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Genetic Diversity of Antifungi-Producing Rhizobacteria of *Pseudomonas* sp. Isolated from Rhizosphere of Soybean Plant

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Antifungi-producing rhizobacteria have been recognized playing an important role in plant disease suppression. In our laboratory, 13 indigenous soybeans' rhizobacteria *Pseudomonas* sp. that showed strong growth inhibition of root pathogenic fungi, *Rhizoctonia solani*, *Fusarium oxysporum* and *Sclerotium rolfsii*, have been isolated from rhizosphere of soybean plant. For further understanding, the genetic diversity of the antifungi-producing *Pseudomonas* sp. was investigated using Amplified 16S rDNA Restriction Analysis (ARDRA) and 16S rRNA gene sequences analysis. 16S rDNA were amplified by PCR technique and digested with restriction endonuclease *Hae*III, *Rsa*I and *Alu*I. Sequences of 16S rRNA gene were analyzed using the BLAST program for similarity searches on sequence databases. ARDRA based dendrogram analysis was carried out by neighbor-joining of TREECON 1.3b software package. ARDRA indicated the variability of *Pseudomonas* sp. based on the digestion sites. Dendrogram clustering analysis based on the restriction enzymes profile of the amplified rDNA distinguished *Pseudomonas* sp. into 7 ribotype groups. The sequences of 16S rRNA gene confirmed that the isolates belonging to *Pseudomonas* sp. and the phylogenetic tree formed 4 clusters. There was a quite overlap among ARDRA groups and 16S rRNA sequence clusters. This finding suggested that antifungal producing *Pseudomonas* sp. were present in the rhizosphere of soybean plant and the level of genetic diversity exist within these species. Sequence analysis of the 16S rRNA gene of the *Pseudomonas* sp. with an identical ARDRA pattern confirmed that members of an ARDRA group were closely related to each other.

Key words: antifungi producing rhizobacteria, ARDRA, genetic diversity, *Pseudomonas* sp., 16S rRNA

Disease suppressive soil suppresses the growth of phytopathogenic fungi or the induction of severe disease on susceptible plants (Schroth and Hancock 1982). This phenomenon, despite rarely happens, has been extensively known and strong evidences show that the disease is suppressed by specific rhizobacteria that have the ability to produce antifungal compounds. Several experiments have demonstrated that a number of *Pseudomonas* sp. strains with the ability to produce antifungal metabolite 2,4-diacetylphloroglucinol (DAPG) can be isolated with high frequency from black root rot disease suppressive soil applied to tobacco (Keel *et al.* 1996). These strains are able to suppress diseases in wheat (Raaijmakers *et al.* 1997). They are also involved in many plant disease suppression that can be related to the presence of phytopathogen-antagonistic functions in the soil microbiota (Garbeva *et al*. 2004; Mazzola 2004; Garbeva *et al*. 2006).

There is a great interest in plant-associated bacteria, particularly in the genus of *Pseudomonas*. Researches aiming to improve crop responses emphasized on antibiotic producing bacteria indigenous to certain rhizosphere. Moreover, the components of suppressiveness have been described for multiple pathosystems, especially for those involving a specific pathogen and microbial antagonist (Weller *et al*. 2002; Borneman and Becker 2007). Recently, antibiotic-producing bacteria have been recognized playing an important role in disease suppression. In our laboratory, we have screened the indigenous soybeans' rhizobacteria *Pseudomonas* sp. CRB (Cirebon) that are potential as a biocontrol for rot root disease caused by pathogenic fungi. Several *Pseudomonas* sp. CRB isolates strongly inhibited growth of root pathogenic fungi, i.e. *Rhizoctonia solani*, *Sclerotium rolfsii* or *Fusarium oxysporum, in vitro*.

Genetic diversity means the total number of genetic characteristics. Molecular tools such as ribotyping, *in-situ* hybridization, DNA sequence analysis and restriction fragment length polymorphism (RFLP) are now in common use to provide accurate genetic diversity information of microbes by using 16S rRNA gene. RFLP analysis on 16S rRNA gene is called amplified rDNA restriction analysis (ARDRA). This method is useful for genotype identification and can be used to infer genetic variability and similarity of microorganisms (Yang *et al*. 2007; Kidd *et al*. 2009). ARDRA can also be applied for characterizing a number of species including *Clostridium botulinum* toxinotype A strains (Pourshafie *et al*. 2005) and mycobacteria (De Baere *et al*. 2002). In this study, we described diversity of antifungi-producing *Pseudomonas* sp. CRB from rhizosphere of soybean plant employing ARDRA and 16S rRNA gene sequences analysis.

MATERIALS AND METHODS

Microorganism and Culture Condition. *Pseudomonas* spp. were isolated from soybean's rhizosphere from a soybean plantation in Cirebon area, West Java, Indonesia. The rhizophere's soil was diluted in 10 mL 0.85% NaCl solution and then serial dilution was made to obtain the appropriate bacterial number so that various individual bacteria would grow separately on agar surface. Diluted bacteria (100 µL) were then spread onto King's agar (King *et al.* 1954) and incubated for 24 hours at room temperature (27-28ºC). Each visible colony with different appearance was picked and streaked on fresh medium to obtain pure

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culture. Identification of *Pseudomonas* sp. was conducted based on morphological and physiological characters. Microgen™ system (GNA and GNB) that employs 24 standardized biochemical substrates in microwell was also used to complete the test. Gram negative, rods, motile, aerobic, catalase positive, and oxidize positive were the characters that lead to *Pseudomonas* identification (Holt *et al.* 1994). Eighty one isolates named *Pseudomonas* sp. CRB were collected. Thirteen isolates that showed strong inhibition of fungal pathogen *in vitro* were used in this study (Table 1). *Pseudomonas* sp. CRB were routinely cultivated on agar plate of King's medium B (20 g L^{-1} bacto peptone, 1.5 g L^{-1} K_2HPO_4, 1.5 g L^{-1} $MgSO_4$, 15 g L^{-1} bacto agar (Difco, France), 15 mL L^{-1} glycerol) in room temperature (27-28°C).

DNA Extraction and PCR Amplification. Genomic DNA was isolated from overnight cultures of *Pseudomonas* sp. CRB by using a cetyl trimethyl ammonium bromide (CTAB)-based protocol (Sambrook and Russell 2001). DNA coding for 16S rRNA of each isolate was amplified with primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Invitrogen, Japan). These primers amplify approximately 1 300 bp of the 16S rRNA gene, specific to consensus regions that are considered as universal bacterial domains (Marchesi *et al*. 1998). PCR reactions (Takara, Japan) were done in a total reaction volume of 50 µL containing 25 µL GC buffer II, 8 µL dNTP mix (2.5 mM each), 20 pmol of each primer, 2.5 unit LA Taq polymerase, 100 ng DNA template and ddH_2O. The thermal cycling (GeneAmp PCR System 2400, Perkin Elmer, USA) was as the following: denaturation at 94ºC for 4 min;, followed by 30 cycles of denaturation at 94ºC for 2 min, annealing at 55ºC for 1 min and extension at 72ºC for 1 min 30 sec; with the final extension at 72ºC for 5 min. PCR-amplified DNAs were visualized in 1% agarose gel electrophoresed in 1X TAE buffer at 100 V for 1 h. The gel then was stained with the ethidium bromide (5 µg mL^{-1}) for 30 min, and visualized with UV transilluminator.

ARDRA. Amplified 16S rDNA was purified from the gel using Wizard SV Gel and PCR Clean-up System (Promega, USA). The purified amplification products (1.5 µg) were single-digested with 5 unit *Hae*III, *Rsa*I and *Alu*I (Fermentas Life Science, EU). Enzyme digestions were carried out at 37ºC for overnight. The restriction fragments were analyzed by electrophoresis at 2% of agarose gel in 1X TAE buffer at 100V for 50 minutes. The gel then was stained with ethidium bromide (5 µg mL^{-1}) for 30 min, and visualized with UV transilluminator. DNA 1 kb ladder (Fermentas Life Science, EU) was used as a DNA molecular size marker. To determine series of bands that appeared in the gel as a result of successful restriction, estimation of the DNA fragment size was done using a mathematical equation that links migration rate to molecular weight. The relevant formula $D = a - b (\log M)$ (Brown 1986); where D is the distance moved, M is the molecular weight, a and b are constants that depend on the electrophoresis condition; was applied for analysis. For each restriction band, binary data matrix was constructed on the basis of the presence or absence of each band, coded as 1 or 0, respectively. The band patterns obtained with each enzyme restriction were combined to obtain a single pattern for each isolate. The patterns were used to construct dendrogram by using the neighbor joining methods as a part of the TREECON 1.3b software package (Van de Peer and De Wachter 1997).

Table 1 *Pseudomonas* sp. producing antifungal compounds used in this study with percentage of inhibition radial growth of phytopathogenic fungi

Isolates of *Pseudomonas*	Growth inhibiton of fungi by *Pseudomonas* sp. CRB (%)		
	Sclerotium rolfsii	*Fusarium oxysporum*	*Rhizoctonia solani*
CRB-3	-	-	56.7
CRB-16	-	24.6	-
CRB-17	-	14.3	-
CRB-31	-	18.7	50.0
CRB-44	-	39.2	-
CRB-75	-	11.1	37.7
CRB-80	20.0	-	52.3
CRB-82	-	11.1	38.9
CRB-86	-	30.3	36.9
CRB-102	25.0	-	60.0
CRB-109	-	-	36.9
CRB-111	-	-	30.0
CRB-112	-	-	48.1

16S rRNA Gene Sequence Analysis. Purified PCR products were subjected to cycle sequencing. Single direction (forward primer) sequencing of PCR product was performed by DNA sequencer (ABI Prism 3100, Applied Biosystems, USA) at PT Charoen Pokphand, Indonesia. Databases search was carried out for similar nucleotide sequences (600 nucleotides) with the BLASTN network server at the NCBI (www.ncbi.nlm.gov/BLAST). For describe their phylogenetic relationship, the partial 16S rRNA gene sequences were aligned using ClustalW, and then analyzed by means of neighbor-joining method according to Jukes and Cantor model using MEGA version 4 (Kimura *et al*. 2007). The robustness of the inferred trees was evaluated by 100 bootstrap resamplings.

RESULTS

Amplified 16S rRNA Gene and ARDRA Pattern. The 16S rDNAs of 13 *Pseudomonas* sp. CRB were amplified, resulting in 1 300 bp bands (data not shown). Different patterns obtained from each of the three endonuclease enzymes, *Hae*III, *Rsa*I and *Alu*I, distinguished the isolates one from to another. In this study, digestion of 16S rDNA of *Pseudomonas sp.* CRB by the three enzymes produced 3 to 5 bands with different sizes for each treatment. Restriction fragment lengths for each of the possible patterns based on distance relative are presented in Table 2. Unique digestion profiles shown by each isolates analyzed could be used as references or as an ARDRA profile library. All of the 13 isolates of *Pseudomonas* sp. CRB have already been identified as biocontrol bacteria. ARDRA profile revealed in this study can be widely used to investigate or identify several *Pseudomonas* isolates that inhabit the rhizosphere and possessing the characters of biocontrol.

Table 2 16S rDNA restriction fragments of antifungi-producing *Pseudomonas* sp. digested with *Hae*III, *Rsa*I and *Alu*I

Strains of *Pseudomonas*	Size of rDNA fragment (bp)		
	*Hae*III	*Rsa*I	*Alu*I
CRB-3	600, 500, 270	540, 480, 380	630, 200, 140
CRB-16	740, 690, 210, 130	720, 440, 230	700, 200, 110, 80
CRB-17	720, 330, 210, 130	720, 440, 230	650, 430, 200, 110, 80
CRB-31	620, 500, 280, 160	590, 520, 480	670, 200, 110, 80
CRB-44	570, 210, 200, 180	520, 480, 400	380, 290, 200, 110, 80
CRB-75	740, 510, 280	590, 520, 480	470, 270, 230, 150
CRB-80	890, 210, 140	720, 440, 230	430, 200, 200, 110, 80
CRB-82	740, 220, 140	720, 440, 230	430, 200, 200, 110, 80
CRB-86	640, 530, 280	480, 440, 380	670, 200, 110, 80
CRB-102	890, 220, 180	720, 440, 230	430, 200, 200, 110, 80
CRB-109	650, 510, 190	520, 440, 410	700, 200, 110, 80
CRB-111	650, 510, 190	520, 440, 410	700, 200, 110, 80
CRB-112	600, 500, 270	480, 440, 340	590, 220, 150, 80

Cluster Analysis Based-ARDRA. The results of ARDRA using *Hae*III, *Rsa*I, and *Alu*I were subjected to cluster analysis. Neighbor-joining method was used to construct the dendrogram of bands resulting from endonuclease digestion. In this method, similar variables were grouped. The ARDRA clustering revealed a considerable level of genetic diversity between the isolates, since 7 clusters, designated as ribotype 1-7, were identified (Fig 1). *Pseudomanas* sp. CRB-16, CRB-17, CRB-80, CRB-82 and CRB-102 are clustered in one group, i.e. ribotype 7. The isolates that occupy the same cluster are considered to share the same attributes. Therefore, isolates belonging to the same cluster are similar in some sense. Other isolates forming other groups differ from ribotype 7. Cluster dendrogram showed that ARDRA pattern of ribotype 1 was similar to ribotype 2 and differ from ribotype 7 (Fig 1). In this result, *Pseudomonas* sp. CRB-3 is the most unique isolate among others.

16S rRNA Gene Sequences and Phylogenetic Relationship. BLASTN results of the partial sequence of 16S rRNA gene (about 600 nucleotides) showed high similarity with *Pseudomonas* spp. (83-100%) (Table 3). The phylogenetic relationships among 13 isolates were represented as a dendrogram using neighbor joining method (Fig 2). Four clusters were generated when *Pseudomonas* sp. CRB isolates were grouped according to their 16S rRNA sequences. Isolates found in the same cluster seem to have close phylogenetic relationship. Many isolates with the same

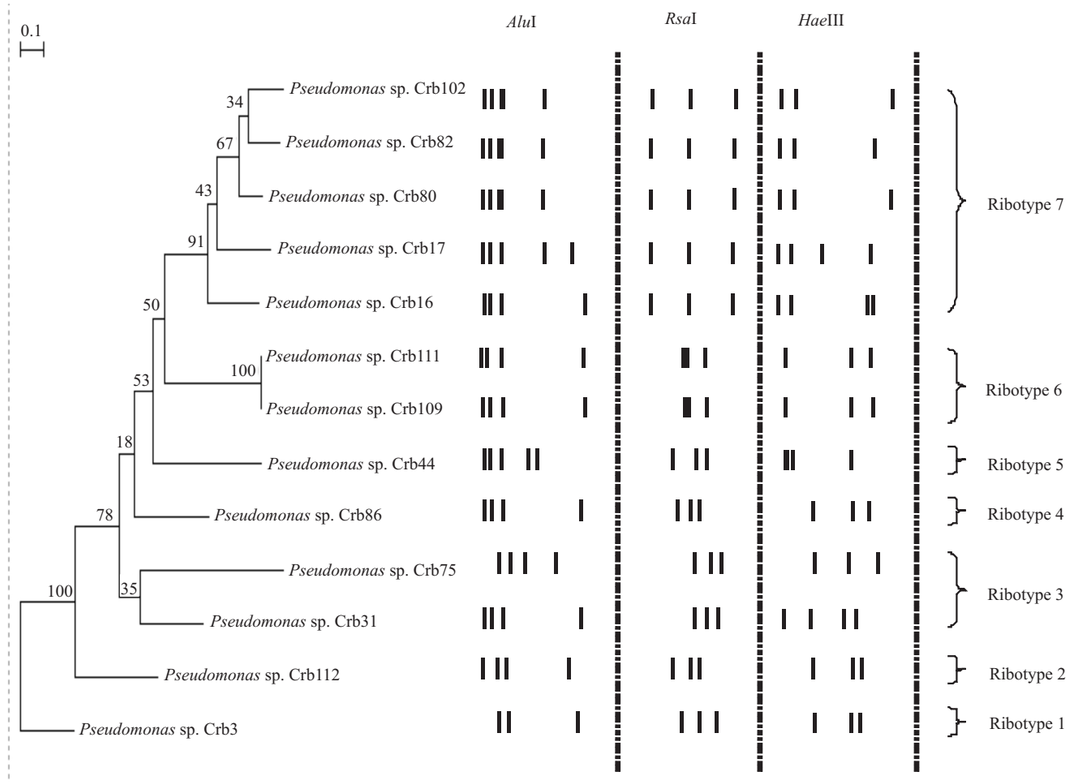

Fig 1 Cluster dendrogram and electrophoregram of antifungi-producing *Pseudomonas* sp. CRB, based on restriction profiles obtained with *Hae*III, *Rsa*I and *Alu*I. Each ribotype is designated by a right brace. The dendrogram was constructed with TREECON and grouped by neighbor-joining method with booststrap analysis of 100 resamples.

Table 3 Homologous searches analysis of 16S rRNA gene sequences of *Pseudomonas* sp. isolates as compared to GenBank data base

Isolate code	Homology	Score (bits)	Identity (%)	Accession number
CRB-3	*Pseudomonas monteilii* strain R23	939	95	DQ095885.1
CRB-16	*Pseudomonas* sp. m41	883	96	EU375659.1
CRB-17	*Pseudomonas plecoglossicida* strain NyZ12	1086	99	EF544606.2
CRB-31	*Pseudomonas* sp. CL3.1	640	83	FM173664.1
CRB-44	*Pseudomonas* sp. YF3	1057	98	EU220236.1
CRB-75	*Pseudomonas putida* strain FWC30	874	92	EU833948.1
CRB-80	*Pseudomonas* sp. RD9SR1	1066	98	AM911646.1
CRB-82	*Pseudomonas fluorescens* strain BFPB92	981	92	EF600890.1
CRB-86	*Pseudomonas* sp. G12	826	91	FJ211222.1
CRB-102	*Pseudomonas* sp. KLP2	1094	100	AM911670.1
CRB-109	*Pseudomonas monteilii* strain SeaH-As4w	760	89	FJ607352.1
CRB-111	*Pseudomonas monteilii* strain SeaH-As4w	734	88	FJ607352.1
CRB-112	*Pseudomonas* sp. BFF-1	957	95	EF031081.1

ARDRA pattern had sufficiently similar 16S rRNA sequence that they were phylogenetically placed in the same evolutionary lineage (Fig 2). For example, cluster 4, represented by *Pseudomonas* sp. CRB-16, CRB-17, CRB-80, CRB-82, and CRB-102, except CRB-3, were found in ribotype 7 group. *Pseudomonas* sp. CRB 109 and 111 those were grouped as ribotype 6 were also found in the same cluster, i.e. cluster 6. It is interesting to note that the *Pseudomonas* sp. CRB (3, 16, 17, 80, 82 and 102) were found in the same cluster with the biocontrol reference strain of *Pseudomonas fluorescens* CHA0 and Pf-5 cluster. Therefore, they were likely to be closely related.

DISCUSSION

Bacteria responsible for soilborne plant disease suppression can be found among microbial community of the rhizosphere. There were 13 isolates of *Pseudomonas* sp. CRB possessing antifungal activity from rhizosphere of soybean plant. The antifungal activity was confirmed *in vitro* antagonist test toward tree target fungal pathogens. Considering that antifungal activity, these bacteria are possibly naturally occurring disease suppressing bacteria. The number of the isolates was not many, but these antifungi-producing *Pseudomonas* sp. CRB isolates are promising for biocontrol agent. For comparison, there were 39 fluorescent pseudomonads producing DAPG (Ramette *et al*. 2001). Among antifungal compounds synthesized by pseudomonads, DAPG is a key secondary metabolite

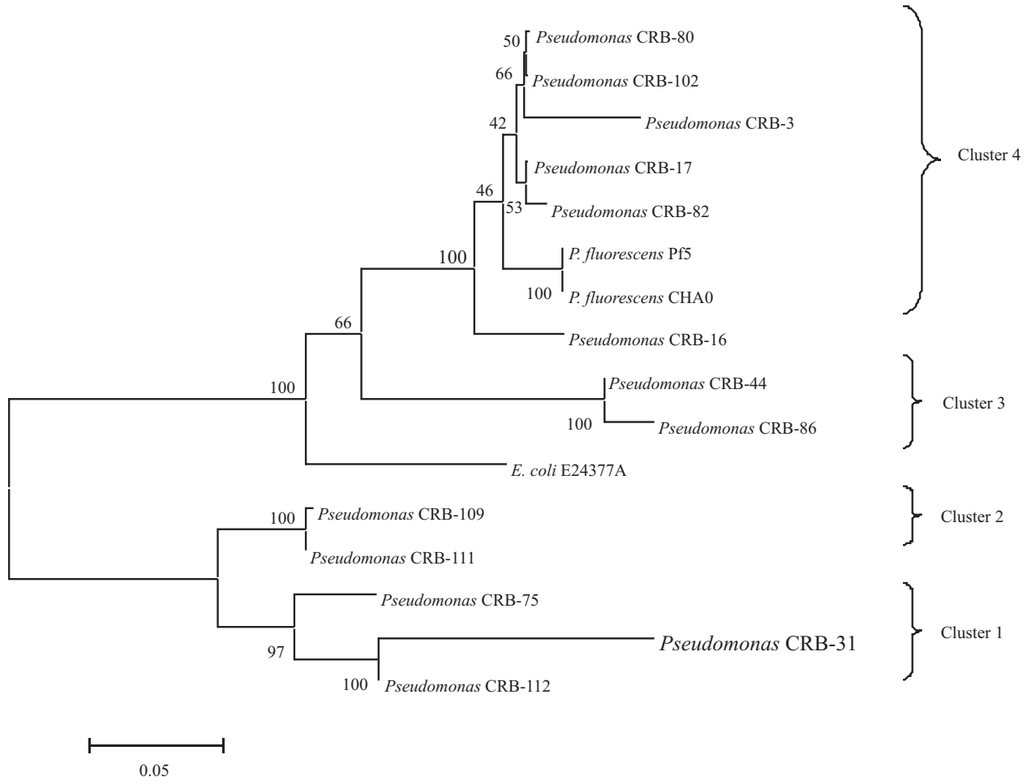

Fig 2 Dendrogram showing genetic relationship among the partial 16S rRNA genes sequences (600 nucleotides) of 13 *Pseudomonas* sp. CRB and their reference strains *P. fluorescens* CHA0 (accession number AJ278812), Pf-5 (CP000076.1) that are known as biocontrol strains and *Escherichia coli* (E24377A), the same group of γ proteobacteria. The neighbor-joining (NJ) tree was constructed using Jukes-Cantor model in MEGA 4, and nodal supports of the inferred tree were evaluated by 100 bootstrap replicates. The scale bar represents the number of substitutions per site.

associated with disease suppression in several pathosystems. Benítez and McSpadden-Gardener (2009) identified novel *Mitsuaria* and *Bulkholderia* species involved in damping-off disease suppression caused by oomycete pathogens toward tomatoes and soybeans.

ARDRA of *Pseudomonas* sp. CRB indicated 7 groups with supposedly high genetic diversity. Genetic diversity plays a very important role in the survival and adaptability of a species because when a species's environment changes, slight gene variations are necessary to produce changes in the organism that enable it to adapt and survive. A species that has a large degree of genetic diversity among its population will have more variations from which to choose the most fit. Moreover, knowledge on the diversity within a group of strain that share a common antifungi production may provide a new approach for identifying the isolates that are superior with respect to rhizosphere competence and ability to suppress soil-borne pathogen (Raijmakers and Weller 2001). In contrast, cluster analysis of restriction patterns of amplified DNA coding for 16S rRNA of 13 fluorescent *Pseudomonas* strains that phenotypically produced antibiotic, DAPG, pyoluteorin and hydrogen cyanide from almost all locations sampled in United State, Europe and Africa were genetically highly similar, clustering in only one group (Keel *et al*. 1996). Furthermore, they stated that, in general, the occurrence of phenotypically and genotypically similar groups of DAPG2,4-diacetylphloroglucinol producers did not correlate with the geographic origin of the isolates, and highly similar strains could be isolated from diverse locations worldwide. ARDRA analysis of 11 plant growth promoting rhizobacteria *Bacillus* sp. by Bahri *et al*. (2009) resulted in 4 phylotypes.

Other researches that employed ARDRA showed more diversity at the genotype level. In Saldana *et al.* (2003), ARDRA analysis revealed 3 different genotypes among fast-growing rhizobia that nodulate soybean, even though all belonged to a subcluster that included *Sinorhizobium saheli* and *S. meliloti*. Chèneby *et al*. (2000) showed that denitrifying isolates from soils were grouped according to the similarity of their restriction patterns into 26 ARDRA types. Interestingly, ARDRA results suggest that some denitrifying isolates are specific to a soil type,while others seems to be geographically widespread. Tian *et al*. (2009) demonstrated that a total of 28 ARDRA patterns were identified among the 299 siderophore-producing bacterial isolates from the rhizosphere of tobacco. These 28 ARDRA patterns represented bacteria of 14 different genera belonging to 6 bacterial divisions, namely β-, γ-, α- Proteobacteria, Sphingobacteria, Bacilli and Actinobacteria. Indeed, from all the examples analyzed, ARDRA could describe the genetic diversity of bacteria studied. If the bacteria have lower genetic diversity, it will be represented by the same band pattern of the enzyme digestion even though many enzymes were used and if the bacteria have greater genetic diversity, different band patterns will be generated.

The 16S rRNA gene sequence is about 1 550 bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient interspesific polymorphisms to provide distinguishing measurement (Clarridge 2004; Woese 2006). The beginning of the gene and at either the 540-bp region or at the end of the whole sequence is the conserved regions. The sequence of the variable region in between is used for comparative taxonomy (Relman 1999). Comparison of the 16S rRNA gene sequences allows differentiation between strains at multiple levels, including what we now call the species and sub-species level. Previous reports showed that the phylogenetic assignments obtained from the partial and full-length sequences were very similar (Massana *et al*. 1997; Xiang *et al*. 2005). Since we were interested only in determining the group of pseudomonas, a partial sequence analysis was justified. In line with this reason, for most clinical bacterial isolates the initial 500-bp sequence provide adequate differentiation for identification (Hall *et al*. 2003). Sequence analysis of approximately 600-bp 16S rDNA fragment allowed the rapid identification of this isolates that lead to genus *Pseudomonas*. However, for discrimination of closely related species sequencing of the entire 16S rRNA gene is essential.

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