Indian Journal of Biotechnology
Vol 7, October 2008, pp. 508-514

PCR fingerprinting for identification and discrimination of plant-associated facultative methylobacteria

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Received 19 September 2007; revised 1 April 2008; accepted 15 June 2008

Pink-pigmented facultative methylotrophic bacteria (PPFMs) belonging to the genus *Methylobacterium*, often associated with phyllosphere and roots and symbiotically benefit the plant species. For identification and discrimination of common plant-associated *Methylobacterium* species, different PCR-based DNA fingerprinting techniques were evaluated along with metabolic divergence. Standard/type strains of 10 species of plant-associated *Methylobacterium* were fingerprinted by means of RAPD, ARDRA, RISA, BOX and ERIC markers. These species showed divergence in carbon-substrates utilization pattern. Among the above PCR techniques evaluated, RAPD, RISA and ERIC fingerprinting had high discriminatory power than ARDRA and BOX-PCR. Being simple, rapid and repeatable, RAPD, RISA and ERIC-PCR techniques could be used for species identification and diversity analysis of plant-associated PPFMs belonging to *Methylobacterium*.

Keywords: ARDRA, BOX-PCR, ERIC-PCR, *Methylobacterium*, RAPD, RISA

Introduction

Methylobacterium is a Gram-negative, rod-shaped, strictly aerobic facultative methylotrophic bacterium, which are able to grow on one-carbon compounds as well as on variety of C_2, C_3, C_4 substrates[1]. It belongs to α_2-*Proteobacteria* of order Rhizobiales and family Methylobacteriaceae with 24 validly published species. Members of the genus *Methylobacterium* are distributed in a wide variety of natural and man-made environments, including soil, air, dust, fresh water and marine water sediments, water supplies, bathrooms, air-conditioning systems and masonry. In addition, many of the methylobacterial species are frequently associated with terrestrial and aquatic plants, where they colonize roots and leaf surfaces[2]. Some of the plant-associated methylobacteria so far reported are: *M. aminovorans* and *M. thiocyanatum* from phyllosphere of maize, soybean and sunflower; *M. extorquens* from phyllosphere of several plants including grapevine[3], clover[3], rice[4]; *M. fujisawaense* from the phyllosphere of rice[4]; *M. mesophilicum* from celandine and clover leaves[3]; *M. populi* in internal tissues of poplar trees[5]; and *M. nodulans* forming root nodules of *Crotolaria glaucoides*[6,7].

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The association of *Methylobacterium* with plant possesses an associative symbiotic relationship in which *Methylobacterium* utilizes the methanol emitted from leaves of plants as sole carbon and energy source. In response, *Methylobacterium* produces cytokinins and auxins like phytohormones, which are known to stimulate plant growth[8], fix the atmospheric nitrogen in nodules[6]; helps plants to induce systemic resistance against pathogens[9] or regulates acetylene by ACC deaminase enzyme[4]. In recent years, due to demonstrated capabilities in different applications in the field of industry, agriculture and bioremediation, *Methylobacterium* is gaining more importance. Hence, in order to discriminate and identify the *Methylobacterium* species associated with plant species, different PCR based fingerprint typing methods were assessed.

Many reports are now available demonstrating the utility of PCR for fingerprinting of several organisms[10] including soil proteobacteria such as rhizobia. Randomly amplified polymorphic DNA (RAPD) is a powerful molecular fingerprinting technique, which is highly discriminative and allows distinction even between closely-related strains[11]. RAPD utilizes PCR to amplify DNA segments with single primer of arbitrary nucleotide sequence generating fragments by hybridizing with compatible

regions of bacterial DNA and amplifying the regions where the primers are in correct orientation and appropriately spaced (100-2500 bp)[12]. Another rapid, reliable and sensitive PCR method to differentiate the species is the amplified ribosomal DNA restriction analysis (ARDRA), which involves PCR amplification of 16S rRNA gene using specific primers followed by digestion with one or more selected restriction enzymes[13]. Although the 16S rRNA gene has been widely used for fingerprinting, the analysis of intergenic spacer region (IGS) between 16S and 23S rRNA gene (referred as ribosomal intergenic spacer analysis, RISA) has also been shown to be species specific and the method has been successfully used to fingerprint simple communities[14]. Enterobacterial repetitive intergenic consensus (ERIC) and BOX elements like repetitive extragenic palindromic elements are described in a vast variety of eubacterial species[15] and now ERIC- and BOX-PCR fingerprinting have been widely used for typing gram-positive and gram-negative bacteria[16,17]. The purpose of the present study was to evaluate the utility of different PCR fingerprinting methods *viz.,* RAPD, RISA, BOX-PCR and ERIC-PCR along with metabolic fingerprinting as differential carbon-substrates utilization pattern of ten different plant-associated *Methylobacterium* species.

Materials and Methods

Methylobacterium Strains and Culture Condition

The type cultures and standard strains of various plant-associated *Methylobacterium* spp. used in this study are presented in Table 1. These cultures were cultivated and maintained in ammonium mineral salt medium supplemented with 0.1% filter-sterilized methanol (MMS medium)[18] at 30°C.

Carbon Utilization Test

All the standard/type strains of *Methylobacterium* species were analyzed for growth in different carbon compounds *viz.,* acetate, betaine, citrate, fructose, D-glucose, L-glutamate, methylamine, D-xylose, thiocyanate, tartarate, arabinose, dimethyamine, trimethylamine and cyanate. These carbon compounds were substituted for methanol in AMS liquid medium at 0.5% (w/v) level. Presence of growth was observed after 15 d of incubation at 30°C in an orbital shaking incubator and growth was compared to a negative control containing no added carbon source.

Table 1—Details of standard/type strains of *Methylobacterium* species used in this study

Methylobacterium species	Strain/type strain	16S rRNA gene GenBank accession number	Source
M. aminovorans	TNAU 9	EF116587	Present study
M. extorquens	DSM1337^T	AB175633	T Sa, Korea
M. fujisawaense	DSM5686^T	AJ250801	T Sa, Korea
M. mesophilicum	DSM1708^T	AB175636	T Sa, Korea
M. nodulans	ORS2060^T	AF220763	P Jourand, France
M. populi	TNAU12	EF116590	Present study
M. radiotolerans	DSM1819^T	AB175640	T. Sa, Korea
M. suomiense	TNAU4	EF116584	Present study
M. sp.	TNAU14	EF116592	Present study
M. thiocyanatum	TNAU8	EF116586	Present study

Genomic DNA Extraction

The strains were grown in MMS broth for 7 d and their genomic DNA extracted by hexadecyltrimethyl ammonium bromide (CTAB) method[19]. The integrity and concentration of purified DNA was determined by agarose gel electrophoresis[20]. The total genomic DNA extracted was dissolved in sterile distilled water and stored at 4ºC. The concentration of total genomic DNA was adjusted to a final concentration of 20 ng/µL for PCR amplification.

RAPD-PCR

The RAPD primers, OPQ1 and OPQ2 (obtained from Bangalore Genei, India) were used in this study and the RAPD-PCR reactions were carried out as described elsewhere[21]. The reaction mixture and PCR conditions are presented in Table 2. The RAPD products resolved in 1.5% metaphor agarose gel electrophoresis were documented in Alpha Imager TM1200 documentation and analysis system.

ARDRA and RISA

The 16S rRNA gene and intergenic spacers region (IGS) between 16S and 23S rDNA were amplified by following the procedures of Sy *et al*[6] and Martin-Laurent *et al*[22], respectively. The reaction mixture and PCR conditions are given in Table 2. Both the PCR

products were purified using PCR clean kit (Sigma GenElute™ PCR clean-up kit, USA) according to the manufacturer's instructions and approximately 1 µg each of PCR amplified 16S rDNA fragments and ITS were restricted with *Hae*III enzyme (Fermentas, USA) separately at 37°C for overnight. The digestion mixtures were separated by electrophoresis in 1.5% metaphor agarose gel and documented.

BOX-and ERIC-PCR

PCR was carried out with BOX and ERIC primers according to the procedure of Versalovic *et al*[15] as modified by Hussain *et al*[23]. The reaction mixture and PCR conditions are given in Table 2. BOX- and ERIC-PCR products were analyzed by electrophoresis in 1.5 % metaphor agarose gels and documented.

Results and Discussion

Discrimination and species identification of *Methylobacterium* that are associated with many plant species is gaining importance for diversity analysis, taxonomical purposes and ecological studies. Hence, we have selected 10 species of *Methylobacterium*, frequently isolated from phyllosphere, root nodules and internal tissues of selected plant species. Among the 10 species, the strain TNAU14 isolated from mentha leaves, capable of fixing atmospheric nitrogen, is yet to be characterized to species level[24]. All these 10 species were subjected for metabolic fingerprinting, by observing the difference of growth in carbon-substrates utilization pattern with 15 different carbon-substrates substituted for methanol in MMS medium. The presence or absence of growth was recorded (Table 3). The results clearly showed the difference among the species of *Methylobacterium* to utilize the carbon compounds as sole source. Providing differential carbon-substrate utilization pattern is one of the characteristics useful for distinguishing the *Methylobacterium* at species level from other species, being essential for any new species description[5,7]. The present results are in accordance with the above works and the new strain TNAU14 could not be differentiated from *M. fujisawaense*. However, the metabolic fingerprint of differential carbon-substrates utilization pattern could discriminate all other species from each other. For molecular fingerprinting and typing of these 10 plant associated *Methylobacterium* species, the common PCR based techniques such as RAPD, ARDRA, RISA, BOX-PCR and ERIC-PCR are evaluated and results are discussed herewith.

RAPD fingerprinting of all the *Methylobacterium* species differentiated them into 10 different distinct groups, when the primer OPQ2 was used; whereas OPQ1 produced too many amplicons that resulted in poor resolution and discrimination. The PCR amplification banding pattern of OPQ1 and OPQ2 primers have been shown in Fig. 1. OPQ2 produced

Table 2—PCR conditions adopted in this study

PCR conditions	RAPD	16S rRNA gene	IGR gene	BOX-PCR	ERIC-PCR
A. Reaction mixture					
Reaction volume	25 µL	20 µL	20 µL	20 µL	20 µL
Taq buffer	1X	1X	1X	1X	1X
$MgCl_2$	1.5 m*M*	1.5 m*M*	1.5 m*M*	1.5 m*M*	1.5 m*M*
dNTP mixture	0.10 m*M* each	0.25 m*M* each	0.25 m*M* each	0.25 m*M* each	0.25 m*M* each
Primers[a]	1 µ*M*	1 µ*M*	1 µ*M*	1 µ*M*	1 µ*M*
Template DNA	25 ng	10 ng	10 ng	50 ng	50 ng
B. Reaction conditions					
Initial denaturation	95°C for 3 min	94°C for 3 min	95°C for 3 min	94°C for 3 min	94°C for 3 min
Denaturation	94°C for 1 min	94°C for 1 min	95°C for 1 min	94°C for 1 min	94°C for 1 min
Annealing	36°C for 45 sec	55°C for 1 min	55°C for 40 sec	55°C for 1 min	55°C for 1 min
Extension	72°C for one min	72°C for 1 min	72°C for 1 min	72°C for 2 min	72°C for 2 min
No. of cycles	35	35	35	30	30
Final extension	72°C for 7 min	72°C for 10 min	72°C for 7 min	72°C for 15 min	72°C for 15 min

[a]The primer sequences used in different PCRs are available in corresponding references. For RAPD and BOX-PCR, single primer is used at 1 µ*M* and all other PCR reactions, forward and reverse primers were used at 1 µ*M* each concentration.

Table 3—Differential carbon-substrate utilization profile standard strains of *Methylobacterium* species

Carbon substrates[a, b]	Stains/type stains of *Methylobacterium* species[b]									
	Ma	Me	Mf	Mm	Mn	Mp	Mr	Ms	Msp	Mt
Methylamine	+	+	-	-	-	+	-	+	-	+
Acetate	+	+	+	-	+	+	+	+	+	+
Citrate	-	-	+	+	+	-	+	-	+	+
L-Glutamate	+	+	+	+	+	-	+	-	+	+
D-Glucose	-	-	+	+	-	-	+	+	+	+
D-xylose	-	-	+	-	+	-	+	-	+	+
Fructose	+	-	+	-	-	+	-	+	+	+
Betaine	+	+	-	-	+	+	+	+	-	+
Thiocyanate	-	-	-	-	-	-	-	-	-	+
Tartarate	-	+	+	-	+	+	-	-	+	+
Arabinose	+	-	+	+	+	-	-	-	+	+
Dimethyl amine	+	-	-	-	-	-	-	-	-	+
Trimethyl amine	+	-	-	-	-	-	-	-	-	-
Cyanate	-	-	-	-	-	-	-	-	-	-
Nutrient broth	+	+	+	-	+	+	+	+	+	+

[a]The carbon substrates were substituted for methanol in MMS medium at 0.5% (w/v)
[b]+, growth; -, No growth; Mean of three replicates
Ma-*M. aminovorans* (TNAU 9); Me-*M. extorquens* (DSM1337^T); Mf-*M. fujisawaense* (DSM5686^T); Mm-*M. mesophilicum* (DSM1708^T); Mn-*M. nodulans* (ORS2060^T); Mp-*M. populi* (TNAU12); Mr-*M. radiotolerans* (DSM1819^T); Ms-*M. suomiense* (TNAU4); Msp-*Methylobacterium* sp. (TNAU14); & Mt-*M. thiocyanatum* (TNAU8)

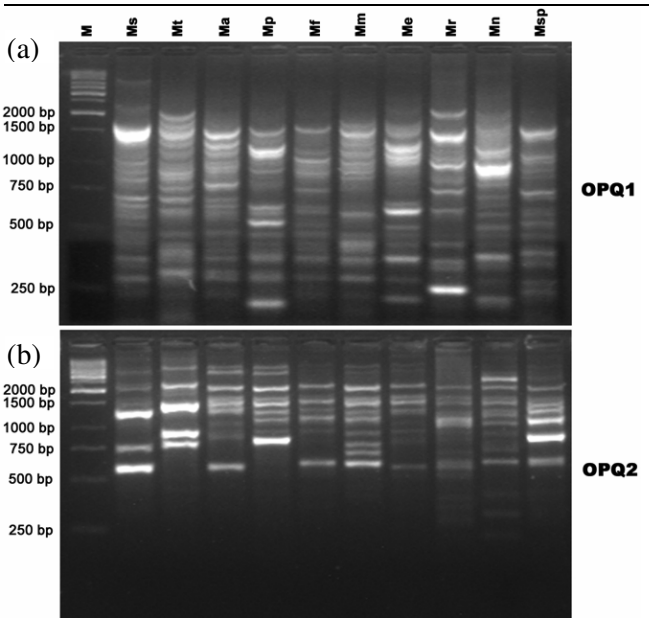

Fig. 1—RAPD fingerprints of standard/type strains of *Methylobacterium* species generated by Primers OPQ1 (a) and OPQ2 (b). M-1 kb DNA marker ladder; Ms-*M. suomiense* (TNAU4); Mt-*M. thiocyanatum* (TNAU8); Ma-*M. aminovorans* (TNAU 9); Mp-*M. populi* (TNAU12); Mf-*M. fujisawaense* (DSM5686^T); Mm-*M. mesophilicum* (DSM1708^T); Me-*M. extorquens* (DSM1337^T); Mr-*M. radiotolerans* (DSM1819^T); Mn-*M. nodulans* (ORS2060^T); & Msp-*Methylobacterium* sp. (TNAU14).

8 to 12 bands from a size of about 500 to 2000 bp and each *Methylobacterium* species fingerprinting profile differentiated well. However, the result revealed that the power of RAPD fingerprinting for discrimination is a primer-dependent one. The present result was in accordance with several reports with different eubacterial genera such as *Salmonella*[25]; *Rhizobium*[16]; *Carnobacteria*[11] and *Helicobacter*[17]. However, the major limitation of RAPD is its poor repeatability due to various reasons such as PCR conditions, genomic DNA integrity, *Taq* polymerase enzyme, etc[12].

The full-length of 16S rRNA gene (about 1500 bp) amplified using universal eubacterial primers and digested with *HaeIII* enzyme, resolved is presented as ARDRA profile in Fig. 2a. The restricted DNA fragments of 3 to 6 with a size range of < 250 bp to 500 bp could be observed in the ARDRA profile. The ARDRA could be able to discriminate the 10 species of *Methylobacterium* into 7 different groups. The closely-related pair of species such as *M. suomiense* and *M. aminovorans*; *M. populi* and *M. thiocyanatum*; *M. fujisawaense* and *M. mesophilicum* showed similar ARDRA profile and could not be differentiated from each other. The result on ARDRA typing of plant-associated *Methylobacterium* species revealed that even though ARDRA could be a valuable PCR based tool for discrimination, it was not able to differentiate the closely-related methylobacterial species. The

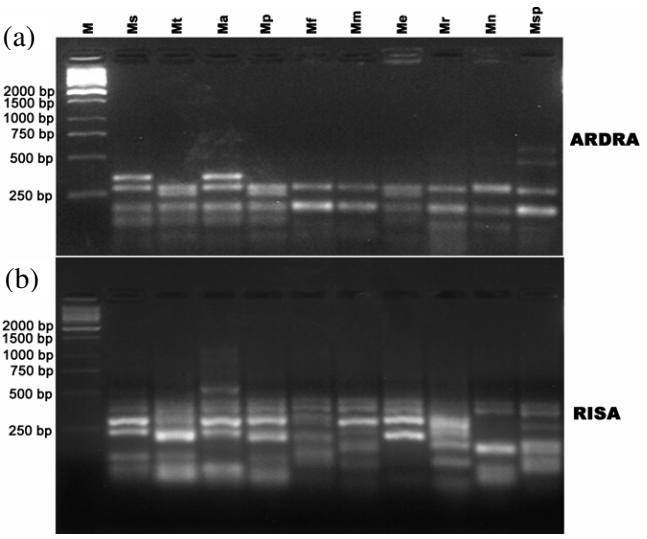

Fig. 2—ARDRA and RISA profile of standard/type strains of *Methylobacterium* species. M-1 kb DNA marker ladder; Ms-*M. suomiense* (TNAU4); Mt-*M. thiocyanatum* (TNAU8); Ma-*M. aminovorans* (TNAU 9); Mp-*M. populi* (TNAU12); Mf-*M. fujisawaense* (DSM5686^T); Mm-*M. mesophilicum* (DSM1708^T); Me-*M. extorquens* (DSM1337^T); Mr-*M. radiotolerans* (DSM1819^T); Mn-*M. nodulans* (ORS2060^T); & Msp-*Methylobacterium* sp. (TNAU14).

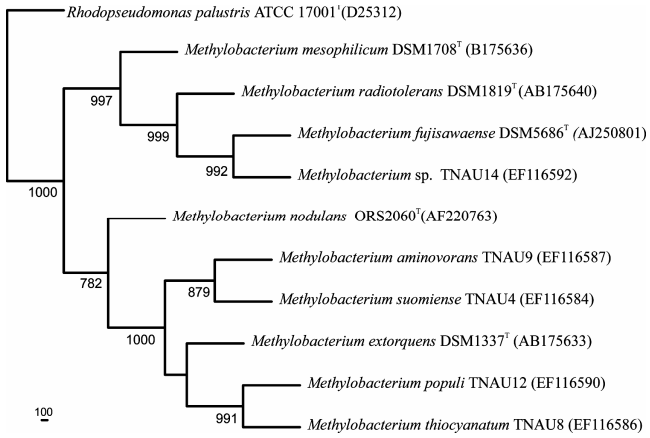

Fig. 3—Phylogenetic relationship of plant associated *Methylobacterium* spp. based on 16S rRNA gene sequence comparisons. The sequence of strains of representative species obtained from GenBank used for the construction of phylogenetic tree. Sequence accession number of 16S rRNA of the strains given in parentheses. The phylogenetic tree was constructed by neighbour-joining method using DNADIST, SEQBOOT, NEIGHBOR and CONSENSE from PHYLIP v3.5c and the tree file was analysed using treeview. Bootstraps values of 500 or more (from 1000 replicates) are indicated at the nodes.

inability of ARDRA to distinguish closely-related species and strains of species, already reported in other bacterial communities[25,26] was confirmed for plant-associated *Methylobacterium* species in the present study.

As an alternative to 16S DNA restriction analysis, the intergenic spacer (IGS) region between 16S and 23S rDNA of 10 *Methylobacterium* species was amplified and analyzed for the power to differentiate. All were able to amplify about 2000 bp sized IGS product and much less size difference was noticed among them. Hence, all the IGS products were digested with *HaeIII* enzyme to reveal the sequence diversity among them. The RISA profile of the 10 species was able to discriminate all of them (Fig. 2b). When comparing ARDRA, *Methylobacterium* spp. produced more distinctive profiles of RISA and could differentiate the closely-related species. The 16S rRNA sequence information obtained from GenBank of the 10 species was subjected to phylogenetic grouping. The phylogenetic tree revealed the close-relatedness of these plant-associated *Methylobacterium* species (Fig. 3). All the species had 97 to 99 per cent similarity with each other. Hence, ribotyping by ARDRA was unable to discriminate these species, while RISA typing could differentiate them. The potential discriminative performance of RISA over ARDRA was well-documented for *Streptococcus*[27]; *Bradyrhizobium* and *Rhizobium*[28] and *Helicobacter, Camphylobacter* and *Aerobacter*[29] and the present results are in conformity with the above findings with reference to plant-associated methylobacterial typing.

Rep-PCR genomic fingerprinting makes use of DNA primers complementary to naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in the genome of most of the Gram-negative and Gram-positive bacteria. Three families of repetitive sequences have been identified, including the 35-40 bp repetitive intergenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence and 154 bp BOX element. These sequences appear to be located in distinct, intergenic positions around the genome. The profile is now widely used for typing the organisms[10,15]. Among the three elements, BOX and ERIC elements of the plant-associated *Methylobacterium* species were amplified and the fingerprinting patterns of BOX and ERIC-PCR products are presented in Figs 4a & b. Amplification of genomic DNA of methylobacterial species with BOX primer generated 8 to 14 DNA fragments and the size of DNA band ranged from 0.1 to 1.5 kb (Fig. 4a). The methylobacterial species could not be differentiated using BOX-PCR profile, as they

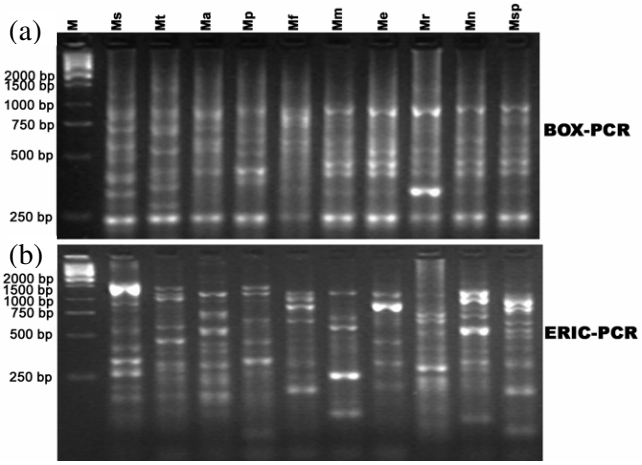

Fig. 4—BOX-and ERIC-PCR fingerprints of standard/type strains of *Methylobacterium* species. M-1 kb DNA marker ladder; Ms-*M. suomiense* (TNAU4); Mt-*M. thiocyanatum* (TNAU8); Ma-*M. aminovorans* (TNAU 9); Mp-*M. populi* (TNAU12); Mf-*M. fujisawaense* (DSM5686^T); Mm-*M. mesophilicum* (DSM1708^T); Me-*M. extorquens* (DSM1337^T); Mr-*M. radiotolerans* (DSM1819^T); Mn-*M. nodulans* (ORS2060^T); & Msp-*Methylobacterium* sp. (TNAU14).

segregate the species into only 4 different groups. Interestingly, the ERIC-PCR genomic fingerprinting of methylobacterial species generated 8 to 15 bands that ranged from 0.1 to 2.0 kb and were able to discriminate all the species of *Methylobacterium* including the strain TNAU14 (Fig. 4b). The banding patterns ranging in size from 1.5 kb to 0.25 kb are highly discriminative and were able to generate the efficient DNA fingerprint profile for the above species. The ERIC-PCR is commonly applied for typing the pathogenic bacteria like *Francisella*[30], *Salmonella*[25], *Helicobacter*[17] and for soil microbial ecological studies[31,16]. In the present study also it was confirmed that this PCR technique could be used for the identification of plant associated *Methylobacterium* species.

In summary, among the 5 different PCR fingerprinting techniques evaluated in plant-associated methylobacterial species, RAPD, RISA and ERIC-PCR could be used as tools for rapidly identifying and discriminating *Methylobacterium* species.

Acknowledgement

The authors are thankful to Dr T Sa, Chungbuk National University, Republic of Korea and Dr P Jourand, Campus International de Baillarguet, France for providing type strains of *Methylobacterium* species.

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