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Genetic Diversity of Plant Growth Promoting Rhizobacteria of *Bacillus* sp. Based on 16S rRNA Sequence and Amplified rDNA Restriction Analysis

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Plant-growth promoting rhizobacteria (PGPR) are rhizosphere associated soil-borne bacteria that can enhance plant growth and inhibit the development of root pathogens. Many soil bacteria have been used as PGPR, and one of them is *Bacillus* sp. The implementation of PGPR is constrained by genotype fluctuation that makes it inactive on the rhizosphere. Our previous study had characterized and revealed that 11 *Bacillus* sp. isolated from the soybean plant rhizosphere were PGPR. To asses and compare the genetic diversity of these isolates, Amplified Ribosomal DNA Restriction Analysis (ARDRA) and DNA sequence analysis of 16S rRNA were conducted. The construction of Neighbor-joining trees and bootstrap analysis of 100 resamples of ARDRA and 16S rRNA gene sequences were performed using Treecon software for windows ver. 1.3b. ARDRA analysis was done by using four restriction enzymes (*Rsa*I, *Hae*III, *Cfr*I and *Hinf*I), resulting in four phylotypes, respectively phylotype I (*Bacillus* sp. Cr24, Cr33, Cr64 and Cr68), phylotype II (*Bacillus* sp. Cr 31 and Cr66), phylotype III (*Bacillus* sp. Cr44 and Cr71) and phylotype IV (*Bacillus* sp. Cr67, Cr28 and Cr69). Results of BLASTN from 16S rRNA gene sequences showed that these isolates are genetically diversed. The evolution relationship of *Bacillus* sp. could be shown by the 16S rRNA gene sequences analysis, while ARDRA based on the digestion sites showed their variability.

Key words: *Bacillus* sp., plant-growth promoting rhizobacteria, 16S rDNA, ARDRA, genetic diversity

The slow growth of plant is a major handicap that can decrease food production and ecosystem stability. Synthetic fertilizers have been used to solve this problem. However, it can give negative effects on human and environment, such as pollution of the surrounding ecosystem and the death of non-target microbes. Therefore, efforts to find alternative solutions, among others by using plant-growth promoting rhizobacteria (PGPR), i.e. associated soil-borne bacteria on the rizosphere that can enhance plant growth and inhibit the growth of root pathogens, such as *Bacillus* sp.

Bacillus is a motile, catalase-positive, Gram-positive rod, with 40-60% GC content. It forms endospore that is very resistant to extreme environmental conditions. *Bacillus* has been known for the production of phytohormone such as indole acetic acid (Glick 1995), siderophores (Compant *et al*. 2005) and antibiotics such as zwittermicin A (Silo-suh *et al*. 1994), bacilin, clorotetain and Iturin A (Phister *et al*. 2004). These compounds are natural and beneficial to promote plant growth. Hence, it is potential to utilize those mechanisms in agriculture. Its implementation, however, is constrained by the genotype fluctuation.

The 16S rRNA gene has been routinely used as a reliable molecular marker for phylogeny identification. It contains conserved region, a unique array of sequences that are relative among species or different species (Woose 1987; Moyer *et al*. 1994). It is the basis of molecular tools such as ribotyping, *in-situ* hybridization, DNA sequence analysis and restriction fragment length polymorphism (RFLP), which are now proposed to provide accurate genetic diversity information of microbes. Based on the use of the 16S rRNA, the DNA sequence analysis is used in phylogenic studies (Lagace *et al*. 2004). RFLP is used to identify the difference of DNA fragment length (polymorphism) by digesting with restriction enzymes. RFLP analysis on 16S rRNA gene or amplified rDNA restriction analysis (ARDRA) is a useful technique for genotype identification, to infer genetic variability and similarity of microorganisms (Yang *et al*. 2007).

This study was conducted on 11 *Bacillus* sp. isolates from the rhizosphere of soybean plant, with a focus on the analysis of their genetic diversity based on ARDRA and 16S rRNA sequences analysis.

MATERIALS AND METHODS

Bacterial Strains and Growth Condition. All plant growth promoting *Bacillus* sp. isolates were maintained in Nutrient Broth medium and grown at room temperature (28 °C) for 24 h. Characteristics of bacterial strains of *Bacillus* sp. strains used in this study are listed in Table 1.

Isolation of *Bacillus* sp. Genome. Eleven isolates of *Bacillus* sp. were cultured in 25 mL nutrient broth and incubated for 24 h at room temperature (28 °C). Genomic DNA isolation was carried out by using standard protocol as described by Sambrook and Russel (2001).

Amplification of 16S rRNA by PCR. The amplified 16S rRNA genes were obtained by PCR with forward primer 63f (5'-CAGGCCTAACACATGCAAGTC-3') and reverse primer 1387r (5'-GGGCGGWGTGTACAAGGC-3') (Marchesi *et al*. 1998), which are targeted to the conserved region of bacterial 16S rRNA genes and permit the amplification of an approximately 1300-bp fragment. The PCR mixture with a total volume of 50 µL was composed of 5 µL DNA template, 1 µL each primer (10 pmol) , 8 µL dNTPs, 25 µL *Taq* polymerase buffer, 0.5 µL LA *Taq* polymerase, and 9.5 µL ddH_2O (Takara, Japan). Amplification was done in a Thermal Cycler 2 400 (Perkin-Elmer, USA). Initial denaturation at 94 °C for 2 min was followed by 30 cycles of denaturation at 92 °C for 30 sec, annealing at 55 °C for 30 sec, and elongation at 75 °C for 1 min, then the final extension was carried out for 5 min at 75 °C. The presence and yield of specific PCR products were visualized by 1% agarose (w/v) gel electrophoresis for 45 min at 70 V cm^{-1} in TAE 1X buffer. Amplicons were further purified

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Table 1 The characteristics of eleven plant growth promoting of *Bacillus* sp. isolates (unpublished data)

Strain	IAA Production (ppm)	Phosphate solubilization	Siderophore production	Chitinase production
Cr24	15.16	+	+	-
Cr28	12.16	+	+	-
Cr31	5.45	+	+	-
Cr33	3.25	+	+	+
Cr44	3.73	+	+	-
Cr64	7.56	+	+	+
Cr66	3.02	+	+	-
Cr67	0.81	-	+	-
Cr68	0.87	+	+	+
Cr69	4.32	+	+	+
Cr71	9.63	+	+	-

IAA, indole acetic acid

by Gel/PCR DNA fragments extraction kit according to the manufacturer's instructions (Geneaid, USA).

ARDRA. Each purified PCR products of 16S rDNA were digested by four restriction enzymes, *Rsa*I, *Hae*III, *Cfr*I and *Hinf*I in separated reaction. The DNA digestion were performed for 3 h at 37 °C in 20 µL of reaction volumes containing 5 µL of amplicon (1.5 µg), 2 µL of buffer Tango 10X, ddH_2O and 2 Units of the restriction enzyme (Fermentas, USA). Restriction was inactivated by heating at 65 °C for 20 min. Restriction products were electrophoresed on a 1% agarose (w/v) gel electrophoresis in TAE 1X buffer. The sizes of the fragments were converted into binary data and analyzed by using Treecon software for Windows ver. 1.3b (van de Peer and de Watcher 1994).

Sequence Analysis of 16S rRNA Gene. The PCR products of 16S rDNA were purified by Gel/PCR DNA fragments extraction kit (Geneaid, USA), sequenced and further analyzed for bioinformatics analysis. The BLASTN program (www.ncbi.nlm.nih.gov) was used to find the identity and similarity of each sequences compared to the GenBank database. Furthermore, the ClustalW program (www.ebi.ac.uk) was used in order to align those sequences. The construction of Neighbor-joining tree and bootstrap analysis of 100 resamples were performed using Treecon software for Windows ver 1.3b (van de Peer and de Watcher 1994).

RESULTS

Amplification of 16S rRNA by PCR. PCR amplification of 16S rRNA gene yielded DNA fragments of single bands at 1300 base pairs for each *Bacillus* sp. isolates (Fig 1). These amplified DNA can be used as a genetic tool to identify and classify the diversity of *Bacillus* sp.

ARDRA. Four restriction enzymes (*Hae*III, *Rsa*I, *Cfr*I and *Hinf*I) resulted in small variability of digestion profiles for each *Bacillus* isolates. The length of DNA fragment that were obtained from digestion by four restriction enzymes are shown in Table 2. According to the digestion profiles, those *Bacillus* sp. isolates were grouped into four different phylotypes as follows: phylotype I consisted of *Bacillus* sp. Cr24, Cr33, Cr64 and Cr68; phylotype II consisted of *Bacillus* sp. Cr31 and Cr66; phylotype III consisted of *Bacillus* sp. Cr44 and Cr71; and phylotype IV consisted of *Bacillus* sp. Cr67, Cr28, and Cr69. Most of the isolates (four isolates: Cr71, Cr44, Cr66 and Cr31) were found to be distributed within the phylotype I (Fig 2), which mean their digestion sites have been evolving with the same direction.

Partial Sequencing of 16S rRNA Gene and Sequence Analysis. There was no dominant species within 11 isolates of *Bacillus* sp. PGPR based on bioinformatics analysis using BLASTN program (Table 3). Maximum identities for each isolate were more than 85% with E-value 0. The distributions were genetically diversed on several species of *Bacillus* sp., such as *B. subtilis*, *B. shandongensis*, *B. pumilus*, *B. cereus* and *B. thuringiensis*. The phylogenetic tree based on 16S rRNA gene partial sequences showed the evolutionary relationship among the isolates (Fig 3). The phylogenetic tree exhibited that the 11 isolates were divided into three major groups. Group I (Cr66, Cr33, Cr24, Cr 69 and Cr 68); Group II (Cr64 and Cr71); and Group III (Cr44). Isolate Cr44 was closely related to the reference strains. There was a significant difference between groups (phylotypes) of the isolates based on ARDRA compared to those obtained by phylogenetic tree analysis.

DISCUSSION

Amplified rDNA restriction analysis (ARDRA) is a method to analyze 16S rRNA gene fragments that are produced by digestion enzymes. This method has been used for genetic analysis and diversity studies of many bacteria such as *Streptococcus* (Sasaki *et al*. 2004), *Lactobacillus* (Moreira *et al*. 2005), *Mycobacterium* (Baere *et al*. 2002; Kurabachew *et al*. 2003), and type A toxin-producing *Clostridium* (Pooeshafie *et al*. 2005). Furthermore, Vaneechoutte *et al*. (1995) revealed that ARDRA can avoid DNA contamination in pure culture. The restriction profiles produced by ARDRA can be used as a robust library for particular species (Hall *et al*. 2001).

In this study, the digestion of 16S rDNA of *Bacillus* sp. by *Hae*III, *Hinf*I, *Rsa*I and *Cfr*I produced 1 to 3 bands for each treatment (Table 2). Genetic diversity of *Bacillus* sp. species as revealed by ARDRA was low. In fact, a number of studies reported that there was no intraspecies variability and diversity (Schlegel *et al*. 2003). The best explanation for this condition was that they had the same digestion sites in their conserved region. This has been proven by means of *Rsa*I digestion that showed homogenous pattern for all isolates with no diversity at all (Table 2). Meanwhile, the digestions by the other 3 restriction enzymes produced small diversity. It means, genetically, the isolates were evolving with the same direction.

This study has also shown that some isolates were closely related and grouped each other as exhibited in the phylogenetic tree. Isolates in the same phylotype according

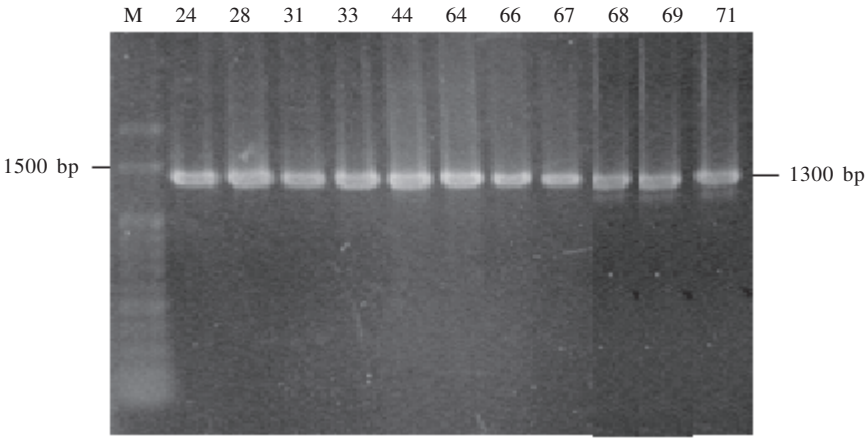

Fig 1 PCR amplification of the 16S rRNA of each *Bacillus* sp. indicated by a single band at ~1300 bp. Marker (M): 100 bp ladder.

Table 2 The sizes of 16S rDNA fragments resulted from digestion with four restriction enzymes, *Hae*III, *Rsa*I, *Hinf*I and *Cfr*I

Isolate	The size of DNA fragment (bp)			
	Hae III	*Rsa*I	*Hinf*I	*Cfr*I
Bacillus sp. Cr24	600, 500, 200	650, 650	1000, 300	550, 550, 200
Bacillus sp. Cr33	600, 500, 200	650, 650	1000, 300	550, 550, 200
Bacillus sp. Cr64	600, 500, 200	650, 650	1000, 300	550, 550, 200
Bacillus sp. Cr68	600, 500, 200	650, 650	1000, 300	550, 550, 200
Bacillus sp. Cr31	700, 600	650, 650	1000, 300	1100, 200
Bacillus sp. Cr66	700, 600	650, 650	1000, 300	1100, 200
Bacillus sp. Cr44	600, 500, 200	650, 650	700, 300, 300	1100, 200
Bacillus sp. Cr71	600, 500, 200	650, 650	700, 300, 300	1100, 200
Bacillus sp. Cr28	620, 480, 200	650, 650	1000, 300	550, 550, 200
Bacillus sp. Cr67	620, 480, 200	650, 650	1000, 300	550, 500, 200
Bacillus sp. Cr69	620, 480, 200	650, 650	1000, 300	550, 500, 200

Phylotype I (*Bacillus* sp. Cr24, Cr33, Cr64 and Cr68), phylotype II (*Bacillus* sp. Cr31 and Cr66), phylotype III (*Bacillus* sp. Cr44 and Cr71), and phylotype IV (*Bacillus* sp. Cr67, Cr 28 and Cr69)

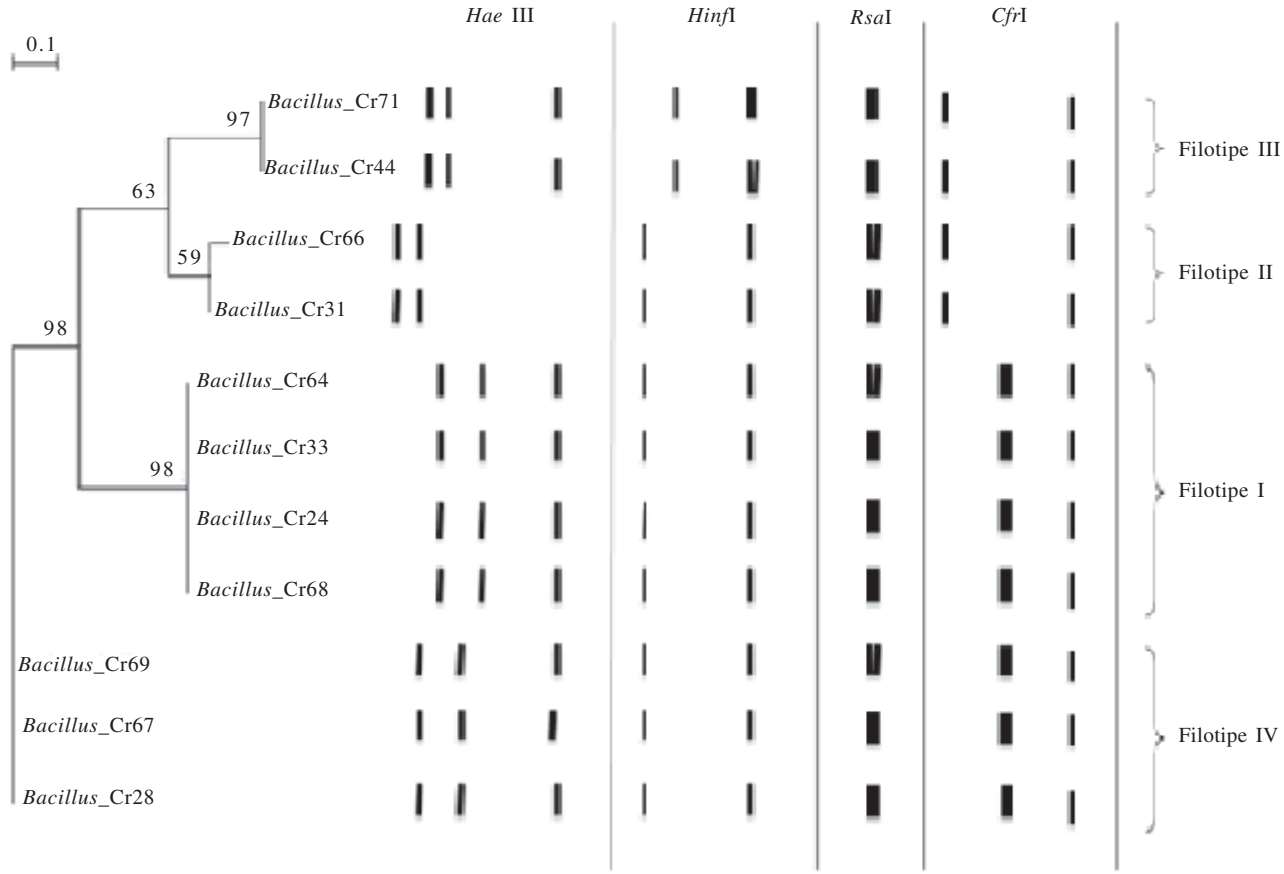

Fig 2 Dendrogram of phylogenetic and electrophoregram of *Bacillus* sp. isolates by ARDRA. The dendrogram was constructed with Treecon software for windows ver. 1.3b and grouped by Neighbor-joining method with bootstrap analysis of 100 resamples.

Table 3 Similarity analysis based on 16S rRNA partial gene sequences of each 11 isolates *Bacillus* sp. by comparing to the GenBank data

Isolate	Homology	Maximum Identity	Access Num.
Bacillus sp. Cr24	*Bacillus* sp. 1Re28	94%	EF178451.1
Bacillus sp. Cr28	*Bacillus pumilus* strain S2	91%	EF439667.1
Bacillus sp. Cr31	*Bacillus* sp. B-3(2008)	98%	EU862293.1
Bacillus sp. Cr33	*Bacillus cereus* strain IBT016	95%	AY296806.2
Bacillus sp. Cr44	*Bacillus subtilis* strain CICC10166	92%	DQ055129.1
Bacillus sp. Cr64	*Bacillus sphaericus* strain NUC-5 16	85%	DQ833758.1
Bacillus sp. Cr66	*Bacillus thuringiensis* strain W64	94%	EU874887.1
Bacillus sp. Cr67	*Bacillus pumilus*	94%	AB048252.1
Bacillus sp. Cr68	*Bacillus* sp. NIOT-3	94%	AM981260.1
Bacillus sp. Cr69	*Bacillus cereus* strain AD2	97%	DQ298080.1
Bacillus sp. Cr71	*Bacillus shandongensis* strain SD	99%	EU046267.1

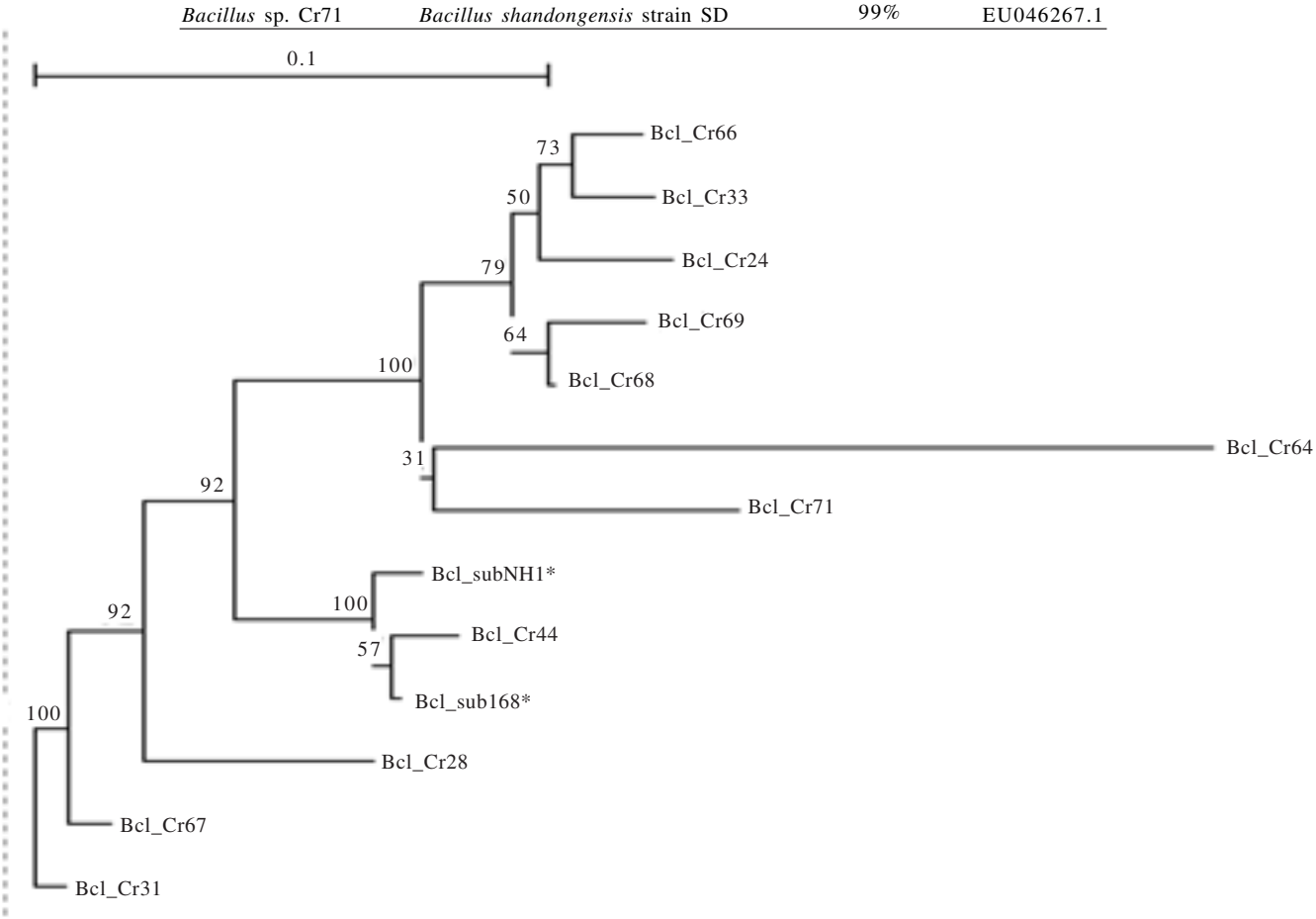

Fig 3 Dendrogram of 11 *Bacillus* sp. isolates based on partial sequences 16S rRNA gene constructed with Treecon software for windows ver. 1.3b and grouped by Neighbor-joining method with bootstrap analysis of 100 resampling. Bcl, *Bacillus*; *reference strains.

to the ARDRA profile would emerge in different groups in the phylogenetic tree. It might be due to the difference of the methods of analysis. For example, Cr68 and Cr69 were closely related to each other as shown in the phylogenetic tree, but these two isolates were separated from each other in the ARDRA profile. Cr68 belongs to the phylotype I, whereas Cr69 belongs to the phylotype IV. ARDRA profiles would much depend on the utilization of restriction enzymes. From the genotypic approach point of view we suggest the use of 16S ARDRA technique as tool to initially characterize *Bacillus* sp. isolates and the use of total DNA restriction profile and other molecular approaches, such as fragment selective amplification of chromosomal DNA (AFLP), for typing and singling out uncommon *Bacillus* sp. strains. The diversity within species was shown by isolate Cr33 and Cr69. These two isolates were identified as *Bacillus cereus* based on partial sequence analysis, although they were exhibited different ARDRA profile. Therefore they splited into two different phylotypes and were designated as different strains.

The similar characteristics between ARDRA and sequencing methods are actually unambiguous: a large number of characters collected from each organism and the information obtained could be used to identify the abundance and diversity of two DNA sequences. The main difference between them is the asymmetry of the evolution of restriction sites vs nucleotide positions. The 4-bp recognition site for a restriction enzyme can be inactivated by any of the 12 different nucleotide substitutions. If the sequence differs from the recognition site only at a single site, then only one substitution can occur to produce that site. As a consequence, convergent losses of a restriction site are less than convergent gains and the ratio of convergent losses to

convergent gains increases as taxa become more divergent (Moyer *et al*. 1996).

The unique digestion profiles shown for each isolate can be used for reference purpose. This study has also defined the taxonomic status at the species level of all isolates following the comprehensive examination of the DNA/DNA similarity level of *Bacillus* species type strains (GenBank data). Thus, ARDRA can be widely used to investigate or identify several *Bacillus* sp. isolates inhabiting the rhizosphere possessing plant growth promoting characters. Moreover, because of its simplicity and cheapness, ARDRA analysis will be of practical value than the more laborious sequencing analysis.

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