ISOLATION AND CHARACTERIZATION OF *BACILLUS THURINGIENSIS* STRAINS FROM DIFFERENT GRAIN HABITATS

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October, 2004

Isolation and Characterization of *Bacillus thuringiensis* Strains from Different Grain Habitats

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A Dissertation Submitted to
the Graduate School in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

Department: Biotechnology and Bioengineering
Major: Biotechnology

İzmir Institute of Technology
İzmir, Turkey

October, 2004

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ACKNOWLEDGEMENTS

I deeply thank my advisor Assoc. Prof. Dr. HaticeGÜNEŞ for her precious help, contributions, support, encouragement, confidence and endless patience throughout this study.

I want to add my thanks to my co-advisor Assist. Prof. Dr. Ali Fazıl YENİDÜNYA for his valuable help, contributions and support.

I also would like to thank my co-advisor Prof. Dr. Şebnem HARSA for her advice, support and confidence.

I want to thank greatfully my friends F.Tuba ÇETİNKAYA, Çelenk ÇINAR, Z.Seda ELMACI, Mert SUDAĞIDAN, Elif YAVUZ, Güney AKBALIK, Burcu OKUKLU and Seçil CERTEL for their kind support and help.

I also want to express my thankfulness to Turan AKBAŞ and Şevket APAYDIN for their help and support in Taşkale / Karaman.

Finally, I want to thank specially to my family; my sister Sinem APAYDIN, my mother Nuray APAYDIN and my father Gürses APAYDIN for their endless support, patience, encouragement and love during the preparation of this thesis-work.

Anyone else I should have thanked, but have forgotten.

ABSTRACT

Bacillus thuringiensis is a Gram positive, facultative anaerob bacteria that produces proteins toxic against different insect species. This feature makes it the most widely used biological control agent in agriculture. Since *B. thuringiensis* strains have great genetic diversity, the toxic behaviours of these strains differ from region to region. Native *B. thuringiensis* strains are isolated from different habitats and characterized to determine their toxic potential all over the world.

The aim of this study was to isolate *B. thuringiensis* strains from different grain habitats in Central Anatolia and Aegean Regions, and to investigate their phenotypic and genotypic characterizations. Total 96 samples containing soil, grain, stored product dust, straw and various residues were collected from wheat farms, grain silos, haylofts and caves in Ereğli/Konya, Taşkale/Karaman, Nikfer/Denizli, and Bozbük/Söke under aseptic conditions. Seven hundred bacteria were isolated from these samples by sodium acetate selection and heat treatment. For phenotypic characterization, 500 of these isolates were grown for 48 h and crystal protein production was observed by phase contrast microscobe during spore formation. One hundred and sixty three of the bacterial colonies were identified as *B. thuringiensis*. The isolates were divided into 5 different groups based on the shape of the crystals that they produced. Spherical type crystal morphology was mostly observed type among the others. For genotypic characterization, the *cry* gene content of the isolates were screened by polymerase chain reaction (PCR) analysis. In addition, chromosomal DNA analysis of 34 isolates by Pulsed Field Gel Electrophoresis (PFGE) as well as plasmid DNA profiling for all isolates were also carried out.

One hundred and three isolates were positive for 5 different *cry* genes (*cry1, cry2, cry3, cry4, cry9*) examined by PCR. Among all *cry* genes examined, *cry1* and *cry9* genes were mostly found in the isolates. Morover, plasmid profiling of the isolates indicated that a 15 kb DNA band was present in all the isolates; however, some of them had more than one DNA band at different sizes. Finally, chromosomal DNA profiling by PFGE showed different DNA patterns for isolates containing the same *cry* gene which suggest a high level of diversity among the *B. thuringiensis* strains isolated. Further studies related with extensive genetic characterization and toxic activity of each *B. thuringiensis* strain will give more comprehensive results on biodiversity of *B. thuringiensis* strains in Anatolia.

ÖZ

Bacillus thuringiensis Gram pozitif, fakültatif anaerob bir bakteri olup, sporlanma evresinde ürettiği proteinler ile bir çok böcek türü üzerinde toksik etki gösterir. Bu özelliğinden dolayı, tarımsal mücadelede en yaygın kullanılan biyolojik kontrol ajanıdır. *B. thuringiensis* suşları geniş bir genetik çeşitliliğe sahip olup, bu suşların toksik davranışları bulundukları coğrafik bölgelere göre farklılık göstermektedir. Dünyanın çeşitli yerlerinde, değişik ortamlardan doğal *B. thuringiensis* suşları izole edilmekte ve toksik potansiyellerinin belirlenmesi amacıyla karakterizasyonları yapılmaktadır.

Bu çalışmanın amacı, İç Anadolu ve Ege Bölgelerindeki çeşitli tahıl ortamlarından *B. thuringiensis* suşları izole ederek, bunların fenotipik ve genotipik olarak karakterizasyonlarını yapmaktır. Ereğli/Konya, Taşkale/Karaman, Nikfer/Denizli, Bozbük/Söke' de bulunan buğday tarlaları, tahıl ambarları, samanlıklar ve mağaralardan aseptik koşullar altında toprak, tahıl, depo tozu, saman ve çeşitli kalıntılardan oluşan toplam 96 adet örnek toplandı. Bu örneklerden, sodyum asetat seleksiyonu ve ısıl işlem uygulanarak 700 civarında bakteri izole edildi. Fenotipik karakterizasyon için, bu izolatların 500 tanesi 48 saat uygun katı besi ortamında büyütüldü ve faz kontrast mikroskobu ile spor oluşumu sırasındaki kristal protein üretimi incelendi. Bakteri kolonilerinin 163 tanesi *B. thuringiensis* olarak tanımlandı. İzolatlar ürettikleri kristal protein şekillerine göre 5 farklı gruba ayrıldılar. Kristal protein ürettiği gözlemlenen izolatlarda, çoğunlukla küresel tipte kristal morfolojisine rastlandı. Genotipik karakterizasyon için, bu izolatların *cry* gen içeriği PCR analiziyle tarandı. Buna ek olarak, izolatların plazmid DNA profilleri çıkarılırken, 34 adet izolatın kromozomal DNA analizleri *Pulsed Field Gel* Elektroforez (PFGE) ile incelendi.

Kristal genlerinden *cry1, cry2, cry3, cry4* ve *cry9* için yapılan PCR analizlerinde, 103 tane izolat pozitif sonuç verdi. Taranan bütün *cry* genleri arasında en çok *cry1* ve *cry9* genlerinin varlığına rastlandı. Plazmid profilleri incelendiğinde bir çok izolat için farklı büyüklüklerde bantların yanında, hepsinde 15 kb'lık DNA bantları saptandı. Son olarak, PFGE analizlerinden alınan sonuçlar, izole edilen *B. thuringiensis* suşları arasında yüksek oranda farklılık olduğunu ifade etmektedir. Gelecek çalışmalarda, her bir *B. thuringiensis* suşu için yapılacak olan daha geniş genotipik karakterizasyon ve toksik aktivite testleri *B. thuringiensis* suşlarının Anadolu'daki biyoçeşitliliği hakkında daha detaylı sonuçlar verecektir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

bp : Base pair

Bt : *Bacillus thuringiensis*

CHEF : Clamped Homogeneous Electrical Field

cry : Crystal

DNA : Deoxyribonucleic Acid

dNTP : Deoxynucleotide triphosphate

EDTA : Ethylenediamine tetra acetic acid

h : Hour

kb : Kilo base

Mb : Mega base

µl : Microliter

µM : Micromolar

mM : Milimolar

PCM : Phase Contrast Microscopy

PCR : Polymerase Chain Reaction

PFGE : Pulsed Field Gel Electrophoresis

PMSF : Phenyl Methyl Sulfonyl Floride

subsp. : Subspecies

sp. : Species

TAE : Tris Acetate EDTA

TBE : Tris Borate EDTA

TE : Tris EDTA

U : Unit

UV : Ultra Violet

Chapter 1

INTRODUCTION

Insects are the most abundant groups of organisms on earth. They often negatively affect humans in a variety of ways. They cause massive crop damage and act as vectors of both human and animal diseases, such as malaria and yellow fever (Glazer and Nikaido, 1994). Therefore, human have desired to control insects. As being parallel to development of chemistry, chemical substances had been started to be used for controlling of pests in the mid 1800s. The use of inorganic chemicals and organic arsenic compounds were followed by organochlorine compounds, organophosphates, carbomates, pyrethroids and formamidines (Glazer and Nikaido, 1994). These chemicals were very effective in killing and controlling of many species of pests. However, they have many direct and indirect adverse effects on ecosystem including accumulation of toxic residues in nature, leading health problems in mammals and development of insect resistance (Glazer and Nikaido 1994). The problems related with chemical pesticides oriented human to find out safer and natural alternative ways of pest control.

In nature, some microorganisms have the potential to produce some biological agents capable of infecting other living organisms including insects. Many of these infectious agents have a narrow host range and, are not toxic to beneficial insects or vertebrates (Glazer and Nikaido 1994). Therefore, the use of these non-pathogenic microorganisms have been developed as the biological way of pest control. Insect viruses (baculoviruses), some fungi, protozoa and bacteria have been used as biological pest control agents. Among all, *Bacillus thuringiensis* is the most important microorganism with entamopathogenic activity against certain insect orders. It is ubiquitous, gram-positive and spore-forming bacterium which produces insecticidal crystal proteins during sporulation. The toxic activity due to proteins produced by plasmid encoded *cry* genes, varies with insect type. The native strains of this bacterium have been used nearly for 50 years safely, as an alternative to chemical pesticides. Bt preparations account for 80-90% of world biopesticide market (Kumar *et al*., 1997). By contrast, it represents only 2% of the total global pesticide market with $90 million worlwide sales (Lambert and Peferoen 1992; Schnepf *et al*., 1998).

1.1. Pests in Croplands

Human population is estimated to increase to 7.7 billion by the year 2020 (United Nations, 1996). This increased population will cause an increase in the demand for agricultural production. However, the land suitable for agricultural production is limited due to restricted water availability, depletion of land sources and already cultivated highly productive soils. Under these limitations, it is important to develop the yield of agricultural production (Oerke and Dehne 2004). It has been estimated that upto 15% of crops worldwide are lost due to insect damage only (Boulter *et al*., 1989). Therefore, the need to exterminate insects that are destroying crops becomes urgent.

Wheat, rice, maize and barley are the primary source for human nutrition worlwide and cover more than 40% of global cropland (Tilman, 1999). Most of the pests giving damage to these grains belong to Coleoptera and Lepidoptera orders. In addition, some species of Arachnida, Orthoptera, Hymenoptera, Diptera and Psocoptera can also cause damage in stored grain products.

1.1.1. Pesticides

Early pesticides were the chemical substances. Certain properties made them useful, such as long residual action and effective toxicity to a wide variety of insects. However, the use of them may lead to negative outcomes. The chemical insecticides used today are considered as presumably safer than those used in the past, but there are still some concerns. Long-term exposure to these chemicals can cause cancer, liver damage, immunotoxicity, birth defects and reproductive problems in humans and animals (Kegley and Wise 1998). Also, they can cause accumulation and persistance of toxic residues in soil, water and food; toxicity aganist beneficial insects and development of pest resistance (Marrone and Macintosh 1993; Van Frankhuyzen, 1993; Glazer and Nikaido, 1994). Nevertheless, chemical insecticides have a large market volume, and global sales of them are about $5 billion a year (Glazer and Nikaido, 1994).

By contrast, microbial pesticides are safe for ecosystem. They are non-toxic and non-pathogenic to wildlife and humans. The toxic action of them is often specific to a single group or species of insects, so they do not affect the other insect population in treated areas. Because they have no hazardous residues to humans or animals, they can also be applied when crop is almost ready for harvest (Neppl, 2000). In spite of these

attractive features, microbial pesticides represent about 2% of global insecticide sales. *Bacillus thuringiensis* based pesticides account major share of the bioinsecticide market with 80-90% (Glazer and Nikaido, 1994).

For several reasons, the use of biopesticides as insecticide has grown slowly when compared with chemicals. Microbial pesticides are generally more expensive to produce than many chemicals. Large quantities of toxins have to be applied to the field to ensure that each larvae will ingest a lethal dose. However, the cost can be decreased by increasing demands. Many chemical pesticides have broad spectrum of toxicity, so pesticide users may consider microbial pesticides with a narrower range to be less convenient. In addition, microbial pesticides kill the insects in a slower speed and thus, this contributes users that they are less effective than the traditional chemical agents (Glazer and Nikaido, 1994). Nevertheless, the use of biological pest control agents have been considered to be much safer than chemical ones for the ecosystem. Moreover, the future prospects of them seem to be positive. It is estimated that, the growth rate of usage of biopesticide use over the next 10 years will be 10-15% compared with 2% for chemical pesticides. Also, the cost of development of *Bacillus thuringiensis* insecticides is predicted to be $3-5 million, compared with $50-80 million for chemical insecticides. In addition, the use of chemical insecticides seems likely to decline in the future, restrictions for their registration will increase resulting in a smaller chemical pesticide market (Navon, 2000).

1.2. History of *Bacillus thuringiensis*

The entamopathogenic bacterium *B. thuringiensis* was first isolated by the Japanese scientist S. Ishiwata, in 1901, from silkworm larvae (*Bombyx mori*) exhibiting the sotto disease and named as *Bacillus sotto* (Ishiwata, 1901). In 1911, Berliner formally described the species from a diseased Mediterranean flour moth larvae (*Anagasta kuehniella*) collected in a German town, Thuringia which gave the name to the species (Berliner, 1911). *B. thuringiensis* first became available as a commercial insecticide, against flour moth, in France in 1938 (Neppl, 2000). In 1956, the main insecticidal activity of *B. thuringiensis* against Lepidopteran insects was found to be due to parasporal crystals by the researchers Hanay and Fitz-James Angus. This discovery increased the interest of other researchers in crystal structure, biochemistry and action mechanism of toxins. In 1950s, *B. thuringiensis* has been started to be used

commercially in US. By 1961, *B. thuringiensis* has been registered as biopesticide to the United States Enviromental Protection Agency (EPA).

Upto 1976, *B. thuringiensis* has been available only for control of Lepidoptera (butterflies and moths), with a highly potent strain *B. thuringiensis* supsp. *kurstaki* (Dulmage, 1970). This strain still forms the basis of many *B. thuringiensis* formulations. In 1976, with the discovery of *B. thuringiensis* supsp. *israelensis* by Margalit and Tahori in Israel, disease causing dipteran insect pests such as mosquitoes and blackflies have been taken under control (Margalit and Dean 1985). In 1980s, developments in biotecnology have stimulated researchers to screen large number of natural *B. thuringiensis* isolates to find different strains toxic against other insect orders. In 1983, *B. thuringiensis* supsp. *tenebrionis* has been described effective against the larvae of coleopteran insects (Krieg *et al*., 1983). *B. thuringiensis* supsp. *aizawai* active against both Lepidoptera and Diptera orders (Glazer and Nikaido 1994) and *B. thuringiensis* supsp. *sandiego* active against beetles (Hernstadt *et al*., 1986) has been introduced into markets.

At the end of the 1980s, the first report came on the insertion of genes encoding *B. thuringiensis* toxic proteins into plants. The first transgenic plants expressing *B. thuringiensis* toxins were tobacco and tomato (Van Frankenhuyzen, 1993). *B. thuringiensis* field cotton was the first *B. thuringiensis* plant (Bt plant) pesticide registered by United States EPA (USEPA, 1999). Rapidly developing recombinant DNA technology after 1990 became an important tool to develop genetically manipulated Bt pesticides. Today, major Bt transgenic crops include corn, cotton, potatoes and rice. They have been commercialized and are in use widely in Canada, Japan, Mexico, Argentina, Australia and United States (Frutos *et al*., 1999). Both *B. thuringiensis* in the form of transgenic crop and spray formulations are still being widely used (Lui and Tabashnik 1997). Furthermore, many research centers focus on the collection of native strains from different environments to find novel strains with high toxic potential to wider insect spectra.

1.3. Commercial *Bacillus thuringiensis* Products

Some representative examples of natural and genetically modified commercial *B. thuringiensis* products used in agriculture were given in Appendix A.

1.3.1. Formulations of *Bacillus thuringiensis* Preparats

Commercially available *B. thuringiensis* preparats (Bt preparats) contain both spore and toxic crystal protein (δ-endotoxin). In the production, spores and crystals obtained from fermentation are mixed with the additives including wetting agents, stickers, sunscreens and synergists (Burges and Jones, 1999).

It is excepted that UV inactivation of the crystal toxin is the major cause for the rapid loss of *B. thuringiensis* activity. Several approaches such as the use of some chromophores to shield Bt preparats against sunlight (Dunkle and Shasha, 1989; Cohen *et al*., 1991) and enhancing the melanin-producing mutants of the organism, increase UV resistance and insecticidal activity (Patel *et al*., 1996). Besides, encapsulation of *B. thuringiensis* in biopolymers reduce washing of the product from the plant by rain (Ramos *et al*., 1998).

In the development of new formulations and optimization of the utilization of biopesticides, knowledge of insect feeding behaviour is a fundamental requirement (Navon, 2000). Some formulations used to stimulate feeding, such as the use of a phagostimulant mixture or a yeast extract in a dustable granular form have been proposed to increase residual toxic activity and to attract to the feed selectively on the *B. thuringiensis* product than the feed on the plant (McGuire and Shasha, 1995; Navon *et al*., 1997). These approaches can help to increase the effectiveness of the new *B. thuringiensis* formulations.

1.3.2. Applications of *Bacillus thuringiensis* Preparats

In agricultural use, Bt preparats are mostly applied with ground sprayers. Since high volumes of aqueous spray per unit area are needed for adequate coverage of the plant, ground spraying can be impractible in some cases. In recent years, air spraying have been applied from a helicopter have reduced spray volume and made more effective and beter controlling of the droplets (Wysokis, 1989). Also the use of air-assisted sleeve boom have increased spray penetration, plant coverage and reduce the drift (Navon, 2000).

Low persistance of the spore-crystal product on the plant is an important problem in *B. thuringiensis* applications. When the products of *B. thuringiensis* were applied to cotton (Fuxa, 1989) and potato (Ferro *et al*., 1993), persistance was observed

as 48 hours. Therefore, timing is the major factor for determining the effectiveness of *B. thuringiensis* applications. Application early in the season, according to monitoring egg hatching and after sunset instead of in the morning can increase the persistance of Bt preparats (Navon, 2000).

Laboratory and field assays have showed that younger larvae are more susceptible to Bt preparats than older ones (Navon *et. al*., 1990; Ferro and Lyon, 1991). Therefore, larval age is an important aspect in *B. thuringiensis* applications.

1.3.3. Safety of *Bacillus thuringiensis* Products

The primary advantage of *B. thuringiensis* products is their safety resulting from their selectivity which is affected by several factors. The δ-endotoxins are activated by alkaline solutions and different varieties may require different pH values. Also, crystals need to be broken down to toxic elements by certain enzymes that should be present in the insect's gut. In addition, certain cell characteristics in the insect gut encourage binding of the endotoxin and leading to pore formation (Gill *et.al*., 1992). Therefore, each strain is capable of producing toxic proteins effective on one or few specific groups of insect. Non-target species such as beneficial insects and wildlife pets are not affected by these toxins.

According to oral mammalian toxicology and *in vitro* digestibility studies which are demanded by the Environmental Protection Agency (EPA), cry proteins (cry1Ab, cry1Ac, cry3A) have not shown toxicity to mammals and they are rapidly degraded in simulated gastric fluid (EPA, 1998). Additionally, *B. thuringiensis* toxins are biodegradable and do not persist in the environment (Van Frankenhuyzen, 1993).

1.4. Development and Management of Pesticide Resistance

Insects can develop resistance to nearly every type of insecticides due to genetic variation in large insect population. Besides, there are several other factors increasing the rate of resistance development, which are related to the insect population and insecticide usage. Species with higher reproductive rates, shorter generation times, greater numbers of progeny, and more genetically varied local populations develop a large resistance in the population more quickly (Pimental and Burgess 1985). Also, resistance develops more rapidly against more persistant insecticide due to increasing

the time of exposure of susceptible larvae to the toxin Similarly, frequent application of non-persistent insecticides can have the same effect (Wood, 1981).

Insecticide resistance is a major problem for agriculture, health and economics. The first reported case of resistance to chemical insecticides has occured over 50 years ago. Since then, pesticide resistance has become one of the world's most serious environmental problems because of the concerns on human nutrition due to crop loss, spread of disease by resistant insects, environmental risks in the application of greater amounts of chemicals to the pests which are already gained resistance (Pimental and Burgess 1985). In 1990s, much evidence on the resistance development of different pests against Bt preparats have been reported from Hawaii, Florida, Newyork, Japan, China, the Philippines, Thailand and Malaysia (Iqbal *et al*., 1996; Lui and Tabashnik, 1997). As a result, insecticide resistance appeared the negative outcome of insecticide usage. In order to overcome resistance problem against *B. thuringiensis* based pesticides, different management strategies have been developed. Basically, it has been aimed to slow down resistance development as much as possible and to make resistant populations revert to susceptibility (Croft 1990). Generally , three main approaches are involved in resistance management programs. One approach targets to minimize exposure to toxins and allow for mating between resistant and susceptible insects, thus susceptible traits continue for the next generations. Different strategies based on this approach include tissue-specific and time-specific expression of toxins, mixtures, mosaics, rotations, refuges and occasional release of susceptible males into the field (Wood, 1981). Other approach focuses on combining pest control techniques to provide synergy and improve the efficiency of Bt preparats against pests. This includes the strategies of gene stacking, high doses, combination of toxins with completely different modes of action and combination of low toxin dose, other entomopathogenic microbes, plant allochemicals and natural enemies (Navon, 1993; Trumble and Alvaro-Rodriguez, 1993; Murray *et al*., 1993). Another approach developed for only transgenic Bt plants, not spray form, uses trap plants to lure pests away from productive crops (Alstad and Andow 1995).

1.5. General Characteristics of *Bacillus thuringiensis*

B. thuringiensis, a member of the genus *Bacillus*, is a rod shaped, motile, Gram-positive, facultative anaerob and spore-forming bacterium. In a standard liquid media,

the size of the rods varies between 3 and 5 µm. The endospores of the organism like those of other spore-forming species are more resistant than vegetative cell to heat, drying, disinfection and other destructive agents, thus may remain viable for centruies.

B. thuringiensis is characterized by the formation of intracellular parasporal crystal proteins during the sporulation period of stationary phase of its growth cycle (Schnepf *et al*., 1998) as shown in Figure 1. These parasporal crystal proteins (cry proteins) are synthesized by plasmid encoded *cry* genes and exhibit toxic activity on certain insect groups (Gonzales and Carlton 1980). Because of this important feature, *B. thuringiensis* have been widely used as bioinsecticide for nearly 50 years.

B. thuringiensis is very closely related to *Bacillus cereus* and *Bacillus anthracis*. (Helgason *et al*., 2000). Formation of crystal proteins is the key feature discriminating *B. thuringiensis* from related species. However, plasmid encoded genes could be transferred to other related species (e.g. *B. cereus, B. anthracis* and *B. mycoides*) by conjugation and it has been observed that these relatives could express the toxin and produce crystal protein (Hu *et al*., 2004). Also, *B. thuringiensis* has been observed to produce *B. cereus* type enterotoxin, suggesting the conjugative transfer of enterotoxin producing genes from *B.cereus* to *B. thuringiensis* (Carson and Kolstø, 1993).

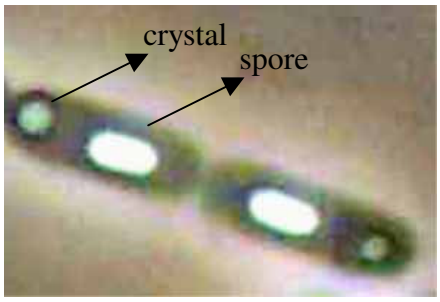

Figure 1.1. Crystal protein formation of a *B. thuringiensis* cell. Phase contrast microscope image of 48 hour-grown *B. thuringiensis* culture, isolated in this current study.

1.5.1. Morphological Features of *Bacillus thuringiensis*

B. thuringiensis forms white and rough colonies which spread out and can expand over the plate very quickly. The spores of the organism are elipsoidal, unswollen and lie in the subterminal position in the cell (Figure 1). The best criteria to distinguish *B.*

thuringiensis from other *Bacillus* species is the presence of parasporal crystal inclusions which can be easily observed under phase contrast microscobe. Morphology, size and number of crystal inclusions may vary among *B. thuringiensis* strains. There are five distinct crystal morphologies: bipyramidal crystals, related to Cry1 proteins; cuboidal crystals, related to Cry2 proteins; amorphous and composite inclusions, associated with Cry4 and Cyt proteins; flat-square crystals, typical of Cry3 proteins; and bar-shaped inclusions, related to Cry4D proteins (Lopez-Meza and Ibarra, 1996; Schnepf et al., 1998).

1.6. Ecological Role of *Bacillus thuringiensis*

B. thuringiensis is mainly a soil bacterium living as both saprophytic, digesting organic matter derived from dead organism, and parasitic, colonizing within living insects (Glazer and Nikaido, 1994). It can be present naturally in many different habitats such as soil, stored product dust, insect cadavers, grains, agricultural lands, olive tree related habitats, different plants, and aquatic environments (Martin and Travers 1989; Meadows *et al*., 1992; Ben-Dov *et al*., 1997; Theunis *et al*., 1998; Bel *et al*., 1997; Mizuki *et al*., 1999; Iriarte *et al*., 2000).

The true ecological role of *B. thuringiensis* is poorly understood. Meadows *et al*., (1992) has analyzed *B. thuringiensis* as an entomopathogen, as a phyloplane inhabitant and a soil microorganism. Although it is known that *B. thuringiensis* produces different toxic proteins effective against many different insect orders, some strains show no toxicity (Maede *et al*., 2000).

1.7. Insecticidal Crystal Proteins (ICP)

1.7.1. Crystal Protein Structure

During sporulation, *B. thuringiensis* produces one or more large protein containing crystalline inclusions, delta (δ) endotoxins, which are easily observed under phase contrast microscobe. There are two types of δ-endotoxins; highly specific cry (crystal) toxins which act through specific receptors and the non-specific cyt (cytolytic) toxins with no known receptors (Höfte and Whitely, 1989; de Maagd *et al*., 2000). Both are classified on the basis of their amino acid sequence identity. Four hierarchical ranks

have been defined depending on its place in a phylogenetic tree. Proteins less than 45% sequence identity differ in primary rank, and 78% and 95% identities are the boundaries of secondary and tertiary ranks, respectively (de Maagd *et al*., 2001).

The three dimensional structures of activated forms of toxic proteins cry1A, cry2, cry3A and cyt2A have been solved by X-ray crystallography (Grochulski *et al*., 1995; Li *et al*., 1991; Li *et al*., 1996). Cry proteins are remarkably similar, each has three domains (Figure 1.2). The N-terminal domain I consists of six amphipatic helices around a central core helix and involved in membrane insertion and pore formation. Domain II has three β-sheets with three-fold symmetry in 'Greek key' conformation. The C-terminal domain III consists of two antiparallel β-sheets in a 'jelly-roll' formation. Both domain II and III are involved in receptor recognition and binding. Additionally, pore formation function of domain III has been found recently (de Maagd *et al*., 2001).

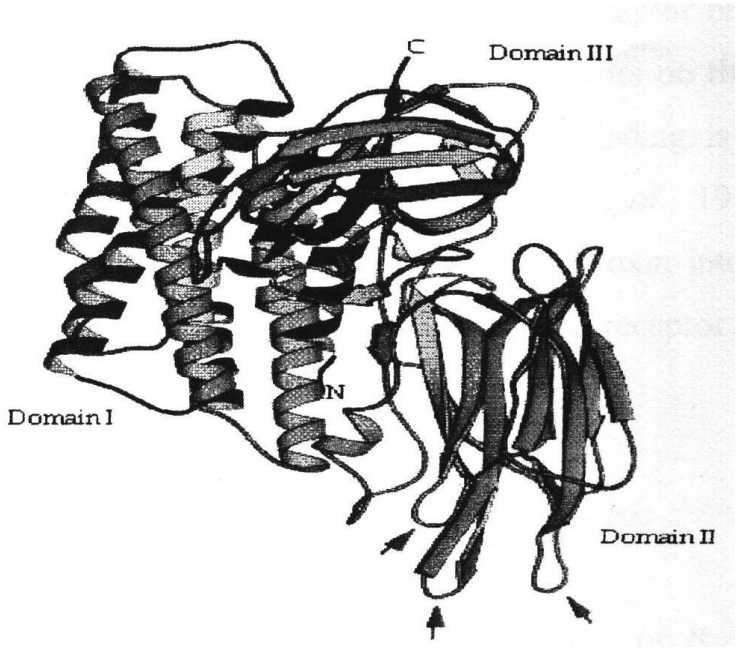

Figure 1.2. The structure of Cry 3A protein
(http://www.bioc.cam.ac.uk/UTOs/Ellar.html)

In contrast, cyt2A protein has a single domain in which two outer layers of α-helix wrap around a mixed β-sheet (Schnepf *et al*., 1998) (Figure 1.3). Unlike cry

proteins, cyt proteins do not recognize specific receptors on the epithelium and exhibit hemolytic activity (Crickmore *et al*., 1998).

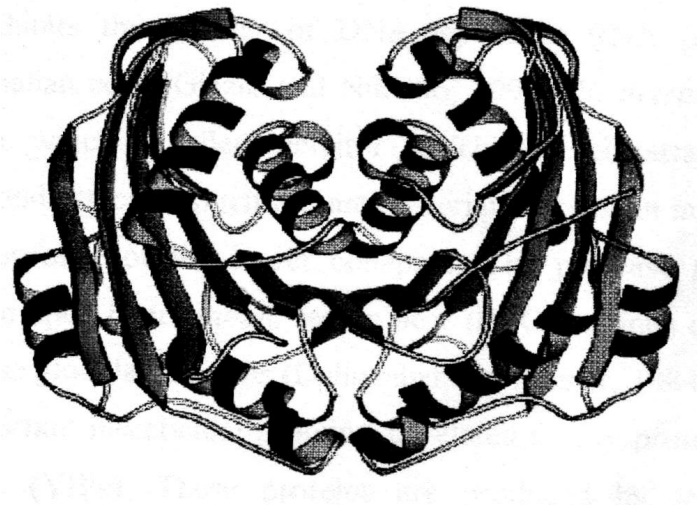

Figure 1.3. The structure of Cyt 2A protein
(http://www.bioc.cam.ac.uk/UTOs/Ellar.html)

When the sequences of crystal proteins are aligned, five conserved sequence blocks are common in the majority of them (de Maagd *et al*., 2001). Conserved block 1 is in the central helix of domain I, block 2 is at the domain I-II interface, block 3 is at the boundary between domains II and III, block 4 is in the central β-strand of domain III and block 5 is at the end of domain III (Figure 1.4).

1.7.2. Action Mechanism

Crystal proteins are synthesized as protoxins which must be converted to active toxins. After ingestion of cry proteins by the susceptible insects, they are solubilized in the alkaline environment (pH 10-12) in the insect midgut, and activated by gut proteases (Höftee and Whitely, 1989). The activated toxin binds to specific receptors located in the apical microvilli of susceptible larval midgut epithelia (Hofmann *et al*., 1988; Van Rie *et al*., 1990). After binding, toxin inserts itself into cell plasma membrane and forms pores or ion channels (Van Rie *et al*., 1989). These pores lead to osmotic shock. At the

end, midgut cells lyse, feeding activity is paralysed, and insect dies from starvation (Knowless and Dow, 1993).

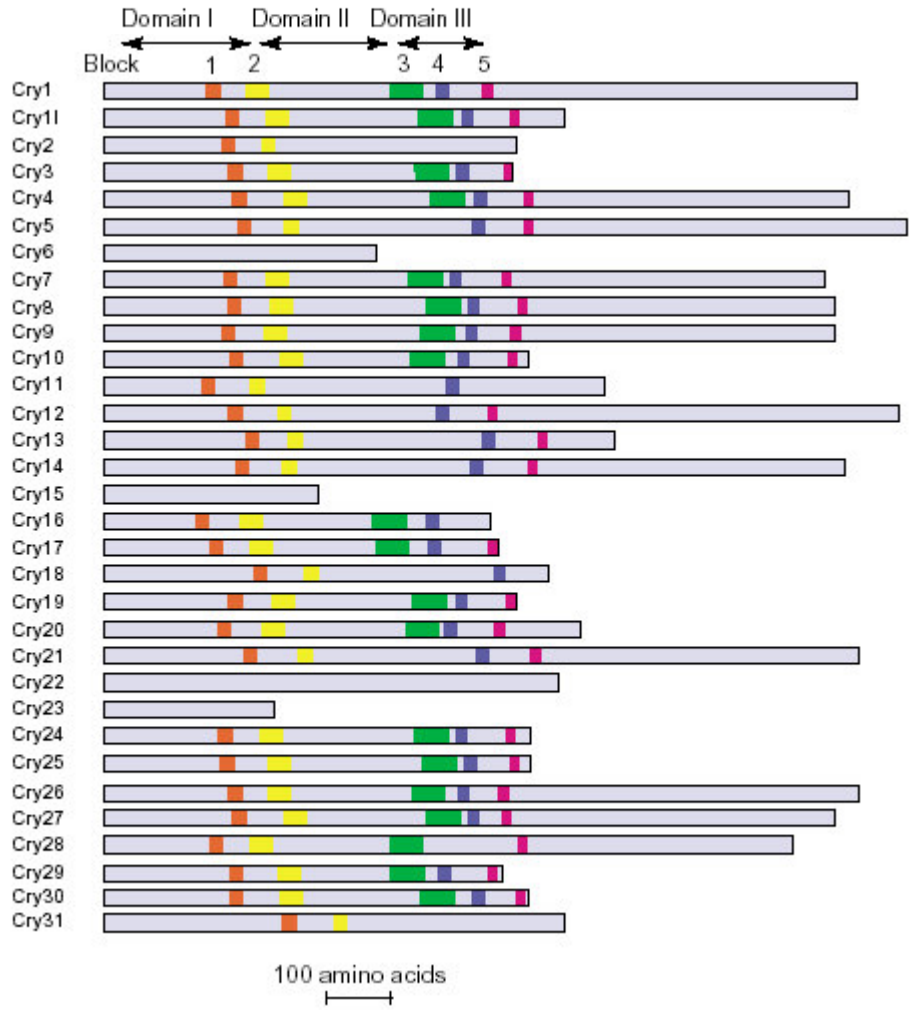

Figure 1.4. Relative lengths of cry protoxins and position of the five conserved sequence blocks (de Maagd *et al*., 2001).

1.7.3. Insect Spectrum of *Bacillus thuringiensis* δ-endotoxins

The toxic proteins of *B. thuringiensis* are classified according to amino acid sequence identity and insect specifity. Each group of toxin shows a wide range of toxicity for different insect orders such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Mallophage and Acari (Feitelson, 1993). In addition, it was

reported that *B. thuringiensis* toxins are also able to control some invertabrates such as Nemathelminthes, Platyhelminthes and Sarcomastigorphora (Feitelson, 1993).

The proteins toxic for lepidopteran insects belong to the Cry1 and Cry9 groups. Toxin active against coleopteran insects are the Cry3, Cry7 and Cry8 proteins. Nematocidal toxic proteins are Cry5, Cry12, Cry13, Cry14, and dipteran active toxins are the Cry4, Cry10, Cry11, Cry16, Cry17, Cry19, and Cyt proteins. The Cry2 group proteins are active against both lepidopteran and dipteran insects. Also, the Cry1B and Cry1I proteins which are subgroups of Cry1 proteins have dual activity against lepidopteran and coleopteran insects (Bravo *et al*., 1998).

1.8. Other Pathogenic Features of *Bacillus thuringiensis*

B. thuringiensis produces various virulance factors other than δ-endotoxins. Vegetative insecticidal proteins (VIP) expressed and screeted during vegetative growth and sporulation, were described as toxic against lepidopteran insects (Estruch *et al*.,1996). Beside VIP, a series of extracellular compounds synthesized and contribute virulence, such as β-exotoxins, phospholipases, proteases, and chitinases (Levinson, 1990; Lövgren *et al.*, 1990; Zhang *et al*., 1993; Sonngay and Panbangred, 1997). Also, the spores themselves contribute to pathogenity, often synergizing the activity of the crystal proteins (Johnson *et al*., 1996).

1.9. Genetic Features of *Bacillus thuringiensis*

1.9.1. *Bacillus thuringiensis* Genome

B. thuringiensis strains have a genome size of 2.4 to 5.7 million base pairs (Carlson *et al*., 1994). Physical maps have been constructed for two *B. thuringiensis* strains and compared with *B. cereus* chromosomal maps. It has been shown that chromosomes have a similar organization in the half near the replication origin while displaying greater variability in the terminal half (Carlson and Kostø, 1993; Carson *et al*., 1996). Most *B. thuringiensis* strains contain several circular and linear extrachromosomal elements (plasmid DNA) ranging from 2 kb to greater than 200 kb (Carlton and Gonzalez 1985). They make up to 20% of the total DNA (Aronson, 2002). The genes (*cry* genes) encoding crystal proteins are mostly carried on large plasmids (Li

et al., 1991). Sequence hybridization studies have shown that these genes are also found in the *B. thuringiensis* chromosome (Carlson *et al*., 1994).

B. thuringiensis and its subspecies also contain a large variety of transposable elements including insertion sequences and transposons (Mahillon *et al*., 1994). It is postulated that they are involved in the amplification of the *cry* genes in the cell. A second possibility for their role is mediating the transfer of plasmid between self-conjugative plasmids and chromosomal DNA or non-conjugative plasmids (Schnepf *et al*., 1998).

1.9.2. The *cry* Genes

The genes coding insecticidal crystal proteins are mostly located on large plasmids (Gonzales *et al*., 1982). Many toxin genes (*cry* and *cyt*) have been cloned and sequenced. Upto now, more than 200 insecticidal crystal protein (ICP) genes have been described and classified into 32 groups of *cry* genes and 2 groups of *cyt* genes (Crickmore *et al*., 1998).

Many *B. thuringiensis* strains can contain multiple *cry* genes often flanked by transposons or insertion sequences. Therefore, the strains are able to synthesize more than one crystal protein. This diversity in toxin genes are due to plasmid transfer among *B. thuringiensis* strains (Thomas *et al*., 2001).

1.9.3. The *cry* Gene Expression

The expression of *cry* genes occurs during the stationary phase of bacterial growth. The *cry* gene products generally accumulated in the mother cell compartment and form crystal inclusions that forms upto 20 to 30 % of the dry weight of sporulated cell (Schnepf *et al*., 1998).

Sporulation and crystal protein synthesis are syncronic processes. In order to coordinate sporulation with the synthesis of the protoxins and their assembly into inclusions, crystal protein synthesis is controlled by a variety of mechanisms occuring at the transcriptional, posttranscriptional and posttranslational levels (Agaisse and Lereclus, 1995; Baum and Malvar, 1995).

The *cry* gene expression can be activated by both sporulation-dependent and sporulation-independent mechanisms. The *cry1Aa* gene is generally expressed during

sporulation, as a typical example for sporulation-dependent control. However, *cry3A* gene is expressed during vegetative growth but it can also be expressed in much lower amounts during sporulation (Schnepf *et al*., 1998).

The expression level of a gene may also be influenced by its copy number. In fact, the production of *B. thuringiensis* toxins is not strictly proportional to the *cry* gene copy number. However, it can be decleared that the capacity of *B. thuringiensis* strains to produce crystal proteins may reach maximum at a certain number of *cry* gene copies in the cell (Agaisse and Lereclus, 1995).

The stability of mRNA is another important contributor to the high level of toxin production in *B. thuringiensis*. It has been reported that, the mRNAs half-life encoding the crystal proteins are relatively longer than normal mRNAs (Glathorn and Rapoport, 1973). The determinants of mRNA stability are generally part of untranslated regions of the molecule and classified as 3' and 5' terminal structures according to their location in the mRNA. The fusion of 3' terminal fragment acting as positive retroregulator, with the 3' end of heterologous genes increases the half-life of their transcripts and consequently their expression levels (Wong and Chang, 1986). Also, the Shine-Dalgarno (SD) sequence present close to 5' end may be a general determinant of mRNA stability in *Bacillus* species (Agaisse and Lereclus, 1995).

The ability of protoxins to crystallize may decrease their susceptibility to premature proteolytic degradation; however, efficient and rapid solubility of the crystals in the larval gut depends on the secondary structure of the protoxin, the energy of the disulfide bonds and the presence of additional *B. thuringiensis* specific components (Schnepf *et al*., 1998).

1.10. Strain Collections of *Bacillus thuringiensis*

Recent developments suggest that biological control with *B. thuringiensis* based products will become increasingly important. This prompts many researchers to focus on the isolations of native strains from different environments. It is also necessary to find out novel *B. thuringiensis* strains with higher toxicity to help coping with the problem of insect resistance especially with regard to transgenic Bt plants (Van Rie *et al*., 1991).

B. thuringiensis has great strain diversity with different toxic potential according to regions where they are isolated (Thomas *et al*., 2001). Worldwide, many screening

programs have been performed to establish *B. thuringiensis* strain collections in different countries such as Antartica (Forsyth and Logan, 2000), China (Hongyu *et al*., 2000) Colombia (Uribe *et al*., 2003), Japan (Mizuki *et al*., 1999), Mexico (Bravo *et al*., 1998), Philippines (Theunis *et al*., 1998), Spain (Bel *et al*., 1997; Iriarte *et al*., 2000), Taiwan (Chack *et al*., 1994), United Kingdom (Meadows *et al*., 1992; Bernhard *et al*., 1997), United States (Martin and Travers, 1989), and some Asian countries (Ben-Dov *et al*., 1997). These collections have great importance in analyzing the distribution of toxin producing strains in nature and evaluating their toxic potentials against various insect orders. Moreover, they may help to understand the role of *B. thuringiensis* in the environment.

1.11. Isolation and Characterization Methods of *Bacillus thuringiensis* to Establish *Bacillus thuringiensis* Strain Collections

1.11.1. Isolation Methods of *Bacillus thuringiensis*

B. thuringiensis can be present in many different habitats containing different spore-forming bacterial species. There are some selective techniques to isolate *B. thuringiensis* from these environments. Acetate selection method developed by Travers *et al*., 1987 has been widely used by the researchers for *B. thuringiensis* isolation (Martin and Travers, 1989; Carrozi *et al*., 1991; Ben-Dov *et al*., 1997; Bravo *et al*., 1998; Hongyu *et al*., 2000). Sodium acetate at 0.25 M concentration inhibits the germination of spores of *B. thuringiensis* and some relative species. After a period of bacterial growth, the vegetative cells are eliminated by heat treatment and only spore-formers stay alive. They were then plated on nutrient medium without acetate. After an incubation period, *B. thuringiensis* colonies can be distinguished from the others by colony morphology and microscobic observation.

Another method for isolation of *B. thuringiensis* is based on antibiotic selection. Yoo *et al*., (1996) used the antibiotics polymyxin B sulfate and penicilin G in isolation to eliminate the cells which have not resistance to these antibiotics. This method however is not used as often as the acetate selection.

1.11.2. Characterization Methods of *Bacillus thuringiensis*

The characterization of *B. thuringiensis* strains has great imprortance. It may help to analyze distribution of *cry* genes and to understand the role of *B. thuringiensis* in nature. Moreover, it is also important in evaluating toxic potential of the strains against insect orders.

The main point in establishing *B. thuringiensis* strain collections is to have a rapid and accurate characterization method. Upto now, many different methods have been developed to characterize *B. thuringiensis* strains. The toxicity analysis of the proteins against insect orders, so-called biossay, is one of them. It is necessary to test each isolate for all target insects, thus it is a long and exhaustive process in screening large number of natural isolates (Ceron *et al*., 1994). Southern blot analysis to search for known homologous genes (Kornstad and Whiteley, 1986) and analysis of reactivity to different monoclonal antibodies (Höfte *et al*., 1988) have been used to characterize novel *B. thuringiensis* isolates. Flagellar (H) antigen serotyping was established for intraspecific classification of *B. thuringiensis* strains (de Barjac and Bonnefoi, 1973). However, they are imprecise predictors of insecticidal activity, expensive and time-consuming methods for the identification of novel toxins. In addition, biochemical tests, DNA fingerprinting, utilization of oligonucleotide probes specific to the *B. thuringiensis* toxin genes are possible but they are very expensive and time-consuming characterization methods for the identification of new strains from large numbers of environmental samples (Bourque *et al*., 1993).

The use of PCR has been a milestone for the analysis of *B. thuringiensis* strain collections (Carozzi *et al*., 1991). It is highly sensitive, relatively fast and can be easily used on a routine basis (Ceron *et al*., 1994). PCR has been used to predict insecticidal activities (Carozzi *et al*., 1991), to identify *cry*-type genes (Bourque *et al*., 1993; Glaeve *et al*., 1993; Ceron *et al*., 1994, 1995), to determine the distribution of the *cry* genes (Chak *et al*., 1994) and to detect novel *cry* genes (Kalman *et al*., 1993; Kuo and Chak, 1996). Recently, PCR based different methods have been developed for further characterization of the strains, such as PCR-RFLP which is a two-step strategy where group specific primers are used first, followed by enzymatic digestion of the produced amplicons (Kuo and Chak, 1996); E-PCR based on the use of two sequencial PCR reactions, using a multiplex PCR with specific and universal primers (Juarez-Perez *et al*., 1997); and RT-PCR (Shin *et al*., 1995).

Another approach, pulsed field gel electrophoresis of chromosomal DNA digested with an appropriate restriction enzyme recognizing rare sites in the DNA is considered as an accurate typing procedure for closely related bacteria. Thus, it is used for subspecific classification of *B. thuringiensis* strains and provides more discriminative typing of *B. thuringiensis* strains than H-serotyping (Rivera and Priest, 2003).

Although bioassay remains as an essential tool to determine insecticidal activity exactly, other methods such as serotyping, analyzing of DNA profiles or protein profiles are still necessary for subspecific classification of *B. thuringiensis* strains. PCR analysis of new isolates of *B. thuringiensis* provides valuable prescreening opportunity that is followed by subsequent insect toxicity assays or other subspecific classifications.

1.12. Thesis Objectives

The genetic diversity and toxic behaviours of *B. thuringiensis* strains vary with the geographical conditions of the regions where the *B. thuringiensis* strains are isolated. Because each habitat may contain novel *B. thuringiensis* isolates with more effective toxic potential to a wide insect spectra, it is important to screen *B. thuringiensis* isolates from diverse geographical regions. Therefore, the main objectives of this study were;

1) to isolate novel *B. thuringiensis* strains from different grain related habitats
2) to characterize isolates phenotypically based on colony morphologies and parasporal crystal protein formation by phase contrast microscopy
3) to characterize the isolates genotypically based on crystal protein gene content by polymerase chain reaction (PCR) analysis, plasmid profiles, and chromosomal DNA profiles by pulsed field gel electrophoresis (PFGE).

Chapter 2

MATERIALS AND METHODS

2.1. Materials

See Appendix B for growth medium and chemicals used.

2.2. Methods

2.2.1. Sample Collection

Ninety-six samples including soil, grain, stored product dust, straw, insect cadaver and various residues were collected from grain silos, crop fields, farms, caves, haylofts where Bt preparats have not been applied before, in central Anatolia (Ereğli/Konya, Taşkale/Karaman) and Aegean region (Nikfer/Denizli, Bozbük/Söke). The collected samples were summarized in Table 2.1. Samples were taken from the places not exposed to sunlight or 5 cm below the surface and were placed into plastic bags aseptically. All samples were stored at + 4 ºC until processed.

2.2.2. *Bacillus thuringiensis* Isolation

B. thuringiensis strains were isolated from collected samples, based on acetate selection method. First, 0.25 gr of each sample were suspended in 10 ml nutrient broth medium containing 0.12 M and 0.25 M sodium acetate [pH 6.8]. Then, suspensions were vortexed vigorously and incubated overnight for microbial growth at 37 ºC in a shaking water bath. Next, heat treatment was applied for 5 min at 80 ºC to eliminate vegetative and non-sporeforming cells. After that, they were plated on nutrient agar plates and incubated overnight at 37 ºC. Finally, *B. thuringiensis* like colonies which are white, spread out and seems to fried egg on plate (Travers *et al.*1987) were labelled and subcultured. Subculturing from one individual colony was repeated until pure culture obtained.

Table 2.1. Locations, types and numbers of collected samples

Location	type of sample	number of sample
Ereğli / Konya	soil	3
	grain	3
	stored product dust	2
Ayranlı / Konya	soil	9
İvriz / Konya	soil	7
Üçharman / Konya	soil	7
	various residues	1
Natural Grain Silos Taşkale / Karaman	grain	9
	stored product dust	16
	animal faeces	1
Manazan Caves Taşkale / Karaman	soil	9
	stored product dust	5
	animal faeces	1
	various residues	5
Bozbük Söke / Aydın	soil	5
	grain	1
	animal faeces	1
	dead insect	1
	straw	1
Nikfer / Denizli	soil	3
	stored product dust	4
	straw	2
		Total: 96

2.2.3. Crystal Morphology Analysis

Each pure culture was grown on T3 agar plates for 48 – 72 h at 37 ºC. A colony from each culture was dissolved in sterile distilled water and examined with phase contrast microscope for crystal production and morphology. All isolates were recorded according to presence of crystal protein and crystal shape. Then, each crystal producing isolate was defined as *B. thuringiensis* and stored in stock solution containing 25 % glycerol in nutrient broth medium, at -80 ºC for further studies.

2.2.4. *Bacillus thuringiensis* Strains

B. thuringiensis reference strains, shown in Table 2.2, were kindly supplied by Bacillus Genetic Stock Center (Ohio,USA).

Table 2.2. Reference strains of *B. thuringiensis*

Strains	BGSC Code	Original Code	Genotype	*cry* Genes
B. thuringiensis subsp. *kurstaki*	4D1	HD1	serotype 3a3b	*cry 1,2*
B. thuringiensis subsp. *aizawai*	4J3	HD133	serotype 7	*cry 1,2,9* *cry 7,8*
B. thuringiensis biovar. *tenebrionis*	4AA1	tenebrionis	serovar tenebrionis	*cry 3*
B. thuringiensis biovar. *israelensis* ONR60A	4Q2	HD500	serotype 14	*cry 4,11*

2.2.5. DNA Isolation

DNA isolation was performed by the method of Bravo *et al*. (1998). Reference *B. thuringiensis* strains, which were used as positive controls, and *B. thuringiensis* isolates were grown overnight on nutrient agar plates at 37 ºC. A loopfull of cells were transferred into 0.2 ml of sterile distilled water and mixed. After freezing the mixture for 20 min at -80 ºC, it was transferred into boiling water for 10 min. Then the cell lysate was centrifuged (Henttich, Micro 12-24 Eppendorf Model) at 10,000 rpm for 10 s and 15 µl of supernatant was used as DNA template in PCR analysis.

2.2.6. Oligonucleotide Primers for Polymerase Chain Reactions (PCR)

In this study, 5 pairs of universal primers reported by Bendov *et al*. (1997, 1999), for *cry1, cry2, cry3, cry4* and *cry9* genes were used. Their sequences and the expected sizes of their PCR products were shown in Table 2.2. These primers were synthesized by Integrated DNA Technologies, INC.

Table 2.3. Universal Primers

Universal Primers	Expected PCR Product Size
for *cry 1* genes Un1, D_1 5'- CATGATTCATGCGGCAGATAAAC -3' R_1 5'- TTGTGACACTTCTGCTTCCCATT -3'	274-277 bp
for *cry 2* genes Un2, D_2 5'- GTTATTCTTAATGCAGATGAATGGG -3' R_2 5'- CGGATAAAATAATCTGGGAAATAGT -3'	689-701 bp
for *cry 3* genes Un3, D_3 5'- CGTTATCGCAGAGAGATGACATTAAC -3' R_3 5'- CATCTGTTGTTTCTGGAGGCAAT -3'	589-604 bp
for *cry 4* genes Un4, D_4 5'- GCATATGATGTAGCGAAACAAGCC -3' R_4 5'- GCGTGACATACCCATTTCCAGGTCC -3'	439 bp
for *cry 9* genes Un9, D_6 5'- CGGTGTTACTATTAGCGAGGGCGG -3' R_6 5'- GTTTGAGCCGCTTCACAGCAATCC -3'	351-354 bp

2.2.7. *cry* Gene Identification by Polymerase Chain Reactions (PCR)

All PCR reactions were carried out in 50 µl reaction volumes. DNA template, 15 µl, was mixed with reaction buffer containing 200 µM deoxynucleotide triphosphate mix, 0.5 µM each primer, 3 mM MgCl and 2 U of Taq DNA polymerase. Amplifications were carried out in a DNA thermal cycler (Techne Progen). For all *cry* genes, an initial denaturation step was applied for 1 min at 94 ºC and followed by denaturation for 1 min at 94 ºC, annealing for 1 min at 54 ºC (for *cry1*) and 60 ºC (for

cry2, cry3, cry4 and *cry9*), then extention for 1 min at 72 ºC. Thirty-five cycles were carried out for the amplification of *cry* gene fragments. Finally, an extra extension step was applied for 10 min at 72 ºC.

After amplifications, 10 µl of each PCR product was electrophorased on 1 % agarose-ethidium bromide gel in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA [pH 8.0]) at 95 V for 40 min. Gels were visualized in a gel documentation system (Vilber Lourmat, France).

2.2.8. Plasmid Profiling

Plasmid isolation was performed with minor modifications of the method described by O'Sullivan *et al.*, (1993). Bacterial cultures were grown overnight on nutrient agar plates at 37 ºC and transferred into eppendorf tubes by scraping gently with the help of sterile distilled water. After pelleting the cells , they were resuspended in 200 µl of a solution containing 25 % sucrose and 30 mg/ml lysozyme and incubated for 15 min at 37 ºC. The sample was mixed with 400 µl alkaline SDS solution (3 % SDS, 0.2 N NaOH) and incubated for 7 min at room temperature. Then, 300 µl ice-cold 3 M sodium acetate (pH 4.8) was added, mixed and spinned at 10,000 rpm for 20 min at 4 ºC. Supernatants were transferred into new eppendorf tubes, mixed with 650 µl of isopropanol and centrifuged again at 10,000 rpm for 20 min at 4 ºC. After discarding all liquid, pellets were resuspended in 320 µl sterile distilled water. They were mixed with 200 µl 7.5 M ammonium acetate containing 0.5 mg/ml ethidium bromide and 400 µl phenol/chloroform, then centrifuged at 10,000 rpm for 10 min, at room temperature. Upper phases were transferred to new eppendorf tubes and mixed with 1 ml ethanol at -20 ºC. After centrifugation at 10,000 rpm for 20 min at 4 ºC, pellets were washed with 70 % ethanol. All liquid were discarded and the pellets were dissolved in 25 µl TER solution (TE, pH 7.8 and RNase, 0.1 mg/ml). After incubation at for 20 min 37 ºC, plasmid samples were electrophoresed on 0.8 % agarose-ethidium bromide gel in TAE buffer at 80 V for 2 h and visualized with gel documentation system (Vilber Lourmat, France).

2.2.9. Pulsed Field Gel Electrophoresis (PFGE) Analysis

PFGE analysis of *B. thuringiensis* isolates was performed according to Rivera and Priest (2003) with some modifications. Bacterial strains were grown overnight in 10 ml NB at 37 ºC and cells were harvested by centrifugation at 4,500 rpm for 2 min at 4 ºC. Cells were washed once with 500 µl TE (50 mM Tris, 1 mM EDTA, pH 8.0) and SE (10 mM NaCl, 30 mM EDTA, pH 7.5) buffer respectively. Then, the cells were resuspended in 50 µl SE buffer mixed with 50 µl 2 % agarose (low melt) at 50 ºC and dispensed into the slots of plug mold. The plugs were allowed to set at room temperature. The cells embedded into agarose were allowed to lyse in lysis buffer (30 mM Tris, 50 mM NaCl, 5 mM EDTA, pH 8.0) containing 2 mg/ml lysozyme for 18 h at 37 ºC. Bacterial plugs then were washed three times with 5 ml of buffer containing 20 mM Tris, 50 mM EDTA, pH 8.0. Proteins were digested with 2 ml of proteinase K solution (0.5 mg proteinase K/ml and 0.1 % N-laurolysarcosine-EDTA, 50 mM, pH 8.0) at 50 ºC overnight. Then plugs were washed twice with 5 ml of buffer containing 20 mM Tris, 50 mM EDTA, 1 mM NaCl, pH 8.0; once with buffer containing 20 mM Tris, 50 mM EDTA, 1 mM PMSF, pH 8.0, and once with buffer containing 20 mM Tris, 50 mM EDTA, pH 8.0. After equilibrated the plugs with 1 ml restriction enzyme buffer, the plugs were digested with 40 U of *SmaI* for 20 h at 30 ºC. Then the plugs were electrophorased on 1 % agarose in TBE buffer in a CHEF-DRII system for 40 h at 4 V/cm and 14 ºC with pulse times of 15 s rising to 60 s. After staining of the gel in ethidium bromide (1 µl/ml) for 45 min and destaining in distilled water for 1h, DNA profiles were recorded in a gel documentation system (Vilber Lourmat, France).

Chapter 3

RESULTS AND DISCUSSION

3.1. Isolation of *Bacillus thuringiensis*

Total 96 samples, 78 from Konya and Karaman in Central Anatolia region and 18 from Söke and Denizli in Aegean region were examined in this study. Number, type and locations of samples were summerized in Table 2.1.

B. thuringiensis was isolated from collected samples by using acetate selection (Travers *et al.,* 1987) and heat treatment methods. Travers and his colleagues tested the strains of spore-forming bacteria in different sodium acetate concentrations (0.06 M, 0.12 M, 0.25 M, 0.5 M) to determine their ability to germinate in acetate-buffered medium. They have reported that all bacterial strains germinated and grew in the absence of acetate buffer and in the medium with 0.06 M sodium acetate. However, none of the strains germinated in the medium containing 0.5 M sodium acetate. The medium buffered with 0.25 M acetate usually inhibited the germination of *B. thuringiensis* strains, while allowed the germination of other spore-formers. The medium with 0.12 M sodium acetate allowed several *B. thuringiensis* isolates to germinate. By considering these isolation data, in this study, two different sodium acetate concentrations (0.12 M and 0.25 M) were used to increase the rate of *B. thuringiensis* isolation and to eliminate more of other spore-formers. After acetate selection, heat treatment was applied to kill non-spore formers and vegetative cells of other spore-formers which was allowed to germinate with sodium acetate.

In the twelve of the samples, no microbial growth was observed after acetate selection and heat treatment procedures. Totally, 700 isolates were obtained from collected samples and checked for their colony morphologies. Fifteen different morphologies were observed for the isolates (Table 3.1).

The isolates were named according to the sample number representing the location of isolation, the colony morpology (Table 3.1), and the sodium acetate concentration (a: 0.12 M and b: 0.25 M). For example, in the isolate named as ‘4Ca’; ‘4’ shows sample number, ‘C’ represents colony morphology and ‘a’ represents sodium acetate concentration.

Table 3.1. The colony morphologies of *B. thuringiensis*-like isolates

Colony Code	Morphological Features
A	White, spread and wavy
B	Yellow, small, round, smooth and bright
C	White, spread, rough
D	White, medium size, rough, opaque and round
F	Spread, dense, dull, rough and round with radiating margin
H	Yellow, round, medium size, dull and smooth
I	White, small, round, bright and runny
J	White, round, runny, larger and less brighter than colony I
K	Resembles to colony D, but brighter than D
L	Resembles to colony H, but has a magrin arround
N	White, small, smooth, dull and round with a transparent margin
P	Resembles to colony C, but more transparent
R	Spread, medium size and transparent
U	White, medium size, runny iner layer with dry outer layer
Y	Yellow, medium size, bright and dense

A large number of *B. thuringiensis* were isolated from 81 % of soil samples collected from agricultural lands (Table 3.2). Indeed, Martin and Travers (1989) reported that soil is the normal habitat of *B. thuringiensis*. The occurance of *B. thuringiensis* in all soil samples collected from Konya was found to be relatively high compared to other soil samples. Especially in İvriz, *B. thuringiensis* was isolated from all of the soil samples. Meadows *et al.* (1992) and Hongyu *et al.* (2000) reported that stored product samples are rich in *B. thuringiensis* strains. In present study, the second highest *B. thuringiensis* isolation was made from stored product dusts. Meadows *et al.,* (1992) isolated *B. thuringiensis* from 78 % of the settled grain dust samples. Similar to their study, our isolation represents 70 % (Table 3.2) of stored product samples.

Table 3.2. *Bacillus thuringiensis* isolation analysis according to sample types

sample type	sample collected	samples yielded Bt	% of samples yielding Bt	no of isolates obtained	no of isolates produced crystal proteins	no of isolates cry gene found	% of isoletes cry gene found	Bt index
soil	43	35	81.4	287	116	74	63.8	0.40
grain	13	3	23.1	15	3	1	33.3	0.20
stored product dust	27	19	70.4	149	38	25	65.8	0.26
animal faeces	3	1	33.3	10	2	1	50.0	0.20
various residues	6	1	16.7	23	1	0	0.0	0.04
straw	3	2	66.7	16	3	2	66.7	0.19
insect cadaver	1	0	0.0	-	-	-	-	-
total	**96**	**61**	**63.5**	**500**	**163**	**103**	**63.2**	**0.33**

After acetate selection no growth was observed in 7 of the grain samples. In all regions, percentage of grain samples yielding *B. thuringiensis* was relatively low, 23 % (Table 3.2). This indicates that grain is not as good source as the others for *B. thuringiensis*. Meadows *et al.,* (1992) also suggested that *B. thuringiensis* multiplied in the cadavers of insects that have been killed by the *B. thuringiensis* toxins, and these cadavers were ingested by birds and mammals who spread spores in their feces. Therefore, three animal feces samples were used in this study and one of them yielded two *B. thuringiensis* isolates.

B. thuringiensis index (Bt index), representing the ratio of *B. thuringiensis* isolates in all isolates, is an important measure of success in isolating *B. thuringiensis*. Distribution of *B. thuringiensis* according to sample types and location is shown in Table 3.3. Percentage of samples yielding *B. thuringiensis* from Nikfer was high, 89 %. This is because of the sampled haylofts which had been used for 65 years. In addition, natural grain silos (NGS) have been used for grain storage for more than 500 years and the percent of samples yielding *B. thuringiensis* was 62 %. In fact, Bt indexes of NGS and Nikfer are very similar with the values of 0.27 and 0.26, respectively. This shows a similar degree of occurance of *B. thuringiensis* in two places with similar background.

An average Bt index was found to be 0.33 for all samples but the index changes according to sample types and origins (Table 3.3). The abundance of *B. thuringiensis* was the highest in all soil samples, with a Bt index of 0.40. It decreased to 0.26 in all stored dust product samples and to 0.20 in all grain and animal faeces. Unlike this study, Bravo *et al.* (1998) collected soil samples from cultivated fields in Mexico and obtained a Bt index of about 0.24, nearly two-fold lower than that of this study. However, Martin & Travers (1989) found the highest Bt index as 0.85 in the soil samples collected from Asia, nearly two-fold greater than the Bt index of this current study. This may be related to climate and geographic conditions. In addition, Hongyu *et. al*. (2000) and Bernhard *et.al*. (1997) reported that *B. thuringiensis* is more abundant in stored product environments than in soil. Taken together, these studies show that the level of Bt index changes from region to region and between types of samples.

Table 3.3. Distribution of *Bacillus thuringiensis* based on sample types and location

Location	Type of sample	No. of sample	No. of sample yielding Bt	No. of isolates obtained	No. of isolates producing crystals	No. of isolates positive for *cry* genes	Bt index
Ayranlı / Ereğli-Konya (CA)	soil	9	7	70	42	33	0.60
Ereğli / Konya (CA)	grain	3	1	4	1	0	0.25
	soil	3	3	19	13	8	0.68
	stored product dust	2	1	6	3	3	0.50
		8	5	29	17	11	**0.59***
İvriz / Ereğli-Konya (CA)	soil	7	7	57	15	14	0.26
Üçharman / Ereğli-Konya (CA)	soil	7	6	52	17	7	0.33
	various residues	1	0	1	0	0	0.00
		8	6	53	17	7	**0.32***
Manazan Caves (MC)	animal faeces	1	0	0	0	0	-
Taşkale-Karaman (CA)	soil	9	5	37	8	3	0.22
	stored product dust	5	2	22	4	1	0.18
	various residues	5	1	23	1	0	0.04
		20	8	82	13	4	**0.16***
Natural Grain Silos (NGS)	animal faeces	1	1	6	2	1	0.33
Taşkale-Karaman (CA)	grain	9	2	11	2	1	0.18
	stored product dust	16	13	101	28	18	0.28
		26	16	118	32	20	**0.27***
Bozbük / Söke (AR)	animal faeces	1	0	4	0	0	-
	dead insect	1	0	0	0	0	-
	grain	1	0	0	0	0	-
	soil	5	4	25	11	6	0.44
	straw	1	0	0	0	0	-
		9	4	29	11	6	**0.38***
Nikfer / Denizli (AR)	soil	3	3	26	10	3	0.38
	stored product dust	4	3	20	3	3	0.15
	straw	2	2	16	3	2	0.19
		9	8	62	16	8	**0.26***
Total		**96**	**61**	**500**	**163**	**103**	**0.33**

Isolates were examined with PCM for crystal formation and cry gene content of crystal positive isolates was screened by PCR. CA: Central Anatolia, AR: Aegean Region. Bt index is the ratio of Bt isolates producing crystal to all isolates in each sample group. * indicates the total Bt index in each geographical location.

3.2. Crystal Protein Composition of *Bacillus thuringiensis* Isolates

Five hundred isolates were examined with the phase contrast microscope (PCM) for spore formation and crystal production and morphology. Among them, 163 isolates produced crystals (Table 3.3). Even though 99 other isolates had *B. thuringiensis* like spore and colony morphology, they did not show crystal formation. The remaining 238 isolates did not exhibit any morphological similarities to *B. thuringiensis* nor produced crystals.

B. thuringiensis strains produce parasporal crystal inclusions with different morphologies, sizes and numbers. Based on literature, some distinct morphologies are apparent; bipyramidal crystals, related to Cry 1 proteins (Aranson *et al.,* 1976); cuboidal inclusions, related to Cry 2 proteins (Ohba and Aizawai, 1986); flat and square crystals, related to Cry 3 proteins (Hernstadt *et al.,* 1986); amorphous and composite crystals, related to Cry 4 proteins (Federici *et al.,* 1990).

Crystal morphology of *B. thuringiensis* can provide valuable information on target insect spectra (Maeda *et al.* 2000). For example, bipyramidal shaped crystals are related to Cry 1 proteins that are toxic against lepidopteran species. Therefore, in order to determine the crystal morphology of each *B. thuringiensis* isolate, all isolates were grown for 48 h and examined with the PCM (Figure 3.1).

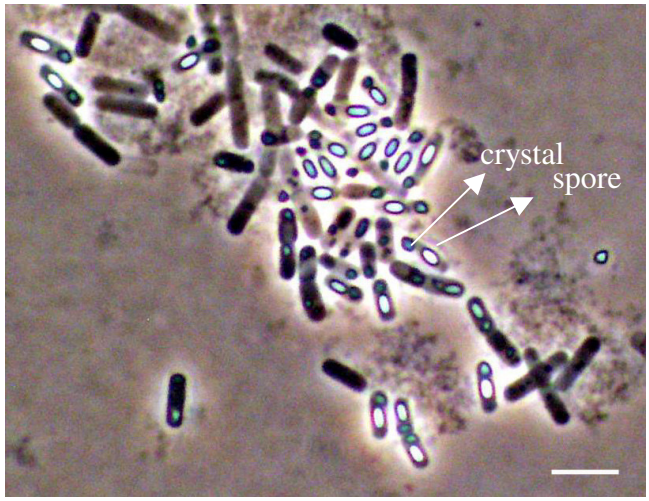

Figure 3.1. Photomicrograph of spores, crystals and vegetative cells of *cry1* positive *B. thuringiensis* isolate (57Hb). Bar represents 2.5µm.

Five different crystal morphology were observed in 163 isolates. Although only one type of crystal morphology was observed in 58% of the isolates, more than one type of crystal morphology was present in other 42% of the isolates (Figure 3.2). Meadows *et al.,*(1992) have obtained isolates producing bipyramidal (53%), irregular pointed (24%) and spherical (9%) crystals. In the present study, the distribution of crystal shapes in 163 isolates was 36% spherical (S), 5% cubic (C), 9% irregular pointed (IP), 2% bipyramidal (B), 19% cubic and spherical (C&S), 22% spherical and irregular pointed (S&IP), 1% cubic and irregular pointed (C&IP), 2% irregular shaped (IS), and 6% not defined (Figure 3.2). Crystal morphologies for all isolates were given in Appendix E.

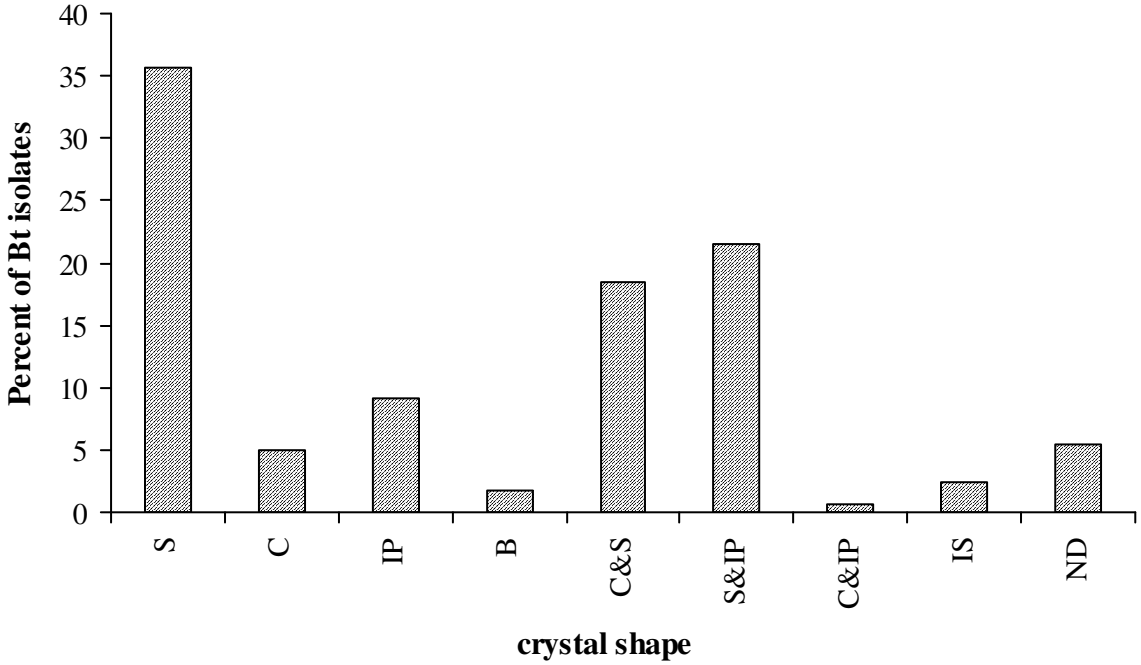

Figure 3.2. Crystal shape distribution of *B. thuringiensis* isolates. After growing the isolates for 48 h, crystal protein formation was observed by using a PCM.

3.3. Characterization of *cry* Gene Content of *Bacillus thuringiensis* Isolates

Toxic crystal proteins are encoded by *cry* genes and one *B. thuringiensis* strain can contain one or more *cry* genes. The *cry* gene contents of 163 crystal producing isolates were determined by PCR analysis of *cry1*, *cry2*, *cry3*, *cry4* and *cry9* genes. Universal primers designed for these genes and their expected PCR product sizes are shown in Table 2.3. Genomic DNA extracted from each *B. thuringiensis* isolate was

used as template in PCR reactions and target gene fragments were amplified by using the universal primers specific to each group of *cry* gene. Each PCR product was checked with the respective reference strains of *B. thuringiensis*, listed in Table 2.2.

One hundred and three of 163 isolates were positive for the *cry* genes examined (Table 3.3). PCR analysis of each isolate with five different *cry* gene primers indicated that 63 of the isolates contained only one type of *cry* gene; however, 40 of them contained more than one type of *cry* gene in which 28 isolates containing 2 different *cry* genes, 8 isolates containing 3 different *cry* genes and 4 isolates containing 4 different *cry* genes (Figure 3.8). Results of all PCR reactions were given in Appendix F.

Ninety-nine isolates exhibited spore and colony morphology similar to that of *B. thuringiensis*, whereas no crystal formation was observed by phase contrast microscobe. On the other hand, when PCR analysis was performed for 9 of them, 7 isolates were positive for *cry* genes examined. This is also related to the absence of gene expression at the protein level. In fact, crystal protein synthesis in *B. thuringiensis* is controlled by a variety of mechanisms at the transcriptional, post-transcriptional or post-translational levels (Agaisse & Lereclus, 1995).

3.3.1. *Cry1* Gene Analysis of *Bacillus thuringiensis* Isolates

The DNA extracted from 163 *B. thuringiensis* isolates was amplified with *cry1* gene primers and 38 of the isolates were obtained as positive for *cry1* gene. Nineteen of *cry1* positive isolates carried only *cry1* gene; however, the other nineteen isolates had more than one type of *cry* gene in addition to *cry1* gene (Figure 3.8). Universal primers for *cry1* gene produced PCR products of expected size at arround 275 bp (Figure 3.3). As being optimized culture in laboratory conditions, *B. thuringiensis* reference strain, *B. thuringiensis* supsp. *aizawai*, (Figure 3.3., lane 1) produced much more amplification product so, gave brighter DNA band than that of environmental isolates.

3.3.2. *Cry2* Gene Analysis of *Bacillus thuringiensis* Isolates

All 163 isolates were screened by PCR for the presence of *cry2* gene and 24 isolates were shown to contain this gene. Six of them contained only *cry2* gene, whereas, 18 of them harboured with the other *cry* genes tested (Figure 3.8). PCR products of *cry2* gene gave bands at expected size about 690 bp for the isolates (Figure

3.4). DNA bands obtained from native strains were also as bright as that of *B. thuringiensis* reference strain.

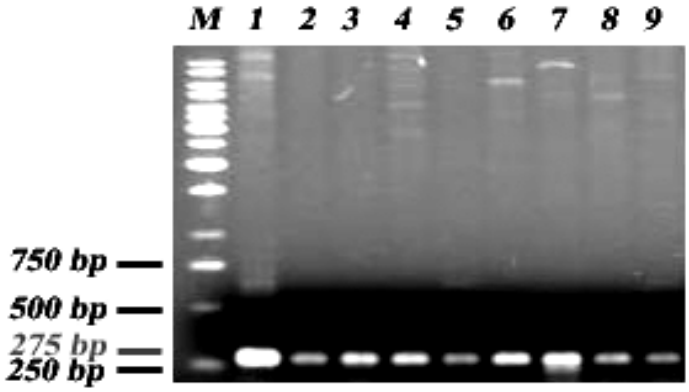

Figure 3.3. Agarose gel (1%) electrophoresis of PCR products of *cry1* gene from different isolates. Lane M: 1 kb DNA MW marker, Lane 1: *B. thuringiensis* supsp. *aizawai* as *cry1* positive control, Lane 2: 35Pb, Lane 3: 35Kb, Lane 4: 13La, Lane 5: 5Ca, Lane 6: 4Cb, Lane 7: 11Ka, Lane 8: 107Fa, Lane 9: 102Fb

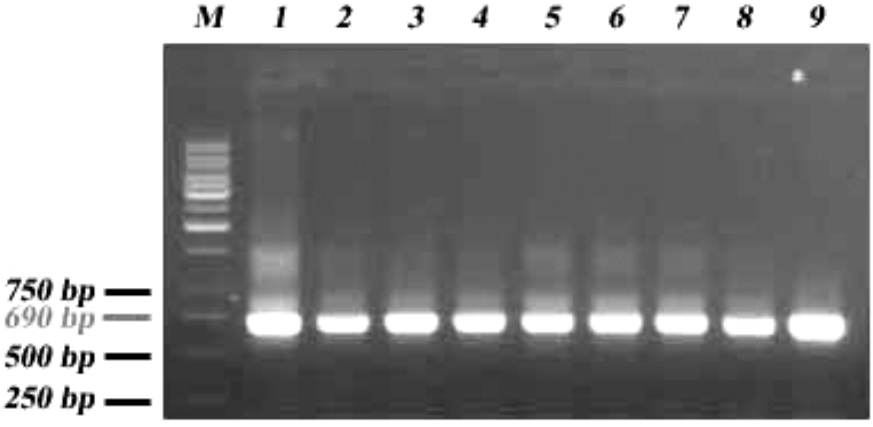

Figure 3.4. Agarose gel (1%) electrophoresis of PCR products of *cry2* gene from different isolates. Lane M: 1 kb DNA MW marker, Lane 1: *B. thuringiensis* supsp. *kurstaki* as *cry2* positive control, Lane 2: 18Fa, Lane 3: 93Ha, Lane 4: 93Da, Lane 5: 93FFa, Lane 6: 27Fb, Lane 7: 19Rb, Lane 8: 19Hb, Lane 9: 85PPb

3.3.3. *Cry3* Gene Analysis of *Bacillus thuringiensis* Isolates

By PCR amplification, 26 of the 163 isolates were obtained as positive for *cry3*. Even though 10 of 26 isolates were carrying only *cry3* gene, 16 isolates were also carrying some of the other *cry* genes examined (Figure 3.8). The expected size of PCR products for *cry3* gene was about 590 bp. Some of the isolates (Figure 3.5., lane 2, 4, 5 and 8) produced PCR products at this expected size, however, the others (Figure 3.5., lane 3, 6, 7 and 9) produced various size of amplification products. This may be due to the genetic diversity of *B. thuringiensis* strains isolated from different environmental samples. These strains may contain different *cry* gene subgroups, including *cry3*, sharing homology with the *cry3* gene primer at binding region. An extraordinary PCR profile obtained for the isolate 98Lb (Figure 3.5., lane 9) which exhibited seven different PCR bands changing in size between 260 and 800 bp approximately. These extra bands produced by the isolates 70Bb, 71Lb and 98Lb (Figure 3.5., lane 6, 7 and 9) might indicates that these isolates may contain other *cry3* subgroups sharing the homology with the primers designed for *cry3* gene amplification. In order to identify such *cry* gene groups, these isolates should be screened by primers specific for subgroups of each *cry* genes. The isolates 24Na and 24Ra (Figure 3.5., lane 4 and 5) gave weak bands compared to others. In fact, the amount of DNA, used as template in PCR reaction mixtures, was not quantified exactly. Therefore, the differences in the intensities of the bands may be arised from the different amount of starting DNA material in gene amplifications. In addition, it may also be arised from plasmids with low copy number.

3.3.4. *Cry4* Gene Analysis of *Bacillus thuringiensis* Isolates

Twenty eight of 163 isolates were found to be positive for *cry4* gene by PCR analysis. Seven of them contained only *cry4*, but the remaining 21 isolates carried some other *cry* genes screened as well (Figure 3.8). PCR products were obtained at expected size about 450 bp for the isolates. The isolate 26Kb (Figure 3.6., lane 4) produced an extra band at arround 650 bp which might indicate that this strain may contain other subgroups of *cry4* gene that might have the homologous sequence with the *cry4* universal primers.

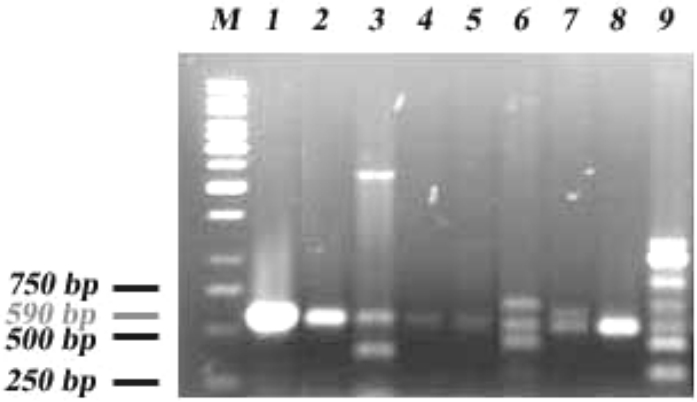

Figure 3.5. Agarose gel (1%) electrophoresis of PCR products of *cry3* gene from different isolates. Lane M: 1 kb DNA MW marker, Lane 1: *B. thuringiensis* biovar. *tenebrionis* as *cry3* positive control, Lane 2: 4La, Lane 3: 13Hb, Lane 4: 24Na, Lane 5: 24Ra, Lane 6: 70Bb, Lane 7: 71Lb, Lane 8: 86Db, Lane 9: 98Lb

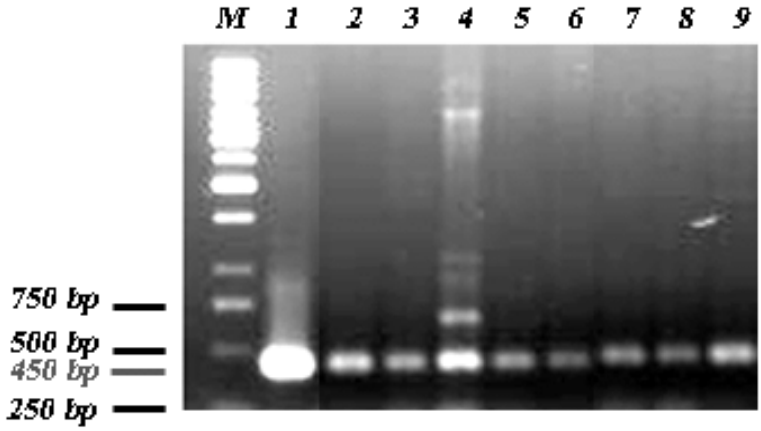

Figure 3.6. Agarose gel (1%) electrophoresis of PCR products of *cry4* gene from different isolates. Lane M: 1 kb DNA MW marker, Lane 1: *B. thuringiensis* biovar. *israelensis* as *cry4* positive control, Lane 2: 19Pb, Lane 3: 24La, Lane 4: 26Kb, Lane 5: 27Ba, Lane 6: 27Pa, Lane 7: 93FFa, Lane 8: 93Fa, Lane 9: 94YYb

3.3.5. *Cry9* Gene Analysis of *Bacillus thuringiensis* Isolates

PCR analysis of 163 isolates with primers for *cry9* gene indicated that 44 isolates were positive for *cry9* gene. Although, half of these 44 isolates were positive

only for *cry9*, the other half were also positive for some other *cry* genes examined (Figure 3.8). The expected size of PCR products for *cry9* gene was 350 bp. Although a single band obtained for some isolates (Figure 3.7., lanes 2,3,6,8 and 9), some isolates such as 25Ca, 94YYb and 93Da (Figure 3.7., lanes 4, 5 and 7) also produced extra amplification products at different sizes. Similar to extra bands observed with other *cry* genes, extra bands obtained with *cry9* gene is most probably due to *cry9* gene subgroups which have homology with the *cry9* gene universal primer.

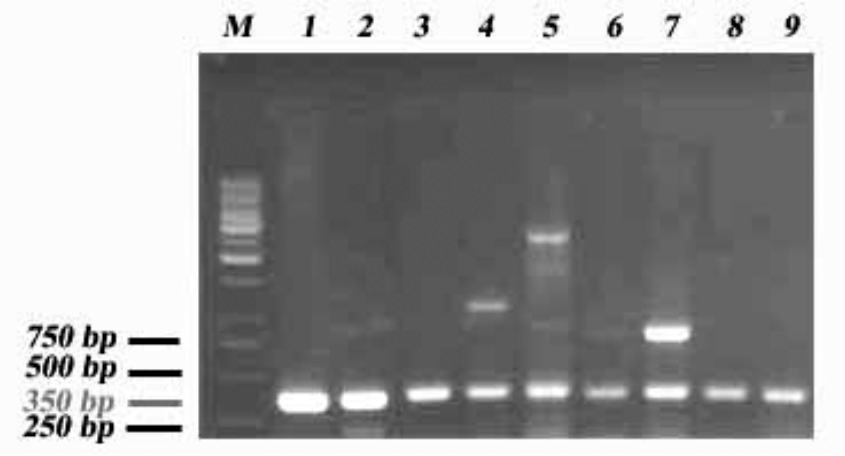

Figure 3.7. Agarose gel (1%) electrophoresis of PCR products of *cry 9* gene from different isolates. Lane M: 1 kb DNA MW marker, Lane 1: *B. thuringiensis* supsp. *aizawai* as *cry9* positive control, Lane 2: 82YYb, Lane 3: 24Ca, Lane 4: 25Ca, Lane 5: 94YYb, Lane 6: 24Nb, Lane7: 93Da, Lane 8: 25Aa, Lane 9: 29Fa

3.4. Analysis of *cry* Gene Distribution

The high number of different *cry* gene profiles are closely related with the environmental diversity of the geographic area surveyed. In this study, the isolates containing *cry9* gene were the most abundant group (28 %) compared to others. However, Bravo *et al*. (1998) have found that *cry9* gene was less abundant (2.6 %) in Mexican soil. Ben-Dov *et al*. (1999) have detected cry genes in 10.2 % of *B. thuringiensis* strains isolated from Israel, Kazakhstan and Uzbekistan.

The most common *cry* genes found in nature belong to *cry1* gene group (Porcar and Juarez-Perez 2002). Bravo *et al*. (1998), Ben-Dov *et al*. (1997) and Wang *et al*. (2003) have reported *cry1* genes were the most frequent in their collections. Similarly,

the isolates containing *cry1* gene were the second most abundant group (23 %) in this current study. In Mexican strain collection (Bravo *et al*., 1998), *cry3* gene abundance has been quite high (21.7 %), whereas in Taiwan (Chak *et al*., 1994) and Israel, Kazakhstan and Uzbekistan (Ben-Dov *et al*., 1997) *B. thuringiensis* collections did not harbor *cry3* gene. These two studies might suggest that *cry3* genes were absent in *B. thuringiensis* strains collected from Asia. However, in the present study, the isolates carrying *cry3* gene were detected as 16 %.

The percentage of *cry4* gene in our *B. thuringiensis* isolates were 18. Bravo *et al*. (1998) have detected *cry4* gene as about 8 % in Mexican soil; however, Chack *et al*. (1994) detected this gene only in 4 of 536 isolates in Taiwan soil. It has been reported that *cry2* gene was found more commonly in isolates from Asia (Ben-Dov *et al*., 1997; Chak *et al*., 1994; Kim *et al*., 2000; Zhang *et al*., 2000) than those from Latin America (Bravo *et al*., 1998). Wang *et al*. (2003) found that 70 % of the isolates obtained from different regions in China contained *cry2* gene. By contrast, 15 % of the isolates were defined as *cry2* gene positive in the present study.

Many studies have reported that *cry1* and *cry2* genes were most often present together (Wang *et al*., 2003; Ben-Dov *et al*., 1997; Zhang *et al*., 2000). Wang *et al*. (2003) have found that among the *cry1* gene containing isolates, 90.7 % strains also harbored a *cry2* gene. Ben-Dov *et al*. (1997) have reported that most of the isolates containing *cry1* gene were also positive for *cry2* gene. The PCR results of the present study showed that only about 10 % of the *cry1* gene positive isolates contained *cry2* gene. In addition, other *cry* genes examined in this study were present with different combinations in the isolates (Figure3.8). For example, 17 % of *cry2* gene positive isolates contained *cry9* gene and 8 % of *cry1* gene positive isolates also contained *cry4* gene.

The reported *cry* gene distribution among the collections is quite variable. Even collections of *B. thuringiensis* strains isolated from the same country may vary in the frequency of observed genes. This shows how different geographic regions affect diversity of *cry* gene content of *B. thuringiensis* strains. As a result, number of the isolates containing *cry9* and *cry1* genes were the most abundant compared to isolates containing other cry genes. In addition, some of the isolates contained 2, 3 or 4 different *cry* genes (Figure 3.8). Name of the isolates containing *cry* genes are in Table 3.4.

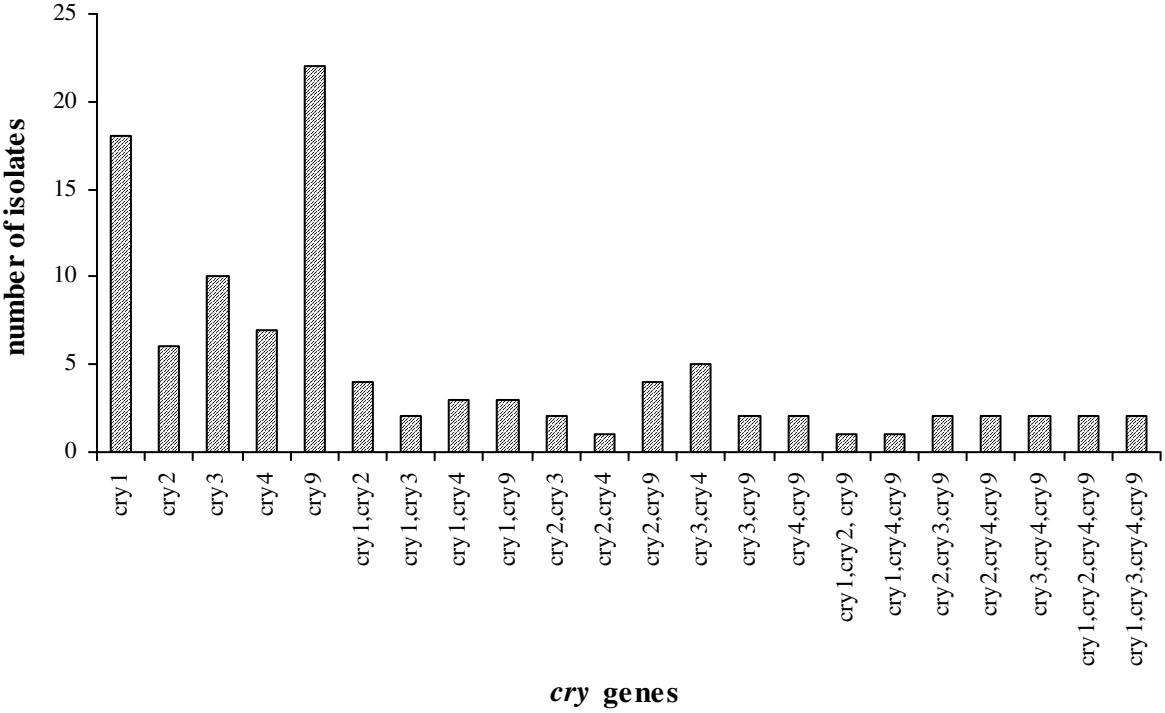

Figure 3.8. *Cry* gene distribution of *B. thuringiensis* isolates based on PCR analysis

When the *cry* gene distribution was examined through sample types, it was found that samples from stored product dust and soil contained *cry* genes at high percentages, 65.8 % and 63,8 % respectively (Table 3.2). When it was examined through sampling locations, almost all screened *cry* genes were present in İvriz/ Ereğli-Konya (Table 3.2).

Sixty of the isolates were negative for *cry* genes examined. In literature, 32 different *cry* gene groups and many subgroups have been defined (Schnepf *et al*., 1998; Crickmore *et al*., 1998); therefore, the remaining 60 isolates may contain different *cry* genes from the ones examined in this study.

Table 3.4. Isolates positive for *cry* genes

type of *cry* gene	name of isolate
cry1	48Ra, 39Ya, 35Pb, 39Yb, 43Db, 71Na, 35Kb, 13La, 5Ca, 4Cb, 11Ka, 107Fa, 102Fb, 1Ab, 1Cb, 60Na, 57Hb, 58Kb
cry2	70Ka, 93Ha, 27Fb, 19Hb, 94Da, 31Fa
cry3	43Ra, 70Bb, 61Kb, 4La, 24Ra, 24Na, 24La, 25Fa, 98Lb, 13Hb, 1Fa
cry4	19Pb, 26Kb, 27Ba, 28Da, 113Ha, 27Pa, 113Ya
cry9	34Bb, 24Nb, 25Aa, 25Ca, 36Ba, 87Fb, 43Fa, 18FFa, 20Rb, 13Nb, 94YYb, 27Cb, 24Pb, 53Yb, 1CCb, 24Ca, 24Fb, 27Fa, 62PPa, 2Ib, 27Ka, 26Ba
cry1, cry2	59Ya, 33Yb, 7Fa, 85PPb
cry1, cry3	71Lb, 55Ka
cry1, cry4	25Ua, 24Fa, 28Ca
cry1, cry9	7Bb, 28Aa, 28Lb
cry2, cry3	1Bb, 24Lb
cry2, cry4	27Kb
cry2, cry9	82YYb, 23Ba, 19Rb, 93Da
cry3, cry4	2Ja, 25Ab, 26Fb, 29Db, 31Na
cry3, cry9	86Db, 2Jb
cry4, cry9	29Fa, 26Pb
cry1, cry2, cry9	25Fb
cry1, cry4, cry9	27Pb
cry2, cry3, cry9	18Fa, 56Kb
cry2, cry4, cry9	93FFa, 8Ba
cry3, cry4, cry9	19Ka, 29Ab
cry1, cry2, cry4, cry9	25Pb, 93Fa
cry1, cry3, cry4, cry9	24La, 1Aa

3.5. Plasmid Profiles of *Bacillus thuringiensis* Isolates

Most *B. thuringiensis* isolates have several extra-chromosomal elements (plasmids) ranging in size from 2 to 200 kb. Some of these plasmids are circular and some are linear, and *cry* genes are generally carried on these plasmids (Carlson *et al*., 1996). Within *B. thuringiensis* species, a large variation of plasmid patterns has been found, reflecting a high strain diversity. Therefore, in this study, plasmids were isolated from *B. thuringiensis* isolates, positive for *cry* genes screened, as well as 4 different *B. thuringiensis* reference strains and differences in plasmid patterns of the isolates in each *cry* gene group were investigated. In addition to different bands varying in size between 15 kb to 22 kb for some isolates, a major DNA band at 15 kb in size was obtained in all isolates (Figure 3.9). Almost all *cry1* gene positive isolates exhibited different plasmid profiles from each other (Figure 3.9., lane 1 to lane 13). Also, some of *cry2* (data not

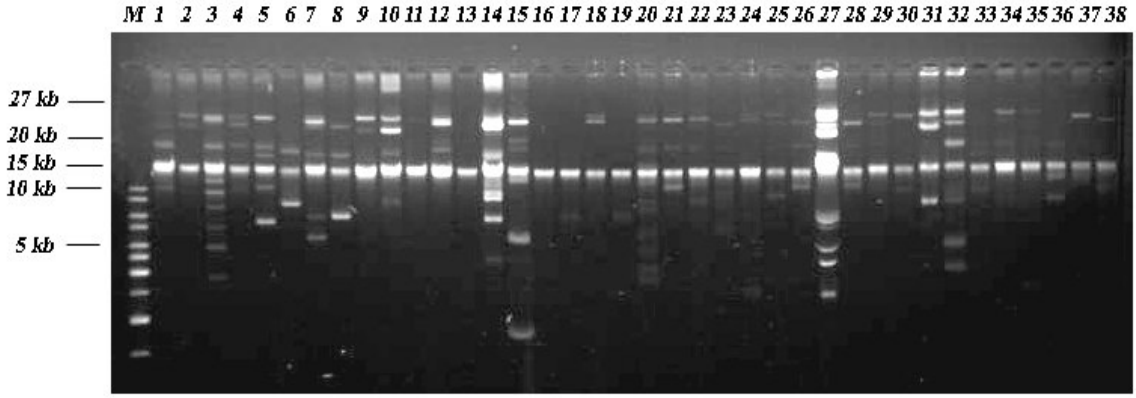

Figure 3.9. Agarose gel (0,8 %) electrophoresis of plasmid preperations of cry1 (lane1-lane14), cry9 (lane 16-29), and cry3 (lane 31-38) positive Bt isolates. LaneM: 1 kb DNA MW marker, Lane 1: 35Pb, Lane 2: 35Kb, Lane 3: 13La, Lane 4: 5Ca, Lane 5: 4Cb, Lane 6: 11Ka, Lane 7: 107Fa, Lane 8: 1Ab, Lane 9: 1Cb, Lane 10: 1Cb, Lane 11: 60Na, Lane 12: 57Hb, Lane 13: 58Kb, Lane 14: *B. thuringiensis* supsp. *aizawai*, Lane 15: 34Bb, Lane 16: 87Fb, Lane 17: 43Fa, Lane 18: 18FFa, Lane 19: 20Rb, Lane 20: 13Nb, Lane 21: 27Cb, Lane 22: 24 Pb, Lane 23: 1CCb, Lane 24: 24Fb, Lane 25: 27Fa, Lane 26: 86Db, Lane 27: 27Ka, Lane 28: 26Ba, Lane 29: *B. thuringiensis* biovar. *tenebrionis*, Lane 30: 43Ra, Lane 31: 70Bb, Lane 32: 61Kb, Lane 33: 4 La, Lane 34: 24Ra, Lane 35: 24Na, Lane 36: 25Fa, Lane 37: 13Hb, Lane 38: 1Fa

shown), *cry4* (data not shown), *cry3* (Figure 3.9., lane 30 to lane 38) and *cry9* (Figure 3.9., lane 15 to lane 28) positive isolates exhibited different plasmid profiles. This may reflect the strain diversity in our isolates.

Iriarte *et al.* (2000) have characterized two novel strains isolated from Spain and reported that these two strains contained two (205 and 160 kb) and three (210, 160 and 80 kb) large plasmids. They have considered the absence of small plasmids as the characteristics of these two strains. However, in our profiles the largest plasmid band was obtained arround 27kb. This may be due to the protocol used for plasmid isolation that was not sufficient for large plasmids.

3.6. PFGE Profiles of *Bacillus thuringiensis* Isolates

Pulsed field gel electrophoresis (PFGE) of chromosomal DNA digested with a restriction enzyme is known to be an accurate and reproducible typing method for closely related bacterial strains (Tenover et al., 1995; Bygraves and Maiden 1992). In a recent study, Rivera and Priest (2003) have examined 70 *B. thuringiensis* strains using PFGE procedure, and investigated the correlation between PFGE type and *cry* gene composition. In this study, PFGE analysis were carried out for 34 environmental *B. thuringiensis* isolates and 4 *B. thuringiensis* reference strains, in order to see if the isolates carrying the same *cry* gene show similar PFGE patterns. Chromosomal DNA from 6 *cry1*, 8 *cry2*, 5 *cry3*, 4 *cry4* and 11 *cry9* gene positive isolates were subjected to PFGE analysis. Although there were some similarities among PFGE patterns of the isolates (data not shown except for *cry9*), mostly no identical patterns were obtained within each of the *cry* gene groups (Figure 3.10). Based on Rivera and Priest (2003), if PFGE patterns differed by changes up to 3 bands and more than three bands, strains are described as closely related and unrelated, respectively. Therefore, results of this study showed that isolates in each group of *cry1, cry2, cry3,* and *cry4* genes could be unrelated strains. However, two patterns in *cry9* positive isolates, 53Yb and 29Fa (Figure 3.10., lane 4 and lane 7) produced the same PFGE profile, indicating that these two were the same strains. The rest of the isolates carrying *cry9* gene were not idendical. Similar to the results of Rivera and Priest (2003), our findings also showed that there is no exact correlation between *cry* gene content and PFGE patterns. This is possible because *cry* genes are often carried on plasmids and plasmid exchange between

strains as well as recombination between *cry* genes from different backgrounds occur in *B. thuringiensis* strains (De Maagd *et al*. 2001).

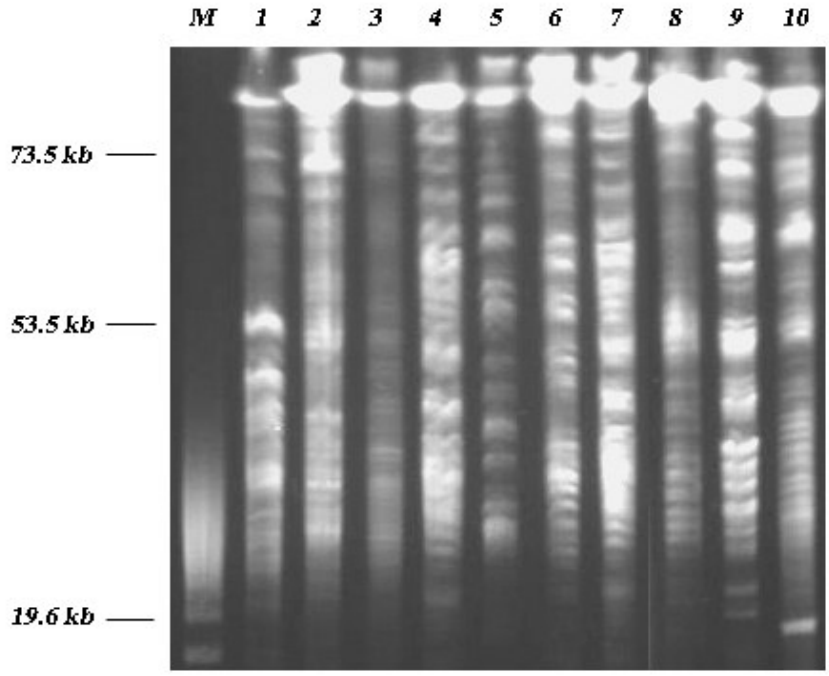

Figure 3.10. PFGE patterns of cry9 positive isolates. Lane M: 5 kb DNA MW marker, Lane 1: 62PPa, Lane 2: 24Ca, Lane 3: 28Aa, Lane 4: 53Yb, Lane 5: 94YYb, Lane 6: 93Da, Lane 7: 29Fa, Lane 8: 25Aa, Lane 9: 82YYb, Lane 10: *B. thuringiensis* supsp. *aizawai*

Chapter 4

CONCLUSION AND FUTURE EXPERIMENTS

B. thuringiensis is a ubiquitous, Gram-positive, spore-forming bacterium that produces intracellular toxic proteins which are active against certain insect species. Because of its insecticidal activity, *B. thuringiensis* has been widely used as biopesticide for more than 40 years, as an alternative to chemical pesticides. The genetic diversity and toxic potential of *B. thuringiensis* strains are closely related to geographic conditions of the regions where the strains are isolated. Many researchers and research centers focus on to isolate and characterize environmental *B. thuringiensis* strains to find novel strains with better toxic activity. Therefore, the objective of this study was to isolate and characterize native *B. thuringiensis* strains in grain related habitats, providing a base to establish a Turkish *B. thuringiensis* culture collection.

Four approaches were taken; analysis of crystal protein production with phase contrast microscopy, detection of *cry* gene content by PCR, plasmid profiling and chromosomal DNA profiling by PFGE. In total 96 samples were collected from certain parts of Central Anatolia and Aegean region. *B. thuringiensis* was isolated from 61 of 96 samples (63.5%). In total 700 bacterial colonies were isolated and defined on the basis of colony morphology. Five hundred of the isolates were examined under phase contrast microscobe and 163 of them (33%) were identified as *B. thuringiensis* based on crystal production. Five different crystal morphologies, spherical (S), cubic (C), irregular pointed (IP), bipyramidal (B), irregular shaped (IS), were determined and spherical type crystals were mostly observed (36%). It was found that 42% of the isolates had more than one crystal morphology, while 58 % of them had only one. In PCR screening, 103 of 163 crystal producing isolates were found to be positive for 5 different *cry* genes (*cry1, cry2, cry3, cry4, cry9*) examined. Plasmid and chromosomal DNA profiling resulted in different patterns of the isolates carrying the same *cry* gene, indicating wide range of diversity among *B. thuringiensis* strains found in Anatolia.

All the data obtained from this study have great importance for the future works. The biological activities of crystal proteins purified from *B. thuringiensis* isolates will be examined on different insects. Protein profiling studies for the strains with high toxic activity will be performed. Serological tests of *B. thuringiensis* isolates will be carried out in order to identify them according to known serotypes, and search for new and

unknown serotypes in our isolates, if present. Identification at subspecies level by detailed genetic characterization such as screening with specific primers and DNA sequencing may be another part of future works. Also, studies on culture conditions of *B. thuringiensis* isolates with higher insecticidal activities will be carried out to large scale crystal protein production. Toxin producing genes, *cry* genes, may also be cloned from plasmids into *E. coli* with a suitable vector in order to produce more target proteins rapidly and economically.

REFERENCES

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

Alstad, D.N. and Andow, D.A., Managing the evolution of insect resistance to transgenic plants, *Science*, **268**, (1995), 1894-1896.

Aronson, A., Sporulation and δ-endotoxin synthesis by *Bacillus thuringiensis, Cellular and Molecular Life Sciences,* **59**, (2002), 417-425.

Baum, J.A. and Malvar, T., Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*, *Mol. Microbiol*., **18**, (1995), 1-12.

Baum, J.A., Johnson, T.B., Carlton, B.C., *Bacillus thuringiensis*: Natural and recombinant biopesticide products, in *Biopesticides: Use and Delivery*, edited by F.R. Hall and J.J. Menn, (Humana Press, Totowa, NJ, 1999), p. 189-210.

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

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

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

Berliner, E., Über die Schlaffsucht der Mehlmottenraupe, *Z. Gesamte Getreidewes*, **3**, (1911), 63-70.

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

Boulter, D., Gatehouse, A. M. R., Hilder, V., Use of crowpea trypsin inhibitor (CPTI) to protect plants against insect predation, *Biotechnol. Adv*., **7**, (1989), 489-497.

Bourque, S.N., Valero, J.R., Mercier, J., Lavoie, M.C., Levesque, R.C., Multiplex polymerase chain reaction for detection and differentiation of the microbial insecticide *Bacillus thuringiensis*, *Applied and Environmental Microbiology*, **59**, (1993), 523-527.

Bravo, A., Sarabia, S., Lopez, L., Ontiveros, H., Abarca, C., Ortiz, A., Ortiz, M., Lina, L., Villalobos, V., Pena, G., Nunez-Valdez, M., Soberon, M., Quintero, R., Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain Collection, *Applied and Environmental Microbiology,* **64**, (1998), 4965-4972.

Burges, H.D. and Jones, K.A., Formulation of bacteria, viruses, and protozoa to control insects, in *Formulation of Microbial Biopesticides*, edited by H.D. Burges (Kluwer Academic Publisher, Dordrecht, The Netherlands, 1999) p. 34-127.

Bygraves, J., Maiden, M.C., Analysis of the clonal relationships between strains of *Neisseria meningitidis* by pulsed field gel electrophoresis, *Journal of Genomic Microbiology,* **138**, (1992), 523-531.

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

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

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

Carlton, B.C. and Gonzalez, J.M., The genetics and molecular biology of *Bacillus thuringiensis*, in *The Molecular Biology of the Bacilli*, edited by D.A. Dubnau, (Academic Pres, New York, 1985), p. 211-249.

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

Ceron, J., Covarrubias, L., Quintero, R., Ortiz, A., Ortiz, M., Aranda, E., Lina, L., Bravo, A., PCR analysis of the cry1 insecticidal crystal family genes from *Bacillus thuringiensis*, *Applied and Environmental Microbiology*, **60**, (1994), 353-356.

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

Chak, K.F., Chao, D.C., Tseng, M.Y., Kao, S.S., Tuan, S.J., Feng, T.Y., Determination and distribution of *cry* – type genes of *Bacillus thuringiensis* isolates from Taiwan, *Applied and Environmental Microbiology* **60**, (1994), 2415-2420.

Cohen, E., Rozen, H., Joseph, T., Margulis, L., Photoprotection of *Bacillus thuringiensis* var. *kurstaki* from ultra-violet irradiation, *Journal of Invertabrate Pathology,* **57**, (1991), 343-351.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van-Rie, J., Lereclus, D., Baum, J.,Dean, D.H., Revision of nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins, *Microbiology and Molecular Biology Review*., **62**, (1998), 807-813.

Croft, B.A., Developing a philosophy and program of pesticide resistance management, in *Pesticide resistance in Artropods*, edited by R.T. Roush and B.E. Tabashnik, (Chapman and Hall, New York, 1990), p. 277-296.

De Barjac, H. and Bonnefoi, A., Mise au point sur la classification des *Bacillus thuringiensis*, *Entomophage*, **18**, (1973), 5-17

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

Dulmage, H.D., Insecticidal activity of HD-1, a new isolate of Bacillus *thuringiensis* var. *alesti*, *Journal of Invertabrate Pathology*, **15**, (1970), 232-239.

Dunkle, R.L. and Shasha, B.S, Response of starch encapsulated *Bacillus thuringiensis* containing UV screens to sunlight, *Environ. Entomol*., **18**, (1989), 1035-1041.

EPA Guidance for the registration of pesticide products containing *Bacillus thuringiensis* as the active ingredient, (Registration Standard 540/RS-89-023, December, 1998).

Estruch, J.J., Warren, G.W., Mullins, M.A., Nye, G.J.,Craig, J.A., Koziel, M.G., Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects, *Proc. Natl. Acad. Sci*., **93**, (1996), 5389-5394.

Federici, B.A., Lüthy, P., Ibarra, J.E., The parasporal body of *Bacillus thuringiensis* subsp. *israelensis*: structure, protein composition and toxicity, in *Bacterial Control of Mosquitos and Blackflies: Biochemistry, Genetics and Applications of Bacillus thuringiensis and Bacillus sphaericus*, edited by H. de Barjac and S. Sutherland, (Rutgers University Pres, New Brunswick, N.J., 1990).

Feitelson, J.S., Payne, J., Kim, L., *Bacillus thuringiensis*: insects and beyond. *Bio/Technology,* **10,** (1992), 271-275.

Feitelson, J.S., The *Bacillus thuringiensis* family tree, in Advanced Engineered Pesticides, edited by L. Kim, (Marcel Dekker, Inc., New York, NY, 1993), p. 63-72.

Ferro, D.H., Lyon, S.M., Colorado potato beetle (Coleoptera: Chrysomelidae) larval mortality: operative effects of *Bacillus thuringiensis* subsp. *san diego*, *J. Econ.Entomol.,* **84**, (1991), 806-809.

Ferro, D.H., Yuan, Q.C., Slocombe, A., Tutle, A., Residual activity of insecticides under field conditions for controlling the Colorado potato beetle (Coleoptera: Chrysomelidae), *J. Econ. Entomol*., **86**, (1993), 511-516.

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

Frutos, R., Rang, C., Royer, M., Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. *Critical Rewievs in Biotechnology*, **19**, (1999), 227-276.

Fuxa, J., Fate of released entomopathogens with reference to risk assessment of genetically engineered microorganisms, *Bull. Entomol. Soc. Am*., **35**, (1989), 12-24.

Gill, S.S., Cowles, E.A., Pietranto, P.V., The mode of action of *Bacillus thuringiensis* endotoxins, *Annu. Rev. Entomol*., **37**, (1992), 615-636.

Glaeve, A.P., Williams, R., Hedges, R.J., Screening by polymerase chain reaction of *Bacillus thuringiensis* serotypes for the presence of *cryV*-like insecticidal protein genes and characterization of a *cryV* gene cloned from *B. thuringiensis* subsp. *kurstaki, Applied and Environmental Microbiology*, **59**, (1993), 1683-1687.

Glathorn, M.F. and Rapoport, G., Biosynthesis of the parasporal inclusions of *Bacillus thuringiensis*: half-life of its corresponding Messenger RNA, *Biochimie*, **54**, (1973), 1291-1301.

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

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

Gonzales, J.M., Brown, B.J., Carlton, B.C., The transfer of *Bacillus thuringiensis* plasmid coding for δ-endotoxin among the strains of *Bacillus thuringiensis* and *Bacillus cereus*, *Proceedings of National Acad. Sci*. USA, **79**, (1982), 6951-6955.

Grachulski, P., Mason, L., Borisova, S., Puzstai-Carey, M., Schwartz, J.-L., Brousseau, R., Cygler, M., *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation, *J. Mol. Biol*., **254**, (1995), 447-464.

Helgason, E., Økstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I., Kolstø, A.-B., *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis*- one species on the basis of genetic events, *Applied and Environmental Microbiology,* **66**, (2000), 2627-2630.

Hernstand, C., Soares, G. G., Wilcox, E. R., Edwards, D. I., A new strain of *Bacillus thuringiensis* with activity against coleopteran insects, *Bio/Technology*, **4**, (1986), 305-308.

Hofmann, C., Lüthy, P., Pliska, V., Binding of the delta endotoxin from *Bacillus thuringiensis* to brush border membrane vesicles of cabbage butterfly (*Pieris brassicae*), *Eur. J. Biochem*., **173**, (1998), 85-91.

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

Höfte, H., Van-Rie, J., Jansens, S., Van Houtven, A., Vanderbruggen, H., Vaeck, M., Monoclonal antibody analysis and insecticidal spectrum of three types of

lepidopteran-specific insecticidal crystal proteins of *Bacillus thuringiensis*, *Appied and Environmental Microbiology*, **54**, (1988), 2010-2017.

Höfte, H. and Whiteley, H.R., Insecticidal crystal proteins of *Bacillus thuringiensis*, *Microbiol. Rev*., **53**, (1989), 242-255.

Hu, X., Hansen, B.M., Eilenberg, J., Hendriksen, B. N., Smidt, L., Yuan, Z., Jensen, G.B., Conjugative transfer, stability and expression of plasmid encoding a cry1Ac gene in *Bacillus cereus* group strains, **231**, (2004), 45-52.

Iqbal, M., Verkerk, R.H.J., Furlong, M.J., Ong, P.C., Rahman, S.A., Wright, D.J., Evidence for resistance to *Bacillus thuringiensis* subsp. *kurstaki* HD-1, *Bacillus thuringiensis* subsp. *aizawai* and Abamectin in field populations of *Plutella xylostella* from Malaysia, *Pestic. Sci*., **48**, (1996), 89-97.

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

Ishiwata, S., On a kind of severe flacherine(sotto disease), *Dainihon Sanshi Kaiho*, **114**, (1901), 1-5.

Johnson, D.E. and McGaughey, H., Contribution of *Bacillus thuringiensis* spores to toxicity of purified Cry proteins towards Indeanmeal moth larvae, *Current Microbiology*, **33**, (1996), 54-59.

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

Kalman, S., Kiehne, K.L., Libs, J.L., Yamamoto, T., Cloning of a novel *cry1C*-type gene from a strain of *Bacillus thuringiensis* subsp. *galleriae*, *Appied and Environmental Microbiology*, 59, (1993), 1131-1137.

Kegley, S. E. and Wise, L. J., *Pesticides in Fruit and Vegetables*, (Sausalito, CA: University Science Books, 1998).

Kim, H.S., Comperative study of the frequency, flagellar serotype, crystal shape, toxicity and cry gene contents of *Bacillus thuringiensis* from three environments, *Journal of Invertabrate Pathology*, 41, (2000), 250-256.

Knowless, B.H. and Dow, J.A.T., The crystal δ-endotoxin of *Bacillus thuringiensis*: models for their mechanism of action on the insect gut, *Bioassays*, **15**, (1993), 469-476.

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

Krieg, A., Huger, A. M., Langenbruch, G. A., Schnetter, W., *Bacillus thuringiensis* var. *tenebrionis*: Ein neuer, gegenüber Glarven von Coleoptaran Wirksamer Pathotype. Z. angew. *Entomology*, **96**, (1983), 500-508.

Kumar, P. A., Sharma, R. P., Malik, V. S., The insecticidal proteins of *Bacillus thuringiensis*, *Advances in Applied Microbiology*, **42**, (1997), 1-43

Kuo, W.-S. and Chak, K.-F., Identification of novel *cry*-type genes from *Bacillus thuringiensis* strains on the basis of restriction fragment length polymorphism of the PCR-amplified DNA, *Appied and Environmental Microbiology*, **62**, (1996), 1369-1377.

Lambert, B., Peferoen, M., Insecticidal promise of *Bacillus thuringiensis*. Facts and mysteries about a succesful biopesticide, *Bioscience*, **42**, (1992), 112-122.

Levinson, B.L., High performance liquid chromatography analysis of two β-exotoxin produced by some *Bacillus thuringiensis* strains, in *Analytical Chemistry of Bacillus thuringiensis*, edited by L.A. Hickle and W.L. Fitch, (American Society, Washington, D.C.,1990), p. 115-136.

Li, J., Carroll, J., Ellar, D.J., Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Aº resolution, *Nature*, **353**, (1991), 81-821.

Li, J., Koni, P.A., Ellar, D.J., Structure of the mosquitocidal δ-endotoxin *cyt B* from *acillus thuringiensis*, *Journal of Molecular Biology*, **257**, (1996), 129-152.

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

Lövgren, A., Zang, M.-Y., Engstöm, A., Dalhammar, G., Landen, R., Molecular characterization of immune inhibitor A, a secreted virulence protease from *Bacillus thuringiensis*, *Molecular Microbiology,***4**, (1990), 2137-2146.

Lui, Y.B. and Tabashnik, B.E., Experimental evidence that refuges delay insect adaptation to *Bacillus thuringiensis*, *Proc. R. Soc. Lond B*, **400**, (1997), 519.

Maeda, M., Mizuki, E., Nakamura, Y., Hatano, T., Ohba, M., Recovery of *Bacillus huringiensis* from marine sediments of Japan, *Current Microbiology,* **40**, (2000), 413-422.

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

Margalit, J. and Dean, D., The story of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*), *J. Am. Mosq. Control Assoc*., **1**, (1985), 1-7.

Marrone, P. G. and Machintosh, S.C., Resistance to *Bacillus thuringiensis* and resistance management, in *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice*, edited by P. F. Entwistle, J. S. Cory, M. J. Bailey, S. Higgs, (John Wiley & Sons, Chichester, UK, 1993), p. 221-235.

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

McGuire, M.R. and Shasha, B.S., Starch encapsulation of microbial pesticide, in *Biorational Pest Control Agents Formulation and Delivery,* edited by F.R. Hall and J.W. Barry, (ACS Symposium Series 595, London, UK, 1995), p. 229-237.

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

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

Murray, K.D., Alford, A.R., Groden, E., Interactive effects of an antifeedant used with *Bacillus thuringiensis* subsp. *san Diego* delta endotoxin on Colorado potato beetle (Coleoptera: Chrysomelidae), *J. Econ.Entomol.,*86, (1993), 1793-1801.

Navon, A., Klein, M., Braun, S., *Bacillus thuringiensis* potency bioassay against Heliotis armigera, Earias insulana, and spodoptera littoralis larvae based on standardized diets, *Journal of Invertabrate Pathology*, **55**, (1990), 387-393.

Navon, A., Control of lepidopteran pests with *Bacillus thuringiensis*, in *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice*, edited by P. F. Entwistle, J. S. Cory, M. J. Bailey, S. Higgs, (Wiley New York, USA, 1993), p. 125-146.

Navon, A., Keren, S., Levski, S., Grinstein, A., Riven, J., Granular feeding baits based on *Bacillus thuringiensis* products for the control of lepidopterous pests, *Phytoparasitica*, **25**, (1997), 101S-110S.

Navon, A., *Bacillus thuringiensis* insecticides in crop protection-reality and prospects, *Crop Protection*, **19**, (2000), 669-676.

Neppl, C. C., Management of Resistance to *Bacillus thuringiensis* toxins, Thesis in The Environmental Studies Program (University of Chicago, 2000).

Oerke, E. C., Dehne, H. W., Safeguarding production-losses in major crops and the role of crop protection, *Crop Protection*, **23**, (2004), 275-285.

Ohba, M. and Aizawai, K., Distribution of *Bacillus thuringiensis* in soils of Japan, *Journal of Invertabrate Pathology,* **47**, (1986), 277-282.

O'Sullivan, D.J. and Klaenhammer, T.R., Rapid mini-prep. isolation of high-quality plasmid DNA from Lactococcus and Lactobacillus spp., *Appied and Environmental Microbiology*, **59, (**1993), 2730-2733.

Patel, K.R., Wyman, J.A., Patel K.A.,Burden, B.J., A mutant of *Bacillus thuringiensis* producing a dark-brown pigment with increased UV resistance and insecticidal activity, *Journal of Invertabrate Pathology,* **67**, (1996), 120-124.

Pimentel, D. and Burgess., Effects of single versus combinations of ınsecticides on the development of resistance, *Environ. Entomol*., **14**, (1985), 582-589.

Porcar, M. and Juarez-Perez, V., PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes, *FEMS Microbiology Reviews,* **757**, (2002), 1-4.

Ramos, L.M., McGuire, M.R., Galan Wong, L.J., Utilization of several biopolymers for granular formulations of *Bacillus thuringiensis, J. of Econ. Entomol*., **91**, (1998), 1109-1113.

Rivera, A.M.G. and Priest, F.G., Pulsed field gel electrophoresis of chromosomal DNA reveals a clonal population structure to *Bacillus thuringiensis* that relates in general to crystal protein gene content, *FEMS Microbiology Letters,* **223,** (2003), 61-66

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

Shah, P.A. and Goettel, M.S., *Directory of Microbial Control Products and Services*, edited by P.A. Shah and M.S. Goettel, (Society of Inveretbrate Pathology, Gainesville, FL 32614-7050, USA, 1999), p. 31.

Shin, B.-S., Park, S.-H., Choi,S.-K., Koo, B.-T., Lee, S.-T., Kim, J.-I., Distribution of *cryV- type* insecticidal protein genes in *Bacillus thuringiensis* and cloning of *cryV-type* genes from *Bacillus thuringiensis* subsp. *kurstaki and Bacillus thuringiensis* subsp. *entomocidus*, *Appied and Environmental Microbiology*, **61**, (1995), 2402-2407.

Sonngay, S. and Panbangred, W., Unpublished observation, (1997)

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelson, P.A., Murray, B.E., Persing, D.H., Swaminathan, B.A., Interpreting chromosomal DNA restriction patterns produced by pulse-field gel electrophoresis: criteria for bacterial typing, *Journal of Clinical Microbiology,* **33**, (1995), 2233-2239.

Theunis, W., Aguda, R.M., Cruz, W.T., Decock, C., Peferoen, M., Lambert, B., Bottrell, D.G., Gould, F.L., Litsinger, J.A., Cohen, M.B., *Bacillus thuringiensis* isolates from the Philippines: habitat distribution, δ-endotoxin diversity and toxicity to rice stem borers (Lepidoptera: Pyralidae), *Bulletin of Entomological Research,* **88**, (1998), 335-342.

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

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

Tilman, D., Global environmental impacts of agricultural expansions: the need for sustainable and efficient practices, *Proc. Natl. Acad. Sci*., **96**, (1999), 5995-6000.

Trumble, J., Alvaro-Rodrigez, B., Development of economic evaluation of an IPM program for fresh market tomato production in Mexico, *Agric. Ecosys. Environ*., **43**, (1993), 267-284.

United Nations, World population prospects: the 1996 revisions, (United Nations, New York, 1996).

USEPA, Meeting summary: EPA-USDA Bt crop insect resistance management workshop, 1999b. (http://www.epa.gov/oppbppd1/biopesticides/summary 826.htm)

Uribe, D., Martinez, W., Ceron, J., Distribution and diversity of *cry* genes in native strains of *Bacillus thuringiensis* obtained from different ecosystems from Colombia, *Journal of Invertebrate Pathology*, **82**, (2003), 119-127.

Van Frankenhuyzen, K., The challenge of *Bacillus thuringiensis*, in *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice*, edited by P. F. Entwistle, J. S. Cory, M. J. Bailey, S. Higgs, (John Wiley & Sons, Chichester, UK, 1993), p. 1-35.

Van Rie, J., Jansens, S., Höfte, H., Degheele, D., Van Mellaert, H., Specificity of *Bacillus thuringiensis* δ-endotoxin: importance of specific receptors on the brush border membrane of the mid-gut of target insects, *Eur. J. Biochem*., **186**, (1989), 239-247.

Van Rie, J., Jansens, S., Höfte, H., Degheele, D., Van Mellaert, H., Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins, *Appied and Environmental Microbiology*, **57**, (1990), 1650-1655.

Van Rie, J., Insect control with transgenic plants: resistance proof?, *Trends in Microbiology*, **9**, (1991), 177-179.

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

Wong, H.C. and Chang, S., Identification of positive retroregulator that stabilizes mRNAs in bacteria, *Proc. Natl. Acad. Sci*. USA, **83**, (1986), 3233-3237.

Wood, R. J., Strategies for conserving susceptibility to insecticides, *Parasitlogy*, **82**, (1981), 69-80.

Wysoki, M., *Bacillus thuringiensis* preparations as a means for the control of lepidopterous pests in Israel, *Isr. J. Entomol*., **23**, (1989), 119-129.

Yoo, K.-H., Kim, S.-Y., Kang, M.-H., Cho, M.-H., Lee, H.-H., Characterization of *Bacillus thuringiensis* isolates from soil in Wonju area, *The Jour.of Microbiol*., **34**, (1996), 370-373.

Zhang, M.-Y., Lövgren, A., Low, M.G., Landen, R., Characterization of a virulent pleiotropic mutant of the insect pathogen *Bacillus thuringiensis*: reduced expression of flagellin and phospholipases, *Infections and Immunity*, **61**, (1993), 4947-4954.

APPENDIX A

Natural and Genetically Modified Bt Products Registered for Agricultural Use

Bt strain	Company	Product	Target insect	Crop
a) Natural				
kurstaki HD-1	Abbott Laboratories Chicago IL, US	Biobit, Dipel, Foray	Lepidoptera	Field and vegetable crops, greenhouse,orchard fruits and nuts, ornamentals, foresty, stored products
kurstaki HD-1	Thermo Trilogy Crop Columbia MD, US	Javelin, Steward, Thuricide, Vault	Lepidoptera	
kurstaki	Abbott	Bactospeine, Futura	Lepidoptera	
kurstaki	Thermo Trilogy	Able, Costar	Lepidoptera	
aizawai	Abbott	Florbac Xentari	Lepidoptera armyworms	Row crops
tenebrionis	Abbott	Novodor	Colorado Potato Beetle,	Potato, tomato, eggplant
			Elm Bark Beetle	Ornamentals, shade trees
tenebrionis	Thermal Trilogy	Trident	Coleoptera	Potato, tomato, eggplant
kurstaki	Bio Dalia, Dalia, Israel	Bio-Ti	Lepidoptera	Avocado, tomato, vineyards, pine forests
kurstaki	Rimi, Tel Aviv, Israel	Bitayon (granular feeding baits)	Btrachedra amydraula	Date palms
galleriae	Tuticorin Alkali Chemicals & Fertilizers Ltd. India	Spicturin	Lepidoptera	Cruciferous crop plants
YB-1520	Huazhong Agric. University, China	Mainfeng pesticide	Lepidoptera	Row crops, fruit trees
-	Scient.& Thechnol. Develop., China	Bt 8010 Rijin	Lepidoptera	Row crops, rice, maize, maize, fruit trees, forests, ornamentals
CT-43	Huazhong Agric. Univ., China	Shuangdu	Lepidoptera, Coleoptera, Diptera	Row crops, garden plants, forests

b) Genetically modified

aizawai recipient *kurstaki* donor	Thermo Trilogy	Agree, Design (transconjugant)	Lepidoptera (Resistant *P.xylostella*)	Row crops
kurstaki recipient *aizawai* donor	Ecogen, Inc. Langhorne PA, US	Condor, Cutlass (transconjugant)	Lepidoptera	Row crops
kurstaki	Ecogen	CRYMAX, Leptinox	Lepidoptera	Vegetables, horticultural, ornamental
kurstaki	Ecogen	Leptinox (recombinant)	Lepidoptera armyworms	Truf, hay, row crops, sweet corn
kurstaki recipient	Ecogen	Raven (recombinant)	Lepidoptera Coleoptera	Row crops Potato, tomato, eggplant
δ-endotoxin encapsulated in *Pseudomonas fluorescens*	Mycogen, Crop. San Diego, CA, US	MVP MATTCH MTRACK (CellCap®)	Lepidoptera Lepidoptera Coleoptera	Row crops-armyworms Potato, tomato, eggplant

Based on Baum *et al*., (1999), Shah and Goettel (1999).

APPENDIX B

Chemicals Used in the Experiments

Nutrient Broth	Merck 1.05443
Sodium Acetate	Sigma S2889
Agar Agar	Merck 1.01613
Yeast Extract	Merck 1.03753
Bacteriological peptone	Oxoid LP037
Sodium Chloride (NaCl)	Applichem A2942
Tryptone	Oxoid L42
Tryptose	Oxoid L47
di-Sodium Hydrogen Phosphate (Na_2HPO_4)	Applichem A2943
Sodium di-Hydrogen Phosphate (NaH_2PO_4)	Merck 1.06346
Magnessium Chloride Hexahydrate ($MgCl_2.6H_2O$)	Merck 1.05832
Mineral Oil	Sigma M5904
Glycerol	Applichem A2926
Agarose (Standard)	Applichem A2114
Agarose (Low Melt)	Appplichem A3762
Agarose (Molecular Biology Certified)	Bio-Rad 162-0134
Trizma Base	Sigma T6066
EDTA	Applichem A2937
Ethidium Bromide	Applichem A1151
D(+)-Sucrose	Applichem A2211
Sodium Dodecyl Sulphate (SDS)	Applichem A2263

Sodium Hydroxide (NaOH)	Merck 1.06498
Ammonium Acetate	Applichem A2936
Hydrochloric Acid (HCl)	Merck. 1.00317
Phenol	Applichem A1594
Chloroform	Applichem A3633
Lysozyme	Applichem A3711
Isopropanol	Applichem A3928
Ethanol	Applichem A3678
N-laurylsarcosine	Applichem A1163
Boric Acid	Applichem A2940
Isoamylalcohol	Applichem A2610
Oil Immersion	Applichem A0699
Proteinase K	Applichem A3830
Ribonuclease A	Applichem A3832
Taq DNA Polymerase	MBI, Fermentas EP0401
dNTP Set	MBI, Fermentas R0181
Gene Ruler^{TM} 1 kb DNA Ladder	Fermentas, SM0313
*Sma*I (Restriction enzyme)	Fermentas, ER0662

APPENDIX C

MEDIA

C.1. Nutrient Agar Medium Used for *B. thuringiensis* Isolation

	g/l
Nutrient broth	13
Agar agar	15

Ingredients are dissolved in 800 ml distilled water by stirring with gentle heating and completed to 1000 ml. Medium is sterilised by autoclaving at 121°C for 15 minutes.

C.2. T3 Agar Medium Used for Sporulation

	g/l
Tryptone	3
Tryptose	2
Yeast extract	1.5
Mangane chloride	0.005
Agar agar	15
and	
Sodium phosphate	0.05 M

All ingredients are dissolved in 800 ml distilled water by stirring with gentle heating and completed to 1000 ml. Medium is sterilized by autoclaving at 121°C for 15 minutes.

APPENDIX D

BUFFERS AND STOCK SOLUTIONS

D.1. 50 X TAE

242 g Tris base is dissolved in deionized water, 57.1 ml glacial acetic acid and 100ml 0.5 M EDTA (pH 8.0) are added. Volume is adjusted to 1000 ml with deionized water.

D.2. 1 X TAE

20 ml of 50X TAE buffer is taken and the volume is adjusted to 1000 ml with deionized water to obtain 1000 ml 1X TAE buffer.

D.3. 5 X TBE

54 g Tris Base and 27.5 g boric acid are weighed and dissolved in nearly 800 ml of deionized water. And 20 ml 0.5 M EDTA pH 8.0 is added. The volume is brought to 1000 ml with deionized water.

D.4. 1X TBE

200 ml 5X TBE is taken and the volume is brought to 1000 ml with deionized water.

D.5. 1X TE (pH 8.0)

10 mM Tris (pH 8.0), 1mM EDTA

D.6. Tris-HCl (1 M, pH 8.0)

121.1 g Tris base is dissolved in 800 ml of deionized water. pH is adjusted to 8.0 with concentrated HCl. Volume is adjusted to 1000 ml with deionized water. The solution is sterilized by autoclaving.

D.7. EDTA (0.5 M, pH 7.5, 8.0 and 9.5)

186.1 g of EDTA is dissolved in 800 ml of deionized water and pH is adjusted to desired value with 10 N NaOH. Volume is brought to 1000 ml with deionized water. The solution is sterilized by autoclaving.

D.8. Sodium Acetate (3M, pH 5.2)

408.1 g sodium acetate (3 H_2O) is dissolved in 800 ml deionized water and pH is adjusted to 5.2 by glacial acetic acid. Volume is brought to 1000 ml. The solution is sterilized by autoclaving.

D.9. Ammonium Acetate (10M)

770 g of ammonium acetate is dissolved in 800 ml of distilled water. Volume is adjusted to 1000ml. The solution is sterilized by filtration.

D.10. Ethidium Bromide (10 mg/ml)

1 g of ethidium bromide is dissolved in 100 ml of deionized water by strring for several hours. The solution is stored in a dark bottle at room temperature.

D.11. Phenol

Phenol should be allowed to warm at room temperature, and it is melted at 68 °C. Equal volume of buffer (usually 0.5 M Tris.Cl, pH 8.0, at room temperature) are added to the melted phenol. The mixture is stirred for 15 minutes and allowed to settle. When the two phases have separated, the aqueous (upper) phase is removed using a separation funnel. Then equal volume of 0.1 M Tris.Cl, pH 8.0, is added to the phenol. The mixture is again stirred for 15 minutes and allowed to settle. The aqueous phase is removed as described before. The extractions are repeated until the pH of the phenolic phase reached to > 7.8. The pH is measured by using pH paper slips. After the phenol is equilibrated, the mixture is divided into aliquots. They are stored under 100 mM Tris.Cl (pH 8.0) at -20ºC. Before use, the phenol is melted at room temperature.

Hydroxyquinoline and β-mercaptoethanol are added to a final concentration of 0.1% and 0.2 %, respectively. The phenol solution can be stored in this form at 4ºC.

D.12. Phenol: Chloroform: Isoamyl Alcohol (25:24:1)

Equal volume of phenol and chloroform isoamyl alcohol (24:1) solutions are mixed. The solution is stored in a light-tight bottle at +4°C for periods up to 1 month.

D.14. Phenyl Methyl Sulfonyl Floride (PMSF) Solution (100Mm)

17.4 mg PMSF is dissolved in 1 ml isopropanol. The solution is divided into aliquots and stored at - 20°C.

D.15. 6X Gel Loading Buffer (20 ml)

2 ml of 10x TBE, 6 ml of glycerol and 12 ml deionized water are mixed. Bromophenol blue is added with toothpick until obtaining sufficient color of the solution.

APPENDIX E

Crystal morphologies of the isolates according to phase contrast microscope

No	Origin of Sample	Isolate Name	Presence of Cry Protein	Shape of Cry Protein
1	free farm soil / Ereğli	1Fa	present	Bipyramidal
2	free farm soil / Ereğli	1Ab	present	Spherical
3	free farm soil / Ereğli	1Cb	present	Spherical
4	free farm soil / Ereğli	1Aa	present	Spherical
5	free farm soil / Ereğli	1Bb	present	Spherical
6	free farm soil / Ereğli	1Db	present	Spherical and Irregular Pointed
7	free farm soil / Ereğli	1CCb	present	Spherical and Irregular Pointed
8	soil from Ayranlı / Ereğli	2Ib	present	Spherical
9	soil from Ayranlı / Ereğli	2Jb	present	Spherical
10	soil from Ayranlı / Ereğli	2Aa	present	Spherical
11	soil from Ayranlı / Ereğli	2Ja	present	Cubic and Spherical
12	soil from Ayranlı / Ereğli	2Da	present	Spherical
13	soil from Ayranlı / Ereğli	2Cb	present	Cubic and Spherical
14	soil from Ayranlı / Ereğli	2Ca	present	Spherical
15	free farm soil / Ereğli	4La	present	Spherical and Irregular Pointed
16	free farm soil / Ereğli	4Lb	present	Cubic and Spherical
17	free farm soil / Ereğli	4Fa	present	Spherical and Irregular Pointed
18	free farm soil / Ereğli	4Cb	present	Cubic and Spherical
19	free farm soil btw Ayranlı and Ereğli	5Ca	present	Spherical and Irregular Pointed
20	soil under nut trees / İvriz	7Fa	present	Cubic and Spherical
21	soil under nut trees / İvriz	7Bb	present	Cubic and Spherical
22	soil from cave mouth (kaynaklar)	8Ba	present	Spherical
23	soil from spring water out / İvriz	9Ka	present	Cubic and Spherical
24	soil from Obruk Cave mouth / Ereğli	10Kb	present	Irregular Shaped
25	soil from Obruk Cave mouth / Ereğli	10Lb	present	Irregular Shaped
26	wheat farm soil / Üçharman	11La	present	Spherical
27	wheat farm soil / Üçharman	11Kb	present	Spherical
28	wheat farm soil / Üçharman	11Ka	present	Cubic and Spherical
29	wheat from silo / Ereğli	12Fb	present	Cubic and Spherical

30	wheat from silo / Ereğli	13La	present	Spherical
31	wheat from silo / Ereğli	13Nb	present	Spherical and Irregular Pointed
32	wheat from silo / Ereğli	13Hb	present	Spherical
33	wheat farm soil / Ereğli-İvriz	15La	present	Cubic and Spherical
34	wheat farm soil / Üçharman	17Ca	present	Irregular Pointed
35	wheat farm soil / Üçharman	17Ka	present	Irregular Pointed
36	wheat farm soil / Üçharman	18FFa	not decided	Spherical and Irregular Pointed
37	wheat farm soil / Üçharman	18Fa	not decided	Cubic and Spherical
38	soil from spring water out / İvriz	19Ab	present	Spherical
39	soil from spring water out / İvriz	19Hb	present	Spherical
40	soil from spring water out / İvriz	19Ka	present	Spherical
41	soil from spring water out / İvriz	19Ca	present	Spherical and Irregular Pointed
42	soil from spring water out / İvriz	19Rb	not decided	not determined
43	soil from spring water out / İvriz	19Pb	not decided	not determined
44	wheat farm soil / Üçharman	20Rb	present	Spherical
45	free farm soil btw Ayranlı and Ereğli	23Ba	present	Spherical
46	free farm soil btw Ayranlı and Ereğli	24Lb	present	Cubic and Spherical
47	free farm soil btw Ayranlı and Ereğli	24La	present	Spherical and Irregular Pointed
48	free farm soil btw Ayranlı and Ereğli	24Ra	present	Cubic and Spherical
49	free farm soil btw Ayranlı and Ereğli	24Pb	present	Spherical and Irregular Pointed
50	free farm soil btw Ayranlı and Ereğli	24Fb	present	Cubic and Spherical
51	free farm soil btw Ayranlı and Ereğli	24Na	present	Cubic and Spherical
52	free farm soil btw Ayranlı and Ereğli	24Pa	present	Spherical and Irregular Pointed
53	free farm soil btw Ayranlı and Ereğli	24Fa	present	Cubic and Spherical
54	free farm soil btw Ayranlı and Ereğli	24Ca	present	Spherical
55	free farm soil btw Ayranlı and Ereğli	24Nb	present	Cubic and Spherical
56	free farm soil btw Ayranlı and Ereğli	25Ra	present	Spherical
57	free farm soil btw Ayranlı and Ereğli	25Fa	present	Spherical and Irregular Pointed
58	free farm soil btw Ayranlı and Ereğli	25Ca	present	Spherical
59	free farm soil btw Ayranlı and Ereğli	25Aa	present	Spherical and Irregular Pointed
60	free farm soil btw Ayranlı and Ereğli	25Ua	present	Spherical
61	free farm soil btw Ayranlı and Ereğli	25Pa	present	Spherical
62	free farm soil btw Ayranlı and Ereğli	25Ab	present	Cubic
63	free farm soil btw Ayranlı and Ereğli	25Pb	present	Spherical and Irregular Pointed

64	free farm soil btw Ayranlı and Ereğli	25Fb	present	Cubic and Spherical
65	free farm soil btw Ayranlı and Ereğli	26Ba	present	Cubic and Spherical
66	free farm soil btw Ayranlı and Ereğli	26Kb	present	Spherical and Irregular Pointed
67	free farm soil btw Ayranlı and Ereğli	26Pb	present	Irregular Pointed
68	free farm soil btw Ayranlı and Ereğli	26Fb	present	Spherical
69	free farm soil btw Ayranlı and Ereğli	26Pa	present	Irregular Pointed
70	free farm soil btw Ayranlı and Ereğli	27Pb	present	Cubic and Spherical
71	free farm soil btw Ayranlı and Ereğli	27Fa	present	Cubic and Spherical
72	free farm soil btw Ayranlı and Ereğli	27Kb	present	Spherical and Irregular Pointed
73	free farm soil btw Ayranlı and Ereğli	27Db	present	Spherical and Irregular Pointed
74	free farm soil btw Ayranlı and Ereğli	27Pa	present	Cubic and Spherical
75	free farm soil btw Ayranlı and Ereğli	27Ba	present	Spherical and Irregular Pointed
76	free farm soil btw Ayranlı and Ereğli	27Fb	not decided	not determined
77	free farm soil btw Ayranlı and Ereğli	27Cb	not decided	not determined
78	free farm soil btw Ayranlı and Ereğli	27Ka	present	Spherical and Irregular Pointed
79	soil under Perçe tree / İvriz	28Bb	present	Spherical and Irregular Pointed
80	soil under Perçe tree / İvriz	28Lb	present	Cubic and Spherical
81	soil under Perçe tree / İvriz	28Da	present	Spherical
82	soil under Perçe tree / İvriz	28Ca	present	Spherical
83	soil under Perçe tree / İvriz	28Ib	not decided	not determined
84	soil under Perçe tree / İvriz	28Aa	present	Spherical
85	soil under willow tree / Üçharman	29Fa	present	Spherical and Irregular Pointed
86	soil under willow tree / Üçharman	29Db	present	Spherical and Irregular Pointed
87	soil under willow tree / Üçharman	29Ab	present	Cubic and Spherical
88	soil under willow tree / Üçharman	29Ca	present	Spherical
89	soil under willow tree / Üçharman	31Na	present	Cubic
90	soil under willow tree / Üçharman	31Rb	present	Irregular Pointed
91	soil under willow tree / Üçharman	31Ca	present	Cubic and Spherical
92	soil under willow tree / Üçharman	31Fa	present	Spherical
93	soil under willow tree / Üçharman	32Fb	present	Irregular Pointed
94	soil under willow tree / Üçharman	32Aa	present	Spherical
95	soil under willow tree / Üçharman	32Fa	present	Spherical
96	dust,grain from grain silos / Taşkale	33Yb	present	Spherical and Irregular Pointed
97	dust,grain from grain silos / Taşkale	34Bb	present	Cubic and Spherical

98	residues from grain silos / Taşkale	35Pb	present	Spherical
99	residues from grain silos / Taşkale	35Kb	present	Spherical
100	dust from grain silos / Taşkale	36Ba	present	Cubic
101	dust,grain from grain silos / Taşkale	39Ya	present	Spherical
102	dust,grain from grain silos / Taşkale	39Yb	present	Spherical and Irregular Pointed
103	dust,grain from grain silos / Taşkale	42Ba	present	Irregular Pointed
104	dust,grain from grain silos / Taşkale	42Ha	present	Irregular Pointed
105	soil from crop field / Bozbük	43Hb	present	Spherical and Irregular Pointed
106	soil from crop field / Bozbük	43Db	present	Spherical and Irregular Pointed
107	soil from crop field / Bozbük	43Ra	present	Spherical
108	soil from crop field / Bozbük	43Fa	present	Irregular Pointed
109	soil from crop field / Bozbük	43Aa	present	Irregular Pointed
110	soil from crop field / Bozbük	48Ra	present	Spherical
111	soil from crop field / Bozbük	48Na	present	Irregular Pointed
112	straw from hyloft / Nikfer	53Yb	present	Irregular Pointed
113	soil from wheet farm / Nikfer	55Ka	present	Irregular Pointed
114	straw from hyloft / Nikfer	56Kb	present	Spherical
115	straw from hyloft / Nikfer	56Hb	present	Spherical
116	straw from hyloft / Nikfer	57Hb	present	Bipyramidal
117	grain from grain silo / Nikfer	58Kb	present	Spherical
118	soil from wheat farm / Nikfer	59Fa	present	cubic
119	soil from wheat farm / Nikfer	59Ra	present	Spherical
120	soil from wheat farm / Nikfer	59Db	not decided	not determined
121	soil from wheat farm / Nikfer	59Ya	present	Spherical
122	soil from wheat farm / Nikfer	59PPa	present	Spherical
123	soil from wheat farm / Nikfer	60Pa	present	Spherical
124	soil from wheat farm / Nikfer	60Na	present	Spherical
125	soil from wheat farm / Nikfer	60Ra	present	Spherical
126	straw from hyloft / Nikfer	61Kb	present	Cubic and Spherical
127	soil from wheat farm / Nikfer	62Lb	present	Spherical
128	soil from wheat farm / Nikfer	62PPa	present	Spherical and Irregular Pointed
129	dust,wheat from grain silos / Taşkale	70Ka	present	Spherical
130	dust,wheat from grain silos / Taşkale	70Yb	present	Spherical and Irregular Pointed
131	dust,wheat from grain silos / Taşkale	70Bb	present	Spherical

132	dust,wheat from grain silos / Taşkale	71Lb	present	Spherical
133	dust,wheat from grain silos / Taşkale	71Na	present	Irregular Shaped
134	dust,wheat from grain silos / Taşkale	71Fa	not decided	not determined
135	soil from grain silos / Taşkale	82FFa	present	Cubic and Spherical
136	soil from grain silos / Taşkale	82YYb	present	Cubic
137	dust,insect from grain silos / Taşkale	85PPb	present	Bipyramidal
138	faces from arround of silos / Taşkale	86Fb	present	Cubic
139	faces from arround of silos / Taşkale	86Db	present	Spherical
140	potato farm soil / Bozbük	87Fb	present	Spherical and Irregular Pointed
141	dust,wheat from grain silos / Taşkale	91Fb	present	Spherical
142	dust from grain silos mouth / Taşkale	93Ha	present	Spherical
143	dust from grain silos mouth / Taşkale	93FFa	not decided	not determined
144	dust from grain silos mouth / Taşkale	93Da	not decided	not determined
145	dust from grain silos mouth / Taşkale	93Fa	present	Spherical
146	dust from grain silos mouth / Taşkale	94Da	present	Spherical and Irregular Pointed
147	dust from grain silos mouth / Taşkale	94YYb	present	Cubic
148	dust from grain silos mouth / Taşkale	94Ab	present	Cubic and Irregular Pointed
149	soil from grain silos / Taşkale	98Fa	present	Spherical
150	soil from grain silos / Taşkale	98Lb	present	Cubic
151	wheat from grain silos / Taşkale	100Aa	present	Spherical
152	dust,residues from Manazan Caves	102Fb	present	Spherical and Irregular Pointed
153	dust,residues from Manazan Caves	102Cb	present	Spherical and Irregular Pointed
154	dust,residues from Manazan Caves	102Da	present	Spherical and Irregular Pointed
155	soil from Manazan Caves	104Db	present	Irregular Pointed
156	soil from Manazan Caves	105Fb	present	Irregular Pointed
157	soil from Manazan Caves	106Da	present	Spherical
158	soil from Manazan Caves	107Fa	present	Spherical and Irregular Pointed
159	dust,residues from Manazan Caves	109Da	present	Spherical and Irregular Pointed
160	soil from Manazan Caves	113Ya	present	Cubic and Spherical
161	soil from Manazan Caves	113Pa	present	Spherical
162	soil from Manazan Caves	113Ha	present	Cubic and Spherical
163	soil, residues from Manazan Caves	114Ya	present	Irregular Shaped

APPENDIX F

Analysis of *cry* gene amplification products

No	Origin of Sample	Isolate Name	*cry 1*		*cry 2*		*cry 3*		*cry 4*		*cry 9*	
			No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands
1	soil btw Ayranlı/Ereğli	25Fb	2	strong, near over and below the ref.line	1	normal	-	-	few	weak bands,one in ref.line	2	strong ones, one in ref.line
2	soil btw Ayranlı/Ereğli	25Pb	2	strong ones, near over and far below the ref.line	1	normal	-	-	1	normal	2	strong ones, one in ref.line
3	insect,grain Taşkale	34Bb	-	-	1	weak	-	-	-	-	2	strong ones, one in ref.line
4	soil in silo Taşkale	82YYb	few	normal, one in ref.line	1	very strong	-	-	few	normal, one in ref.line	1	very strong
5	soil btw Ayranlı/Ereğli	24Nb	few	normal, one in ref.line	-	-	-	-	1	very weak one near over ref.line	1	normal
6	soil btw Ayranlı/Ereğli	25Aa	-	-	-	-	-	-	-	-	1	normal
7	soil btw Ayranlı/Ereğli	25Ca	-	-	2	weak ones very near ref.line	-	-	-	-	1	one strong in ref.line, one weak at 1000bp
8	soil btw Ayranlı/Ereğli	25Ra	-	-	-	-	-	-	-	-	-	-
9	grain (silo) Taşkale	36Ba	-	-	-	-	-	-	1	very weak	1	very strong
10	soil Üçharman	29Fa	-	-	-	-	-	-	1	strong one near over ref.line	1	normal
11	soil (grain farm) Nikfer	59Ya	1	normal	2	normal ones near ref.line	-	-	-	-	-	-
12	soil btw Ayranlı/Ereğli	23Ba	-	-	2	normal ones near ref.line	-	-	-	-	1	normal one near over ref.line
13	soil btw Ayranlı/Ereğli	25Ua	1	normal	-	-	-	-	2	normal ones near over ref.line	-	-
14	soil (nut tree) İvriz	7Bb	1	normal	-	-	-	-	-	-	1	very strong
15	soil (wheet farm) İvriz	15La	-	-	-	-	-	-	-	-	-	-

No	Origin of Sample	Isolate Name	*cry 1*		*cry 2*		*cry 3*		*cry 4*		*cry 9*	
			No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands
16	soil Üçharman	29Ca	-	-	-	-	-	-			-	-
17	soil (potato farm) Bozbük	87Fb	-	-	-	-	-	-	few	normal, one in ref.line	3	strong ones, one in ref.line
18	soil (grain farm) Nikfer	59Db	1	weak	2	weak ones near ref.line	-	-	-	-	-	-
19	soil (crop field) Bozbük	48Ra	1	normal	-	-	-	-	few	normal, one in ref.line	-	-
20	soil (crop field) Bozbük	43Fa	few	normal ones, one in ref.line	few	normal, one in ref.line	1	weak	few	normal, one in ref.line	few	normal, one in ref.line
21	dust, grain Taşkale	70Ka	few	normal ones, one in ref.line	1	normal	1	weak	few	normal, one in ref.line	-	-
22	dust, grain Taşkale	71Fa	1	weak	-	-	-	-	-	-	-	-
23	soil Üçharman	32Fb	few	normal ones, one in ref.line	few	normal, one in ref.line	-	-	few	normal, one in ref.line	-	-
24	dust, grain Taşkale	39Ya	1	normal	-	-	1	weak	-	-	-	-
25	dust, grain Taşkale	42Ha	-	-	-	-	-	-	-	-	-	-
26	soil (crop field) Bozbük	43Ra	-	-	-	-	1	normal	few	normal, one in ref.line	-	-
27	soil (crop field) Bozbük	43Hb	-	-	few	normal, one in ref.line	-	-	1	weak one near ref.line	-	-
28	grain residue Taşkale	35Pb	2	normal	-	-	-	-	-	-	-	-
29	dust, grain Taşkale	71Lb	2	normal, one in ref.line	few	normal, one in ref.line	1	normal one near below ref.line	-	-	-	-
30	straw (hayloft)Nikfer	56Hb	-	-	few	normal, one in ref.line	-	-	-	-	-	-

No	Origin of Sample	Isolate Name	*cry 1*		*cry 2*		*cry 3*		*cry 4*		*cry 9*	
			No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands
31	soil (crop field) Bozbük	43Aa	-	-	-	-	-	-	-	-	-	-
32	dust, grain Taşkale	39Yb	1	very strong	-	-	-	-	-	-	-	-
33	soil (crop field) Bozbük	43Db	1	very strong	-	-	-	-	-	-	-	-
34	dust, grain Taşkale	42Ba	few	normal, one in ref.line	-	-	-	-	-	-	-	-
35	dust, grain Taşkale	71Na	1	very strong	-	-	-	-	-	-	-	-
36	soil İvriz	18Fa	1	weak	1	very strong	few	strong one in ref.line, weak ones in different lines	-	-	2	normal, below and over near the ref.line
37	soil İvriz	18FFa	1	weak	few	normal, one in ref.line	-	-	-	-	1	normal, near over the ref.line
38	dust,grain Taşkale	93FFa	1	weak	1	very strong	-	-	1	normal	few	strong ones, one in ref.line
39	dust,grain Taşkale	93Ha	1	weak	1	very strong	-	-	few	normal ones below and over the ref.line	-	-
40	soil (wheat farm)Üçharman	20Rb	1	very weak	-	-	-	-	2	weak bands near ref.line	few	normal, one in ref.line
41	soil İvriz	19Rb	-	-	1	very strong	-	-	-	-	1	normal
42	soil Ereğli	27Fb	-	-	1	very strong	-	-	-	-	-	-
43	dust,grain Taşkale	93Da	-	-	1	very strong	-	-	-	-	2	strong, one in ref.line, one at nearly 800bp
44	soil İvriz	19Hb	1	weak	1	very strong	-	-	-	-	few	weak ones, one in ref.line
45	Perçe tree İvriz	28Ib	1	weak	-	-	-	-	-	-	-	-

No	Origin of Sample	Isolate Name	*cry 1*		*cry 2*		*cry 3*		*cry 4*		*cry 9*	
			No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands
46	wheat(wheat silo)Ereğli	13Nb	-	-	-	-	-	-	1	very weak	few	normal, one in ref.line
47	soil İvriz	19Ab	-	-	-	-	-	-	-	-	-	-
48	wheat,grain Taşkale	100Aa	1	weak	-	-	-	-	-	-	1	weak
49	dust,grain Taşkale	94YYb	few	normal, one in ref.line	few	weak ones, one in ref.line	few	weak ones,one in ref.line	1	normal	few	strong one in ref.line, weak ones at 1000bp
50	soil Ereğli	27Cb	-	-	1	weak one	-	-	few	very weak, one in ref.line	few	normal, one in ref.line
51	dust,grain Taşkale	94Ab	-	-	few	one weak in ref.line	1	weak	-	-	1	weak, near over ref.line
52	dust,grain Taşkale	93Fa	1	normal	few	two stronger ones near ref.line	-	-	1	normal	few	stronger one is in ref.line
53	soil İvriz	19Ca	-	-	1	weak one	-	-	-	-	-	-
54	soil İvriz	19Pb	-	-	1	weak one	-	-	1	normal	-	-
55	dust, grain Taşkale	70-Bb	1	weak	-	-	3	normal, one in ref.line, two below, over ref.line	-	-	-	-
56	grain residue Taşkale	35Kb	1	normal	-	-	-	-	1	weak	-	-
57	dust, grain Taşkale	70Yb	1	very weak	-	-	1	normal one about 1000 bp	-	-	1	very weak
58	straw (hayloft)Nikfer	56Kb	1	strong near 400 bp	1	normal	1	normal	few	weak, one in ref.line	3	normal ones,one in ref.line
59	soil (crop field) Bozbük	48Na	-	-	-	-	-	-	few	normal, one in ref.line	-	-
60	dust, grain Taşkale	33Yb	1	normal one near over ref.line	1	normal one near oner ref.line	-	-	1	very weak	-	-

No	Origin of Sample	Isolate Name	*cry 1*		*cry 2*		*cry 3*		*cry 4*		*cry 9*	
			No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands
61	soil btw Ayranlı/Ereğli	24Pb	-	-	-	-	1	strong one about 500 bp	few	normal, one in ref.line	4	strongest one in ref.line
62	straw (hayloft)Nikfer	61Kb	-	-	-	-	2	normal ones near over ref.line	1	very weak	-	-
63	soil (wheat farm) Nikfer	55Ka	1	very strong	-	-	2	normal ones near over ref.line	-	-	-	-
64	straw (hayloft)Nikfer	53Yb	-	-	1	weak	few	normal, one in ref.line	-	-	1	normal
65	soil (farm) Ereğli	1CCb	3	normal, one in ref.line	1	weak	-	-	-	-	3	strongest one in ref.line
66	soil Ayranlı	2Da	1	weak	-	-	-	-	-	-	-	-
67	soil (farm) Ereğli	4La	-	-	-	-	1	strong	-	-	-	-
68	soil (farm) Ereğli	1Bb	-	-	1	normal one near over ref.line	1	strong one near over ref.line	-	-	-	-
69	soil Ayranlı	2Ca	-	-	-	-	-	-	-	-	-	-
70	soil btw Ayranlı/Ereğli	24Ra	-	-	-	-	1	normal	-	-	-	-
71	soil btw Ayranlı/Ereğli	24Pa	-	-	-	-	-	-	-	-	-	-
72	soil btw Ayranlı/Ereğli	24Lb	-	-	1	normal, near over ref.line	1	normal,nice,near over ref.line	few	normal, one in ref.line	1	weak
73	dust,wheet in silo/Taşkale	91Fb	-	-	-	-	-	-	-	-	-	-
74	soil (farm) Ereğli	4Lb	-	-	-	-	-	-	-	-	-	-
75	soil (farm) Ereğli	1Db	-	-	-	-	-	-	-	-	-	-

No	Origin of Sample	Isolate Name	*cry 1*		*cry 2*		*cry 3*		*cry 4*		*cry 9*	
			No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands
76	soil Ayranlı	2Cb	-	-	-	-	-	-	-	-	-	-
77	dust,wheat (silo) Taşkale	94Da	-	-	few	normal, one in ref.line ref.line	-	-	few	normal, one in ref.line	-	-
78	soil (cave) Kaynaklar	8Ba	1	strong one btw 500-750 bp	few	strongest one in ref.line	-	-	1	normal,near over ref.line	few	strong,one in ref.line
79	wheat (silo) Taşkale	13La	1	normal	few	normal,one in ref.line	-	-	-	-	-	-
80	soil (Perçe tree) İvriz	28Aa	2	strong, one in ref.line	-	-	-	-	1	weak	1	normal
81	soil Ayranlı	5Ca	1	normal	-	-	-	-	-	-	1	very weak
82	soil btw Ayranlı/Ereğli	24Ca	-	-	-	-	-	-	-	-	1	very strong
83	soil btw Ayranlı/Ereğli	24Fa	1	normal, near over ref.line	-	-	-	-	2	normal, one in ref.line	1	weak
84	(soil) farm Ereğli	4Cb	1	strong	-	-	-	-	1	weak,near over ref.line	-	-
85	soil btw Ayranlı/Ereğli	24Na	-	-	-	-	1	normal	-	-	-	-
86	soil Ayranlı	2Ja	few	normal, one in ref.line	1	nice band near 400 bp	1	normal	1	normal	-	-
87	soil btw Ayranlı/Ereğli	24La	1	normal	-	-	2	normal ones,one in ref.,one over	1	normal	1	normal
88	soil btw Ayranlı/Ereğli	24Fb	1	weak,near over ref.line	1	nice band near 400 bp	-	-	-	-	1	normal
89	soil btw Ayranlı/Ereğli	25Pa	1	weak	-	-	-	-	-	-	-	-
90	soil btw Ayranlı/Ereğli	25Fa	-	-	-	-	1	normal, near over ref.line	-	-	-	-

No	Origin of Sample	Isolate Name	*cry 1*		*cry 2*		*cry 3*		*cry 4*		*cry 9*	
			No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands
91	soil btw Ayrancı/Ereğli	25Ab	-	-	-	-	2	normal, one at 600 bp one at 300 bp	1	normal one near below ref.line	-	-
92	soil İvriz	19Ka	-	-	-	-	1	normal, near over ref.line	1	normal one near below ref.line	3	normal,one in ref.line
93	soil from Obruk cave mouth	10Lb	-	-	-	-	-	-	-	-	-	-
94	soil (wheat farm)Üçharman	11Kb	-	-	-	-	-	-	-	-	-	-
95	wheat (silo) Ereğli	12Fb	-	-	-	-	-	-	-	-	-	-
96	soil, farm btw Ereğli/Ayranlı	26Pb	-	-	-	-	-	-	1	normal one near below ref.line	2	one normal in 200bp one normal in ref.
97	soil, farm btw Ereğli/Ayranlı	26Fb	-	-	-	-	2	one normal	1	normal	-	-
98	soil, farm btw Ereğli/Ayranlı	26Kb	1	weak	-	-	-	-	few	strong one in ref.line, weak ones over 500bp	1	weak
99	soil, farm btw Ereğli/Ayranlı	27Pb	1	normal,near 250 bp	-	-	-	-	1	normal	1	normal
100	soil, farm btw sreğli/Ayranlı	27Fa	-	-	-	-	-	-	-	-	1	normal
101	soil, farm btw Ereğli/Ayranlı	27Kb	few	strong bands,one in ref.line	1	normal	-	-	2	normal,one in ref., one below	1	weak
102	soil, farm btw Ereğli/Ayranlı	27Ba	-	-	-	-	-	-	1	normal	-	-
103	soil (Perçe tree) İvriz	28Lb	1	normal	-	-	-	-	-	-	1	normal
104	soil (Perçe tree) İvriz	28Da	-	-	-	-	-	-	1	normal	-	-
105	soil (Perçe tree) İvriz	28Ca	1	normal	-	-	-	-	1	normal	-	-

No	Origin of Sample	Isolate Name	*cry 1*		*cry 2*		*cry 3*		*cry 4*		*cry 9*	
			No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands
106	soil (willow tree) Üçharman	29Db	-	-	-	-	2	normal,one below one over near ref.line	2	normal,one over and one below near ref.	-	-
107	soil (willow tree) Üçharman	29Ab	1	weak	-	-	1	normal near over ref.line	1	normal near over ref.	1	normal
108	soil (willow tree) Üçharman	31Na	-	-	-	-	2	one weak near over, one normal in ref. line	2	one weak near over, one in ref.line	-	-
109	soil (willow tree) Üçharman	31Ca	-	-	-	-	-	-	-	-	-	-
110	soil (willow tree) Üçharman	31Fa	-	-	3	normal,one at 300bp, one at 500bp, one in ref.line	-	-	1	normal at 300bp	1	weak
111	soil Manazan Caves	113Ha	-	-	-	-	-	-	1	strong at 400bp	-	-
112	soil Manazan Caves	113Pa	-	-	3	normal,one at 300bp, one over, one below near ref.line	-	-	1	strong at 300bp	1	weak
113	soil (Perçe tree) İvriz	28Bb	-	-	lots	one normal at 300bp, one in ref line	-	-	-	-	1	normal at 500bp
114	soil, farm btw Ereğli/Ayrancı	27Pa	-	-	-	-	-	-	1	normal	1	weak
115	soil from Obruk cave mouth	10Kb	-	-	-	-	-	-	-	-	-	-
116	soil (wheat farm)Üçharman	11Ka	1	very strong	-	-	-	-	-	-	-	-
117	soil, farm btw Ereğli/Ayrancı	26Pa	1	weak	-	-	-	-	-	-	-	-
118	soil Manazan Caves	104Db	1	weak	-	-	-	-	-	-	1	weak
119	soil Manazan Caves	107Fa	1	normal	-	-	-	-	-	-	-	-
120	dust,residues Manazan Caves	102Cb	-	-	-	-	-	-	-	-	1	weak

No	Origin of Sample	Isolate Name	*cry 1*		*cry 2*		*cry 3*		*cry 4*		*cry 9*	
			No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands
121	dust,residues Manazan Caves	102Fb	1	normal	-	-	-	-	-	-	-	-
122	soil Manazan Caves	113Ya	-	-	-	-	-	-	1	normal	-	-
123	soil, residues Manazan Caves	114Ya	-	-	-	-	-	-	-	-	-	-
124	dust,residues Manazan Caves	109Da	-	-	-	-	-	-	-	-	-	-
125	dust,residues Manazan Caves	102Da	-	-	4	normal btw 250-500bp	-	-	-	-	-	-
126	soil Manazan Caves	105Fb	-	-	-	-	-	-	-	-	-	-
127	soil Manazan Caves	106Da	-	-	-	-	-	-	-	-	-	-
128	soil (grain farm) Nikfer	62PPa	-	-	-	-	-	-	-	-	1	normal
129	soil (grain farm) Nikfer	59PPa	-	-	-	-	-	-	-	-	-	-
130	soil (silo) Taşkale	98Lb	-	-	-	-	many	one normal in ref.line	-	-	-	-
131	soil (silo) Taşkale	98Fa	-	-	-	-	-	-	-	-	-	-
132	soil (spring water out) İvriz	9Ka	-	-	1	very weak near over ref.line	-	-	-	-	-	-
133	soil (under nut trees) İvriz	7Fa	1	very strong	1	normal near over ref.line	-	-	-	-	-	-
134	dust,insect (silo) Taşkale	85PPb	1	normal	1	normal	-	-	-	-	1	very strong
135	wheat (silo) Taşkale	13Hb	-	-	-	-	3	normal, one in ref.line, two at 400, 1000 bp	-	-	-	-

No	Origin of Sample	Isolate Name	*cry 1*		*cry 2*		*cry 3*		*cry 4*		*cry 9*	
			No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands
136	faces (arround silos) Taşkale	86Fb	3	one weak in ref, one normal at 450bp, one nice at 700bp	-	-	-	-	-	-	-	-
137	faces (arround silos) Taşkale	86Db	3	one weak in ref, one normal at 450bp, one normal at 700bp	-	-	1	very strong, near below ref.line	-	-	4	three strong at 275, 500, 600 and normal one in ref.line
138	soil (willow tree) Üçharman	32Aa	-	-	-	-	-	-	-	-	-	-
139	(soil) farm Ereğli	4Fa	1	normal at 700bp	-	-	-	-	-	-	-	-
140	soil (farm) Ayranlı	2Ib	-	-	-	-	-	-	-	-	2	one strong at 275bp, one normal in ref.
141	soil (farm) Ayranlı	2Jb	2	one normal near ref., one normal at 500bp	-	-	1	normal	2	one weak at 700bp, one strong at 275bp	1	normal, nice, very near to ref.line
142	Soil from Ereğli/Ayrancı	27Ka	2	one normal at 500bp, one weak at 600bp	-	-	1	normal near about 500bp	1	weak at 700bp	1	normal, very near to ref.line
143	soil, farm btw Ereğli/Ayrancı	27Db	-	-	-	-	-	-	-	-	-	-
144	soil, farm btw Ereğli/Ayrancı	26Ba	2	one weak at 500bp, one normal at 600bp	-	-	1	weak at 500 bp	1	normal at 700bp	1	normal, very near to ref.line
145	soil (wheat farm)Üçharman	11La	-	-	-	-	-	-	-	-	-	-
146	soil (willow tree) Üçharman	31Rb	-	-	-	-	-	-	-	-	-	-
147	(soil) farm Ereğli	1Ab	1	very strong	-	-	-	-	-	-	-	-
148	(soil) farm Ereğli	1Aa	1	very strong	-	-	2	one normal at 600bp one strong at 300 bp	1	normal	1	normal

No	Origin of Sample	Isolate Name	*cry 1*		*cry 2*		*cry 3*		*cry 4*		*cry 9*	
			No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands	No.of Bands	Appearence of Bands
149	(soil) farm Ereğli	1Cb	1	normal	-	-	-	-	-	-	-	-
150	(soil) farm Ereğli	1Fa	-	-	-	-	2	one strong at 600bp one normal at 300 bp	1	weak	1	very strong at 275 bp
151	soil (farm) Ayranlı	2Aa	-	-	-	-	-	-	-	-	-	-
152	soil (wheat farm)Üçharman	17Ka	-	-	-	-	-	-	-	-	-	-
153	soil (willow tree) Üçharman	32Fa	1	weak	-	-	-	-	-	-	-	-
154	soil (grain farm) Nikfer	60Pa	-	-	-	-	-	-	-	-	1	very strong at 275 bp
155	soil (grain farm) Nikfer	60Na	1	normal	-	-	-	-	-	-	1	weak
156	soil (grain farm) Nikfer	60Ra	-	-	1	weak	-	-	-	-	-	-
157	soil (wheat farm)Üçharman	17Ca	-	-	1	weak	-	-	-	-	-	-
158	straw (hayloft)Nikfer	57Hb	1	normal	1	weak	-	-	-	-	-	-
159	grain (silo) Nikfer	58Kb	1	normal	-	-	-	-	-	-	-	-
160	soil (grain farm) Nikfer	59Fa	-	-	-	-	-	-	-	-	-	-
161	soil (grain farm) Nikfer	59Ra	-	-	-	-	-	-	-	-	-	-
162	soil (grain farm) Nikfer	62Lb	-	-	-	-	-	-	-	-	-	-
163	soil (silo) Taşkale	82FFa	-	-	-	-	-	-	-	-	-	-