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ORIGINAL PAPER

Distribution and diversity of Dipteran-specific *cry* and *cyt* genes in native *Bacillus thuringiensis* strains obtained from different ecosystems of Iran

Gholamreza Salehi Jouzani · Ali Pourjan Abad · Ali Seifinejad · Rasoul Marzban · Khalil Kariman · Bahram Maleki

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Abstract One hundred and twenty-eight *Bacillus thuringiensis* isolates from fields of different ecological regions of Iran were collected to study the distribution and diversity of Dipteran-specific *cry* and *cyt* genes. The percentage of samples with *Bt* showed significant differences between different regions and also between different fields. The most *Bt* frequency was observed in the soil samples collected from Caspianic zone (7%) and soils of cotton (17%). Characterization of isolates was based on morphological characteristics of crystals, plasmid profiles and protein band patterns as well as PCR analysis using general and specific primers for 22 different *cry* and *cyt* genes encoding proteins active against mosquitoes. Thirty-eight different *cry* gene profiles were detected in this collection. Several of them were found to be different from all previously published profiles and none of the previous researches reported these numbers of profiles. Strains containing *cry2*-type genes were the most abundant and represent 57.1% of the isolates. Strains harboring *cry24* and *cry10* genes were also highly abundant (38.7 and 32.8%, respectively). *cry11*, *cry4*, *cry17*, *cry19*, *cry21*, *cry29*, *cyt1*, and *cry9* genes were less abundant, found in 25.7, 14.3, 11.4, 1.4, 4.3, 1.4, and 10% of the strains, respectively. Among the *cry2* gene containing isolates, 37.5% strains harbored *cry2Aa*, 55% *cry2Ab*, 2.5% *cry2Ac*, and 5% other or novel *cry2*-type genes. Among the *cry4* gene containing isolates, 0% strains harbored *cry4A*, 60% *cry4B*, and 40% *cry4C*, *cry4D* or novel *cry4* type genes. Finally, based on crystal morphology, protein patterns and PCR, 21 strains were selected as potentially high Dipteran-active for bioassays. Also our results showed that some of the isolates may harbor minimum a putative novel *cry* gene.

Keywords *Bacillus thuringiensis* · Diptera · Molecular identification · *cry* genes · *cyt* genes

G. Salehi Jouzani (✉) · K. Kariman
Department of Microbial Biotechnology and Biosafety,
Agricultural Biotechnology Research Institute of Iran (ABRII),
Mahdasht Road, 21525-1897, Karaj, Iran
e-mail: gsalehi@abrii.ac.ir

A. Pourjan Abad · B. Maleki
Department of Biotechnology,
College of Agriculture, Zanjan University,
Zanjan, Iran

A. Seifinejad
Department of Agronomy and Plant Breeding,
College University of Agriculture Natural Resource,
University of Tehran, Tehran, Iran

R. Marzban
Department of Biological Control,
Plant Protection Institute of Iran, Tehran, Iran

Introduction

Over half a century synthetic pesticide applications have led to the emergence of resistance to agricultural pests and vectors of human diseases. The properties that made these chemicals useful—long residual action and toxicity to a wide spectrum of organisms—have brought serious problems [25, 38]. An urgent need has thus emerged for environment-friendly pesticides to reduce contamination and the likelihood of insect resistance [24, 33, 38].

Bacillus thuringiensis (*Bt*) is a gram-positive bacterium that is characterized by the production of insecticidal crystal proteins (Cry proteins or δ-endotoxins that are encoded by *cry* genes). *Bt* has been used as a successful biological

insecticide for more than 40 years and is a uniquely specific, safe, and effective tool for the control of a wide variety of insect pests [7, 28]. Cry toxins constitute a family of related proteins that can kill insects belonging to the Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, and Mallophaga, as well as some invertebrates [5, 15, 34]. These toxins can be grouped according to the degree of amino acid homology. The genes have been classified as *cry1−51*, *cyt1* and *cyt2*, and are ranked according to their homology.

The incorporation of *cry* genes into major crops, producing insect-resistant plants, is the most recent application of *Bt* in agriculture [28, 33]. Intensive screening programs leading to important collections of isolates have been conducted in the last few decades [10, 14, 17, 26, 37]. The need for novel Cry proteins with toxic potential against different organisms with specificity for a much broader range of pests or to provide alternatives after the appearance of insect resistance has resulted in a continuous search for new experimental approaches in order to expand the host ranges of the strains available [11, 12].

The number of known *Bt* strains active on Diptera is increasing. A feature of all mosquitocidal strains is the presence of *Cyt* toxin, which is not very toxic by itself. The mosquitocidal activity of a *Bt* strain is not only the additive effect of each toxin but a complex synergistic interaction among them [2, 31]. *Bt* subsp. *israelensis* produces four Cry toxins (Cry4Aa, Cry4Ba, Cry10Aa, and Cry11Aa) and two Cyt toxins (Cyt1Aa and Cyt2Ba) [18], and synergism between these toxins has been reported [9, 42]. Interestingly, the presence of the Cry toxin delays or prevents the development of resistance to Cry toxins. *Bt* subsp. *israelensis* has been used in the fields for nearly 20 years with no development of insect resistance [30]. The search for native strains with activity against Dipteran species especially plant pests could have an impact on the control of mosquitoes worldwide.

Iran is the 17th-largest country in the world, and its area roughly equals the size of the United Kingdom, France, Spain, and Germany combined. Iran with its broad territory, unique and rich biodiversities, can be divided into three main geographic and climatic regions [Caspianic (temperate and humid), moderate or cold mountainous, and dry and semidry]. In this study, we characterized the native *Bt* strains isolated from different regions of Iran, and their Dipteran-active *cry* and *cyt* genes contents. Characterization included the identification of mosquitocidal *cry* and *cyt* genes (22 *cry* and *cyt* genes) by PCR analysis with general and specific primers, plasmid patterns, crystal protein band patterns and crystal morphology of each isolate. *Bt* strains containing some of the previously described *cry* genes as well as potentially novel Cry proteins were identified.

Materials and methods

Sample collection and bacterial isolation

About 200 g of soil sample scraped from a depth of 10 cm with a sterile spatula was collected randomly from agricultural lands in 28 different provinces of Iran; the samples were placed in sterile plastic bags and stored at 4°C. The fields from which these samples were taken have not been previously sprayed with any commercial formulation of *Bt*. Isolation of bacteria was done according to the modified method of Anwar Hossain et al. [1]. Two hundred grams of sample was suspended in sterile physiological saline (1:5, w/v), mixed vigorously, and left to stand without any disturbance. A total of 2 ml of the clear supernatant was heated at 80°C for 5 min. The heat-treated supernatant was grown on nutrient agar plates at 31°C for up to 5 days. Colonies similar in morphology of bacteria were selected. Colonies were examined under a phase-contrast microscope for the presence of parasporal inclusion bodies and spores. Known strains that served as references were supplied by DSMZ Company (the German Resource Centre for Biological Material) (Table 1).

Preparation and characterization of parasporal inclusions

Sporulating cultures of *Bt* strains were produced in the standard *Bt* medium (UG) containing bactopeptone (7 g l^{-1}), glucose and salts as previously reported [22], at 28−30°C for 72 h, or until more than 90% of the cells had lysed, releasing spores and crystals. Spore crystal mixtures were washed (10,000g for 10 min at 41°C) once in 0.5 M NaCl and then twice in cold sterile water. Two milliliter samples of lysed cultures were washed by centrifugation and resuspended once in 2 ml of 0.5 M NaCl, and then twice in cold sterilized water containing 1 mM of the protease inhibitor phenyl-methane-sulfonylfluoride (PMSF). Parasporal

Table 1 Description of known *Bt* strains used as reference for Dipteran-active genes

No.	Serovar	Subspecies	Genes
1	H7	*aizawai*	*cry39*, *cry40*
2	H10	*darmstadiensis*	*cyt1*, *cyt2*
3	H6	*entomocidus*	*cry30*
4	H14	*israelensis*	*cry2*, *cry4*, *cry10*, *cry11*,
5	H4a4c	*kenyae*	*cyt1*, *cyt2*
6	H3a3b3c	*kurstaki*	*cry2*
7	H8a8b	*morrisoni*	*cry2*
8	–	*wuhanensis*	*cyt1*, *cyt2*
9	H20a20b	*yunnanesis*	*cry2*
10	H28a28c	*jegathesan*	*cry32*, *cry11*, *cry19*, *cry24*

inclusions of each isolate were classified through phase contrast microscopy in one of the following types: bipyramidal (Bp); elliptical (El); cuboidal (Cb); spherical (Sp); spindle (Sl); and irregular (Ir).

Protein electrophoresis

The protein content of spore–crystal mixtures was determined by SDS-PAGE analysis, as described by Laemmli [21], using 10 or 12.5% acrylamide separating gels. Samples (5–15 mg) of washed spore–crystal mixtures, prepared as described by Thomas and Ellar [36], were placed in 2× concentrated sample buffer and heated at 80°C for 10 min, as previously described [23] and loaded onto the gel immediately before electrophoresis. Gels were stained in a solution containing 50% (v/v) ethanol, 10% (v/v) acetic acid, and 0.1% (w/v) Coomasie brilliant blue R250 for 40 min, and then destained in a solution containing 6.7% (v/v) glacial acetic acid and 9.4% (v/v) ethanol.

Plasmid patterns

Bacillus thuringiensis strains were grown to an optical density at 600 nm of 0.8 in Spizizen medium (0.2% NH_4SO_4, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% sodium citrate, 0.02% $MgSO_4(\cdot 7H_2O)$ with 0.5% glucose, 0.1% Casamino acids (DIFCO) and 0.01% yeast extract. Cells were washed in TE (50 mM Tris, 10 mM EDTA [pH 7.8]) and incubated for 30 min at 37°C in 10 mg of lysozyme/ml in 0.5 M sucrose, 25 mM Tris, and 10 mM EDTA (pH 8.0). After 10 min at 4°C, lysis buffer (0.2 M NaOH, 1% sodium dodecyl sulfate [SDS]) was added and the mixture was incubated for 5 min at 4°C. A solution of 3 M sodium acetate, pH 4.8, was added and stored for 20 min at −20°C. Particles were centrifuged at 12,000 rcf (×g) for 20 min in a centrifuge. Two volumes of ethanol were added, and the mixture was incubated for 20 min at −80°C to precipitate DNA. DNA was centrifuged as above, dissolved in distilled water, and visualized in 0.6% agarose gels.

Oligonucleotide PCR primers and PCR analysis

For detection of 22 Dipteran-specific *cry* and *cyt* genes, including *cyt1*, *cyt2*, *cry2* (*cry2Aa*, *cry2Aa*, *cry2Ac*), *cry4* (*cry4A*, *cry4B*, *cry4C*, *cry4D*), *cry10*, *cry11*, *cry17*, *cry19*, *cry21*, *cry24*, *cry25*, *cry27*, *cry29*, *cry30*, *cry32*, *cry39*, *cry40*, two types of primers were synthesized, namely, universal primers (UN) from conserved regions of related genes and specific primers (SP) as previously described [3, 4, 13, 20, 29] (Table 2). Oligonucleotides were synthesized in a DNA synthesizer (Microsyn 1450A; Systec Inc.) as specified by the manufacturer.

Bt strains were grown on LB medium plate for 16–18 h. A loop of cells was transferred to 100 µl ddH_2O and mixed, 5 µl was used as a DNA template. PCR was carried out in a DNA Thermal Cycler (iCycler, Bio Rad). The PCR mixture included 100 mM (each) dNTP, 20 pM each of the primers, 1 U Taq DNA polymerase enzyme, and 2 µl of 10× PCR buffer (Fermentas) in a total volume of 20 µl. The PCR cycling conditions were similar to those described by Wang et al. [39, 40].

The distribution frequency of a *cry* gene in *Bt* strains from a certain origin was defined as the percentage of the *Bt* isolates containing this gene among all the isolates from that origin.

Results

The *Bt* strain collection built up in this study came from 2,292 soil samples that were collected from cultivated fields of 28 provinces of Iran. In total, 128 crystal-forming *Bt* strains upon examining 28,445 *Bacillus* like-colonies were identified. These selected strains were isolated from the temperate and humid zone that occupies about 8% of the country, the moderate or cold mountainous zone that occupies about 42% of the country; and the dry and semidry zone that occupies about 50% of the country (Fig. 1). The overview of the geographical origin of the 128 isolates from different regions and sources is shown in Table 3 and 4. The most *Bt* frequency was observed in the soil samples collected from Caspianic zone (7%), while the lower was found in the moderate or cold mountainous (5.7%), and the dry and semidry zones (4%) (Table 3). The soil samples of over 80% of provinces contained *Bt*, whereas about 20% of provinces did not contain any *Bt* strains (Khorasan, Khuzestan, Lorestan, Semnan, and Zanjan). The higher percentage of samples with *Bt* was observed in the Ardabil (38%), East Azarbaijan (15%), and Charmahal and Bakhtiari (11%), while the lower was found in the other provinces (0–10%). Furthermore, the percentage of samples with *Bt* showed significant differences between different crops. The largest number of *Bt* isolates was collected from the soils of cotton (17%) while the smallest number was from the soils of sesame (0%) (Table 4).

Microscopic studies showed that about 40% of isolates contained more than one type of crystals. The results showed that 47.2% of isolates produce bipyramidal parasporal inclusions, 30% cuboidal in shape, 28.6% elliptical in shape, 10% spindly in shape, 5.7% spherical in shape, and 27.4% of isolates showed irregular forms. The diameter of the crystals was 0.9−1.1 µm. Figure 2 shows the microscopic observation of the crystals produced by some strains. Based on previous studies [19, 20, 41, 43], strains containing

Table 2 Characteristics of general and specific primers for *cyt1*, *cyt2*, *cry2, cry4*, *cry10*, *cry11*, *cry17*, *cry19*, *cry21*, *cry24*, *cry25*, *cry27*, *cry29*, *cry30*, *cry32*, *cry39*, and *cry40* genes

UNcry2Primer	Sequence	Positions	Gene(s) recognized	Product size	GenBank accession No.
UNcry2(+)	5′-gttattcttaatgcagatgaatggg-3′	726–750, 1402–1426	*cry2Aa1*	701	M31738
UNcry2 (−)	5′-cggataaaataatctgggaaatagt-3′	1444–1468, 2120–2144	*cry2Ab2*	701	X55416
		2695–2719, 3359–3383	*cry2Ac*	689	X57252
UNcry2(+)	5′-cggataaaataatctgggaaatagt-3′	726–750,	*cry2Aa1*	498	M31738
SPcry2Aa(−)	5′-gagattagtcgcccctatgag-3′	1203–1223			
UNcry2(+)	5′-cggataaaataatctgggaaatagt-3′	1444–1468,	*cry2Ab2*	546	X55416
SPcry2Ab (−)	5′-tggcgttaacaatgggggggagaaat-3′	1965–1989			
UNcry2(+)	5′-cggataaaataatctgggaaatagt-3′	2695–2719	*cry2Ac*	725	X57252
SPcry2Ac (−)	5′-gcgttgctaatagtcccaacaaca-3′	3396–3419			
UNcry4(+)	5′-gcatatgatgtagcgaaacaagcc-3′	3324–3347, 3738–3762	*cry4A2*	439	D00248
UNcry4(−)	5′-gcgtgacatacccatttccaggtcc-3′	3259–3282, 3673–3697	*cry4B4*	439	D00247
SPcry4A (+)	5′-tcaaagatcatttcaaaattacatg-3′	1706–1731	*cry4Aa*	459	Y00423
SPcry4A (−)	5′-cggcttgatctatgtcataatctgt-3′	2140–2165			Y00423
SPcry4B (+)	5′-cgttttcaagacctaataatataatacc-3′	1868–1896	*cry4Ba*	321	X07423
SPcry4A (−)	5′-cggcttgatctatgtcataatctgt-3′	2164–2189			X07423
SPcry10 (+)	5′-tcaatgctccatccaatg-3′	978–996	*cry10*	348	M12662
SPcry10 (−)	5′-cttgtataggccttcctccg-3′	1306–1326			M12662
UNcry11 (+)	5′-cgcttacaggatggatagg-3′	990–1009, 1308–1332	*cry11Aa*	342	M31737
UNcry11 (−)	5′-gctgaaacggcacgaatataata-3′	1025–1044,1324–1368	*cry11Ba*	343	X86902
		1048–1067, 1376–1400	*cry11Bb*	352	AF017416
SPcry17 (+)	5′-aagtaaagatttctggg-3′	367–384	*cry17Aa1*		X99478
SPcry17 (−)	5′-ctgaggtattttgtgga-3′				
UNcry19 (+)	5′-aggggagtccaggttatgagttac-3′	1287–1310	*cry19Aa1*	331	Y07603
UNcry19 (−)	5′-atttccctagttagttcggttttt-3′	1641–1618	*cry19Ba1*		
UNcry21 (+)	5′-atacagggataggatttcaag-3′	1112–1132	*cry21Aa1*	453	I32932
UNcry21 (−)	5′-atccccattttctataagtgtct-3′	1564–1542	*cry21Aa2*		I66477
			cry21Ba1		AB088406
UNcry24 (+)	5′-agggggcgatggatacgac-3′	1253–1271	*cry24Aa1*	355	U88188
UNcry24 (−)	5′-ggccctgctacaaccgaaacta-3′	1607–1587	*cry24Ba1*		BAD32657
SPcry29 (+)	5′-tcagctccaataactggtg-3′	897–917	*cry29*	451	AJ251977
SPcry29 (−)	5′-gcatgtcatccccttgtcta-3′	1328–1348			
UNcry30 (+)	5′-aactcacacatcctccatcg-3′	2479–2499	*cry30*	265	AJ251978
UNcry30 (−)	5′-atcggaaggcaatcattcg-3′	2725–2744			
UNcry32 (+)	5′-tggtcgggagagaatggatgga-3′	2236–2258, 2893–2913	*cry32Aa*	677	AY008143
UNcry32 (−)	5′-atgtttgcgacaccattttc-3′	2338–2360, 2994–3014	*cry32Ba*	676	BAB78601
		2254–2276, 2910–2930	*cry32Ca*	676	BAB78602
		2218–2240, 2874–2894	*cry32D*	676	BAB78603
Uncry39 (+)	5′-agctgcgaatctgcatttacttttt-3′	597–621	*cry39*	619	BAB72016
UNcry39 (−)	5′-ctcataattttccgtccataaat-3′	1193–1216			
Uncry40 (+)	5′-ttatcaatgttaagggatgc-3′	593–613	*cry40Aa*	366	BAB72018
UNcry40 (-)	5′-ctggatctgtgtatattttcctag-3′	935–959			
UNcyt1 (+)	5′-cctcaatcaacagcaagggttatt-3′	197–221, 647–674	*cyt1Aa*	477	X03182 X98793
UNcyt1 (−)	5′-tgcaaacaggacattgtatgtgtaatt-3′	85–109, 535–565	*cyt1Ab*	480	U37196
		97–121, 547–574	*cyt1Ba*	477	
UNcyt2 (+)	5′-attacaaattgcaaatggtattcc-3′	509–533, 837–865	*cyt2Aa*	356	Z14147 U52043
UNcyt2 (−)	5′-tttcaacatccacagtaatttcaaatgc-3′	529–553, 856–884	*cyt2Ba*	355	U82519
		649–673, 976–1004	*cyt2Bb*	355	AAK50455
		196–218, 523–551	*cyt2Ca*	355	

Fig. 1 The geographic locations of the provinces and three main geographic and climatic regions of Iran

Table 3 Distribution of *Bt* strains in fields of different regions and provinces of Iran

Climates	Soil samples	Bacterial colonies	*Bt* isolates	*Bt* frequency (%)
Caspianic	440	4,961	31	7
Moderate or cold mountainous	1,314	17,493	75	5.7
Dry and semidry	538	5,991	22	4
Total	2,292	28,445	128	5.6

Dipteran-active *cry* and *cyt* genes shows elliptical (very similar to those found in *Bt* subsp *israelensis*), cuboidal, rhomboidal and spherical crystal inclusions. Thus, these results predicted that our isolates contain Dipteran-active *cry* and *cyt* genes.

The plasmid profile analysis detected a great complexity in the content of plasmids, providing 15 different plasmid profiles, with several others being similar, not considered as different (Fig. 3, Table 5). The results showed that isolates contained between one and seven plasmids with estimated molecular masses of 4–130 MDa. The band that corresponds to chromosomal DNA was identified in all isolates, and megaplasmids, i.e., plasmids detected above the chromosomal band, were identified in several isolates. Seven of the investigated isolates showed unique plasmid pattern.

All *Bt* isolates were further characterized by SDS-PAGE of their crystal protein products. We found diversity of protein profiles among isolates, regardless of their origin. The results revealed that the Iranian strains synthesize a protein or group of proteins with a molecular mass between 28 and 140 kDa, and some of them further had a protein of 21–28 kDa (Fig. 4). The isolates produced 1–5 different protein bands and about 70% of the isolates showed more than one band. Based on previous studies [20, 27, 32], major Dipteran-active Cry and Cyt toxins have molecular masses of 17–28 (Cry17, Cyt), 50 (Cry2Ab), 65–75 (Cry2Aa, Cry2Ac, Cry4D, Cry17A, Cry19, Cry24), 80 (Cry4C, Cry11B) and 128–140 kDa (Cry4A, Cry4B, Cry32). Thus, based on these studies, band patterns were divided in to six different specific patterns (Fig. 5). Crystal protein patterns

Table 4 Distribution of *Bt* strains in different fields of Iran

Fields	Soil samples	*Bt* isolates	Frequency (%)
Wheat and barley	785	46	6
Rice	313	22	7
Vegetables	656	19	3
Sugar beet	167	13	7.5
Potato	116	6	5
Sunflower	46	7	15
Cotton	59	10	17
Sesame	21	0	0
Oil seed rape	44	4	9
Corn	27	1	3.5
Total	2,292	128	5.6

of the isolates were comparable with *Bt* subsp. *israelensis*, *aizawai*, *kurstaki, tenebrionis*, and some other known strains. About 8% of the isolates showed pattern similar to that reported for *Bt* subsp. *israelensis*, with major proteins of 130, 70, and 28 kDa.

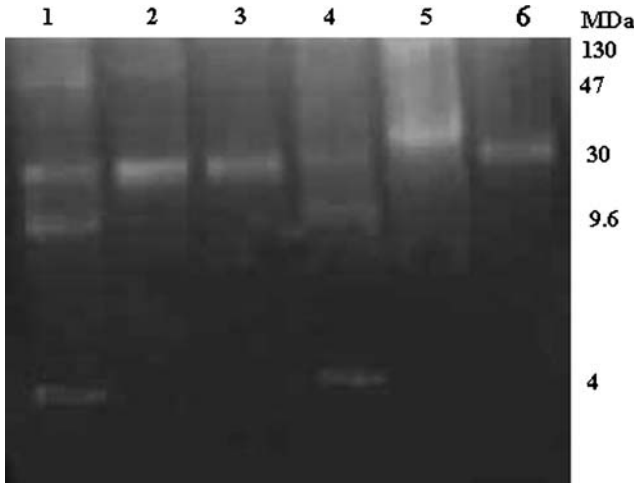

Fig. 3 Plasmid pattern of some Iranian *Bt* isolates. **1–6** YD5, GN5, SN1, KH5, *Bt* subsp. *israelensis*, and KH4, respectively

The *cry* gene composition of the *Bt* isolates was determined by PCR using 37 universal and specific primers to detect 22 different *cry* and *cyt* genes described to codify for

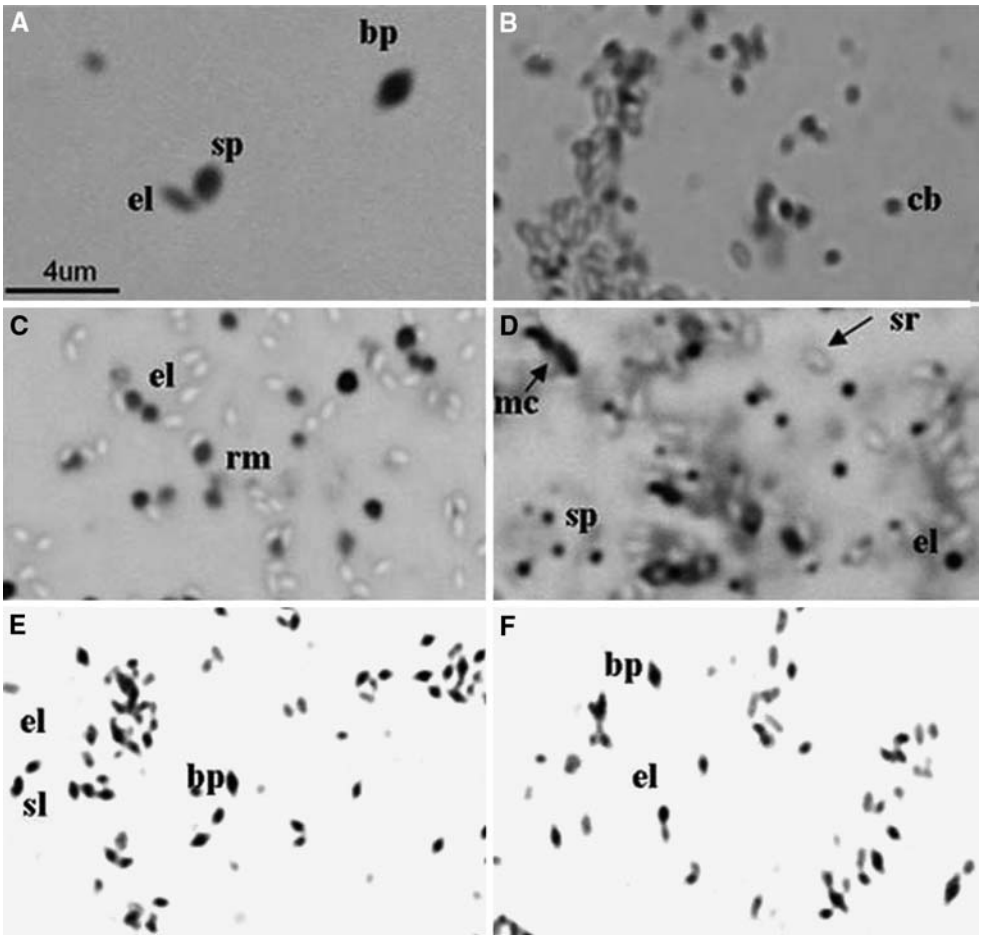

Fig. 2 Photomicrograph of some native *Bt* isolates showing the spherical, bipyramidal, cuboidal, elliptical, and rhomboidal crystals. **a** strain KH7, **b** strain GN1, **c** strain GON6, **d** strain S19, **e** GN4, and **f** YD5. *bp* bipyramidal crystals, *sp* spherical crystal, *cb* cuboidal crystal, *rh* rhomboidal crystals, *el* elliptical crystals, *vc* vegetative cells, and *sr* spore

Table 5 Distribution of plasmid profiles present in the Iranian *Bt* strain collection ($n = 128$)

No.	Profile	Isolates (%)
1	4.9, 9.6, 30, 47	9
2	9.6, 30, 47	7
3	18, 30, 47, 72, 98	5
4	4.9, 47, 72, 98	5
5	9.6, 30, 47	12
6	57, 68	9
7	30	8
8	9.6, 57, 72, 98, 130	5
9	47, 72	9
10	72, 130	3
11	4.9, 9.6, 18, 30, 57, 72, 98	3
12	47	7
13	47, 90	8
14	30, 47	7
15	72	3

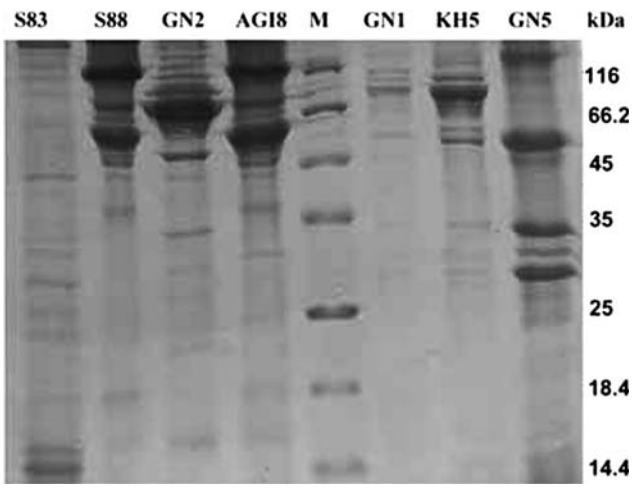

Fig. 4 SDS-PAGE of spore−crystal from some Iranian *Bt* strains

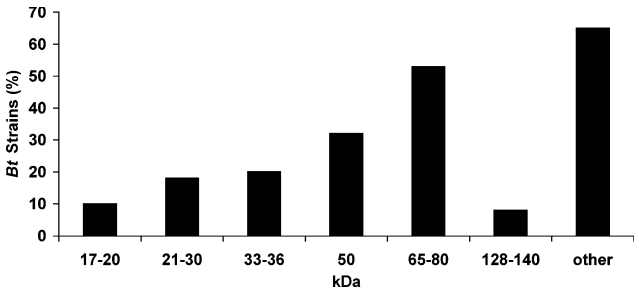

Fig. 5 Protein band patterns in Iranian *Bt* isolates

proteins active against mosquitoes. All known strains showed the expected PCR products when amplified with the universal and specific primers for the mentioned genes (Table 1). Each *cry* or *cyt* gene produced a PCR product with a unique molecular weight. Therefore, strains with PCR products of sizes other than those predicted are also candidates for harboring putative novel *cry* genes. For verification of PCR products, some PCR products (produced with specific primers) were selected and digested with enzymes, their coordinate sites were expected in the interested genes. After that, digested products were observed in agarose gel. Almost in all occasions predicted fragments were observed. Furthermore, some doubtful PCR products with different band sizes from those expected were sequenced. Sequencing of these gene fragments showed that the observed PCR products corresponding to interested gene sequences. Interestingly, sequences of a few PCR products with different size (from expected size) did not correspond to the interested gene sequences (data are not shown). The strains containing these sequences may contain a new gene or genes that seem to be promising for biological control of insects and management of resistance.

Agarose gel electrophoresis of the PCR products obtained with some known and native strains is shown in Fig. 6. Strains containing *cry2*-type genes were the most abundant and represent 73 of the 128 *Bt* isolates (57%). Strains harboring *cry24* and *cry10* genes were also highly abundant (38.75 and 32.85%, respectively), and 28.2% of the strains contained *cyt2* genes. *cry11*, *cry4*, *cry17*, *cry19*, *cry21*, *cry29*, *cyt1*, and *cry9* genes were less abundant, found in 25.7, 14.3, 11.4, 1.4, 4.3, 1.4, and 10% of the strains, respectively. No strains with *cry30*, *cry32, cry39* and *cry40* genes were found. Finally, 16% of the strains did not give any PCR product when assayed with the general primers (Fig. 7).

Thirty-eight different *cry* gene profiles were detected in this collection (Table 6). The most common profile of *cry* genes contained only *cry24* genes (13.3%). We found some new interesting combinations of *cry* and *cyt* genes not reported in previous studies. The most interesting profiles contained "*cry2Ab, cry4B, cry10, cry11, cry17, cry19, cyt1*, and *cyt2*" and "*cry2, cry4B, cry10, cry11, cry24, cyt1*, and *cyt2*". The strains containing these profiles could have high toxicity against a wide spectrum of Dipteran insects.

The strains harboring *cry2* genes selected with universal-*cry2* primers were analyzed with the *cry2* specific primers. Among the *cry2* gene containing isolates, 37.5% strains harbored *cry2Aa,* 55% *cry2Ab* and 2.5% *cry2Ac* (Fig. 8). PCR products of three *cry2* positive isolates were smaller or bigger than standard PCR products for *cry2*-type genes. Furthermore, two *cry2* positive isolates did not amplify any product with specific primers for these three genes, however, these strains produced crystal inclusions, suggesting that they may contain potentially novel Cry2 toxins. Among the *cry4* gene containing isolates, 0% of strains harbored *cry4A*, 60% harbored *cry4B*, and 40% harbored

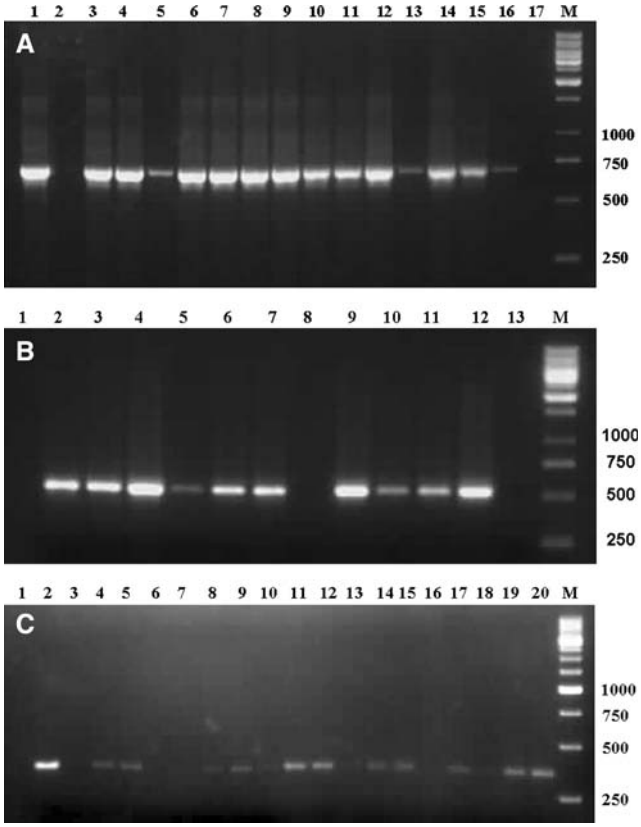

Fig. 6 Agarose gel (1.2%) electrophoresis of PCR products amplified from the native *Bt* strains. **a** With *cry2* universal primers: *1 Bacillus thuringiensis* var. *israelensis* as positive control, *2* negative control, *3–17* the native *Bt* strains (GON6, KH5, GN15, KON1, S19, AGI1, KH5, S55, QM2, GON2, GN5, QM1, GON7, GN2, GON3, respectively), *M* molecular weight marker (kb); **b** with *cry2A*a specific primers. *1* negative control, *2 Bacillus thuringiensis* var. *kurstaki* as positive control, *3–13* the native *Bt* strains (KH7, GN4, GON6, AGI1, AGI2, AGI8, AL11, S2, S8, S11, GN3), *M* molecular weight marker (kb); **c** with *cry24* universal primers. *1* negative control, *2 Bacillus thuringiensis* var. *jegathesan* as positive control, *3–20* the native *Bt* strains (KH3, S19, S54, GN1, GN2, S86, S88, MI1, BR7, QM1, GON7, KH5, KH7, AGI1, GN15, KON5, GON6, KON1), *M* molecular weight marker (kb)

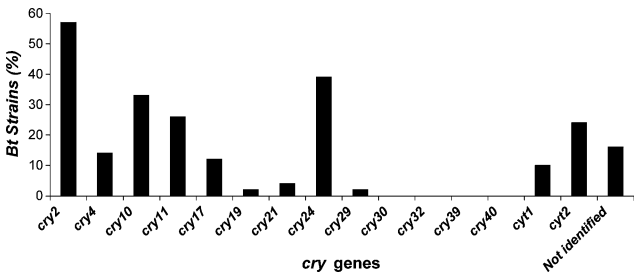

Fig. 7 Distribution of *cry* and *cyt* genes in 128 field-collected strains of *Bt*

cry4C, *cry4D* or novel *cry4* type genes (Fig. 9). Among the *cry4* gene containing isolates, there was a major protein around 80 (Cry4C or *D*) or 130 kDa, a size similar to that of the Cry4 proteins, encoded by *Bt* var. *israelensis*.

Table 6 Distribution of *cry* gene profiles present in the Iranian *Bt* strain collection ($n = 128$)

No.	*cry* gene profiles	No. of isolates	Freq. (%)
1	*cry2Ab*	9	7
2	*cry2Ab,cry10,cyt1*	2	1.6
3	*cry2Ab, cry11*	4	3
4	*cry2Ab, cry24, cry29, cyt1*	2	1.6
5	*cry2Ac*	2	1.6
6	*cry2, cry4B , cry10, cry11 , cry24, cyt1, cyt2*	2	1.6
7	*cry2, cry21, cry24*	1	0.8
8	*cry2Ab, cry10, cyt2*	2	1.6
9	*cry2Ab, cry10*	3	2.3
10	*cry2Ab, cry4B, cry10, cry11, cry24, cyt2*	1	0.8
11	*cry2Ab, cry10,cyt1, cyt2*	2	1.6
12	*cry2Aa, cry4B, cry10, cry11, cry21, cry24*	2	1.6
13	*cry2Ab, cry10, cry17*	2	1.6
14	*cry2Ab, cry4, cry10*	1	0.8
15	*cry2Ab, cry10, cry11, cry17, cyt2*	1	0.8
16	*cry2Ab, cry24, cyt2*	2	1.6
17	*cry2Aa, cry10, cry11, cyt2*	2	1.6
18	*cry2Ab, cry4B, cry10, cry11, cry17, cry19, cyt1, cyt2*	1	0.8
19	*cry2Ab, cry4, cry10, cry11, cry24, cyt2*	2	1.6
20	*cry11, cry24, cyt2*	2	1.6
21	*cry17, cyt1*	2	1.6
22	*cry2Aa*	14	11
23	*cry17*	4	3
24	*cry2Aa, cry4B, cry10, cry11, cry24, cyt2*	2	1.6
25	*cry4B, cry10, cry11, cry24*	1	0.8
26	*cry2Aa, cry10, cry11, cry24, cyt2*	2	1.6
27	*cry 24, cyt2*	2	1.6
28	*cry24*	17	13.3
29	*cry2Aa, cry24, cyt2*	2	1.6
30	*cry10, cry11, cry24*	4	3
31	*cry2Aa, cry4, cry10, cry11, cry24, cyt2*	2	1.6
32	*cry2Ab, cry10, cry11*	2	1.6
33	*cry2Aa, cyt2*	2	1.6
34	*cry2Aa, cry17*	2	1.6
35	*cry2Ab, cry17, cyt1, cyt2*	2	1.6
36	*crt10, cry21, cry24*	2	1.6
37	*cry4, cry10, cry11, cry24*	1	0.8
38	Did not reacted with any of primers	20	16

The distribution of Dipteran-specific *cry* genes in different ecological regions of Iran was analyzed. The summary of the results is shown in Fig. 10. All observed Dipteran-specific *cry* genes can be divided into four groups. The first group consists of *cry2* (54–60%), *cry10* (25–44%), *cry11* (15–34%), and *cry24* (28–48%) genes, which were the four most common genes found in all the three main zones. The

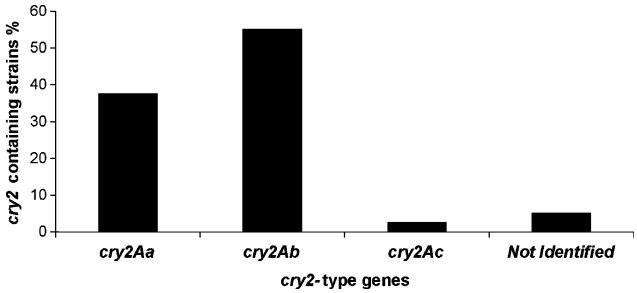

Fig. 8 Distribution of *cry2-type* genes in *cry2* containing strains

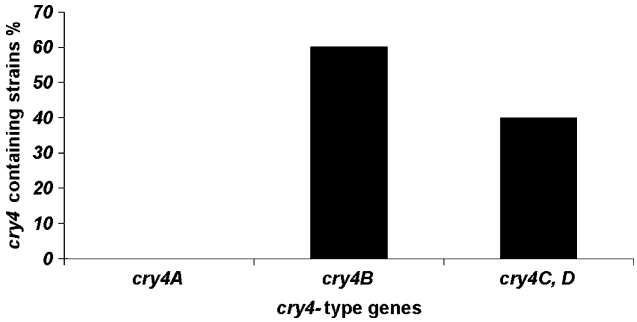

Fig. 9 Distribution of *cry4-type* genes in *cry4* containing strains of *Bt*

second group includes *cry4* (10–16%), *cry17* (4–15%), and *cyt1* (8–12%) genes, which were less abundant and distributed in all the three main zones. The third group includes *cry19*, *cry21*, and *cry29* genes, which were less abundant and distributed only in one or two zones. The last group consists of *cry30, cry32, cry39*, and *cry40* genes which were not found in any of the regions (Fig. 10).

Finally, based on crystal morphology, protein patterns, and PCR 21 native strains containing Dipteran-active gene combinations were selected as potentially high toxic strains for Dipteran pests (Table 7). These strains containing several types of *cry* genes encoding highly active insecticidal crystal proteins (ICPs) might be predicted to have a wider pest spectrum or increased activity. Also our results showed that some of the isolates may harbor minimum a putative novel *cry2* gene.

Discussion

In this paper, about 128 *Bt* strains were isolated from different agro-ecological regions of Iran. The presence of certain Dipteran-active *cry* and *cyt* genes was analyzed in the strains. This study showed that about 6% of the analyzed soil samples contained at least one *Bt* strain, whereas previous studies have shown the presence between 20 and 70% [6, 39]. This may be because of technical problems in isolation from soil samples. Since most *Bt* frequency was observed in Caspianic zone (7%), it can be concluded that *Bt* strains prefer humid and temperate climates. Furthermore, the largest number of *Bt* isolates was collected from the soil of cotton and sunflower preferring humid and temperate climates.

Characterization of the isolates, regardless of their origin, was based on morphological characteristics of crystals, plasmid profiles and protein band patterns as well as PCR analysis using general and specific primers for *cry* and *cyt* genes encoding proteins active against mosquitoes. These studies are useful for understanding the distribution of *cry* genes and may lead to the identification of effective isolates for application in biological control of pests, and novel candidate genes for bioengineered crop protection. Cry proteins are very selectively active against certain insect species, so the strains containing several types of *cry* genes encoding highly active ICPs might be predicted to have a wider pest spectrum or increased activity. This would lead to additional broad spectrum and highly toxic *Bt* insecticides. Previous studies showed that a great variability in *cry* genes coding different mosquitocidal toxins exists in nature, but one of the most Dipteran-active combinations of proteins is that present in the *Bt* strains, containing Cry4, Cry10, Cry11, and Cyt toxins [20]. In this research, we also found such interesting gene combinations which could be

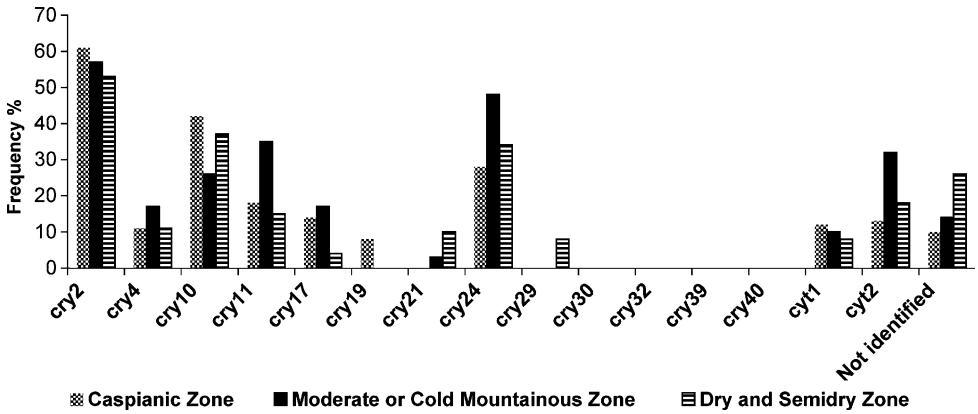

Fig. 10 The *cry* gene distribution in different ecological regions

Table 7 Crystal protein and gene features of 20 selected *Bt* isolates

Strains	Crystal morphologies	Protein size (kDa)[a]	Genes detected by PCR
Bti	El, Rm	28, 70, 135	*cry2, cry4A, cry4B, cry10, cry11, cyt1, cyt2*
S19	Sp, El	28, 70, 135	*cry2Aa, cry4B, cry10, cry11, cry24, cyt2*
S54	Rm	35, 70, 135	*cry4B, cry10, cry11, cry24*
S55	Cb, Rm	135	*cry2Aa, cry10, cry11, cry24, cyt2*
S86	Rm	70	*cry10, cry11, cry24*
S88	Rm, El	35, 50, 90, 120	*cry10, cry11, cry24*
BR7	Sl, Cb	28, 35, 50	*cry2Ab, cry24, cry29, cyt1*
QM1	Sl,Cb, Sp	70, 135	*cry2, cry4B, cry10, cry11, cry24, cyt1, cyt2*
KH5	Rm, Cb	50, 95, 105	*cry2Ab, cry4B, cry10, cry11, cry24, cyt2*
KH6	Rm, Cb	28, 50	*cry2Ab, cry10, cyt1, cyt2*
KH7	Bp, El, Sp	35, 70, 135	*cry2Aa, cry4B, cry10, cry11, cry21, cry24*
GN1	Sp, Cb	50, 95, 105	*cry2Ab, cry4C or D, cry10*
GN2	Cb, Rm	28, 32, 50, 65, 90	*cry2Ab, cry10, cry11, cry17, cyt2*
GN4	El, Bp, Sl	135	*cry2Aa, cry10, cry11, cyt2*
GN5	Cb	28, 30, 35, 50, 135	*cry2Ab, cry4B, cry10, cry11, cry17, cry19, cyt1, cyt2*
GN15	Cb, Rm	28, 35, 50, 70	*cry2Ab, cry4C or D, cry10, cry11, cry24, cyt2*
GON6	El, Rm	28, 65, 135	*cry2Aa, cry4C or D, cry10, cry11, cry24, cyt2*
GON7	Cb	50, 95	*cry2Ab, cry10, cry11*
AGI8	Cb, Bp	28, 50, 70, 120	*cry2Ab, cry17,cyt1, cyt2*
KON1	Rm, Bp	35, 120	*cry10, cry21, cry24*
YD5	El, Bp	70, 105	*cry4C or D, cry10, cry11, cry24*

[a] Major protein bands on SDS-PAGE gels

Bti Bt subsp. *israelensis*

Sp spherical, *Rm* rhomboidal, *Cb* cuboidal, *El* elliptical, *Sl* spindle, *Bp* bipyramidal

used to predict that these isolates will show high toxicity against mosquitoes. Thirty-eight distinct *cry* and *cyt* gene profiles were identified in our collection, which indicates the high diversity in the *cry* gene contents of the *Bt* isolates. The *cry* gene content of the *Bt* isolates exhibited a wide diversity, and most of the Dipteran-active *cry* and *cyt* genes were observed. Among them, the *cry2*-type genes were the most abundant in every region and source (57%). In this context Wang et al. [39] when working with soil samples from China obtained 310 isolates of which 70 (15.5%) contained *cry2* genes. The *cry24* genes represented the second most frequent gene, whereas *cry19* and *cry29* genes displayed the lowest frequency. The analysis of the genes showed that the *cry2* and *cry10*, and *cry24* and *cyt* genes were most often present together and most of the *Bt* isolates contained more than one type of Dipteran-active *cry* gene. The great variability and distribution of *Bt* in each country under study suggest that the ecological differences of the place of origin and/or co-evolutive relations had favored the expression of different patterns of *cry* genes. The above evidences show the importance of establishing collections of this species in different countries for developing strategies of biological pest control.

Most *cry2*, *cry4*, *cry10, cry11*, *cry24* and *cyt* gene containing isolates showed elliptical (very similar to those found in *Bt* subsp. *israelensis)*, cuboidal, rhomboidal and spherical crystal inclusions which coincide with previous results [19, 41]. About 8% of the isolates showed protein band pattern similar to that reported for *Bt* var. *israelensis*, with major proteins of 130, 70, and 28 kDa. Sixteen percent of crystal forming *Bt* isolates did not harbor any Dipteran-active *cry* or *cyt* genes, this implied that they might contain known genes belonging to other families or potentially novel ICP genes.

One of the problems of *Bt* based insecticides is their limited spectrum of action as compared with chemical based insecticides. This is a limiting factor since usually agricultural crops are simultaneously facing different plagues and sometimes different insect orders. An important characteristic found in this study was the presence of more than one *cry* gene in most of the isolates, with differences, at least in theory, in terms of their host range. While other studies have reported the presence of strains with these characteristics [6, 8], these are not above 3.5% of the total isolates, a factor which evidences the great biopesticide potential of the isolates of our collection. It is worth emphasizing that other studies have also showed the presence of *cry* genes from different groups in the same strain [6, 8, 16, 35]. It shows that at least, some *Bt* isolates could be selected to develop biopesticides that potentially control more than one insect pest in a given crop.

Finally, based on results, 20 isolates with potentially high toxicity to mosquitoes; especially to important crop pests were selected for more characterization. The bioassay

of the selected isolates on *Aedes aegypti*, and characterization of the observed potentially novel *cry* genes and the search for additional novel genes will be continued.

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