17	Urease	+	+
18	Nitrate reduction	+	+
19	Casein hydrolysis	+	+
20	Growth with 3% Nacl	+	+
21	Growth with 5% Nacl	+	+
22	Growth with 7% Nacl	-	-

(+) Positive reaction; (-) Negative reaction

Table 11. **Biochemical types of *Bt* isolates[a]**

Biochemical type (described subspecies)	Biochemical and physiological test result [b]				Number of *Bt* isolates and (% frequency)
	Hydrolysis of		Utilization of		
	Esculin	Lecithin	Sucrose	Salicin	
thuringiensis	+	+	+	+	5 (4.67)
aotto	+	+	+	-	0 (0)
alesti	-	+	+	+	0 (0)
Kurstaki/aizawai	+	+	-	+	4 (3.73)
indiana	+	-	+	+	0 (0)
dendrolimus	+	+	-	-	1 (0.93)
galleriae	+	-	-	+	3 (2.80)
morrisoni	+	-	+	-	1 (0.93)
darmstadiensis	+	-	-	-	0 (0)
israelensis	-	+	-	-	33 (30.84)
ostriniae	-	-	-	+	0 (0)
1*	-	+	+	-	10 (9.34)
2*	-	-	-	-	10 (9.34)
3*	-	-	+	-	15 (14)
4*	-	+	+	-	4 (3.73)
5*	-	+	-	+	12 (11.21)
6*	-	-	+	+	9 (8.41)

[a] Martin and Travers, 1989 (modified)

[b] (+) Positive reaction; (-) Negative reaction

* undescribed combinations of biochemical types

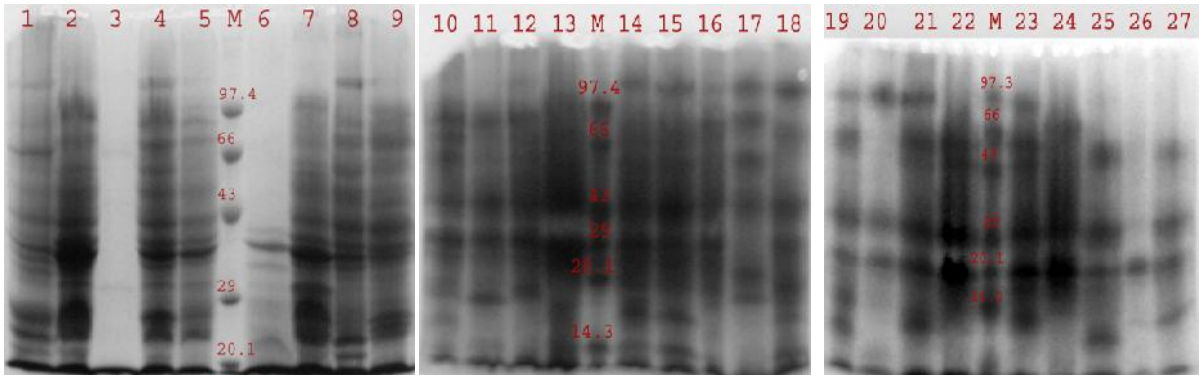


Figure 8. SDS-PAGE gel photo (Lane 1- *Bt* israeliensis. 2-RD2, 3-CHP, 4-RP, 5-SH, 6-CHP-5, 7-CHTP, 8-CAMPUS, 9-SK, 10-KK, 11-LP-02, 12-LP-03, 13-SE, 14-HP, 15-SK, 16-RK, 17-LP-04, 18-WP, 19-SL-02, 20-LL, 21-*ALESTI*, 22-RD, 23-SC, 24-SL-02, 25-TZ, 26-LP-01, 27-LT.) Lane M: Low molecular weight protein marker

Table 12. Grouping of *Bt* isolates of Mizoram based on crystal protein profile

S.No.	Group number	Molecular weight of crystal protein(s) (kDa)	Bt isolates	
			Total	Per cent
1	I	43	9	8.41
2	II	30	4	3.73
3	III	43,30	6	5.60
4	IV	65	20	18.69
5	V	135	18	16.82
6	VI	65,135	22	20.56
7	VII	17, 60	9	8.41
8	VIII	19	2	1.86
9	IX	27,95	5	4.67
10	X	14,25	5	4.67
11	XI	22,106	3	2.80
12		No discrete band	4	3.73
		Total	107	

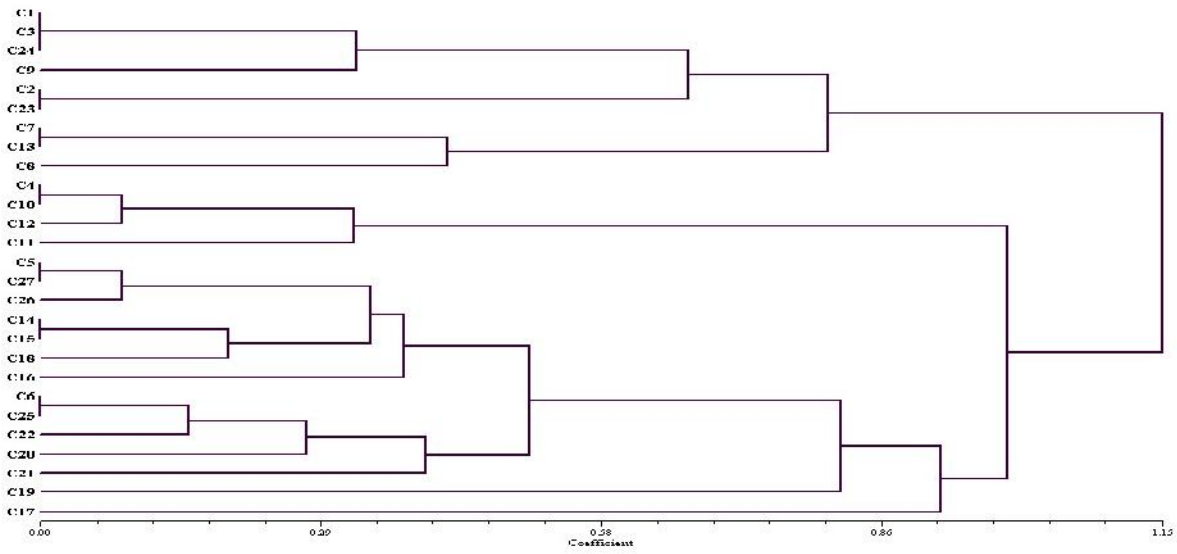


Figure 9. SDS-PAGE analysis of spore–crystal mixture isolated from Mizoram soil isolates of *Bt* showing 30, 43, 135 and 65, 135, 95, and 65 kDa protein(s)

C1: – *Bt alesti* 4C1; C2: Chhippui, CHP 1; C3: Chhippui, CHP5; C4: Chhimtuipui, CHTP1; C5: MZU Campus , CAMP1; C6: Hmunpui, HP7; C7: *Bt israelensis* 4Q1,Israel; C8: Khengkhawng , KK1; C9: Lunglei , LL; C10: LP1, Lengpui; C11: Lengpui, LP2; C12: Lengpui, LP3; C13: Lengpui, LP4; C14: Lengte, LT1; C15: Champhai, CH1; C16: RihDil, RD2; C17: Ramrikawn , RK1; C18: Rawpuichhip, RP1; C19: Serchip, SC1; C20: Seling, SE1; C21: Sihhmui, SH1; C22: Serkawn , SK 1; C23: Sailam, SL1; C24: Sailam SL2; C25: Sairang, SR1; C26: Thenzawl , TZ1; C27: West Phaileng, WP1

From SDS-PAGE results, some isolates was found to be relatively similar to *Bti* in their protein banding patterns. In bioassay, higher mortality rate was also observed with these isolates (Lane 7, 8, 14, 15, 21). Hence, these strains could be used as an alternative to chemical mosquitocides in mosquito control programs.

Cry gene profiling

Each PCR analysis was checked with the appropriate positive control strains of *B. thuringiensis* and a negative control. These strains were *B. thuringiensis* serovar. *alesti, B. thuringiensis* subsp. *kurstaki* for *cry* 1 and *cry* 2 gene groups, *B. thuringiensis* biovar. *tenebrionis* for *cry* 3 gene groups, *B. thuringiensis* serovar. *israelensis for cry 4 group* and *B. thuringiensis* subsp. *aizawai* for *cry* 9 gene groups (Table 1). PCR conditions were optimized using the control strains of *B. thuringiensis* before screening the isolates. All positive controls gave the expected PCR products. DNA ladder used was 100 bp DNA ladder (Bangalore Genei)**.**

The profiles of all PCR products were compared with those of standard strains. An isolate was considered to contain a determined gene only when the amplification product was of the expected size (Table 14, Figure 10). Analysis of the *Bt* isolates indicated that most contained *cry* 9 (25.23%), *cry* 4 (19.62%), as well as *cry* 4 and 9 (14.01%) genes (NCBI Accession No.'s JN215465 and JN596969). Twenty-two of the 107 isolates (10.28 %) have a high tendency to occur in combinations of *cry* 2, 9 and *cry* 2, 3, 9 genes (Table 13). The study of *cry* gene combinations in *cry* gene-containing isolates showed that 55.14 % (59 isolates) harbored more than one type of *cry* gene.

The 107 isolates are later grouped into 29 strains after *cry* gene profiling, SDS-PAGE and from the soil sample collection site. Colonies that developed from the same soil having similar characteristics are placed together.

Table 13. *cry* gene combination profiles present in the *Bt* isolates collected from different soil ecosystem of Mizoram (n = 107)

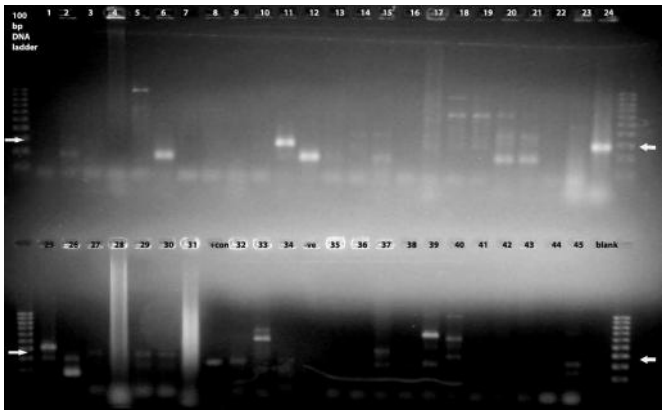
No.	*cry* gene	No. of Bt isolates	Frequency (%)
1	*cry 1, cry2, cry 4*	07	6.54
2	*cry 1, cry4, cry 9*	06	5.60
3	*cry 2, cry3, cry 9*	11	10.28
4	*cry 2, cry9*	11	10.28
5	*cry 3, cry9*	09	8.41
6	*cry4, cry9*	15	14.01
7	*cry4*	21	19.62
8	*cry 9*	27	25.23

Table 14. ***cry*** **gene and protein profiling in** ***Bt*** **isolates and their LC_{50} and LT_{50} against third instar of** ***C. tritaeniorhynchus***

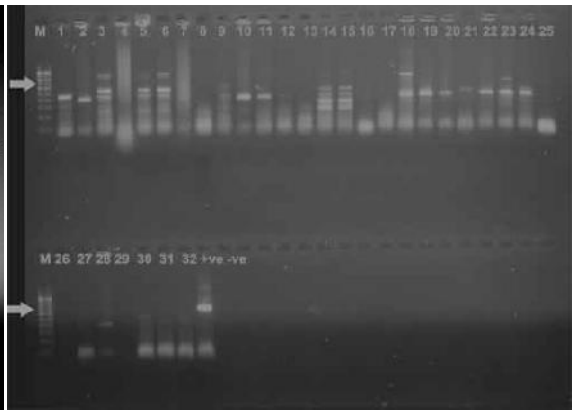
Habitat	*Bt* source	Code	*cry* gene#	Protein (kDa)	M * (%)	LC_{50} ** (cells/ml)	LT_{50} *** (days)
Soil	Bacillus Genetic Stock Centre, Ohio, USA	*Bt alesti* 4C1	*cry 1*	25, 27, 34, 43, 65, 135	90	2.70×10^2	4.11
Soil		*Bt kurstaki* 4D1	*cry 1, 2*	25, 29, 35, 65, 135	55	4.12×10^2	9.98
Soil		*Bt israelensis* 4Q1	*cry 4, 11*	27, 35, 65, 135	90	1.08×10^2	4.03
Soil		*Bt aizawai* 4J3	*cry 1, 2, 7, 8, 9*	14, 19, 45, 65, 135	60	3.67×10^4	7.71
Shifting cultivation	Chhippui	CHP 1	*cry 2, 9*	25, 27, 43, 65	80	3.12×10^3	6.42
		CHP 5	*cry 4,9*	30, 43, 65	95	2.70×10^2	5.71
	Hmunpui	HP 7	*cry 4,9*	23, 27, 35,65,135	100	3.90×10^1	3.69
	Lengpui	LP-1	*cry 2, 9*	14, 17, 19, 27, 43, 65	80	7.19×10^3	6.83
		LP-2	*cry 9*	19, 27, 43	60	5.03×10^4	7.71
		LP-3	*cry 9*	19, 27, 43	60	5.06×10^4	8.00
		LP-4	*cry 9*	14, 27, 30, 65	70	3.08×10^4	7.56
	Rawpuichhip	RP 1	*cry 4,9*	14, 17, 19, 30, 43, 65,106	80	1.02×10^3	6.72
	Seling	SE 1	*cry 4,9*	18, 20, 29, 43, 65	90	1.57×10^2	4.75

	Sihhmui	SH 1	*cry 4*	17, 19, 29, 43, 65	60	5.94×10^4	8.75
	Sairang	SR 1	*cry 9*	17, 27, 43, 65	70	4.35×10^4	8.53
	West Phaileng	WP 1	*cry 4*	17, 27, 43, 65	70	2.69×10^4	8.19
Forest	MZU Campus	CAMP 1	*cry 4*	23, 27, 35,65	95	2.03×10^2	4.51
	Khengkhawng	KK 1	*cry 4*	22, 27, 35, 43, 65	80	4.20×10^3	6.70
	Lengte	LT 1	*cry 9*	14, 17, 19, 30, 43, 65,106	70	4.20×10^4	7.89
	Serkawr	SK 1	*cry 1,2,4*	29, 43, 65	70	3.27×10^4	8.74
Fallow	Lunglei	LL 1	*cry 1, 4, 9*	25, 36, 40, 46, 65	75	1.06×10^4	7.92
Aquatic	Champhai	CH 1	*cry 3,9*	14, 17, 19, 30, 43, 65,106	75	5.72×10^4	8.18
	RihDil	RD 2	*cry 4,9*	19, 29, 43, 65	75	5.13×10^4	8.75
	Ramrikawn	RK 1	*cry 2, 3, 9*	19, 27, 43, 65, 106	95	2.46×10^2	4.64
	Thenzawl	TZ 1	*cry 4*	17, 27, 43, 65	80	3.12×10^3	6.78
	Chhimtuipui	CHTP 1	*cry 2,3,9*	25, 27, 30, 65	75	5.82×10^4	8.30
Agriculture	Serchhip	SC 1	*cry 4,9*	17, 20, 27, 43, 65,135	100	2.46×10^1	2.36
	Sailam	SL-1	*cry 9*	18, 24, 29, 43, 65	85	2.09×10^2	4.75
		SL-2	*cry 4*	29, 43	70	1.94×10^4	8.71

* Mortality at the 5th day after the *Bt* application (8×10^5 cells/ml)
** at the 5th day after the *Bt* application (1,350 insects per *Bt* isolate)
*** *Bt* application (8 x 10^4 *Bt* cells/ml)
# Genbank accession No. Cry 2A - JN215465 and Cry 1 - JN596969

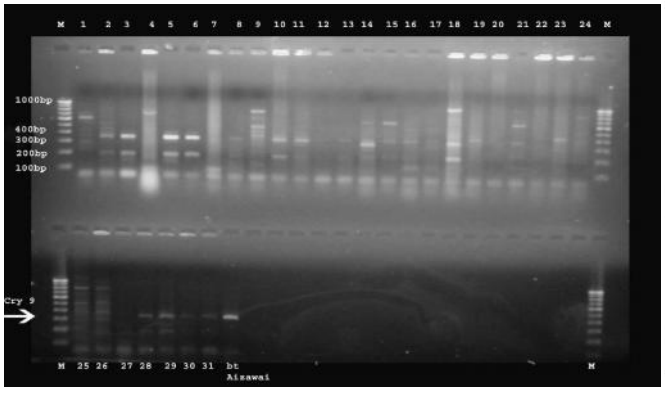


cry 1 gene analysis. Arrow mark set at to 300bp

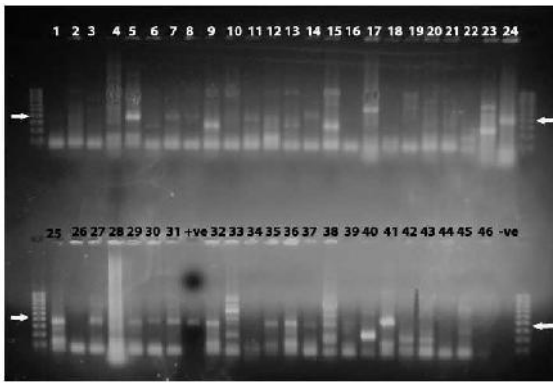


Cry 2 gene analysis. Arrow mark set at to 700bp

Cry 3 gene analysis. Arrow mark set at to 600bp



Cry 9 gene analysis. Arrow mark set at 350bp



Cry 4 genes.Arrow mark set at 400bp.

Figure 10: *cry* gene profiling

Insecticidal activity of *Bt* isolates

Dipteran larvicidal bio-assay

From the 29 strains of *Bt* tested during screening assays against *C. tritaeniorhynchus*, twenty five isolates were pathogenic. The isolates HP7, SC, CHP5, CAMP1, RK1, SE1, SL1, CHP1, KK1, LP1, RP1, TZ1, CHTP1, LL1, CH1, RD2, LP4, LT1, SK1, LP2, LP3 and SH1, which caused 60 - 100% mortality during the pre-selective assays, were used to determine the LC_{50}, LT_{50} and growth curve studies.

Mortality

The isolates HP7 and SC1 showed 100% of mortality [1.34 - 2.95 X 10^7 cells/ml], followed by CHP5, CAMP1 and RK1 with 95% [1.62 – 7.53 X 10^6 cells/ml], SE1 with 90% [2.13 X 10^6cells/ml], SL1 with 85% [2.66 X 10^6cells/ml], CHP1, KK1, LP1, RP1 and TZ1 with 80% [1.17 - 8.05 X 10^5 cells/ml], CHTP1, LL1, CH1, and RD2 with 75% [1.51 - 7.03 X 10^4 cells/ml], LP4, LT1, SK1, SL2, SR1 and WP1 with 70% [3.51 - 7.96 X 10^4 cells/ml] and LP2, LP3 and SH1 with 60% [1.42 - 4.04 x 10^4 cells/ml] of mortality at the fifth day after the treatment application. The reference strains *Bt.al* 4C1 [2.09 X 10^6 cells/ml] and *Bti* 4Q1 [1.85 X 10^6 cells/ml] showed 90% of mortality, while 55% and 60% of mortality by *Btk* 4D1 [5.78 X 10^6 cells/ml] and *Btai* 4J3 [2.37 X 10^4cells/ml] (Table 14).

Lethal concentration

The virulence assays showed that SC1 and HP7 were the most active isolates, with an LC_{50} of 2.46 X $10^1$and 3.90 X 10^1 cells/ml. *Bt* isolates CHP5, CAMP1, RK1, SE1 and SL1 presented an LC_{50} between 1.57 X 10^2 and 2.7 X 10^2 cells/ml (Table 14). LC_{50} of 1.02 X 10^3 - 7.19 X 10^3 cells/ml was observed in CHP1, KK1, LP1, RP1 and TZ1

isolates. Thirteen *Bt* isolates (CHTP1, LL1, CH1, RD2, LP4, LT1, SK1, SL2, SR1, WP1, LP2, LP3 and SH1) showed a lethal concentration ranging between 1.06 X 10^4 – 5.94 X 10^4 cells/ml (Table 14).

Lethal time

The median lethal time (LT_{50}) for *Bt* varies according to the concentrations (cells/ml), being inversely proportional to the concentration. The LT_{50} for the twenty five *Bt* isolates ranged between 2.36 and 8.75 days(Table 14). The LT_{50} for 1 x 10^4 *Bt* cells/ml concentration was 2.36 and 3.69 days for *Bt* isolates SC1 and HP7, followed by 4.51 – 5.71 days for the isolates *CAMP1*, RK1, CHP5, SE1 and SL1, 6.42 – 6.83 days for CHP1, TZ1, KK1, LP1, and RP1 isolates and finally by 7.56 – 8.75 days for the isolates RD2, CH1, CHTP1, LL1, SL2, WP1, LP4, SK1, LT1, SR1, LP3, SH1, and LP2. The pathogenicity of *Bt* isolates against *C. tritaeniorhynchus* was confirmed by presence of its cells under microscope observation of mixed larval guts, at the fifth day after the treatment.

Lepidopteran larvicidal bio-assay

Out of the 29 isolates of *Bt* tested against *Galleria melonella*, 16 isolates were found to be pathogenic. Ten isolates along with the standard gave 100% mortality at 1x10^6 spores and crystal per ml concentration. None of the screened cultures gave 100% mortality at a low dose of 1×10^4 and 1x10^5 spores and crystal per ml (Table15, Figure 11,12).

Table15. Mortality of *G. melonella* at different concentrations of *Bt* spores and crystal.

Culture code	Diet incorporation method					
	1×10^{-4}		1×10^{-5}		1×10^{-6}	
	% of mortality	Time of host death(days)	% of mortality	Time of host death(days)	% of mortality	Time of host death(days)
KK1	75.25	4.4-5.1	85.63	3.1-4.2	100.00	0.5-1
CHP5	77.14	4.3-5.4	86.92	3.6-4.3	100.00	0.5-1.0
CHTP	-	-	55.25	7.6-8.1	63.43	6.5-7.3
RRK	74.13	4.1-5.5	88.15	2.5-3.6	99.25	0.5-1.0
SL1	76.87	2.8-3.5	85.25	1.2-2.3	100.00	0.5-1.1
SK1	72.16	6.3-7.2	83.26	5.1-6.2	98.14	3.2-4.1
SC1	70.34	2.5-3.5	84.17	1.3-2.1	100.00	0.5-1.1
SR1	75.76	2.1-2.7	83.71	1.2-1.8	100.00	0.5-1.0
CH1	76.13	2.5-3.3	87.63	1.7-2.1	100.00	1-1.5
LT1	79.26	2.5-3.3	83.46	1.2-2.3	100.00	0.5-1.0
LP2	71.61	3.6-4.2	82.71	23.34	97.68	1.1-2.2
LP3	77.93	2.6-3.2	86.83	1.2-2.3	100.00	0.5-1.0
LP4	78.56	2.3-3.0	87.68	1.2-1.8	100.00	0.5-1.0
RP1	74.87	3.3-4.2	86.37	1.2-3.2	100.00	0.5-1.1
SL2	71.68	9.3-9.9	83.17	8.1-9.5	97.32	7.1-8.6
TZ1	77.16	7.2-8.4	81.43	6.2-7.4	95.16	5.3-6.1
Control	-	-	-	-	-	-
Bt k	79.16	1.3-2.2	88.68	0.9-1.5	100.00	0.5-1.1

Figure 11. Toxicity assay against G. melonella (A,B, C, D denotes three different doses 1×10^{-4} ,1×10^{-5} , 1×10^{-6} & control)

Figure 12. Dead cadavers of G.melonella larvae

***cry* gene and protein profiling with respect to dipteran larvae toxicity**

Significant difference in toxicity (mortality, LC_{50} and LT_{50}) was observed between 25 isolates in relation to habitat and *Bt* source. ANOVA analysis of twenty five *Bt* isolates screened from agricultural and shifting cultivation habitats showed higher mortality (F value – 88.526, df 14, $p<0.0001$), lower lethal dose (F value – 5.39×10^{10}, df 14, $p<0.0001$), and lesser time to kill (F value – 691.87), df 14, $p<0.0001$) than forest, aquatic and fallow habitats. All the 25 *Bt* isolates possess Cry4 and Cry9 genes and shared 43 and 65 kDa proteins(Table 13). Significant differences in terms of toxicity and Cry gene and protein profiling were observed in the *Bt* isolates screened from the same habitat (e.g. Lengpui, Sailam, Chhippui) (Table 14).

Growth curve studies

The bacterial growth curve with identifiable lag, exponential and stationary phases were obtained as OD was measured at regular intervals. The maximum OD (referred as log phase) was seen between 24-42 h for *Bt* standard strains and between 18- 24 h for *Bt* natural isolates. The *Bt* cultures reached the stationary phase between 24 and 48 hours. But after 48 hours *Bt* growth was inhibited, due to the depletion of media components and may be due to release of secondary metabolites (Table 14).

The growth of the bacterium was monitored for 48 h, as *Bt* reached its sporulating stage within that time. Further, positive correlation was observed during log phase in all the chosen *Bt* cultures (r – 0.9752-0.9990 and r^2 – 0.9511-0.9909) (Table 14). In Btk and Btal, the log phase was observed between 24 and 42 h with OD values

of 1.139-1.251 and 0.766-1.251, respectively. The log phase was attained within 18-21 h in SC1 (OD value – 0.66-0.67) and 21-24 h in KK1 (OD value – 0.66-0.79) and SL2 (OD value – 0.742-0.78), respectively. Higher growth index (0.76) and lower mean generation time (0.9) and growth rate constant (0.01) were registered in SC1 and HP7 whereas, lower growth index (0.2) and higher mean generation time (3.32) and growth rate constant (2.18) were observed in KK1,SK1 and CHTP1 isolates. SC1 and HP7 *Bt* isolates took lesser time to reach stationary phase and crystal production whereas, longer time (2.65 -3.32 h) was observed in KK1, SK1, CHTP1, SC2 and LT1 isolates (Table 15).

Table 15. Mean generation time (g), growth rate constant (μ) and growth index (c) of *Bt* isolates

Name of the isolate/strain	*Bt* cells/ml	Mean generation time(g)	Growth rate constant (μ) in 39 hrs.	Growth index(C)	Correlation coefficient	
					r	r^2
Bt. kurstaki 4D1	5.78 x 10^6	1.48 ± 0.30	0.02 ± 0.63	0.46 ± 0.72	0.9955	0.9909
Bt. Israelensis 4Q1	1.85 x 10^6					
Thenzawl TZ1	1.66 x 10^5					
Bt. alesti 4C1	2.09 x 10^6	2.03 ± 0.65	0.03 ± 0.60	0.34 ± 0.19	0.9752	0.9511
Sailam SL1	2.66 x 10^6					
Chhippui CHP1	2.02 x 10^5					
Khengkhawng KK1	1.17 x 10^5	3.32 ± 0.22	2.18 ± 0.75	0.20 ± 0.85	0.9920	0.9841
Serkawr SK1	3.51 x 10^5					
Chhimtuipui CHTP1	2.36 x 10^5					
Sailam SL2	6.96 x 10^5	2.65 ± 0.74	0.04 ± 0.72	0.26 ± 0.07	0.9830	0.9663
Lengte LT1	3.65 x 10^5					
Serchhip SC1	7.53 x 10^6	0.90 ± 0.53	0.01 ± 0.60	0.76 ± 0.54	0.9909	0.9819
Hmunpui HP7	1.62 x 10^6					

RAPD – PCR

Out of the 26 random primers screened (Table 3), only 15 primers produced clear and highly reproducible amplicons and were considered for further analysis (Table 16, Figure 13). The total number of polymorphic bands observed was 1292, with BT15 primer producing the maximum of 134 bands, whereas BT7 primer with a minimum of 45 bands. Polymorphism was found between *Bt* isolates from different habitats. The percent polymorphism ranged from 75 to 100%. The average PIC - 0.412 (0.296 – 0.478); RP - 0.602 (0.36 – 0.858); EMR - 0.877 (0.750 – 1.00) and MI - 0.362 (0.222 – 0.430) values were high showing better discriminatory power and marker efficiency. The polymorphism between isolates was high and 15 primers, used in the present study, were efficient in differentiating the *Bt* isolates (Table 2). The chosen primers produced a range of 3 to 14 bands, allowing confirming at least eight different groups per primer. RAPD reactions with the 15 primers yielded characteristic products ranging from 150 to 2500 bp. High number of amplification products was obtained with the primers BT1,10,11,15,18 and 24 followed by moderate (BT 5,6,8,12,16,17 and 23) and low by BT3 and 7(Table 16).

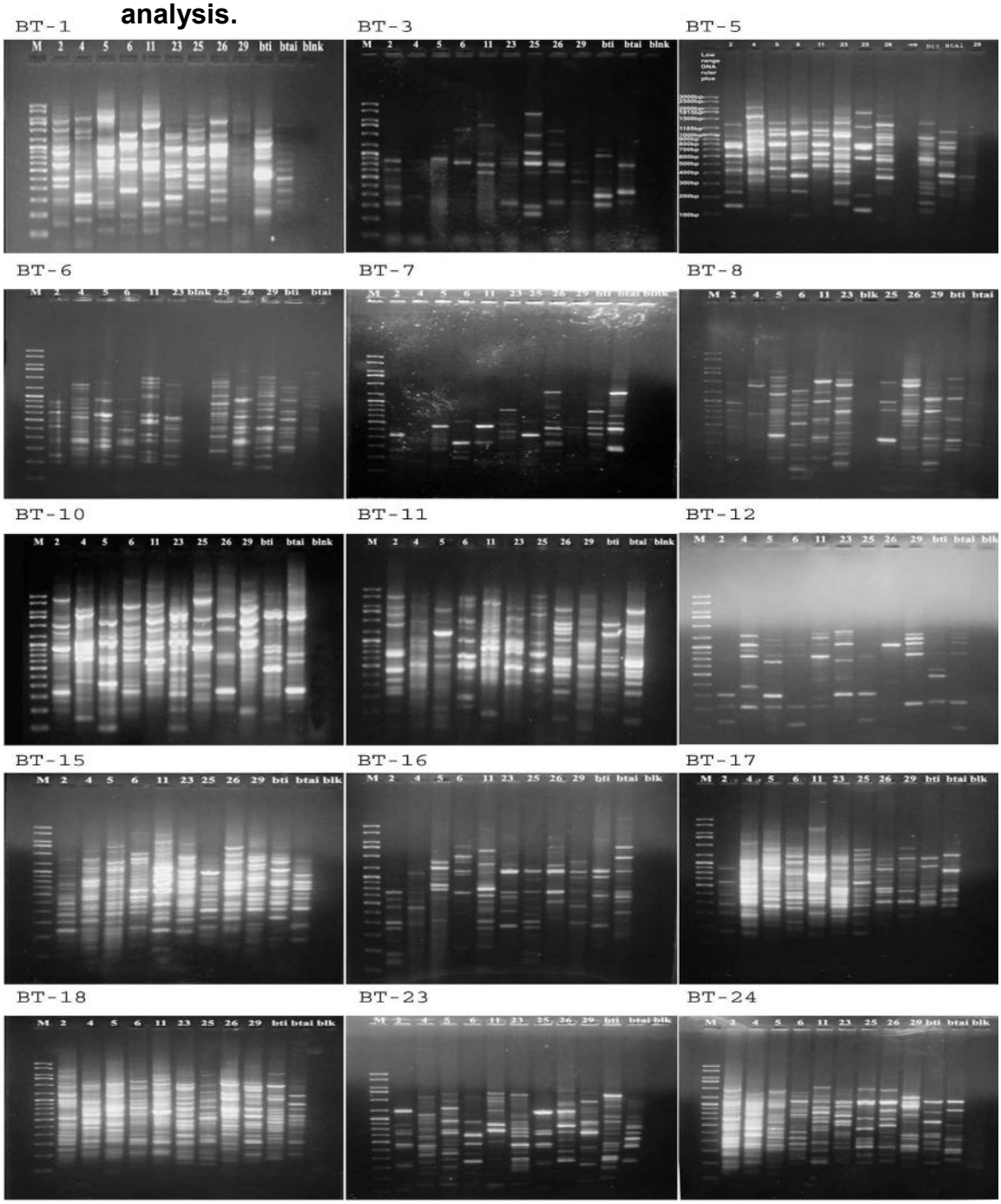
The dendrogram obtained using Darwin with a bootstrap value of 5000 is shown in Figure 14. The overall topology of majority of *Bt* strains was dissimilar. The dendrogram represents three major clades wherein polymorphism was observed between cluster 1 (garden, wayside, barren land) and cluster 2 (jhum, shrub, grass, aquatic habitats). The first cluster was again divided into four sub clusters, namely Btaiz

and Mzubt26; Bti; Mzubt29; and Mzubt25. The second sub-cluster was also again divided into three subclusters namely Mzubt23 and Mzubt4; Mzubt 2; and Mzubt 11 and Mzubt5. The third cluster contained the isolate Mzubt6 (Figure 13). It was observed that the clades were clustering based upon the source of isolation habitat irrespective of the *cry* gene content. The *cry* gene combination does not play a role in the arrangement of the tree.

Table 16. RAPD analysis of *Bt* isolates and standard strains.

S. No.	Primer used	Total no. of bands	Polymorphic bands	Monomorphic band(s)	Polymorphism %	Frequency	PIC	RP	EMR	Marker Index
1	BT-1	112	101	1	90.17	0.299	0.419	0.598	0.901	0.378
2	BT-3	44	33	1	75	0.181	0.296	0.362	0.750	0.222
3	BT-5	119	108	1	90.75	0.373	0.467	0.746	0.907	0.424
4	BT-6	96	85	1	88.54	0.241	0.365	0.482	0.885	0.323
5	BT-7	45	45	0	**100**	0.240	0.364	0.480	1.000	0.364
6	BT-8	94	72	2	76.59	0.294	0.415	0.588	0.765	0.317
7	BT-10	93	82	1	88.17	0.242	0.366	0.484	0.881	0.323
8	BT-11	109	98	1	89.9	0.267	0.391	0.534	0.899	0.351
9	BT-12	66	55	1	83.34	0.272	0.396	0.544	0.833	0.330
10	BT-15	156	134	2	85.89	0.429	0.489	0.858	0.858	0.420
11	BT-16	86	75	1	87.2	0.289	0.410	0.578	0.872	0.358
12	BT-17	108	97	1	89.81	0.350	0.455	0.700	0.898	0.408
13	BT-18	123	112	1	91.05	0.338	0.447	0.676	0.910	0.407
14	BT-23	100	89	1	89	0.303	0.422	0.606	0.890	0.375
15	BT-24	117	106	1	90.59	0.397	0.478	0.794	0.905	0.433
Total		1468	1292	16	88.01	4.515	6.188	9.030	0.880	0.363
						Average	0.412	0.602	0.877	0.362

Figure 13: Gel electrophoresis photo of the 15 primers selected for RAPD analysis.



M = Low range DNA ruler plus (Marker sizes in base pairs – 3000, 2500, 1815, 1500, 1185, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100)

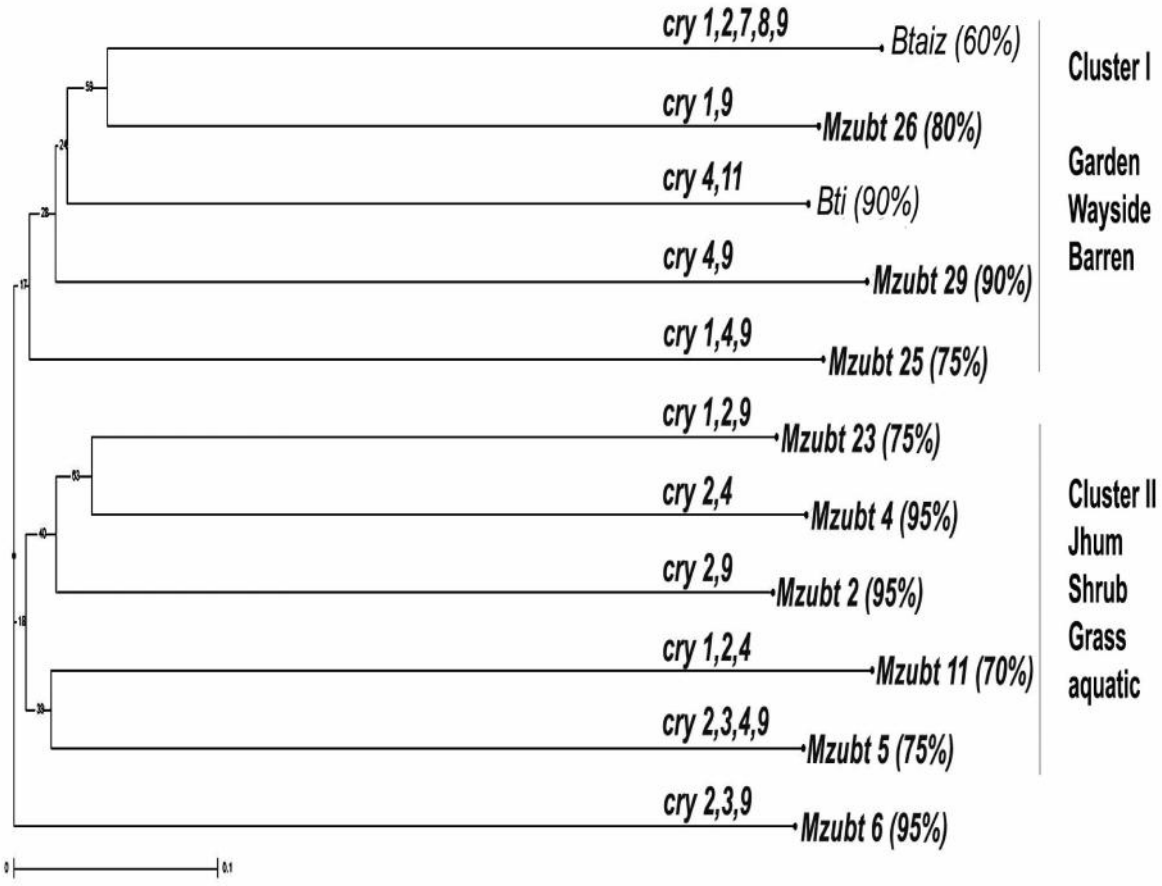
Strain no *Mzubt* = 2, 4, 5, 6, 11, 23,25,26,29

Bti = *Bacillus thuringiensis israelensis*

Btai = *Bacillus thuringiensis aizawai*

Blnk or Blk or –ve = blank

Figure 14: Phylogenetic tree constructed using RAPD-PCR analysis of bacillus thuringiensis isolates with two standard strains *Bt. Israeliensis and Bt. Aizawai* showing mortality percentage against Dipteran larvae.



16s rRNA gene characterization through PCR

All samples gave a clear band when run in 1.5 % Agarose gel (Figure 15). Low range DNA ruler plus (Bangalore's genei) which contains 16 bands of double stranded linear DNA fragments ranging from 100 bp to 3 kb was used as a marker. The PCR product was around 1500bp. Two 16s rRNA of local isolates mzubt 6 and mzubt 29 (ramrikawn,RRK and serkawr2) was sequenced and a phylogenetic tree was buit using MEGA5.0. The tree with the highest log likelihood (-11431.0700) was chosen (Figure 16). The sequence of RRK and SK2 was found to be highly similar with the sequences obtained from NCBI database.

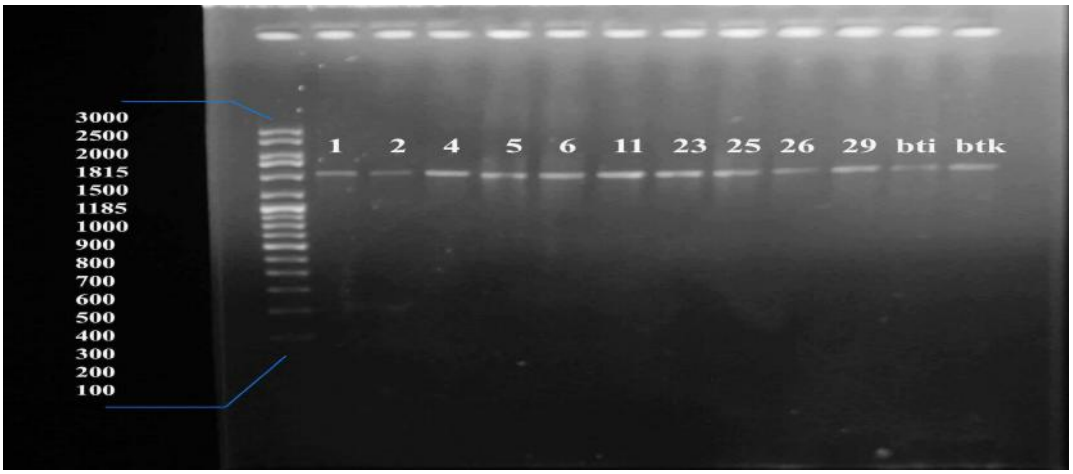


Figure 15. 16sRNA gene identification of selected strains.

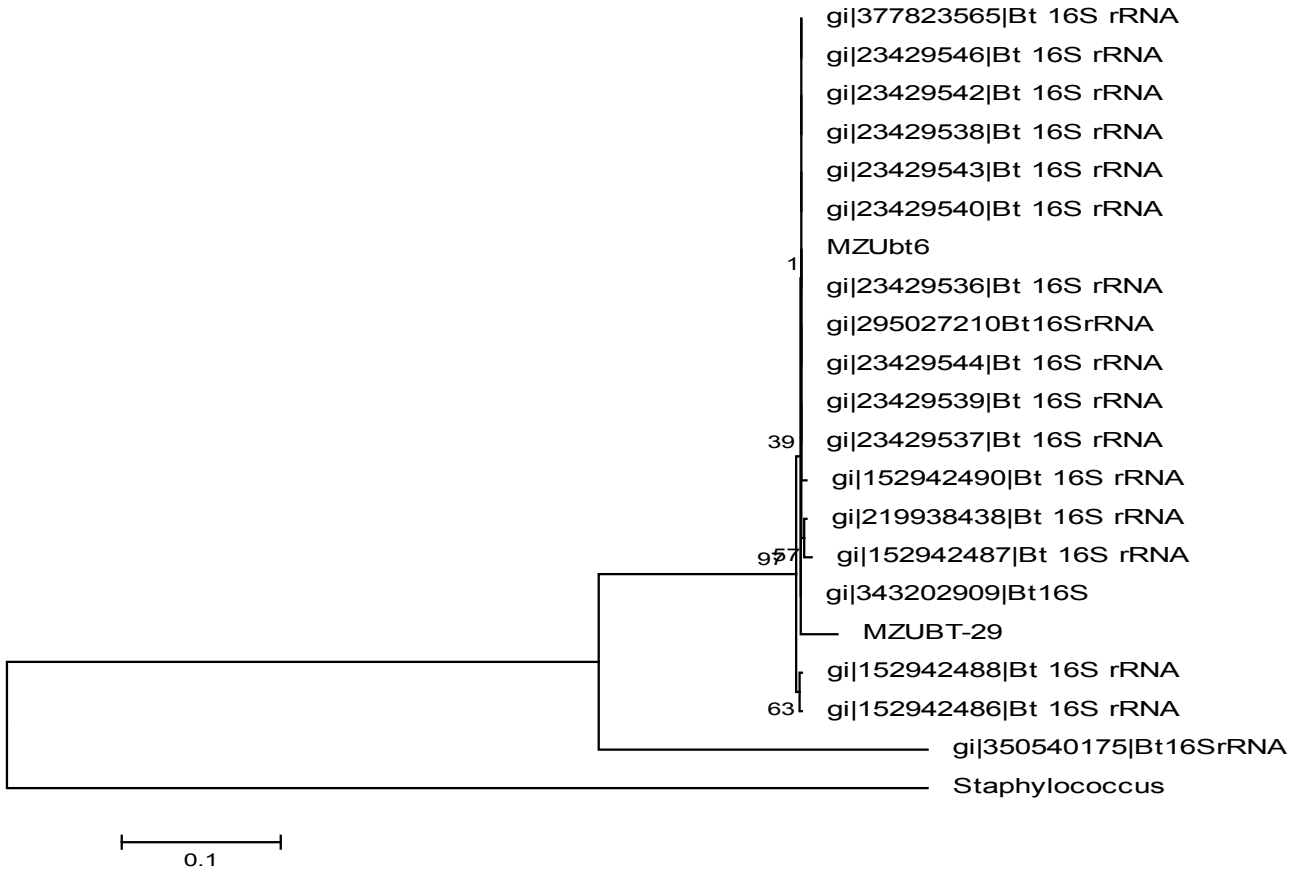


Figure16. Molecular Phylogenetic anaylsis by Maximum Likelihood method
The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-2421.2749) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

5. DISCUSSIONS

Population growth rates globally have so outstripped the linear rate of increases in food production that the Food and Agriculture Organization of the United Nations (FAO) estimated that 70% more food must be produced over the next four decades in order to nourish adequately a human population projected to exceed 9 billion by the year 2050 (FAO, 2009). The odds for attaining such an unprecedented increase, which would require the raising of the historically linear increases in annual food production by 37% (Tester *et al*., 2010), is substantially lessened by the consequences of climate change and variations on crop production systems (Beddington *et al*., 2011).

Providing adequate supply of food and improving the health of a rapidly increasing human population are two of the greatest challenges of today. The annual rate of food production increase in tropical developing nations is less than 1.0% while in most of these countries the population is growing at an annual rate of 2.0% (Youdeowei and Service, 1983). Thus there is a serious gap between food supply and demand. Crop losses to weeds, animal pests, pathogens and viruses continue to reduce available production of food and cash crops worldwide. Actual crop protection depends on the importance of pest groups or its perception by farmers and on the availability of crop protection methods. Actual loss rates show higher coefficients of variation than absolute losses in kg/ha (Oerke, 2000).

Without pesticides, an estimated two-thirds of all crops would be lost, depriving millions of peoples of food. Agricultural economists have estimated that in the absence of pesticides, production costs could increase by between 60 and 200% depending on the nature of the crop (Thygarajan, 1988). Of all the pesticides, insecticides are the most used. The proportion of pesticides used in 38 developing nations in 1973 was 60.2% insecticides, 30.4% fungicides and 3.4% herbicides (Youdeowei and Service, 1983).

Excessive use of insecticides for pest control has resulted in resistance development, pest resurgence, residual toxicity, environmental pollution, replacement of beneficial and non target species, outbreak of secondary pests and accumulation of harmful levels of pesticide residues in food and fodder (Devine and Furlong, 2007). To overcome these problems, eco-friendly approaches are being advocated. Synthetic chemical insecticides provide many benefits to food production and human health, but they also pose some hazards. Because of broad spectrum of toxicity many conventional insecticides raise concerns about human safety and the environment. Further, evolution of resistance to insecticides has occurred in more than 500 species of insects (Georghiou and Lagunes, 1991).

These problems with conventional insecticides are spurring the search for alternatives. One such alternative is the use of microbial insecticides- insecticides that contain microorganisms or their by-products. Microbial insecticides are especially valuable because their toxicity to non-target animals and humans is extremely low.

Compared to other commonly used insecticides, they are safe for both the pesticide user and consumers of treated crops. Microbial insecticides also are known as biological pathogens, and biological control agents. The most successful insect pathogen used for insect control is the bacterium *Bt*, which presently is 2% approximately of the total insecticidal market. *Bt* is almost exclusively active against larval stages of different insect orders and kills the insect by disruption of the midgut tissue followed by septicemia caused probably not only by *Bt,* but probably also by other bacterial species (Raymond *et al.,* 2010).

Bacillus thuringiensis (*Bt*) is the oldest commercial biopesticide and has been in use under various formulations since 1938 (Weiser, 1961), the first commercial product being released in 1959. There is a long history of assessment of toxicology and environmental impact associated to the use of *Bt* insecticidal proteins, which led to the conclusion that these proteins are safe for non-target organisms and especially for mammals. The overall broad spectrum of *Bt* as a species associated with the narrow host range of each individual toxin also make this group of insecticidal proteins very attractive with respect to both efficiency and environmental safety.

Bt is the most promising and widely used microbial control agent because of its activity against important Lepidopteran and Dipteran pests and its relatively easy production in fermentors. *Bt* is an aerobic gram positive spore-forming bacteria characterized by the production of proteinaceous crystalline inclusions (crystals during sporulation.). A *Bt* strain can harbour more than one crystal and some crystals are

found to be comprised of several proteins (Endotoxins) are valued for their specific insecticidal activity and non-toxicity towards mammals. Variation of a single amino acid in the endotoxins can significantly influence the level of toxicity. Hence, it is essential to isolate and screen new *Bt* strains against pest insects. *Bt* is a major source for transfer of genes into plant genomes to develop insect resistant transgenic crop plants. Despite an earlier view that insects would not develop resistance to microbial insecticides, now it is realized that insect resistance to *Bt* can evolve rapidly under situations of selection pressure. However, the insect lines which developed resistance to a particular kind of *Bt* crystal proteins were found to be sensitive to a different kind of *Bt* crystal protein. Therefore, searches are continued throughout the world to isolate novel *Bt* crystal proteins, which might be effective killing the *Bt*-resistant insects (Ferre *et al.,* 1995).

India is considered as one of the centers of biodiversity (Khoshoo, 1994). This study is a part of the larger effort to sample biodiversity of *Bt* from Mizoram to contribute the diversity in India. Search for novel *Bt* strains may lead to the discovery of additional insecticidal proteins with higher toxicity and/or wider spectrum (Ramalakshmi and Udayasuriyan, 2010; Martin and Travers, 1989). New variants of the already known *cry* gene subgroups could encode crystal proteins with significant difference in the level and spectrum of toxicity due to variation in their sequences (Xue *et al.,* 2008). The soil samples of Mizoram (one of the biodiversity hotspots) may yield new isolates of *Bt* with novel Cry proteins which could be used for control of insect pests. In the present study, 55 soil samples from five different habitats of the Mizoram were used as a source material for isolation of indigenous *Bt* strains.

The taxonomy of *B. thuringiensis* has been the subject of study since the late 1950s. Methods of identifying *Bt* varieties were proposed, based on morphological and biochemical characterization, using conventional microbiological techniques (Heimpel and Angus, 1958). H-serotyping, the immunological reaction test to the flagellum antigen (de Barjac and Bonnefoi, 1962; 1973) was also developed early and is still widely accepted for classification and identification of *Bt* (de Barjac and Frachon, 1990; Lecadet *et al*., 1999). Morphological and biochemical characterization of *Bt* isolates from Mizoram resulted in proper identification and classification, in accordance with the standard strains used, and they produced oval, bipyramidal and spherical crystals. The present study is the first scientific documentation of *Bt* from the soils of Mizoram.

Results of the present study showed that about 52.72% of the 29 samples were positive for *Bt* and yielded 107 isolates. Earlier studies reported varied frequency for isolation of *Bt* from soil samples ranging from 3 to 85% (Ramalakshmi and Udayasuriyan 2010; Martin and Travers 1989; Wang *et al*., 2003). Moderate frequency (52.72%) for isolation of *Bt* from the five different habitats, in the present study, may be due to large amount of nutrients in the soil itself, allowing optimum survival and enrichment in the soils. Soil is very important source of *Bt* strain providing a large genetic resource for its use in the development of bioinsecticide to control insect pests (Quesada-Moraga *et al.,* 2004). The *Bt* index from Mizoram ranged from 0.010 to 0.015 in the soil samples studied. Extreme values were frequently reported in several studies to range from 0 to 0.2 in United States (DeLucca *et al*., 1981), 0.2 to 0.5 in New Zealand

(Chilcott and Wigley, 1993), 0.75 in Bangladesh (Hossain *et al*., 1997), 0.009 to 0.380 in Thailand (Martin and Travers, 1989) and 0.15 to 0.18 in Western Ghats, India (Ramalakshmi and Udayasuriyan, 2010). Vilas-Bôas and Manoel (2004) suggested the *Bt* index may be a consequence of biotic environmental factor, e.g., microorganism in the soil, the type of insect commonly found in the area, or the vegetation besides, abiotic factors such as the pH, texture, oxygen and nutrient availability, temperature, and humidity.

In the present study, 107 of the 8,676-stained bacterial colonies observed through phase contrast microscopy showed the presence of crystalline inclusions, and were characterized into three major groups viz., spherical, oval and bipyramidal. These findings differed from the earlier reports (Bernhard *et al.,* 1997; Ramalakshmi and Udayasuriyan 2010; Martin and Travers 1989), wherein strains with bipyramidal (46%) and cuboidal (26.9%) crystals were predominant. Differences observed in the morphology of crystalline inclusions of *Bt* suggested presence of diversity in the *Bt* isolates of Mizoram.

Grouping of *Bt* isolates according to crystal protein(s) profile studied by SDS-PAGE will give a prelude for the presence of diversity in *cry* genes. The lepidopteran-active *cry*1 (130–140 kDa), lepidopteran and dipteran-active *cry*2 (65-75 and 135 kDa), coleopteran active - *cry*3 (66-73 and 70-75 kDa), dipteran-active *cry*4 (125-145, 68, 72,78,128, 130-140 and 135 kDa), *cry*10 (80 kDa) and *cry*11 (67-94 and 80 kDa), *cry* 5-9 gene (129, 73, 75, 35 and 38 kDa) show toxicity to different insect orders were

described by Crickmore *et al*.,(2010). Therefore, analysis of crystal proteins(s) profile could be useful to predict the presence of *cry* genes. In the present study, 60 of the 107 isolates are having 135 and/or 65 kDa proteins suggesting the presence of genes related to *cry*4 and *cry*9 families. Other isolates showed that the presence of 43 (group I) or 30 (group II) or 43 and 30 (group III) or 17 and 60 (group VII) or 19 (group VIII) or 27 and 95 (group IX) or 14 and 25 (group X) or 22 and 106 (group XI) kDa proteins indicating the presence of other novel *cry* genes also. Among the eleven groups identified, groups IV-VI are more predominant (56.07%) and group VII-XI are novel *Bt* isolates (22.42%). These results led us to suggest the presence of diversity in *Bt* isolates of Mizoram.

Cry proteins that could be of particular interest for this study are: Cry4 and Cry9 since they have been reported as effective against dipterans especially mosquitoes (Schnepf *et al.,* 1998) and most abundant in Mizoram soils. The characterizations for most of the *Bt* collections were based on bioassays against different insect larvae without identification of the Cry genes present in the *Bt* strains. In this study, 25 isolates were selected for toxicity, Cry gene and protein profiling. All the *Bt* isolates showed 60 - 100% mortality, low LC_{50} values and possess Cry 4 and/or 9 genes.

Optical Density is an indication of cell numbers in liquid media, enables the time necessary to reach stationary phase and crystal formation to be determined. The growth curve analysis was performed to determine the time required by the bacterium to reach its vegetative or exponential phase in a batch culture with shaking. In growth curve

studies, culturing conditions such as incubation time (24-48 h) and growth medium (LB agar) significantly influenced final yield. Further, log phase was varied between *Bt* cultures which is the major variable that determines the population size, production of cry toxin and their toxicity (Crickmore *et al*., 2010). From the growth curve it is evident that the bacterium remains in its vegetative or exponential phase from 21- 42 h. The result of this experiment was very much in accordance to the growth pattern demonstrated by Sattar *et al*., (2008) for a different *Bt* strain. Thus the protein was harvested from the culture supernatant after 24 h of culture, expecting maximum yield of vegetative proteins secreted by the *Bt* strain. The time taken to harvest the fermentation varied among the *Bt* cultures used.

The *cry* gene and protein profiling and toxicity (mortality, LC_{50} and LT_{50}) of *Bt* isolates significantly varied within habitats. The differences in toxicity of *Bt* isolates to *C. tritaeniorhynchus* may be related to the composition of *cry* gene and protein, crystal production and their toxic potential. *Bt* isolates SC1 and HP7 have Cry4 and Cry9 genes related to high toxicity (low LC_{50}, lesser LT_{50}, lesser time for crystal production and harvesting and 100% mortality), while CHTP1, LL1, CH1, RD2, LP4, LT1, SK1, SL2, SR1, WP1, LP2, LP3 and SH1 isolates have one to three cry gene combinations (Cry4/Cry9/Cry3,9/Cry4,9/ Cry1,2,4/ Cry1,4,9 and Cry2,3,9) and showing high LC_{50}, longer LT_{50}, longer harvesting time and 60 - 75% mortality. Distribution of *cry* gene and proteins and their toxicity to *C. tritaeniorhynchus* varied between *Bt* isolates which may be influenced by habitat, interaction between *cry* genes, expression of *cry* gene, crystal morphology and production and bacterial growth conditions. . The presence of different

cry genes in the same *Bt* strain has been reported, for example Aronson (1994) and Ben-Dov *et al*. (1997) reported the presence of *cry*1 and *cry*3, *cry*8, or *cry*7 genes in the same *Bt* strain. Chowanadisai *et al*. (1995) found highly effective isolates for controlling *Aedes* mosquitoes larvae, namely S-KB1802, S-KB1001 and S-KB2701, which showed LC50 at 1.28x102, 3.59x102 and 9.80x102 spores/ml, respectively. The importance of the isolation of native strains of *Bt* in the mosquito management program in Mizoram is important based on the findings.

Jansen *et al.,* (1997) reported Cry9 was toxic to *S. litura, S. exigua, H. armigera* especially *P. xylostella* that is resistant to *cry*1 gene groups. The toxicity of *Bt* did not depend on cry gene content only because factors other than Cry proteins may contribute to toxicity as well as spore interaction with crystal protein and the other soluble toxins such as β-exotoxin (Porcar *et al*., 2000). The identification of known cry genes in the *Bt* strains is important, since the specificity of action is known for many of the Cry toxins. This fact allows the possibility of selecting native strains that could be used in the control of some targets and of selecting strains with the highest activity. The PCR screening is a rapid method for detecting and differentiating *Bt* field strains by their PCR product profiles and for predicting their insecticidal activities in order to direct them for subsequent toxicity assays against lepidoptera, coleoptera and diptera.

RAPD analysis of *Bt* isolates in Jordan revealed high polymorphism between isolates which is in accordance with the present study (Sadder *et al.,* 2006). RAPD-PCR assay has been optimized that discriminate *Bt* isolates from nine different habitats soil

covering five districts of Mizoram. All the studied isolates showed a diverse RAPD patterns and were different from each other in relation to habitats, toxicity and type of *cry* gene present. High polymorphism was observed between *Bt* isolates which was authenticated through high PIC, RP, EMR and MI values. Further, three major clusters were identified through dissimilarity analysis. No relationship found between the type of cry gene in *Bt* and their toxicity against mosquitoes. Markers generated by RAPD were used to fingerprint and elucidate phylogenetic relationships of many microorganisms such as *Aphelenchus avenae* (Ali *et al.,* 1999)**;** *Renibacterium salmoninarum* (Grayson *et al.,* 2000); *Eschericia coli* (Aslam *et al.,* 2003); *Loctobacillus plantarum* (Elegado *et al.,* 2004); *Serratia marcescens* (Enciso-Moreno *et al.,* 2004); *Xanthomonas axonopodis* (Khoodoo and Jaufeerally-Fakim, 2004) and *Staphylococcus aureus* (Casey *et al.,* 2007).

The advantage of RAPD analysis in this study is that it covers the entire genome; therefore it provides sufficient information about differences that might be present inside the genome. Williams *et al.,* (1990) showed that RAPD markers cover the entire genome, revealing coding or non-coding regions, repeated or single-copy sequences. Also, Schnell *et al.,* (1995) reported that the arbitrary nature of the primer resulted in amplified DNA products representing random samples of the entire genome. This interpretation was reinforced by Michelmore *et al.,* (1991) who reported that polymorphism in RAPD profile might be resulted from base changes that alter primer-binding sites. Similarly, Lu *et al.,* (1996) and Martin *et al.,* (2000) revealed that polymorphism might be due to structural changes in the genomic DNA that alter the

distance between two annealing sites, delete an existing site or insert a new one, insertion of a DNA segment that render priming sites to distant to support amplification or insertions that increase the distance between two priming sites without preventing its amplification. Yang and Quiros (1993) reported that the intensity of DNA bands depends on the starting copy number of a particular DNA sequence within the genome. Therefore, the differences in band's intensity could be interpreted on the basis of alterations of some DNA sequences.

Konecka *et al.* (2007) used RAPD analysis to estimate phylogenetic relationships among twelve *Bacillus thuringiensis* strains isolated from intestinal tracts of *Cydia pomonella* larvae. The result indicated a tendency of bacterial strains to cluster according to their source which in similar to the results of the findings in the present study. The *Bt* isolates from agricultural soil, aquatic and barren land formed three separate clusters and branched out according to their source or habitat. Molecular typing methods, such as Arbitrary Primer- PCR technology (Brousseau *et al*., 1993), DNA re-association measurements (Nakamura, 1994), ribosomal RNA gene b restriction fragment length polymorphism (Priest *et al*.,1994; Akhurst *et al*., 1997), ribosomal RNA gene intergenic spacer sequences comparison (Bourque *et al*., 1995), and DNA-colony hybridization and random amplified polymorphic DNA (RAPD) analysis (Hansen *et al*., 1998), have also been applied to limited numbers of *B. thuringiensis* strains.

Genotype-based molecular typing methods, 16s rRNA gene polymorphism studies (ribotyping), has proved to be very effective as a molecular taxonomic tool for estimating chromosomal genetic diversity and relationships among various bacterial species and subspecies (Saunders *et al*., 1988; Grimont and Grimont, 1991; Hernandez *et al*., 1991; Williams and Collins, 1991; Jacquet *et al*., 1992; Mugnai *et al*., 1994; Okwumabua *et al*., 1995). In the present study, minor sequence differences among the 16s rRNA gene sequences of *B. thuringiensis* strains was observed. Phylogenetic analysis using 16s rRNA sequence showed that it is highly conserved; and is a reliable method for species identification as well as authenticity of its use in molecular biology.

16S rRNA gene restriction band pattern analysis is not subjected to the constraints associated with the *B. thuringiensis* serotyping system. Using the *B. subtilis* 16S rRNA gene as a probe, Priest *et al*. (1994) examined 43 *B. thuringiensis* strains from 10 serovars by ribotyping with Hind III. The diversity of the 16S rRNA gene restriction patterns was higher for *B. thuringiensis* than for any other species examined by same technique (Verger *et al*., 1987; Saunders *et al*., 1988; De Buyser *et al*., 1989; Aquino de Muro *et al*., 1992;Jacquet *et al*., 1992). Bourque et al., (1995) examined 24 strains of *B. thuringiensis* belonging to seven serovars and two closely-related species, *B. anthracis* and *B. cereus*, by comparative analysis of the 16S to 23S ribosomal intergenic spacer sequences.

In conclusion, high *Bt* index was recorded in the shifting cultivation while lowest frequency was observed in the forest habitats of Mizoram. The *Bt* isolates of Mizoram

exhibited eleven different types of crystal protein profile revealing the molecular diversity of this bacterium in different habitats of Mizoram. *cry*4 and *cry*9 genes were found to be the most abundant in *Bt* isolates of Mizoram soils, which have strong activity against dipteran insects (*C. tritaeniorhynchus*). Genetic characterization of *Bt* isolates revealed polymorphism and exhibited diversity according to their habitat. Due to their distinctive characteristics, CHP5 , RRK, HP7 ,RP, LP1, LP4, SL1, SC1, CHTP isolates are impressive candidates for the development of new larvicidal formulations. The isolation of native strains with activity against dipteran and lepidopteran pests, gives us new tools to be introduced into the pest management program.

6. SUMMARY

- The diversity, toxicity, growth, crystal protein and gene profiling of Bt were carried out in five natural habitats (forest, agriculture, aquatic, fallow and shifting cultivation) in Mizoram state, Northeast India.

- 107 Bt colonies, which are later placed into 29 strains were isolated from a total of 55 soil samples from five different habitats covering eight districts of Mizoram.

- The isolates were morphologically, biochemically and physiologically characterized and they produced oval spherical and bipyramidal crystals.

- The highest frequency of *Bt* was recorded in the shifting cultivation (88.88%) while lowest frequency was observed in the forest habitats (31.57%).

- The Bt index of the soil samples from five different habitats in Mizoram was 0.012. *Bti* was observed at higher frequency (30.84%) and six different undescribed biochemical type combinations were observed (3.73 – 14.0%).

- Eleven different types of crystal protein profile were observed using SDS-PAGE from the isolates of *Bt* revealing the molecular diversity of this bacterium in different habitats of Mizoram.

- *cry* 4 and *cry* 9 genes were found to be the most abundant in *Bt* isolates of Mizoram soils.

- Among the *Bt* isolates against *Culex tritaeniorhynchus*, SC1 and HP7 showed high toxicity (low LC_{50}), 100% mortality, less time to kill (shorter LT_{50}).

- Ten isolates have 100% mortality along with the standard at a concentration of 1×10^{-6} against *G. mellonella.*

- Culture no.'s MZUbt-6, 15, 21, 22 (RRK, SR1, LP4 and RP1) require less time at a concentration of 1×10^{-6} than standard and promises a great value in IPM.

- In RAPD analysis, it was concluded that *cry* gene content is not involved in determining polymorphism and locus linked genes instead the source or habitat from where *Bt* is isolated determines the polymorphism.

- Phylogenetic analysis using 16s rRNA sequence showed that it is highly conserved; and is a reliable method for species identification as well as authenticity of its use in molecular biology.

1. Luria Bertani (broth)

Tryptone	:	10 g
Yeast extract	:	5.0 g
Sodium chloride	:	5.0 g
Distilled water	:	1000 ml
pH	:	7.0 -7.2

2. Luria Bertani (agar)

Tryptone	:	10 g
Yeast extract	:	5.0 g
Sodium chloride	:	5.0 g
Distilled water	:	1000 ml
Agar	:	1.5 %
pH	:	7.0 -7.2

3. T_3 medium (Travers *et al*., 1987)

Tryptone	:	3.0 g
Tryptose	:	2.0 g
Yeast extract	:	1.5 g
Sodium phosphate	:	0.05m (pH 6.8)
Manganous chloride	:	0.005 g
Distilled water	:	1000 ml
Agar	:	15.0 g

4. Nutrient agar

Tryptone	:	5.0 g
Yeast extract	:	2.50 g
Glucose	:	1 g
Agar	:	15 g
Distilled water	:	1000 ml
pH	:	7.0

5. Methyl red agar (1 liter)

Peptone : 5 gram

Beef extract : 3 gram

Sodium chloride : 3 gram

Agar : 2%

Methyl red : 10 ml.

pH : 7.0-7.2

0.2 gram of methyl red was dissolved in 10.0 ml distilled water.

6. MR-VP Broth

Peptone - 0.5gm

Di-potassium hydrogen phosphate - 0.5gm

Glucose-10%solution - 5ml (filter sterilized)

Distilled water - 100ml

pH - 7.6

Barrit's Reagent

Solution-A

Alpha-napthol - 50.0gm

Ethanol (absolute) - 95.00ml

Dissolve the alpha-napthol in ethanol with constant stirring.

Solution-B

Potassium hydroxide

Creatinine

Distilled water

Dissolve the potassium hydroxide in 75ml of water. The warm solution was cooled, creatinine added and stirred. Remaining water was added and stored.

7. Composition of Starch hydrolysis media

Peptone - 0.5gm

Beef extract - 0.38gm

Soluble starch	-	0.2gm
Agar	-	1.5gm
Distilled water	-	100ml
pH	-	7.0

8. Nitrate Broth

Beef extract	-	0.3gm
Peptone	-	0.5gm
Potassium nitrate	-	0.1gm
Distilled water	-	100ml
pH	-	7.6

Reagents

Solution-A

Alpha-napthalamine	-	0.5gm
Acetic acid(5N)30%	-	100ml

Solution-B

Sulfanilic acid	-	0.8gm
Acetic acid(5N)30%	-	100ml.

The reagents were stored in brown bottles.

9. Christensen's Urea Agar

Peptone	: 1 g
Dextrose	: 1 g
Sodium chloride	:5 g
Potassium phosphate, monobasic	:2 g
Urea	:20 g
Phenol red	:0.012 g

To prepare the urea base, dissolve the first six ingredients in 100 ml of distilled water and filter sterilize (0.45-mm pore size). Suspend the agar in 900 ml of distilled water, boil to dissolve completely, and autoclave at 121^{o}C and 15 psi for 15 minutes. Cool the agar to 50 to 55^{o}C. Aseptically add 100 ml of filter-sterilized urea base to the cooled

agar solution and mix thoroughly. Distribute 4 to 5 ml per sterile tube (13 x 100 mm) and slant the tubes during cooling until solidified. It is desirable to have a long slant and short butt. Prepared media will have a yellow-orange color. Store the prepared media in the refrigerator at 4 to 8^{o}C until needed. Once prepared, do not reheat the medium as the urea will decompose.

10. Tryptophan broth

Tryptone :10.0 g
L-Tryptophan :1.0 g
Sodium chloride :5.0 g
Distilled water : 1000 ml

Ferric chloride solution

Ferric chloride - 10.0gm
Distilled water - 95.0ml
Conc. HCl - 5.0ml
pH - 6.8

11. Arginine Dihydrolase Broth

Arginine dihydrolase - 19.3gm
Distilled water - 100ml
pH - 6.0

12. Peptone Broth

Peptone - 2g
Sodium chloride - 0.5gm
Distilled water - 100ml
pH - 7.4.

Kovac's Reagent

Amyl/isoamyl alchol - 150.0gm

Para-dimethyl aminobenzaldehyde	-	10.0gm
Concentrated HCl	-	50.0ml

13. Skimmed Milk Agar

Skim milk powder	-	100gm
Peptone	-	5gm
Agar	-	15gm
Distilled water	-	1000ml
pH	-	7.2

14. Ammonium salts basal medium for fermentation test

Ammonium dihydrogen phosphate	1.0 g
Potassium chloride	0.2 g
Magnesium sulphate	0.2 g
Agar	10.0 g
Distilled water	1000 ml

15. Nutrient Yeast Salt Medium (NYSM)

Glusose	: 10 g
Peptone	: 5.0 g
Sodium chloride	: 5.0 g
Beef extract	: 3.0 g
Yeast extract	: 0.5 g
Magnesium chloride	: 0.203 g
Calcium chloride	: 0.102 g
Manganese chloride	: 0.01 g
Distilled water	: 1000 ml
pH	: 7

16. Esculin

Esculin	-	0.1gm
Ferric citrate	-	0.05gm
Distilled water	-	100ml
Agar	-	1.5%
pH	-	7.6

17. Salicin fermentation test

Salicin 10% stock solution (10g sugar was dissolved in 100 mL distilled water) filter sterilized.

Peptone water

Peptone	1g
NaCl	0.5g
Distilled water	100 mL
pH	7
phenol red indicator	0.01%

5 ml of petone broth mixed with 0.5 ml of Salicin stock solution after sterilization of peptone broth.

18. Sucrose fermentation test

Sucrose 10% stock solution (10g sugar was dissolved in 100 mL distilled water) filter sterilized.

Peptone water

Peptone	1g
NaCl	0.5g
Distilled water	100 mL
pH	7
phenol red indicator	0.01%

5 ml of petone broth mixed with 0.5 ml of sucrose stock solution after sterilization of peptone broth.

19. Egg yolk powder agar (for Lecithinase production test)

Egg powder	0.5%
NaCl	0.5%
Agar	1.5%
pH	7

Agaisse, H., Lereclus, D. (1995). How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? Journal of Bacteriology. 177: 6027-6032.

Akhurst, R.J., Lyness, E.W., Zhang, Q.Y., Cooper, D.J., Pinnock, D.E. (1997). A 16S rRNA oligonucleotide probe for identification of *Bacillus thuringiensis* isolates from sheep fleece. Journal of Invertebrate Pathology. 69: 24-31.

Ali, M.R., Yamaguchi, Y., Ishibashi, N. (1999). RAPD and PCR-RFLP analysis on genetic diversity of *Aphelenchus avenae* isolates collected from Kyushu and some other districts of Japan. Japanese Journal of Nematology. 29: 24-34.

Allwin, L., Kennedy, J.S. and Radhakrishnan V. (2007). Characterization of Different Geographical Strains of *Bacillus thuringiensis* from Tamil Nadu. Research Journal of Agriculture and Biological Sciences. 3(5): 362-366.

Andrews, R.E., Faust R.M.Jr., Wabiko H., Raymond K.C., and Bulla L.B., (1987).The biotechnology of *Bacillus thuringiensis*. CRC Critical Reviews of Biotechnology. 6: 163-232.

Aquino de Muro, M., Mitchell, W.J., Priest, F.G. (1992). Differentiation of mosquito-pathogenic strains of *Bacillus sphearicus* from non-toxic varieties by ribosomal RNA gene restriction patterns. Journal of General Microbiology. 138:1159-1166.

Aronson, A.I., (1994). *Bacillus thuringiensis* and its use as biological insecticide. Plant Breeding Review. 12: 19-45.

Aslam, M., Nattress, F., Greer, G., Yost, C., McMullen, L. (2003). Origin of contamination genetic diversity of *Eschericia coli* in beef cattel. Applied and Environmental Microbiology. 69: 2794-2799.

Bartlett, J.M.S., Stirling, D. (2003). A Short History of the Polymerase Chain Reaction. PCR Protocols. 226. pp. 3–6.. ISBN 1-59259-384-4.

Beddington, J., Asaduzzaman, M., Fernandez, A., Clark, M., Guillou, M., Jahn, M., Erda, L., Mamo, T., Van Bo, N., Nobre, C.A., Scholes, R., Sharma, R., Wakhungu, J. (2011).Achieving food security in the face of climate change: Summary for policy makers from the Commission on Sustainable Agriculture and Climate Change. Copenhagen, Denmark: CGIAR Research Program on Climate Change. Copenhagen: Agriculture and Food Security (CCAFS).

Berber, I. (2004). Characterization of *Bacillus* species by numerical analysis of their SDS-PAGE protein profiles. Journal of Cell and Molecular Biology. **3**:33-37.

Beegle, C. C. and Yamamoto, T. (1992).Invitation paper (C. P. Alexander fund): History of *Bacillus thuringiensis* Berliner research and development. Canadian Entomologist. 124: 587-616.

Bel, Y.F., Granedo, T.M., Alberola, M.J., Martinez-Sebastian, Ferrė, J. (1997). Distribution, frequency and diversity of *Bacillus thuringiensis* in olive tree environments in Spain. Systematic of Applied Microbiology. 20: 652-658.

Ben-Dov, E., Zaritsky, A., Dahan, E., Barak, Z., Sinai, R., Manasherob, R., Khamraev, A., Troitskaya, E., Dubitsky, A., Berezina, N., Margalith, Y. (1997). Extended screening by PCR for seven *cry* group genes from field-collected strains of *Bacillus thuringiensis*. Applied and Environmental Microbiology. 63: 4883-4890.

Ben-Dov, E., Wang, E.Q., Zaritsky, A., Manasherob, R., Barak, Z., Schneider, B., Khamraev, A., Baizhanov, M., Glupov, G., Margalith, Y. (1999).Multiplex PCR screening to detect *cry9* genes in *Bacillus thuringiensis* strains. Applied and Environmental Microbiology. 65: 3714-3716.

Berliner, E, (1915), Ueber die schlaffsucht der Ephestia kuhniella und *Bacillus thuringiensis* n. sp. Z Angew Entomol. 2:21–56.

Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D.J., Roberts, G.M., Pauli, S., Rodgers, P., and Burges, H.D. (1997). Natural isolates of *Bacillus thuringiensis*: Worldwide distribution, characterization, and activity against insect pests. Journal of Invertebrate Pathology. 70: 59-68.

Bobrowski, V.L., Pasquali, G., Bodanese-Zanettini, M. H., Pinto, L.M.N.and Fiuza L.M. (2001). Detection of Cry1 genes in *Bacillus thuringiensis* isolates from South of Brazil and activity against *Anticarsia gemmatalis* (lepidoptera:noctuidae).Brazilian Journal of Microbiology. 32:105-109.

Bourque, S.N., Valero, J.R., Lavoie, M.C., LeÂvesque, R.C. (1995). Comparative analysis of the 16S to 23S ribosomal intergenic spacer sequences of *Bacillus thuringiensis* strains and subspecies and of closely related species. Applied and Environmental Microbiology 61: 1623-1626.

Bravo, A., Gomez, I., Conde, J., Muñoz-Garay, C., Sánchez, J., Miranda, R., Zhuang, M., Gill, S.S., Soberón, M. (2004). Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. Biochimica et Biophysica Acta. 1667:38–46.

Bravo, A., Gill, S.S., Soberón, M. (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon. 49:423–435

Brousseau, R., Saint-Onge, A., PreÂfontaine, G., Masson, L., Cabana, J. (1993). Arbitrary primer polymerase chain reaction, a powerful method to identify Bacillus thuringiensis serovars and strains. Applied and Environmental Microbiology 59, 114-119.

Bulla, L.A., Faust, R.M.Jr, Andrews, R, Goodman, N. (1985).Insecticidal *bacilli*, in the Moleculer Biology of the *Bacilli*. vol 2. Academic Press, Inc., New York. 185-209.

Cadavos, C.F.G., Fonseca, R.N., Chaves, J.Q., Rabinovitch, L., and Araúŕjo- Coutinho, J.P.C. (2001).Identification of entomopathogenic *Bacillus* isolated from *Simulium* (Diptera, *Simuliidae)* larvae and adults. Mem Inst Oswaldo Cruz. Rio de Janerio. 96: 1017-1021.

Carlson, C.R., Caugant, D.A., Kolstø, A.B. (1994). Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. Applied and Environmental Microbiology. 60: 1719–1725.

Carlson, C.R., Johansen, T., Lecadet, M.M., Kolstø, A.B. (1996). Genomic organization of the entomopathogenic bacterium *Bacillus thuringiensis* subsp. *Berliner* 1715. Microbiology. 142, 1625-1634.

Carson, C.R., Kolstø, A.B. (1993). A complete physical map of *Bacillus thuringiensis* chromosome. Journal of Bacteriology. **175**: 1053-1060.

Carozzi, N.B., Kramer, V.C., Warren, G.W., Evola, S., Koziel, M.G. (1991). Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Applied and Environmental Microbiology. 57: 3057-3061.

Case, R.J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W.F., Kjelleberg, S. (2007). Use of 16S rRNA and rpoB Genes as Molecular Markers for Microbial Ecology Studies. Applied and Environmental Microbiology. 73 (1): 278–88. PMC 1797146.

Casey, A.L., Worthington, T., Caddick, J.M., Hilton, A.C., Lambert, P.A., Elliott, T.S.J. (2006). RAPD for the typing of coagulase-negative staphylococci implicated in catheter-related bloodstream infection. Journal of infection. 52: 282-289.

Ceron, J., Ortiz, A., Quintero, R., Güereca L., Bravo, A. (1995). Specific PCR pimers directed to identify *cryI* and *cry III* genes within a *Bacillus thuringiensis* strain collection. Applied and Environmental Microbiolgy. 61: 3826-3831.

Ceron, J., Covarrubias, L., Ortiz, A., Quintero, Ortiz, M., Aranda, E., Lina, L., Bravo, A. (1994). PCR analysis of the *cryI* insecticidal rytal family genes from *Bacillus thuringiensis*. Applied and Environmental Microbiolgy. 60: 353-356.

Chambers, J.A., Jelen, A., Gilbert, M.P., Jany, C.S., Johnson T.M., and Gawron- Burke C. (1991). Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. *aizawai*. Journal of Bacteriology, 173: 3966-3976.

Chilcott, C.N., Wigley, P.J. (1993). Isolation and toxicity of *Bacillus thuringiensis* from soil and insect habitats in New Zealand. Journal of Invertebrate Pathology 61: 244-247.

Chaowanadisai, L., Chanpaisaeng, J., Sirirote, P.,Attathom, T. (1995). Selection of pathogenic Bacilli in Thailand against *Aedes aegypti* (L.). Mosquito-Borne Disease Bulletin. 12: 92-96.

Cocconcelli, P.S., Porro, D., Galandini, S., Senini, L. (1995). Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci. Letters in Applied Microbiology. 212: 336– 379.

Coenye, T., Vandamme, P. (2003). Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. FEMS Microbiology Letters. 228 (1): 45–49. PMID 14612235.

COHN. (1872) Untersuchungen über Bakterien. Beitrage zur Biologie der Pflanzen Heft 2. 1: 127-224.

Crickmore, N., Zeigler, D.R., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Bravo, A., Dean, D.H. (2010). Bacillus thuringiensis toxin nomenclature. http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html.

De Barjac, H. (1981). Identification of H-serotypes of *Bacillus thuringiensis.* In: Burges HD ed. Microbial control of pests and plant diseases. 1970-1980. New York, London, Academic Press Inc., pp 35-43.

De Barjac, H., Bonnefoi, A. (1962). Essai de classification biochimique et sérologique de 24 souches de *Bacillus* de type *thuringiensis.* Entomophaga, 7: 5-31.

De Barjac, H., Franchon, E. (1990). Classification of *B.t.* strains, *Entomophaga*, 35: 233-240.

De Buyser, M.L., Morvan, A., Grimont, F., Solh, N. (1989).Characterization of *Staphylococcus* species by ribosomal RNA gene restriction patterns. Journal of General Microbiology. 135: 989-999.

De Lucca, A.J., Simonson, J.G., Larson, A.D. (1981). *Bacillus thuringiensis* distribution in soil of the United States. Canadian Journal of Microbiology. 27:865-870.

de Maagd R.A., Bravo, A., Crickmore, N. (2001). How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends in Genetics 17:193–199.

De Maagd, R., Van Der Klei, H., Bakker, P.L., Stiekema,W.J., D. Bosch, (1996). Different domains of *Bacillus thuringiensis* δ-endotoxins can bind to insect midgut membrane proteins on ligand blots. Applied and Environmental Microbiology. 62: 2753-2757.

de Maagd, R.A., Bravo, A., Berry, C., Crickmore, N., Schnepf, H.E. (2003). Structure, diversity, and evolution of protein toxins from spore forming entomopathogenic bacteria. Annual Review of Genetics. 37:409–420.

De Souza, M.T., Lecaded, M.M., Lereclus, D. (1993). Full expression of the Cry IIIA toxin gene of *Bacillus thuringiensis* requires a distant upstream DNA sequence affecting transcription., Journal of Bacteriology. 175: 2952-2960.

Devine, G.J., Furlong, M.J. (2007). Insecticide use: Contexts and ecological consequences. Agriculture and Human Values. 24: 281-306.

Elegado, F. B., Guerra, M.A.R.V, Macayan, R.A., Mendozo, H.A., Lirazan, M.B. (2004). Spectrum of bacteriocin activity of *Loctobacillus plantarum* BS and fingerprinting by RAPD-PCR. International Journal of Food Microbiology. 95: 11-18.

Entwistle, P.F., Cory, J.S., Bailey, M.J., Higgs, S. (1993). *Bacillus thuringiensis*, An Environmental Biopesticide: Theory and practice, John Wiley, Chichester.

Feitelson, J.S. (1993). The *Bacillus thuringiensis* family tree, p. 63–71. In: L.Kim (ed.), Advanced engineered pesticides. Marcel Dekker, Inc., New York, N.Y.

Ferré, J., Escriche, B., Bel, Y., Van Rie, J. (1995). Biochemistry and genetics of insect resistance to *Bacillus thuringiensis* insecticidal crystal proteins. FEMS Microbiology Letters. 132:1-7.

Finney, D.J. (1971). Probit Analysis. Cambridge, London: Cambridge University Press.

Food and Agriculture Organization of the United Nations. (2009). How to Feed the World in 2050. Rome: FAO.

Forsty, G.,: Logan, N.A . (2000). Isolation of *Bacillus thuringiensis* from Northern Victoria Land, Antartica. Letters in Applied Microbiology. 30: 263- 266.

Georghiou, G.P., Lagunes, T.A. **(**1991). The occurrence of resistence to pesticides in Arthropods. FAO, United Nations, Rome.

Gerhardt, P.R., Murray, G.E., Costelow, R.N, Nexter, E.W., Wood W.A., Kreig, N.P, Phelleps G.G. (1981). Manual of methods for general bacterial. American Society of Microbiology. Washington D.C.

Gill, S.S., Sing, G.J.P., Hornung, J.M. (1987). Cell membrane activation of *Bacillus thuringiensis* subsp. *israelensis* cytolytic toxins. Infections and Immunity. 55: 1300-1308.

Glazer, A.N., Nikaido, H. (1995). Microbial insecticides, in Microbial Biotechnology Fundamentals of Applied Microbiology. W.H. Freeman and Company. New York, p. 209-229.

Goldberg, L.J., Margalit, J. (1977). A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii, Uranotaenia unguiculata, Culex univitattus, Aedes aegypti* and *Culex pipiens.* Mosquito News. 37: 355-358.

Gonzales, J.M., Carlton, B.C. (1980). Patterns of plasmid DNA in crystalliferous strains of *B thuringiensis*. Plasmid. 3: 92-98.

Grayson, T. H., Atienzar, F.A., Alexander, S.M., Cooper, L.F., Gilpin, M.L. (2000). Molecular diversity of *Renibacterium salmoninarum* isolates determined by randomly amplified polymorphic DNA analysis. Applied and Environmental Microbiology. 66 (1): 435-438.

Griffiths, J.S., Haslam, S.M., Yang, T., Garczynski, S.F., Mulloy, B., Morris, H., Cremer, P.S., Dell, A., Adang, M.J., Aroian, R.V. (2005). Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. Science. 307:922–925.

Grimont, F., Grimont, P.A.D. (1991). DNA fingerprinting. In Nucleic Acid Techniques in Bacterial Systematics ed. Goodfellow, M. and Stackebrandt, E. pp. 249-280. Chichester: John Wiley.

Hansen, B., Damgaard, P.H., Eilenberg, J., Pedersen, J.C. (1998). Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated from leaves and insects. Journal of Invertebrate Pathology 71: 106-114.

Hastowo, S., Lay, B.W., Ohba, M. (1992). Naturally occurring *Bacillus thuringiensis* in Indonesia. Journal of Applied Bacteriology. 73: 108-113.

Heimpel, A.M. (1967). A critical review of *Bacillus thuringiensis* var *thuringiensis Berliner* and other crystalliferous bacteria. Annual Review of Entomology. 12: 287-322.

Heimpel, A.M., Angus, T.A. (1958). The taxonomy of insect pathogens related to *Bacillus cereus*. Frankland & Frankland. Canadian Journal of Microbiology. 4: 531-541.

Hernandez, J., Owen, R.J., Costas, M., Lastovica, A. (1991). DNA-DNA hybridization and analysis of restriction endonuclease and rRNA gene patterns of atypical (catalase-weak/negative) *Campylobacter jejuni* from paediatric blood and faecal cultures. Journal of Applied Bacteriology. 70: 71-80.

Hiren, K. Patel , Janardan J. Jani , Harendra G. Vyas. (2009). Isolation and characterization of Lepidopteran specific *Bacillus thuringiensis* .International Journal of Integrative Biology. Vol. 6, No. 3, 121.

Herrnstand, C., Soares, G.G., Wilcox, E.R., Edwards, D.I. (1986).A new strain of

Bacillus thuringiensis with activity against coleopteran insects. *Biotechnology,* 4: 305-308.

Höfte, H., Van-Rie, J., Jansens, S., Van Houtven, A., Vanderbruggen, H., Vaeck, M., (1988). Monoclonal antibody analysis and insecticidal spectrum of three types of lepidopteran-specific insecticidal crystal proteins of *Bacillus thuringiensis*. Applied and Environmental Microbiology. 54: 2010-2017.

Höfte, H., Whiteley, H.R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiological Reviews*,* 53: 242-255.

Hossain, M.A., Sohe,l A., Siraju,l H., Ahmed, S., Hoque, S. (1997). Abundance and distribution of *Bacillus thuringiensis* in the agricultural soil of Bangladesh. Journal of Invertebrate Pathology. 70: 221-225.

Hongyu, Z., Ziniu, Y., Wangxi, D. (2000). Isolation, distribution and toxicity of *Bacillus thuringiensis* from warehauses in China. Crop Protection. 19: 449- 454.

Holt, J.G., (1984). Bergey's manual of systematic Bacteriology. Vol. I. Williams and Wilkins,Baltimore. Maryland.

Ichimatsu,T., Mizuki, E., Nishimura, K., Akao, T., and Saitoh, H. (2000). Ocurance of *Bacillus thuringiensis* in fresh waters of Japan. Current Microbiology. 40: 217-220.

Iriarte, M., Porcar, M., Lecadet, M.M, Caballero, P.(2000). Isolation and characterization of *Bacillus thuringiensis* strains from aquatic environments in Spain. Current Microbiology. 40: 402-408.

Ishiwata, S. (1901). On a new type of severe flacherie (sotto disease) (original in Japanese). Dainihon Sansi Kaiho. 114:1–5

Ishiwata, S. (1905). About “Sottokin,” a *bacillus* of a disease of the silk-worm. Dainihon Sanshi Kaiho. Report Association Sericulture. Japan. 161:1–5

Itoqou-Apoyolo, C., L. Drift, J. M., Vassal, H., DeBarjac, Bossy, J. P., Leclant, F., Frutos, R. (1995). Isolation multiple subspecies of *Bacillus thuringiensis* from a population of the europian sunflower moth, Homoesoma nebulella. Applied and Environmental Microbiolgy, 61: 4343-4347.

Jacquet, C., Aubert, S., Solh, N., Rocourt, J. (1992). Use of rRNA gene restriction patterns for the identification of Listeria species. Systematic and Applied Microbiology. 15: 42-46.

Jansen, S., vanVliet, A., Dickburt, C., Buysse, L., Piens, C., Saey, B., De-Wulf, A., Gossele, V., Paez, A., Goebel, E., and Peferoen, M. (1997). Transgenic corn expressing a Cry9c insecticidal protein from *Bacillus thuringiensis* protected from European corn borer damage. Crop Science. 37, 1616-1624.

Joung, K.B., Côté, J.C. (2001). Phylogenetic analysis of *Bacillus thuringiensis* serovars based on 16S rRNA gene restriction fragment length polymorphisms. Journal of Applied Microbiology. 90: 115–122*.*

Juarez-Perez, V.M., Ferrandis, M.D., Fructos, R. (1997).PCR-based approach for detection of novel *Bacillus thuringiensis cry* genes. Applied and Environmental Microbiology. 63: 2997-3002.

Jurat-Fuentes, J.L., Adang, M.J. (2004). Characterization of a Cry1Ac receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. European Journal of Biochemistry. 271:3127–3135.

Kaelin, P., Morel, P., Gadani, F. (1994).Isolation of *Bacillus thuringiensis* from stored tobacco and *Lasioderma serricorne* (F.). Applied and Environmental Microbiolgy. 60: 19-25.

Khoodoo, M.H.R., Jaufeerally-Fakim, Y. (2004). PARD-PCR Fingerprinting and Southern analysis of *Xanthomonas axonopodis* Pv. Dieffenbachiae strains isolated from different aroid hosts and location. Plant disease. 88 (9): 980-988.

Khoshoo, T.N. (1994). Current Science. 67 (8): 577- 582.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution. 16:111-120.

Knowles,B.H., (1994). Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxins. Advances in Insect Physiology. 24: 27-308.

Konecka E., Kaznowski A., Ziemnicka J., Ziemnicki K. (2007). Molecular and phenotypic characterisation of *Bacillus thuringiensis* isolated during epizootics in *Cydia pomonella* Larvae . Journal of Invertebrate Pathology. 94: 56-63.

Krieg, A., Huger, A.M., Langenbruch, G.A., Schnetter, W. (1983). *Bacillus thuringiensis* var. *tenebrionis,* a new pathotype effective against larvae of Coleoptera. Zeitschrift für Angewandte Entomologie 96(5): 500-508.

Kronstad, J.W., Whiteley, H.R. **(**1986). Three classes of homologous *Bacillus thuringiensis* crystal-protein genes. Gene 43**:**29–40.

Krywienczyk, J., Dulmage, H.T., Fast, P.G. (1978). Occurrence of two serologically distinct groups within *Bacillus thuringiensis* serotype 3ab variety *kurstaki.* Journal of Invertebrate Pathology. 37: 372-375.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Journal of Applied Microbiology. 59: 109-111.

Lacey, L.A., (1997). Biological Techniques-Manual of Techniques in insect pathology. Academic press. New York, U.S.A.

Lecadet, M.M., Frachon, E., Cosmao Dumanoir, V. (1999) Updating the H-antigen classification of *Bacillus thuringiensis*. Journal of Applied Microbiology. 86: 660-672.

Lee, M.K., You, T.H., Young, B.A., Cotrill, J.A., Valaitis, A.P., Dean, D.H. (1996). Aminopeptidase N purified from gypsy moth brush border membrane vesicles is a specific receptor for *Bacillus thuringiensis* Cry1Ac toxin. Applied and Environmental Microbiology. 62:2845–2849.

Lereclus, D., Lecadet, M.M., Ribier, J., Dedonder, R. (1982). Molecular relationships among plasmids of *Bacillus thuringiensis*: conserved sequences through 11 crystalliferous strains. Molecular and General Genetics. 186: 391-398.

Lopez-Meza, J. E., Ibarra, J. E. (1996).Characterization of a novel strain of *Bacillus thuringiensis*. Applied and Environmental Microbiology. 62:1306-1310.

Lu, J., kno,x M.R. Ambrose, M.J., Brown, J.K.M., Ellis, T.H.N. (1996). Comparative analysis of genetic diversity in pea assessed by RFLP– and RAPD-based methods. Theory of Applied Genetics. 93: 1103-1111.

Lynch, M.J., Baumann, P. (1985) Immunological comparisons of the crystal protein from strains of *Bacillus thuringiensis*. Journal of Invertebrate Pathology 46: 47-57.

Madigan, M., Martinko, J. (2005). Brock Biology of Microorganisms (11th ed.). Prentice Hall. ISBN 0-13-144329-1.

Maeda, M. E., Mizuki, Y., Nakamura, T., Hatano,. Ohba, M. (2000). Recovery of *Bacillus thuringiensis* from marine sediments of Japan. Current Microbiology, 40: 418-422.

Mahillon, J., Rezsöhazy, R., Ballet, B., Delcour, J. (1994). *IS231* and other *Bacillus thuringiensis* transposable elements: a review. Genetica. 93**:** 13-26.

Martin, P.W., Travers, R.S. (1989).Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Applied and Environmental Microbiology. 55: 2437-2442.

Martin, P., Dary, A., Andre, A., Decaris, B. (2000). Identification and typing of *Streptomyces* strains: evaluation of interspecific, intraspecific and intaclonal differences by RAPD fingerprinting. Research in Microbiology. 151 (10): 853-864.

Meadows, M.P., Ellis, D. J., Butt, J., Jarret, P., Burges, H. D. (1992) .Distribution, frequency, and diversity of *Bacillus thuringiensis* in an animal feed mill. Applied and Environmental Microbiology. 58: 1344-1350.

Michelmore, R.W., Paran, I., Kesseli, R.V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detected markers in segregating populations. Proceedings of National Academy of Sciences. 88: 9828-9832.

Mizuki,E., Ichimatsu, T., Hwang, S.H., Park, Y.S., Saitoh, H., Higuchi K., Ohba, M. (1999). Ubiquity of *Bacillus thuringiensis* on phylloplanes of arboreous and herbaceous plants in Japan. Journal of Applied Microbiology. 86: 979 - 984.

Moraga, E.Q., Tovar, E.G. (2004).Isolation, geographical diversity and insecticidal activity of *Bacillus thuringiensis* from soil in Spain. Microbial Research. 130: 515-537.

Mugnai, L., Giovannetti, L., Ventura, S.,Surico, G. (1994). The grouping of strains of *Pseudomonas syringae* subsp. *savastanoi* by DNA restriction fingerprinting. Journal of Phytopathology. 142: 209-218.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., Fonseca, G.A.B., Kent, J. (2000). Biodiversity hotspots for conservation priorities. Nature. 403: 853-858.

Myers, P., Yousten, A.A. (1978). Infection and Immunology. 19: 1047-1053.

Nakamura, L.K. (1994). DNA relatedness among *Bacillus thuringiensis* serovars. International Journal of Systematic Bacteriology 44: 125-129.

Oerke, E.C., (2000). Infection, damage, loss - An assessment of crop losses to pests and their avoidance. Habilitation, University of Bonn, 313pp (in German).

Ohba, M., Aizawa, K. (1986).Distribution of *Bacillus thuringiensis* in soils of Japan. Journal of Invertebrate Pathology. 47: 277-282.

Okwumabua, O., Staats, J., Chengappa, M.M. (1995). Detection of genomic heterogeneity in *Streptococcus suis* isolates by DNA restriction fragment length polymorphisms of rRNA genes (ribotyping). Journal of Clinical Microbiology. 33: 968-972.

Pelczar, M.J., Bard, R.C., Burnett, G.W., Conn, H.J., Demoss, R.D., Euans, E.E., Weiss, F.A., Jennison, M.W., Meckee, A.P., Rike,r A.J., Warren, J., Weeks, O.B. (1957). Manual of microbiological methods. Society of American Bacteriology. New York, USA : McGraw Hill Book Company, Inc., p. 315.

Perrier, X., Jacquemoud-Collet, J.P. (2006). DARwin software.

Priest, F.G., Kaji, D.A., Rosato, Y.B., Canhos, V.P. (1994). Characterization of *Bacillus thuringiensis* and related bacteria by ribosomal RNA gene restriction fragment length polymorphisms. Microbiology. 140:1015-1022.

Porcar, M., Martinéz, C., and Caballero, P. 2000. Host range and gene contents of *Bacillus thuringiensis* strains toxic towards *Spodoptera exigua*. Entomologia Experimentalis et Applicata. 97, 339-346.

Prescott M. Lansing, Harley P. John, Donald, Klein A.(2002). Microbiology, 5th edition. McGraw-Hill Higher Education.

Prevost, A., Wilkinson, M.J. (1999). A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theory of Applied Genetics. 98:107–112.

Priest, F.G., Goodfellow, M., Todd, C. (1988). A numerical classification of the genus *Bacillus*. Journal of General Microbiology. 134: 1847-1882.

Quesada-Moraga, E., Garcia-Tovar, E., Valverde-Garcia, P., Santiago-Alvarez, C. (2004). Isolation, geographical diversity and insecticidal activity of *Bacillus thuringiensis* from soils in Spain. Microbiology Research. 159: 59-71.

Ramalakshmi, A., Udayasuriyan, V. (2010). Diversity of *Bacillus thuringiensis* Isolated from Western Ghats of Tamil Nadu State, India. Current Microbiology. 61: 13–18.

Raymond, B., Johnston, P.R., Nielsen-LeRoux, C., Lereclus, D., Crickmore, N., (2010). *Bacillus thuringiensis*: an impotent pathogen? Trends in Microbiology. 18: 189-194.

Rodriguez, H., Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances.17:319–339.

Roholf, F.J. (1998) .NTSYS-pc Numerical Taxonomy and Multivariate Analysis System. Version 2. 00. Setauket, New York, USA: Exeter Software.

Roldan-Ruiz, I., Dendauw, J., VanBockstaele, E., Depicker, A., Loose, M.D. (2000). AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium spp*.). Molecular Breeding. 6:125–134.

Sadder, M.T.,Hala, K.H., Luma, A.B. (2006) Application of RAPD technique to study polymorphism among *Bacillus thuringiensis* isolates from Jordan. World Journal Microbiology and Biotechnology. 22:1307–1312.

Sambrook Joseph, Russell David W. (2001). Molecular cloning: A laboratory manual. New York; Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Saiki, R., Gelfand, D., Stoffel, S., Scharf, S., Higuchi, R., Horn, G., Mullis, K., Erlich, H. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 239 (4839): 487–491.

Sansinenea, E. (2012.). *Bacillus thuringiensis* Biotechnology, DOI 10.1007/978-94-007-3021-2_1, © Springer Science Business Media B.V.

Sattar, S., Biswas, K.P., Hossain, A.M., Maiti, K.M., Sen, K.S., Asitava Basu. (2008). Search for vegetative insecticidal proteins (VIPs) from local isolates of *Bacillus thuringiensis* effective against lepidopteran and homopteran insect pests. Journal of Biopesticides. 1(2): 216 – 222.

Saunders, N.A., Harrison, T.G., Kachwalla, N., Taylor, A.G.(1988). Identification of species of the genus Legionella using a cloned rRNA gene from *Legionella pneumophila*. Journal of General Microbiology. 134: 2363-2374.

Schaeffer, A.B., Fulton, M. (1933). A simplified method of staining endospores. Science 77**:**194.

Sekar, V. (1988).The insecticidal crystal protein gene is expressed in vegetative cells of

Bacillus thuringiensis subsp. *tenebrionis*. Current Microbiology. 17: 347-349.

Sneath, P.H.A. (1986). Endospore forming gram positive rods and cocci, In J. G. Holt (ed.)Bergey's Manual of systematic bacteriology, Springer New York. N.Y., p. 1104-1139.

Schnell, R.J., Ronning C.M., Knight, R.J. (1995). Identification of cultivars and validation of genetic relationships in *Mingifera indica* L. using RAPD markers. Theory of Applied Genetics. 90: 269-274.

Schnepf, E., Crickmore, N., Van-Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler D.R., Dean, D.H. (1998). *Bacillus thuringiensis* and its insecticidal proteins. Microbiology and Molecular Biology Reviews. 62: 774-806.

Schnepf, H.E., Whiteley, H.R. (1981). Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. Proceeding of the National Academy of Sciences USA, 57: 2893-2897.

Smith, R.A., Couche, G.A. (1991). The phylloplane as a source of *Bacillus thuringiensis* variants. Applied and Environmental Microbiology. 57: 311-315.

Stahly, D. P., Andrews, R. E., Yousten, A. A. (1991). The genus *Bacillus*- insect pathogens, in The Prokaryotes. 2nd edition. Edited by Ballows, A., Springer Verlag, New York. 1097-1745.

Smith, R.A. (1987). Use of crystal serology to differentiate among varieties of *Bacillus thuringiensis.* Journal of Invertebrate Pathology. 50: 1-8.

Suzuki, E. (2002). High-resolution scanning electron microscopy of immunogold-labelled cells by the use of thin plasma coating of osmium. Journal of Microscopy. 208: 153–157:1365-2818.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution (In Press).

Tester, M., Langridge, P. (2010). Breeding technologies to increase crop production in a changing world. Science. 327:818–822.

Thomas, D.J., Morgan, A.W., Whipps, J.M., Saunders, J. R. (2001). Plasmid transfer between *Bacillus thuringiensis* subsp. *israelensis* strains in labrotory culture, river water, and dipteran larvae. Applied and Environmental Microbiology. 67: 330-338.

Thyagarajan, G. (1988). Pesticide safety, storage and disposal. In Crop Production for Small-scale Farms in East and central Africa- a Review (Edited by Prinley R. T. and Terry P.G), Commonwealth Science Council of London, pp.89-96.

Travers, R.S., Martin, P.A., Reichelderfer, C.F. (1987). Selective process for efficient isolation of soil *Bacillus* spp. Applied and Environmental Microbiology. 53: 1263-1266.

Verger, J.M., Grimont, F., Grimont, P.A.D., Grayon, M. (1987). Taxonomy of the genus Brucella. Annales de l'Institut Pasteur Microbiology. 138: 235-238.

Vilas-Böas, G.T., Manoel, V.F.L. (2004). Diversity of *cry* genes and genetic characterization of *Bacillus thuringiensis* isolated from Brazil. Canadian Journal of Microbiology. 50: 605-613.

Visser, B. (1989). A screening for the presence of four different crystal protein gene types in 25 *Bacillus thuringiensis* strains. FEMS Microbiologie Letters 58: 121-124.

Wang, J., Boets, A., Van Rie, J., Ren, G. (2003). Characterization of *cry1, cry2 and cry9* genes in *B. thuringiensis* isolates from China. Journal of Invertebrate Pathology. 82: 63–71.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology. 173 (2): 697–703.

Weiser, J. (1961). Die Mikrosporidien a ls Parasiten der Insekten. *Monographien Zeitschrift fur Angewandte Entomologie*, 17: 149 pp.

Welsh, J., McClelland, M. (1990). Fingerprint genomes using PCR with arbitrary primers. Nucleic Acids Research. 18: 7213-7218.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research. 18: 6531-6535.

World Health Organisation. (1999). Environmental Health Criteria no: 217.

WHO/CDS/WHOPES/GCDPP/2005.13

www.greengenes.lbl.go (16s rRNA data base)

Xavier, R., Nagarathinam, P., Gopalakrishnan, Jayaraman K. (2007). Isolation of Lepidopteran Active Native *Bacillus thuringiensis S*trains Through PCR Panning. Asia Pacific Journal of Molecular Biology and Biotechnology. Vol. 15 (2): 61-67.

Xue, J., Liang, G., Crickmore, N., Li, H., He, K., Song, F., Feng, X., Huang, D., Zhang, J. (2008). Cloning and characterization of a novel *cry1A* toxin from *Bacillus thuringiensis*

with high toxicity to the Asian corn borer and other lepidopteran insects. Microbiology Letters. 280: 95–101.

Yang, X., Quiros, C. (1993). Identification and classification of celery cultivars with RAPD markers. Theory of Applied Genetics. 86: 205-212.

Youdeowei, A., Service, M.W. (1983). Pest and Vector Management in the Tropics. Longman, London.

Zeigler, D.R., (1999). *Bacillus* Genetic Stock Center Catalog of Strains, Seventh Edition,Part 2: *Bacillus thuringiensis and Bacillus cereus*, 37.