HAYATI Journal of Biosciences September 2010
Vol. 17 No. 3, p 145-150
EISSN: 2086-4094

Available online at:
http://journal.ipb.ac.id/index.php/hayati
DOI: 10.4308/hjb.17.3.145

Diversity of Antifungal Compounds-Producing *Bacillus* spp. Isolated from Rhizosphere of Soybean Plant Based on ARDRA and 16S rRNA

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Received March 8, 2010/Accepted August 18, 2010

Plant growth promoting rhizobacteria (PGPR) play an important role in improvement of seed germination, root development, and water utilization by plants. These rhizobacteria can stimulate plant growth directly by producing growth hormones or indirectly by producing antifungal compounds/antibiotics to suppress phytopathogenic fungi. The objective of this research was to analyze the diversity of 22 antifungal-producing rhizobacteria of *Bacillus* sp. isolated from rhizosphere of soybean plant based on Amplified rDNA Restriction Analysis (ARDRA) and 16S rRNA Sequence. Restriction enzymes in ARDRA analysis, *Hin*fI, *Hae*III, and *Rsa*I were used to digest 22 16S rDNA amplified from *Bacillus* sp. genomes. Based on this analysis, genetic diversity of 22 *Bacillus* sp. producing antifungal compounds were classified into eight different groups. Moreover, six selected isolates randomly from each ARDRA group that have strong activity to suppress fungal growth were analyzed for their 16S rDNA sequences compared with reference strains. The distributions of these isolates were genetically diverse on several species of *Bacillus* sp. such as *B. subtilis, B. cereus*, and *B. fusiformis*. ARDRA is a reliable technique to analyze genetic diversity of *Bacillus* sp. community in the rhizosphere.

Key words: *Bacillus* sp., biodiversity, ARDRA, sequence analysis, antifungal

INTRODUCTION

Utilization of antagonistic bacteria against plant pathogens in agricultural crops has been reported and proposed as a biocontrol agent to alternate chemical pesticides. *Bacillus* sp. has been known to play a role in suppression of pathogenic microbes. These bacteria enforce suppression of plant pathogen by secretion of extracellular metabolites functioning as antimicrobial compounds or antibiotics. Many antibiotics produced by *Bacillus* sp. have an important role to suppress soil borne phytopathogenic fungi.

Bacillus sp. strains have been reported to produce a broad spectrum of antibiotics such as kanosamine (Milner *et al*. 1996), Zwittermycin A (Silo-Suh *et al.* 1998), Iturin A (Phister *et al*. 2004), Bacillomycin (Moyne *et al.* 2001), and plipastins (Volpon *et al*. 2000). These antibiotics produced by *Bacillus* sp. have strong antifungal action, one of them is iturin A suppressing *Rhizoctonia solani*, *Fusarium oxysporum*, and *Sclerotium* sp. Our previous study reported that *Bacillus* strains isolated from rhizosphere of soybean plant produced antifungal compounds/antibiotics against *F. oxysporum*, *R. solani*, or *Sclerotium rolfsii* (unpublished data). One of strains is *Bacillus* sp. CR64 having zwittermycin A gene identified through cloning and sequence analysis of the *zma*R gene (Wahyudi *et al*. 2010). Molecular technique such as ARDRA and 16S rRNA gene sequences are required to study the genetic diversity of those antifungal producing-*Bacillus* strains.

The 16S rRNA gene has been widely used as a reliable molecular marker for phylogeny identification. The 16S rRNA gene contains conserved region as unique sequence which is relative among species or different species (Moyer *et al*. 1996). Restriction Fragment Length Polymorphism (RFLP) is a method which is used to determine the difference of DNA fragment length (polymorphism) digested by restriction enzymes. RFLP analysis on 16S rRNA gene is called amplified rDNA restriction analysis (ARDRA). ARDRA is a useful method for identification and can be used to infer genetic variability and similarity of microorganisms (Yang *et al*. 2008). This method has also been used for genetic analysis and diversity of many bacteria such as *Streptococcus* (Sasaki *et al*. 2004), *Lactobacillus* (Moreira *et al*. 2005), *Mycobacterium* (Kurabachew *et al*. 2003), and *Clostridium* toxinotipe A (Pooeshafie *et al*. 2005). Furthermore, a report revealed that ARDRA can also be used to identify the bacterial community of Maple Sap (Lagace *et al*. 2004).

This study was conducted on 22 *Bacillus* sp. isolates producing antifungal compounds isolated from rhizosphere of soybean plant, especially to analyze their biodiversity based on ARDRA and 16S rRNA sequence analysis.

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MATERIALS AND METHODS

Isolation of *Bacillus* sp. Genome. Twenty two rhizobacterial isolates of *Bacillus* sp. were cultured in 5 ml nutrient broth. Incubation was performed at 37 °C for 24 h on an incubator shaker at 100 rpm. The DNA genome of *Bacillus* strains was isolated according to the procedure of Sambrook and Russel (2001). The extracted DNA was treated with RNA-se and incubated at 37 °C for one hour, and stored at -20 °C. Characteristics of bacterial strains of antifungal producing-*Bacillus* sp. used in this study are shown in Table 1. The isolates were chosen to represent their ability to inhibit pathogenic fungi *F. oxysporum*, *R. solani*, and *S. rolfsii*.

Amplification of 16S rRNA Gene by PCR. The 16S rRNA gene of the *Bacillus* sp. strains were amplified by PCR using the universal primers, comprises the forward primer 63f (5'-CAGGCCTAACACATG-CAAGTC-3') and the reverse primer 1387r (5'-GGGCGGWGTGTACAAGGC-3') (Marchesi *et al*. 1998). The primers are targeted to conserved regions and permitted the amplification of an approximately 1,300 bp rDNA fragment. PCR amplification was carried out using GeneAmp PCR system 2,400 thermal cycler (PerkinElmer, USA). Total volume of PCR mixture was 50 µl which contained 2 µl DNA template (25 ng), 2 µl of each primer (10 pmol), 8 µl dNTPs, 25 µl of 2 X GC Buffer II, 0.5 µl *TaKaRa LA Taq* (5 u/ µl), and 10.5 µl ddH_2O (Takara Bio Inc., Japan). Initial denaturation at 94 °C for 4 minutes was followed by 30 cycles of denaturation at 94 °C for 2 minutes, annealing at 55 °C for 1 minutes, and polymerization at 72 °C for 1 minutes, the final extension was carried out for 10 minutes at 72 °C. The PCR products of 16S rRNA gene were visualized by 1% agarose (wt/vol) gel electrophoresis at 70 V for 45 minutes in *Tris buffer acetate EDTA* (TAE) 1X buffer. The PCR products were further purified by DNA purification Kit according to the manufacturer's instructions (Promega Corporation, USA).

Table 1. The characteristics of 22 *Bacillus* sp. strains used in this study which able to inhibit the growth of pathogenic fungi based on Wahyudi and Rachmania (2008, unpublished data)

Strains	Antagonism test*		
	SR	FO	RS
Bacillus Cr7	-	-	+++
Bacillus Cr11	-	++	++
Bacillus Cr24	-	++	+++
Bacillus Cr39	-	+	+++
Bacillus Cr40	-	+	+
Bacillus Cr42	-	+	+
Bacillus Cr43	-	+	+
Bacillus Cr44	+	++	+
Bacillus Cr45	-	+	+++
Bacillus Cr46	-	+	+
Bacillus Cr55	+++	++	+
Bacillus Cr57	-	++	++
Bacillus Cr58	-	+	+
Bacillus Cr60	-	-	+++
Bacillus Cr64	-	-	++
Bacillus Cr66	-	++	+++
Bacillus Cr67	-	-	+++
Bacillus Cr68	-	-	+++
Bacillus Cr76	-	+	+++
Bacillus Cr77	-	+	+++
Bacillus Cr78	-	-	+++
Bacillus Cr79	+	++	+

*On antagonism test, the level of inhibition activity of 22 *Bacillus* sp. strain Cr toward fungi were divided into four classes which are showed by symbols: +++ means strong (> 40%); ++ means moderate (40% ≤ x ≥ 30%); + means weak (< 40%); and – means not antagonist (0%). Note: (SR): *Sclerotium rolfsii*, (FO): *Fusarium oxysporum*, and (RS): *Rhizoctonia solanii*.

Amplified rDNA Restriction Analysis (ARDRA). Each purified PCR products of 16S rDNA from *Bacillus* strains were digested by three restriction enzymes (RE), i.e. *Rsa*I, *Hae*III, and *Hinf*I (Promega Corporation, USA), in separated reaction. The DNA digestion was performed for 3 hours at 37 °C in 20 µl reaction volume containing 10 µl of 16S rDNA (1 µg), 2 µl of RE 10X Buffer, 7.3 µl of ddH_2O, 0.2 µl of Bovine Serum Albumin (BSA), and 0.5 µl of the restriction enzyme (10 u/µl). Restriction was inactivated by heating at 65 °C for 15 minutes for *Rsa*I and *Hae*III. Meanwhile, the *Hinf*I reaction was performed without inactivation as mentioned on the manufacture's instruction. Reaction products were run on a 2% agarose (wt/vol) gel electrophoresis at 100 V for 60 minutes in 1X TAE buffer. The length and number of DNA fragments (bp) were entered into binary data (.txt) and analyzed using Treecon software for windows ver 1.3b. Construction of Neighbor-joining tree and bootstrap analysis of 1,000 re-sampling were also performed using Treecon software (Van de Peer & De Watcher 1994).

Amplification of 16 rRNA Gene and Sequence Analysis. The PCR products of 16S rRNA from *Bacillus* sp. Cr24, Cr55, Cr64, Cr66, Cr67, and Cr68 which were randomly selected were purified by DNA purification kit (Promega Corporation, USA) and sequenced using DNA Sequencer ABI Prism 310 (PerkinElmer, USA). According to Table 1, six isolates were selected from each ARDRA group that have strong activity to suppress fungal growth. Sequences of the PCR products were further analyzed by bioinformatics analysis. The BLASTN program was used to search homology (identity and similarity) of each sequences compared to the GenBank databases. On this research, six *Bacillus* sp. isolates were aligned with some reference strains from GenBank as the producers of antifungal compound and capable to suppress the growth of plant pathogenic fungi. We used *B. cereus* ATCC 53522 which produced Zwittermicin A (Silo-Suh *et al*. 1998; Kevany *et al*. 2009), *B. subtilis* 168 which produced Bacilysocin (Tamehiro *et al*. 2002) and Surfactin (Tsuge *et al*. 2001b), and *B. subtilis* RB14 which produced Iturin (Tsuge *et al*. 2001a; Tsuge *et al*. 2005). Furthermore, the ClustalW program was used in order to align the sequences. Sequence dissimilarities were converted to evolutionary distances according to the method of Jukes and Cantor. The construction of Neighbor-joining tree and bootstrap analysis of 1,000 re-sampling were performed using Treecon software for windows ver 1.3b (Van de Peer & De Watcher 1994).

RESULTS

Amplification of 16S rRNA Gene and ARDRA. Genome of Twenty two isolates of *Bacillus* strains were successfully isolated from cell cultures using CTAB method (Sambrook & Russell 2001). PCR amplification of 16S rRNA gene yielded DNA fragments with single band at 1,300 bp for each *Bacillus* sp. strain (Figure 1). As many as 22 isolates of *Bacillus* sp. previously determined as biocontrol agent against phytopathogenic fungi were subjected to ARDRA analysis by digesting the amplified 16S rRNA gene with *Hae*III, *Hinf*I, and *Rsa*I (Figure 2). In silico restriction mapping analysis using WebCutter program also indicated that digestion with three restriction enzymes may generate same digestion profiles (data not shown) and the lengths of DNA fragment which were resulted from digestion by three restriction enzymes are shown in Table 2.

According to the digestion profiles, those *Bacillus* sp. isolates were grouped into eight different groups as follows: group I consisted of *Bacillus* Cr64; group II consisted of *Bacillus* Cr60 and Cr67; group III consisted of *Bacillus* Cr44, Cr55, and Cr79; group IV consisted of *Bacillus* Cr78; group V consisted of *Bacillus* Cr11, Cr40, Cr42, Cr43, Cr46, Cr58, and Cr66; group VI consisted of *Bacillus* Cr24; group VII consisted of *Bacillus* Cr7; and group VIII consisted of *Bacillus* Cr39, Cr45, Cr57, Cr68, Cr76, and Cr77 (Figure 3). The groups indicated that 22 isolates of *Bacillus* sp. strains were diverse.

There was no relation between the ability of 22 *Bacillus* sp. isolates to suppress the growth of plant pathogenic fungi with the relationship of ARDRA profile. For example,

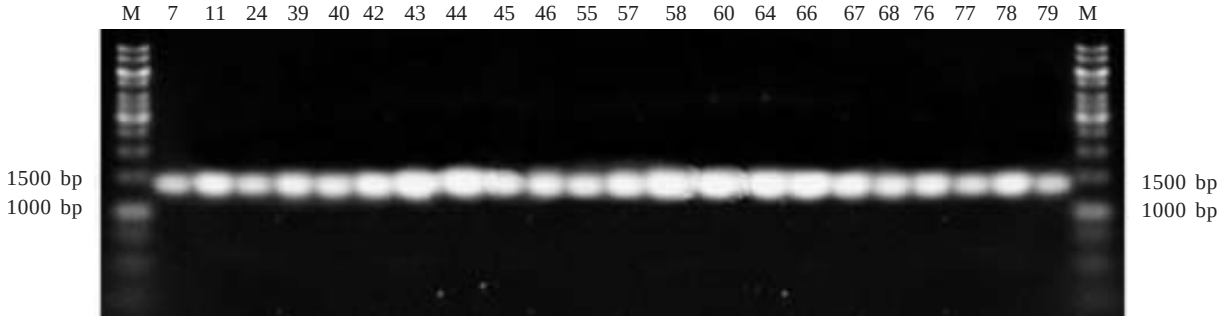

Figure 1. PCR amplification of the 16S rRNA gene of 22 antifungal-producing *Bacillus* sp. strains indicated by a single band at ~1300 bp. Marker (M): 1 kb ladder.

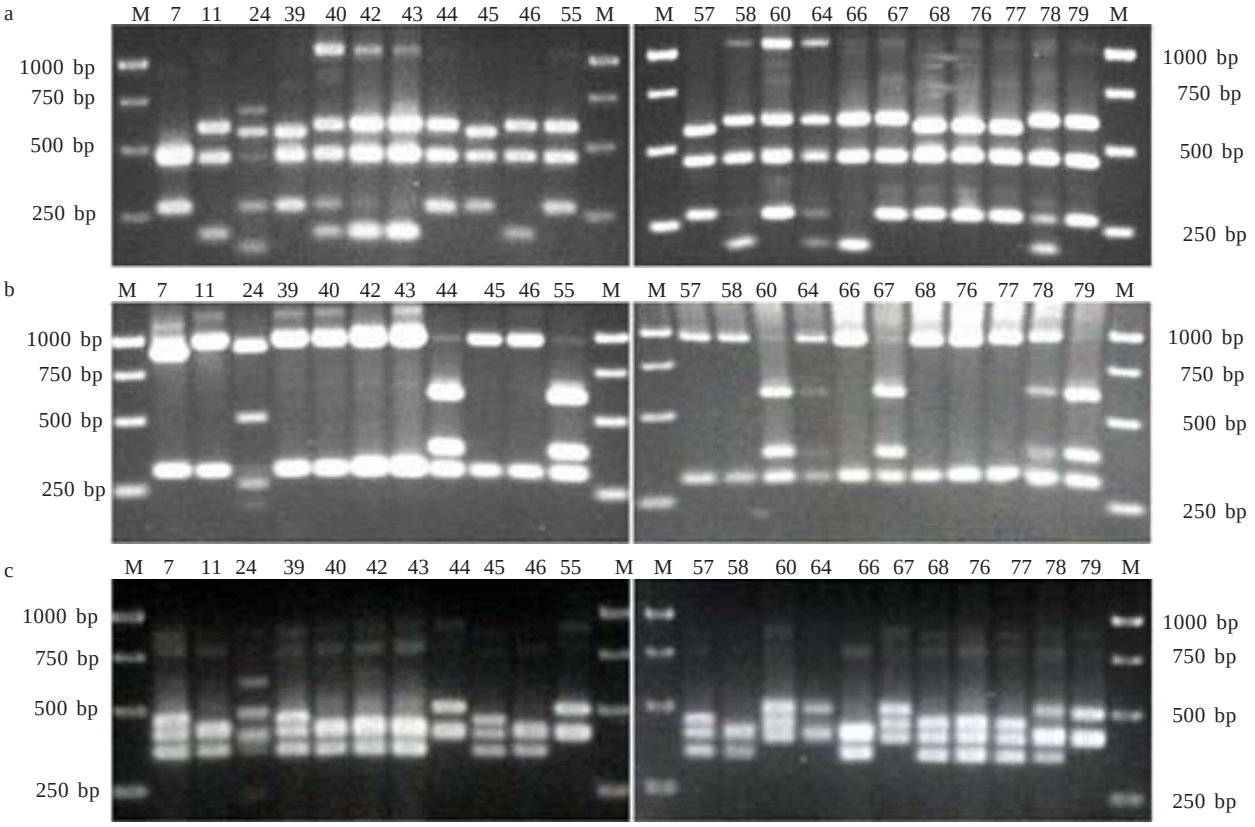

Figure 2. The restriction patterns of amplified 16S rRNA genes from 22 antifungal producing-*Bacillus* sp. strains using (a) restriction enzyme *HaeIII*, (b) restriction enzyme *HinfI*, and (c) restriction enzyme *RsaI*. Marker (M): 1 kb ladder.

Table 2. The sizes of 16S rDNA fragments digested with three different restriction enzymes, *Hae*III, *Hinf*I, and *Rsa*I

Strain	The sizes of DNA fragment (bp)		
	*Hae*III	*Hinf*I	*Rsa*I
Bacillus Cr7	530, 480, 290	900, 400	470, 440, 390
Bacillus Cr11	660, 470, 170	980, 320	460, 450, 390
Bacillus Cr24	580, 470, 250	980, 320	470, 430, 400
Bacillus Cr39	580, 470, 250	980, 320	470, 440, 390
Bacillus Cr40	660, 470, 170	980, 320	460, 450, 390
Bacillus Cr42	660, 470, 170	980, 320	460, 450, 390
Bacillus Cr43	660, 470, 170	980, 320	460, 450, 390
Bacillus Cr44	660, 420, 220	620, 360, 320	470, 430, 400
Bacillus Cr45	580, 470, 250	980, 320	470, 440, 390
Bacillus Cr46	660, 470, 170	980, 320	460, 450, 390
Bacillus Cr55	660, 420, 220	620, 360, 320	470, 430, 400
Bacillus Cr57	580, 470, 250	980, 320	470, 440, 390
Bacillus Cr58	660, 470, 170	980, 320	460, 450, 390
Bacillus Cr60	660, 420, 220	620, 360, 320	470, 430, 400
Bacillus Cr64	660, 470, 170	620, 360, 320	470, 430, 400
Bacillus Cr66	660, 470, 170	980, 320	460, 450, 390
Bacillus Cr67	660, 420, 220	620, 360, 320	470, 430, 400
Bacillus Cr68	580, 470, 250	980, 320	470, 440, 390
Bacillus Cr76	580, 470, 250	980, 320	470, 440, 390
Bacillus Cr77	580, 470, 250	980, 320	470, 440, 390
Bacillus Cr78	660, 470, 170	620, 360, 320	460, 450, 390
Bacillus Cr79	660, 420, 220	620, 360, 320	470, 430, 400

based on data in Table 1, *Bacillus* Cr7, Cr60, Cr67, Cr68, and, Cr78 have same strong inhibition activity against *Rhizoctonia solani* about more than 40% (data not shown). However, the result has differed from phylogenetic tree generated by ARDRA.

Sequence Analysis of 16S rRNA Gene. Homologous search analysis of 16S rRNA gene sequence of *Bacillus* sp. strains using BLASTN program was shown that dominant species within six isolates of *Bacillus* sp. based on bioinformatics analysis. The distributions were genetically diverse only on several species of *Bacillus* sp. such as *B. subtilis*, *B. fusiformis*, and *B. cereus*. Maximum identities for each isolate were in range of 92-96% with E-value 0 (Table 3). Phylogenetic tree based on 16S rRNA gene sequences showed the relation among the isolates (Figure 4). Phylogenetic tree that was made on the basis of 16S rRNA sequence exhibited that six isolates of *Bacillus* sp. were divided into three major groups. Group I consisted of *Bacillus* Cr24, Cr66, and Cr68 which were quite related to any *B. cereus* reference strains, and Group II consisted of *Bacillus* Cr55 and Cr67 which were also relatively related to any *B. subtilis*

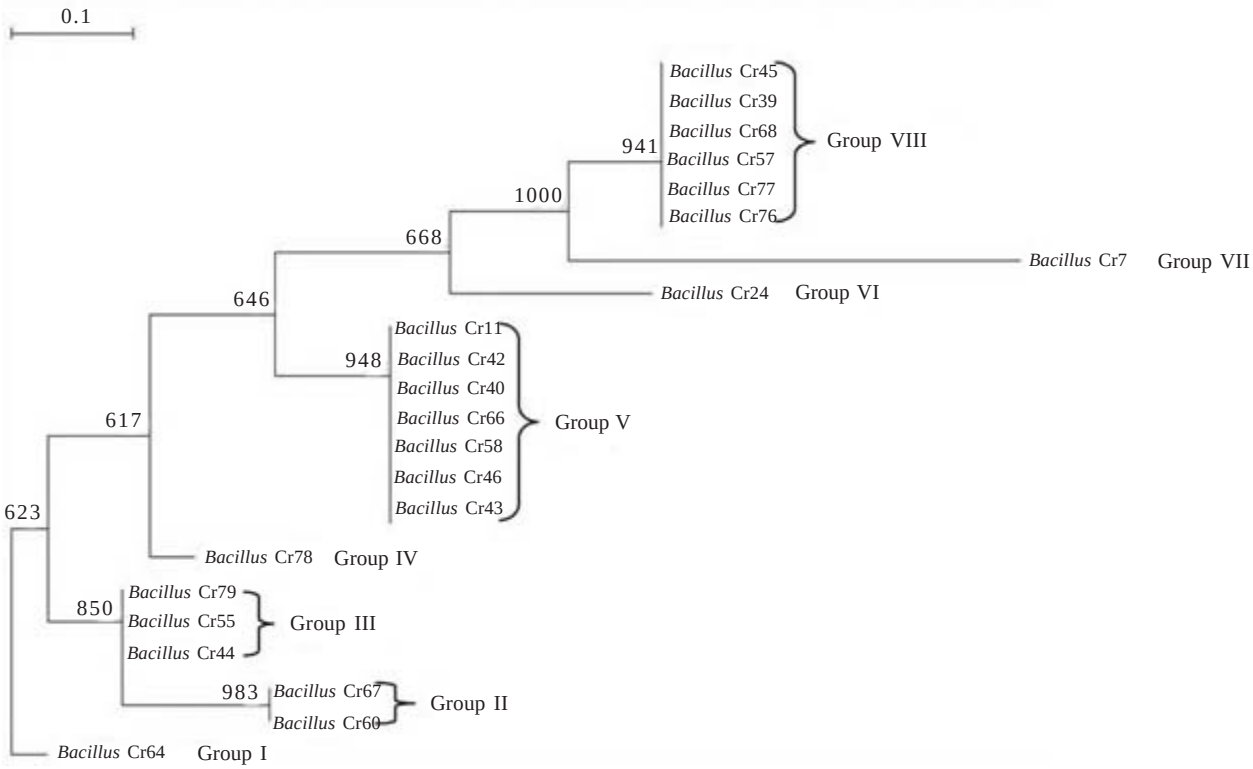

Figure 3. Dendrogram of 22 antifungal producing-*Bacillus* sp. strains based on ARDRA profile. The scale bar corresponds to 0.1 substitutions per presence of pattern in binary data file.

Table 3. Sequence analysis of the 16S ribosomal RNA gene of *Bacillus* sp. isolates compared to the GenBank database

Isolate	Accession number	Homology	Total score	Maximum identity (%)
Bacillus sp. Cr24	FJ859693.2	*B. cereus* strain BIHB 348	793	94
Bacillus sp. Cr55	EU373438.1	*B. subtilis* strain YRR10	861	92
Bacillus sp. Cr64	AY548950.1	*B. fusiformis* strain Z1	835	92
Bacillus sp. Cr66	GQ375259.1	*B. cereus* strain ANY	872	94
Bacillus sp. Cr67	GQ452909.2	*B. subtilis* strain N-6	924	94
Bacillus sp. Cr68	GQ344805.1	*B. cereus* strain DC3	965	96

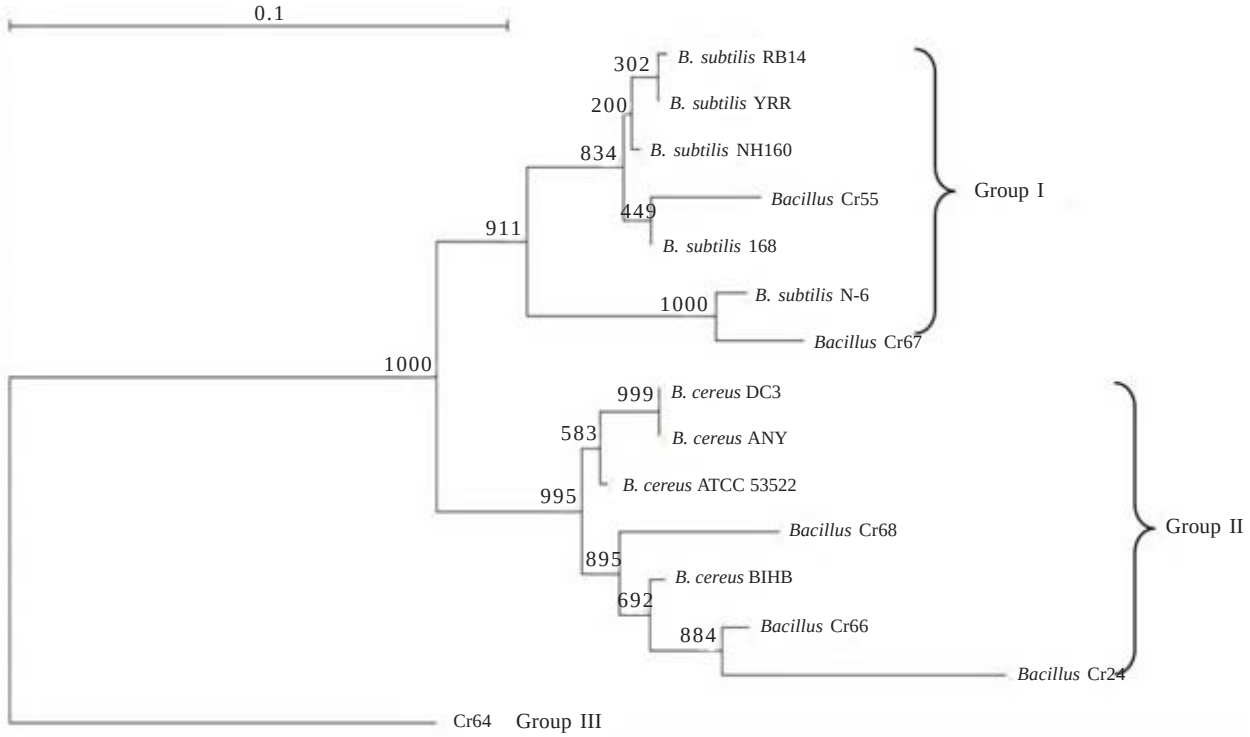

Figure 4. Phylogenetic dendrogram of six *Bacillus* sp. strain Cr based on partial sequences 16S rRNA gene with reference strains. The scale bar corresponds to 0.1 substitutions per nucleotide position.

reference strains. *Bacillus* Cr64 as a group III was the most distant strain compared with all strains, and it was performed by its BLASTN analysis which was 92% identical with *B. fusiformis*.

DISCUSSION

The eight groups of ARDRA profiles successfully represented 22 antifungal compound-producing rhizobacteria of *Bacillus* sp. strains using three restriction enzymes for digestion. It showed that the utilization of three restriction enzymes could distinguish within the isolates, and simplify the earlier working which used four restriction enzymes. It also revealed that digestion profile by *Rsa*I produce two to three DNA fragments (Figure 2) which is contrary to earlier result that digestion profile using *Rsa*I only produce homogenous pattern for all isolates of *Bacillus* sp. that promote soybean plant growth (Bahri *et al.* 2009). Since, Bahri *et al.* (2009) used different isolates of *Bacillus* sp. mainly showing plant growth promoting activity. Therefore, the different *Rsa*I digestion profile compared to our *Rsa*I digestion profile which derived from *Bacillus* sp. isolates showing antifungal activity was reasonably occured. *Bacillus* Cr7 was the most distant isolate compared with all isolates, and it was performed from its digestion profiles.

The 16S rRNA sequences also generated three major groups within the isolates. Group I was dominated by *B. subtilis* strain, group II was dominated by *B. cereus*, and group III was dominated by *B. fusiformis*. *Bacillus* species are known to produce various antibiotics and antifungal compounds that inhibit phytopathogenic fungi. *B. cereus*, *B. subtilis*, and *B. fusiformis* e.g. have been reported to produce antifungal molecules, which are mostly found in the rhizosphere. The dendogram based on 16S rRNA sequences and the dendrogram based on ARDRA profiles showed a same position of each isolate of *Bacillus* sp., it is due to the similarity level of nucleotide, as input, among the six isolates. Here, in this study we found that the isolates which are in the same group based on 16S rRNA are not clustered in the same group based on ARDRA. It was due to the number of isolates which were used for ARDRA analysis was different to those for 16S-rRNA analysis. We used all isolate tested (22 isolates) for ARDRA analysis, meanwhile only six isolates were used for 16S rRNA analysis. Therefore, the input data between those two analysis were different, further implicated to each phylogenetic profile.

Based on the data analysis, *Bacillus* Cr55 was closely related with *B. subtilis* 168 and *Bacillus* Cr67 was also closely related with *B. subtilis* N-6. Therefore, it can be revealed that *Bacillus* Cr55 and Cr67 may have the same antifungal activity with reference strain by producing same antifungal compound (Figure 4). According to Tamehiro *et al.* (2002), Antifungal Bacilyosocin from *B. subtilis* 168 suppressed the growth of *Candida* and *Aspergillus niger*. From the other research, *B. subtilis* N-6 showed an antagonistic interaction against *Fusarium oxysporum* on banana. In addition, *B. cereus* ATCC 53522 produced antifungal zwittermicin A (Kevany *et al.* 2009) which suppressed the growth of *Fusarium* and *Rhizoctonia solani* (Silo-Suh *et al.* 1998).

ARDRA analysis revealed in this study can be widely used to investigate or identify other *Bacillus* sp. strains

that were inhabit in the rhizosphere of soybean root and may also possess the characters that able to produce antifungal compounds. Combination of ARDRA and 16S rRNA sequence technique provided an optimal result for genetic diversity analysis of *Bacillus* sp. strains, both grouping and identifying. The combination technique was more advance, because the previous study (Yusuf *et al.* 2002) had only recognized the number of groups or phylotypes without knowing the genus of its samples. Moreover, ARDRA analysis will be practically used based on practical matters such as the simplicity and the lower price compared to the sequencing analysis, which is more expensive.

ACKNOWLEDGEMENT

This research was supported by Incentive Program for Basic Research from the Ministry of Research and Technology (Program Insentif Riset Dasar, RISTEK) 2008 to ATW. We are therefore grateful for this funding and support of this research.

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