

## Isolation and Screening of *cry* and *parasporin* Genes of Native *Bacillus thuringiensis* from Cherry Gardens in Izmir

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### Research Article

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### ABSTRACT


*Bacillus thuringiensis* (Bt), which produces various proteins, is a versatile microorganism. In this study, we performed Bt isolation from cherry orchards in İzmir (Türkiye) and characterization of *cry* and *parasporin* (*ps*) genes related to the proteins that are insecticidal and cytotoxic, respectively. Forty-one soil samples were collected, and 314 Bt-like colonies were obtained from these samples. According to phase contrast microscopy examination, 80 colonies showing crystal formation were identified as 'Bt'. Polymerase chain reaction (PCR) was performed to determine the *cry* and *ps* gene profiles of the isolates. It was found that 66 out of the 80 samples carried *cry1*, *cry2*, or *cry3* genes. The most common of these genes was the *cry1* (68%). In the case of *parasporin* gene screening, the existence of *ps1*, *ps2*, *ps3*, *ps4*, *ps5*, and *ps6* genes was investigated. Seventeen of the 80 isolates were found positive for *ps* genes. Among these, the *ps6* gene showed the highest frequency with 29%, followed by the *ps3* gene (24%). Partial DNA sequence analysis of seven *ps*-positive isolates showed that 5.4, 5.7, 5.10, 7.1, and 7.3 showed 99% identity with *ps6*, and the other isolates 2.1 and 40.4, matched with 98% and 97% identity with *ps1* and *ps2*, respectively. In conclusion, this study reports a new Bt collection from Türkiye. The native Bt isolates carrying *cry* and/or *ps* genes will help us select potential ones with insecticidal and cytotoxic effects that will form the basis for the production of recombinant parasporal proteins planned in future studies.



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**Keywords:** *Bacillus thuringiensis*, *cry* gene, DNA sequencing, *parasporin* gene, PCR

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## 1. Introduction

*Bacillus thuringiensis* (Bt) is a bacterium that has been used as a successful source in biopesticide production for many years. Its entomopathogenic property is based on insecticidal proteins synthesized during the vegetative and/or stationary phases of bacterial growth [1]. Due to its advantages as a natural and harmless product to other organisms in biocontrol compared to harmful chemical pesticides, there have been so many studies in literature indicating Bt isolation from different sources and locations and determination of insecticidal activities of these isolates against important pests [2-5]. The gaining of new native isolates to Bt collections has contributed to genetic and therefore protein diversity. It has also been seen as a way of solution to overcome the resistance developed against existing toxins [6].

Cry (crystal) proteins, a group of delta endotoxins, are the most researched and clarified insecticidal protein family synthesized by this bacterium, with its structure, mechanism of action, and insecticidal activities [1]. So far, 800 cry genes encoding Cry proteins have been classified into 75 classes [7]. Cry proteins with a target-specific activity have been toxic to different orders of insects, such as Lepidoptera-specific Cry1, Lepidoptera and Diptera-

specific Cry2, Coleoptera-specific Cry3 and Diptera-specific Cry4 [8]. The studies on mode of action showed that Cry proteins, synthesized in the form of protoxins, are solubilized in alkaline conditions and then activated by proteolytic cleavage. This toxin is then expected to bind to the receptors of the target cell, create ion channels and pores in the membrane, cause osmotic shock, paralysis, and finally death of the insect due to starvation [7].

The fact that most of the screened parasporal proteins on bioassays that did not have insecticidal activities made researchers search for the roles of those non-insecticidal proteins. Thus, a new Cry protein family, parasporins (PS), was discovered as non-insecticidal parasporal proteins that had cytotoxic effects on cancer cells [9]. This definition was changed later to '*B. thuringiensis* and related bacterial parasporal proteins that are non-hemolytic but capable of preferentially killing cancer cells' with the findings that insecticidal proteins could have cytotoxic activity as well [10]. Parasporins are classified in 6 families, from PS1 to PS6, and so far 19 parasporin proteins have been identified [11]. Their cytotoxic activities on various cancer cells were demonstrated [12-14].

In this study, native Bt isolates were obtained from cherry orchards in Kemalpaşa, İzmir, for the first time. *cry1*, *cry2*, and *cry3* genes were screened in order to detect insecticidal genes. The isolates were then investigated to see whether they contained all identified parasporin genes in literature: *ps1*, *ps2*, *ps3*, *ps4*, *ps5*, and *ps6*. DNA sequence analysis determined their gene profiles. The data obtained from this study enabled us to identify Bt isolates that have the potential to be effective for producing biopesticides against agricultural pests and with cytotoxic potential toward cancer cells.

## 2. Materials and Methods

### 2.1. Sample Collection and Bt Isolation

Soil samples were collected from 41 different locations of cherry orchards in Kemalpaşa, İzmir, Türkiye. They were taken from approximately 10 cm below the soil at the tree roots with a sterile spatula and stored in sterile bags. Bt isolates from soil samples were isolated according to Santana et al. [15]. The Bt-like colonies examined under a phase contrast microscope (Olympus) were identified as 'Bt' if they harbored parasporal inclusions. They were stocked in 25% glycerol at -80°C.

### 2.2. Bacterial Strains

The reference strains for *cry* and *ps* genes, *Bacillus thuringiensis* subs. *kurstaki* (*cry1* and *cry2*), *Bacillus thuringiensis* subs. *tenebrionis* (*cry3*), and *Bacillus thuringiensis* subs. *dakota* 4R2 (*ps2*), were obtained from the Bacillus Genetic Stock Center (Ohio, USA).

### 2.3. Screening of *cry* and *parasporin* Genes

Genomic DNA was isolated following the method by Cinar et al. [16]. The detection of *cry1*, *cry2*, *cry3*, *ps1*, *ps2*,

*ps3*, *ps4*, *ps5*, and *ps6* genes was performed by PCR analysis in a total volume of 25 µl containing 100 ng DNA, 1X PCR buffer, 1 U Taq DNA polymerase (Fermentas), 2 mM MgCl<sub>2</sub>, 200 µM dNTP, and 0.2 µM of each primers for *cry* genes and 0.5 µM of each primer for *ps* genes. The primer pairs used in this study are shown in Table 1. PCR conditions for *cry* genes consisted of initial denaturation at 94°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for *cry1* and *cry2* and 60°C for *cry3*, and extension at 72°C for 1 minute. In the case of *parasporin* genes, PCR conditions consisted of initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for *ps1*, 54°C for *ps2*, *ps5*, and *ps6*, 65°C for *ps3*, and 59°C for *ps4* for 30 seconds, and extension at 72°C for 2 minutes. Amplifications were completed in the final stage with extension at 72°C for 7 minutes. PCR was performed using the Advanced Primus 96 Thermal Cycler (PeQLab). After amplification, the PCR products were electrophoresed on 1% agarose gel at 90 V for 1 hour and visualized in the Bioprint Vilber documentation system. PCR products sent by Dr. Rampersad (The University of Texas Rio Grande Valley, USA) were used as positive controls in *ps* amplifications.

### 2.4. DNA Sequence Analysis and Construction of Phylogenetic Tree

Bt isolates that were shown to carry parasporin genes were selected, and their relevant genes were amplified and extracted from the gel using the gel extraction kit (Geneall Expin Gel SV). The samples were sent for DNA sequence analysis (Sentegen Biotech). BioEdit Sequence Alignment Editor and BlastN programs were used to edit DNA sequences. The phylogenetic analyses were performed using the MEGA7 program.

Table 1. Primers used for detection of *cry* and *parasporin* genes in the Bt isolates.

Gene	Primers	Nucleotide sequence (5'-3')	PCR product (bp)	References
<i>cry1</i>	Un1(F)	CATGATTCATGCGGCAGATAAAC	274-277	[17]
	Un1(R)	TTGTGACACTTCTGCTTCCCATT		
<i>cry2</i>	Un2(F)	GTTATTCTTAATGCAGATGAATGGG	689-701	[17]
	Un2(R)	CGGATAAAATAATCTGGGAAATAGT		
<i>cry3</i>	Un3(F)	AGTTATCGCAGAGAGATGACATTAAC	589-604	[17]
	Un3(R)	CATCTGTTGTTTCTGGAGGCAAT		
<i>ps1</i>	ps1 (F)	CCCAGATTCAAATAATAACCAAGA	511	[13]
	ps1 (R)	AGCACCTAATGATGATAGAGGAA		
<i>ps2</i>	ps2 (F)	TGTTGGGACTGTTTCAGTACGT	503	[18]
	ps2 (R)	CGTCACGGTACCTCTTAGTGT		
<i>ps3</i>	ps3 (F)	GGAATCCAGGTGCACTGCT	701	[18]
	ps3 (R)	GTCCCGGATCATACTGGGA		
<i>ps4</i>	ps4 (F)	AGTGGTCTCCAGGCTCATACTGG	681	[18]
	ps4 (R)	TGATATCCCGAACCTGCCCT		
<i>ps5-1</i>	ps5-1 (F)	ATGGTACGCCGAACAAAA	487	[13]
	ps5-1 (R)	TACTGGGACTACAACCTGGTCCT		
<i>ps5-2</i>	ps5-2 (F)	CCGACCTGTCCTTGTCT	384	[13]
	ps5-2 (R)	GAGTACGTACGGCCATTACCT		
<i>ps6</i>	ps6 (F)	TGTTTACTATGTGAAAGGTGGAGA	446	[13]
	ps6 (R)	CAATAGTGGTTCCTATTGGACCT		

### 3. Results and Discussion

#### 3.1. Bt Isolation

The identification of Bt isolates was performed according to the presence of parasporal inclusions. As a result of the isolation from 41 soil samples, 314 Bt-like colonies were detected. Phase contrast microscopy examination of these colonies after 48 hours of incubation in sporulation medium revealed that 80 of them showed crystal formation as well as spore formation, and these isolates were identified as Bt. (Figure 1).

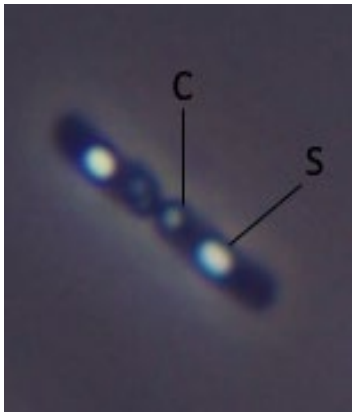


Figure 1. Spore-crystal formation of Bt isolates under a phase contrast microscope (1000×). (C) Crystal, (S) Spore.

Bt has been researched in the agricultural world for decades, and its insecticidal properties have been well known. In the last 25 years, the cytotoxic properties of this bacterium have also gained importance, and the parasporal proteins obtained from this bacterium are being investigated for their usability as anti-cancer agents. Isolation of native Bt isolates from different ecosystems in the world and characterization of genes are the first steps to achieve this goal [9, 13,14].

Bt has a wide distribution in nature. It can be isolated from many different ecosystems: soil, insects, stored grain residues, and plant leaves [19]; however, it has been proven by various studies that its main habitat has been soil [20]. The method that allows distinguishing Bt from other bacteria of the *B. cereus* group, to which it is quite similar in terms of biochemical properties and DNA homologies, is the observation of crystals produced with sporulation [19]. This method is generally based on the elimination of vegetative bacteria with high temperature and the selection of spore-crystal forming cells. In this study, 41 soil samples were examined for Bt isolation, and the Bt index was found to be 0.25. Likewise, Iriarte et al. [21] and Şahin et al. [22] found 0.22 and 0.24 Bt indexes from soil samples, respectively. On the other hand, there have been some studies showing Bt indexes much lower than our findings, such as 0.047 [23], or much higher, such as 0.62 [24]. The reason for different rates of Bt isolates obtained from soil may be due to the chemical properties of the soil. While some elements in the soil allow Bt reproduction, others may have the opposite effect [25].

#### 3.2. PCR Analysis of *cry* and *parasporin* Genes

In this study, the presence of *cry* and *parasporin* genes in native Bt isolates was determined by PCR. As a result of PCR analyses performed with *cry* primers, 66 isolates carried one or more of the *cry1*, *cry2*, and *cry3* genes, while 14 of the isolates did not contain any of them. The *cry1* gene, which produces a 277 bp product, was detected in 68% of the isolates. Thirty-five (44%) of the isolates were positive for the 690 bp *cry2* gene. The 590 bp *cry3* gene was detected in 32 isolates (40%). (Figure 2). The distribution of isolates carrying only a single *cry* gene or several *cry* genes together is shown in Figure 3.

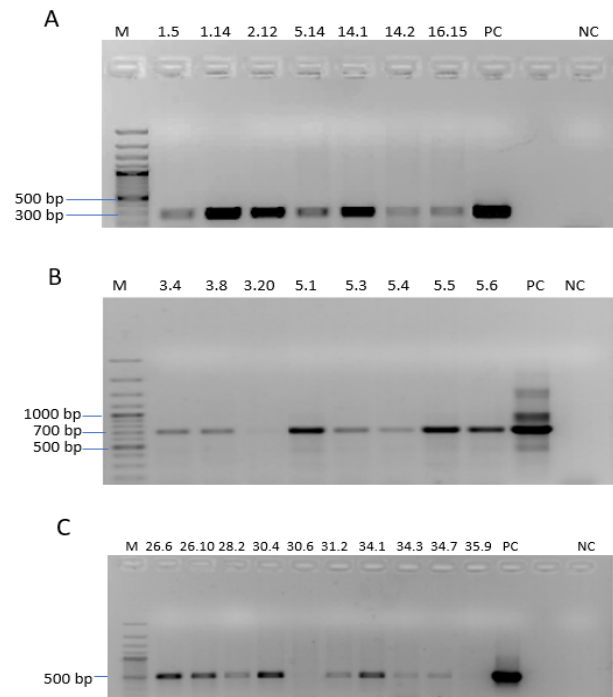


Figure 2. Agarose gel electrophoresis of PCR products of Bt isolates for (A) *cry1* gene, (B) *cry2* gene, (C) *cry3* gene. M: DNA Ladder, PC: Positive control, NC: Negative control.

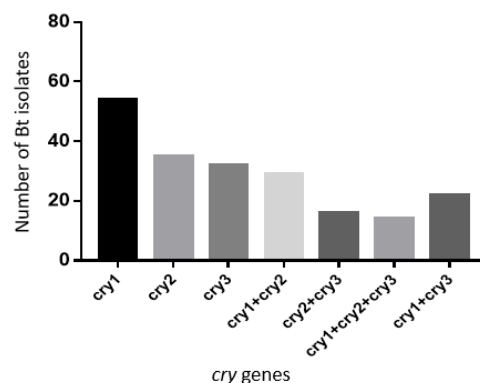


Figure 3. *cry* gene distributions of Bt isolates

In case of determining the presence of *parasporin* genes in Bt isolates, 7 primer pairs were used. Seventeen of 80 isolates were positive for one of the *parasporin* genes (Table 2). None of the isolates tested were positive with the *parasporin-4* primers.

**Table 2. Bt isolates positive for *parasporin* gene**

<i>parasporin</i> gene	Bt isolates
<i>ps1</i>	2.1
<i>ps2</i>	40.4
<i>ps3</i>	17.7, 19.2, 2.7, 2.12
<i>ps5-1</i>	1.9, 17.2, 30.4
<i>ps5-2</i>	13.10, 15.4, 15.8
<i>ps6</i>	5.4, 5.7, 5.10, 7.1, 7.3

PCR is a rapid and sensitive method for identifying new Bt isolates with crystal-producing genes [26]. Therefore, in this study, the presence of *cry* and *parasporin* genes in native Bt isolates was determined by PCR analyses. In the *cry* gene screenings, it was seen that Bt isolates contained *cry1*, *cry2*, and *cry3* genes at 68%, 44%, and 40%, respectively. It was not surprising that the highest frequency was found in the *cry1* gene, followed by the *cry2* gene, as reported in previous studies [24, 27]. The *cry3* gene, which showed different distributions such as 22.2% [28] and 62% [4], was found to be 40% in our study. In addition, some isolates were found to carry more than one *cry* gene. These isolates may have the potential to exhibit insecticidal activity against more than one order of agricultural pests.

Screening of *parasporin* genes is an important preliminary study to find out isolates with anti-cancer potential. Of the 80 Bt isolates screened, 21% were positive for one of the *parasporin* genes (1.25% for *ps1*, 1.25% for *ps2*, 5% for *ps3*, 0% for *ps4*, 3.75% for *ps5-1*, 3.75% for *ps5-2*, and 6.25% for *ps6*). Among these, *ps6* was found to be at the highest percentage, while *ps1* [29, 30] and *ps2* [31, 32] were present at the lowest percentage. The frequency of *parasporin* genes varies among studies [13, 18, 30-32]. Low percentages of *ps* genes, as in the case in our study, could be because of some limitations in determining parasporin classes by PCR. Due to the lack of identified *parasporin* genes in the literature, primers to be used in PCR are sometimes designed according to a single gene. This situation prevents the discovery of isolates that have not yet been identified and actually contain parasporin [13].

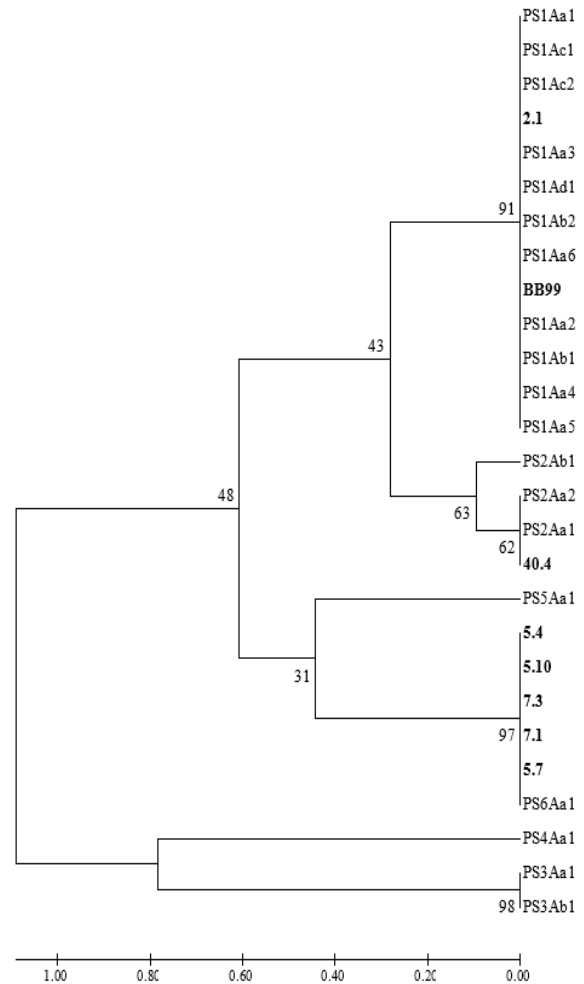
**3.1. Sequence Analysis and Phylogenetic Tree**

Partial sequence analysis was performed with PCR products of isolates that gave positive results in PCR analyses for *parasporin* genes. PCR products of isolates 5.4, 5.7, 5.10, 7.1, and 7.3 overlapped 99% with the *parasporin 6* gene reported in the literature. PCR products of 40.4 and 2.1 showed 97% and 98% identity with *parasporin2* and *parasporin1*, respectively (Table 3). Phylogenetic analyses were performed based on the comparison of the similarities of the DNA sequences obtained with the existing parasporins (Figure 4).

DNA sequence analysis of some isolates determined 97-99% identity with the known genes in the database. Phylogenetic analyses were performed based on partial DNA sequences; therefore, DNA sequence analysis performed after cloning all of the relevant genes will provide more descriptive information on whether they contain new genes.

**Table 3. Sequence similarity of Bt *parasporin* genes with matched genes in the database**

Isolate	Queried sequence (%)	Identity (%)	Relevant match
5.4	100	99	M019CP84/PS6
5.7	100	99	M019CP84/PS6
5.10	100	99	M019CP84/PS6
7.1	100	99	M019CP84/PS6
7.3	100	99	M019CP84/PS6
40.4	100	97	PS2
2.1	100	98	PS1



**Figure 4. Phylogenetic analysis of Bt isolates based on *parasporin* gene sequences. Phylogenetic analyses were performed using the MEGA 7 program, ClustalW and UPGMA methods with 1000 repetitions, and bootstrap values shown as percentages.**

In conclusion, this study presented a new Bt collection so that we could screen more isolates to find out new *cry* and *parasporin* genes and effective proteins against pests or cancer cells. In future studies, testing the parasporal proteins of our *cry* and *ps* gene-positive isolates in bioassays and cytotoxicity assays, respectively, will enable us to reveal their activities.

**Conflict of Interest**

There are no conflicts of interest in this work.

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## Ethical Approval Statement

The authors of this article declare that the materials and methods used in this study do not require ethics committee approval.

## Declaration of Generative AI

The authors did not use any generative AI or AI-assisted technologies in the preparation of this manuscript, including the data analysis and writing stages.

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