J. Ecol. Field Biol. 31 (4): 309~316, 2008

Comparison of Soil Bacterial Community Structure in Rice Paddy Fields under Different Management Practices using Terminal Restriction Fragment Length Polymorphism (T-RFLP)

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ABSTRACT: To develop a monitoring method for soil microbial communities in rice paddy fields, we used terminal restriction fragment length polymorphism (T-RFLP) to compare soil bacterial community structure in rice paddy fields experiencing different management practices: organic practices, conventional practices without a winter barley rotation, and conventional practices with a winter barley rotation. Restriction fragment length profiles from soils farmed using organic practices showed very different patterns from those from conventional practices with and without barley rotation. In principal component analyses, restriction fragment profiles in organic practice samples were clearly separated from those in conventional practice samples, while principal component analysis did not show a clear separation for soils farmed using conventional practices with and without barley rotation. The cluster analysis showed that the bacterial species compositions of soils under organic practices were significantly different from those under conventional practices at the 95% level, but soils under conventional practice with and without barley rotation did not significantly differ. Although the loadings from principal component analyses and the Ribosomal DNA Project II databases suggested candidate species important for soils under organic farming practices, it was very difficult to get detailed bacterial species information from terminal restriction fragment length polymorphism. Rank-abundance diagrams and diversity indices showed that restriction fragment peaks under organic farming showed high Pielou's Evenness Index and the reciprocal of Simpson Index suggesting high bacterial diversity in organically farmed soils.

Key words: Diversity indices, Organic farming practice, Rice paddy fields, Soil bacterial community, Terminal restriction fragment length polymorphism (T-RFLP)

INTRODUCTION

In recent years, molecular techniques using the polymerase chain reaction (PCR) to amplify the 16S rRNA gene, such as the terminal restriction fragment length polymorphism (T-RFLP) technique, have become popular for determining whole soil microbial communities (Liu et al. 1997, Marsh 1999). T-RFLP has several advantages over similar PCR-based techniques for the 16S rRNA gene such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE): direct reference to sequence databases such as the Ribosomal DNA Project (RDP), greater resolution than DGGE or TGGE, and output as digital profiles that make comparisons among different soil communities easy (Marsh 1999). Our recent risk assessment for a transgenic watermelon (Park et al. 2006a, Park et al. 2006b, Park 2007) resulted in the development of a molecular approach to elucidate soil bacterial community structure using terminal restriction fragment length polymorphism (T-RFLP).

As global interest in organic farming as an alternative to conventional farming practices has increased, much research has focused on the impact of farming practices on soil fertility and biodiversity (Drinkwater et al 1995, Mäder et al. 2002, Van Diepeningen et al. 2006). Since the 1970s, organic farming practices have developed in South Korea to produce safer foods free from chemical fertilizers and pesticides (Chung 2003). Soil bacterial communities play a major role in energy flow and nutrient cycling in soil food webs (Neher 1999). Therefore, the diversity of soil bacterial communities must be determined to study the relationship between soil diversity and function (Nannipieri et al. 2003).

In this study, we compared soil bacterial communities under organic and conventional management practices using T-RFLP. Our purposes were to identify characteristic bacterial species for soils being farmed using organic and conventional practices by analyzing T-RF profiles through principal component analysis (PCA) and to

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describe the diversity of soil bacterial communities using T-RF as representatives of each bacterial genotype.

METHODS

Site Description and Sample Collection

We collected soil samples from rice paddy fields in Hodong-ri, Beolgyo-eup, Boseong-gun, and Jeollanam-Do, Korea in 2004. Samples were collected from depths of 0～10 cm and 0～30 cm from rice paddy fields under organic practices (O-10, O-30), conventional practices (C-10, C-30) and conventional practices with crop rotation (CB-10, CB-30). Crop rotation with winter barley had already been conducted for 3 years in the crop rotation field when this study began. Only compost had been applied as fertilizer and no pesticides had been applied for 10 years in the organic paddy field when this study began. The field had been certified as "organic" by the National Agricultural Products Quality Management Service (NAQS, government certification body) of the Ministry of Agriculture and Forestry, Korea since 2001.

T-RFLP

Total DNA was extracted from the soils for T-RFLP analyses using the FastDNA SPIN KIT For Soil (Qbiogene, USA). The concentration of DNA was estimated using an UV spectrometer (Bio Rad Smart Spec 3000). We amplified the 16S rRNA gene using PCR using the extracted DNA as templates with fluorescence-dye-labeled 8F-FAM (5'-AGAGTTTGATCCTGGCTCAG-3') and unlabeled 1492R (5'-TACGGTTACCTTGTTACGACTT-3') primers (Bioneer, Korea). The reactions were conducted using 50 μL (final volume) mixtures containing 10× *Taq* buffer (Neutrotics, Korea), 1 μL of each deoxyribonucleoside triphosphate (Promega, USA) at a concentration of 10 pmol and 2 U of *Taq* DNA polymerase (Neurotics Inc., Korea). Conditions for PCR were as follows: an initial denaturation step of 94℃ for 3 min, 25 amplification cycles of denaturation (45 s at 94℃); annealing (45 s at 55℃); and elongation (2 min at 70℃); and a final extension step of 7 min at 72℃ (Ritchie et al. 2000). We combined products from six PCR runs (total volume: 300 μL), followed by a purification step using Qiaquick PCR Purification Kits (Qiagen, Germany). One microgram of purified PCR product was digested with the restriction endonucleases *Hae*III (Promega, USA) and *Hha*I (Promega, USA) at 37℃ for 2 h. The reactions were conducted using 20 μL (final volume) mixtures containing 2 μL of 10× buffer, 2 μL of 10× Acetylated Bovine Serum Albumin supplied by the manufacturer (Promega, USA), and 1 μL of the restriction endonuclease (10 U). Digests (1～2 μL) were mixed with 12 μL of formamide and 0.5 μL of size standard (GeneScan-1000 ROX, Applied Biosystems). The samples were denatured at 60℃ for 20 min and then placed on ice. Lengths of the restricted fragments were determined using an automated ABI DNA sequencer (Model 3100, Applied Biosystems, USA) for 1 h 32 min 10 s. The fluorescently labeled 5'-terminal restriction fragments (T-RFs) were detected and analyzed using GeneScan 3.7 software (Applied Biosystems, USA) with size markers ranged between 29 and 677 which covered most of the major T-RFs.

Principal Component Analyses and Cluster Analysis

T-RF peaks identified by GeneScan 3.7 software from individual T-RFLP profiles were compiled and aligned to produce large data matrices (6 observations × 89 peak variables for the *Hae*III dataset and 6 observations × 80 peak variables for the *Hha*I dataset). Centered T-RFLP profile data were used in PCA without any further normalization. We assigned a value of 0 if there was no matching peak. We then applied PCA to the weighted covariance data matrices to reduce their dimensionality. All statistical analyses except for cluster analysis were performed with S-Plus 6 for Windows (Insightful Corp., USA). Cluster analysis was conducted on candidate bacterial species lists obtained from the TAP-TRFLP menu on the RDP (Ribosomal DNA Project) II site (http://rdp.cme.msu.edu) using BOOTCLUS program (McKenna 2003). We used the Bray-Curtis Coefficient for the similarity index and UPGMA as the linkage method.

Diversity Indices

We used genotype richness (S), the Shannon diversity index (H'), Pielou's evenness index (E) and the reciprocal of Simson index ($1/D$) as indices of diversity. S was defined as the number of unique T-RF peaks for each T-RFLP profile. The Shannon diversity index was calculated as follows:

$$H' = -\sum (p_i) (\ln p_i) \quad (1)$$

where the summation is over all values of p_i, the relative abundance of the unique restriction fragment i. The abundance of a particular T-RF peak can be determined using the intensity of fluorescence in fluorescence units. Pielou's evenness index was calculated as follows:

$$E = H' / (\ln [S]) \quad (2)$$

The reciprocal of Simpson index ($1/D$) was calculated as follows:

$$1/D = 1 / (\sum p_i^2)$$

Diversity indices were compared among bacterial communities using the Tukey multiple comparison test.

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RESULTS

Restriction Fragment Length Profiles of Soils Under Different Farming Practices

T-RF profiles from soils of paddy rice fields under conventional farming practices with and without barley rotation and under organic farming appeared to be different for both restriction enzymes (Fig. 1 and Fig. 2). In particular, the T-RF profiles from soils farmed using organic practices showed very different patterns from those farmed using conventional practices with and without barley rotation. Restriction fragment profiles produced using the *Hha*I enzyme showed very different patterns from profiles produced using the *Hae*III enzyme. T-RF peaks in the *Hha*I dataset were closely aggregated relative to the T-RF peak patterns in the *Hae*III dataset.

Comparison of Bacterial Communities Using T-RFLP Profile Data

We conducted PCA to summarize the T-RF profile data and to identify the most important T-RF peaks (Fig. 3). For the *Hae*III enzyme dataset, PCA explained 83% of the total variation with the first and the second principal components. Restriction fragment profiles in organic-practice samples were clearly separated from those in conventional-practice samples along the first principal component, which explained 70.3% of the total variation. For the *Hha*I enzyme dataset, PCA explained 89.6% of the total variation with the first and the second principal components. Restriction fragment profiles in the organic-practice samples were clearly separated from those in conventional-practice samples along the second principal component, which explained 21.7% of the total variation. In both datasets, PCA scores of conventional-practice samples with and without barley rotation were not clearly separated.

Based on predicted lengths of T-RF peaks for *Hae*III and *Hha*I enzymes from the RDP II database (http://rdp8.cme.msu.edu/html), we identified a total of 129 bacterial strain candidates including 52 genera and 87 species for the studied soils (data are not shown). We included species candidates only if the predicted lengths of T-RF peaks were found in the T-RF profiles for both *Hae*III and *Hha*I. We performed a cluster analysis on a species presence/absence matrix for 6 soil samples. The cluster analysis showed that the bacterial species compositions of soils under organic practices

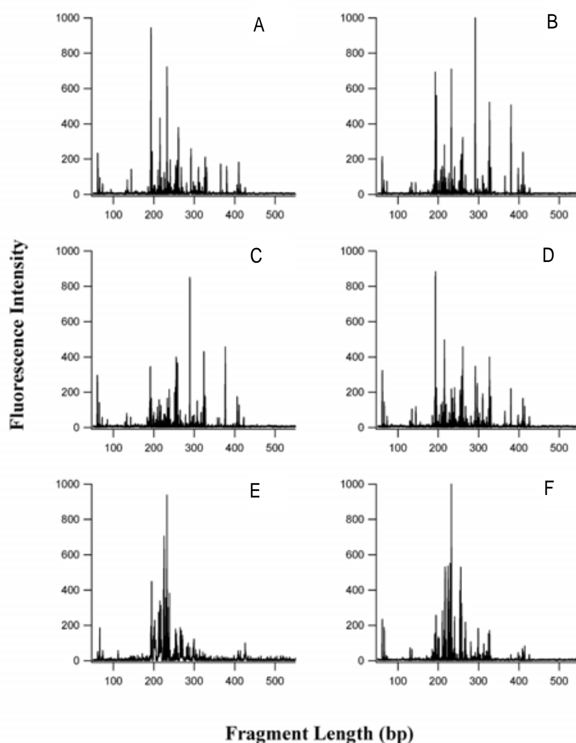

Fig. 1. *Hae*III restriction enzyme T-RFLP profiles of soils from rice fields under conventional farming (A and B), conventional farming with barley rotation (C and D), and organic farming (E and F). A: C-10 sample, B: C-30 sample, C: CB-10 sample, D: CB-30 sample, E: O-10 sample, F: O-30 sample.

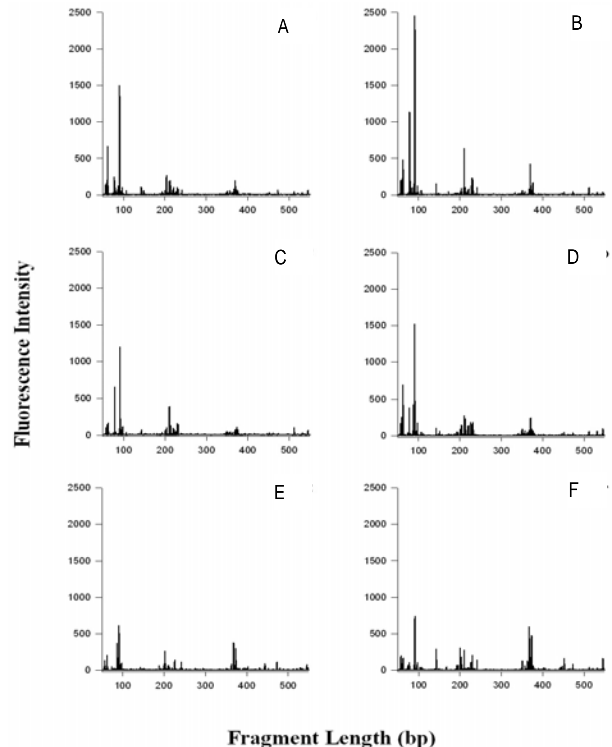

Fig. 2. *Hha*I restriction enzyme T-RFLP profiles of soils from rice fields under conventional farming (A and B), conventional farming with barley rotation (C and D), and organic farming (E and F). A: C-10 sample, B: C-30 sample, C: CB-10 sample, D: CB-30 sample, E: O-10 sample, F: O-30 sample.

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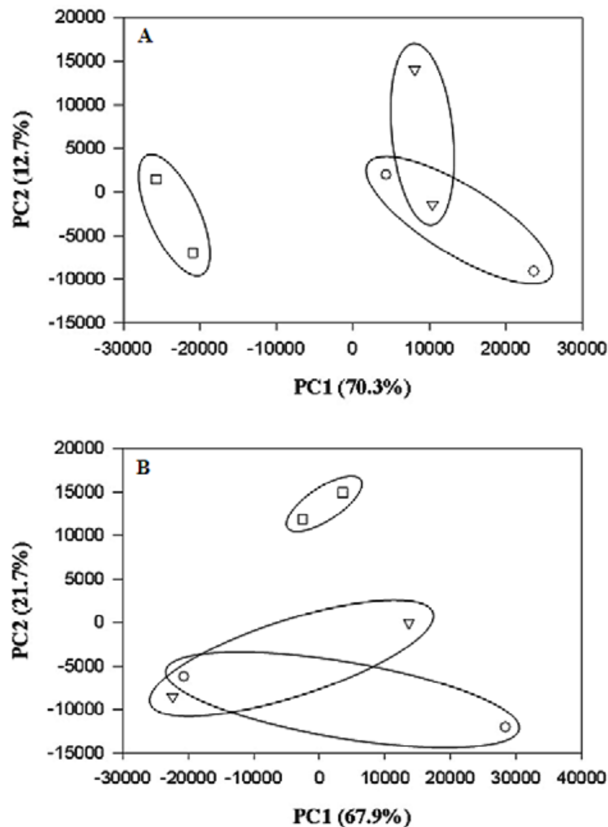

Fig. 3. Scores from a principal component analysis (PCA) on T-RF profiles of rice field soils under conventional farming (○), conventional farming with barley rotation (△), and organic farming (□) for the *Hae*III dataset (A) and the *Hha*I dataset (B). The ellipses were drawn arbitrarily for grouping.

were significantly different from those under conventional practice (Fig. 4). However, soils under conventional practices with and without barley rotation did not differ at the 95% significance level.

Characteristic Species Candidates for Each Community

To identify characteristic species for soils under organic farming practice, we investigated the loadings for the first principal component (PC1) of the PCA for the *Hae*III dataset and the second principal component (PC2) of the PCA for the *Hha*I dataset. We used significant loading values and their corresponding T-RF peaks to identify bacteria species candidates (Table 1). Since scores for T-RF profiles of soils under organic farming are located on the negative side on the first principal component in the *Hae*III dataset and on the positive side on the second principal component in the *Hha*I dataset (Fig. 3), peaks with negative loadings for the *Hae*III dataset and positive loadings for the *Hha*I dataset were expected as characteristic peaks for soils under organic farming. Only two T-RF length pairs (219/367 bp and 225/373 bp) showed significant (> 0.1) negative loadings for PC1 from the *Hae*III dataset and significant positive loading for PC2 from the *Hha*I dataset. The RDPII database suggested 5 species candidates (*Idobacter fluviatile*, *Methylophylus methylotrophus*, *Mycobacterium intracellulare*, *Mycobacterium marium*, and *Telluria mixta*) for the 219/367 pair and 4 species candidates (*Kingella denitrifians*, *Methylobacillus flagellatum*, *Neisseria denitrifians*, and *Vitreoscilla strecoraria*) for the 225/373 pair. Three T-RF length pairs (233/210, 233/63, and 233/91 bp in the *Hae*III and *Hha*I datasets, respectively) showed significant positive loadings for both PC1 from the *Hae*III dataset and PC2 from the *Hha*I dataset. Only one significant loading was found for 13 and 12 T-RF length.

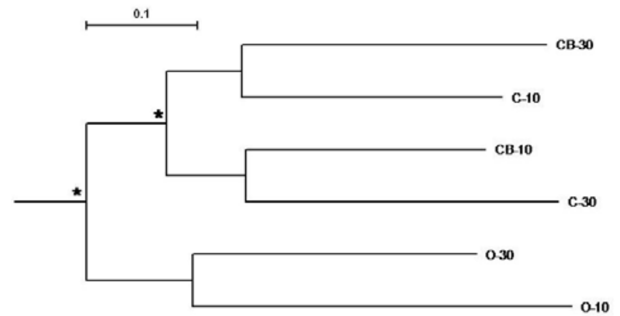

Fig. 4. A dendrogram from a cluster analysis of the projected bacterial community structures of soils under conventional farming (C-10 and C-30), conventional farming with barley rotation (CB-10 and CB-30), and organic farming (O-10 and O-30). * indicates significant linkages based on bootstrap resampling tests. The scale indicates distance on Bray-Curtis similarity.

Comparisons of Bacterial Species Diversity

Rank-abundance diagrams showed that soil bacterial communities under organic practices had a less steep slope than those under conventional practices with and without barley rotation in both the *Hae*III and *Hha*I datasets (Fig. 5). Soils under organic practices had fewer *T-RF* peaks (47 ± 1.4, mean ± SD) than soils under conventional practices with barley rotation (55 ± 2.8) but more than soils under conventional practices without barley rotation (46 ± 2.1) in the *Hae*III dataset. In the *Hha*I dataset, soils under organic practices displayed more *T-RF* peaks (43 ± 5.7, mean±SD) than soils under conventional practices with (37 ± 8.5) and without barley rotation (42 ± 2.8).

T-RF peaks in soils under organic farming showed significantly higher diversity than those in soils under conventional farming (Table 2). The Pielou's Evenness Index and the reciprocal of the Simpson index for organic-farming soils were significantly higher than those of soils under conventional practices with and without barley rotation for both the *Hae*III and *Hha*I datasets.

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Table 1. Bacteria species list from the RDPII site based on terminal restriction fragment (T-RF) lengths from T-RFLP using *Hae*III and *Hha*I enzymes and loadings from principal component analyses on T-RFLP profiles. Loadings for the first principal component (PC1) are shown for the *Hae*III dataset and loadings for the second principal component (PC2) are shown for the *Hha*I dataset

Species	T-RF length		Loadings	
	*Hae*III	*Hha*I	*Hae*III (PC1)	*Hha*I (PC2)
Iodobacter fluviatile ATCC 33051 (T)	219	367	−0.164	0.281
Methylophilus methylotrophus str. AS1 ATCC 53528 (T)	219	367	−0.164	0.281
Mycobacterium intracellulare CDC1214	219	367	−0.164	0.281
Mycobacterium marium	219	367	−0.164	0.281
Telluria mixta ACM 1762 (T)	219	367	−0.164	0.281
Kingella denitrifians str. UB-38 CCUG 30450 (T)	225	373	−0.153	0.249
Methylobacillus flagellatum str. KT1	225	373	−0.153	0.249
Neisseria denitrifians str. M37 ATCC 14686 (T)	225	373	−0.153	0.249
Vitreoscilla stercoraria ATCC 15218 (T)	225	373	−0.153	0.249
Kurthia zopfii str. F64/100 ; K5 ATCC 33403 (T)	233	210	−0.179	−0.162
Kurthia zopfii str. F64/100 ; K5 NCIMB 9878 (T)	233	210	−0.179	−0.162
Salinicoccus roseus DSM 5351(T)	233	63	−0.179	−0.297
Bdellovibrio stolpii str. UKi2 ATCC 27052 (T)	233	91	−0.179	−0.476
Eikenella corrodens FDC 373	225	554	−0.153	
Listeria grayi CIP 6818	233	578	−0.179	
clone CO28	233	211	−0.179	
clone OCS24	226	81	−0.186	
Thermobispora bispora str. R51 ATCC 19993 (T) [gene=rrnD]	226	376	−0.186	
Bacillus megaterium DSM 32 (T)	233	578	−0.179	
Erythrobacter longus str. Och 101 ATCC 33941 (T)	193	82	0.184	
Flavobacterium johnsoniae str. MVX. 1.1.1 ATCC 17061 (T)	262	90	0.184	
Methylocystis M str. M	193	229	0.184	
Porphyrobacter KK351 str. KK351	193	82	0.184	
Porphyrobacter neustonensis DSM 9434	193	82	0.184	
Porphyrobacter tepidarius str. OT3 DSM 10594 (T)	193	82	0.184	
str. JTB20	193	95	0.184	
str. LSv54	193	95	0.184	
Paenibacillus macquariensis str. 673 DSM 2 (T)	293	241	0.346	
Sphingomonas DhA-95 str. DhA-95	293	82	0.346	
str. CD	293	82	0.346	
unnamed organism	200	367		0.281
clone Sva0864	259	373		0.249
Anaerobranca horikoshii str. JW/YL-138 DSM 9786 (T)	230	369		0.190
Anaerobranca horikoshii str. JW/YL-138 DSM 9786 (T)	230	369		0.190
Desulfovibrio desulfuricans str. E1 Agheila Z NCIMB 8380	230	369		0.190
Micrococcus luteus str. Hucker S66 ATCC 381	228	369		0.190
clone SJA-102	214	202		0.131
Listeria ivanovii subsp. *Ivanovii* str. SLCC 2379 NCTC 11846 (T)	232	577		0.115
Listeria seeligeri NCTC 11856 (T)	232	577		0.115
Thermodesulfovibrio TGL-LS1 str. TGL-LS1	228	550		0.112
Clostridium cochlearium ATCC 17787 (T)	224	363		0.107
clone SJA-121	232	213		−0.127
str. JTB255	325	213		−0.127
str. NKB18	232	213		−0.127
Agromyces mediolanus str. CNF 186 ; K. AN-15 JCM 9632	229	370		−0.284
Arthrobacter sp. Str. RC100	229	370		−0.284
Clavibacter xyli subsp. *Cynodontis* str. Cxc	230	370		−0.284
clone Adriatic33	211	91		−0.476
Myxococcus coralloides str. M2 ATCC 25202 (T)	203	91		−0.476

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DISCUSSION

Our results showed that T-RFLP could detect differences in bacterial communities among soils of farms using different farming practices. PCA scores and cluster analysis indicated that soil bacterial communities under organic farming were clearly different from those produced by conventional farming with and without barley rotation, whereas T-RFLP could not detect the impacts of barley rotation on soil bacterial communities under conventional practices. Our findings are consistent with the results of previous studies that found significant differences between soil bacterial communities under conventional and organic farming practices using phospholipid fatty acids (PLFA) (Bossio et al. 1998, Esperschütz 2007) and using both community level substrate utilization (CLSU) and T-RFLP (Widmer et al. 2006). Hartmann et al. (2006) reported that the differences in soil bacterial communities under organic practices and conventional practices were mainly due to the application of manure to organically farmed soils, which suggests a possible mechanism underlying our results.

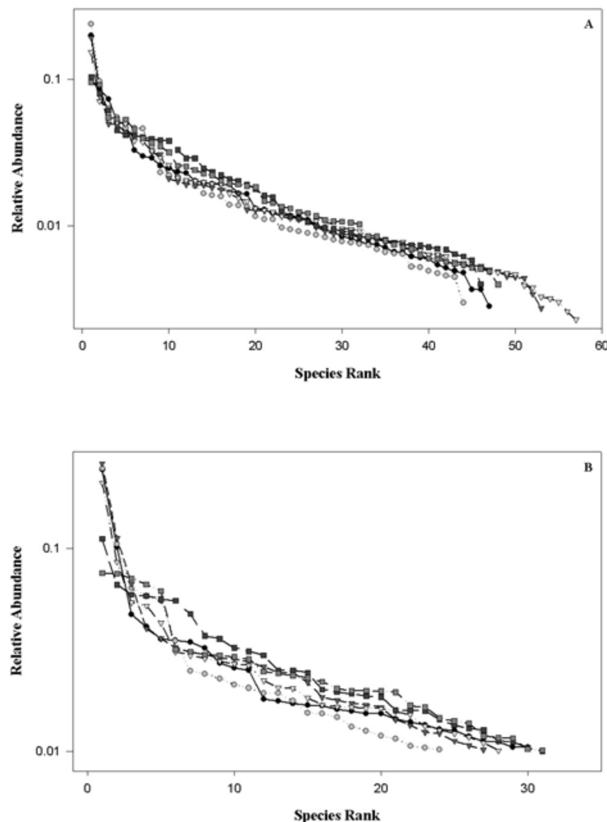

Fig. 5. Rank-abundance diagram for genotypes based on restriction fragments from the six soil bacteria communities under conventional (●), conventional with barley rotation (▲), and organic farming practices (■) for *Hae*III dataset (A) and *Hha*I dataset (B).

Our PCA loading results suggest candidates for the bacterial species with high abundance in organic-farm soil (Table 1). Among the candidates for the 219/367 pair, *Mycobacterium* has been known to occur in acid soils or in soils with high organic contents (Iivanainen et al. 1993, Iivanainen et al. 1999) and *Telluria mixta* was reported to be related to peat (Green et al. 2004). Therefore, *Mycobacterium* and *Telluria mixta* appear to be reasonable candidate bacteria for rice paddy field sediments with high organic matter supplies. However, we were not able to find any relevant literature for 4 candidates for the 225/373 pair that would suggest their suitability as candidates for soils under organic farming practices. It is notable that the candidate bacterial species were only identified based on the 16S rRNA gene sequence database at RDP II site. Our approach in identifying candidate bacterial species based on T-RF length has several limitations. First, it is very hard to assign a single species candidate to a given T-RF pair. In our case, two meaningful T-RF pairs were assigned to 4 or 5 candidates. Also, it is possible that multiple species in the soil may produce the same T-RF peaks

Table 2. Comparison of species richness (*S*), Shannon diversity index (*H'*), Pielou's evenness index (*E*), and reciprocal of Simpson index (*1/D*) for each farming practice: conventional, conventional with barely rotation, and organic. Average values (n = 2) for each site are shown with standard deviations in parentheses. Superscripts (a and b) indicate results from Tukey multiple comparisontests among the samples (95% significance level)

Endonuclease	Sites	*S*	*H'*	*E*	*1/D*
*Hae*III	Conventional	46 (2.1)[a]	4.618 (0.1686)[a]	0.838 (0.0203)[a]	15.27 (1.030)[a]
	Conventional with barley rotation	55 (2.8)[a]	4.998 (0.1239)[a]	0.864 (0.0103)[ab]	23.58 (3.630)[ab]
	Organic	47 (1.4)[a]	5.049 (0.0187)[a]	0.909 (0.0037)[b]	25.76 (1.676)[b]
*Hha*I	Conventional	42 (2.8)[a]	4.481 (0.0382)[a]	0.832 (0.0221)[a]	11.77 (0.566)[a]
	Conventional with barley rotation	37 (8.5)[a]	4.442 (0.3621)[a]	0.855 (0.0148)[a]	12.50 (3.098)[a]
	Organic	43 (5.7)[a]	4.994 (0.1730)[a]	0.921 (0.0004)[b]	28.04 (2.083)[b]

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with a given restriction enzyme. While finding important T-RF peaks using loading values may be useful in T-RFLP analysis as well as PLFA (Esperschütz et al. 2007) we obtained only two T-RF peaks that permitted further identification, partly due to statistical software that provided loadings only above a certain threshold. Overall, it appears to be very difficult to get detailed bacterial species information from T-RFLP and the 16S rRNA gene sequence database at this time. Recent reports showed variation between observed and true T-RF lengths depending on the purine content, which supports our conclusion that T-RFLP provides poor results for species identification (Kaplan and Kitts 2003, Takeshita et al. 2007).

T-RF peaks in T-RFLP can be interpreted as genotypes of the 16S rRNA gene, and several diversity indices using T-RF peaks in T-RFLP have been used to assess microbial diversity patterns (Morris et al. 2002, Denaro et al. 2005, Noguez et al. 2005, Saikaly et al. 2005). Our results indicate that relative abundances of bacterial genotypes were more evenly distributed in soils under organic practices than conventional practices (Fig. 5, Table 2). However, diversity indices such as the Shannon diversity index (H') and genotype richness (S) did not show any differences among soil bacterial genotypes under different farming practices. The genotype richness index may have low resolving power because T-RF peaks with low fluorescence intensity would be easily ignored in T-RFLP. Also, genotype richness indices exhibited variability depending on the restriction enzymes used. Variability in genotype richness was also reported in other studies (Moeseneder et al. 2001, Saikaly et al. 2005).

Although T-RFLP has several merits over other PCR related methods (Marsh 1999), our results revealed that T-RFLP has limited ability to generate bacterial species lists for different communities through reference to the sequence database. Recent studies have shown that some limitations of T-RFLP can be overcome by combining it with cloning (Moesceneder et al. 2001, Wang et al. 2004, Kibe et al. 2004, Widmer et al. 2006).

In conclusion, our results showed that T-RFLP could detect differences in the bacterial community of soils under different farming practices. In addition, we were able to identify some important bacterial species candidates occurring under organic farming practices. However, T-RFLP and reference to the sequence database allowed us to identify only a limited list of candidate bacterial species for different communities. In addition, rank-abundance diagrams and diversity indices using T-RF peaks revealed that organic soil bacterial genotypes were more evenly distributed than soil bacterial genotypes under conventional farming practice.

To further our understanding of soil microbial community structure and function under different management practices, we will need to obtain more information about microbial species and functional groups of soil ecosystems in rice paddy fields.

ACKNOWLEDGMENTS

This research was supported by the grants from the Crop Functional Genomics Center, the Korea Science and Engineering Foundation (KOSEF) funded by the Ministry of Science and Technology, and Intramural Research Funds by Ajou University granted in 2005.

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(Received October 16, 2008; Accepted November 5, 2008)

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