Tropical Ecology **58**(3): 605–610, 2017 ISSN 0564-3295

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Diversity and distribution of *cry* genes in native *Bacillus thuringiensis* strains isolated from wild ecological areas of East-Mediterranean region of Turkey

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Abstract: *Bacillus thuringiensis* (*Bt*) crystal (Cry) proteins have specific insecticidal activity against insect pests and extensively used in biological control applications worldwide. In the present study, native *Bt* strains were screened for frequency and distribution profile of their *cry* genes encoding Cry proteins toxic to insect pests. A total of 120 strains from 80 different ecological locations with altitudes ranging from 0 to 1600 m were used to investigate *cry* gene contents. *cry1Ab/ Ac* (47.72%), *cry1Aa/Ad* (35.50%), *cry2* (31.82%), *cry5* (28.41%), and *cry9C* (27.27%) were among the most abundant genes in strains. Frequency of *cry1C, cry1Ad, cry1Ac, cry1D, cry1B, cry3-7-8, cry4A, cry9A,* and *cry11A/B* were, respectively, estimated as 20.68, 5.75, 2.27, 2.27, 5.68, 4.55, 1.14, 4.55, and 7.95%. Results provide useful information with regard to analyzed *cry* gene prevalence of *Bt* strains in the region.

Key words: *Bacillus thuringiensis*, *cry* gene frequency, distribution, diversity.

Introduction

Bacillus thuringiensis (*Bt*) is known to be an important microbial entomopathogen for the biological control of the agricultural insect pests and disease vectors (dos Santos *et al.* 2009). *Bt* is characterized by its production of different insecticidal proteins as parasporal crystals during sporulation (Crickmore *et al.* 1998; Rowe & Margartis 1987). Their specific toxicity exerted on various insect orders is due to Cry (the most common), Cyt and Vip proteins encoded especially in plasmid DNAs (Sanahuja *et al.* 2011). Crystal proteins of *Bt* are toxic to a wide variety of lepidopteran, dipteran and coleopteran insects causing serious damage to economically important crops (Wang *et al.* 2003). Diversity of *Bt* and its *cry* genes are widely studied throughout the world. The aim of the present study was to analyze the distribution of *cry* genes with selected primer pairs, and to determine any change in their frequency in *Bacillus thuringiensis* strains from East-Mediterranean region of Turkey. The presence of *cry1C*, *cry1Ad*, *cry1Ac*, *cry1Ab*/*Ac*, *cry1Aa*/*Ad*, *cry1D*, *cry1B*, *cry2*, *cry3-7-8*, *cry4A*, *cry5*, *cry9A*, *cry9C* and *cry11A*/*B* genes were screened in 120 isolates using PCR analysis.

Methods

Collection of samples

Different agricultural and wild ecological areas of East-Mediterranean region of Turkey (Table S1) were screened. To eliminate confusion, samples were collected from areas with no *Bt* based product application.

Bacterial isolation

One g of soil sample was added to 20 ml of Luria Bertani Broth buffered with 0.25M sodium acetate (pH 6.8), and incubated at 200 rpm for 4 h (30 °C).

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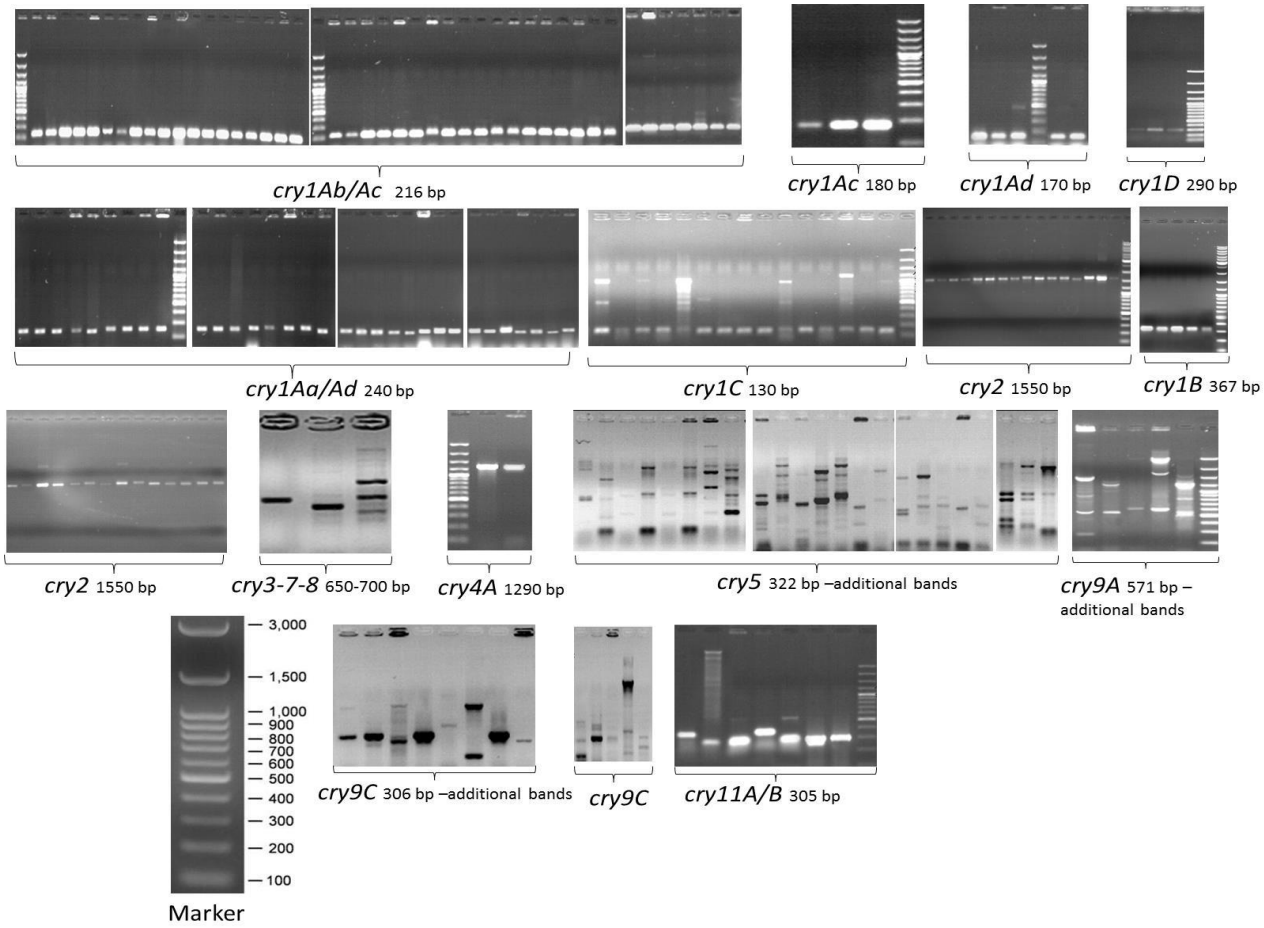

Fig. 1. Agarose gel electrophoresis of the PCR products amplified by using *cry* primers.

One ml of sample was then heated at 80 °C for 5–10 min. The 50 µl aliquot was spread on nutrient agar plate and incubated overnight at 30 °C (Travers *et al.* 1987). From each location, at least 10 colonies were morphologically selected for *Bt* determination. After microscopic and morphological analysis, 120 isolates were cultured for screening *cry* genes with selected primers.

Characterization of parasporal inclusions

Effective *Bt* strains were incubated in 150 ml T3 medium (3 g triptone, 2 g triptose, 1.5 g yeast extract, 0.005 g $MnCl_2$, 6 g NaH_2PO_4, 7.1 g Na_2HPO_4) at 30 °C and 200 rpm during 7 days to induce sporulation (Travers *et al.* 1987). Suspensions were centrifuged at 4 °C and 15000 ×g for 10 min to harvest spore-crystal mixtures. Spore-crystal samples were spread on a microscope slide and fixed. Later, they were sputter coated with 10 nm Au/Pd using a SC7620 Mini-sputter coater and viewed using a LEO440 scanning electron microscope at 20kV beam current.

cry *gene analysis*

Molecular characterization of the strains was performed by PCR analysis using primers specific to *cry* genes (Table S2). Each reaction contained the reagents at a final concentration as 2.3 mM $MgCl_2$, 1x Taq buffer, 0.2 mM dNTP mix, 0.3 pmol primers (each), 0.5 U Taq DNA polymerase, and 30–100 ng template DNA. The PCR amplification was performed under the following conditions: Initial denaturation at 95 °C for 2.5 min followed by 30 cycles at 95 °C for 1 min (Tm for each primer pairs were given in Table S2), 72 °C for 1 min and a final extension step at 72 °C for 5 min.

Results

Bt strains collection

According to the PCR analysis, out of 120 *Bacillus* sp. samples isolated, 88 were determined as *Bt* based on *cry* gene analysis (Fig. 1). Spore-crystal samples were examined under scanning

Table 1. Distribution of *cry* genes after PCR analysis.

Cry gene	*Cry* forms*	Protein sizes (kDa)	Insect order specificity	Isolates	Total Number
cry1C	Bp	130-140	Lepidoptera	SY49.1, SY65.1A, SY45.4, SY73.4, SY56.3, SY48.1, SY2.1, SY6.1, SY1.6, SY16.6, SY65.1B, SY25.1, SY2.3, SY39.6, SY28.5, SY39.5, SY1.1, SY74.3	18
cry1Ad	Bp	130-140	Lepidoptera	SY27.1, SY78.1, SY2.1, SY52.1, SY64.2	5
cry1Ac	Bp	130-140	Lepidoptera	SY27.1, SY73.2	2
cry1Ab/Ac	Bp	130-140	Lepidoptera	SY27.1, SY65.1A, SY45.4, SY69.7, SY62.1A, SY73.4, SY26.3, SY27.3,SY33.3 SY52.1, SY10.5, SY41.4, SY64.4, SY15.1, SY58.5, SY38.4, SY46.2, SY2.3, SY39.6, SY74.2, SY6.1, SY46.6, SY48.1, SY25.1, SY50.4, SY56.2, SY23.1 SY74.3, SY1.5, SY1.6, SY25.2, SY49.4, SY73.3, SY24.2, SY56.4, SY8.2, SY8.4, SY69.5, SY1.8, SY65.1B, SY6.2, SY4.1	42
cry1Aa/Ad	Bp	130-140	Lepidoptera	SY49.1, SY26.3, SY27.3, SY56.3, SY26.2, SY33.3, SY73.2, SY64.4, SY36.7, SY58.5, SY38.4, SY54.6, SY39.6, SY8.2, SY42.2, SY47.1, SY42.3, SY79.3, SY74.2, SY78.3, SY6.2, SY48.3, SY10.5, SY46.6, SY48.1, SY25.1, SY69.4, SY56.2, SY74.3, SY53.4, SY16.6, SY24.2, SY65.1	34
cry1D	Bp	130-140	Lepidoptera	SY64.4, SY73.6	2
cry1B	Bp	130-140	Lepidoptera,Coleoptera, Diptera	SY27.1, SY49.1, SY73.2, SY6.1, SY41.4	5
cry2	Cb	65-70	Lepidoptera, Diptera	SY27.1, SY65.1A, SY45.4, SY69.7, SY62.1A, SY56.3, SY33.3, SY73.2, SY2.1, SY52.1, SY41.4, SY48.2, SY36.7, SY15.1, SY58.3, SY3.1, SY58.5 SY55.3, SY24.1, SY45.1, SY54.6, SY46.6, SY48.3, SY52.2, SY48.1, SY25.2, SY49.4, SY65.1	28
cry3-7-8	S	75	Coleoptera	SY56.3, SY35.3, SY61.6, SY26.2	4
cry4A	Cb	130	Diptera	SY50.4	1
cry5	Rb	78-85	Nematod	SY49.1, SY26.3, SY27.3, SY73.2, SY55.3, SY46.2, SY39.6, SY10.5, SY46.6 SY5.2, SY73.6, SY79.3, SY51.1, SY73.5, SY15.3, SY4.3, SY23.1, SY16.3, SY3.2, SY74.3, SY39.5, SY59.5, SY1.1, SY16.6, SY24.2	26
cry9A	Bp	130-140	Lepidoptera	SY49.1, SY52.2, SY3.2, SY72.2	4
cry9C	Bp	130-140	Lepidoptera	SY27.1, SY49.1, SY69.7, SY62.1A, SY27.3, SY26.2, SY36.7, SY45.1, SY55.3 SY42.2, SY73.6, SY79.3, SY73.5, SY15.3, SY39.5, SY59.5, SY69.3, SY69.1, SY62.3, SY73.1, SY1.5, SY42.4, SY53.4, SY31.5, SY55.9	25
cry11A/B	Cb	65-70	Diptera	SY73.4, SY58.3, SY74.3, SY53.4, SY31.5, SY1.6, SY16.6	7

*Bp: Bipyramidal, Cb: Cubic, S: Spherical, Rb: Rhomboid.

electron microscope to determine their morphology. Bipyramidal, cubic, spherical, rhomboidal, irregular shaped spherical parasporal inclusions and spores were analyzed by electron microscopy (Table 1).

Characterization of the Bt strains

The number of strains determined to carry *cry1C, 1Ac, 1Ad, 1Ab/Ac, 1Aa/Ad, 1D, 1B, cry2, cry5, cry9A* and *cry9C* primers were 18, 2, 5, 42, 34, 2, 5, 28, 26, 4 and 25, respectively (Table 1). Also, some strains produced positive results with *cry4A*, *cry3-7-8* and *cry11A/B* primers. Most of the strains contained more than one type of *cry* gene (Table 1). It was seen that 69.3% of the strains carried *cry1* type genes. When the sub types of *cry1* gene was considered, the frequency of *cry1Ab/Ac*, *cry1Aa/Ad, cry1C, cry1Ad, cry1Ac, cry1D* and *cry1B* were found to be 47.72, 37.50, 20.45, 5.68, 2.27, 2.00 and 5.68%, respectively. The frequency of *cry1* type genes were markedly higher compared to other *cry* genes tested.

Discussion

In the current study, amplification of *cry1C*, *1Ac, 1Ad, 1Ab/Ac*, *1Aa/Ad, 1D, 1B*, *cry2 cry5, cry9A,* and *cry9C* genes were carried out on 120 strains and observed that respectively 18, 2, 5, 42, 34, 2, 5, 28, 26, 4 and 25 strains produced expected band size. Also 1, 4 and 7 strains yielded positive results with *cry4A, cry3-7-8* and *cry11A/B* primer pairs, respectively. Some of the strains yielded additional bands with *cry1Ab/Ac*, *cry1Ad*, *cry3-7-8*, *cry5, cry9C, cry2*, *cry9A,* and *cry11A/B* primers. More detailed investigations are required on these strains in order that the extra bands may be a sign of new *cry* genes rather than non-specific bands. Most of the strains carried more than one *cry* gene (varying from 2 to 6) suggesting that *Bt* strains have high frequency of genetic information exchange (Arrieta *et al.* 2004). Approximately one fourth of the strains did not produce any bands. However, the strains should be tested with other primer pairs in case they might contain other genes of interest. In a *cry* gene screening study carried out by Bravo *et al.* (1998) the highest frequency was seen in *cry1* genes with a value of 49.5%, followed by *cry3* (21.7%) and *cry11* (7.9%). The researchers calculated the frequency of *cry1B*, *cry1D* and *cry1Ac* as 77, 24 and 12%, respectively. In another screening study, incidence of *cry1Ac*, *cry1B*, *cry1Ad, cry1Aa*, *cry1Ab* and *cry2* were respectively found as 24, 13.8, 5, 48, 47, and 74% (Martinez *et al.* 2005). Those findings, except for *cry2,* were coherent with the current findings. Wang *et al.* (2003) estimated the frequency of *cry9* as 15%, however in the current study *cry9A* and *cry9C* were found to be 4.55 and 27.27%, respectively. In another study *cry1Ab* and *cry2Aa* are found in all strains, however *cry1Aa* was only seen in 6, *cry1Ac* and *cry1B* in 5 and *cry1D* in 1 isolate (dos Santos *et al.* 2009). Their observation, except for *cry2* and *cry1Aa,* were compatible with our findings. Valicente *et al.* (2010) reported the most commonly seen gene as *cry1D* with a rate of 57.5%, whereas *cry1Aa/Ad* and *cry1C* genes frequency was only 1.2%. However, at present study, the incidence of *cry1D* and *cry1Aa/Ad* were found as 2.27 and 35.5%, respectively. The results of our study indicated that most of the strains carrying *cry1* were also found to harbor *cry2* as also indicated by López-Pazos *et al.* (2009).

Researchers noted that *Bt* is commonly found in natural soils. However, no satisfactory correlation was reported regarding the habitat and distribution of bacteria (DeLucca *et al.* 1981; Martin & Travers 1989). It is well known that great variability and distribution of *cry* gene content in *Bt* strains were likely associated with differences in the biological, geographical and ecological properties of the collection sites (Chak *et al.* 1994). Some studies supporting the finding that the frequency of *cry1* gene were quite different in different habitats being 19.5, 98.2, 33.5 and 76.5% in Mexican, Taiwan, Uzbekistan and Colombia collections, respectively (Ben Dov *et al.* 1997; Ceron *et al.* 1994; Chak *et al.* 1994; Uribe & Ceron 2003). At present study, when categorizing the fields in three different ranges of altitudes as 0–600, 600–1200 and 1200–1600 m, and analyzing the number of genes in each range per number of bacteria, no correlation was also observed between locations with regard to distribution of *cry* genes. Yet, the frequency of genes in interest was higher (163gene/67 bacteria) in *Bt* inhabiting fields with an altitude of less than 600 m.

Conclusions

In conclusion, *cry* gene spectrum among isolates were found to be quite different. The abundance of genes only specific to Lepidoptera, Coleoptera and Nematode was estimated as 5.68, 45 and 23%, respectively. While the ratio of Lepidopter-Dipter and Lepidopter-Coleopter-Dipter specific *cry* genes were found in order of 32.95 and 5.68%. Results provide useful information with regard to analyzed *cry* gene prevalence of *Bt* strains in the region.

Acknowledgement

This project was funded by the Erciyes University Scientific Project Unit under the codes of FBD-08-540.

References

Arrieta, G., A. Hernández & A. M. Espinoza. 2004. Diversity of *Bacillus thuringiensis* strains isolated from coffee plantations infested with the coffee berry borer *Hypothenemus hampei*. *Revista de Biología Tropical* **52:** 757–764.

Ben-Dov E., A. Zaritsky, E. Dahan, Z. E. Barak, R. Sinai, R. Manasherob, A. Khamraev, *et al.* 1997. Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. *Applied and Environmental Microbiology* **63:** 4883–4890.

Bravo, A., S. Sarabia, L. Lopez, H. Ontiveros, C. Abarca, A. Ortiz, M. Ortiz, *et al.* 1998. Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Applied and Environmental Microbiology* **64:** 4965–4972.

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

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

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

De Lucca, A. J., J. G. Simonson & A. D. Larson. 1981. *Bacillus thuringiensis* distribution in soils of the United States. *Canadian Journal of Microbiology* **27:** 865–870.

dos Santos, K. B., P. Neves, A. M. Meneguim, R. B. dos Santos, W. J. dos Santos, G. V. Boas, V. Dumas, *et al.* 2009. Selection and characterization of the *Bacillus thuringiensis* strains toxic to *Spodoptera eridania* (Cramer), *Spodoptera cosmioides* (Walker) and *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). *Biological Control* **50:** 157–163.

López-Pazos, S. A., J. W. Martínez, A. X. Castillo & J. A. Cerón Salamanca. 2009. Presence and significance of *Bacillus thuringiensis* Cry proteins associated with the Andean weevil *Premnotrypes vorax* (Coleoptera: Curculionidae). *International Journal of Tropical Biology and Conservation* **57:** 1235–1243.

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

Martinez, C., J. E. Ibarra & P. Caballero. 2005. Association analysis between serotype, *cry* gene content, and toxicity to *Helicoverpa armigera* larvae among *Bacillus thuringiensis* isolates native to Spain. *Journal of Invertebrate Pathology* **90:** 91–97.

Masson, L., M. Erlandson, M. Puzstai-Carey, R. Brousseau, V. Juarez-Perez & R. Frutos. 1998. A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. *Applied and Environmental Microbiology* **64:** 4782–4788.

Rowe, G. E. & A. Margaritis. 1987. Bioprocess developments in the production of bioinsecticides by *Bacillus thuringiensis* CRC. *Critical Reviews in Biotechnology* **6:** 87–107.

Sanahuja, G., B. Raviraj, R. M. Twyman, T. Capell & P. Christou. 2011. *Bacillus thuringiensis*: A century of research, development and commercial applications. *Plant Biotechnology Journal* **9:** 283–300.

Travers, R. S., P. A. W. Martin & C. F. Reichelderfer. 1987. Selective process for efficient isolation of soil *Bacillus* sp. *Applied and Environmental Microbiology* **53:** 1263–1266.

Uribe, W. M. & J. Ceron. 2003. Distribution and diversity of *cry* genes in native strains of *Bacillus thuringiensis* obtained from different ecosystems from Colombia. *Journal of Invertebrate Pathology* **82:** 119–127.

Valicente, F. H., E. A. de Toledo Picoli, M. J. de Vasconcelos, N. P. Carneiro, A. A. Carneiro, C. T. Guimarães & U. G. Lana. 2010. Molecular characterization and distribution of *Bacillus thuringiensis cry1* genes from Brazilian strains effective against the fall armyworm, *Spodoptera frugiperda*. *Biological Control* **53:** 360–366.

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

(Received on 22.11.2016 and accepted after revisions, on 04.04.2017)

Supporting Information

Additional Supporting information may be found in the online version of this article.

Table S1. Locations and types of *cry* genes found in isolates from soil samples.

Table S2. Specifications of *cry* primers.