Ann Microbiol (2012) 62:1643–1650
DOI 10.1007/s13213-012-0421-z

ORIGINAL ARTICLE

Diversity of free-living nitrogen-fixing bacteria associated with Korean paddy fields

Md. Rashedul Islam · Tahera Sultana · Jang-Cheon Cho · M. Melvin Joe · T. M. Sa

Received: 21 September 2011 / Accepted: 20 January 2012 / Published online: 18 February 2012

Abstract Nitrogen (N)-fixing microorganisms play a major role in maintaining soil fertility and are thereby important for sustainable rice production. Among a total of 165 bacterial isolates recovered from seven paddy field soils through an isolation process on four N-free media, 32 were found to be positive for PCR amplification of *nif*H gene. On screening, the BOX-PCR fingerprint technique grouped the *nif*H gene positive isolates into seven clusters. Clustering of bacteria revealed a very low level of similarity (20%), indicating the existence of a high degree of genetic diversity among the N-fixing isolates. Further characterization based on fatty acid methyl ester (FAME) showed that the isolates were members of 16 different genera, with maximum number belonged to the genus *Burkholderia* followed by *Sphingomonas*. Our results provide evidence for wide diversity of free-living N-fixing bacteria that can be used in future as a feasible alternative to N fertilizers in rice-paddy ecosystems.

Keywords Nitrogen fixation · *nif*H gene · BOX-PCR · Diversity · FAME · Paddy soil

Electronic supplementary material The online version of this article (doi:10.1007/s13213-012-0421-z) contains supplementary material, which is available to authorized users.

M. R. Islam · M. M. Joe · T. M. Sa
Department of Agricultural Chemistry,
Chungbuk National University,
Cheongju, Chungbuk 361-763, Republic of Korea

M. R. Islam (✉) · T. Sultana · J.-C. Cho
Department of Biological Sciences, Inha University,
Incheon 402-751, Republic of Korea
e-mail: rashed@inha.ac.kr

Introduction

The nitrogen (N) requirement of paddy rice is well known and rice production today depends on the application of large quantities of nitrogenous fertilizers (Choudhury and Kennedy 2004). Unfortunately, besides increasing production costs, these fertilizers also cause severe environmental pollution in rice-producing environments (Wartiainen et al. 2008). Biological nitrogen fixation (BNF) is considered as a suitable alternative in the development of sustainable agriculture, satisfying human needs, and conserving natural resources (Giller and Cadisch 1995; Vance 1997). BNF is performed by phylogenetically diverse groups of bacteria that harbor *nif*H genes that encode the Fe-protein subunit of nitrogenase. Among nitrogen fixers, free-living N-fixing bacteria are important contributors to soil N, reaching up to 60 kg ha^{-1} $year^{-1}$ (Cleveland et al. 1999). It has been reported that soil N availability is linked to the presence of N-fixing organisms (Bormann and Sidle 1990; Matthews 1992), and this plays a vital role for successive microbial colonization (Walker and del Moral 2003). Wartiainen et al. (2008) reported the genetic diversity of free-living N-fixing bacteria in paddy soils based on *nif*H gene sequences, and addressed their contribution to the N input in rice-paddy ecosystems.

BOX-PCR analysis is a highly effective approach for determining genetic relationships among bacterial isolates (Versalovic et al. 1991). A high correlation exists between BOX-PCR fingerprinting and DNA-DNA homology data, and this technique has been applied in numerous taxonomic studies on plant-associated, environmental, medical and food-associated bacteria (Rademaker et al. 1998; Lanoot et al. 2004). Fatty acid methyl ester (FAME) analysis is another well-established method for identification and classification of bacteria based on whole cellular fatty acids derivatized to methyl esters (Sasser 2009). Differences in

chain length, positions of double bonds and the binding of functional groups make them very useful taxonomic markers (Dawyndt et al. 2006). Because of cheap, fast, automated and high throughput process, FAME analysis has revolutionized bacterial identification in a way that has now increased the efficiency of many laboratories around the world (Kunitsky et al. 2005) and, as a result, libraries of fatty acid profiles for thousands of bacteria have been developed to aid in identification (Kloepper et al. 1992, Slabbinck et al. 2008).

Although numerous studies have suggested that free-living heterotrophic N-fixers are a potentially important source of N fixation in rice fields, knowledge of the ecology of N-fixers has not been well documented and cannot be enhanced by studying only laboratory microorganisms (Boddey et al. 1998; Mahadevappa and Shenoy 2000). It is therefore necessary to study the naturally occurring diversity of N-fixers in a large-scale soil ecosystem in order to understand phenotypic variation (Balandreau 1986). The objective of the present study was to assess genetic diversity among the N-fixing isolates recovered from seven different paddy soils by analyzing *nif*H gene and BOX-PCR genomic DNA fingerprinting, and to identify the isolates by FAME analysis. In addition, the isolates were characterized physiologically, and the effects of different fertilizer treatments on the occurrence and distribution of *nif*H gene were also investigated.

Materials and methods

Site and soil sampling

Soil samples were collected from the National Institute of Agricultural Science and Technology located at Suwon city of the Republic of Korea in October 2007 (after harvesting the rice crop), where research plots were established in 1954. Rice straw, prepared by fermenting for 5 months, was used as compost. Compost with and without nitrogen–phosphorus–potassium (NPK) fertilizer was applied to soil. Since 1986, chemical fertilizers and compost have been applied at the rates noted in Table 1. While CNPK received ammonium sulfate, all the other treatments received urea as N source. The control treatment received neither chemical fertilizer nor compost amendments. The sampling was done by collecting soils (0–20 cm depth) from nine randomly selected points within each plot using a 1.45 cm diameter soil core. Samples from each plot were then combined to form one composite sample and stored at 4°C.

Isolation and phenotypic characterization

N-fixing bacteria were isolated using the serial dilution technique on four N-free selective media viz., NFMM (Piao et al. 2005), LGI-P (Reis et al. 1994), BAz (Estrada-De Los Santos et al. 2001) and JNFb (Kirchhof et al. 1997). The pure cultures of the isolates were maintained in nutrient broth containing 50% (w/v) glycerol and refrigerated at −80°C.

Table 1 Different paddy soil treatments used in the present study

Soil sample	Treatment[a]
Control	No fertilizers including rice straw
NPK	N-P_2O_5-K_2O at 110:70:80 kg/ha
CNPK	N-P_2O_5-K_2O at 110:70:80 kg/ha+7.5 Mg/ha rice straw
NPKC750	N-P_2O_5-K_2O at 110:70:80 kg/ha+7.5 Mg/ha rice straw
NPKC750	N-P_2O_5-K_2O at 110:70:80 kg/ha+15.0 Mg/ha rice straw
NPKC2250	N-P_2O_5-K_2O at 110:70:80 kg/ha+22.5 Mg/ha rice straw
NPKC3000	N-P_2O_5-K_2O at 110:70:80 kg/ha+30.0 Mg/ha rice straw

[a] Chemical fertilizer was applied at the rate of 75-75-75 from 1954 to 1970, 100-75-75 from 1971 to 1978, 150-86-86 from 1979 to 1985 and 110:70:80 kg/ha from 1986 to present. Rice straw was used as organic compost by fermenting 5 months of period

Physiological and biochemical characters of N-fixing bacterial isolates were examined according to *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994). Gram staining was performed with a Gram stain kit (Difco, Detroit, MI). Qualitative tests for the production of oxidase and catalase were performed by using Difco strips as recommended by the manufacturer. The strains were also characterized biochemically for the following basic reactions: nitrate reduction, gelatin hydrolysis, starch, casein and urea hydrolysis (Stanier et al. 1966; Bossis et al. 2000). The results of these tests were scored as either positive or negative (Table 2).

Amplification of nifH gene

The presence of the *nif*H gene in the N-fixing bacterial isolates was determined by amplifying a 390-bp fragment through PCR using a pair of specific primers, 19F (5′-GCIWTY TAYGGIAARGGIGG-3′) and 407R (5′-AAICCRCCR CAIACIACRTC-3′) (Ueda et al. 1995) directed against conserved sequences of the *nif*H gene. The amplified products were resolved on a 1% agarose gel in 1x TBE buffer and visualized under UV light (Bio-Rad Laboratories, Richmond, CA).

PCR amplification with specific BOX A1R primer

The DNA of the bacterial isolates was amplified by PCR using primer BOX A1R (5′-CTACGGCAAGGCGACGCTGACG-3′, Invitrogen, Life Technologies, Carlsbad, CA) (Versalovic et al. 1994). The PCR cycling protocol was as follows: 1 cycle

Table 2 Biochemical characteristics of the N-fixing bacteria isolated from paddy field soils. *1* Gram's reaction; *2* motility; *3* casein hydrolysis; *4* starch hydrolysis; *5* gelatine hydrolysis; *6* nitrate reduction; *7* catalase production; *8* urease activity; *9* oxidase activity; *10* V-P test; *11* polysaccharide production

Isolate name	Soil sample	Isolation medium	1	2	3	4	5	6	7	8	9	10	11
RFNB1	Control	NFMM	-[a]	-	-	-	+	+	+	-	+	+	-
RFNB2	NPK	NFMM	+	-	+	+	+	+	+	+	+	-	+
RFNB3	CNPK	NFMM	-	+	+	+	+	+	+	-	-	+	-
RFNB4	NPKC750	NFMM	+	+	-	-	+	+	+	-	-	-	+
RFNB5	NPKC750	NFMM	+	+	-	+	+	-	-	-	-	-	-
RFNB6	NPKC2250	NFMM	+	-	+	+	+	+	+	+	+	-	+
RFNB7	NPKC3000	NFMM	-	-	-	-	+	+	+	-	+	-	-
RFNB8	CNPK	LGI-P	-	+	+	+	+	+	+	-	+	+	-
RFNB9	CNPK	LGI-P	-	-	-	-	+	+	+	-	-	+	-
RFNB10	NPKC1500	LGI-P	+	-	+	+	+	+	+	+	+	+	-
RFNB11	Control	BAz	-	-	-	+	+	-	-	-	-	-	-
RFNB12	Control	BAz	-	+	-	+	+	-	-	-	-	+	-
RFNB13	Control	BAz	-	+	-	+	+	-	-	-	-	+	-
RFNB14	Control	BAz	-	+	+	+	+	+	+	+	+	+	-
RFNB15	CNPK	BAz	-	+	+	+	+	+	+	-	+	-	-
RFNB16	NPKC1500	BAz	-	-	-	+	+	-	-	-	-	-	-
RFNB17	NPKC2250	BAz	-	+	+	+	+	+	+	+	+	-	-
RFNB18	NPKC2250	BAz	-	-	-	+	+	-	-	+	-	-	-
RFNB19	NPKC3000	BAz	-	+	+	+	+	+	+	+	+	-	-
RFNB20	NPKC3000	JNFb	-	-	-	-	+	+	-	-	-	-	-
RFNB21	Control	JNFb	-	+	-	-	+	-	-	-	-	+	-
RFNB22	Control	JNFb	-	+	-	-	+	-	-	-	-	-	-
RFNB23	Control	JNFb	-	+	-	-	+	-	+	-	-	-	-
RFNB24	Control	JNFb	-	-	+	+	+	+	+	+	+	-	+
RFNB25	NPK	JNFb	-	-	-	+	+	-	+	-	-	+	-
RFNB26	NPK	JNFb	-	-	-	-	+	+	-	-	-	+	-
RFNB27	NPK	JNFb	-	+	-	+	+	+	-	-	+	+	-
RFNB28	CNPK	JNFb	-	-	-	-	+	-	-	-	-	-	-
RFNB29	NPKC1500	JNFb	-	-	-	+	+	+	-	-	-	+	-
RFNB30	NPKC2250	JNFb	-	-	-	+	+	+	+	-	-	-	-
RFNB31	NPKC3000	JNFb	-	-	-	-	+	+	-	-	-	-	-
RFNB32	NPKC2250	JNFb	-	-	-	-	+	+	-	-	-	+	-

[a]+ Positive for the reaction/motile, - negative for the reaction/non-motile

of denaturation at 95°C for 7 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 65°C for 8 min; 1 cycle of final extension at 65°C for 16 min; and a final soak at 4°C. The amplified fragments were separated by horizontal electrophoresis on a 1.5% agarose gel (15 cm×15 cm), at 75 V, for 5 h. Gels were stained with ethidium bromide, visualized under UV light and photographed. Cluster analyses of the BOX-PCR products were performed using the method of unweighted pair grouping with mathematic average (UPGMA) in the Gelcompar II 3.5 software package (Applied Math, Austin, Texas).

Fatty acid methyl ester profiles

FAME profiles were obtained by saponification, methylation and extraction following the MIDI system (Microbial Identification System, Newark, NJ) (Sasser 2009). Briefly, strains were grown overnight on nutrient agar plates. One loopful of cells was harvested and transferred to a culture tube; 1 ml saponication reagent added. Tubes were sealed tightly with a teflon-lined screw cap and vortexed for 5–10 s. The tubes were placed in water bath at 100°C for 5 min. They were then taken out of the boiling water bath and cooled slightly, vortexed for 10 s and incubated in a water bath for an additional 25 min. Each tube received 2 ml methylation reagent, was capped tightly and vortexed for 10 s. The tubes were placed in a water bath at 80±1°C for 10 min. Then, 1.25 ml extraction reagent was added to the cooled tubes followed by gentle tumbling on a rotator for 10 min. The bottom phase was removed by using a pipette. Finally, 3 ml base reagent was added to upper phase remaining in the tubes and tumbled for 5 min. The upper solvent

phase was transferred to vials for fatty acid analysis. FAME data were analyzed with Sherlock 6.0 MIDI TSBA60 library (see Sasser 2009), and bacteria were identified based on similarity index value.

Results and discussion

Biochemical characterization

In the present study, the composition of the N-fixing bacteria associated with paddy soils was assessed by phenotypic characterization and by genomic DNA fingerprinting analysis to evaluate genetic variation among isolates. With the exception of RFNB2, RFNB4, RFNB5, RFNB6 and RFNB10, all tested isolates were Gram negative; 14 isolates exhibited motility and the remaining 18 isolates were non-motile. RFNB10 and RFNB14 were positive for all biochemical tests including casein, starch, gelatine, nitrate reduction, catalase, urease, oxidase and V-P test. The isolates RFNB2, RFNB4, RFNB6 and RFNB24 were able to produce polysaccharide (Table 2).

Amplification of nifH gene and its distribution

Out of a total of 165 N-fixing isolates, 32 were positive for amplification of a 390-bp *nif*H gene fragment (Supplemental Fig. S1). Amplicons of the *nif*H gene were obtained to evaluate the diversity of potential N-fixing bacteria in the rice-paddy system. Among isolates containing the *nif*H gene, most were isolated from JNFb medium (41%), followed by BAz (28%), NFMM (22%) and LGI-P (9%), respectively. It should be noted that the isolates obtained from different media represent only the culturable fraction. However, when comparison was made based on various soil treatments, the highest number (28%) of *nif*H-gene-positive isolates was obtained in control plots (Table 3). This is not surprising, because the control plots never received any fertilizer, and thus might allow a higher number of N-fixing bacteria to grow. However, we found no differentiation between the occurrence of *nif*H isolates in plots amended with different levels of fertilizers, supporting the observations of Ogilvie et al. (2008) and Wakelin et al. (2007) that long-term application of N fertilizers does not have much impact on *nif*H abundance within agricultural soils.

Genomic finger printing by BOX-PCR

The BOX-PCR technique is a multilocus analysis used to elucidate the phylogenetic relatedness among different isolates (Martin et al. 1992; Cottyn et al. 2001). To access genetic relationships, the N-fixing bacterial isolates were submitted to DNA analysis with the BOX A1R-PCR method to amplify the DNA with primers (BOX A1R) of repetitive and evolutionary conserved regions. BOX sequences are dispersed in the genomes of diverse bacteria (Lupski and Weinstock 1992) and the primers amplify genomic regions between the two BOX elements. Successful amplification was achieved from isolates with high polymorphism in the well resolved bands, with 3–10 amplification products ranging in the size from 200 to 1,350 bp. Information about the FAME analysis (Table 4) was used for the description of BOX-PCR results (Fig. 1).

Based on the BOX-PCR banding pattern, the isolates were grouped into seven clusters (Fig. 1); 34% of the isolates were grouped into cluster III with 45% similarity within the group. This cluster was most diverse, comprising

Table 3 Distribution of the isolates showing *nif*H gene amplifications to various paddy soils and the four types of N-free medium used for isolation purposes

Soil sample	Medium				Type of bacteria identified
	NFMM	LGI-P	BAz	JNFb	
Control	1 (8)[a]	0 (0)	4 (5)	4 (9)	*Methylobacterium, Burkholderia, Roseomonas*, *Klebsiella*, *Sphingomonas*, *Stenotrophomonas*
NPK	1 (7)	0 (4)	0 (6)	3 (8)	*Mycobacterium*, *Grimontia*
CNPK	1 (9)	2 (2)	1 (1)	1 (4)	*Paucimonas*, *Enterobacter*, *Novosphingobium*
NPKC750	2 (10)	0 (5)	0 (3)	0 (6)	*Paenibacillus*, *Brochothrix*
NPKC1500	0 (5)	1 (6)	1 (3)	1 (8)	*Novosphingobium*, *Burkholderia*
NPKC2250	1 (6)	0 (6)	2 (6)	2 (7)	*Bacillus, Burkholderia*, *Pseudomonas*, *Sphingomonas*
NPKC3000	1 (9)	0 (8)	1 (6)	2 (8)	*Methylobacterium, Burkholderia*, *Pseudomonas*, *Phyllobacterium*

[a] Values in parenthesis indicate the total number of N-fixing bacteria obtained on the respective medium from seven soil samples

five different genera viz. *Burkholderia* (RFNB11, RFNB12, RFNB16, RFNB17, RFNB18), *Mycobacterium* (RFNB2), *Novosphingobium* (RFNB10), *Klebsiella* (RFNB14) and *Enterobacter* (RFNB15). While isolate RFNB11 shared an identical electrophoretic profile with RFNB12, isolate RFNB2 was only 45% similar with other members of this group. Isolates RFNB17 and RFNB18 also shared indistinguishable electrophoretic profiles. Cluster IV formed the second largest group, with 22% of isolates sharing approximately 43% similarity among them. *Sphingomonas* (RFNB22, RFNB23), *Burkholderia* (RFNB19), *Grimontia* (RFNB25) and *Phyllobacterium* (RFNB31) formed part of this group. Five isolates of cluster I, comprising different genera, viz. *Methylobacterium* (RFNB1, RFNB7), *Brochothrix* (RFNB5) and *Stenotrophomonas* (RFNB24), shared a low level of similarity (29%). Group II has three isolates, viz. *Paucimonas* (RFNB9), *Pseudomonas* (RFNB30), with 42% similarity within the group and approximately 40% similarity with group III. Group VII clustered with all the other groups with a very low level similarity (20%). These results confirmed the existence of a wide genetic diversity among N-fixing bacterial isolates recovered from paddy soils.

Table 4 Taxonomic identification of N-fixing isolates as determined by fatty acid methyl ester (FAME) profiles

Isolate code	FAME identification	Similarity index
RFNB1	*Methylobacterium mesophilicum*	0.457
RFNB2	*Mycobacterium aichiense*	0.152
RFNB3[a]	Not matched	-
RFNB4	*Paenibacillus azotofixans*	0.515
RFNB5	*Brochothrix campestris*	0.417
RFNB6	*Bacillus megaterium*	0.388
RFNB7	*Methylobacterium rhodesianum*	0.631
RFNB8	Not matched	-
RFNB9	*Paucimonas lemoignei*	0.729
RFNB10	*Novosphingobium capsulatum*	0.291
RFNB11	*Burkholderia gladioli*	0.471
RFNB12	*Burkholderia gladioli*	0.224
RFNB13	*Roseomonas* sp.	0.594
RFNB14	*Klebsiella pneumoniae*	0.308
RFNB15	*Enterobacter hormaechei*	0.631
RFNB16	*Burkholderia gladioli*	0.173
RFNB17	*Burkholderia gladioli*	0.214
RFNB18	*Burkholderia cenocepacia*	0.518
RFNB19	*Burkholderia gladioli*	0.214
RFNB20	*Pseudomonas pertucinogena*	0.221
RFNB21[a]	Not matched	-
RFNB22	*Sphingomonas paucimobilis*	0.590
RFNB23	*Sphingomonas paucimobilis*	0.475
RFNB24	*Stenotrophomonas maltophilia*	0.179
RFNB25	*Grimontia hollisae* (*Vibrio*)	0.175
RFNB26	Not matched	-
RFNB27[a]	Not matched	-
RFNB28	*Novosphingobium capsulatum*	0.130
RFNB29[a]	Not matched	-
RFNB30	*Pseudomonas pertucinogena*	0.213
RFNB31	*Phyllobacterium myrsinacearum*	0.579
RFNB32	*Sphingomonas paucimobilis*	0.369

[a] Isolates not matched with information available in MIDI Aerobic Bacteria Library TSBA60

Specific primers of the *nif*H genes and BOX-PCR were used in the present study, which allowed the genetic structure of N-fixers in paddy fields to be assessed through the diversity of *nif*H genes. Our result revealed that many N-fixing bacteria isolated from different fertilization treatments shared identical BOX-PCR patterns, indicating similarity among isolates. However, the fingerprinting pattern was complex, with seven major clusters joining with a very low final level of similarity (20%) indicating a degree of diversity among N-fixers. This fingerprinting technique has proven valuable in studies of N-fixing bacteria and has been used extensively in ecology, genetic and taxonomic studies, as well as for identification of N-fixing bacterial isolates (e.g., Versalovic et al. 1994; Chen et al. 2000; Kaschuk et al. 2006). From our results, it appears that BOX analysis of *nif*H PCR products from environmental sample is a powerful tool for assessing the presence and diversity of N-fixing microorganisms in ecosystems (Sato et al. 2009), and the technique is rapid and simple. The number and positions of the BOX fragments reflect the diversity and heterogeneity of the bacteria in a sample. Although this approach does not directly allow evaluation of functional aspects of the N-fixers in a sample, genetic information on the gene pool and the potential for N fixation may be assessed.

Identification of isolates by FAME analysis

The MIDI-FAME technique was used to determine the whole-cell cellular fatty acid profiles of N-fixing bacterial isolates (Table 4). In the present work, this analysis identified more than 81% of the bacteria with varied levels of confidence value, for a total of 16 different genera, viz., *Burkholderia* sp., *Sphingomonas* sp., *Methylobacterium* sp., *Pseudomonas* sp., *Novosphingobium* sp., *Bacillus* sp., *Paenibacillus* sp., *Enterobacter* sp., *Klebsiella* sp., *Mycobacterium* sp., *Roseomonas* sp., *Brochothrix* sp., *Paucimonas* sp., *Stenotrophomonas* sp., *Phyllobacterium* sp., *Grimontia* sp., etc. The highest number of isolates was assigned to *Burkholderia* sp. and *Sphingomonas* sp. Isolates RFNB3, RFNB8, RFNB21, RFNB26, RFNB27 and RFNB29 did not give a match with the MIDI Aerobic

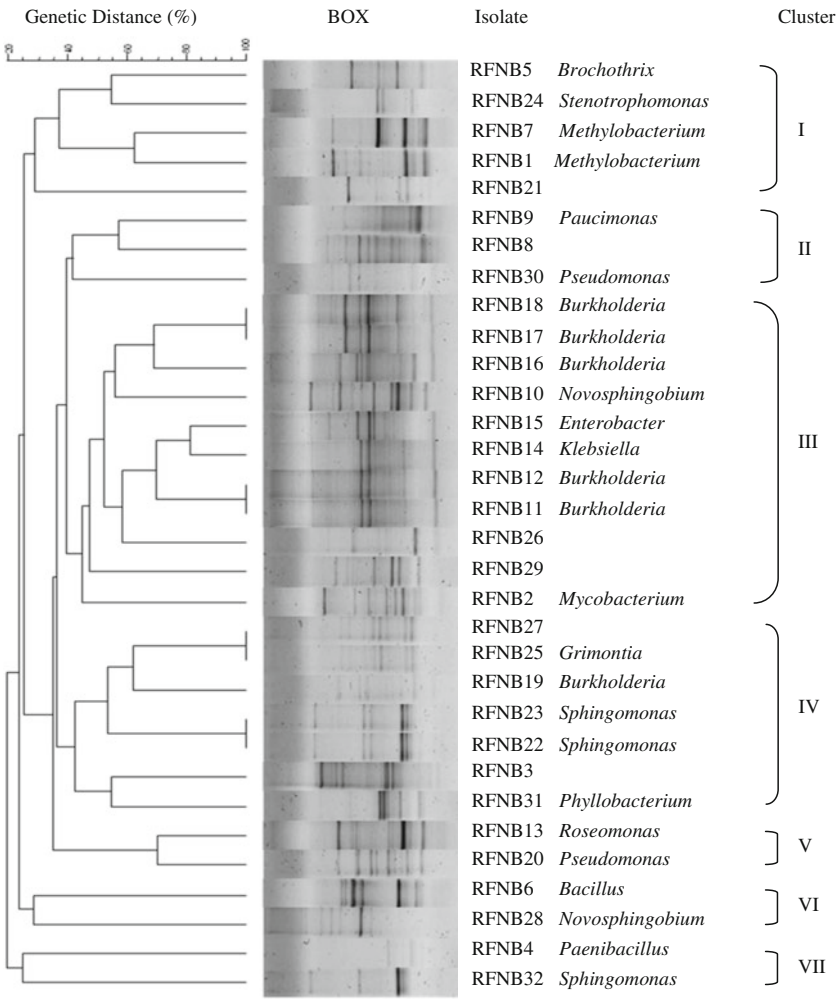

Fig. 1 BOX-PCR fingerprinting gel and unweighted pair grouping with mathematic average (UPGMA) dendrogram showing genetic relationship as estimated by the cluster analysis of products of N-fixing isolates obtained from different paddy soil samples. *Scale bar* Percent similarity

Bacteria Library TSBA60 and therefore we were not able to identify them. Earlier studies by McInroy and Kloepper (1995) and Lilley et al. (1996) found that MIDI-FAME systems could identify 95% and 80% of their isolates, respectively. These differences may be due to the different similarity standards used: 0.1 in McInroy and Kloepper (1995), 0.3 in Poonguzhali et al. (2006) and unknown in Lilley et al. (1996).

DNA-based technology, which typically uses only the 16S rRNA gene as the basis for microbial identification, has the advantage of identifying difficult-to-cultivate strains and is growth independent. As the 16S rRNA gene is highly conserved at the species level, speciation is normally quite good, but subspecies and strain level differences are not shown (Kunitsky et al. 2005). Some other problems with 16S rRNA technology include the requirement for a high level of technical proficiency, and the cost per sample, as well as equipment costs, are high (Slabbinck at al. 2010). Therefore, this technology is not well suited for routine microbial quality control in the pharmaceutical and other sectors (Sutton and Cundell 2004). On the other hand, FAME profiles are used routinely to identify genera, species and strains of bacteria (Cavigelli et al. 1995; Ibekwe and Kennedy 1999). This analysis was developed to identify bacterial species more quickly and easily, and is currently able to identify accurately over 1,500 species of bacteria, many to the subspecies or strain level (Kunitsky et al. 2005). Earlier, Cottyn et al. (2001) used FAME analysis successfully to classify phenotypic diversity among bacterial isolates.

In conclusion, the classification of N-fixing isolates according to biochemical analyses and generated BOX-PCR fingerprints supported the observation that there is a high degree of *nif*H gene diversity in paddy field soils. The information on the N-fixing bacteria of paddy soils obtained in the present report provide a framework that will aid in their DNA signature sequences for further ecological and taxonomic studies.

Acknowledgments This work was supported by grants from the Korea Research Foundation (KRF), Republic of Korea. We thank the anonymous reviewers for their thorough evaluation and constructive

suggestions for improving the quality of the original manuscript. The partial support from the research grant of Inha University, Republic of Korea, is also acknowledged.

References

Balandreau J (1986) Ecological factors and adaptive processes in N_2-fixing bacterial populations of the plant environment. Plant Soil 90:73–92

Boddey RM, Alves B, Urquiaga S (1998) Evaluation of biological nitrogen fixation associated with non-legumes. In: Malik KA, Mirza MS, Ladha JK (eds) Nitrogen fixation with non-legumes. Kluwer, Dordrecht, pp 287–305

Bormann BT, Sidle RC (1990) Changes in productivity and distribution of nutrients in a chronosequence at Glacier Bay National Park, Alaska. J Ecol 78:561–578

Bossis E, Lemanceau P, Latour X, Gardan L (2000) The taxonomy of *Pseudomonas fluorescens* and *Pseudomonas putida*: current status and need for revision. Agronomie 20:51–63

Cavigelli MA, Robertson GP, Klug MK (1995) Fatty acid methyl ester (FAME) profiles as measures of soil microbial community structure. Plant Soil 170:99–113

Chen LS, Figueredo A, Pedrosa FO, Hungria M (2000) Genetic characterization of soybean rhizobia in Paraguay. Appl Environ Microbiol 66:5099–5103

Choudhury ATMA, Kennedy IR (2004) Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. Biol Fertil Soils 39:219–227

Cleveland CC, Townsend AR, Schimel DS (1999) Global patterns of terrestrial biological nitrogen (N_2) fixation in natural ecosystems. Global Biogeochem Cycles 13:623–645

Cottyn B, Regalado E, Lanoot B, De Cleene M, Mew TW, Swings J (2001) Bacterial populations associated with rice seed in the tropical environment. Phytopathology 91:282–292

Dawyndt P, Vancanneyt M, Snauwaert C, De Baets B, De Meyer H, Swings J (2006) Mining fatty acid databases for detection of novel compounds in aerobic bacteria. J Microbiol Methods 66:410–433

Estrada-De Los Santos P, Bustillos-Cristales R, Caballero-Mellado J (2001) *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl Environ Microbiol 67:2790–2798

Giller KE, Cadisch G (1995) Future benefits from biological nitrogen fixation: an ecological approach to agriculture. Plant Soil 174:255–277

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th edn. Williams and Wilkins, Baltimore

Ibekwe AM, Kennedy AC (1999) Fatty acid methyl ester (FAME) profiles as a tool to investigate community structure of two agricultural soils. Plant Soil 206:151–161

Kaschuk G, Hungria M, Andrade DS, Campo RJ (2006) Genetic diversity of rhizobia associated with common bean (*Phaseolus vulgaris* L.) grown under no-tillage and conventional systems in Southern Brazil. Appl Soil Ecol 32:210–220

Kirchhof G, Reis VM, Baldani JI, Eckert B, Döbereiner J, Hartmann A (1997) Occurence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. Plant Soil 194:45–55

Kloepper JW, McInroy JA, Bowen KL (1992) Comparative identification by fatty acid analysis of soil, rhizosphere, and geocarposphere bacteria of peanut (*Arachis hypogaea* L.). Plant Soil 139:85–90

Kunitsky C, Osterhout G, Sasser M (2005) Identification of microorganisms using fatty acid methyl ester (FAME) analysis and the MIDI Sherlock Microbial Identification System. Encyclopedia of Rapid Microbiological Methods, MIDI, Newark, DE

Lanoot B, Vancanneyt M, Dawyndt P, Cnockaert M, Zhang J, Huang Y, Liu Z, Swings J (2004) BOX-PCR fingerprinting as a powerful tool to reveal synonymous names in the genus *Streptomyces*. Emended descriptions are proposed for the species *Streptomyces cinereorectus*, *S. fradiae*, *S. tricolor*, *S. colombiensis*, *S. filamentosus*, *S. vinaceus* and *S. phaeopurpureus*. System Appl Microbiol 27:84–92

Lilley AK, Fry JC, Bailey MJ, Day MJ (1996) Comparison of aerobic heterotrophic taxa isolated from four root domains of mature sugar beet (*Beta vulgaris*). FEMS Microbiol Ecol 21:231–242

Lupski JR, Weinstock GM (1992) Short, interspersed repetitive DNA sequences in prokaryotic genomes. J Bacteriol 174:4525–4529

Mahadevappa M, Shenoy VV (2000) Towards nitrogen fixing rice (Oryza sativa). Adv Agric Res India 13:131–139

Martin B, Humbert O, Camara M, Guenzi E, Walker J, Mitchell T, Andrew P, Prudhomme M, Alloing G, Hakenbeck R, Morrison DA, Boulnois GJ, Claverys J-P (1992) A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Res 20:3479–3483

Matthews JA (1992) The ecology of recently-deglaciated terrain: a geoecological approach to Glacier Forelands and primary succession. Cambridge University Press, Cambridge, UK

McInroy JA, Kloepper JW (1995) Survey of indigenous bacterial endophytes from cotton and sweet corn. Plant Soil 173:337–342

Ogilvie LA, Hirsch PR, Johnston AWB (2008) Bacterial diversity of the broadbalk 'Classical' winter wheat experiment in relation to long-term fertilizer inputs. Microb Ecol 56:525–537

Piao Z, Cui Z, Yin B, Hu J, Zhou C, Xie G, Su B, Yin S (2005) Changes in acetylene reduction activities and effects of inoculated rhizosphere nitrogen-fixing bacteria on rice. Biol Fertil Soils 41:371–378

Poonguzhali S, Madhaiyan M, Sa TM (2006) Cultivation-dependent characterization of rhizobacterial communities from field grown Chinese cabbage *Brassica campestris* ssp. pekinensis and screening of potential plant growth promoting bacteria. Plant Soil 286:167–180

Rademaker JL, Louws FJ, de Bruijn FJ (1998) Characterization of the diversity of ecological important microbes by rep-PCR genomic fingerprinting. In: Akkermans ADL, van Elsas JD, de Bruijn FJ (1998) Molecular ecology manual. Kluwer, Dordrecht, pp 1–26

Reis VM, Olivares FI, Döbereiner J (1994) Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. World J Microbiol Biotechnol 10:101–104

Sasser M (2009) (revised from 1990) Microbial identification by gas chromatographic analysis of fatty acid methyl esters (GC-FAME). Technical Note # 101; http://www.midi-inc.com/pdf/MIS_Technote_101.pdf

Sato A, Watanbe T, Unno Y, Purnomo E, Osaki M, Shinano T (2009) Analysis of diversity of diazotrophic bacteria associated with the rhizosphere of a tropical arbor, *Melastoma malabathricum* L. Microbes Environ 24:81–87

Slabbinck B, De Baets B, Dawyndt P, De Vos P (2008) Genus-wide Bacillus species identification through proper artificial neural network experiments on fatty acid profiles. Antonie van Leeuwenhoek 94:187–198

Slabbinck B, Waegeman W, Dawyndt P, De Vos P, De Baets B (2010) From learning taxonomies to phylogenetic learning: Integration of 16S rRNA gene data into FAME-based bacterial classification. BMC Bioinforma 11:69

Stanier RY, Palleroni NJ, Doudoroff M (1966) The aerobic pseudomonads a taxonomic study. J Gen Microbiol 43:159–217

Sutton SVW, Cundell AM (2004) Microbial identification in the pharmaceutical industry. Pharmacopeial Forum 30:1884–1894

Ueda T, Suga Y, Yahiro N, Matsuguchi T (1995) Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. J Bacteriol 177:1414–1417

Vance CP (1997) Enhanced agricultural sustainability through biological nitrogen fixation. In: Legocki A, Bothe H, Pühler A (eds) Biological fixation of nitrogen for ecology and sustainable agriculture. Springer, Berlin, pp 179–186

Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19:6823–6831

Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5:25–40

Wakelin SA, Colloff MJ, Harvey PR, Marschner P, Gregg AL, Rogers SL (2007) The effects of stubble retention and nitrogen application on soil microbial community structure and functional gene abundances under irrigated maize. FEMS Microbiol Ecol 59:661–670

Walker LR, del Moral R (2003) Primary succession and ecosystem rehabilitation. Cambridge University Press, Cambridge, UK

Wartiainen I, Eriksson T, Zheng W, Rasmussen U (2008) Variation in the active diazotrophic community in rice paddy—*nifH* PCR-DGGE analysis of rhizosphere and bulk soil. Appl Soil Ecol 39:65–75