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Distribution and diversity analysis of *Bacillus thuringiensis cry* genes in different soil types and geographical regions of India

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ABSTRACT

Molecular characterization of 117 *Bacillus thuringiensis* (*Bt*) isolates from various geographical locations was previously done by PCR amplification of *cry* genes. In present investigation, diversity of *cry* genes from different soil types and climatic environments was studied using rarefaction method. Presence of *cry1, cry2, cry3, 7, 8, cry4, cry5, 12, 14, 21, cry11, cry13* and *cyt1* genes from *Bt* strains isolated from various regions of India was determined by PCR amplification. A varied distribution of *cry* genes and their profiles was found in four soil types. The *cry1* gene was the most abundant in the isolates from four soil types and geographical regions. A higher degree of *cry* gene diversity was observed in isolates from alluvial soil. Rarefaction analysis indicated that more *cry* genes could be found from various soil types. Distribution of *cry* genes in semi arid, subtropical humid and tropical dry regions was varied but the degree of *cry* gene diversity determined by rarefaction analysis was similar. No major difference in distribution and diversity of *cry* genes was found in agricultural and non-agricultural samples except the absence of *cry3* and *cry13* genes in isolates of non-agricultural samples. We report the utility of rarefaction analysis to compare *cry* gene diversity from different geographical regions.

1. Introduction

Diversity of any organism is important in order to understand its role in nature, interaction with other organisms and its effect in various habitats. Diversity of *Bacillus thuringiensis* and its *cry* genes is widely studied throughout the world. Thereby, distribution of *Bt* as a natural inhabitant in various geographical regions, ecosystems and diverse habitats was identified (Bernhard et al., 1997; Martin and Travers, 1989).

Distribution and diversity study has been addressed using different statistical methods. Rarefaction is one of the statistical methods often used in paleontology and ecology to compare species diversity among different regions when number of samples varied. Rarefaction method was initially developed by Sanders (1968). It was revised by Hurlbert (1971); Heck et al. (1975) and later on modified to compare genetic diversity. Different authors have used it to study genetic diversity on basis of allelic richness or number of operational taxonomic units (OTUs) defined as group of similar sequences (Kalinowski, 2004; Urakawa et al., 2008). In present study, rarefaction method was used to compare diversity of *cry* genes in *Bt* isolates from different soil types and climatic regions of India.

The diversity of flora and fauna follow geographic pattern, and thus could be expected for the microbiota. Heterogeneous distribution of *Bt* and its *cry* genes have been observed (Martin and Travers, 1989; Bernhard et al., 1997; Chen et al., 2004). However, geographically related distribution of *cry* genes has been limited to several reports (Chak et al., 1994; Bravo et al., 1998; Wang et al., 2003). Soil type also plays an important role to determine the microbial community of a habitat. Though *Bt* being a soil inhabitant organism, effects of soil types on *Bt* and *cry* gene distribution has not been studied in detail. A detailed study is thus required in order to establish a strong correlation of *cry* gene distribution with geographical regions and soil type.

Indian regions with diverse soil types and climatic environments are ideal to study effect of these factors on distribution of microbes. Indian regions possess rich and unique biodiversity. Diversity of *cry* genes of *Bt* in some geographical regions of India was investigated by Prabagaran et al. (2002). However, distribution and diversity of *cry* genes in *Bt* isolates from different regions of India has not been yet approached in detail. Considering these aspects, present study describes distribution and diversity of *cry* gene in Indian regions. This study would be helpful to understand distribution and diversity of *cry* genes in various soil types and geographically regions.

Abbreviations: Bt, Bacillus thuringiensis; OTU, operational taxonomic unit; RFLP, restriction fragment length polymorphism.

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2. Materials and methods

2.1. Isolation of Bt from different soil types

Indian regions spans into three major soil types. The southern region represents red and yellow soil. The central and north-western regions represent black and alluvial soil types respectively. Soil samples were collected from agricultural fields of these three soil types where *Bt* based biopesticides were not employed as described in previous study (Patel et al., 2011b). River sedimentary soil was the fourth type collected from Mahi river basin, Gujarat, India (Patel et al., 2011a). A clear demarcation in color and texture of four soil types was observed. Non-agricultural soil samples were collected from Western Ghats mountains (Maharastra state), Khordung La mountain (Jammu and Kashmir state) and barren land soil from Dharwar (Karnataka state).

Isolation of *Bt* strains from total 94 soil samples and characterization of isolates was performed as described in previous study (Patel et al., 2011b). Identification and characterization of *Bt* isolates was done by colony morphology observation, phase contrast microscopy to observe crystal inclusions, SDS-PAGE analysis for Cry proteins and PCR amplification for detection of *cry* genes (Patel et al., 2011a,b). Isolates from same soil sample and sampling site were differentiated for sibling species/clones/replicates by vegetative whole cell protein profile as described by Konecka et al. (2007). Isolates showing similar patterns were considered sibling species and only one isolate was considered for PCR amplification of *cry* genes.

2.2. Allocation of isolates to three climatic regions

Bt isolated from the distinct soil types were allocated according to the climatic region they belong (http://www.indianetzone.com/40/types_indian_climate.htm). *Bt* isolates from soil samples of north Gujarat, central Gujarat and Punjab states were allocated to semi-arid region. Similarly isolates from south Gujarat, Andhra Pradesh and Karnataka states were allocated to tropical wet and dry region, and isolates from Madhya Pradesh and Uttar Pradesh states to sub-tropic humid region.

2.3. PCR amplification of Bt cry genes

Bacterial total DNA extraction was performed as described in previous study (Patel et al., 2011a). Total DNA (~25 ng) was used as template DNA in a 30 μL PCR reaction mixture with final 1X buffer, 250 μM dNTPs each, 0.2 μM primers each and 1.5 units of Taq DNA polymerase (Banglore Genei, India). PCR amplification was carried out for *cry1, cry2, cry3, 7, 8, cry4, cry5, 12, 14, 21, cry11, cry13* and *cyt1* genes using different primers as described in Table 1. A total of 117 *Bt* isolates from various soil types were characterized by PCR analysis for presence of different *cry* genes.

2.4. Rarefaction analysis for comparison of cry gene diversity

A set of numbers of isolates showing amplification for different *cry* genes and *cry* gene profiles from alluvial soil (Supplementary Table S1) was used in Analytical Rarefaction program (version 1.3, http://www.uga.edu/strata/software) to generate *E*-values. In similar manner, *E*-values were generated for other soil types (Supplementary Table S2). *E*-values representing number of *cry* genes and *cry* gene profiles for each soil type were plotted against the number of isolates to generate rarefaction curves and compare degree of *cry* genes diversity. Accordingly, rarefaction curves were generated to compare *cry* gene diversity in isolates from different climatic environments and agricultural soil type (Supplementary Tables S3 and S4).

3. Results

3.1. PCR analysis

Bacillus thuringiensis strains were isolated from four distinct soil types and characterized for crystal inclusion, Cry protein content, serotypes and insect toxicity as described in previous study (Patel et al., 2011a,b). Thirty-six isolates from alluvial soil, 34 isolates from black soil and 25 isolates from red/yellow soil and 22 isolates from river sedimentary soil samples were used for PCR analysis. Expected PCR amplification products of sizes 270–290 bp for *cry1* type genes and ~1.5 kb for *cry2* type genes were observed (Fig. 1). Similarly, amplification products of sizes ~700 bp for *cry3, 7, 8* group of genes, ~500 bp for *cry5, 12, 14, 21* group of genes, ~300 bp for *cry13* and *cry11* genes and ~525 bp for *cyt1* genes were observed. Sequence of *cry1* genes from isolates *Bt* C13 and *Bt* Upa (Accession numbers EU906915, EU906916) matched 99% to that of *cry1Ac* gene and *cry2* gene from *Bt* KN4 (JN257713) matched 95% to *cry2Ah* gene. Non-specific amplification or no amplification was observed for *cry4* genes and did not include in this study.

As reported earlier, Lepidoptera specific *cry1* genes were found to be the most abundant in *Bt* isolates from all soil types except red and yellow soil (Fig. 2) (Chak et al., 1994; Bravo et al., 1998; Wang et al., 2003; Chen et al., 2004; Jara et al., 2006; Jouzani et al., 2008). The *cry2* genes were found second most abundant followed by *cry11* and *cyt1* genes. Coleoptera specific *cry3* genes were observed only in isolates from alluvial soil at low percentage (14%). Isolates from red and yellow soil showed PCR amplification with primers for nematode specific *cry5, 12, 14, 21* group of genes whereas that for *cry13* gene was observed in isolates from black and red and yellow soil types. The *cyt1* gene was not found in isolates from red and yellow soil type.

The *cry1* and *cry2* combination of gene profile was found in isolates from all soil types. Isolates from four different soil types were found to be harbored different profiles of *cry* gene combinations. Genes like *cry11*, *cry3* and *cyt1* were found along with *cry1* genes. Isolates from alluvial soil contained most diverse profiles of *cry* genes. Gene profiles *cry1-cyt1* and *cry2-cyt1* was identified in isolates from alluvial soil. The *cry1-cry2-cry11-cyt1* gene profile was found unique in isolates *Bt* MP2 and *Bt* MP10 from black soil, while *cry1-cry3,7,8-cry11-cyt1* gene profile was unique to isolates from alluvial soil. The *cry1-cry3,7,8-cry11-cyt1* gene profile was found unique in isolates from alluvial soil, while *cry1-cry2-cry11-cyt1* gene profile was unique to isolates from black soil. Isolates from river sedimentary soil harbored unique profiles of *cry1-cry2-cry11* and *cry1-cry2-cyt1*. Overall eleven different *cry* gene profiles were observed from four soil types.

3.2. Distribution and diversity of cry genes

Isolates from four soil types showed heterogeneous distribution of *cry* genes. Isolates from alluvial soil harbored maximum while isolates from red and yellow soil contained minimum number of *cry* genes and *cry* gene profiles (Fig. 2). Alluvial soil represented the highest rarefaction curve indicating the degree of *cry* gene diversity among the isolates used in present study was more than other soil types (Fig. 3). Isolates from red and yellow soil showed lowest degree of *cry* gene diversity. A plateau in rarefaction curves (asymptote) denoting full diversity coverage, was not reached for any of the soil types.