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Molecular characterisation and screening for *cry* genes of native *Bacillus thuringiensis* strains from Kazakhstan

Alnura Tursunova, Ainura Adilkhankyzy, Shyryn Turbekova, Ulzhalgas Abylayeva, Ainaz Balabek, Alibek Uspanov, Bakhytzhan Duisembekov*

Kazakh Research Institute of Plant Protection and Quarantine named after Zh. Zhiembayev, Almaty, Kazakhstan

**Corresponding author: alnura89.12.12@mail.ru*

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Abstract: The current study aimed to characterise indigenous *Bacillus thuringiensis* (Bt) strains for their potential use in agricultural broad-spectrum pest control. Twenty-nine Bt strains were isolated from soil in southeastern Kazakhstan. All isolates were Gram-positive and formed endospores. Species identification was conducted by sequencing the *gyrase B* (*gyrB*) gene. The nucleotide sequences of the amplified *gyrB* gene regions were compared with those in the NCBI database, confirming that the isolates were native Bt strains with high homology to known Bt strains (99–100%). In addition, the strains were screened for the presence of genes encoding 11 different crystalline endotoxins using PCR with universal primer pairs. The PCR results showed the distribution frequencies of *cry*, *cyt*, and *vip* genes among the strains: *cry1* (100%), *vip3* (100%), *cry2* (83.3%), *cry4* (20%), and *cyt1* (30%). PCR revealed diverse gene profiles among the Bt strains, with 5 distinct profiles identified. Regarding insecticidal activity, strains Bt8, Bt11, Bt26, and Bt28 demonstrated high pathogenicity, with mortality rates ranging from 97% to 100% against codling moth caterpillars, outperforming other Bt isolates.

Keywords: biological control; insecticidal activity; local strains; identification; endotoxins profiling; *gyrB*; PCR-screening

Modern pest control strategies rely heavily on chemical insecticides, leading to negative side effects from accumulating pesticide residues in different ecosystems, developing insecticidal resilience, and reducing beneficial insects' abundance, disrupting the natural balance. Consequently, the research was forced to focus on alternative, ecologically benign pest control methods (Abo-Bakr et al. 2020).

The entomopathogenic bacterium *Bacillus thuringiensis* (Bt) encompasses a large family of subspecies highly specialised as insect pathogens in various habitats (Seifinejad et al. 2008). Research on Bt strains has focused on the production of insecticidal crystalline proteins during the stationary and sporulation phases of their growth cycle, such as *cry* and *cyt* (σ-endotoxins), as well as proteins released during vegetative growth, such as *vip* and *sip* (Estruch et al. 1996; Donovan et al. 2006; Bravo et al. 2007).

Exploration Bt crystal toxins have classified endotoxin proteins into 73 families and 6 groups (Palma et al. 2014). Furthermore, the genes responsible

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for encoding the above crystal proteins are found on plasmids and chromosomal DNA (Höfte et al. 1989), varying among strains. Given the high practical importance of Bt strains for biological plant protection, expanding the range of environmentally friendly methods for controlling crop pests is essential. Using native Bt strains in biological plant protection offers several advantages over commercial or introduced strains. Firstly, native strains are better adapted to the specific ecological conditions of the region, including climate, soil-biotic factors, and associated microflora, which can enhance their survival and effectiveness in natural habitats (Bravo et al. 2010; Palma et al. 2014). Secondly, strains isolated from local populations of insect pests often exhibit high specificity. They may produce novel types of δ-endotoxins (Cry and Cyt proteins) not previously described, making them promising candidates for controlling resistant insect populations (Van Frankenhuyzen 2009). The isolation and molecular identification of such strains contribute to the expansion of the *B. thuringiensis* gene pool and the enrichment of biotechnological platforms. Therefore, researching their toxin formation, broadening their spectrum of action, and assessing their safety are urgent tasks. Notably, Kazakhstan currently lacks a system for producing biological products, which could enhance phytosanitary control.

Bt strains are characterised using various methods to determine their toxicity against different insect species. The identification of genes encoding protein insecticidal toxins through PCR has been widely used for screening large native collections and predicting the insecticidal activity of individual strains (Ben-Dov et al. 1997; Porcar et al. 2003).

Understanding Bt isolates with specific insecticidal genes becomes even more complex, considering these genes are primarily expressed on plasmids, which can be transferred whole or partially between bacteria (Rolle et al. 2005). Consequently, each ecological niche may contain Bt isolates with unique combinations of insecticidal genes. Research groups worldwide are continually testing different geographic regions to discover highly active novel Bt isolates (Campanini et al. 2012; Soares-da-Silva et al. 2015; El-Kersh et al. 2016; Jain et al. 2017; Lobo et al. 2018; Nair et al. 2018; Hassan et al. 2021).

Considering numerous reports on Bt crystal protein genes, exploring indigenous isolates remains important. Therefore, the current study aimed to characterise Bt strains isolated in Kazakhstan at the molecular level, considering their potential to serve as highly effective biological plant protection agents.

MATERIAL AND METHODS

Soil sampling and microbial isolation. Soil samples were collected from two districts in the Almaty region: Karasai and Enbekshikazakh. To isolate Bt strains, 0.5 g of the soil sample was suspended in 10 mL of culture medium containing 0.25 M $C_2H_3NaO_2$ (Travers et al. 1987; Ammouneh et al. 2011). The obtained soil solutions were stirred at 180–200 rpm for 4 h at 30 °C, followed by heat treatment at 80 °C for 3 min. The samples were then plated onto meat peptone agar (MPA) and incubated at 30 °C for 72 h to allow the growth of Bt-like colonies. *B. thuringiensis kurstaki* 2127-3K was used as a reference strain. Pure cultures obtained from subculturing were investigated for the production of parasporal crystalline proteins and stored at –80 °C in 20% glycerol broth.

***GyrB* gene sequencing.** Bacterial isolates were grown overnight in Luria-Bertani broth at 28 °C with constant shaking at 200 rpm. A 2 mL culture was used for cells to be pelleted by centrifugation at 5 000 rpm for 10 min. Genomic DNA was isolated using the GeneJET Genomic DNA Purification Mini Kit (Thermo Fisher, USA) protocol.

PCR amplification was performed with two pairs of primers: gyrB-F1 (5'-ATGGAACAAAAGCAAATGCA-3') and gyrB-R1 (5'-TTAAATATCAAGGTTTTTCA-3'); gyrB-F2 (5'-CCTTGYTTTGCWGAWCCDCC-3') and gyrB-R2 (5'-ACWCGTATGCGTGARYTRGC-3') (Baragamaarachchi et al. 2019).

The amplified *gyrB* gene fragment was sequenced using the Sanger method. Primers at a concentration of 0.8 pmol and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) were used for sequencing. The reaction mixture was prepared according to the manufacturer's instructions. PCR products were purified using ExoSAP-IT™ Express PCR Product Cleanup Reagent (Thermo Fisher, USA), and the BigDye® XTerminator™ Purification Kit (Thermo Fisher, USA) was used to purify samples after sequencing. Nucleotide sequences were determined on a 3500xL genetic analyser (Applied Biosystems, USA).

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The quality of the nucleotide sequences was assessed using the Sequencing Analysis software. Homologous nucleotide sequences were identified using the BLAST (Basic Local Alignment Search Tool) program in the International Gene Bank database of the US National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). Sequence analysis, editing, and alignment were performed using the MEGA software (version 11) (Kumar et al. 2018; Tamura et al. 2021).

Screening indigenous Bt strains for the *cry* gene via PCR. Table 1 demonstrates eleven pairs (forward and reverse) of primers used to investigate 11 genes encoding *cry*, *cyt*, and *vip* (Jain et al. 2017).

The reaction mixture (25 µL) contained 4 µL HF Buffer (Thermo Scientific), 0.5 µL 2 mM deoxyribonucleoside triphosphate (dNTP), 10 pmol of each primer, 0.2 µL Phusion High-Fidelity DNA Polymerase (Thermo Scientific), and 2 µL of DNA as template. PCR was performed in a SimpliAmp™ thermal cycler (Life Technologies Corporation). The PCR regime was as follows: an initial denaturation step at 98 °C for 30 s, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at the optimal temperature for each primer pair for 30 s, and extension at 72 °C for 30 s. A final extension step was performed at 72 °C for 10 min. After PCR, all samples were analysed by electrophoresis on a 1% agarose gel containing ethidium bromide ($C_{21}H_{20}BrN_3$) in a horizontal electrophoresis chamber. GeneRuler™ 1 kb Plus DNA Ladder (Thermo Fisher Scientific, USA) was used as a DNA ladder.

Insect cultures and bioassays. The bio-insecticidal potential of the studied indigenous Bt strains was tested against 3rd instar *Yponomeuta malinellus* L. *caterpillars*. A prepared spore-crystalline suspension (SCS) (1 × 108 spores/mL) was utilised to assess the bacterium's pathogenicity towards insects. The suspension was applied to the leaves of the caterpillar's food plant at a dose of 0.2 mL per leaf. The insects were maintained at 25 °C for 5–7 days, during which the mortality rate was recorded as a percentage. Insects were orally infected through

Table 1. Oligonucleotide primers used for PCR-based screening of insecticidal toxin genes in native *Bacillus thuringiensis* isolates

Primer pair	Sequence of primer	Gene recognised	Expected size (bp)
Un1F	CATGATTCATGCGGCAGATAA AC	*cry1*	274–277
Un1R	TTGTGACACTTCTGCTTCCCATT		
Un2F	GTTATTCTTAATGCAGATGAATGGG	*cry2*	689–701
Un2R	CGGATAAAATAATCTGGGAAATAGT		
Un3F	CGTTATCGCAGAGAGATGACATTAAC	*cry3*	589–604
Un3R	CATCTGTTGTTTCTGGAGGCAAT		
Un4F	GCATATGATGTAGCGAAACAAGCC	*cry4*	439
Un4R	GCGTGACATACCCATTTCCAGGTCC		
Un5F	TTACGTAAATTGGTCAATCAAGCAAA	*cry 5, 12, 14, 21*	474–489
Un5R	AAGACCAAATTCAATACCAGGGTT		
Un7-8F	AAGCAGTGAATGCCTTGTTTAC	*cry 7–8*	420
Un7-8R	CTTCTAAACCTTGACTACTT		
Un9F	CGGTGTTACTATTAGCGAGGGCGG	*cry9*	351–359
Un9R	GTTTGAGCCGCTTCACAGCAATCC		
Un11F	TTCCAACCCAACTTTCAAGC	*cry11*	305
Un11R	AGCTATGGCCTAAGGGGAAA		
VipF	CCTCTATGTTGAGTGATGTA	*vip3*	1 000
VipR	CTATACTCCGCTTCACTTGA		
Cyt1F	AACCCCTCAATCAACAGCAAGG	*cyt1*	522–525
Cyt1R	GGTACACAATACATAACGCCACC		
Cyt2F	AATACATTTCAAGGAGCTA	*cyt2*	469
Cyt2R	TTTCATTTTAACTTCATATC		

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their food source. In particular, the leaves of the preferred food plant were sprayed on both sides with SCS using a fine mist until they began to drip from the surface. Subsequently, the leaves were air-dried. The experiment was conducted in 4 replicates, each consisting of 10 insects. Insect mortality was recorded post-infections on the 3rd, 5th, and 7th days.

Statistical analysis. The data obtained were analysed using analysis of variance (ANOVA) with a 95% confidence interval. Means (M) and standard errors (± SE) were calculated.

RESULTS

Microbial isolation and identification. 198 samples were examined during the study, comprising 177 soil samples and 21 insects showing signs

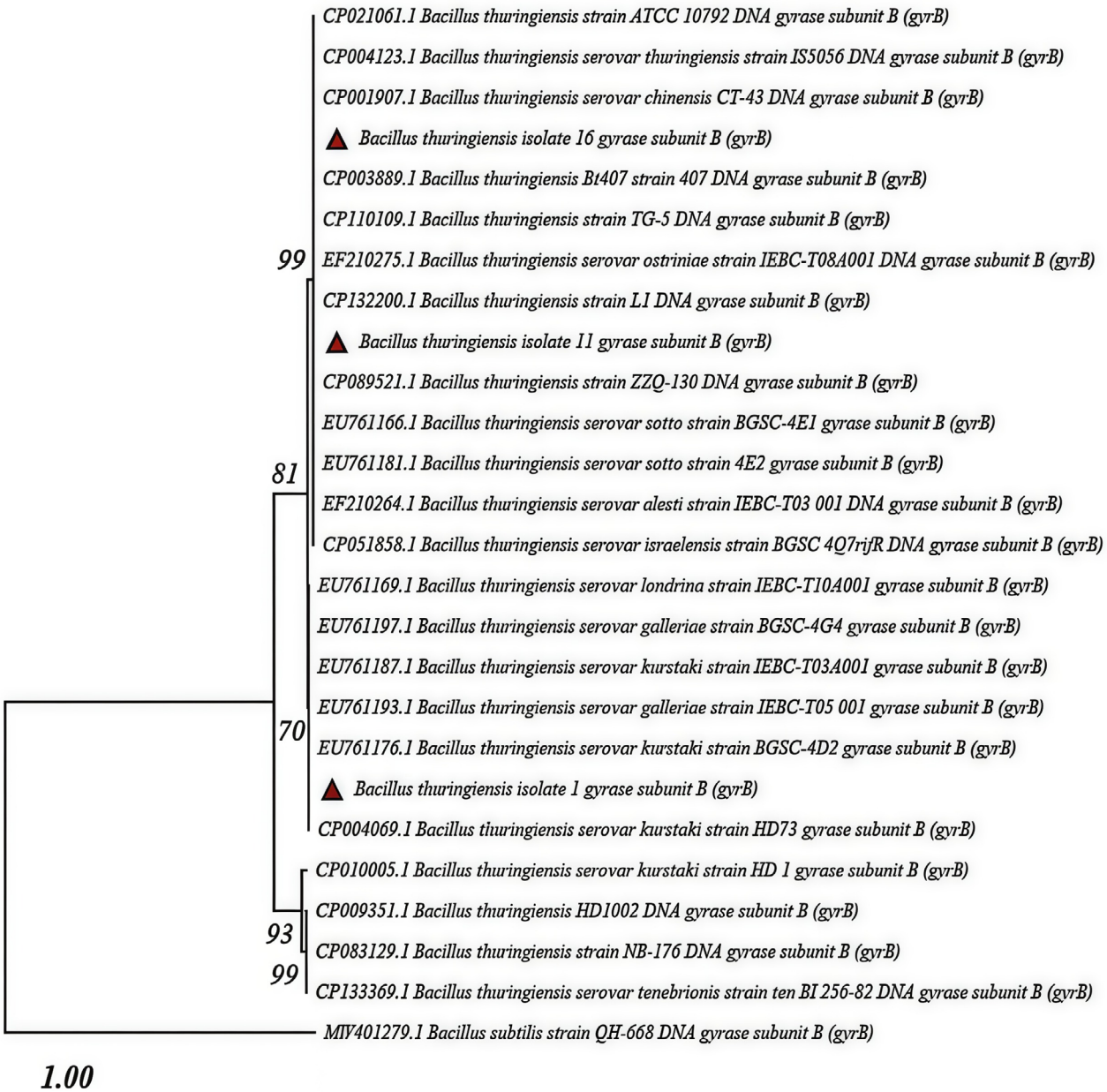

Figure 1. Phylogenetic analysis for the DNA gyrase gene of *Bacillus thuringiensis* strains

Evolutionary relationships of taxa. The evolutionary history was inferred using the neighbour-joining method. The percentage of replicate trees where the associated taxa clustered together in the bootstrap test (1 000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree

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Table 2. Distribution of *cry*, *vip* and *cyt*-type genes within the indigenous isolated Bt strains

Gene frequency (%)	Bt1	Bt2	Bt3	Bt4	Bt5	Bt6	Bt7	Bt8	Bt9	Bt10	Bt11	Bt12	Bt13	Bt14	Bt15
cry 1 (100%)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
cry 2 (83.3%)	+	+	+	+	+	+	+						+	+	+
cry 3															
cry 4 (20%)				+	+	+	+					+			
cry 5, 12, 14, 21															
cry 7–8															
cry 9															
cry 11															
vip 3 (100%)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
cyt 1 (30%)	+			+	+	+	+					+			
cyt 2															
Gene frequency (%)	Bt16	Bt17	Bt18	Bt19	Bt20	Bt21	Bt22	Bt23	Bt24	Bt25	Bt26	Bt27	Bt28	Bt29	Bt30
cry 1(100%)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
cry 2 (83.3%)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
cry 3															
cry 4 (20%)															+
cry 5, 12, 14, 21															
cry 7–8															
cry 9															
cry 11															
vip 3 (100%)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
cyt 1 (30%)						+	+					+		+	
cyt 2															

of bacteriosis from natural sites in the Almaty region of the Republic of Kazakhstan. 29 microbial strains were isolated from the above substrates with Bacillus thuringiensis (Bt) characteristics.

Vegetative cells appear as large bacilli, found singly or in smear chains; spores are oval-shaped. Spore and crystal precipitation occur around days 4–5 when cultivated in a thermostat at 28–30 °C. The cultural and morphological properties of the strains were assessed on meat peptone agar (MPA). When grown on MPA, cultures exhibited varied morphologies typical of Bt: *(i)* colonies were irregular, amoeboid, or rhizoid in shape, ranging in colour from beige to cream; *(ii)* colonies were rounded with scalloped or rolled edges, sometimes wavy or smooth, and comprised of large, slightly rounded or flattened margins with distinct configurations. The colonies had a fine-grained or homogeneous appearance with smooth, rough, or wrinkled surfaces. The texture was typically pasty and easily lifted from the medium surface, often exhibiting a shiny appearance. All selected bacterial samples were gram-positive. During sporulation, crystals in rhomboid and bipyramidal shapes were observed.

***GyrB* gene sequencing.** Isolates were identified to the species level by sequencing the *gyrB* gene and comparing the sequences using BLAST software with the data from the NCBI GenBank database. The nucleotide sequencing of the amplified *gyrB* gene region from the examined samples revealed 3 representative sequences, leading to the selection of isolates *Bt1*, *Bt11*, and *Bt16* for further analysis. The amplified *gyrB* sequences closely matched *gyrB* gene sequences of known Bt strains available in the GenBank database, showing 99–100% sequence similarity. The sequence data have been deposited in GenBank. Aligned sequences underwent neighbour-joining analysis to construct phylogenetic relationships (Figure 1).

The analysis demonstrated that all studied isolates (indicated by red triangles) clustered with representatives of the species *Bacillus thuringiensis*, confirming their species-level identification.

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According to the phylogenetic tree, *B. thuringiensis* isolate 1, including Bt4, Bt5, Bt6, Bt7, Bt12, Bt21, Bt22, and Bt29, grouped with the subspecies *B. thuringiensis kurstaki*, along with reference strains CP004069.1 and EU761716.1, with a bootstrap support value of 70. Isolate 16 (Bt25, Bt27, and Bt28) formed a clade with reference strains of *B. thuringiensis* (CP001907.1, CP003889.1, and CP110109.1), supported by a high bootstrap value (Bootstrap = 99). Isolate 11 was closely related to *B. thuringiensis* strain L1 (CP132200.1), confirming its affiliation with this cluster. The remaining isolates were also included within this clade. The reference strain *Bacillus subtilis* (MW401279.1) was used as an outgroup, which enabled accurate interpretation of the phylogenetic relationships within the genus *Bacillus*.

PCR-based screening of indigenous Bt strains for cry genes. The presence of various *cry*, *cyt*, and *vip* crystalline genes in the isolated strains was analysed to examine differences in gene profiles and to assess whether these differences correlated with variations in insecticidal activity. Among the native Bt strains, genes like *cry1* and *vip3* were most prevalent (100% – all strains contained these genes), followed by *cry2* (83.3%), *cyt1* (30%), and *cry4* (20%) (Figure 2). Agarose gel electrophoresis revealed nonspecific amplification alongside a specific partial amplicon of the cry gene, in correspondence with the literature that exploited the same primers. Screening for the *cry3*, *cry5*, *cry7*, *cry8*, *cry9*, and *cry11* genes showed no amplification. The combination of genes revealed 5 distinct profiles (Table 2).

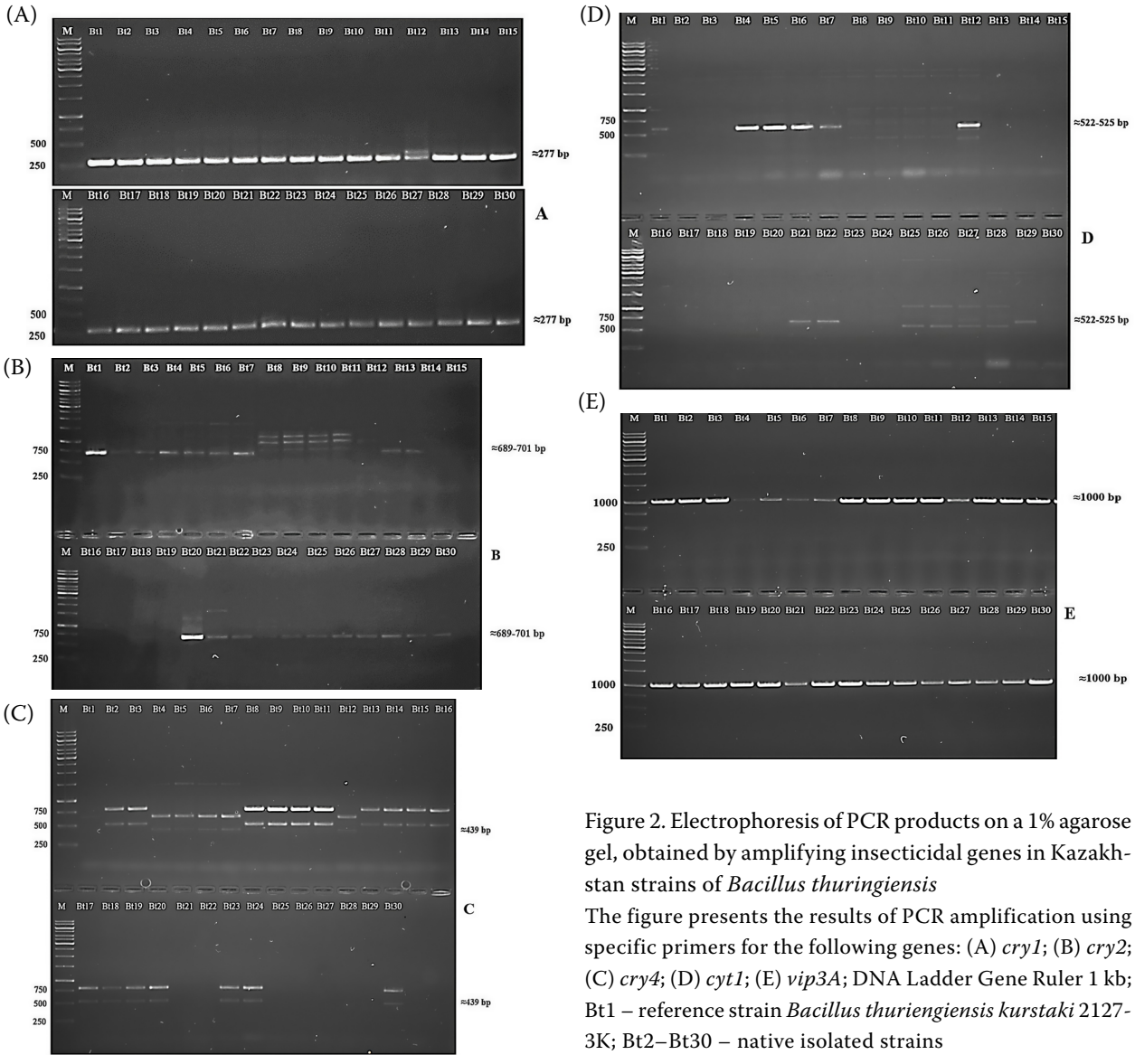

Figure 2. Electrophoresis of PCR products on a 1% agarose gel, obtained by amplifying insecticidal genes in Kazakhstan strains of *Bacillus thuringiensis*

The figure presents the results of PCR amplification using specific primers for the following genes: (A) *cry1*; (B) *cry2*; (C) *cry4*; (D) *cyt1*; (E) *vip3A*; DNA Ladder Gene Ruler 1 kb; Bt1 – reference strain *Bacillus thuriengiensis kurstaki* 2127-3K; Bt2–Bt30 – native isolated strains

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Table 3. Mortality rates of 3rd instar caterpillars of *Y. malinella* induced by Bt isolates

Strain	Day 3	Day 5	Day 7
Bt 8	77.5 ± 2.4	87.5 ± 2.5	97.5 ± 2.5
Bt 11	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Bt 26	85.0 ± 1.5	100.0 ± 0.0	100.0 ± 0.0
Bt 28	97.5 ± 2.5	100.0 ± 0.0	100.0 ± 0.0
Control (reference *Bt1*)	72.5 ± 0.9	97.0 ± 1.0	97.0 ± 1.0
Control (dH_2O)	0.0	0.0	0.0

Bioassays. A bioassay of 29 isolated Bt strains for bio-insecticidal activity against third-instar caterpillars of *Y. malinella* revealed that strains Bt8, Bt11, Bt26, and Bt28 were more efficient in exterminating the caterpillars compared to the reference strain *Bacillus thuringiensis kurstaki* 2127-3K (Bt1). All these Bt strains exhibited significantly higher mortality rates ($P < 0.05$) compared to the control within the tested post-inoculation time points. After 3 days, the average mortality rate ranged from 72.5% to 100%. The highest mortality was observed in Bt11 (100%), followed by Bt28 (97.5%), Bt26 (85%), and Bt8 (77.5%), while the reference strain exhibited a mortality rate of 72.5%. By day 5, the average mortality ranged from 87.5% to 100%. Similarly, the highest mortality was recorded in strains Bt11, Bt26, and Bt28 (100%), followed by the reference strain (97%) and strain Bt8 (87.5%). By the 7th day, mortality rates for all strains ranged from 97.5% to 100% (Table 3).

DISCUSSION

In this study, *B. thuringiensis* isolates were characterised from various natural sources collected in two districts of the Almaty Region, Republic of Kazakhstan. From 198 collected samples, 29 isolates exhibiting morphological and cultural characteristics consistent with *B. thuringiensis* were obtained. Molecular typing based on the *gyrB* gene confirmed the taxonomic affiliation of the isolates to various subspecies of Bt, indicating notable phylogenetic diversity even within a single region.

PCR screening revealed the presence of key insecticidal genes in most isolates. The *cry1* and *vip3* genes were detected in 100% of strains, *cry2* in 83.3%, *cyt1* in 30%, and *cry4* in 20%. In contrast, genes such as *cry3*, *cry5*, *cry7-8*, *cry9*, and *cry11* were not amplified, aligning with findings from other international studies (Salekjalali et al. 2012; Jain et al. 2017). These results demonstrate a gene profile typically associated with toxicity against Lepidoptera, while suggesting limited potential against Diptera and Coleoptera. However, the high prevalence of *vip3*, which encodes proteins with a different mode of action from Cry proteins, indicates a potentially broader insecticidal spectrum of the studied strains.

The identified cry gene profiles revealed five distinct genetic combinations. Such diversity may result from the ecological and geographical conditions of the region, as well as the adaptation of Bt strains to the local entomofauna.

The cry gene screening results for Kazakhstani isolates are generally consistent with findings from other countries. For example, in a study by Thammasittirong & Attathom (2008) on Thai strains, *cry1* and *cry2* were the most prevalent (81.3% and 80.6%, respectively), similar to our findings. In India, Patel et al. (2012) reported a similar predominance of *cry1*; in Iran, *cry1* was detected in 47% of strains (Salekjalali et al. 2012). Notably, the 100% frequency of *vip3* among our isolates significantly exceeds reports from other regions, where this gene is less common or absent (Salama et al. 2015). This may suggest an adaptive advantage under the specific biocenotic conditions of the local environment and warrants further investigation.

Bioassays on third instar larvae of *Y. malinellus* revealed that four strains (Bt8, Bt11, Bt26, Bt28) exhibited high insecticidal activity, surpassing the reference strain *B. thuringiensis kurstaki* 2127-3K. Particularly, strain Bt11 induced 100% mortality by the third day post-inoculation. All highly active strains harboured a combination of *cry1*, *cry2*, and *vip3* genes, suggesting a possible synergistic effect of their toxic actions.

Notably, variations in cry gene profiles correlated with differences in insecticidal activity. This underscores the importance of a comprehensive strain evaluation approach, integrating molecular-genetic and biological characterisation. Our findings are consistent with those of Ferrandis et al. (1999), who highlighted the potential inactivation of individual genes and the influence of regulatory mechanisms on toxin expression.

The identified strains possess strong applied potential for developing domestic biopesticides targeting *Y. malinellus*, a pest causing significant damage

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to Kazakhstan's fruit production. Using local Bt isolates as active components of bioinsecticides offers several advantages, including environmental adaptation and a diversity of cry profiles enabling the design of formulations with combined modes of action. Moreover, developing local products reduces reliance on imported bioinsecticides and supports the advancement of national biotechnology. Strains Bt11, Bt26, and Bt28, which demonstrated 100% mortality by day 5, are priority candidates for prototype development. Subsequent steps should include production standardisation, determination of effective dosages, toxicological safety assessment, and field trials.

This study contributes to understanding the *B. thuringiensis* gene pool by incorporating strains from Central Asia, a region previously underrepresented in global collections. The presented data may enrich international databases and support the creation of multinational strain panels for comparative studies and strain improvement.

Furthermore, the presence of different subspecies among the Kazakhstani isolates confirms that the country represents a rich reservoir of Bt genetic diversity, the full potential of which remains largely untapped.

CONCLUSION

In the current research, 29 isolated indigenous Bt strains were screened for novel Bt genes possessing variable sequences that may be highly effective for pest control. Universal primers were used to investigate *8 cry* genes, *2 cyt* genes, and the *vip3* gene across Bt strains isolated from southeastern Kazakhstan. The *cry1* and *vip3* genes were the most common among the indigenous Bt strains, followed by *cry2, cyt1*, and *cry4*. In addition, it was concluded that strains exhibiting similar toxicity to codling moths possessed different gene profiles. Hence, characterising cry genes in indigenous strains may facilitate the development of highly potent isolates as biocontrol agents targeting indigenous insect pests. Further research is required to evaluate the insecticidal potential and cultivability of the studied strains.

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