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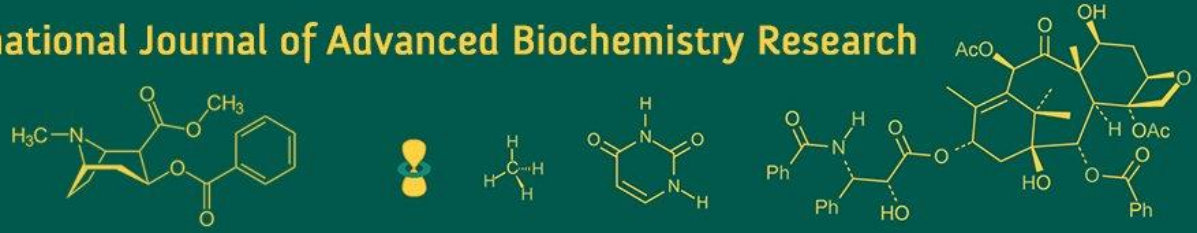

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Akshay Bharat Ghodake
MSc. Research scholar, Division of Molecular Biology and Biotechnology, ICAR-Indian Agricultural Research Institute, New Delhi, India

Suraj Mishra
Ph.D. Research scholar, Division of Genetics and Plant Breeding, ICAR-Indian Agricultural Research Institute, New Delhi, India

Omkar Sunil Karale
MSc. Research scholar, Division of Agricultural Engineering, ICAR-Indian Agricultural Research Institute, New Delhi, India

Aditi Tayade
Ph.D. Research scholar, Division of Agricultural Microbiology, ICAR-Indian Agricultural Research Institute, New Delhi, India

Dr. Sarvjeet Kaur
Prof. UG, Principal Scientist, ICAR-National Institute for Plant Biotechnology, New Delhi, India

Corresponding Author:
Akshay Bharat Ghodake
MSc. Research scholar, Division of Molecular Biology and Biotechnology, ICAR-Indian Agricultural Research Institute, New Delhi, India

Genetic diversity of Indian *Bacillus thuringiensis* isolates through a comparative study of ARDRA and 16S rRNA sequencing methods

Akshay Bharat Ghodake, Suraj Mishra, Omkar Sunil Karale, Aditi Tayade and Sarvjeet Kaur

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Abstract
Bacillus thuringiensis (Bt) is a widely recognized biocontrol agent known for its insecticidal activity and production of bioactive compounds, including Cry toxins and chitinases. The genetic diversity of Bt plays a critical role in its adaptability, efficacy, and biocontrol potential. This study explores the genetic diversity of 33 Bt isolates collected from diverse agroclimatic zones in India, along with three reference strains. Amplified Ribosomal DNA Restriction Analysis (ARDRA) and 16S rRNA gene sequencing were employed to assess genetic variability and phylogenetic relationships among the isolates. ARDRA, using four restriction enzymes (*AluI*, *HaeIII*, *HinfIII*, and *TaqI*), generated unique banding patterns, facilitating effective strain differentiation. The 16S rRNA gene sequences were analyzed to construct phylogenetic relationships using the Maximum Likelihood method. The results revealed significant genetic diversity among the isolates, with distinct clustering patterns observed in the dendrogram. The findings highlighted the robustness of ARDRA as a reliable molecular tool for studying bacterial diversity and reinforce the potential of Bt isolates as effective candidates for sustainable pest and disease management in agriculture. This study enhances the understanding of Bt isolates by combining ARDRA for preliminary microbial typing and genetic polymorphism detection with 16S rRNA sequencing for deeper evolutionary insights.

Keywords: *Bacillus thuringiensis*, DNA, ARDRA, 16S rRNA, diversity, agriculture

1. Introduction
India stands as a global agricultural powerhouse, ranking second in the production of key crops like rice, wheat, and sugarcane. Agriculture forms the backbone of the nation's economy, employing nearly 60% of the population and contributing 17% to the Gross Domestic Product (GDP) [1, 2]. However, this critical sector faces persistent challenges from pests, diseases, and environmental factors, which significantly reduce productivity. Integrated management strategies, particularly biological control, offer sustainable solutions to these challenges [3]. Among the various biocontrol agents, *Bacillus thuringiensis* (Bt) has emerged as a cornerstone in pest management due to its ability to produce δ-endotoxins like Cry and Cyt, which target pests while minimizing environmental impact [4, 5, 6]. Beyond its insecticidal capabilities, Bt exhibits remarkable adaptability and versatility, thriving in diverse habitats such as soil, water, and the phyllosphere [7]. Its dynamic genome, comprising chromosomal DNA and plasmids, facilitates rapid evolution and functional diversification, enabling Bt to produce bioactive molecules like chitinases and siderophores. These attributes not only enhance its biocontrol potential but also extend its applications to antifungal activity, bioremediation, and plant growth promotion, making Bt a pivotal tool in sustainable agriculture [8, 9, 10, 11].
The genetic diversity of Bt plays a crucial role in its adaptability and effectiveness as a biocontrol agent. Understanding this diversity is essential to unlock its full potential and identify novel traits that enhance its functionality in agriculture and environmental management. Bt's genomic plasticity allows for the evolution of new traits and the production of diverse bioactive compounds that are vital for its biocontrol mechanisms [12, 13]. Studies on genetic diversity enable the identification of unique bacterial isolates with enhanced production of Cry toxins and secondary metabolites, paving the way for innovative, sustainable pest management strategies. This exploration is critical for addressing emerging agricultural challenges, such as pest resistance and climate change, and developing tailored solutions for

diverse environmental conditions. By mapping genetic variations and understanding the evolutionary mechanisms underlying Bt's efficacy, researchers can refine its applications, ensuring its continued relevance in integrated pest and disease management strategies.

Various molecular techniques have been employed to assess the genetic diversity of Bt, each offering unique advantages and limitations. Techniques like Randomly Amplified Polymorphic DNA (RAPD), Repetitive Element Polymorphism PCR (rep-PCR), and Amplified Ribosomal DNA Restriction Analysis (ARDRA) have been extensively used [14, 15, 16]. RAPD, which amplifies random genomic regions using arbitrary primers, has proven useful in differentiating native strains based on geographic origin but is hindered by reproducibility challenges. Rep-PCR, on the other hand, targets repetitive DNA sequences to generate unique banding patterns, enabling effective strain differentiation but requiring careful optimization of primers and data analysis. ARDRA, which involves amplification of the conserved 16S rRNA gene followed by restriction enzyme digestion, has emerged as the most consistent and reliable method. This technique generates unique restriction patterns that provide high specificity and resolution in distinguishing closely related strains. By leveraging these molecular tools, researchers have gained valuable insights into Bt's genetic diversity and its implications for ecological adaptability and biocontrol efficacy.

Building on these methodologies, the current research aims to assess the genetic diversity of *Bacillus thuringiensis* isolates and evaluate the effectiveness of the ARDRA technique in distinguishing genetic variability. By analyzing isolates from diverse agroclimatic zones in India, the study uses ARDRA to generate unique restriction patterns that reveal the genetic relationships among the isolates. This approach highlights the robustness and reliability of ARDRA as a molecular tool for studying bacterial diversity. The findings provide critical insights into the genetic adaptability of Bt isolates, offering valuable information for optimizing their application in biocontrol strategies and integrated pest management systems.

2. Materials and Methods

2.1 Bt isolates, reference strains and culture conditions

Thirty-two Bt isolates representing six agroclimatic zones of India and isolated from diverse sources such as cattle sheds, orchard soils, grain dust, kitchen garden soils, desert soils, and agricultural fields with crops like chickpea, cotton, sorghum, and chili, have been used for this study. These isolates were provided by Dr. Sarvjeet Kaur from the ICAR-National Institute for Plant Biotechnology, New Delhi, India. Along with this, three Bt reference strains *i.e.* HD1 (*B. thuringiensis* subsp. *Kurstaki*), HD22 (*B. thuringiensis* subsp. *Kurstaki*), and 4G6 (*B. thuringiensis* subsp. *Galleriae*), obtained from the Bacillus Genetic Stock Centre (BGSC), Department of Biochemistry, Ohio State University, USA, through Dr. Sarvjeet Kaur also were included in this study. The isolates and reference strains were grown in nutrient-rich Luria Bertani (LB) Broth and LB Agar (Miller) (HIMEDIA Laboratories, Mumbai, India). All the media were prepared based on the manufacturers' instructions and the bacterial cultures incubated at 28 °C to allow the proper growth of the cultures. Table 1 gives description of the selected Bt isolates for this study along with their respective agro-climatic zones from where they have been isolated.

Table 1: Selected Indian Bt isolates for this study with their respective agro-climatic zones from where they have been isolated

No.	Agro-climatic zone	Name of Indian Bt isolates	Total
1	Trans Gangetic Plain Region	SK-3, SK-6, SK-211, SK-223, SK-229, SK-306, SK-921, SK-928, SK-962, SK-935, SK-936, SK-943, SK-955, SK-957, SK-958, SK-966, SK-977, SK-980, SK-996	19
2	Upper Gangetic Plain Region	SK-9, SK-678	2
3	Western Himalayan Region	SK-20, SK-63, SK-84, SK-94	4
4	East Coast Plains and Hills Region	SK-617, SK-700, SK-754, SK-768, SK-783, SK-792	6
5	Lower Gangetic Plains Region	SK-853, SK-870	2
	Total		33

2.2 Genomic DNA isolation and PCR conditions

Genomic DNA was isolated by using GeneJET Genomic DNA Purification Kit (Thermo Scientific™, MA, USA) from Thirty-two Bt isolates and three Bt reference which were selected for this study. The genomic DNA banding pattern were analyzed on agarose-gel after electrophoresis. The isolated genomic DNA used as a template for amplifying *16S rRNA* gene by using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′) according to Jain *et al.* (2017) [21]. Each reaction mixture, with a final volume of 40 µL, comprised 50 ng of genomic DNA from Bt, 50 ng of each primer, 200 µM of each dNTP, 1 U of Taq DNA polymerase, and 1× Taq buffer containing 15 mM $MgCl_2$. Amplification was performed in a Gradient Thermal Cycler (iGene Labserve, New Delhi, India) under the following conditions: an initial denaturation at 94 °C for 1 minute, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 45 seconds, and extension at 72 °C for 1 minute. A final extension step was carried out at 72 °C for 10 minutes. The PCR-product were analyzed on 1% agarose gel for expected band size of 1881 bp.

2.3 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The PCR products of 16S rDNA were digested with FastDigest restriction enzymes, *AluI*, *HaeIII*, *HinfIII*, and *TaqI* (Thermo Scientific™, MA, USA), for the ARDRA. For all reactions, an aliquot of 10 µL of PCR product (~0.1-0.5 µg DNA) was taken and mixed with 18 µL of nuclease-free water, 2 µL of 10X Buffer Tango, and 1-2 µL of the respective restriction enzyme. The mixture was gently mixed, briefly centrifuged, and incubated under specific conditions as follows: *AluI* at 37 °C for 15 minutes, *HaeIII* at 37 °C for 5 minutes, *HinfIII* at 37 °C for 5 minutes, and *TaqI* at 65 °C for 5 minutes. Following digestion, the reaction products were analyzed via 1.5% agarose gel prepared in 1X TAE buffer containing 0.5 mg/ml of ethidium bromide. Electrophoresis was carried out at 60 V for 2 h in 1X TAE electrophoresis buffer. The banding patterns were analyzed and ARDRA pattern were prepared for each isolate for specific restriction enzyme. Data analysis was done using Past 5 software (Version 5.0.2, 64-bit Windows) and dendrogram was prepared by using UPGMA method with Jaccard's similarity coefficient.

2.4 16S rRNA sequencing and dendrogram preparation

The 16S rRNA sequencing of 32 Bt isolates in this study were done by Barcode Biosciences (BBS), Bengaluru, India. The multiple sequence alignment of all 16S rRNA sequence were done by ClustalW in MEGA11 software and dendrogram was prepared by using Maximum likelihood tree with 100 Bootstrap replication. The 16S rRNA sequence from few Bt isolates were submitted to NCBI GenBank for accession number.

3. Results and Discussion

3.1 Genomic DNA isolation and 16S rDNA amplification

Genomic DNA was isolated from 32 selected Bt isolates and three Bt reference strains by using the GeneJET Genomic DNA Purification Kit. The isolated DNA was analyzed on 0.7% agarose gel containing 0.5mg/ml ethidium bromide. Electrophoresis was done at 60 V and for 2 h. Gel doc image showed a high-molecular-weight bands for all isolates and Bt reference strains (Fig. 1). The isolated genomic DNA showed a high molecular weight, which is consistent with the typical genome size of Bt, generally ranging from 5.0 to 7.9 Mbp. This observation aligns with previous reports on the genomic characteristics of Bt, supporting the integrity and completeness of the extracted DNA [17]. The high molecular weight may also indicate minimal fragmentation, ensuring reliable downstream applications such as PCR amplification. The 16S rRNA gene from selected Bt isolates and three Bt reference strains was successfully PCR-amplified using universal primers as mentioned in materials and methods (2.2). The PCR product was analyzed by 0.7% agarose gel electrophoresis and expected amplicon size of 1881 bp was observed in Gel doc image (Fig. 2) which is very similar to the banding patterns observed in previous studies during molecular characterization of Bt strains [15].

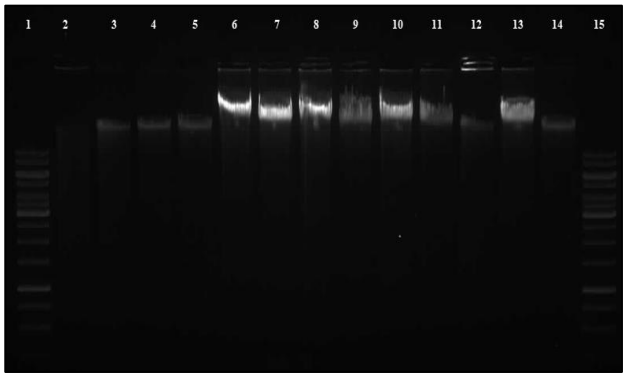

Fig 1: Agarose gel electrophoresis of isolated genomic DNA from Bt isolates, showing high molecular weight bands. Lane 1 and Lane 15 shows 1 kb DNA marker (MBI Fermentas, Germany) and the remaining Lanes depict Bt isolates

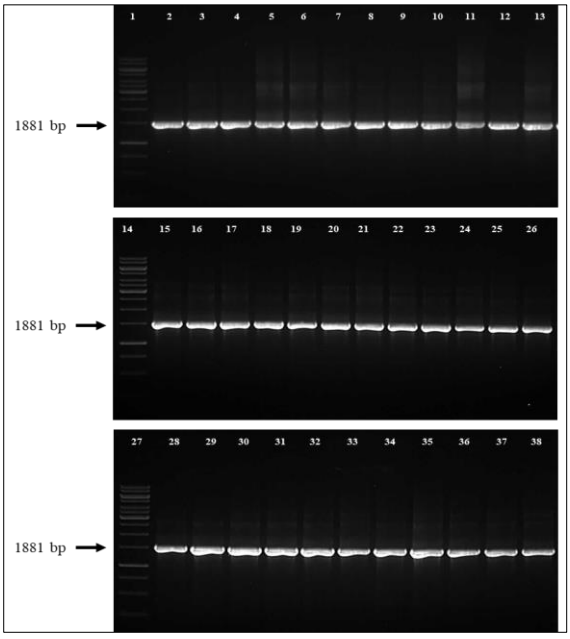

Fig 2: Gel electrophoresis of PCR-amplified 16S rRNA gene products, showing expected band size of 1881 bp from Bt isolates. Lane 1, 14, and 27: 1 Kb DNA ladder. Bt strains used as reference: in Lane 2 (HD1), Lane 15 (HD22), and Lane 28 (4G6). Lanes 3 to 13 correspond to Bt isolates SK-6, SK-9, SK-20, SK-63, SK-84, SK-94, SK-211, SK-223, SK-229, SK-306, and SK-617. Lanes 15 to 26 include Bt isolates SK-678, SK-700, SK-754, SK-768, SK-783, SK-792, SK-853, SK-870, SK-921, SK-935, and SK-936. Lanes 28 to 38 represent Bt isolates SK-943, SK-955, SK-957, SK-958, SK-962, SK-966, SK-977, SK-980, SK-996, and SK-997, respectively.

3.2 ARDRA-based restriction profiling and band analysis

ARDRA is a widely used molecular technique based on the digestion of amplified 16S rRNA gene sequences with specific restriction endonucleases to generate distinct banding patterns, enabling bacterial characterization at the species and subspecies levels [18, 19]. Its applicability extends to resource-limited laboratories, where it offers an efficient alternative for microbial profiling, enabling broad-scale genetic comparisons with minimal technical and financial investment. This approach provides a cost-effective and rapid method for genetic differentiation among bacterial isolates, particularly for taxa with highly conserved 16S rRNA sequences, such as *B. cereus* group members or closely related Bt isolates [20]. In this study, ARDRA produced distinct banding patterns based on length polymorphism, aiding in the molecular characterization of Indian Bt isolates. The 16S rRNA PCR-amplified product were digested with four different restriction enzymes *i.e. AluI*, *HaeIII*, *HinfI*, and *TaqI* and banding patterns were analyzed after gel electrophoresis (Fig. 3). The gel picture analysis showed 24 types of banding patterns with different sizes of bands. Similarly previous studies also reported the occurrence of about 20 different banding patterns after restriction digestion of 16S rDNA from 28 Bt strains with these four-restriction enzyme [15]. It shows the presence of more genetic variability in our Indian Bt isolates as it gives more number of banding patterns than reported in previous studies. Out of 24 different banding patterns, seven distinct band sizes were observed with *AluI* restriction digestion, whereas *HaeIII* digestion resulted in six different band sizes.

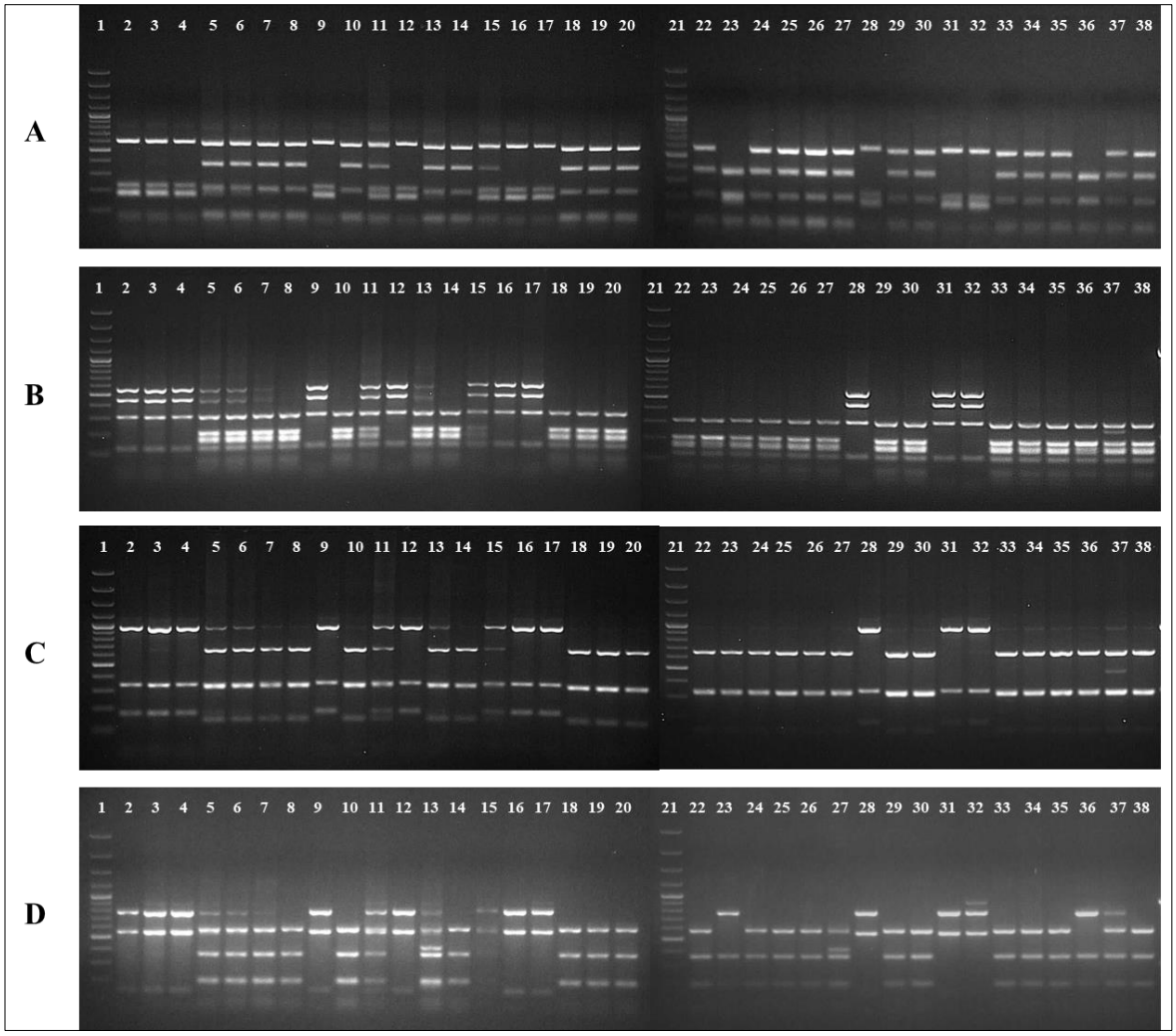

Fig 3: Banding patterns after restriction digestion of 16S rRNA amplified product with four restriction enzymes A) *AluI*, B) *HaeIII*, C) *HinfI*, and D) *TaqI*. Showing distinct fragment patterns corresponding to each enzyme's specific cleavage site. Lane 1 and 2 from each image shows 100 bp Pluse DNA marker (MBI Fermentas, Germany). Lane 2 to 20 depicts HD1, SK-3, SK-84, SK-223, SK-306, SK-617, SK-678, SK-700, SK-783, SK-792, SK-936, SK-957, SK-966, SK-980, HD22, SK-6, SK-9, SK-20, and SK-63 respectively. Lane 22 to 38 depicts SK-94, SK-211, SK-229, SK-754, SK-768, SK-853, 4G6, SK-870, SK-921, SK-928, SK-935, SK-943, SK-955, SK-958, SK-962, SK-977, and SK-996 respectively.

Similarly, *HinfI* and *TaqI* digestion produced six and five distinct band sizes, respectively.

3.3 Genetic diversity of Indian Bt isolates based on ARDRA

Genetic similarity among the 33 Indian *Bacillus thuringiensis* (Bt) isolates and three Bt reference strains was estimated based on ARDRA banding patterns using Jaccard's similarity coefficient. The similarity matrix generated was analyzed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm in PAST 5 software to construct a dendrogram (Fig. 4). The dendrogram analysis shows the similarity index values ranged from 0.30 to 0.97, which indicates considerable genetic variation among the isolates. The pattern of clustering into two major clades with further sub-grouping demonstrates that, although some of the isolates are genetically highly similar, others show considerable divergence. Out of 33 Bt isolates and 3 Bt strains, total 25 Bt isolates were clustered in a first major cluster. In that cluster, Isolates SK-678, SK-783, SK-966, SK-9, SK-20, and SK-63 formed a distinct sub-cluster, suggesting a high degree of genetic similarity. Similarly, another major sub-cluster comprised SK-229, SK-754, SK-768, SK-870, SK-921, SK-943, SK-955, SK-958, and SK-966 which exhibited genetic similarity within them but genetically diverse with other sub-clustered isolates. The isolates SK-3, SK-84, SK-700, SK-936, SK-6, and Bt reference strains HD-22, and HD-1 were sub-clustered together in a second major cluster shows their genetic similarity, while clustering of Bt reference strains with specific Indian Bt isolates, indicating their genetic relatedness. However, isolates SK-211, SK-962, SK-853, SK-977, SK-94, SK-223, SK-306, SK-617, SK-957, and SK-792 from the first major cluster showed genetic differences than the other group, while is isolates SK-980, SK-928, SK-935, and Bt strain 4G6 from second major cluster demonstrated genetic differences in comparison to others.

3.4 16S rRNA sequencing and phylogenetic relationships among Indian Bt isolates

In this study the 16S rRNA sequencing of 31 Bt isolates were done to study their genetic variation. The phylogenetic analysis was done by using Maximum likelihood method with 100 Bootstrap replication by MEGA 11 software (Fig. 5). The phylogenetic tree is divided mainly into three major clusters, with bootstrap values indicating the robustness of each branch. Higher bootstrap values (e.g., 100) suggest

strong support for the grouping of isolates, while lower values indicate relatively weaker relationships. The observed clustering highlights genetic diversity among these Bt isolates, which is crucial for understanding their evolutionary relationships and potential functional differences. The first major cluster includes isolates such as SK-306, SK-617, SK-63, SK-678, SK-977, SK-6, SK-870, SK-20, SK-229, SK-853, SK-957, SK-211, SK-980, SK-936, SK-792, and SK-962. The second major cluster include isolates SK-921, SK-9, SK-94, SK-943, SK-754, and SK-996. Similarly, the third major cluster consist of isolates SK-223, SK-935, SK-84, SK-700, SK-768, SK-783, SK-955, SK-958, and SK-966. The group of isolates present in respective clusters shows relatively close genetic similarity. However, all the isolates clustered in each major cluster are placed in a distinct sub-cluster with high bootstrap values, indicates unique evolutionary characteristics. The separation of these groups demonstrates genetic variation among Bt isolates, which may be linked to differences in their biocontrol potential or ecological adaptation. The 16S rRNA sequences of few Bt isolates were submitted to NCBI GenBank for accession numbers (Table 2).

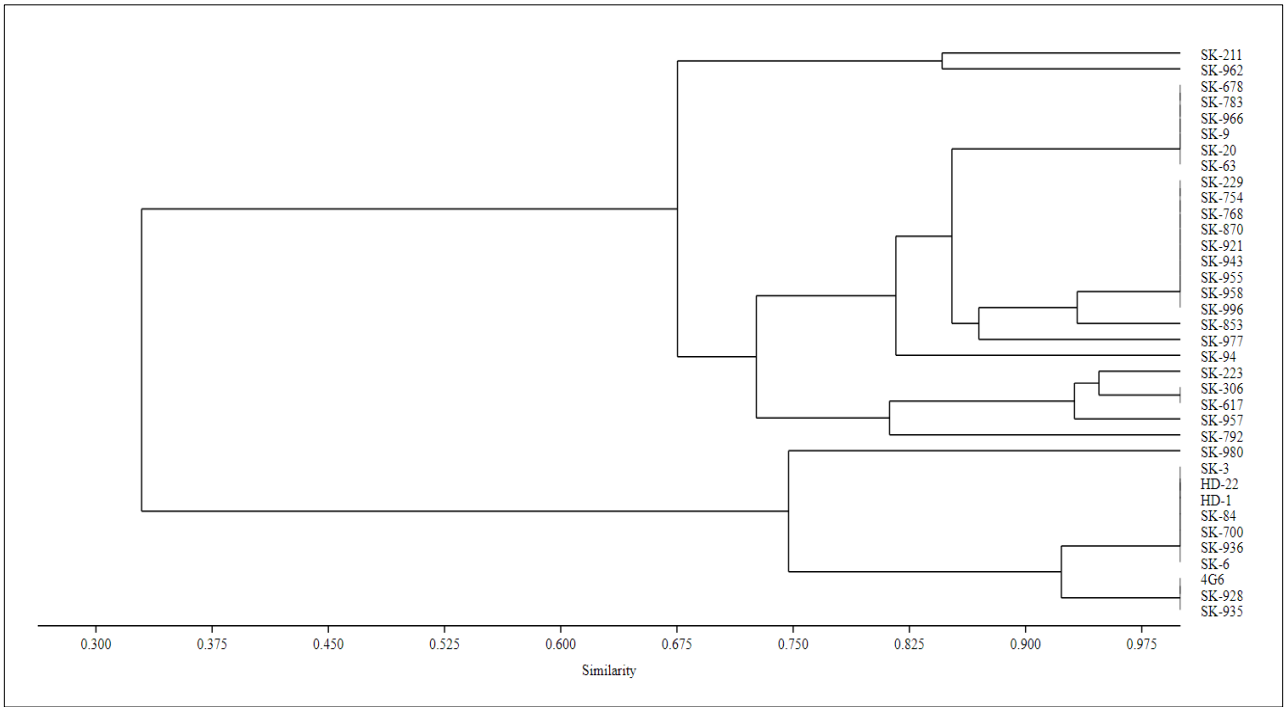

Fig 4: Dendrogram showing genetic diversity based on ARDRA pattern of 33 Indian Bt isolates and three Bt reference strains

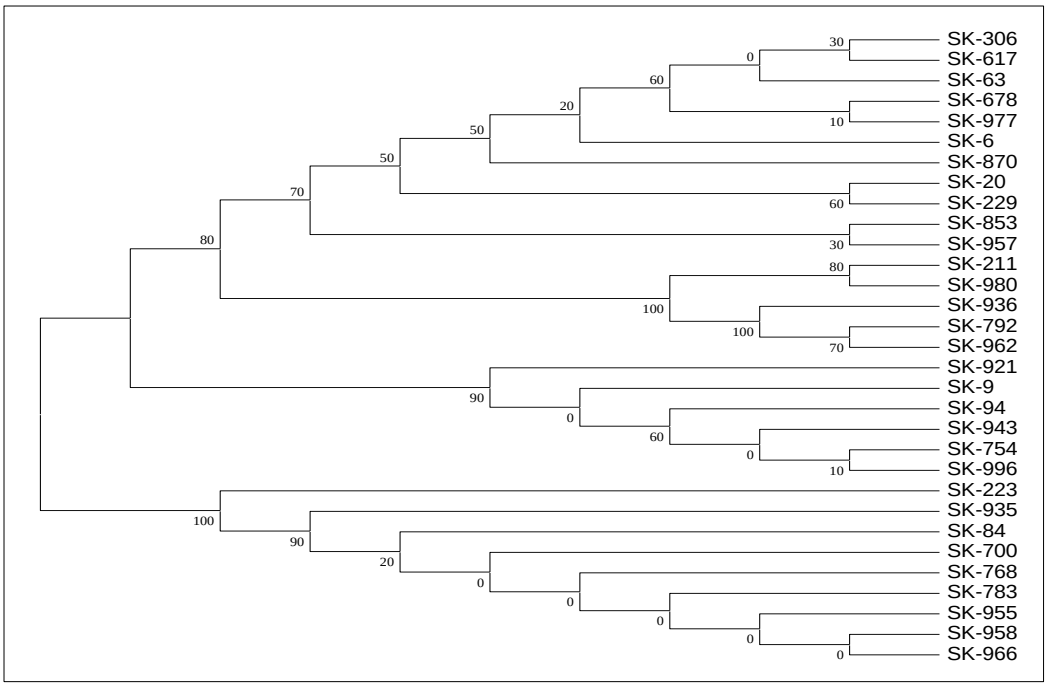

Fig 5: Phylogenetic tree showing genetic diversity of Indian Bt isolates based on 16S rRNA sequencing

Table 2: NCBI accession numbers of submitted 16S rRNA sequences from Indian Bt isolates

Sr. No.	Bt isolate	NCBI accession number
1.	SK-223	PQ670993
2.	SK-768	PQ670998
3.	SK-783	KX595331*
4.	SK-935	PQ670999
5.	SK-955	PQ671001
6.	SK-966	PQ671003

* Submitted during previous studies in our laboratory, rest all were submitted during this study

3.5 Comparative assessment of ARDRA and 16S rRNA analysis for genetic diversity of Bt isolates

Both ARDRA and 16S rRNA sequencing offer valuable insights into the genetic diversity of *Bacillus thuringiensis* (Bt) isolates, each with distinct advantages. ARDRA, based on restriction digestion patterns, effectively differentiates isolates through high-resolution banding profiles, revealing intra-species genetic variation and strain-level differences as studied in former research [21]. However, its resolution is limited, as it cannot detect subtle genetic variations like single nucleotide polymorphisms (SNPs), and clustering results can be influenced by enzyme selection, potentially leading to mis-grouping [21, 22, 23]. Additionally, ARDRA lacks phylogenetic depth, making it unsuitable for precise evolutionary studies. In contrast, 16S rRNA sequencing provides a robust phylogenetic framework by analyzing the complete 16S rRNA gene, allowing for accurate strain differentiation and evolutionary assessments [25, 26]. These findings aligned with the former studies that shows, 16S rRNA sequencing enables the detection of SNPs and other sequence variations, making it superior for advanced microbial studies, including taxonomy, biodiversity assessment, and evolutionary research [27, 28]. While ARDRA is useful for preliminary microbial typing and detecting genetic polymorphisms, 16S rRNA sequencing provides deeper evolutionary insights, making both methods complementary for a comprehensive understanding of Bt isolates.

4. Conclusion

This study comprehensively analyzed the genetic diversity and biocontrol potential of *Bacillus thuringiensis* (Bt) isolates from diverse agroclimatic regions of India, utilizing ARDRA and 16S rRNA sequencing. The ARDRA-based clustering revealed significant intra-species genetic variation, effectively differentiating the isolates based on restriction digestion patterns, while 16S rRNA sequencing provided deeper phylogenetic insights, establishing evolutionary relationships with high confidence. The observed genetic diversity among the isolates underscores their adaptability and potential for biocontrol applications. The comparative analysis of these methods demonstrated their complementary nature in assessing genetic polymorphism and taxonomic classification. The findings highlight the genetic diversity within Bt isolates, emphasizing their potential for further exploration in microbial ecology, taxonomy, and agricultural applications. This study lays the foundation for future research on the functional characterization of Bt isolates, particularly their role in biocontrol and agricultural sustainability.

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