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ORIGINAL ARTICLE

Isolation and characterization of *Bacillus thuringiensis* strains from olive-related habitats in Turkey

C. Cinar[1], O. Apaydin[1], A.F. Yenidunya[2], S. Harsa[3] and H. Gunes[4]

1 Biotechnology Programme, Izmir Institute of Technology, Urla, Izmir, Turkey
2 Department of Biology, Cumhuriyet University, Sivas, Turkey
3 Department of Food Engineering, Izmir Institute of Technology, Urla, Izmir, Turkey
4 Department of Biology, Mugla University, Mugla, Turkey

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Bacillus thuringiensis, characterization, crystal protein, olive-related habitats, polymerase chain reaction, 16S-internal transcribed spacer rDNA restriction fragment length polymorphism.

Correspondence
Hatice Gunes, Department of Biology, Mugla University, Kotekli 48000 Mugla, Turkey.
E-mail: haticegunes@mu.edu.tr

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Abstract

Aims: To isolate *Bacillus thuringiensis* strains from different olive-related habitats (olive groves and olive oil factories) in Turkey and to characterize these strains by molecular methods.

Methods and Results: A total of 150 samples, consisting of olive grove soil, green olive leaves, olive leaf residues, animal faeces, olive pomace and dust, were examined for the presence of *B. thuringiensis.* One hundred *B. thuringiensis* strains were isolated from 54 environmental samples (36%) and characterized in terms of crystal morphology, *cry* and *cyt* gene content by polymerase chain reaction, plasmid profiles and 16S-internal transcribed spacer ribosomal DNA restriction fragment length polymorphism (16S-ITS rDNA RFLP). The highest percentage of samples containing *B. thuringiensis* was found in 38 out of 54 total soil samples (70%). Of the 100 *B. thuringiensis* isolates, the most frequent crystal shapes were irregularly shaped (24%), spherical-irregular pointed (19%), cuboidal (17%) and spherical (16%). The *cry1* plus *cry4* genotype was the most abundant genotype in our collection (21%). RFLP analysis of the amplified 16S-ITS rDNA revealed 11 distinct patterns for the isolates and 10 reference strains.

Conclusions: *Bacillus thuringiensis* isolates showed a great genetic diversity and crystal shape heterogeneity.

Significance and Impact of the Study: This is the first study on the isolation and characterization of *B. thuringiensis* from olive-related habitats in Turkey. No correlation was observed between the *cry* genotypes and insecticidal crystal shapes of the isolates. Restriction profiles of 23% of the isolates were found to be different from those of the 10 reference strains used.

Introduction

Bacillus thuringiensis is characterized by its ability to produce crystalline inclusions during sporulation and exhibiting highly specific insecticidal activity against different insect orders, including Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Mallophaga and Acari (Höfte and Whiteley 1989; Feitelson 1993). The crystals are made of proteins – the Cry and Cyt proteins – and encoded by *cry* and *cyt* genes, respectively (Crickmore *et al.* 1998a; Schnepf *et al.* 1998). *Bacillus thuringiensis* has been isolated from different natural samples, such as soil (Martin and Travers 1989), phylloplanes (Smith and Couche 1991; Mizuki *et al.* 1999) and stored products (Delucca *et al.* 1982). Till date, more than 200 insecticidal crystal protein genes have been cloned, sequenced and classified into 32 groups of *cry* genes and two groups of *cyt* genes based on the amino acid homology of the corresponding proteins (Crickmore *et al.* 1998b).

Olive trees have been traditionally grown, mainly in the Mediterranean region for olive oil and table fruit consumption in the world (Alcaide and Nefzaoui 1996). The olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae) is a major insect pest of olives in the region (Cabras *et al.* 1997). Because *B. thuringiensis*-based biopesticides have been used for the control of many other insect species, it is important to isolate and analyse the diversity of *B. thuringiensis* in olive tree-related areas for the future development of effective biopesticides against the olive fruit fly. Therefore, the aim of this present study is to examine the occurrence of *B. thuringiensis* in the environments of olive trees and the olive oil processing sites in the Aegean region of Turkey and to characterize the isolates in terms of crystal morphology, *cry* and *cyt* gene contents using polymerase chain reaction (PCR), plasmid profiles and 16S-internal transcribed spacer rDNA restriction fragment length polymorphism (16S-ITS rDNA RFLP) analysis.

Materials and methods

Bacterial strains

Reference strains *B. thuringiensis* ssp. *kurstaki* (BGSC 4D1), *B. thuringiensis* ssp. *aizawai* (BGSC 4J3), *B. thuringiensis* biovar. *tenebrionis* (BGSC 4AA1), *B. thuringiensis* ssp. *israelensis* (BGSC 4Q2), *B. thuringiensis* ssp. *kumamotoensis* (BGSC 4W1) were kindly supplied by Prof. Zeigler (Bacillus Genetic Stock Centre, Columbus, Ohio, USA). *Bacillus thuringiensis* ssp. *galleriae*, *B. thuringiensis* ssp. *alesti*, *B. thuringiensis* ssp. *finitimus*, *B. thuringiensis* ssp. *thompsoni* and *B. thuringiensis* ssp. *morrisoni* were provided by Ege University, Izmir, Turkey.

Sample collection

A total of 150 samples were collected from different olive groves of six locations and two olive oil factories in the Aegean region. These samples included olive grove soil, green olive leaves, olive leaf residues, animal faeces, olive pomace and dust (Table 1). The source of these samples had not been previously treated with any *B. thuringiensis* biopesticides. About 200 g of soil samples were collected with a sterile spatula at 5–10 cm depth. During leaf sampling, soil contamination was avoided and leaf samples were collected from trees according to the method of Smith and Couche (1991). Dust samples were obtained by scraping from the ground of olive oil factories. All samples were placed in sterile plastic bags aseptically, sealed and stored at 4°C until processed.

Table 1 Sampling sites, origin of samples collected and number of samples yielding *Bacillus Thuringiensis* (*Bt*)

Sampling site	Source	No. samples collected	No. samples yielding *Bt*	% *Bt* isolates	Type of insecticidal gene
Aliaga	Soil	3	3	8	*cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cyt1*, *cyt2*
Bergama					
Tunc Yag olive oil factory	Dust	9	2	7	*cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cyt1*, *cyt2*
	Olive pomace	13	3	5	*cry1*, *cry4*, *cyt1*, *cyt2*
Sulu olive oil factory	Dust	8	1	1	*cry1*, *cry4*
Dikili	Green olive leaves	7	2	2	*cry4*, *cry9*, *cry11*, *cyt1*, *cyt2*
	Olive leaf residue	8	1	1	*cyt2*
	Soil	17	5	10	*cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cyt1*, *cyt2*
Foca	Green olive leaves	10	3	8	*cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cyt1*, *cyt2*
	Olive leaf residue	8	3	8	*cry1*, *cry2*, *cry4*, *cry9*, *cyt1*, *cyt2*
	Soil	17	9	13	*cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cyt1*, *cyt2*
Izmir					
Kucukkaya village	Soil	4	3	5	*cry1*, *cry2*, *cry4*, *cry11*, *cyt1*, *cyt2*
Tirazli village	Soil	2	2	6	*cry1*, *cry4*, *cry9*, *cyt1*, *cyt2*
Kemalpasa	Soil	16	3	5	*cry1*, *cry2*, *cry4*, *cyt1*, *cyt2*
Kimiz farm	Faeces	3	1	2	*cry1*, *cry4*, *cyt1*, *cyt2*
	Soil	4	4	8	*cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cyt1*, *cyt2*
Turgutlu					
Dalbahce village	Soil	9	4	5	*cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cyt1*, *cyt2*
Irlamaz village	Soil	12	5	6	*cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cyt1*, *cyt2*
Total		150	54	100	

Isolation of *Bacillus thuringiensis* and analysis of parasporal crystal formation

Isolation of bacteria was carried out using two different methods with some minor modifications. The shaken-flask technique (Smith and Couche 1991) was used for the leaf samples. Briefly, 5–6 mm leaf sections were placed in an Erlenmeyer flask containing 10 ml of Luria-Bertani (LB) broth and shaken at 250 rev min^{-1} for 4 h at 30°C. Subsequently, 200 μl aliquots of the resulting suspensions were heated for 3 min at 80°C. The heat-treated suspension was cooled on ice, and a 100 μl aliquot was spread on nutrient agar (NA) plates (Applichem). The acetate selection method described by Travers *et al.* (1987) was used to screen the remaining of the samples for the presence of *B. thuringiensis* strains. The samples (0·25 g) were suspended in 10 ml LB broth containing 0·25 mol l^{-1} sodium acetate (pH 6·8), vortexed vigorously, shaken for 4 h at 250 rev min^{-1} at 30°C. The suspension was pasteurized at 80°C for 10 min and streaked on NA. The colonies were purified by subculturing three times on NA plates. Each pure culture was plated on NA and incubated at 37°C for 48 h. All bacterial colonies with morphology similar to *B. thuringiensis* were examined under a phase-contrast microscope (×1000). Isolates producing parasporal inclusions were identified as *B. thuringiensis* and stored in sterile nutrient broth (Applichem) containing 20% glycerol at −80°C for further analysis.

Plasmid profiling

The *B. thuringiensis* isolates were streaked onto NA plates and incubated overnight at 37°C. The cells were scraped off the plates and suspended in 1·5 ml sterile water. The plasmid DNA of the isolates was extracted according to the method described by O'Sullivan and Klaenhammer (1993). Plasmid profiles were obtained by running 10 μl undigested total plasmid DNA on a 0·7% agarose gel (low electroendoosmosis, EEO, Applichem) at 70 V for 3–4 h in TAE (Tris-acetate-EDTA) buffer (40 mmol l^{-1} Tris acetate and 1 mmol l^{-1} EDTA, pH 8·0) containing 0·02% ethidium bromide (10 mg ml^{-1}).

Determination of *cry* and *cyt* gene content

Polymerase chain reaction conditions and universal primers reported by Ben-Dov *et al.* (1997) and Bravo *et al.* (1998) were used to detect the presence of *cry1*, *cry2*, *cry4*, *cry9* and *cry11* genes. Bacterial strains which served as control for each *cry* gene were: BGSC 4D1 for *cry 1* and *2*; BGSC 4J3 for *cry 9*; and BGSC 4Q2 for *cry 4 and 11*. The amplification conditions and primers reported by Ibarra *et al.* (2003) were used for *cyt1* and *cyt2* genes. Each amplification process was performed in a 50 μl reaction mixture containing 250 ng of plasmid DNA as the template, 5 μl 1× PCR buffer, 1·5 mmol l^{-1} $MgCl_2$, 0·2 mmol l^{-1} each of dNTP, 10 pmol of each of the primers, 2 U of *Taq* DNA polymerase (Fermentas, MBI, Lithuania) in a Techne Progene thermal cycler (Progene Scientific, South Yorkshire, UK). After amplification, the PCR products were electrophoresed on 1–1·5% TAE-agarose gels (40 mmol l^{-1} Tris acetate and 1 mmol l^{-1} EDTA, pH 8·0) at 70 V for 40 min and the bands were recorded in a gel documentation system (CN-3000 WL; Vilber-Lourmat, Torcy, France).

Genomic DNA extraction

Genomic DNA was extracted by combining two methods (Ausubel *et al.* 1994; Cardinal *et al.* 1997). Cells grown in 10 ml of nutrient broth (NB) at 37°C overnight were harvested and resuspended in 200 μl sucrose solution (25% sucrose and 30 mg lysozyme per ml in TE (Tris-EDTA) buffer (10 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, pH 8·0). The samples were incubated for 1 h at 37°C. After lysis, 370 μl TE containing proteinase K (1 mg ml^{-1}) and 30 μl 10% SDS (w/v) were added and incubated for 1 h at 37°C. Then, 100 μl NaCl (5 mol l^{-1}) and 80 μl CTAB/NaCl solution (10% cetyltrimethylammonium bromide, 0·7 mol l^{-1} NaCl) were added and incubated for 10 min at 65°C. The solution was extracted twice with one volume of chloroform:isoamyl alcohol (24 : 1). DNA in the aqueous phase was precipitated by adding one volume of isopropanol. Following this, the precipitate was washed in 500 μl of 70% v/v ethanol. The pellet was dried and dissolved in 100 μl TE supplemented with 100 μg ml^{-1} RNase A and kept for 1 h at 37°C. The sample volume was adjusted to 400 μl with TE. The solubilization of DNA was achieved by alternating heat shocks (10 min at 80°C, and 20 min at −20°C). The lysate was extracted with phenol : chloroform : isoamylalcohol (25 : 24 : 1) to remove impurities. Then, NaCl (0·5 mol l^{-1} of final concentration) and two volumes of 99% v/v ethanol were added to precipitate the DNA. The pellets were washed again in 500 μl 70% v/v ethanol. Finally, the DNA pellets were dried and resuspended in 50–200 μl TE, depending on the amount of the DNA pellet. The samples were stored at −20°C until further use.

Amplification of the 16S rDNA-ITS region

DNA fragments containing 16S rRNA gene and ITS sequences were amplified using genomic DNA as template. PCR mixtures and amplifications were performed as previously described (Akbalik *et al.* 2004). Forward and reverse primers used for the amplification were 5′-AGAGTTTGATCCTGGCTCAG-3′ (Mora *et al.* 1998)

and 5′-CAAGGCATCCACCGT-3′ (Jensen *et al.* 1993), respectively. PCR products were extracted twice with chloroform : isoamylalcohol (24 : 1) and precipitated with two volumes of 95% v/v ethanol and resuspended in TE buffer.

Restriction fragment length polymorphism.

The resulting amplicons were digested by *Taq*I restriction endonuclease (Fermentas). The digestion mixture included 6 μl of purified PCR product, 5 U of the enzyme and 5 μl of 10× restriction enzyme buffer (Fermentas) in a total volume of 50 μl. The samples were overlaid with a few drops of mineral oil and incubated at 65°C in a water bath overnight. After incubation, the digestion products were purified and electrophoresed in 1·6% agarose ethidium bromide gel. The electrophoresis was performed for 1 h at 40 mA and 3 h at 60 mA in TAE buffer containing 0·02% ethidium bromide (10 mg ml^{-1}). RFLP fragmentation patterns were recorded and then analysed with 13% homology coefficient in a Bio-ID++ software (CN-3000 WL; Vilber-Lourmat). The similarity between the strains was determined automatically by specifying the formula of Nei and Li, and clustering was performed by the unweighted pair group method with arithmetic means, UPGMA (unweighted pair group method with arithmetic mean) (Nei and Li 1979).

Results

Isolation of *Bacillus thuringiensis*

In total, 150 samples consisting of 84 soil, 17 green olive leaves, 16 olive leaf residues, 17 dust, three animal faeces and 13 olive pomace from olive-related areas were examined and 100 isolates were identified as *B. thuringiensis* based on the crystal protein production determined using a phase-contrast microscopy (Table 1). *Bacillus thuringiensis* isolates were found in 54 samples out of the 150 samples analysed. Soil samples from olive groves rendered the highest percentages of *B. thuringiensis* isolates – 66 of the 100 isolates were obtained from the soil. However, the lowest percentage of samples containing the isolates was obtained from olive leaf residues in Dikili and dust samples from Sulu Olive Factory (Table 1). The frequency of *B. thuringiensis* isolates varied from 29% in Foca to 8% in Aliaga.

Crystal morphology of the isolates

Because crystal morphology of *B. thuringiensis* may indicate the target insect spectra (Maeda *et al.* 2000), the crystal shape of each isolate was determined using a phase-contrast microscope. Twelve different parasporal

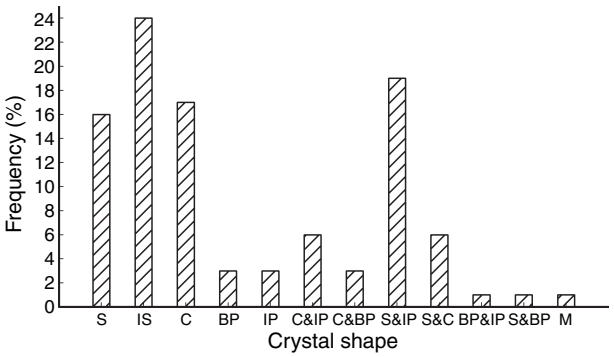

Figure 1 Frequency of crystal protein morphologies among *Bacillus thuringiensis* isolates obtained from olive-related habitats. The description of the crystal shapes is as follows: C, cuboidal; S, spherical; BP, bipyramidal; IP, irregular pointed; IS, irregular shaped; M, multiple.

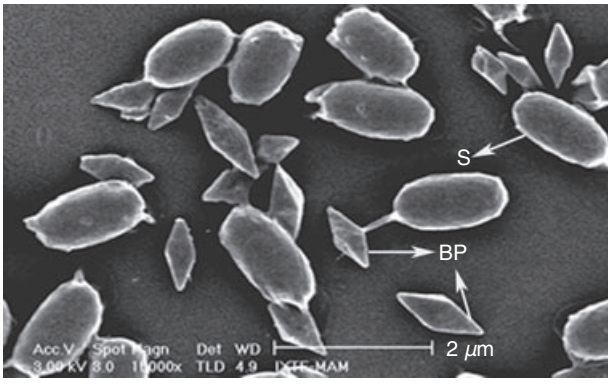

Figure 2 Scanning electron micrographs of the spores and crystals of the isolate *Bacillus thuringiensis* 50Q1 after cell lysis. Arrows indicate spores and crystals. BP, bipyramidal, S, spore.

morphologies were found in the 100 isolates (Fig. 1). Even though only one type of crystal shape was observed in 63% of the isolates, two different crystal morphologies were present in the remaining 37%. The percentages of the isolates containing irregular shaped, cuboidal and spherical type were 24, 17 and 16, respectively (Fig. 1). Each of the bipyramidal and irregular-pointed crystal shapes was observed only in 3% of the isolates. The highest percentage of the isolates containing two crystal morphologies, spherical plus irregular pointed, was 19; however, the isolates possessing other combinations of crystal shapes were six and less than 6%. A scanning electron micrograph of the spores and bipyramidal crystals of the isolate *B. thuringiensis* 50Q1 is shown in Fig. 2.

Plasmid patterns

Bacillus thuringiensis has been known to have several circular/linear plasmids, and *cry* genes are generally found

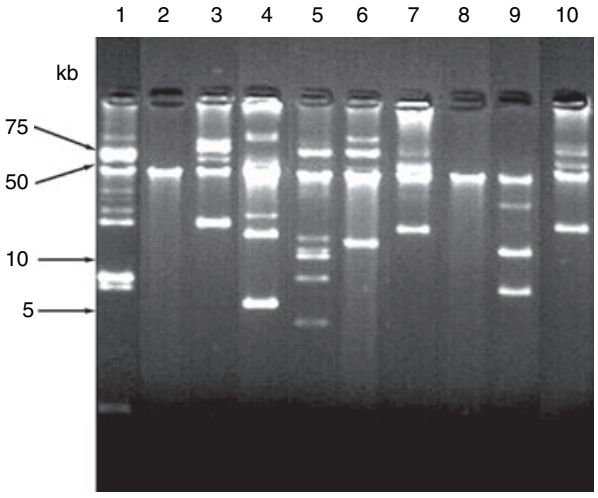

Figure 3 Plasmid profiles of *Bacillus thuringiensis* isolates. Plasmid DNA was prepared from *cry* gene-positive isolates and subjected to 0·7% agarose gel electrophoresis. Molecular masses of plasmid bands were estimated by comparison with *B. thuringiensis* standard strain *kurstaki* HD-1 (Porcar and Caballero 2000). Lane 1, *B. thuringiensis* ssp. *kurstaki* (HD-1); lane 2, 54Q2; lane 3, 14Q5; lane 4, 19Q3; lane 5, 169Q1; lane 6, 60Q2; lane 7, 14Q2; lane 8, 59Q1; lane 9, 44Q2; lane 10, 63Q1.

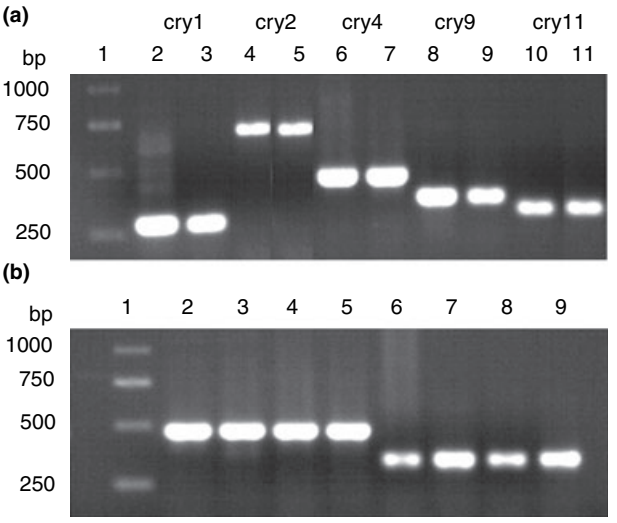

Figure 4 Agarose gel electrophoresis of PCR products for *cry* and *cyt* genes. (a) *cry* PCR products of some of the isolates. Lanes 1, molecular weight standard (1 kb DNA ladder, GeneRuler; Fermentas); lane 2, 53Q1; lane 3, *B. thuringiensis* ssp. *kurstaki*; lane 4, 125Q1; lane 5, *B. thuringiensis* ssp. *kurstaki*; lane 6, 41Q2; lane 7, *B. thuringiensis* ssp. *israelensis*; lane 8, 24Q1; lane 9, *B. thuringiensis aizawai*; lane 10, 36Q2; lane 11, *B. thuringiensis* ssp. *israelensis*. (b) *cyt* PCR products of some of the isolates. Lane 1, molecular weight standard (1 kb DNA ladder, GeneRuler; Fermentas). *cyt1* PCR products (lanes 2–5): lanes 2, 59Q3; lane 3, 174Q1; lane 4, 74Q1; lane 5, *B. thuringiensis* ssp. *israelensis*. *cyt2* PCR products (lanes 6–9): lane 6, 50Q3; lane 7, 59Q1; lane 8, 56Q1; lane 9, *B. thuringiensis* ssp. *israelensis.*

in these extra-chromosomal elements (Carlson *et al.* 1996). Therefore, plasmid profiling of all *B. thuringiensis* isolates and the reference strains was carried out. Agarose gel electrophoresis of plasmid DNA preparations revealed that all isolates carried extra-chromosomal elements. The plasmid bands of the isolates were compared with those of *B. thuringiensis* standard strain *kurstaki* (HD1) (Fig. 3). Similar to the results of Porcar and Cabellaro (2000) and Gonzáles *et al.* (1982), the number of plasmid bands of ssp. *kurstaki* was found to be around nine in this present study (Fig. 3). A common characteristic most of the isolates was the presence of a plasmid band around 50 kb. In addition, plasmid profiling indicated that the plasmid bands ranged from 1 to 9.

Determination of *cry* and *cyt* gene content

Polymerase chain reaction analysis was carried out in order to identify the *cry* and *cyt* gene content of *B. thuringiensis* isolates. Amplification products of *cry* and *cyt* genes of representative isolates are shown in Fig. 4a and b, respectively. It was found that among 100 isolates, 13 isolates contained *cyt1*; 52 isolates had *cyt2*; and 28 isolates included both *cyt1* and *cyt2* genes. However, no *cyt* gene was detected in the remaining seven isolates. The *cry* genotype is defined as the set of *cry* genes found in a given *B. thuringiensis* isolate (Maduell *et al.* 2002). Based on the PCR analysis results, the type of *cry* gene content of the isolates varied from 1 to 5, and 21 groups of *cry* genes (genotypes) were identified (Fig. 5). Sixty-five of the 100 isolates possessed more than one type of *cry* gene; however, 12, 10 and four isolates contained only *cry4*, *cry1* or *cry2* genes, respectively. The percentage of isolates containing both *cry1* and *cry4* genes was the highest (21%) compared with other *cry* genotypes (Fig. 5). In addition, nine strains did not contain any of the *cry* genes even though they had crystal proteins, indicating that they may contain other *cry* genes not examined here. Finally, the distribution of the crystal shapes among *cry* genotypes indicated that there was no correlation between *cry* gene and crystal morphology (Fig. 5).

16S-ITS rDNA RFLP

The PCR analysis of the 16S-ITS rRNA of all isolates and 10 reference strains showed amplification in the size range of *c.* 1·8–2·0 kb. The PCR products gave different patterns after digestion with *Taq*I restriction endonuclease. The dendogram created by the UPGMA analysis showed 11 distinct phylogenetic groups consisting of clusters A to M. Eighteen representative isolates and reference strains indicating each cluster are shown in Fig. 6. Cluster A con-

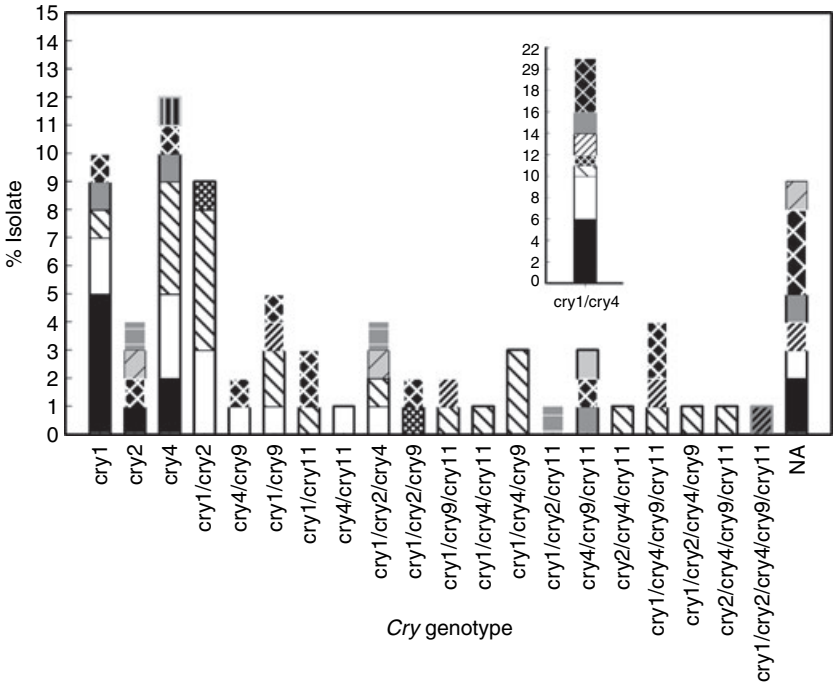

Figure 5 Frequency of *cry* genotypes in *Bacillus thuringiensis* strains obtained from olive-related habitats. NA, no amplification. (■) S; (□) C; (▨) IS; (▩) BP&C; (▨) C&IP; (▩) C&S; (▩) S&IP; (▩) IP; (▩) BP; (▩) BP&IP; (▩) BP&S; (▩) M.

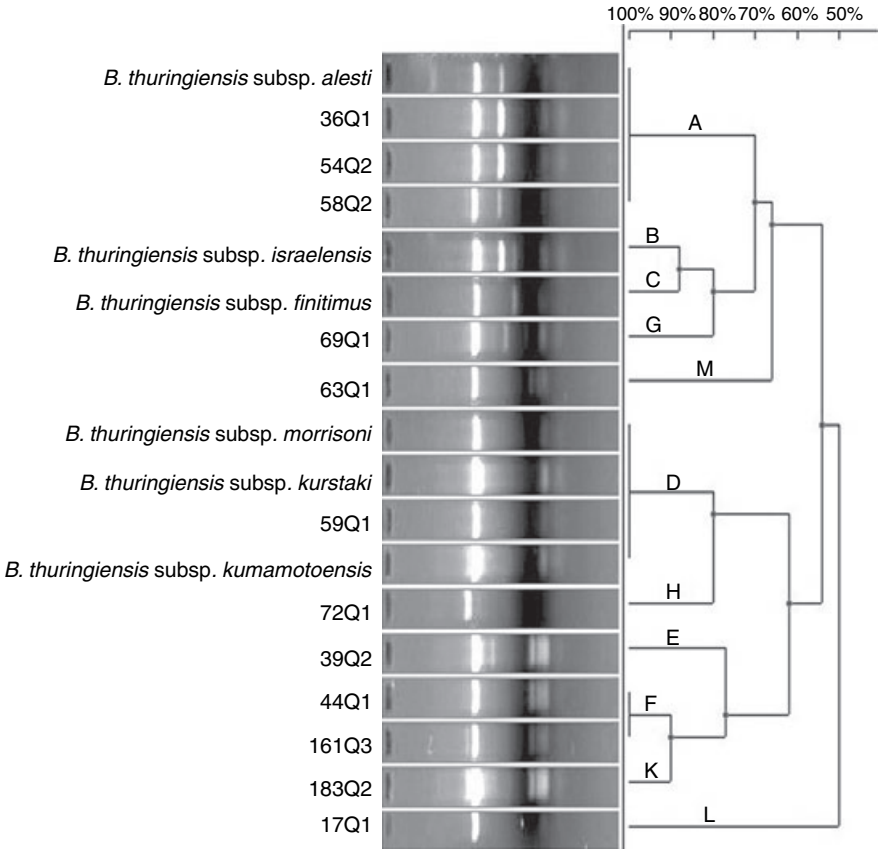

Figure 6 Dendogram of 16S-internal transcribed spacer rDNA restriction fragment length polymorphism homology clusters of representative isolates and reference strains obtained by *Taq*I restriction enzyme. Restriction patterns of each homology group were normalized against a DNA size marker (100 bp DNA ladder; Fermentas) and analysed with 13% homology coefficient in Bio-1D++ computer program (CN-3000 WL; Vilber-Lourmat, France). The names of the phylogenetic groups are indicated on the right (A–M).

tained 19 isolates and ssp. *alesti*. Reference strains ssp. *israelensis* and ssp. *finitimus* were found in clusters B and C, respectively. The largest cluster, designated as group D, includes 58 isolates and the remaining seven reference strains, and share 80% DNA relatedness. Only four isolates were present in cluster E and 14 isolates were represented in cluster F. Five isolates did not cluster in any of the major groups and clustered in different groups; the isolates 69Q1, 72Q1, 183Q2, 17Q1 and 63Q1 were included in cluster G, cluster H, cluster K, cluster L, cluster M, respectively.

Distribution of the insecticidal crystal protein genes and shapes among homology groups

The number of crystal protein shapes observed was different among three different homology groups. For example, clusters A, D and F contained seven, nine and six different crystal shapes, respectively (Table 2). In addition, unlike the isolates in clusters A and F, isolates in cluster D did not exhibit cuboidal plus spherical crystal shape (C&S). Clusters A and F were similar in certain aspects; for instance, the isolates in both groups contained crystal shapes of C, S and IS, whereas they did not have those of BP, IP, C&BP and S&BP. In addition, isolates within each homology group were found to contain all types of *cry* and *cyt* genes examined. The most frequent occurrence of individual *cry* and *cyt* genes among homology groups was *cry1* and *cyt2* in both clusters A and D, and *cry1*, *cry4* and *cyt2* in cluster F. Taken together, these results indicate that there is no correlation between *cry* gene content and crystal protein shapes. However, homology group D exhibited more different crystal shapes than those of homology groups A and F (Table 2).

Table 2 Distribution of insecticidal crystal shapes and genes in homology groups A, D and F

Homology groups	C	S	IS	BP	IP	C&BP	S&BP	C&S	C&IP	S&IP	BP&IP	Gene frequency
Cluster A (19 isolates)												
cry1	2	4	3	–	–	–	–	1	–	2	–	0·63
cry2	–	1	1	–	–	–	–	–	–	–	–	0·11
cry4	2	1	1	–	–	–	–	2	–	3	1	0·53
cry9	–	–	2	–	–	–	–	–	–	1	–	0·16
cry11	1	–		–	–	–	–	–	–	1	–	0·11
cyt1	2	–	2	–	–	–	–	2	–	3	–	0·47
cyt2	2	4	2	–	–	–	–	1	1	3	1	0·74
Total isolates*	3	5	3					2	1	4	1	
Cluster D (58 isolates)												
cry1	5	4	11	2	1	3	1	–	3	7	–	0·64
cry2	2	–	6	3	2	2	–	–	–	2	–	0·29
cry4	6	4	9	1	1	1	–	–	2	4	–	0·48
cry9	–	–	5	–	–	1	1	–	2	4	–	0·22
cry11	–	–	5	1	–	–	1	–	1	3	–	0·19
cyt1	2	3	4	2	–	1	1	–	2	4	–	0·33
cyt2	8	4	15	2	3	3	1	–	2	8	–	0·79
Total isolates*	10	8	16	3	3	3	1		3	11		
Cluster F (14 isolates)												
cry1	2	2	2	–	–	–	–	1	2	1	–	0·71
cry2	–	–	2	–	–	–	–	–	–	–	–	0·14
cry4	3	1	2	–	–	–	–	1	1	1	–	0·64
cry9	–	–	–	–	–	–	–	1	1	–	–	0·14
cry11	–	–	–	–	–	–	–	1	1	–	–	0·14
cyt1	2	1	1	–	–	–	–	1	1	1	–	0·50
cyt2	2	2	–	–	–	–	–	2	2	2	–	0·71
Total isolates*	3	2	3					2	2	2		

Crystal shapes C, S, IS, BP and IP are described in the legend of Fig. 1. Three major homology groups A, D and F (left) on the basis of 16S-internal transcribed spacer rDNA restriction fragment length polymorphism patterns and their crystal shapes in addition to crystal protein gene frequency are indicated (right). Gene frequency indicates the ratio of the number of occurrences of an individual *cry* or *cyt* gene in all of the crystal shapes to the number of the total isolates in each homology group. The number of the isolates carrying the individual *cry* genes is shown in the middle. Most of the isolates contain more than one type of crystal protein gene.

*Total isolates describe the number of isolates in each crystal shape.

Discussion

This is the first study to isolate and characterize *B. thuringiensis* from olive-related habitats in Turkey. Olive groves and olive-processing sites have not previously been sprayed with any commercially available *B. thuringiensis* strains to control insect pests in Turkey. Chemical insecticides are mostly used to control olive pests because of their effectiveness and low cost. The development of *B. thuringiensis* as a microbial insecticide has driven the establishment of several screening programmes for novel *B. thuringiensis* strains that could be used against the olive pests instead of chemicals. In this study, *B. thuringiensis* strains were characterized on the basis of crystal morphology, *cry* and *cyt* gene content, plasmid profiling and 16S-ITS rDNA RFLP.

Olive groves and olive-processing sites were found to be good sources for the isolation of *B. thuringiensis*. This finding was in agreement with that of other workers (Karamanlidou *et al.* 1991; Bel *et al.* 1997; Alberola *et al.* 1999) who have shown that *B. thuringiensis* is a common member of the microbiota in the olive tree environments. We collected soil samples from different olive groves in the Aegean region of Turkey (Table 1), as soil is shown as one of the principal sources of novel *B. thuringiensis* isolates (Dulmage and Aizawa 1982; Smith and Couche 1991). This bacterium was isolated from 54 samples (36%), and the highest percentage was found in soil samples (70%). In a previous study, Bel *et al.* (1997) studied the occurrence, distribution and genetic diversities of *B. thuringiensis* in olive storage warehouses, mills and olive groves of Spain and showed that *B. thuringiensis* was present in 92% of samples analysed and the highest percentage was observed in soils from olive groves.

There is a possible relationship between the crystal protein shapes and toxicity (Ohba and Aizawa 1986). Karamanlidou *et al.* (1991) recovered *B. thuringiensis* from Greek soil samples of olive groves (30%) and found that the isolates contained different shape of crystals (spherical, cuboidal, irregular and bipyramidal). The most frequent crystal type was spherical and most of the toxic strains against the olive fruit fly *Ba. oleae* (Diptera: Tephritidae) were found to contain spherical inclusions. In this present study, phase-contrast microscopy revealed the production of the same types of crystal proteins, but the irregular-shaped crystals were produced at the highest frequency (24%, Fig. 1).

The isolation of *B. thuringiensis* strains from different habitats and geographical locations indicates differences in the occurrence and diversity of *cry* gene content (Chak *et al.* 1994; Bravo *et al.* 1998). Therefore, environmental samples had been collected from wild olive groves and closed areas in different geographical regions. Comparison of the insecticidal gene content of the same sample type from different regions indicated that six out of eight soil samples contained all the insecticidal genes examined; however, either *cry9* or *cry2* gene was absent in two soil samples from Izmir (Table 1). Even though there is a high degree of similarity in the *cry* gene content of samples from different geographical origins, we expect to see a wide variation in the subgroups of each *cry* gene and insecticidal toxic potency as shown in the previous studies (Hongyu *et al.* 2000; Wang *et al.* 2003; Chen *et al.* 2004; Martinez *et al.* 2005). Moreover, these isolates may contain other different *cry* genes as well, not examined here. Furthermore, different *cry* gene profiles of the same sample from different areas (Table 1) may be attributed to plasmid exchange, which has been shown to occur between *B. thuringiensis* strains in soil, insect bodies and water (Thomas *et al.* 2000; Vilas-Bŏas *et al.* 2000). Similarly, Armengol *et al.* (2007) studied the distribution of *cry* genes throughout the Colombian natural regions and revealed that *B. thuringiensis* presents great genetic and molecular diversity even in isolates from the same Colombian soil sample.

The distribution of *cry* and *cyt* genes was different from that of other strain collections. Apaydin *et al.* (2005) isolated *B. thuringiensis* in grain-related habitats of central Anatolia and Aegean region in Turkey and characterized them by phenotypic and genotypic methods. It was found that the most frequent crystal shape was spherical (36%) and most isolates had *cry9* (Lepidoptera-specific) genotype (21%). Our present characterization contributes to the understanding of the occurrence and diversity of this bacterium in Turkey. *cry* and *cyt* gene content determined by PCR showed a high genetic diversity and the most frequent *cry* genotype (21%) identified was a combination of Lepidoptera-active *cry1* and Diptera-active *cry4* genes (Fig. 5). These differences may be related to the climatic and ecological conditions chosen for sampling; there is a mild Mediterranean climate in western Anatolia (Aegean region), whereas the climate of the central Anatolian region is a steppe climate. Interetingly, *cyt2* was the most common gene found in local isolates. This is in agreement with the results of Guerchicoff *et al.* (2001) who revealed that *cyt2* genes are widely distributed among *B. thuringiensis* strains. Alberola *et al.* (1999) performed PCR using 39 specific primers on 72 selected isolates which were found toxic against *Ba. oleae* (Diptera: Tephritidae). Unlike our findings, they did not observe *cry4*, *cry9*, *cry11* and *cyt1* genes in their isolates; however, *cry1*, *cry2* and *cyt2* genes were detected. In addition, our results indicated that some of the isolates originating from different samples did not harbour some of the genes; for instance, the strains isolated from green olive leaves of Foca did not contain *cry1* and *cry2* genes, and

strains obtained from Kemalpasa soil did not contain *cry9* and *cry11* genes (Table 1). Furthermore, it is noteworthy that *cry1* and *cry2* genes were most often detected together, which is similar to the results of other workers (Ben-Dov *et al.* 1997; Kim 2000; Zhang *et al.* 2000; Wang *et al.* 2003).

Based on Maeda *et al.* (2000), there is a correlation between toxic activity and crystal shape of *B. thuringiensis* isolates. Therefore, in this present study, crystal morphology of each isolate was determined using a phase-contrast microscope. In most of the isolates, there was no correlation between crystal shapes and *cry* gene profiles (Fig. 5). It is most probable that crystal proteins could be encoded by other *cry* genes that were not examined in this study or detected genes could not be expressed at the protein level. Similar to our findings, Uribe *et al.* (2003) reported different crystal morphologies for the same subgroups of *cry1*, *cry3* or *cry7* gene profiles of the different isolates. Because there is no accurate relationship between a specific *cry* gene and its toxic activity (Shisa *et al.* 2002), bioactivity assay is required for more definitive results about the toxic potency of the isolates. In addition, PCR analysis of *cry* genes had indicated a great deal of diversity of *cry* gene combinations among the isolates. The number of different *cry* gene in an isolate ranged from one to as many as five (Fig. 5) and 65% of the isolates revealed the 18 different combinations of *cry* genes. Similar results were also reported by other studies (Bravo *et al.* 1998; Ferrandis *et al.* 1999; Uribe *et al.* 2003). These findings indicate that some *B. thuringiensis* isolates possibly have the potency to develop biopesticides that control more than one insect group in a certain crop.

In previous studies, 16S-ITS rDNA RFLP method had been used as a molecular identification method of lactobacilli (Yavuz *et al.* 2004b), alkalophilic bacilli (Akbalik *et al.* 2004) and thermophilic bacilli (Yavuz *et al.* 2004a). Therefore, we carried out the same analysis to reveal the phylogenetic relationships among local isolates and reference strains. *Taq*I restriction located the isolates and reference strains into 11 distinct clusters (Fig. 6). Most of the environmental isolates (58%) and seven reference strains grouped together with a similarity of 80% in cluster D. In other words, strains were found to be relatively homogenous and to share a high degree of DNA relatedness. Other researchers (Joung and Côté 2002) studied 80 different *B. thuringiensis* serovars by 16S rRNA gene RFLP using *Hin*dIII and *Eco*RI restriction enzymes. They found that all 80 *B. thuringiensis* serovars were clustered together in four distinct groups at a DNA similarity rate of 93%. Serovars *kurstaki*, *aizawai*, *thompsoni*, *kumamotoensis* and *galleriae* were grouped together, whereas serovars *alesti*, *morrisoni* and *israelensis* were found together in the other cluster. Unlike the method used by Joung and Côté (2002), 16S-ITS rDNA RFLP method was able to differentiate some of the *B. thuringiensis* reference strains in different groups. For example, serovars *alesti*, *israelensis* and *finitimus* were replaced in clusters A, B and C, respectively (Fig. 6). The remaining seven reference strains *kurstaki*, *aizawai*, *tenebrionis*, *kumamotoensis*, *galleria*, *thompsoni* and *morrisoni* were found in the same cluster D. When the 16S-ITS rDNA RFLP analysis of 80 *B. thuringiensis* serovars is carried out, it will be appropriate to compare the results with those of Joung and Côté (2002). A combination of different molecular characterization tools may enable more accurate classification of *B. thuringiensis* strains.

The distribution of crystal protein shapes and genes among three major homology groups were analysed (Table 2). It is attributed to the fact that most of the *cry* genes were carried on the plasmids, and horizontal transfer of *cry* genes might occur among natural isolates of *B. thuringiensis*. No correlation was observed between the homology groups identified by 16S-ITS rDNA RFLP and *cry* genes of the isolates. However, the number of different crystal shapes was the highest in the homology group D compared with that of clusters A and F (Table 2).

In conclusion, this study demonstrates that *B. thuringiensis* strains isolated from olive-related habitats in Turkey were genotypically heterogenous. A future study related with serological characteristics and insecticidal activity of isolates would likely give more definitive results about the biodiversity of *B. thuringiensis* strains in olive-related areas in Turkey.

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