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COMPARISON OF DNA FINGERPRINTING ANALYSIS FOR IDENTIFICATION OF *BACILLUS* SPECIES

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ABSTARCT:

Identification of *Bacillus* species using conventional sequencing methods can divulge their taxonomic affiliation but there are certain groups of *Bacillus* where alternate methods like ARDRA and PCR fingerprinting canexpose their exact lineage of the species rapidly.In this study, a collection of 171 soil bacterial isolates was analyzed for the occurrence of genus *Bacillus* using group specific primers.Amplified Ribosomal DNA Restriction Analysis (ARDRA)and rep-PCR fingerprinting were performed for the *Bacillus* positive isolates and standard *Bacillus* strains.Cluster analysis of these two fingerprintingpatterns revealed the grouping of isolates with *B. thuringiensis, B. megaterium*, *B. pumilus* and *B. subtilis*.Sequencing of selective isolatesshowed that the identification of*Bacillus* upto species level,isalso possible with DNA fingerprinting techniques rapidly and efficientlyespecially in the case of *Bacillus megaterium*. Compared to ARDRA, rep-PCR fingerprinting has the potential to be developed into a rapid method to identify *Bacillus* species.

Keywords: *Bacillus*; ARDRA; rep-PCR.

1. INTRODUCTION

The genus *Bacillus* are ubiquitous in nature, having been isolated from environments as diverse as freshwater, saline water, soil, plants, animals and air [Pignatelli *et al.*(2009)]. The *Bacillus* group comprises numerous species of industrial, biotechnological and environmental significance, besides clinical value, various studies have been made on its genetic diversity [Freitas *et al.*(2008)]. The *Bacillus* group were one of the first bacteria that were characterized, however their relationships to one another remain incomprehensible [Maughan and Van der Auwera (2011)]. Limitations in phenotypic variance while identifying the bacteria, have led to the development of molecular techniques based on the bacterial genotype [Olive and Bean (1999)].

Comparison of the 16S rRNA gene sequences is one of the most powerful tools for the classification of microorganisms [Woese *et al.*(1990)]. Use of group specific primers for the identification of pure cultures of *Bacillus* species such as *B. subtilis* [Wattiau *et al.*(2001)] *B. cereus* and *B. thuringiensis* [Hansen *et al.*(2001)] and *Paenibacillus alvei* [Djordjevic *et al.*(2000)] have been explored to the limited extent including clinically important species. However, environmental samples such as sewage, water, soil, and feces usually contain mixtures of *Bacillus* species. Presence of such clusters can be detected with the use of a group-specific primer that discriminates as many member species as possible within the genus. Although genus-specific primers have been successfully developed for many bacteria, primers capable of amplifying a specific sequence of 16S rDNA from all *Bacillus* taxa has not been developed [Wu *et al.*(2006)].

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In this study, we have focused on a group of soil bacterial isolates that were capable of growing aerobically on nutrient agar between 25–45°C. The genus *Bacillus* includes Gram-positive rod-shaped, endospore-forming aerobic or facultative anaerobic bacteria [Ash *et al.*(1991)] and thus a group-specific primer pair that was designed to amplify the 16S rRNA gene of *Bacillus* taxa were used, which generated an 1114 bp amplicon but did not amplify 16S rRNA gene ofother non-*Bacillus* species [Freitas *et al.*(2008)]. The specificity of our target PCR primers was also validated against related Gram-positive but non-*Bacillus* species. *Bacillus* species identification was carried out by, ARDRA of the 1114bp PCR product along with rep-PCR fingerprinting using BOX-A1R primer and comparing them with the standard strains.

2. MATERIAL AND METHODS

2.1. *Sampling and Bacterial isolates*

Bacteria subjected for this study were isolated from soil samples from Tamil Nadu and Kerala regions of Western Ghats. The type strains of *Bacillus* were obtained from NRRL, USA.

2.2. *Genomic DNA isolation and Bacillus group specific PCR amplification*

Genomic DNA was extracted from bacterial cultures grown in LB broth (Hi Media, India) using standard protocol [Sambrook *et al.*(1989)]. The internal regions of 16S rRNA genes starting at position 255 and position 1350 (*E. coli* 16S rRNA gene numbering) were amplified using a 19-mer forward primer B-K1/F (5` TCA CCA AGG CRA CGA TGC G 3`) and an 18-mer reverse primer B-K1/R (5`CGT ATT CAC CGC GGC ATG -3`) [Wu *et al.* (2006)]. Universal Eubacterial primers 27F and 1492R [Lane *et al.*(1985)] were used as a PCR control to ensure the quality of the template DNA. The oligonucleotide primers were synthesized commercially (IDT, Bangalore). Amplification was carried out using a Master thermal cycler (Eppendorf, Germany) with a reaction volume of 20 µl. A 2 µl sample of DNA template was added to a mixture containing 4 mM dNTP's, 10 X buffer solution, 15 mM $MgCl_2$, 10 pmol of each primer (B-K1/ F and B-K1/R1) and 1 U of Taq DNA polymerase. Each PCR program was conducted using a denaturation step of 3 min at 94°C, followed by 25 cycles of 94°C for 30 s, 63°C for 30 s and 72 °C for 2 min, with a final extension step at 72 °C for 10 min. Products were separated by electrophoresis in 1.5 % agarose in 1 X TAE buffer for 1 h at 65 V, and visualized by staining with Ethidium Bromide (0.5 mg/mL). The fingerprints generated were compared manually.

2.3. Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The 16S rRNA gene product of 1114 bp was digested with 3 U of *Msp*I restriction enzyme in a total volume of 10 µl for 4-16 h at 37°C. The restricted products were electrophoresed in 2% agarose gel. [Divya *et al.*(2010)]. The fingerprints generated for isolates and type strains were compared manually.

2.4. rep-PCR using BOX-A1R primer analysis

The BOX element (BOX1A) was amplified using the BOX-A1R primer 5'-CTACGGCAAGGCGACGCTGACG-3' [Versalovic *et al.*(1991)]. Amplification was carried out using a

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Master thermal cycler (Eppendorf, Germany) with 10X Taq buffer, 15 mM $MgCl_2$, 4 mM dNTPs, Taq polymerase 1.0 U and primers at 10 pmol concentration, template DNA 50-100 ng (Initial denaturation at 96^{o}C for 5 min, denaturation at 94^{o}C for 1 min, annealing at 52^{o}C for 1 min, Extension at 72^{o}C for 1 min, repeated for 40 cycles and final extension of 72^{o}C for 10 min). Products were separated by electrophoresis in 2% agarose in 1X TAE buffer for 1 h at 65 V, and visualized by staining with Ethidium Bromide (0.5 mg/mL). The fingerprints generated were compared manually.

2.5. Cluster analysis

For cluster analysis, the data were converted to a binary matrix, where the digits "1" and "0" represent the presence or absence of DNA band separately. The similarity matrix was generated by Euclidean distances, which were used to build a tree with the unweighted pair group mean averages (UPGMA) algorithm. Analysis of data was performed using NTsys software (Version 2.1).

3. RESULTS

3.1. Group specific primer validation

The group specific primers (B-K1/F and B-K1/R) designed earlier by [Wu *et al.*(2006)] for *Bacillus* group amplification was validated against a set of *Bacillus* and non-*Bacillus* species (Table 1). The 16S rRNA gene sequences of reference strains retrieved from GenBank were used in the multiple alignments, which displayed the mismatch in their sequence towards the primer sequence (Fig. 1a & b). This confirms that the group specific primers amplify only *Bacillus* genus.

Table 1. List of *Bacillus* and non-*Bacillus* genera providing the 16S rRNA gene sequences from GenBank for the validation of group specific primers

Genera	Species
Bacillus	*B. subtilis, B. acidiceler, B. anthracis, B. cereus, B. flexus, B.megaterium, B. pumilus, B. safensis, B.simplex, B. subtilis, B. thuringiensis, B. sphaericus*
non-*Bacilllus*	*Agrobacterium tumefaciens, Brachybacterium rhamnosum, Micrococcus luteus, Pseudomonas stutzeri, Staphylococcus sciuri, Stenotrophomonas maltipholia*

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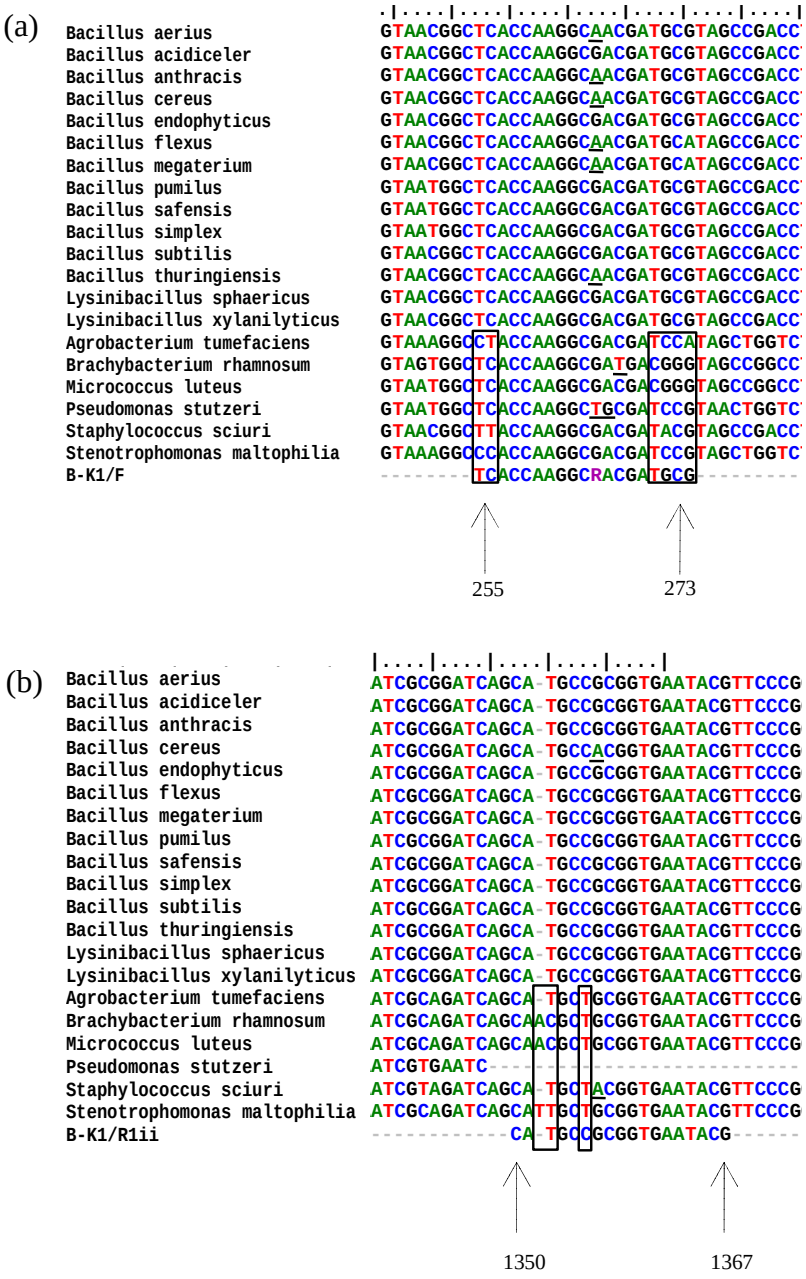

Fig. 1. (a) Alignment of primer B-K1/F with homologous target sequences of 16S rDNA (*E. coli* numbering scheme—nucleotides 255 and 273) from *Bacillus*, *Lysinibacillus* and other genera, sourced from GenBank. (b) Alignment of primer B-K1/R1 with homologous target sequences of 16S rDNA (*E. coli* numbering scheme nucleotides 1350 and 1368). The box inset shows the difference in nucleotide sequencebetween *Bacillus* and non-*Bacillus*.

3.2. PCR amplification using group specific primer

The results of PCR amplification against 255F and 1350R primers yielded an 1114bp product. Among the 171 isolates screened, 76 isolates showed positive for *Bacillus*, identified by the presence of band (Fig. 2), whereas absence of band indicates that the isolate may be non-*Bacilli*.

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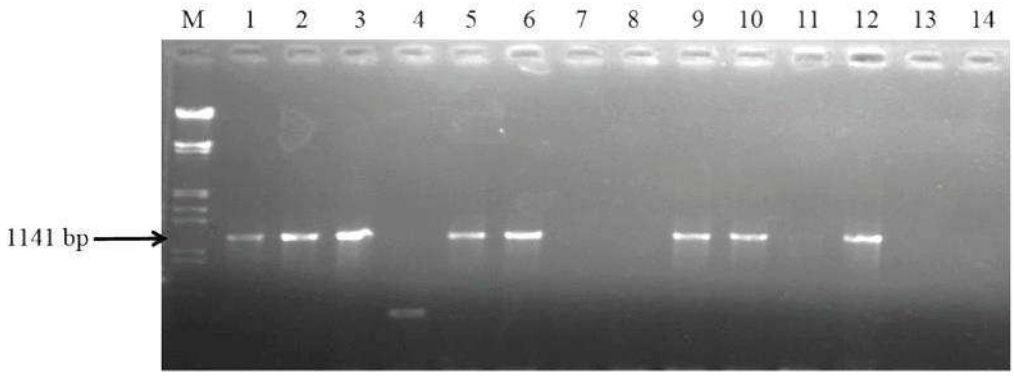

Fig. 2. Representative picture for *Bacillus* genus amplification using group specific primers. Lane M Marker - Lambda/*Eco*RI/*Hind*III digest; Lane 1 - BVP2; Lane 2 - BVP3; Lane 3 - BVP4; Lane 4 - BVP5; Lane 5 - BVP7; Lane 6 - BVP8; Lane 7 - BVP9; Lane 8 - BVP11; Lane 9 - BVP12; Lane 10 - BVP14; Lane 11 - BVP16; Lane 12 - BVP18; Lane 13 - BVP19; Lane 14 - BVP20.

*3.3. ARDRA analysis using Msp*1 *enzyme*

Bacillus positive Isolates identified using group specific primer and standard strains were subjected to restriction digestion with *Msp*1 enzyme. The ARDRA patterns obtained, consistsof fewer fragments and moderate differences were observed among the samples (Fig. 3 & 4). The dendrogram obtained through NTSys-PC, showed 11 clusters (coefficient 65), each cluster consists of about 3 to 13 isolates,of these only 5 clusters aligned with standard strains.

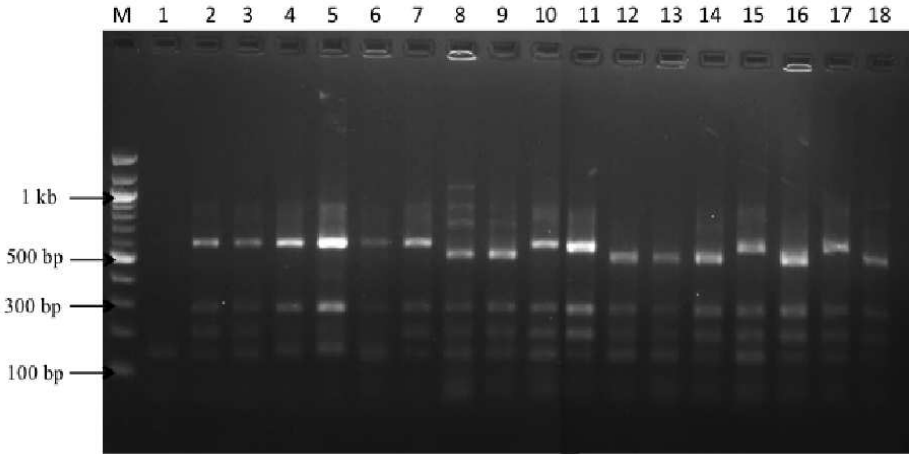

Fig. 3. Representative picture for ARDRA pattern of isolates using *Msp*1 enzyme. Lane M - 100 bp Marker; Lane 1 - BWG2; Lane 2 - BVS1; Lane 3 - BKB12; Lane 4 - BKA4; Lane 5 - BMU20; Lane 6 - BKB14; Lane 7 - BBKB2; Lane 8 - BVP7; Lane 9 - BVP8; Lane 10 - BVP4; Lane 11 – *B. thuringiensis*; Lane 12 – *B. niacin*; Lane 13 – *B. sphaericus*; Lane 14 - *B. subtilis*; Lane 15 – *B. megaterium*; Lane 16 – *B. badius*; Lane 17 – *B. pumilus;* Lane 18 – BKB10.

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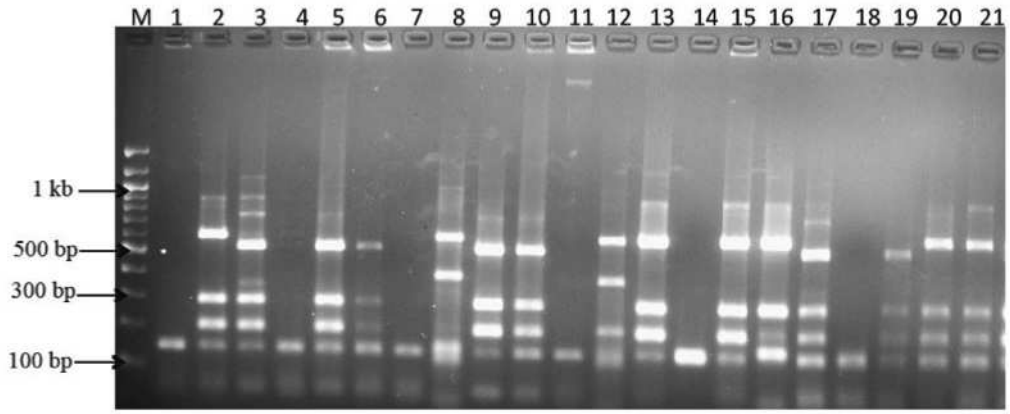

Fig. 4. Representative picture for ARDRA pattern of isolates using *Msp*1 enzyme. Lane 1 - BMU14; Lane 2 - BMU15; Lane 3 - BMA7; Lane 4 - BMU13; Lane 5 - BMU7; Lane 6 - BMA6; Lane 7 - BMA5; Lane 8 - BMA10; Lane 9 - BMA8; Lane 10 - BMU11; Lane 11 - BKS13; Lane 12 - BKS8; Lane 13 - BKS6; Lane 14 - BKS5; Lane 15 - BKS14; Lane 16 - BVP14; Lane 17 - BVP2; Lane 18 - BVS7; Lane 19 - BMU16; Lane 20 - BMU6; Lane 21 - BMU2.

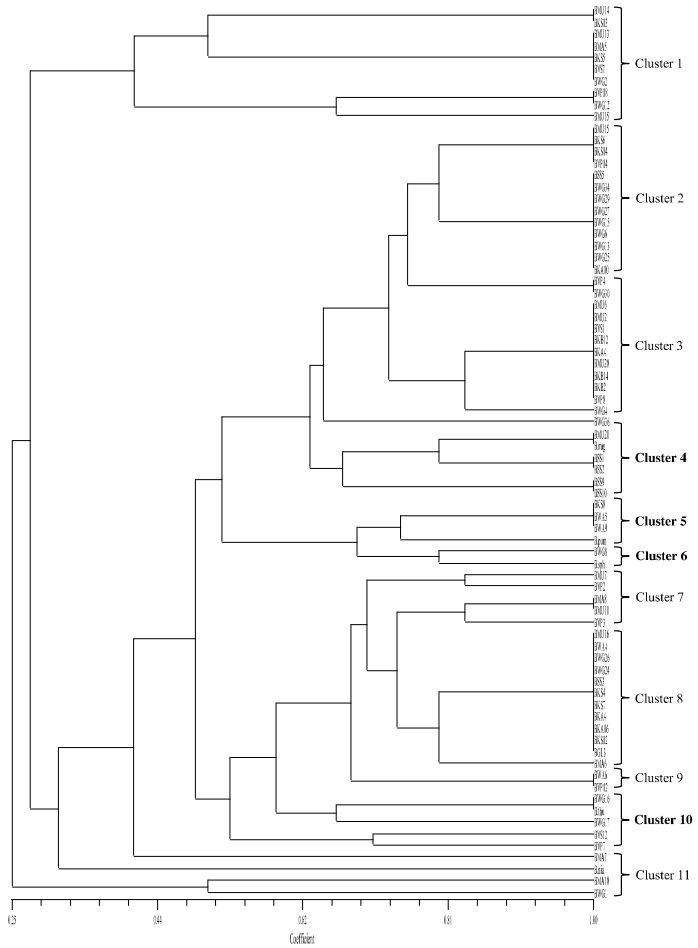

Fig. 5. Similarity Jaccords's Coefficient – UPGMA based dendrogram showing cluster analysis of soil bacterial isolates on the basis of ARDRA using *Msp*1 enzyme

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Table 2. Bacterial isolates clustered against standard *Bacillus* strains after ARDRA using *Msp*1 enzyme

Standard strain	Isolates
Bacillus thuringiensis	BWGA16, BWGA17, BVS12, BVP7
Bacillus pumilus	BWA5, BWA9, BKS8
Bacillus megaterium	BMU21, BSS1, BSS2, BSS9 & BSS10
Bacillus sphaericus	BWGA8

ARDRA analysis using *Msp*1 enzyme was able to group the *Bacillus* positive isolates with standard strains especially *B. megaterium* (n = 5) followed by *B. thuringiensis* (n = 4), *B. pumilus* (n = 3) and then *B. sphaericus* (n = 1) (Table 2). The success of ARDRA technique relies on the selection of appropriate restriction enzyme combination for the analysis of the group of interest. This clearly validates ARDRA method with respect to its main taxonomic level [Heyndrickx *et al.*(1996)]. Therefore, ARDRA at the outset of a taxonomic study is recommended to obtain indicative phylogenetic and taxonomic information, which can be used to select strains for detailed polyphasic taxonomic studies.

3.4. rep-PCR analysis using BOX-A1R primer

DNA Fingerprinting analysis using rep-PCR of BOX1A element has been used in many organisms to understand its phylogenetic relationships among isolates, and to study their diversity in a variety of environments [Cherif *et al.* (2003)]. The fingerprinting patterns for the isolates in our study gave large number of polymorphic bands of variable intensity. The bands ranged between 100-1500 bp. The reference strains fingerprinting profiles matched with severalof the *Bacillus* positive isolates.

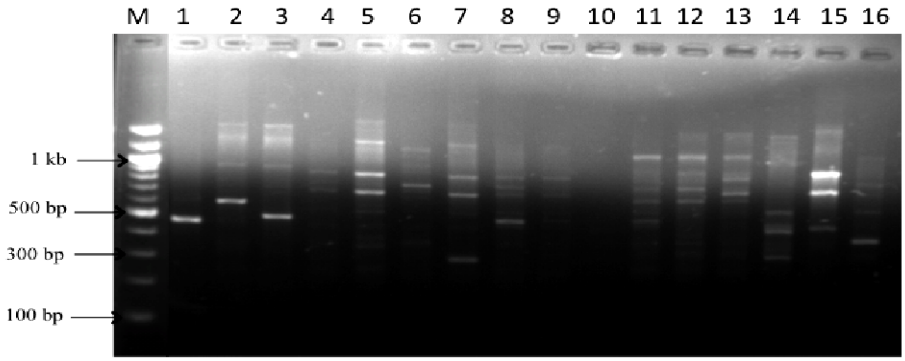

Fig. 6. Representative picture for rep-PCR using BOX-A1R primer for *Bacillus* positive isolates. Lane M - Marker; Lane 1 - BGL20; Lane 2 - BSS14; Lane 3 - BSS3; Lane 4 - BWGA1; Lane 5 - BWGA2; Lane 6 - BWGA3; Lane 7 - BWGA4; Lane 8 - BWGA8; Lane 9 - *B. subtilis*; Lane 10 –BWGB30; Lane 11 - *B. thuringiensis*; Lane 12 – *B. niacin*; Lane 13 - *B. sphaericus*; Lane 14 – *B. megaterium*; Lane 15 - *B. pumilus*; Lane 16 - *B. badius*

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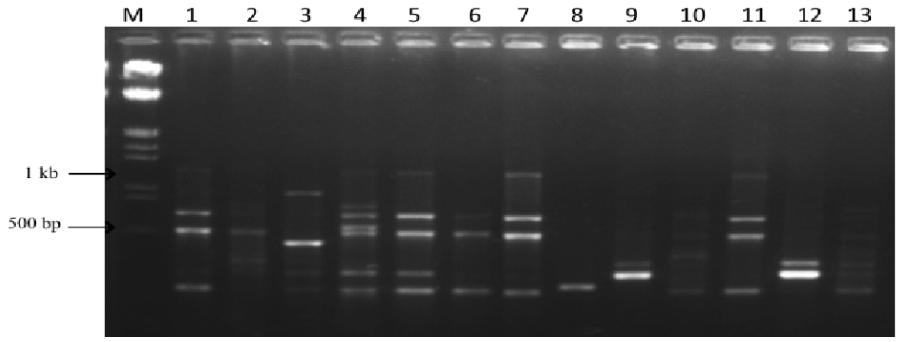

Fig. 7. Representative picture for rep-PCR using BOX-A1R primer for *Bacillus* positive isolates. Lane M - Marker; Lane 1 - BMU9; Lane 2 - BMU10; Lane 3 - BMA2; Lane 4 - BMA5; Lane 5 - BMA6; Lane 6 - BMA7; Lane 7 – BMA13; Lane 8 – BMA14; Lane 9 - BMA15; Lane 10 - BMA16; Lane 11 - BWA15; Lane 12 - BWA16; Lane 13 - BGL3

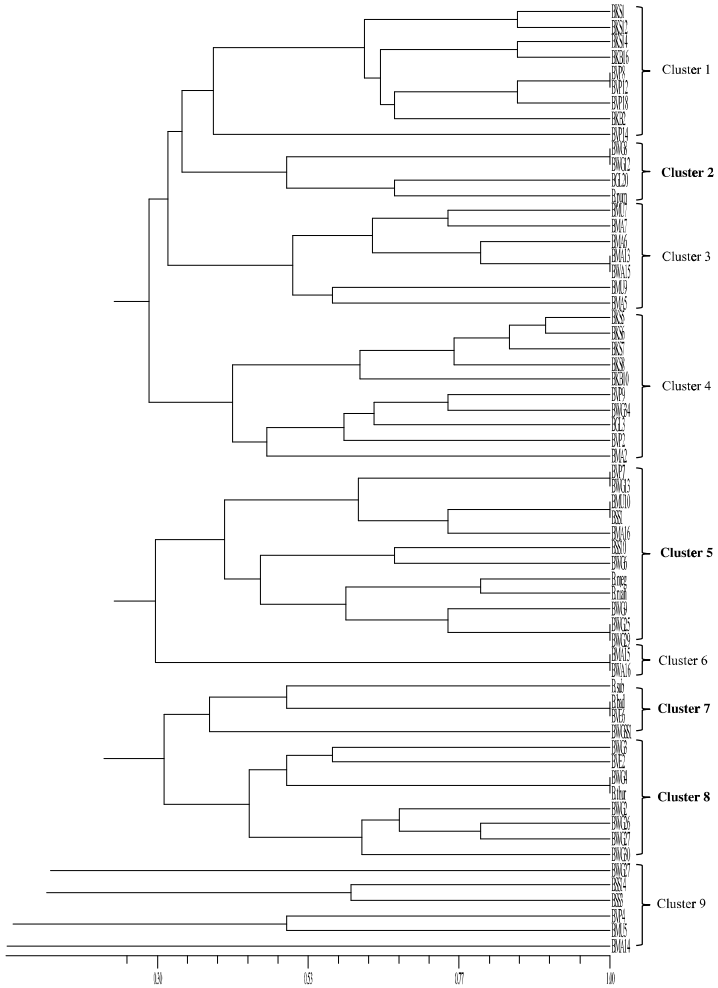

Fig. 8. Similarity Jaccords's Coefficient – UPGMA based dendrogram showing cluster analysis of soil bacterial isolates on the basis of rep-PCR using BOX-A1R primer

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Banding patterns of rep-PCR of BOX1A element amplified an adequate number of bands [de Bruijn (1992)], indicating that they are applicable to discriminate *Bacillus* species. The dendrogram based on BOX-PCR fingerprint analysis (Fig. 8) showed 9 clusters (coefficient 65), each having 2 to 10 isolates.

Among these, 4 clusters included standard strains along with other isolates that have formed cluster. Majority of the isolates clustered with *B. megaterium* (Cluster 5; n = 10)and *B. thuringiensis* (Cluster 8; n = 7), and few clustered with *B. pumilus* (Cluster 2; n = 3)and *B. subtilis* (Cluster 7; n = 2). The rest of the isolates did not match with any of the standard strains, indicating that these isolates may belong to some other species of *Bacillus*.

Table 3. Bacterial isolates clustered against standard *Bacillus* strains after rep-PCR using BOX-A1R primer

Standard strain	**Isolates**
Bacillus thuringiensis	BWGA3, BWGA4, BVE2, BWG2, BWG26, BWG27 & BWG30
Bacillus megaterium	BWGA9, BWGB25, BWGB29, BWG9, BWG13, BVP7, BSS1, BSS10 & BMA16
Bacillus pumilus	BWGA8, BWGA12, BGL20
Bacillus subtilis	BVE6, BSS1

The results revealed that *B. thuringiensis, B. megaterium and B. pumilus* could be detected from the two fingerprinting analysis while the detection of other species of *Bacillus*needs combination of the techniques used. Previous reports also suggests that rep-PCR protocols as well as ARDRA techniques have been applied in the closely related *Bacillus* genus exhibiting high sensitivity in discrimination at the strain level for mesophilic and thermophilic species [Ronimus *et al.*(1997); Mora *et al.*(1998); Giuliano *et al.*(1999)].

By 16S rRNA gene sequence analysis, the isolates used for the study were identified as *Bacillus*. The results of sequencing matched with that of the fingerprinting analysis upto species level. 16S rDNA based taxonomy is thegold standard method except in the case of closely related species groups such as *Bacillus*, where insufficient divergence in 16S rRNA gene prevents the resolution of strain and species relationships [Maughan and Van der Auwera,(2011)]. Further, it has often been proved to show limited variation for the discrimination of closely related taxa and strains [Nubel *et al.*(1996)].

It is unlikely that truly relevant data will be obtained by present culturing methods, thus fingerprinting can be used for initial screeningfollowed by sequencing of 16S rRNA gene and identification of closely related strains/species, which could then be followed by genomic characterization of isolates of our interest. Recently such work has been done in *B. subtilis* using microarray and sequencing technologies and has uncovered a great deal of genomic diversity within this group of closely related *B. subtilis* strains [Earl *et al.*(2007), (2008)].

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Fingerprinting analysis coupled with advanced sequencing technologies should enable the identification and also clear discrimination of species belonging to the genus *Bacillus*.

4. CONCLUSION

Between the two DNA fingerprinting methods, rep-PCRproduced differential fingerprinting patterns and were able to identify a relatively large number of isolates than ARDRA. In conclusion, it has been shown that rep-PCR fingerprinting analysis will be a useful and a rapid tool to identify isolates upto species level belonging to *Bacillus* group.

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