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Microbial community structure and diversity in the soil spatial profile of 5-year-old *Robinia pseudoacacia* 'Idaho,' determined by 454 sequencing of the 16S RNA gene

Yanping Chang,[1,2,**] Xiangpan Bu,[1,**] Weibo Niu,[1] Yu Xiu,[1] and Huafang Wang[1,*]

[1] *College of Biological Sciences and Biotechnology, Beijing Forestry University, Beijing 100083, China*
[2] *College of Life Sciences, Hebei University, Baoding 071002, Hebei, China*

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Relatively little information is available regarding the variability of microbial communities inhabiting deeper soil layers. We investigated the distribution of soil microbial communities down to 1.2 m in 5-year-old *Robinia pseudoacacia* 'Idaho' soil by 454 sequencing of the 16S RNA gene. The average number of sequences per sample was 12,802. The Shannon and Chao 1 indices revealed various relative microbial abundances and even distribution of microbial diversity for all evaluated sample depths. The predicted diversity in the topsoil exceeded that of the corresponding subsoil. The changes in the relative abundance of the major soil bacterial phyla showed decreasing, increasing, or no consistent trends with respect to sampling depth. Despite their novelty, members of the new candidate phyla OD1 and TM7 were widespread. Environmental variables affecting the bacterial community within the environment appeared to differ from those reported previously, especially the lack of detectable effect from pH. Overall, we found that the overall relative abundance fluctuated with the physical and chemical properties of the soil, root system, and sampling depth. Such information may facilitate forest soil management.

Key Words——bacterial abundance; forest soil; 454 pyrosequencing; 16S RNA gene; soil spatial profile

Introduction

Soils cover almost all of the terrestrial area on Earth and play an indispensable ecological role in the global cycles of carbon, nitrogen, and sulfur. Because of their physicochemical complexity in many micro niches, they teem with biodiversity, both phylogenetically and functionally. Microorganisms are vital for the forest ecosystem because of their roles in nutrient cycling and their associations with other organisms, including plants. Kennedy and Smith suggested that stress or change in ecosystems can affect biodiversity, and soil microorganisms can be used as sensitive biological indicators to document the changes (Kennedy and Smith, 1995). Soil microbial communities are an important factor for agriculturally managed systems, as they are responsible for most nutrient transformations in soil and influence aboveground plant diversity and productivity (Van Der Heijden et al., 2008). DNA-based approaches have increasingly been used to study the genetic structure of the total bacterial community and the impact of environmental disturbance on the soil bacterial community (Axelrood et al., 2002).

The abundance, composition, and diversity of microbial communities within soils are strongly depth de-

* Corresponding author: Dr. Wang Huafang, College of Biological Sciences and Biotechnology, Beijing Forestry University, Beijing 100083, China.
Tel/Fax: +86 10 6233 8249
E-mail: hfwang@bjfu.edu.cn
** These authors contributed equally to this work.

pendent. Most soil microbiology studies have focused exclusively on the surface 25 cm of soil where microorganism densities are highest. However, soil profiles are often many meters deep and many microorganisms reside in subsurface layers (Blume et al., 2002; Dodds et al., 1996; Fritze et al., 2000; Van Gestel et al., 1992). Little is known about the microbial communities residing in deeper soil layers. Bacterial biomass (Fierer et al., 2003b), concentration of bacterial 16S rRNA genes (Kemnitz et al., 2007; Zhou et al., 2004), number of terminal restriction fragment length polymorphism peaks (LaMontagne et al., 2003), denaturing gradient gel electrophoresis bands (representative of richness), and proportion of gram-negative to gram-positive bacteria are lower for subsurface soils than topsoils (Fierer et al., 2003b).

Changes in microbial community structure with soil depth have been attributed to the microbial response to contrasting physical and chemical conditions associated with surface, vadose zone, and saturated soils (Holden and Fierer, 2005). Environmental factors that influence microbial community composition and diversity include (but are not limited to) pH (Eichorst et al., 2007), particle size (Sessitsch et al., 2001), organic carbon content (Zhou et al., 2002), nutrient availability (Fierer et al., 2003a), water content (Treves et al., 2003), and oxygen concentration (Van Der Heijden et al., 2008). The magnitude and variation of these parameters vary between surface and subsurface soils.

Here, we investigated the vertical distribution of microbial biomass and specific microbial populations in 5-year-old *R. pseudoacacia* 'Idaho' soil. We investigated whether surface microbial communities are fundamentally distinct from the communities inhabiting the deeper soil layers and how the diversity levels within microbial communities change with soil depth down to a depth of 1.2 m by 454 sequencing of the 16S RNA gene.

Materials and Methods

Site situation and soil sampling. The test field was located in Yanqing, Beijing, China, (115°44′～116°34′E; 40°16′～40°47′N; altitude, 500 m), which is in the marginal site of the North China plain and Inner Mongolia loess plateau and has a continental monsoon climate in the north temperate zone. The climate is relatively dry with strong wind and low temperatures and is characterized by an average annual precipitation of 493 mm and an average temperature of 8.5°C, with minimum and maximum temperatures of −27.3°C and 39°C, respectively.

The deciduous arbor trees of *R. pseudoacacia* 'Idaho' at the site are 5 years old. They were artificially sucked with root cuttings and planted in the spring with a row spacing of 1.5 m × 3 m. The plant height was over 5 m, the canopy density was 0.6−0.8, and all litters were decayed into the soil. The plant root system grew horizontally, extending 20−30 cm under the soil surface and sprouting downward into soil that had a depth of 80−120 cm.

Five replicate soil samples were collected from the surface depth increments of 10−15, 25−30, 40−45, 55−60, 70−75, 85−90, 100−105, and 115−20 cm at five different points (40 sampling locations in total) (Table 1). The soils were homogenized and pooled into one composite sample by mixing equal amounts of the five individual soil sampling locations belonging to the corresponding soil depth. Coarse roots and stones (>2 mm) were removed from the samples. The soil

Table 1. Physicochemical and coordinate characteristics of the forest soil samples.

Sample depth (cm)	Sample ID	AHN ($mg \cdot kg^{-1}$)	AP ($mg \cdot kg^{-1}$)	AK ($mg \cdot kg^{-1}$)	pH	OM (%)
10−15	2_1	78.53 (1.07)	1.49 (0.12)	138.00 (2.65)	8.11 (0.23)	1.75 (0.10)
25−30	2_2	82.08 (1.08)	0.89 (0.09)	110.00 (1.73)	8.00 (0.31)	1.27 (0.02)
40−45	2_3	29.40 (0.52)	0.51 (0.04)	108.00 (1.73)	7.99 (0.10)	0.71 (0.04)
55−60	2_4	30.00 (0.90)	0.52 (0.04)	100.00 (2.00)	8.06 (0.017)	0.79 (0.12)
70−75	2_5	40.60 (1.66)	0.20 (0.01)	120.00 (5.29)	8.02 (0.026)	1.00 (0.02)
85−90	2_6	34.20 (0.95)	0.20 (0.01)	110.00 (3.00)	8.06 (0.05)	0.79 (0.03)
100−105	2_7	31.30 (0.63)	0.40 (0.01)	90.00 (1.73)	8.04 (0.05)	0.90 (0.04)
115−120	2_8	34.20 (2.10)	0.39 (0.02)	92.00 (5.00)	8.03 (0.01)	0.91 (0.02)

Values are means + (SD).

samples were stored at 4°C before biochemical characterization or frozen at −80°C for DNA extraction.

Chemical and physical soil characteristics. The physicochemical properties of the soil such as soil pH, alkali-hydrolyzable nitrogen (AHN) content, available phosphorus (AP) content, available potassium (AK) content, and organic matter (OM) content were determined per the standard method (Shidan, 2008). Select physical and chemical characteristics of the soil samples are shown in Table 1.

DNA extraction, PCR, and pyrosequencing. Soil DNA was extracted and purified using the E.Z.N.A.™ Soil DNA Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's protocol (see Supplemental Method in Supplementary Information). The size and yield of the extracted DNA were estimated by comparison with standards on 1% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) (Sambrook et al.). Gels were stained with ethidium bromide (0.5 $\mu g \cdot ml^{-1}$) and visualized under UV light. The extracted DNA was quantified by agarose gel electrophoresis using a calibrated set of standard DNAs of known concentrations. Extracted community DNAs were stored frozen (−20℃) until further processing.

The bacterial 16S rRNA gene, specifically an ~545 bp region in the 16S rRNA gene covering the V1-V3 region, was selected to construct a community library through tag pyrosequencing. The barcoded broadly conserved primers 8F and 533R containing the A and B sequencing adaptors (454 Life Sciences) were used to amplify this region (see Supplementary Information). The PCR was conducted in triplicate 25 μl reactions with 0.4 μl (5 μM) of each primer, 2.5 ng of the template DNA, 1× PCR reaction buffer, and 1.25 U of Pfu DNA polymerase (MBI Fermentas, Burlington, ON). Cycling conditions were as follows: initial denaturation at 95°C for 2 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and final 2-min extension at 72°C. During amplification, negative controls were also used. Replicate PCR products of the same sample were assembled within a PCR tube, visualized on agarose gels (2% in TAE buffer) containing ethidium bromide, and purified using a DNA gel extraction kit (Axygen, China).

Before sequencing, the DNA concentration of each PCR product was determined using a Quant-iT PicoGreen double-stranded DNA assay (Invitrogen, Darmstadt, Germany), and quality control was performed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Following quantification, the amplicons from each reaction mixture were pooled in equimolar ratios based on concentration and subjected to emulsion PCR to generate amplicon libraries, as recommended by 454 Life Sciences (Margulies et al., 2005). Amplicon pyrosequencing was performed from the A end by using a 454/Roche A sequencing primer kit and a Roche Genome Sequencer GS FLX Titanium platform at Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China. All the sequences used in this study are available from the NCBI sequence read archive under run accession numbers SRR617987-SRR617994.

Sequence analysis. The sequences aligned using the "align.seqs"command and compared with the bacterial SILVA database were further trimmed for subsequent analysis (SILVA version 108; http://www.arb-silva.de/documentation/background/release-108/) (Pruesse et al., 2007). The remaining high-quality reads were used to generate a distance matrix and clustering with the furthest neighbor algorithm. Representative sequences for shared operational taxonomic units (OTUs) as defined by 100% (unique), 97% (species level), 95% (genus level), and 90% (family level) similarity were obtained (http://www.mothur.org/wiki/Main_Page)(Chao, 1984). The estimates were calculated by employing the tools Aligner, Complete Linkage Clustering, and Rarefaction of the RDP pyrosequencing pipeline (Cole et al., 2009). *R* (http://www.mothur.org/wiki/) was used to calculate the α-diversity indices (Shannon index, Simpson's Diversity index, and Chao index). Rarefaction analysis for the 8 libraries was performed; heatmap figures, Venn diagrams, and species rank abundance distribution curves (Whittaker plots) were generated using custom Perl scripts; and ∫-LIBSHUFF analysis was performed using the libshuff command. Furthermore, multivariate analyses were performed using canonical correspondence analysis (CCA). In the present study, data preprocessing, OTU-based analysis, and hypothesis testing were performed on Mothur (Schloss et al., 2009).

Results

Bacterial richness and diversity indices

In total, 113,582 sequences were generated from 8 samples by 454 pyrosequencing. After filtering, 102,414 effective sequences, i.e., nearly 91% of the total sequences, remained. The estimates still increased even for the highest numbers of OTUs analyzed, which

indicates that substantial numbers of undetected OTUs exist in the samples and would only be detected after including more sequences (Fig. 1). Separation of the communities according to sample type indicated that the upper samples from the soils became gradually more diverse than the lower groups with decreasing depths of sampling, except for sample 2_5 (70–75 cm layer). The estimated diversity in all 8 samples was compared by computing the Shannon Diversity Index from the OTU data for each sample (Fig. S1). To investigate the relative diversity at different anatomical sites, the 8 communities were grouped by sample type, and their relative diversity was compared. Rarefaction analysis indicated that most of the Shannon Diversity estimates had reached stable values. The similarities in community structures before and after sequence trimming were also determined through different indices (Table 2). The data in Fig.1 and Table 2 suggest that the sample soils have extensive microbial diversity.

Comparison of rarefaction analyses with the number of OTUs estimated by the Chao1 richness estimator revealed that 90–100% of the estimated taxonomic richness was covered by the sequencing effort. The observed richness was only 39–62% and 72–88% of that predicted by the Chao1 richness estimator at a genetic distance of 0% and 3%, respectively, as the richness predicted by the Chao1indicator was approximately 90% at 10% genetic distance (Table 2). Thus, we did not survey the complete extent of taxonomic diversity at the species level.

The Shannon index of diversity was determined for all samples and was 6.65–7.97 at a genetic distance of 3% and was 7.74–8.38 at a genetic distance of 0%. The predicted diversity in the topsoil exceeded that of the corresponding subsoil, except in sample 2_5 (70–75 cm layer) (Table 2). At the highest resolution (0% dissimilarity), the maximum number of OTUs in any 1 soil with any of the 3 estimators used was just under 52,000 (Fig. S2), and 85% of the pairs displayed $<$3% difference at the nucleotide level (Fig. S2). Data for 0%, 3%, 5%, 10%, and 20% differences have been presented (Table 2).

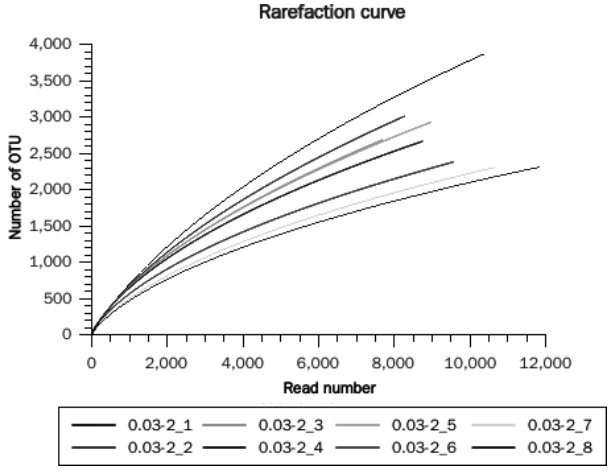

Fig. 1. Rarefaction analysis of the different samples.

Rarefaction curves of OTUs clustered at 97% sequence identity across different spatial environmental samples.

Microbial community in all samples

For different sampling depths, the microbial community structures at the phylum level were basically similar in terms of both the most predominant phylum and the content of each phylum (Fig. 2). The 5 major phyla constituted 73.6% of the total sequences for the soil samples investigated here and included the phyla *Proteobacteria* (20.9%), *Acidobacteria* (20.1%), *Actinobacteria* (13.4%), *Planctomycetes* (10.5%), and *Chloroflexi* (8.7%).The findings for these predominant phyla were consistent with the findings of previous studies.

The changes in the relative abundance of the major soil bacterial phyla followed 3 distinct trends with respect to sampling depth (Fig. S3): (i) decreasing (*Bacteroidetes* and *Proteobacteria*), (ii) increasing (*Actinobacteria*, *Firmicutes,* and *Nitrospirae*), or (iii) no great change or no consistent trend (*Acidobacteria* and *Planctomycetes*) (Fig. S3 in the Supplemental Information). No phylum dominated the soils down to a sampling depth of 1.2 m (Fig. 2).

The unusual and distinctive aspects of the soil biota that we sampled include an abundance of apparently novel phyla whose ecological role is unknown (Fig. 2). We found 16 phylogenetically distinct groups of bacteria that belong to novel lineages at the phylum or subphylum level with no close relatives in the currently available 16S rRNA gene databases. Many of these bacteria are in candidate subphyla that are most closely related to recently identified candidate bacterial phyla rather than to previously cultured phyla. Despite their novelty, members of the new candidate phyla OD1 and TM7 were widespread and were found across all depths in our sampling. The relative abundance of TM7 reached 3.8% in the surface soil sample (10-cm layer).

The overall relative abundance of detectable phyla

Table 2. Intermediate values of richness and diversity for multiple OTU definitions for the soil sample communities.

Dissimilarity (%)	Sample ID	Chao 1(no. of OTUs) [a]	Shannon's index [b]	Simpson's index [c]	Coverage (%)[d]
0	2_1	38,624	8.74	0.0002	38.85
	2_2	32,476	8.52	0.0002	38.32
	2_3	30,478	8.38	0.0004	41.17
	2_4	29,639	8.37	0.0004	47.31
	2_5	32,880	8.43	0.0001	45.01
	2_6	26,823	8.04	0.001	55.24
	2_7	28,198	7.86	0.0014	57.95
	2_8	31,880	7.79	0.0023	60.24
3%	2_1	10,573	7.97	0.0007	72.76
	2_2	8,469	7.79	0.0007	72.17
	2_3	7,883	7.61	0.0009	73.97
	2_4	6,970	7.48	0.0011	80.14
	2_5	7,958	7.6	0.0009	77.62
	2_6	5,687	7.1	0.0022	84.76
	2_7	5,565	6.81	0.0038	86.38
	2_8	5,908	6.65	0.0058	87.54
5%	2_1	7,854	7.64	0.001	79.49
	2_2	6,771	7.49	0.0011	78.02
	2_3	6,135	7.3	0.0014	79.70
	2_4	5,258	7.17	0.0016	84.83
	2_5	6,241	7.31	0.0014	82.50
	2_6	4,265	6.75	0.0032	88.45
	2_7	4,231	6.49	0.0052	89.72
	2_8	4,476	6.32	0.0084	90.53
10%	2_1	4,680	6.88	0.0024	88.36
	2_2	3,797	6.84	0.0026	87.11
	2_3	3,903	6.64	0.0032	87.34
	2_4	3,129	6.52	0.0034	91.13
	2_5	3,638	6.63	0.0032	89.60
	2_6	2,387	6.17	0.0053	93.75
	2_7	2,662	5.91	0.0111	93.84
	2_8	2,665	5.71	0.0184	94.43

[a] Nonparametric richness estimator based on the distribution of singletons and doubletons.
[b] A higher number indicates more diversity.
[c] Simpson's diversity index. A higher number represents less diversity.
[d] A higher number represents deeper sequencing.

extracted from the soil samples decreased with soil depth (Fig. S4), although slight fluctuation was detected in the 70−75 cm layers. This result was completely consistent with those mentioned above. Approximately 17.1% of the relative abundance was obtained from soil depths of 10−15 cm, whereas only 10.5% was obtained from 110−115 cm; addition of each new sampling depth resulted in a 1.65-fold decrease in the relative abundance of bacteria. A decrease in total microbial biomass with soil depth has been previously reported (Blume et al., 2002; Cho et al., 2008; Federle et al., 2006; Fierer et al., 2003b).

Community similarities and differences

Category-based clustering of the functions from each microbiome was performed using principal component analysis (PCA) and hierarchical clustering (de Hoon et al., 2004). Hierarchically clustered heatmap analysis based on bacterial community profiles at the sampling depth level indicated that all samples were gathered into 2 obvious large groups and that the topsoil sample (2_10) was independent from the other

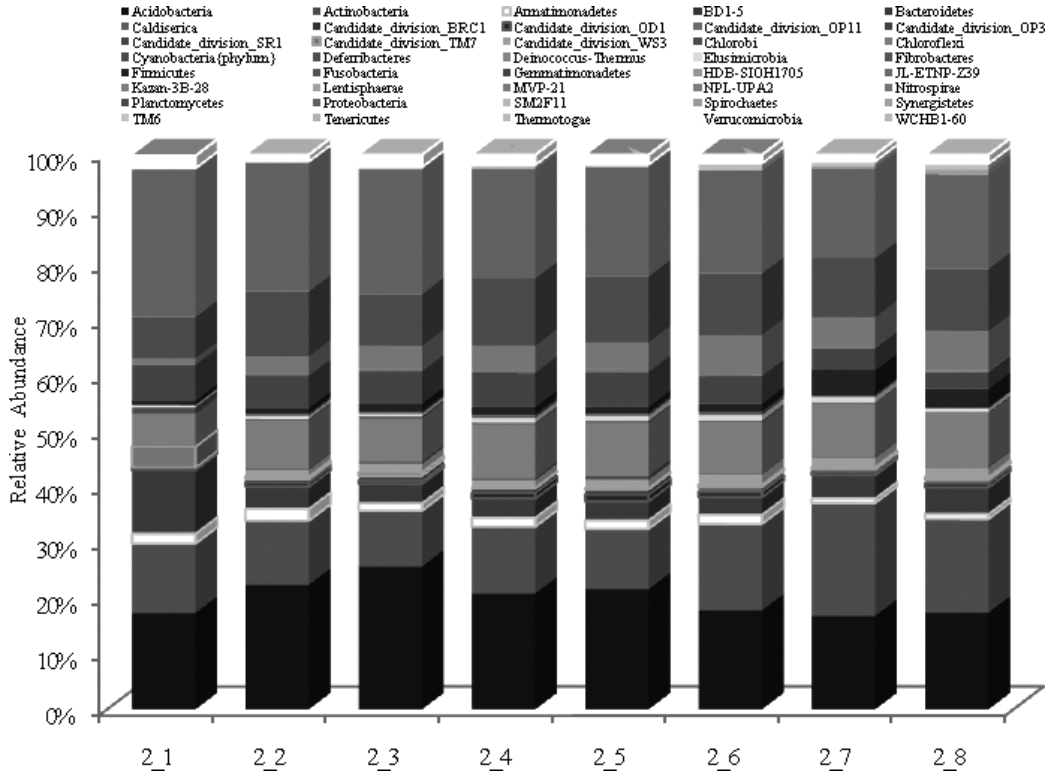

Fig. 2. Relative abundance of bacteria phyla identified in all samples. Different colors represents different bacteria phyla.

soil samples. Figure 3 shows mid-depth soil samples, i.e., samples 2_2 to 2_5 (25–70 cm layers) grouped together and then clustered with sample 2_6 (85 cm layer), 2_7 (100 cm layer), 2_8 (115 cm layer), and 2_1 (10 cm layer), in that order (Fig. 3). The differences in soil microbiota among soil samples gradually decreased with sampling depth, indicating that the soil sampling depth influences microbial structure and diversity distribution.

To determine the effect of soil properties on microbial communities, the geochemical variables were analyzed using CCA to determine the correlation with bacterial community structure. The soil samples were well separated by CCA and showed a signficant correlation between bacterial community structure and environmental factors (Fig. 4). Figure 4 shows that the variability in soil bacterial community composition was related to sampling depth and that there were 3 dispersive groups, the surface soil layer (10-cm layer), mid-depth soil layers (15- to 85-cm layers), and deeper soil layers (>100-cm layers), which was consistent with the findings described in Fig. 3. The first canonical axis explained 24.2% of the detected microbial diversity and was positively correlated with AK, AHN, AP, and OM. The second axis represented 11.3% of the variance. Overall, the CCA biplot indicated that the 8 soil samples were differentiated mainly with respect to

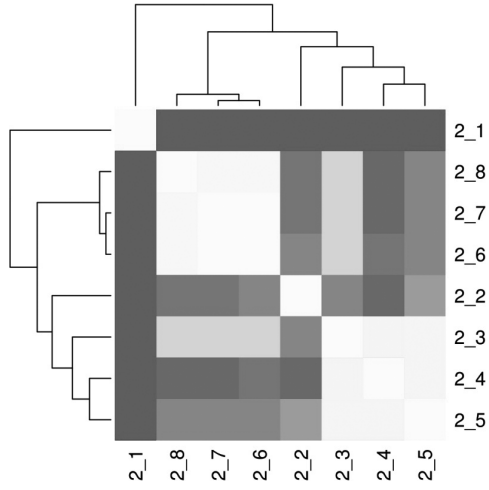

Fig. 3. Clustering and analysis of the 8 samples at operational taxonomic units (OTUs) from pyrosequencing (97% identify threshold).

All pairwise comparisons were made by calculating each R^2 value. The relative similarities for bacteria are depicted by color. A deeper color represents higher dissimilarity.

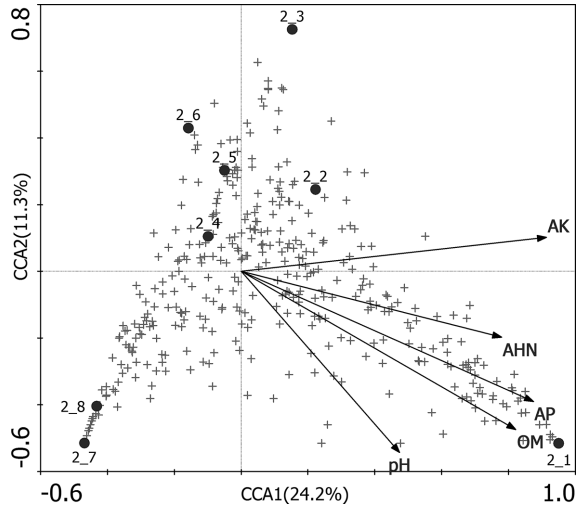

Fig.4. Canonical correspondence analysis (CCA) of bacterial species abundance and soil features.

The solid circles and crosses indicate samples and bacterial phylogenetic species, respectively.

AK and AHN but that the surface soil layer (samples 2_1) (10-cm layer) and shallower soil layers (samples 2_2) (25-cm layer) were additionally distinguished from deeper soil layer (>40-cm layer) groups by AP, OM, and AN, respectively. Thus, while AK was the fundamental regulator, microbial community structure was also influenced by AHN, AP, and OM. However, there was no significant relationship between pH and the evaluated characteristics.

Discussion

Although we obtained an average of 14,192 sequences per soil, there was a lack of asymptotes in the rarefaction curves at the 97% sequence similarity level for the tested soils (Fig. 1 and Table S1). The numbers of quality-filtered and chimera-free reads in each sample are provided in Table S1and are comparable to those in other studies that also adopted 454 pyrosequencing (McLellan et al., 2009; Ye et al., 2011). Because of the nature of 454 pyrosequencing, the number of sequences obtained varied among samples. For example, in this study, 16,596 sequences were obtained from sample 2_1, whereas only 11,994 sequences were obtained from sample 2_3. The remaining sequences were assigned to different groups by the RDP classifier using the same parameters described above, and the classification results before and after sequence trimming were compared. Although the sequencing effort in this study was not very deep, it has been shown that β-diversity-based analyses can be electively performed without requiring deep sequencing (Pinto and Raskin, 2012; Reeder and Knight, 2010). Specifically, deeper sequencing does not improve the accuracy of β-diversity estimates; it only improves precision (Pinto and Raskin, 2012).

At genetic distances below 5%, rarefaction analyses underestimate the bacterial richness, whereas the Chao 1 estimator overestimates it (Roesch et al., 2007); therefore, a substantial fraction of the bacterial diversity at the species level was assessed by this survey. Notably, pyrosequencing provides an unprecedented sampling depth relative to traditional Sanger sequencing of 16S rRNA genes (Sogin et al., 2006). Furthermore, to minimize the overestimation of rare phylotypes, clustering and diversity estimates were performed only at genetic divergences of ≥3%. Acosta-Martínez et al. previously postulated that in managed soils, the maximum number of OTUs is less than 3,400 at a genetic distance of 3% (Acosta-Martinez et al., 2008). This is in contrast to our results, as up to 2,884 and 4,800 OTUs were predicted for all samples (Table S1). Several studies have shown that the number of analyzed sequences per sample affects the predicted number of OTUs (Dunbar et al., 2002; Morales et al., 2009; Roesch et al., 2007; Schloss and Handelsman, 2005; Youssef and Elshahed, 2008). Generally, fewer sequences result in lower curve progression and number of predicted OTUs. Furthermore, comparison of richness estimates between different surveys might be hampered by differences in sequence conservation and sequence length of the analyzed 16S rRNA gene regions. Recently, Engelbrektson et al. showed that amplicon length and differences in the analyzed 16S rRNA gene regions markedly influenced estimates of richness and evenness (Engelbrektson et al., 2010).

Overall, the deepest soils showed the lowest bacterial species richness and higher microbial diversity was observed in the rhizospheres of upper soils. To our knowledge, no other study assessing bacterial diversity along a soil profile has been conducted with a comparable survey effort. However, a significant decrease in bacterial diversity with soil depth was also recorded by community analysis using terminal restriction fragment length polymorphisms (LaMontagne et al., 2003) and phospholipid fatty acid analysis of soil profiles derived from Californian grassland and soil

samples of the Sedgwick Reserve (California) (Fierer et al., 2003b).

The distribution of the phyla *Bacteroidetes* and *Nitrospirae* is notable because it decreased and increased with soil depth, respectively. *Bacteroidetes* contains mostly chemoheterotrophic bacteria whose nutritional requirements are stringent, which may account for the decrease in the number of *Bacteroidetes* with sampling depth. In contrast, *Firmicutes* mostly comprises bacteria that can produce spores, which is favorable for distribution in various extreme environments (Jiang et al., 2006; Kwon et al., 2010; Xia et al., 2010; Ye et al., 2011; Yu et al., 2011). Members of *Nitrospirae* are found in interspace soils and rarely in the rhizosphere (Dunbar et al., 2002). In the rhizosphere, heterotrophic root-associated microorganisms suppress the growth of autotrophic *Nitrospirae*, which may explain why members of *Nitrospirae* were more frequent in subsoil than in topsoil in our study. Thus, hemolithoautotrophic organisms that have adapted to darkness (e.g., *Nitrospirae*) have a selective advantage in subsoil samples. *Acidobacteria* is widely distributed in nature, plays important roles in various ecosystems, and had a relative abundance that was stable and did not fluctuate with sampling depth in our samples. To our knowledge, this is the first study to report that the relative abundance of *Proteobacteria* decreases obviously with soil depth, possibly because *Proteobacteria* comprises a major phylum of bacteria, all of which are gram negative (Lee et al., 2012). In other studies, the proportional abundance of gram-positive bacteria increased with depth, whereas the proportional abundances of gram-negative bacteria generally decreased with soil depth (Fierer et al., 2003b). The proportional abundance of *Actinobacteria* was lowest in the top 5 cm of the profiles and generally increased at greater soil depths in other studies (Fierer et al., 2003b; Shahnavaz and Geremia, 2012), but it showed the opposite pattern in the current study. Additionally, changes were observed in the relative abundance of the major soil bacterial phyla but not in the diversity of major soil bacterial phyla concomitantly with sampling depth (Fig.2 and Fig. S3), partly because of homogeneity and the stability of the deeper underground space. These findings should be explored in future research. Our findings show that the relative abundance of microbial communities decreased with sampling depth, which was consistent with previous studies. Furthermore, phylum diversity was not affected by the deeper sampling.

Precipitation, vegetation cover, and pH have thus far been found to be the main factors that control bacterial community structure in arid and semi-arid environments (Angel et al., 2009; Fierer and Jackson, 2006). Recently, it was clearly shown that soil pH is one of the major drivers that control soil bacterial community structure on a regional scale (Fierer and Jackson, 2006; Lauber et al., 2009; Tripathi et al., 2012). However, our study showed no differences in overall bacterial community across a range of pH values, which is similar to the findings of Kim (Kim et al., 2012). This effect may have been observed because the pH range in the sampled environment is relatively narrow (pH 7.97−8.11) and lacks acidic pH values. However, many previous studies have shown a strong peak around neutral pH, followed by a decline in bacterial diversity at pH values similar to or greater than 8 (Fierer and Jackson, 2006; Lauber et al., 2009; Tripathi et al., 2012). In our samples, no such decline was evident (Table S2). Soil texture has also been noted to have a strong association with below-ground microbial communities (Girvan et al., 2003; Lauber et al., 2008). Horizontal roots of *Robinia* are mainly concentrated at a depth of 10−20 cm, whereas vertical roots are seen at a depth of 50−60 cm. Therefore, the content of organic matter (SOM) is higher in the upper soil and has the greatest impact on surface-sampled microorganisms (10-cm layer) (Fig. 4). The above analysis shows that the overall relative abundance of detectable phyla extracted from the soil samples decreased with soil depth (Fig. S4) and fluctuated with soil physical and chemical properties, root system, and sampling depth (Fig. S4 and Fig. 2). Sampling across the world's land environments is still not complete; therefore, the extent to which soil texture, pH, or other factors influence the composition of soil bacterial communities remains unclear. Determining the factors that lead to these differences remains an important area for future investigation. In conclusion, our results obtained for deep forest soil provide insights regarding the effect of soil properties on the variability of the microbial communities. Such information may facilitate forest soil management.

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Supplementary Materials

Fig. S1. Rarefaction curves to estimate the diversity of taxa present in individual samples, using the Shannon index at 97% sequence identity.

Fig. S2. Estimated number of OTUs for each sample using nonparametric estimators (Figure S2a for ACE and Fig. S2b for Chao1) compared to the observed OTUs resolved from the sequences.

Fig. S3. Changes in relative abundance of individual soil bacterial phyla exhibiting a decreasing trend, increasing trend, or no change or trend with sampling depth.

Fig. S4. Relative abundance of detectable phyla extracted from soil samples with soil depth.

Table S1. Data summary and phylotype OTUs.

Table S2. Changes in physicochemical parameters across different sampling depths of the two soil use types.

Table S3. Relative abundance of the phylogenetic groups. Values are given as percentage.

Supplementary material is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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