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Research Article

Diversity of coleopteran-specific *cry* genes of *Bacillus thuringiensis* strains isolated from soil of some east and south regions of Iran

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Abstract: Isolates were identified by molecular and morphological tests, including coleopteran-specific cry genes in the Iranian native Bacillus thuringiensis collection. Spherical and irregular shapes were observed to be the most frequent shapes using Coomassie brilliant blue staining. PCR analysis with universal and specific primer pairs was used to detect coleopteran-specific *cry* genes such as *cry1I*, *cry3*, *cry7*, *cry18*, and *cry26*. All the isolates contained at least one active coleopteran-*cry* gene, while the most abundant isolates had *cry26* and *cry18* genes. The patterns of protein size were characterized in addition to their insecticidal activity against third-instar larvae of *Tribolium castaneum*. Protein profiles produced bands that varied from 14-180 kDa. Four native isolates containing coleopteran-active *cry* genes displayed higher activity against *T. castaneum* larvae than *B. thuringiensis* subspecies *galleriae* as a reference strain. The median lethal concentration (LC_{50}) of the most pathogenic isolate, PS1078, was 2.72×10^6 spores/ml. Its *16S rDNA* gene sequence analysis demonstrated similarity to *B. thuringiensis*s subspecies *galleriae*. The characterization of isolates provided useful data for selecting new isolates to expand novel bio-insecticidal products.

Keywords: *Bacillus thuringiensis*, coleopteran-specific *cry* genes, *Tribolium castaneum*, *plasmid*, protein profiles

Introduction

Grains are an important dietary component of human food in many countries. As the demand for grains is rising along with the increase in the human population, food security requires global strategic actions (Poutanen, 2012). The annual yield loss caused by insect pests is estimated at around 20-40% in some countries, especially in tropical and subtropical conditions (Bergvinson and García-Lara, 2004; Upadhyay and Ahmad, 2011; Elgizawy and Ashry, 2019).

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), is one of the most common stored product insect pests which feed on grain. Both larvae and adult beetles prefer flour, milled products, and broken grains. However, they feed on dry fruits, beans, and seeds as well (Weston and Rattlingourd, 2000; Yilmaz *et al*., 2012; Taban *et al*., 2017). The yield losses of grain by red flour beetle are due to direct consumption and

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the contamination of the stored product with their feces, exuviae, and webbing, which lead to dissatisfaction and marketing problems.

Exclusive usage of synthetic insecticides in grain storage facilities resulted in residual effects, the appearance of resistant insect biotypes, and the inherent cost of application of conventional insecticides, especially for small producers (Pooja *et al*., 2013). Recently, there has been an increasing interest in developing alternative control and biological control agents due to the environmental issues of synthetic insecticides (Pérez-Guerrero *et al.*, 2011; Da Costa *et al*., 2014).

Bacillus thuringiensis (*Bt*) is a Gram-positive and spore-producing bacterium. It is an entomopathogenic agent that produces delta endotoxins and crystal proteins during sporulation (Anitha *et al*. 2011, Li *et al*. 2014). The crystal proteins of B. thuringiensis are mainly encoded in plasmid DNAs applied against different insect orders (Yilmaz *et al*., 2017). Extensive studies were conducted worldwide on the diversity of *B. thuringiensis cry* genes (Yilmaz *et al*., 2017; Doolotkeldieva *et al*., 2018; Carvalho *et al*., 2020; Domínguez-Arrizabalaga *et al*., 2020). Proteins of cry1I, cry3, cry6, cry7, cry8, cry18, and cry43 classes, as well as the binary toxins cry34A-cry35A, are toxic against coleopteran pests (Li *et al*., 2014; Elgizawy and Ashry, 2019). The results reported by several authors indicated the efficiency of new strains by novel *cry* genes to control the coleopteran pests (Yilmaz *et al*., 2017; Zorzetti *et al*., 2018; Rajchanuwong *et al*., 2019).

Some commercial *B. thuringiensis* formulations have been imported to Iran and evaluated in semi-field and field conditions. Due to the storage or environmental conditions and resistance of pests to these products (Gezelbash *et al*., 2014), further evaluations are needed. Characterizing the indigenous strains of *B. thuringiensis* will help overcome the problems mentioned above in *B. thuringiensis* formulations (Fakhrudin *et al*., 2003). The current study was conducted to isolate and characterize the native strains of *B. thuringiensis* from the soil of different regions of Iran based on morphological and molecular techniques to obtain coleopteran-specific *cry* genes. Moreover, the most pathogenic isolate was characterized and introduced as a highly effective and toxic *B. thuringiensis* strain.

Materials and Methods

Soil sample collection and isolation of *B.thuringiensis* isolates

Eleven soil samples were collected from agricultural and non-agricultural soil from seven provinces of Iran. The details of the collected samples are presented in Table 1. The samples were sieved using fine mesh, then packed and kept at 4 °C. The B. thuringiensis isolates were isolated according to the Travers et al. (1987) method. Briefly, 1 g of the sieved soil was added to the mixture of Luria Bertani culture medium (20 ml) and 0.25 M of sodium acetate and then incubated for 4 h at 30 °C at 200 rpm. The mixture was heated at 70 °C for 10 min. For sporulation, 50 µl of the solution was added to the T3 culture medium for seven days at 30 °C and 200 rpm, as explained by Anitha *et al*. (2011). *B. thuringiensis* subspecies *morrisoni* (*Btm*) and *B. thuringiensis* subspecies *galleriae* (*Btg*) were used as reference strains prepared by the Iranian Research Organization for Science and Technology, Tehran, Iran (IROS).

Morphological characterization of *B. thuringiensis* isolates

B. thuringiensis endospores and spore-crystals were characterized based on morphological tests such as Gram staining and Coomassie brilliant blue under phase-contrast microscopy (Zeiss Axioplan 2, Germany). Moreover, the spores-crystals of indigenous and reference strains of *B. thuringiensis* were purified (Yilmaz *et al*., 2012). Then, the pelleted spore-crystal mixtures were freeze-dried using a freeze-drier (Edwards Freeze-Dryer, UK) according to the manufacturer's instructions and kept at 4 °C for further use. Scanning electron microscopy (SEM) was used to visualize the freeze-dried spores-crystals of the PS1078 isolate (the highest pathogenic strain) at the 15 kV beam.

Table 1 Sampling sites and Crystal forms of *Bacillus thuringiensis* isolates from soil samples collected in Iran.

Isolates	Location	Coordinates	Source	Crystal form
PS1053	Razavi-Khorasan: Chenaran	36˚37'20"N 59˚13'12" E	Pear	spherical-ovoid
1055	Kordestan: Saqqez	36˚15'02"N 46˚16'53" E	Urban	cuboidal-irregular- spherical
1062	Kerman: Rafsanjan	30º22'49"N 55º00'40" E	Urban-no vegetation	spherical- irregular
PS1066	South-Khorasan: Birjand	32˚52'5"N 59˚07'20" E	Pomegranate-berry	spherical- irregular
PS1068	Fars: Sepidan	30°01'23"N 52°08'31" E	Pine tree	bipyramidal-ovoid-cuboidal- diamond
1074	Kerman: Ravar	31˚14'10"N 56˚47'24" E	Levant wormseed	irregular– bipyramidal- spherical
PS1078	Hormozgan: Haji Abad	28º19'05"N 55º53'40" E	Orange	bipyramidal- ovoid- spherical
1080	Razavi-Khorasan: Chenaran	36˚65'52"N 59˚09'14" E	Apple	bipyramidal– diamond- irregular
1084	Kohgiluyeh and Boyer-Ahmad: Yasouj	30°39'10"N 51°36'15" E	Plane tree	spherical- irregular
1087	Razavi-Khorasan: Akhlamad	36˚65'82"N 59˚00'59" E	Rangeland	cuboidal-irregular- spherical
PS1090	Razavi-Khorasan: Chenaran	36˚37'07" N59˚09'21" E	Apple	irregular- bipyramidal

Plasmid DNA extraction

Plasmid DNA has been extracted for screening coleopteran-specific *cry* and *16S rDNA* genes. For plasmid DNA extraction, after growing *B. thuringiensis* isolates on the 10 ml LB culture medium for 20 h at 37 °C, they were centrifuged for 10 min, then the supernatant was removed. Subsequently, plasmid DNA was extracted according to the plasmid DNA extraction Mini Kit (Favor Prep™, Taiwaninstruction. Plasmid profiles were obtained by running 3 µl of the total undigested plasmid DNA on a 0.5% agarose gel (YTA, Iran) containing the safe dye nucleic acid staining solution (Gel Red™ Nucleic acid stain, Biolium Company, US) and visualized in a gel documentation system (Syngene, England) as described by Guneş *et al*. (2016).

PCR amplification

To detect the presence of coleopteran-specific *cry* and *16S rDNA* genes, polymerase chain reaction (PCR) assay was conducted using 2 µL of the template DNA, 12.5 µl of Super PCR MasterMix (YTA, Iran), 2 µL of each primer (10 µM) (Table 2), and 8.5 µl of distilled water at the total reaction volume of 25 µL. PCR details and annealing temperature used to amplify coleopteran-specific *cry* genes using specific primers are given in Table 2. The PCR amplification of coleopteran-specific *cry* genes, excluding UNcry18 and SPcry26 primers, was performed as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 5 min. In the case of UNcry18 and SPcry26 primers, the PCR procedure was as follows: the initial denaturation at 95 °C for 3 min; five cycles of denaturation (94 °C, 1 min), annealing (five degrees above the annealing temperature, 20 s), and elongation (72 °C, 20 s); 30 cycles of denaturation (94 °C, 1 min), annealing (annealing temperature, 20 s), elongation (72 °C, 20 s), and final extension at 72 °C for 5 min. PCR products were subjected to electrophoresis using 1% agarose gel containing safe dye nucleic acid staining solution (Guneş *et al*., 2016).

Sequencing and molecular phylogenetic analysis of *16S rDNA* gene

The PCR amplicon of the *16S rDNA* gene for the most toxic *B. thuringiensis* strain, PS1078, was purified and bi-directionally sequenced at Beijing Genomics Institute (Beijing, China). The forward and reverse nucleotide sequences representing the specimens were assembled, edited, and manually aligned using BioEdit7.1. Then, the amplicon sequence was submitted to GenBank with accession number MW644646. The 18 sequences from NCBI with more identity to PS1078 were applied, and *Escherichia coli* was used as an outgroup. The chosen sequences from NCBI and PS1078 were aligned by using MAFFTv.7 (Katoh and Standley, 2013), and then the result was adjusted manually by Mesquite v. 3.10 (Maddison and Maddison, 2015). Then, they were aligned for phylogenic analyses following the maximum likelihood method. The IQ-TREE (Nguyen *et al*., 2015) was used to create a reconstructed tree, and the phylogenetic relationship was analyzed among aligned sequences. Finally, the tree was visualized using Figtree v.1.4. (http://tree.bio.ed.ac.uk/software/figtree/).

Table 2 The universal and specific primers used in PCR to detect Coleopteran-specific *cry* genes.

Primers		Sequence (5' ------ 3')	Melting temperature (°C)	Reference
UNcry18	F	CCGAGGCGATTTGGATAGAT	58	Nazarian *et al*. (2009)
	R	TGCCGGTGTAAACAAAGAAGG	59	
SPcry26	F	CGCGCTGTTCAATTATCAAGTGC	63	Nazarian *et al*. (2009)
	R	ATATGGAAAGAAAAGGCGTGTGGA	62	
SPcry1I	F	ACAATTTACAGCTTATTAAG	48	Nazarian *et al*. (2009)
	R	CTACATGTTACGCTCAATAT	52	
COL1	F	GTCCGCTGTATATTCAGGTG	58	Saadaoui *et al*. (2010)
	R	CACTTAATCCTGTGACGCCT	58	
Spe-cry8B	F	ATGAGTCCAAATAATCTAAATG	53	Bravo *et al.* (1998)
	R	GAACATCTCGTAAGGCTC	54	
Un7	F	TGATCCAGCAACTATAACACGAGTGATAGA	68	Baig and Mehnaz, (2010)
	R	CACTTAACGCAATGGTTAGAATATTCAGAG	68	
Set 1	F	TATGCWCAAGCWGCCAATYTWCATYT	63	Noguera and Ibarra, (2010)
	R	GGRATAAATTCAATTYKRTCWA	53	
Set 2	F	TTTAGATATTGTTGCAWTATKKYC	56	Noguera and Ibarra, (2010)
	R	GGRATAAATTCAATTYKRTCWA	53	
Set 3	F	TATGCWCAAGCWGCCAATYTWCATYT	63	Noguera and Ibarra, (2010)
	R	CATAACGTAGWYTTAYCTKAWT	54	
16S rDNA	F	AGAGTTTGATCCTGGCTCAG	60	Yılmaz *et al*. (2012)
	R	CAAGGCATCCACCGT	53	

Protein profiles of *B. thuringiensis* isolates

The spore-crystals of all native isolates and reference strains were subjected to SDS electrophoresis gel as described by Sambrook *et al*. (1989) and Yilmaz *et al.* (2012).

Insect colony

The original population of red flour beetle was prepared from the Graduate University of Advanced Technology, Kerman, Iran. To obtain the same aged colony, adults of *T.castaneum* were separated and released inside the oviposition plastic containers with a two-liter capacity containing a sterilized mixture of 90% wheat flour and 10% yeast extract as a diet. The insects were maintained under controlled conditions (27 ± 1 °C, 60 ± 5% RH, and L: D, 16: 8 h) (Rizwana, 2014).

Toxicity survey of *B. thuringiensis* isolates against *T. castaneum*

To estimate the median lethal (LC_{50}) concentration, four *B. thuringiensis* isolates (1055, PS1066, PS1078, 1080, and *B. thuringiensis* subspecies *galleriae* as the reference strain) were selected from among the 11 isolates mentioned above due to their most pathogenicity. These isolates were cultured for seven days at 30 °C on a nutrient agar (NA) medium; then, the surface of the medium was scraped to make an aqueous suspension containing Tween 80 (0.02%). Next, the serial dilution of each isolate was prepared. One ml of the spore-crystal suspension from each isolate concentration was mixed with 1 g of wheat flour as a diet. Afterward, 15 third instar larvae were transferred into a sterile Petri dish containing the diet. The bioassay was performed in three replicates, and mortality was recorded after 14 days. Distilled water containing 0.02% Tween 80 was used as the control (Saadaoui *et al*., 2010; Khodabandeh *et al*., 2014; Guneş *et al*., 2016). Probit analysis was performed to estimate the LC_{50} value, and the statistical analyses were conducted in POLO-PC (2002).

Results

Morphological characterization

The morphology of *B. thuringiensis* spore-crystals was visualized using Gram and Coomassie brilliant blue staining under phase-contrast microscopy. *B. thuringiensis* isolates were gram-positive and rod-shaped. Our results revealed the isolates of irregular and spherical shapes had the highest frequency (16.36%), followed by the isolates comprising spherical

and irregular-pyramidal shapes (Table 1). Scanning electron microscopy of the PS1078 strain as the most toxic isolate (based on the results of bioassay and toxicity tests), included bipyramidal, ovoid, and spherical parasporal inclusions. This result is similar to morphological characterization using Gram staining and Coomassie brilliant blue (Fig. 1).

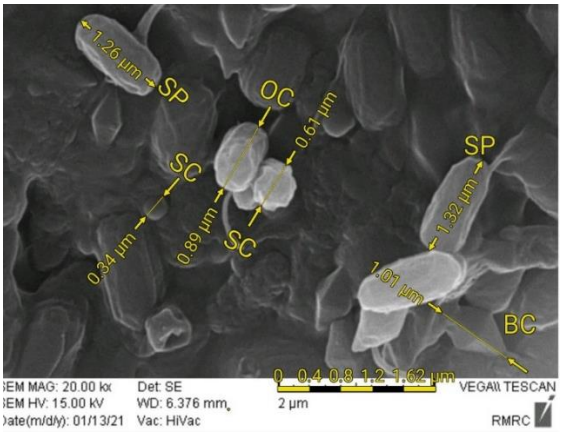

Figure 1 Scanning electron microscopy (SEM) of *Bacillus thuringiensis* PS1078 isolate. The description of the crystal-spore is as follows: SP: Spore, SC: Spherical Crystal, BC: Bipyramidal Crystal, and OC: Ovoid Crystal.

The plasmid DNA profile of *B. thuringiensis* isolates

The *B. thuringiensis* strains were further characterized based on the variations in the number and molecular weights of the plasmid components. Only one native isolate, 1055, matched the pattern of the *B. thuringiensis* subspecies *morrisoni* reference strain. However, its pattern was different from that of *B. thuringiensis* subspecies *galleriae*. The other isolates exhibited a distinct pattern of the reference bacteria. Each isolate included 2 to 10 plasmids with varying molecular weights, and the plasmid sizes ranged from 4 to 20 kb (Fig. 2). Most strains shared some bands with the *B. thuringiensis* subspecies *galleriae*. Furthermore, except for 1080 and 1087, all the reference and native isolates exhibited the chromosomal DNA band (20 kb); there was a clear band as a megaplasmid for 1062, PS1066, 1074, PS1078, and the 1084 isolates above the chromosomal band. No plasmid was extracted from the 1051 isolate.

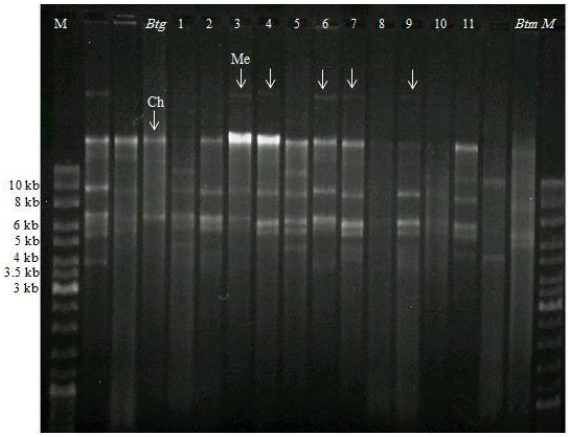

Figure 2 Plasmid patterns of native isolates of *Bacillus thuringiensis*, with 0.5% agarose gel. Columns 1 to 11, native isolate (PS1053, 1055, 1062, PS1066, PS1068, 1074, PS1078, 1080, 1084, 1087, and PS1090, respectively). *B. thuringiensis* subspecies *galleriae* (*Btg*), and subspecies *morrisoni* (*Btm*) as positive control. Me: Megaplasmid. Ch: Chromosomal band and. M: Molecular weight marker (10 kb).

PCR amplification

The plasmid genes of the aforementioned isolates were characterized using coleopteran-specific *cry* and *16S rDNA* genes and reference strains, *B. thuringiensis* subspecies *galleriae*, in which two specific *cry* genes, *cry18* and *cry26*, were determined in most of *B. thuringiensis* isolates (Fig. 3). The results indicated that seven isolates (PS1053, 1055, PS1066, PS1068, PS1078, 1080 and 1084) produced amplicon with the UNcry18 primer pairs, and PS1053, 1055, PS1066, PS1078, and 1084 were only similar to *B. thuringiensis* subspecies *galleriae* (Fig. 3, A). Although, four isolates (1062, 1074, 1087, and PS1090) did not produce any amplicon with the UNcry18 primer pairs. The acceptable band for the *cry26* gene was visible in the two isolates, 1062 and PS1066 (Fig. 3, B). Only one isolate, PS1090, contained none of the coleopteran-specific genes.

Protein profiles of *B. thuringiensis* isolates

The protein profiles of *B. thuringiensis* isolates were visualized using SDS-PAGE analysis in the range of 14 to 180 kDa (Fig. 4). The density of protein bands was high, between 72 to 180 kDa. Two isolates, PS1090 and 1087, showed a band with 10 kDa. The reference strain *B. thuringiensis* subspecies *galleriae* displayed different protein patterns around 20 to 130 kDa.

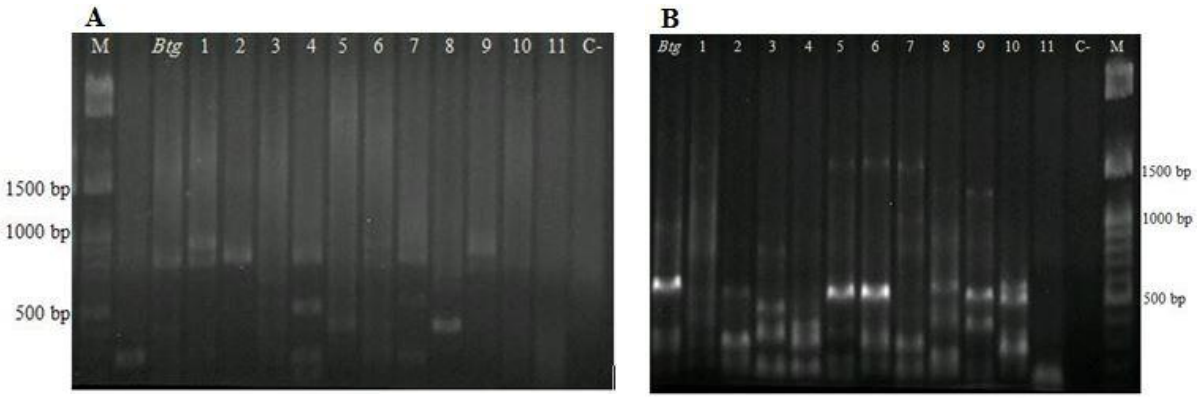

Figure 3 PCR detection of *cry18* (A) and *cry26* (B) genes in native isolates of *Bacillus thuringiensis* using agarose gel electrophoresis. Columns 1 to 11, native isolate (PS1053, 1055, 1062, PS1066, PS1068, 1074, PS1078, 1080, 1084, 1087, and PS1090, respectively). M: molecular weight marker, *B. thuringiensis* subspecies *galleriae* (*Btg*): reference strains, C-: negative control.

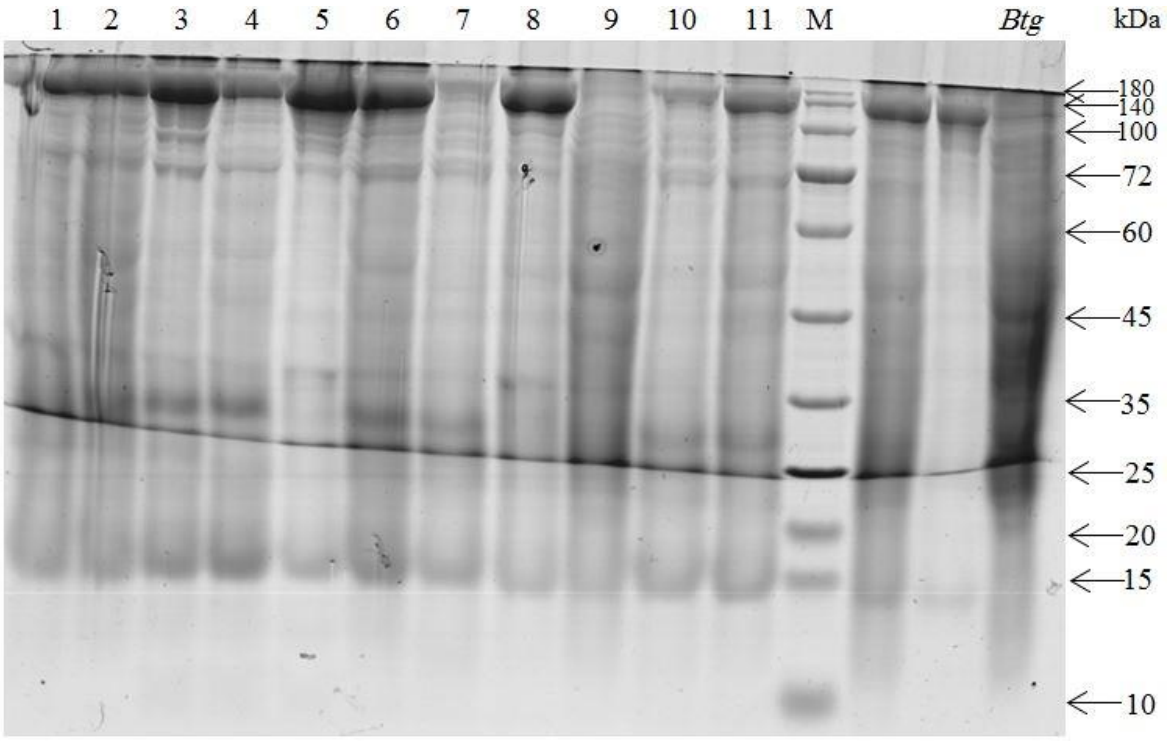

Figure 4 SDS-PAGE protein profile of native *Bacillus thuringiensis* mixture spore-crystal and the reference strain (*B. thuringiensis* subspecies *galleriae* (*Btg*)). Columns 1-11 were defined as PS1053, 1055, 1062, PS1066, PS1068, 1074, PS1078, 1080, 1084, 1087, and PS1090 isolates, respectively.

Toxicity survey of *B. thuringiensis* isolates against *T. castaneum*

The bioassays against the third instar larvae of *T. castaneum* revealed differences among the isolates (Table 3). According to the LC_{50} values, the PS1078 isolate was the most toxic against larvae compared to other native and reference strains. The other isolates showed similar toxicity to the *B. thuringiensis* subspecies *galleriae* as *a* reference strain against the *T. castaneum* larvae.

Sequencing and molecolar phylogenetic analysis of *16S rDNA* gene

The *16S rDNA* fragment of the PS1078 isolate was amplified by PCR and sequenced using the *16S rDNA* primers pair. According to the NCBI database, the BLAST results of the PS1078 sequence showed more than 99% identity to all used *B. thuringiensis* strains. A maximum-likelihood phylogenetic tree based on the PS1078 sequence was reconstructed to distinguish the phylogenetic relationship between the studied isolate and the other 18 *B. thuringiensis* strains. The IQ-TREE study demonstrated the similarity between the isolate PS1078 and the *B. thuringiensis* subspecies *galleriae* (Fig. 5).

Table 3 Median lethal concentration (LC_{50}) of some *Bacillus thuringiensis* isolates against third instar larvae of *T. castaneum* after 14 days.

Isolates	LC_{50} (spores/ml)	95% FL		x^2	Heterogeneity
		lower	upper		
1055	5.03×10^7	1.56×10^7	2.41×10^8	1.46	0.49
PS1066	1.19×10^8	3.78×10^7	6.69×10^8	0.83	0.28
PS1078	2.72×10^6	2.00×10^5	1.50×10^7	4.18	1.39
1080	2.20×10^7	4.47×10^6	1.47×10^8	3.58	1.19
Btg	5.48×10^7	2.23×10^7	1.69×10^8	1.37	0.46

FL: fiducial limit; x^2: chi-square.

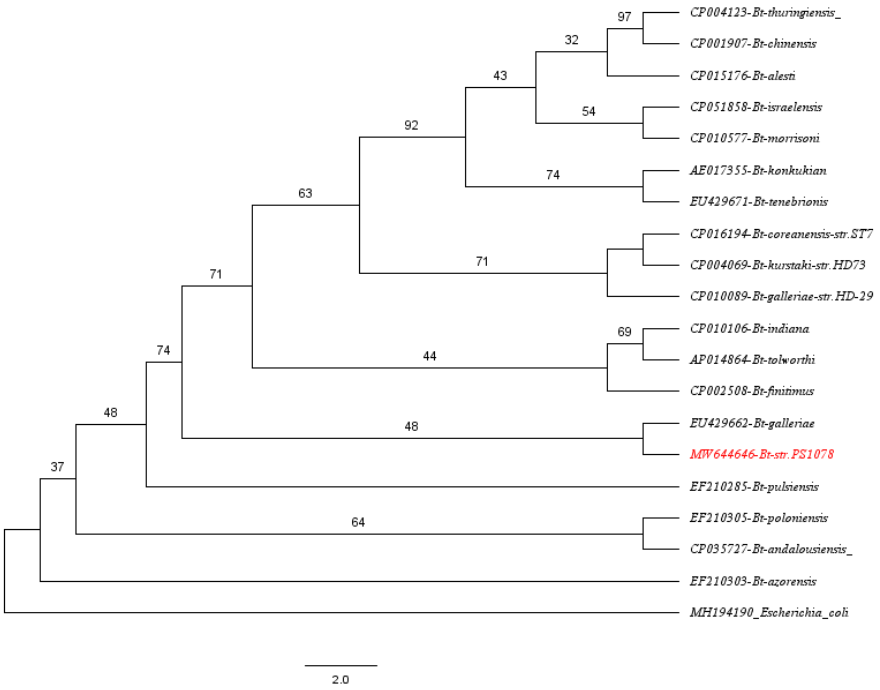

Figure 5 Maximum-likelihood phylogenetic tree reconstruction performed with IQ-TREE according to the *16S rDNA* gene of the most toxic isolates, PS1078.

Discussion

B. thuringiensis isolates could be candidates for biocontrol (Renganathanbr *et al*., 2011). Isolation and characterization of crystal protein genes could help predict the pathogenicity of *B. thuringiensis* isolates in different orders of insects (Konecka *et al*., 2012). In the present research, morphological, molecular survey, and bioassay were performed to characterize the native isolates and the most toxic isolate of *B. thuringiensis* from the soil in Iran. The results revealed that the native isolates containing the irregular and spherical shape had the highest frequency (16.36%), and electron microscopy analysis revealed that the most pathogenic isolate, PS1078, contained bipyramidal, ovoid, and spherical crystals. In comparison to those of Cinar *et al*. (2008) who presented that 19% of native isolates had a spherical and irregular pointed form. Also, Zorzetti *et al*. (2018) exhibited that all isolates toxic to *Hypothenemus hampei* Ferrari, 1876 (Coleoptera: Curculionidae: Scolytinae) had spherical crystals.

Analysis of the plasmid profile is another way of describing *B. thuringiensis* isolates and *cry* genes are found mostly on plasmids of various sizes (4–150 MDa) (Valicente and da Silva, 2017). Our results detected 10 specific plasmid profiles. Each strain appears to have a typical plasmid profile. Similarly, Seifinejad *et al*. (2008) demonstrated 12 distinct plasmid profiles. In the current investigation, only one native isolate, 1055, perfectly matched the pattern of the *B. thuringiensis* subspecies *morrisoni* as the reference strain. This study reported a plasmid with a molecular weight of 16 kb in two reference strains and all the native isolates except for 1080, 1087, and PS1090. Fagundes *et al.* (2011) reported *B. thuringiensis* subspecies *galleriae* with six plasmids of 4.3 to 23 kb and one megaplasmid similar to two strains, 348L and 462A from the United States. However, studies emphasized the plasmids that moved below the chromosomal DNA band; the megaplasmids were a backup option for identifying *B. thuringiensis* isolates *(*Fagundes *et al*., 2011). In agreement with Fagundes *et al*. (2011), in the present study, the chromosomal DNA band was observed in all native isolates except for 1080 and 1087. Nevertheless, the megaplasmids migrated only in 1062, PS1066, 1074, PS1078, and 1084 isolates.

The PCR technique was used to examine the existence of coleopteran *cry* genes using nine specific primers. The PCR analysis indicated two *cry18* and *cry26* genes in most isolates. In this study, 36.36% of the isolates were similar to *B. thuringiensis* subspecies *galleriae*. Five isolates, including PS1053, 1055, PS1066, PS1078, and 1084 contained the *cry18* gene yielding the PCR product around 750 bp, and for two isolates, PS1068 and 1080, the related amplicon produced around 419 bp. Similarly, Nazarian *et al*. (2009) found two products (419 and 750 bp) called *cry18*. Rajashekhar *et al*. (2018) detected the bands of 492 and 594 bp for *cry18*. Similarly, these two bands were observed in the PS1066 and PS1078 isolates.

Moreover, the *cry26* gene was detected in two isolates, 1062 and PS1066, like the study corresponding to Nazarian *et al*. (2009), which demonstrated a band of 362 bp. Yu *et al*. (2015) observed genetic polymorphisms in isolates by several genotypes such as *cry1*, *cry3*, *cry9*, *cry18*, and *cry26*, and they found that strains carrying *cry26* were more prevalent than *cry18* (26.23 and 2.5%, respectively). It was in disagreement with our results that showed *cry18* was more widespread than *cry26* (63 and 18%, respectively).

SDS-PAGE analysis is usually used to compare the protein profiles of *B. thuringiensis* isolates. The B. thuringiensis isolates protein profile analysis showed that the banding patterns ranged from 14 to 180 kDa. Nazarian *et al*. (2009) observed protein bands with molecular weights between 20 and 135 kDa. The expressed protein profiles were almost similar in all the strains. Haggag and Yousef (2010) categorized the expressed proteins into three main groups: group I (14-45 kDa), group II (45-100 kDa), and group III (> 100 kDa). Furthermore, in our study, there were three main categories for protein profile, including 14-35 kDa, 35-72 kDa, and 72-

180 kDa. The previous research demonstrated that the molecular mass of cry34 and cry35 were from 14 to 44 kDa (da Silva *et al*., 2010; Mukhija and Khanna, 2018). This range of protein band was also observed in the present research. Therefore, the presence of the encoded genes should be demonstrated. Gorashi *et al*.(2014) showed that the most toxic Sudanese isolate was St-6, which possessed the *cry1* gene as the Coleopteran active gene. The PS1078 isolate was the most effective isolate due to the lowest LC_{50} values (2.72×10^6 spores/ml) and without the *cry1* gene. Furthermore, according to Elgizawy and Ashry (2019), the most toxic protein (cry3Aa) against the third instar larvae of the red flour beetle had the lowest LC_{50}. But, the protein of the *cry3* was not detected in our findings.

In another study, the lowest LC_{50} of strain BR58 for first instar larvae of *H. hampei* was assessed as 0.037×10^9 spores/ml (Zorzetti *et al.*, 2018). However, the LC_{50} of PS1078 was much lower than BR58. This discrepancy in the toxicity results may be due to the type of the *cry* genes and their expression in the protein level, the sensitivity of target insects, variations in the temporal and spatial conditions, strain preparation, and the bioassay method (Mohan and Gujar, 2002; Carrière *et al*., 2004; Guneş *et al*., 2016).

Previous investigations employed *16S rDNA* gene analysis to identify native *B. thuringiensis* isolates (Yilmaz *et al.*, 2012; El-Kersh *et al*., 2016; Banik *et al*., 2019). This study adopted the same procedure to determine the phylogenetic connections between native isolates and reference strains. The sequence analysis of the most pathogenic isolate, PS1078, revealed more than 99% identity with all the *B. thuringiensis* strains utilized. Cinar *et al*. (2008) created *B. thuringiensis* dendrogram with 11 distinct phylogenetic groups and elicited that the largest group shared 80% DNA. The *16S rDNA* gene sequence analysis of PS1078 demonstrated its similarity to *B. thuringiensis* subspecies *galleriae*. Likewise, Yilmaz *et al*. (2012) displayed a high level of identity (98%) between the Turkish strain, SY49.1, and the reference strains *B. thuringiensis* subspecies *andalousiensis* and *B. thuringiensis* subspecies *monterrey*. El-Kersh *et al*. (2016) analyzed the BLAST of 24 examined isolates from Saudi Arabia and revealed they are highly homologous to the *B. thuringiensis* genome (99.5%).

The bipyramidal and spherical shape of *B. thuringiensis* crystal proteins had molecular mass proteins with molecular weights of 65-130-145 and 44 kDa, respectively, and could be toxic against coleopterans (Itoua-Apoyolo *et al*., 1995; Schnepf *et al.*, 1998; Arrieta *et al*., 2004; Monnerat *et al*., 2007; da Silva *et al*., 2010). Similarly, the native isolates PS1068, 1074, and 1080 produced proteins with a molecular weight of around 130 kDa and created a bipyramidal shape. While two isolates, PS1078 and PS1090, produced bipyramidal crystals but did not show 70 or 130 kDa bands. Four native isolates, PS1053, 1074, and 1084 showed spherical crystal proteins and exhibited 44 kDa, protein bands. However, the three toxic isolates, PS1055, PS1066, and PS1078 harboring two coleopteran-specific *cry* genes, *cry18* and *cry26*, showed spherical crystal protein and exhibited important coleopteran protein bands of 14, 29, 44, 73, 76, and 130 kDa. Arrieta *et al*. (2004) reported some different cry proteins that could be toxic against coleopterans, such as cry3 (73 kDa), cry7 (130 kDa), cry8 (130 kDa), cry1B (140 kDa), cry34 (14 kDa), cry37 (14 kDa), cry23 (29 kDa), cry22 (76 kDa) and cry35 (44 kDa). In contrast, the PS1090 isolate had irregular and bipyramidal crystal proteins and exhibited 14, 30, 70, and 140 kDa protein bands but possessed none of the coleopteran-specific genes and demonstrated the lowest toxicity on *T. castaneum*.

The most pathogenic isolate, PS1078, containing *cry18* and *cry26* genes encoded bipyramidal, ovoid, and spherical crystal protein bands of 29, 70, 73, 76, and 140 kDa, showed efficient toxicity against the coleopteran pest. Similar to the present study, Yu *et al*. (2015) discovered that *B. thuringiensis* isolates with *cry18* genotypes were efficient against Coleoptera.

Conclusion

In the present study, the molecular and morphological characterization and efficiency of some native isolates matched the presence of some *cry* genes encoded toxic genes against coleopteran pests. This is significant since novel toxins with more toxicity for new targets can be found. These native isolates are suggested for application as gene sources for constructing transgenic plants.

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تنوع ژن‌های cry اختصاصی سخت‌بالپوشان سویه‌های *Bacillus thuringiensis* جداشده از خاک برخی مناطق شرق و جنوب ایران

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چکیده: جدایه‌های ایرانی *Bacillus thuringiensis* جمع‌آوری و با روش‌های مولکولی و مرفولوژیکی شناسایی و ژن‌های اختصاصی سخت‌بالپوشان در آن‌ها بررسی شدند. با استفاده رنگ‌آمیزی کوماسی بلو، اشکال کروی و فاقد شکل خاص بیش از سایر اشکال مشاهده شدند. روش PCR با استفاده از آغازگرهای عمومی و اختصاصی برای شناسایی برخی ژن‌های اختصاصی سخت‌بالپوشان از جمله *cry1I*، *cry3*، *cry7*، *cry18* و *cry26* به‌کار گرفته شد. تمام جدایه‌ها حداقل یک ژن فعال اختصاصی سخت‌بالپوشان را نشان دادند درحالی‌که بیشترین فراوانی مربوط به ژن‌های *cry26* و *cry18* بود. علاوه بر توانایی حشره‌کشی جدایه‌ها در برابر لاروهای سن سوم شپشه آرد *Tribolium castaneum*، الگوی اندازه پروتئین‌ها نیز مطالعه شد. الگوی پروتئینی، باندهای متنوعی در محدوده ۱۴ تا ۱۸۰ کیلودالتون نشان داد که برای سخت‌بالپوشان کشنده بودند. چهار جدایه بومی دارای ژن‌های اختصاصی سخت‌بالپوشان تأثیر حشره‌کشی بیشتری در مقایسه با سویه مرجع *B. thuringiensis* subspecies *galleriae* نشان دادند. میانگین غلظت‌کشنده (LC_{50}) برای کشنده‌ترین جدایه، PS1078، ۱۰^۶ × ۲/۷۲ اسپور/میلی‌لیتر بود. بررسی توالی ژن *16S rDNA* این جدایه، شباهت آن با باکتری *B. thuringiensis* subspecies *galleriae* را نشان داد. خصوصیات جدایه‌ها اطلاعات مفیدی در انتخاب جدایه‌های جدید برای بهبود و گسترش حشره‌کش‌های بیولوژیک جدید ارائه کرد.

واژگان کلیدی: *Bacillus thuringiensis*، ژن‌های اختصاصی سخت‌بالپوشان، *Tribolium castaneum*، پلاسمید، الگوی پروتئین