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Changes in soil bacterial community profiles associated with deforestation in the Sakaerat Environmental Research Station, Thailand: comparisons between soils of the original forest and bare ground

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ABSTRACT Multivariate profiling of soil bacterial communities provides information that will possibly contribute to the conservation and rehabilitation of the environment. In the Sakaerat Environmental Research Station, Thailand, the land degradation gradient, represented by a dry evergreen forest (DEF) and bare ground (BG) as a result of deforestation and subsequent land degradation was determined. Profiling was done with the antibiotic resistance most probable number (MPN) method (method 1), the antibiotic disc diffusion method (method 2) and the Biolog method. Compread to the DEF soil, the BG soil had significantly lower moisture content and pH values (at $p=0.05$), and total C and bacterial MPN count (at $p=0.10$). Profiles of the soils obtained by the antibiotic and the Biolog methods were analyzed. The profiling methods scored Wilk's lambda values of 0.001 (method 1, $p=0.086$), 0.004 (method 2, $p=0.157$) and 0.000 to 0.110 (Biolog, $p=0.040$ to 0.708 at 0.2 to 1.4 average well color development values, AWCDs), then gave principal component score plots showing that all the methods successfully determined the degradation gradient with comparable efficiency. Redundancy analysis ordination diagrams for the above data sets indicated that the soil environmental factors that significantly correlated to the bacterial community profiles were: moisture content ($p<0.05$, method 1); moisture and total nitrogen (TN) contents ($p<0.05$, method 2); moisture content and C/N ratio ($p<0.05$, Biolog 0.2 AWCD); moisture and TN contents ($p<0.05$) and bulk density (BD, $p<0.1$, 0.6 AWCD); pH ($p<0.05$, 1.0 AWCD); and moisture and TN contents ($p<0.05$, 1.4 AWCD). The soil moisture gradient was shown to be the most decisive determinant of the changes in soil bacterial community profile associated with deforestation. The changes in TN, pH and BD were thought to be possible causes and/or effects of the variations of multivariate soil bacterial profiles in association with the degradation.

Key words: deforestation, multidimensionality, multivariate analysis, soil bacterial community profile, soil degradation

INTRODUCTION

The conservation of ecosystems is an important environmental issue. The soil plays important roles in terrestrial ecosystems (Beare *et al.* 1995). In the tropics, deforestation often leads to soil degradation, which proceeds rapidly under tropical climatic conditions (Eden & Parry, 1996). Soil degradation has various aspects such as physical, chemical and biotic ones, and it is important to monitor changes in soil quality (Mausbach & Seybold, 1998). Soil variables respond differently to degradation (e.g., Jha *et al.,* 1992), and therefore, each aspect may reflect the degradation differently (Oline & Grant, 2002). Thus, obtaining multivariate data sets on aspects of soil degradation is advantageous (Sena *et al.* 2000).

Multivariate profiling of soils under a vegetative type and its degraded status gives profiles of the soils (e.g., Jha *et al.* 1992). Differences between profiles of original and degraded soils show the effects of the degradation. To measure such effects, the distribution pattern of soil biotic variables, as shown in a multivariate profile, can be used to create a compositional data set (ter Braak & Šmilauer, 1998). Soil microbial profiling provides compositional data sets on soils, and is a strategy that may contribute to monitoring soil quality in the tropics. Sole carbon source utilization profiling (e.g., Garland & Mills, 1991), characterizing soil bacterial isolates (e.g., Doyle & Stotzky, 1993) and soil biotic molecular profiling (e.g., Yang *et al.* 2001) provide compositional data sets on soils.

Different soil microbial aspects may be independent from one another (van Straalen 2002), hence comparing the aspects is meaningful. For example, soil microbial information may provide a unique integrated measure of soil quality, and may specify which soil environmental factor is the most significant for conservation of the ecosystem (Pankhurst *et al.* 2001). However, multivariate microbial profiling of soil for monitoring soil degradation after elimination of the original vegetation has seldom been attempted in the tropics (Borneman & Triplett, 1997). Antibiotic resistance profiling (e.g., Doyle & Stotzky, 1993) and sole carbon source utilization profiling (e.g., Westover *et al.,* 1998) show changes in multivariate soil bacterial

community profiles. Thus, the primary objective of this research was the determination of a degradation gradient as a result of the elimination of the tropical forest by multivariate profiling of soil bacterial communities using antibiotics and carbon sources.

The original vegetative type of the Sakaerat Environmental Research Station (SERS), Thailand is dry evergreen forest (DEF), but there are other vegetative types reflecting anthropologic degradation (Kaeoniam *et al.,* 1976). Bare ground (BG) is the most degraded status in the SERS (Doi & Sakurai, 2003). Compositional data sets on soil bacterial communities were obtained for DEF and BG soils. The multivariate profiling methods, the antibiotic resistance MPN method (method 1), the antibiotic disk diffusion method (method 2) and the Biolog method (Garland & Mills, 1991) were applied. To investigate the possible utilization of the information given by the above profiling methods, the compositional data sets were analyzed by principal component analysis, Wilk's lambda determination and redundancy analysis.

MATERIALS AND METHODS

Site description

The Sakaerat Environmental Research Station (SERS), Wang Nam Kiao district, Nakhon Ratchasima (14°30'N, 101°55E) was established in 1967. At the time of establishment, some parts of the area had already been disturbed and deforested by human activities (Kaeoniam *et al.,* 1976).

The area is 7,808 hectares, and the altitude ranges from 250 to 762 m above sea level. The soil is classified as an Ultisol according to the USDA scheme (USDA Soil Conservation Service, 1975). The major vegetative types include dry evergreen forest (DEF), dry deciduous forest (DDF) and plantation plots, and others, such as bare ground (BG) and grassland. These vegetative types form a mosaic as shown in Fig. 1 (Kaeoniam *et al.,* 1976). The climate is classified as Aw according to the Köppen classification (Köppen, 1931). The annual precipitation is 1,260 mm and the average temperature is 26°C (Bunyavejchewin, 1986). Approximately, the dry season starts in November and ends in late April. The site receives most of the annual precipitation during the rainy season, approximately May to October.

Soil sampling was done at the DEF and the BG. The DEF is primarily dominated by *Hopea ferrea* and *Shorea* spp. that form the upper story, 20 to 40 m above ground. A typical DEF fosters more than 1,000 trees (trunk diameter at breast height, DBH>5 cm) ha^{-1}, and the total basal area at 1.3 m height exceeds 30 m^2 ha^{-1} and the above ground biomass is over 200 tons ha^{-1} (Kanzaki *et al.,* 1995). The BG, having no vegetation as a result of the past deforestation and subsequent human activities, is also scattered in the plot in a mosaic configuration.

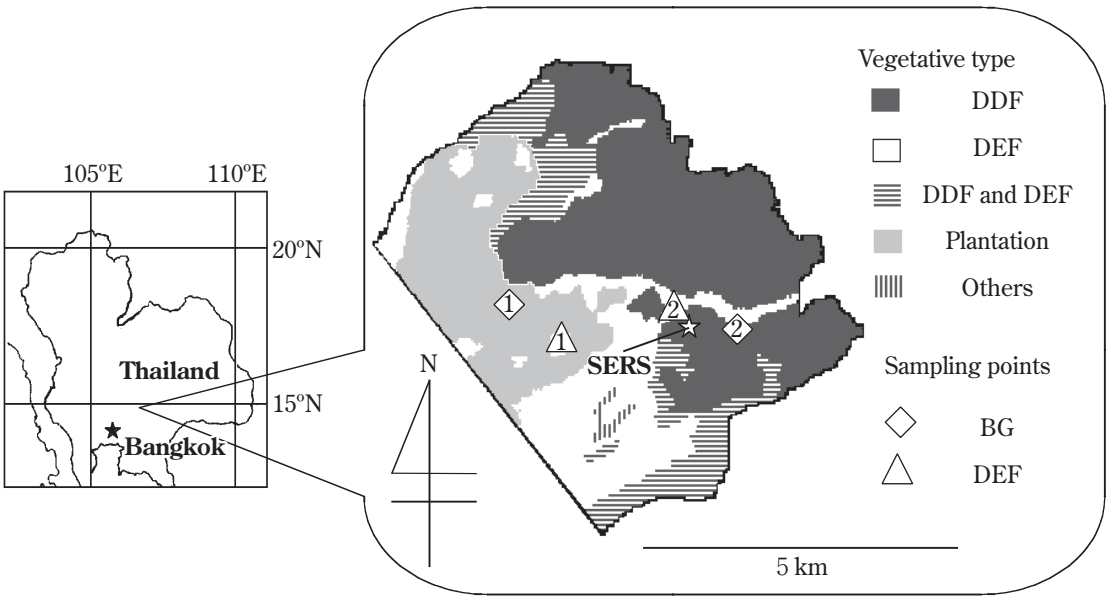

Fig. 1. Map of the Sakaerat Environmental Research Station and the sampling points. DEF, DDF and BG indicate dry evergreen forest, dry deciduous forest and bare ground, respectively. The triangle and diamond indicate sampling points at the DEF and the BG, respectively. The figures in the symbols are replication numbers.

Soil sampling

We chose 2 sampling points in the DEF and the BG, as shown in Fig. 1, because the soils taken at these sampling points were consistently characterized as the most conserved (for DEF) and degraded (for BG), throughout a research period of 5 months in 2001 (Doi & Sakurai, 2003). All four sampling points were on slight slopes (less than 10°). Altitudes for DEF 1, DEF 2, BG 1 and BG 2 were 490, 370, 470 and 350 m above sea level, respectively. Soils were sampled on November 4, 2002. An aerial photo taken in September 1983 by the LANDSAT™ shows that BG1 and BG2 at that time already had no vegetation, and newer photos taken in the 1990s also show the same situation as previously reported (Doi & Sakurai, 2003). Hence, these sampling points had been bare for at least 19 years. At each sampling point, a circle, 10 m in diameter was set, and 6 soil cores were randomly taken in the circle. Hundred mL core samplers, 5 cm in diameter, were inserted from the surface to a depth of 5.1 cm. The 6 soil cores were immediately put into a plastic bag, mixed, passed through a 2 mm screen and kept moist at 5°C for 10 days before the bacterial communities were analyzed in the laboratory.

Soil physico-chemical analyses

Soil moisture content and bulk density (BD) were determined after oven drying at 105°C for 48 h. The air-dried soils were suspended in water at a soil to solution ratio of 1:5 and reciprocally shaken (120 rpm) at room temperature for 1 h to determine their pH and electrical conductivity (EC). Total C (TC) and N (TN) were determined using a CN analyzer (Sumigraph model NC-80, Sumitomo Kagaku Kogyo, Japan). Exchangeable Al and H were determined by the titration method.

Antibiotic resistance MPN method (Method 1)

We applied the methods to profile culturable soil bacterial communities using antibiotics (Doi & Sakurai, 2002) and compared these with the Biolog method (Garland & Mills, 1991). Profiling soil bacterial communities was demonstrated to be possible by using the resistance pattern of each individual bacterial isolate to a variety of antibiotics (Brønstad *et al.,* 1996; Doyle & Stotzky, 1993). These previous findings implied that differences among soil bacterial communities should be detected by (a) determining the most probable numbers (MPNs) of bacteria resistant to antibiotics (method 1), and (b) profiling the soils by directly exposing the bacterial communities to antibiotics diffused from paper disks on an agar plate (method 2).

The most probable number (MPN) method developed by Wren & Venosa (1996) was used with a modification. The four composite samples from the DEF and the BG were profiled by this method with 3 replications for each sample. The basal medium reported by Doyle & Stotzky (1993) was slightly modified to 5.75 mM K_2HPO_4, 4.95 mM KNO_3, 0.82 mM $MgSO_4 \cdot 7H_2O$, 0.90 mM $CaCl_2$, 1.72 mM NaCl, 12.3 μM $FeCl_3$ and 5.56 mM glucose per liter. The pH was adjusted to 6.0. Antibacterials (Acar & Goldstein, 1996) were chosen to profile the soils based on the MPNs of the soil bacterial communities. Final concentrations of the antibiotics were: ampicillin (2.87 mM); chloramphenicol (1.56 mM); dapson (2.02 mM); erythromycin (0.68 mM); kanamycin sulfate (34.3 μM); lasalocid (0.85 mM); nafcillin (2.42 mM); nalidixic acid (0.43 mM); neomycin·HCl (22.0 μM); novobiocin (1.89 mM); penicillin G (3.00 mM); spectinomycin·2HCl (0.25 mM); streptomycin sulfate (68.6 μM); sulfamethoxazole (0.40 mM); tetracycline (0.23 mM); and trimethoprim (1.72 mM). Ampicillin was dissolved in 1 N NH_3 solution and the pH was adjusted to 6.0. Chloramphenicol, dapson, erythromycin, lasalocid, nafcillin, nalidixic acid, novobiocin and sulfamethoxazole were dissolved in 50% (v/v) ethanol. The other antibiotics were dissolved in water. The dissolved antibiotics were filter-sterilized using a cellulose acetate membrane filter (0.20 μm, Toyo Roshi Kaisha, Ltd., Japan), then, added to the basal medium previously autoclaved and cooled to room temperature. Cycloheximide and 2,3,5-triphenyltetrazolium chloride (TTC) were filter sterilized and added to the antibiotic media at final concentrations of 0.20 and 0.36 mM, respectively. Cycloheximide was added as a fungicide. TTC which produces a deep red color in response to oxidation of the substrate was added to aid in the detection of physiological activity of the inoculated microbial communities in the wells. The media were added to microtiter plates, which were sterilized in 70% (v/v) ethanol for 30 min in advance, at 0.15 mL/well.

Five grams of the soil sample were suspended in 9 volumes of sterilized water, reciprocally shaken at room temperature for 1 h at 120 rpm and diluted 10 to 10^7-fold. Fifty μL of each dilution was then added to the microtiter plate well for MPN determination, with 5 replicate wells. The inoculated plates were incubated at 28°C in the dark for 14 days, then TTC reduction was visually observed. During incubation, the plates were wrapped in a plastic film to avoid desiccation. A preliminary test using the basal medium without antibiotics resulted in no significant increase in the MPN after an incubation period longer than 14 days. In the same preliminary test, the method scored a coefficient of variance (CV) of 0.31

(n=4) after incubation for 14 days. The accuracy was comparable to that reported by Wren & Venosa (1996) who observed a CV of around 0.3 (n=5) in determination of MPNs on aromatic and aliphatic hydrocarbons. Another preliminary test was done using the basal medium containing no antibiotics as the control and the antibiotic media mentioned above. Approximately 1/100 to 1/10 of bacterial population from a forest soil was resistant to the antibiotics at those concentrations and the condition mentioned above. The following equation gave log-ratio transformed values (ter Braak & Šmilauer, 1998), and standardized the raw MPNs:

$$\text{Log-ratio transformed value for the } i\text{-th antibiotic} = \mathrm{Log}_{10}\mathrm{MPN}i/\sum \mathrm{Log}_{10}\mathrm{MPN} \quad (1)$$

where, MPNi is the raw MPN for the i-th antibiotic. The transformed values were used for statistical analyses.

Disk diffusion method (Method 2)

Each soil sample was profiled by this method with 3 replications. The basal medium containing 1.5% (w/v) agar was autoclaved, cooled to 55°C, and supplemented with filter sterilized TTC and cycloheximide at final concentrations of 0.20 and 0.36 mM, respectively. Twenty mL of this medium was poured into a petri dish (87 mm in diameter). Ten grams of the soil was suspended in 90 mL of sterilized water and reciprocally shaken at room temperature for 1 h at 120 rpm. The suspension was left for 20 sec, and 60 mL of the upper phase was centrifuged at 1,000 g for 5 min. The supernatant was decanted and the pellet was re-suspended in 3 mL sterilized water. One mL of this soil suspension was poured onto the agar plate, spread and incubated at 28°C in the dark for 10 h. The plates were then aseptically placed under an airflow at room temperature for 1 h to expel excessive moisture. The following antibiotic solutions were prepared: Ampicillin (in 25% 1 N NH_3 solution, the pH was adjusted to 6.0); chloramphenicol (in 50% ethanol at 155 mM); dapson (in 50% ethanol at 66.7 mM); erythromycin (in 50% ethanol at 68.1 mM); kanamycin sulfate (in water at 85.8 mM); lasalocid (in 50% ethanol at 8.46 mM); nafcillin (in 50% ethanol at 242 mM); nalidixic acid (in 50% ethanol at 8.62 mM); neomycin·HCl (in water at 44.0 mM); novobiocin (in water at 63.0 mM); penicillin G (in water at 300 mM); spectinomycin·2HCl (in water at 247 mM); streptomycin sulfate (in water at 137 mM); tetracycline (in water at 180 mM); trimethoprim (in 50% ethanol at 68.9 mM); spectinomycin·2HCl and streptomycin sulfate (mixture 1, in 25% ethanol at 68.6 and 19.8 mM, respectively) and tetracycline and trimethoprim (mixture 2, in 25% ethanol at 90.0 and 34.5 mM, respectively). These mixtures were prepared to investigate the hypothesis that the average of loadings on the first axis given by principal component analysis (PCA) for the mixed antibiotics nearly equals the loading for the mixture. If this hypothesis is not supported, it means that the mixture has another role independent of the two antibiotics. If so, a variable may be added by mixing antibiotics.

Paper disks 6 mm in diameter were prepared from Whatman No. 2 filter paper and autoclaved. Four μL of the filter sterilized antibiotic solution was loaded onto a disk. The disks were air dried for 30 min, and placed onto the plates (6 disks/plate). The agar plates were incubated at 28°C in the dark for 24 h, before the distance between disk and the edge of the inhibitory zone was measured. Duplicate zones of inhibition around a disk were observed in some cases with tetracycline. In these cases, the inner arc was used for the measurement. A ratio transformation was employed, i.e., each observation was divided by the sum of all the observations for the sample and used for statistical analyses.

Biolog method

The bacterial communities in the four composite samples from the DEF and the BG were profiled with the Biolog method. Five grams of each soil sample were suspended in 45 mL of sterilized water and reciprocally shaken at room temperature for 1 h at 120 rpm. The suspension was centrifuged at 1,000 g for 5 min, and the pellet was re-suspended in 45 mL of sterilized water. The soil suspensions were then diluted 40 times with sterilized water. Cycloheximide (0.36 mM) was added to this suspension which was then used to inoculate Biolog GN plates at a rate of 0.1 mL/well. Three plates per soil sample were used. The plates were incubated at 28°C in the dark and absorbance at 405 nm was read using an ELISA plate reader (Model 550, Bio-Rad, California) at 4 to 16 h intervals for 4 days. During the incubation, the plates were wrapped in a plastic film to avoid desiccation. The average well color development (AWCD) method proposed by Garland & Mills (1991) was used for analyses of the Biolog data. AWCD at a particular time was calculated using the following equation:

$$\mathrm{AWCD}t = \sum(\mathrm{ABS}i t - \mathrm{ABS}ct)/95 \quad (2)$$

where ABS*i*t is the absorbance at 405 nm for the *i*-th carbon source at the time (t) and ABSct is the absorbance for the control well, not including any carbon sources, at the time (t). This resulted in some negative values, which were regarded as 0 in the following computation. The increase in AWCD after this process was less than 2% of the AWCD for the sample.

Lindstrom *et al.* (1998) pointed out that rapidly and slowly metabolized carbon sources may contribute differently to discrimination of soil microbial communities. Depending on AWCDs, such differential rates of utilization might result in success or failure in soil discrimination. To investigate this possibility, data sets on AWCD values of 0.2, 0.6, 1.0 and 1.4 were statistically analyzed. Under our experimental conditions, the AWCD for each of the samples exceeded 1.5 after 80 hours of the incubation period, and started to converge. Therefore, the above AWCD values represented stages of color development.

Data analyses

The one-way ANOVA to test the significance of deforestation on each soil characteristic and PCA to elucidate patterns of variation in soil bacterial profiles were performed using the computer software, SPSS 10.0.5J (SPSS Japan Inc., Tokyo). To compare the discriminatory power of the profiling methods, Wilk's lambda statistic and its significance were determined with the SPSS software. Wilk's lambda is the most widely used statistic in determining the difference between multivariate data sets (Zar, 1999). If the means among compared groups for each variable are equal, Wilk's lambda becomes 1. The more different the data sets are, the closer Wilk's lambda converges to 0.

Redundancy analysis (RDA) and summarizing the result as an RDA ordination diagram were performed using CANOCO for Windows 4.02 and CanoDraw 3.10 (Microcomputer Power, NY), respectively. RDA is a multivariate statistical technique to relate species distribution patterns and environmental factors in decreased dimensionality. Hence, this kind of statistical technique is categorized as the direct gradient analysis. RDA specifies statistically more or less significant environmental factors as gradients in relation to a species distribution pattern. RDA detects linear species distribution patterns against significant environmental gradients (ter Braak & Šmilauer, 1998). The significant environmental gradients are shown as vectors from the origin of the ordination diagram. Thus, significant environmental gradients and some bacterial variables have linear correlations. In the same diagram, the soil samples are located according to their scores on the ordination axes. Regarding each soil variable as the abundance of a species, RDA can be applied to analysis of distribution patterns of soil inorganic molecules (Odeh *et al.* 1991), sole carbon source utilization (Pankhurst *et al.* 2001) or soil biotic molecules (Blackwood *et al.* 2003). To determine the significance of the soil environmental factors, a Monte Carlo permutation test was done at 199 random permutations. Another Monte Carlo permutation test was done to examine the significance of extracted ordination axes at 199 random permutations. In RDA, soil physico-chemical characteristics in Table 1 were designated as the environmental factors.

RESULTS

Soil characteristics

The DEF and the BG soils were easily distinguished by color. The DEF soil was dull yellow brown (10YR 5/3), while the BG soil was reddish brown (5YR 4/8). The properties of the soils are summarized in Table 1. According to the ANOVA, soil moisture content and pH significantly reflected the effects of deforestation at $p=0.05$. Deforestation was a marginally significant source of variation of TC and MPN counts ($0.05 < p < 0.10$), and a less significant one of BD and TN ($0.10 < p < 0.125$).

Profiles of the soil bacterial communities

Fig. 2 indicates the relative abundance of the soil bacteria that are resistant to the antibiotics. Relative abundance of bacteria resistant to each antibiotic was determined by the log-ratio transformation as described above, and the results are shown as ratios in the graph. The BG soil had relatively large numbers of bacteria that are resistant to dapson, nafcillin, nalidixic acid, neomycin, novobiocin, spectinomycin and trimethoprim. The soil sample taken at BG 2 was especially rich in bacteria resistant to dapson. The DEF soil had an apparently different distribution pattern from the BG soil. The DEF soil samples had relatively large numbers of bacteria that are resistant to dapson, nafcillin, nalidixic acid, neomycin, novobiocin and trimethoprim. Both the DEF samples were especially rich in bacteria that are resistant to nafcillin. The soil sample taken from DEF 1 had a smaller number of ampicillin-resistant bacteria and a higher number of nalidixic acid-resistant bacteria

Table 1. Soil characteristics

Vegetative type	Replicates	Moisture content (%)	Bulk density (kg L^{-1})	pH	EC (dS m^{-1})	Total C (g kg^{-1} dry soil)	Total N (g kg^{-1} dry soil)	C/N	Exch H (m eq kg^{-1} dry soil)	Exch Al (m eq kg^{-1} dry soil)	Soi color	MPN count† (x 10^8 g^{-1} fresh soil)
Dry evergreen forest	1	25.2	0.85	6.20	10.64	35.4	3.83	9.24	3.42	0.00	Dull yellow	4.83
	2	21.9	1.09	5.82	6.83	21.6	2.23	9.69	1.57	0.00	brown	4.80
Bare ground	1	14.0	1.57	5.05	2.27	7.85	0.84	9.35	5.50	14.0	Reddish	3.30
	2	11.8	1.30	5.12	4.06	7.58	0.68	11.2	2.77	1.03	brown	2.18
ANOVA‡	—	0.033	0.124	0.041	0.118	0.095	0.106	0.488	0.425	0.366	—	0.066

†: The MPN counts for the basal medium having no antibiotics are indicated.
‡: The one-way ANOVA was performed hypothesizing vegetative type to be the significant source of variation. The p value for each soil characteristic is indicated.

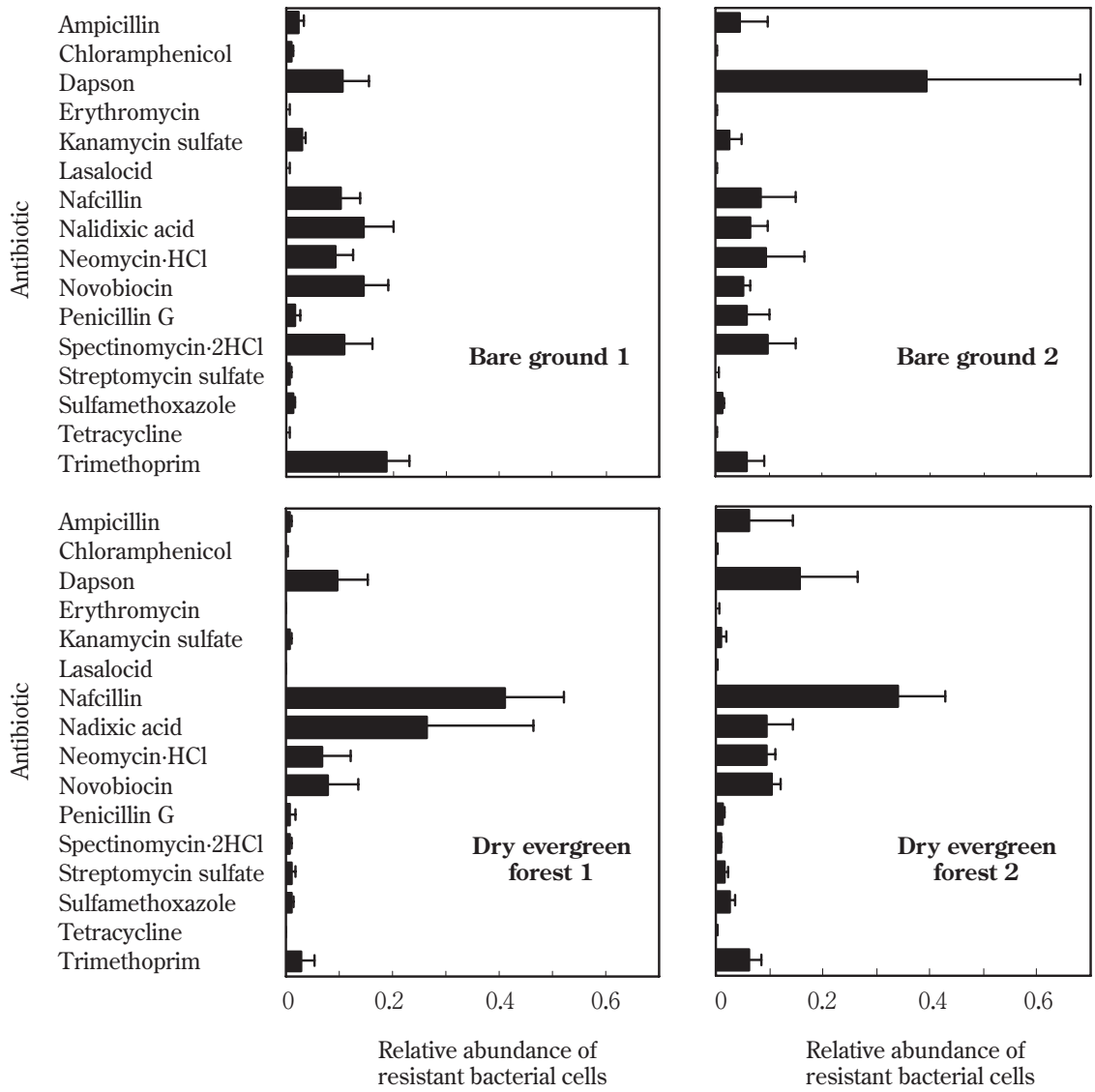

Fig. 2. Profiles of the soil samples based on most probable numbers of resistant bacteria to the antibiotics. The error bars indicate standard deviations (n=3). The horizontal axes indicate the relative abundance of the bacteria resistant to each antibiotic determined by the log-ratio transformation (see text).

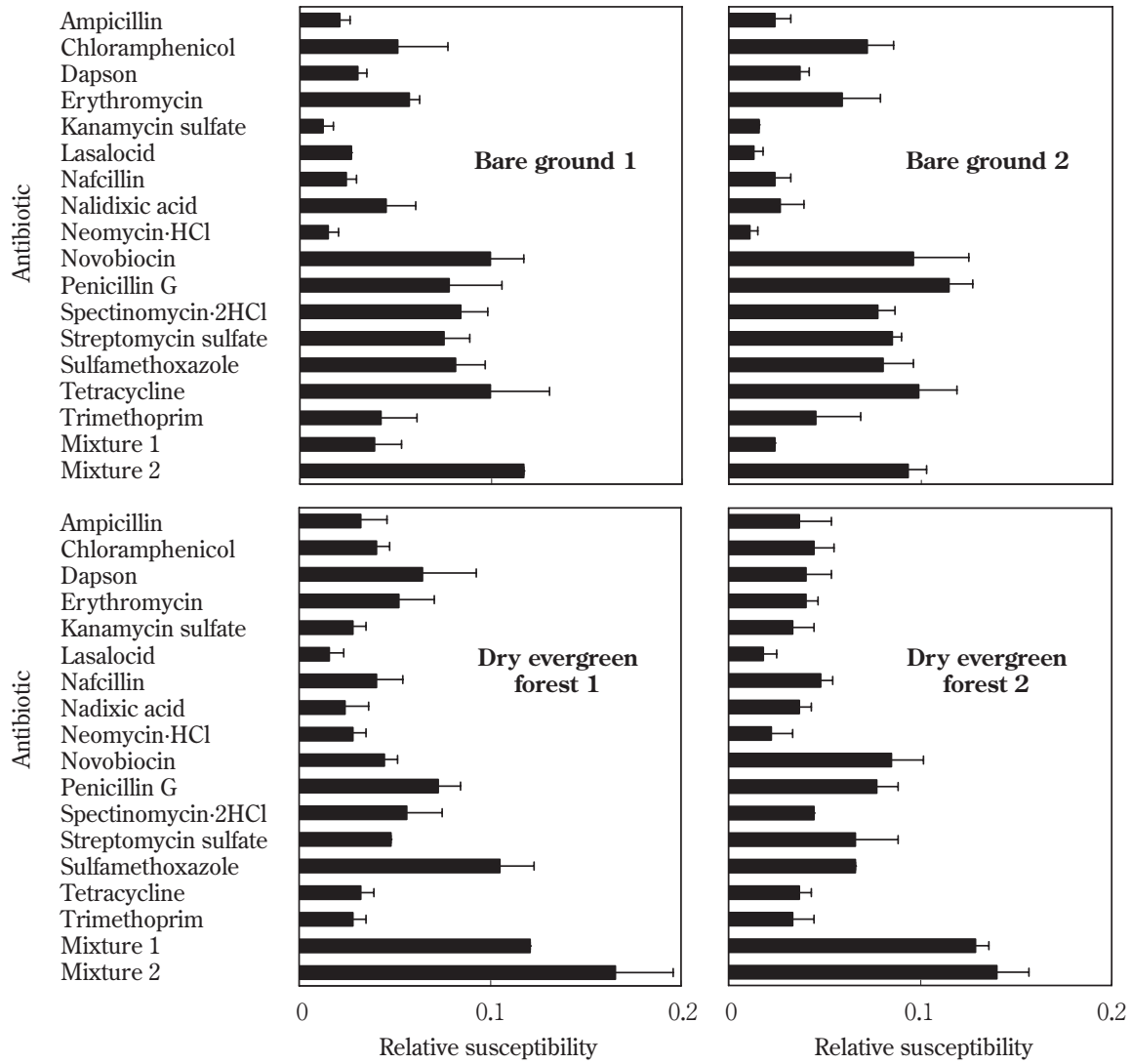

Fig. 3. Profiles of the soil samples based on susceptibility of the soil bacterial communities to the antibiotics. The error bars indicate standard deviations (n=3). The horizontal axes indicate the relative susceptibility of the bacterial community to each antibiotic determined by the ratio transformation (see text).

than that from DEF 2.

Susceptibility patterns of the bacterial communities also reflected effects of deforestation (Fig. 3). The bacterial community in the BG soil had high susceptibility to novobiocin, penicillin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline and tetracycline-trimethoprim mixture (mixture 2). The DEF bacterial community showed high susceptibility to penicillin, sulfamethoxazole and the two mixtures. The DEF soil samples suggested differences in bacterial community susceptibility patterns between them: the DEF 1 sample had high susceptibility to dapson, while the DEF 2 sample to novobiocin and streptomycin.

PCA

Method 1 discriminated between the sample groups as shown in Fig. 4a. The first and second PCs explained more than half of the variation; 44 and 13%, respectively. The sample groups were separated by the first PC axis. The second PC axis did not separate the sampling points under each of the vegetative types.

Method 2 also discriminated between the soil sample groups (Fig. 4b). The first and second PCs explained 30 and 19%

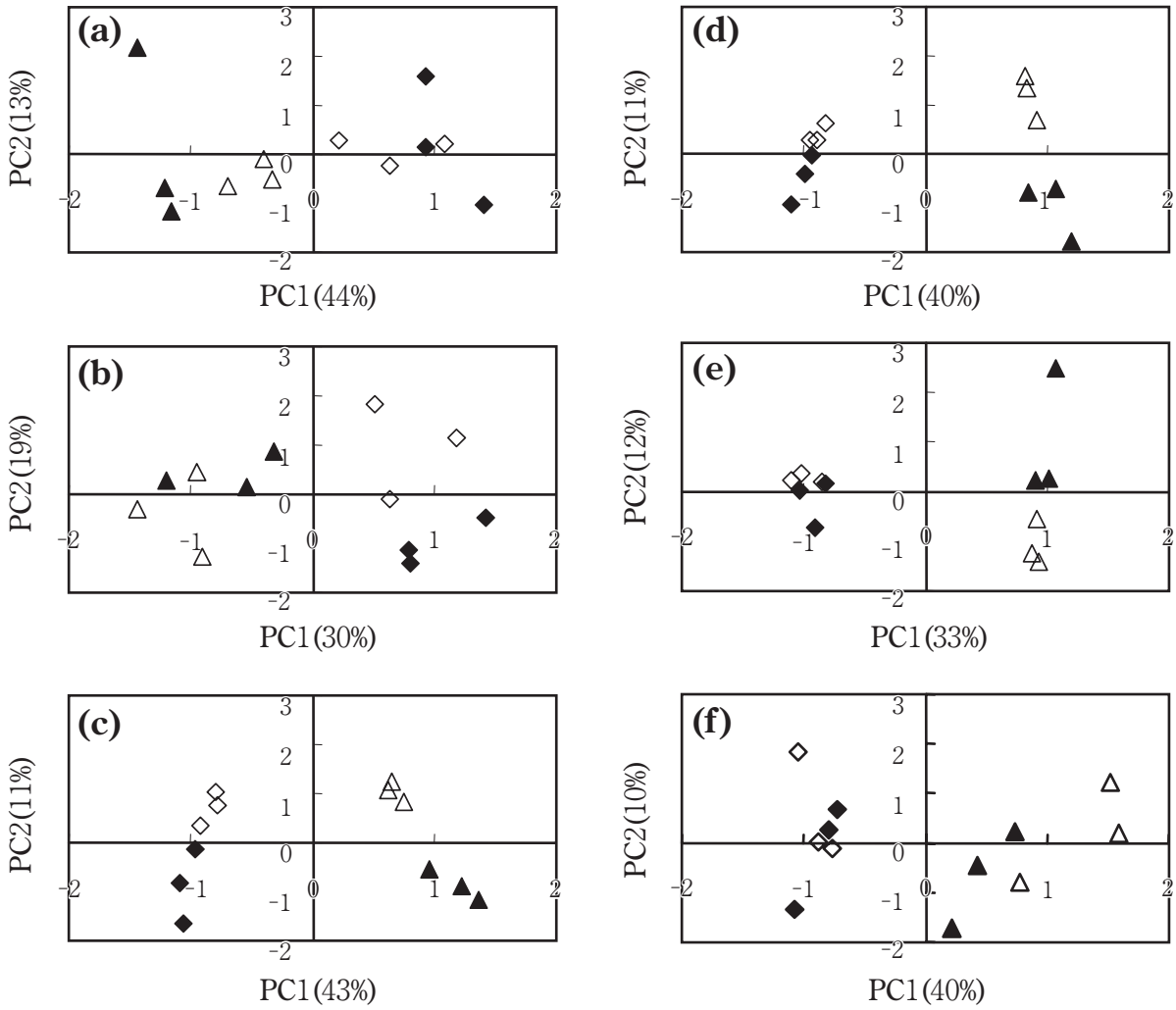

Fig. 4. Principal component score plots for the methods and the AWCDs. The triangle and diamond indicate DEF and BG, respectively. The solid and blank symbols indicate replications 1 and 2, respectively. (See Fig. 1 for the locations.) The diagrams are based on: a, antibiotic resistance MPN method; b, disk diffusion method; c, 0.2 AWCD (Biolog); d, 0.6 AWCD; e, 1.0 AWCD; f, 1.4 AWCD.

Table 2. Comparison of discriminatory power of the tested profiling methods

Method	Condition	Wilk's lambda	Significance
Antibiotic resistance MPN (Method 1)	—	0.001	0.086
Disk diffusion (Method 2)	—	0.004	0.157
Biolog	0.2 AWCD*	0.001	0.078
	0.6 AWCD	0.000	0.040
	1.0 AWCD	0.110	0.708
	1.4 AWCD	0.003	0.134

*Average well color development

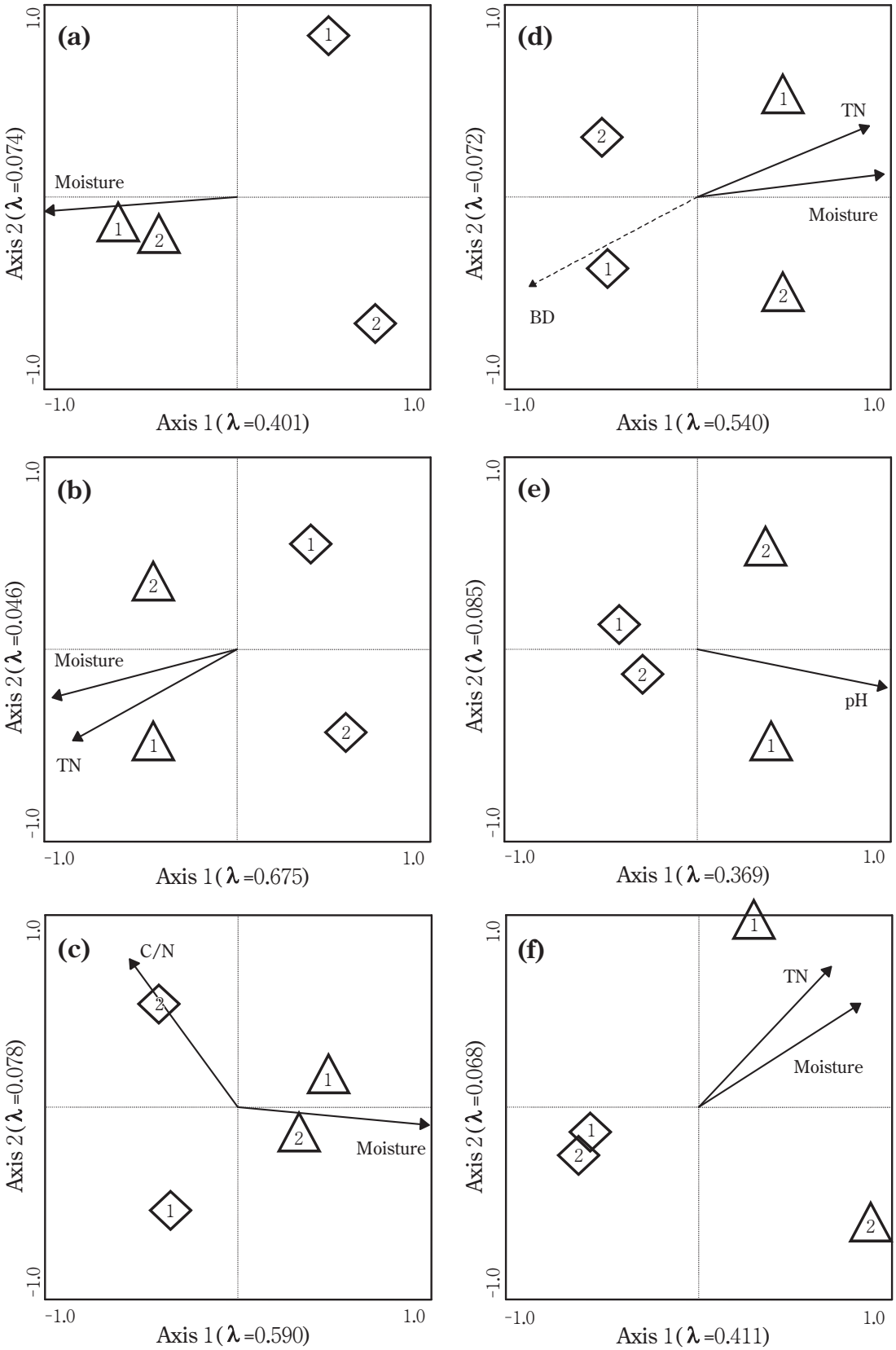

Fig. 5. RDA ordination diagrams for the methods and the AWCDs. The triangle and the diamond indicate DEF and BG, respectively. The figures in the symbols are replication numbers, correponding to that in Fig. 1. The solid and broken arrows indicate significant environmental gradients at p=0.05 and 0.10, respectively. The diagrams are based on: a, antibiotic resistance MPN method; b, disk diffusion method; c, 0.2 AWCD (Biolog); d, 0.6 AWCD; e, 1.0 AWCD; f, 1.4 AWCD.

of the variation, respectively. The sample groups were differentiated by the first PC axis. The second PC axis separated the BG sampling points in the ordination space. On the other hand, an ambiguous separation was seen for the DEF replicates. Loadings on the first axis for mixtures 1 and 2 were -0.938 and -0.865, while that for streptomycin, sulfamethoxazole, tetracycline and trimethoprim were 0.703, -0.376, 0.918 and 0.670. The data did not support the hypothesis that the average of the loadings for the mixed antibiotics nearly equals the loading for the mixture.

At all the AWCD values in the Biolog method, the first and second PCs explained around 50% of the variation. In all of the PC score plots (Fig. 4c, d, e, f), the soil sample groups from the BG and the DEF were separated by the first PC axes. The replicates for each vegetative type tended to be separated by the second PC axis until the AWCD value reached to 1.0, but the separation became ambiguous when the AWCD values reached to 1.4.

Wilk's lambda statistic

The antibiotic methods (methods 1 and 2) showed discriminatory power comparable to that of the Biolog method, scoring low values of Wilk's lambda (Table 2). The discriminatory power of the Biolog method decreased once during the color development, but was restored during the convergence stage.

RDA ordination diagrams

RDA ordination diagrams in Fig. 5 indicate relationships between the bacterial profiles and the soil environmental factors. Only the significant environmental factors at $p=0.05$ and $p=0.1$ are shown, as solid and broken arrows, respectively. No multi-colinearity was detected among the environmental factors. All the pairs of axes were significant at $p=0.01$ according to the Monte Carlo permutation test. Method 1 resulted in Fig. 5a, indicating that soil moisture content is the most significant soil environmental factor related to the MPN profiles. The DEF soil was explained by its relatively high moisture content. Eigenvalues for the first and second axes were 0.401 and 0.074, respectively. Method 2 gave another pattern of RDA ordination. Using method 2, soil moisture content and TN were specified to be the significant environmental factors related to the soil bacterial profiles (Fig. 5b). Two replicates of the BG soil were located far from each other, and the replicates of the DEF soil were also. Eigenvalues for the first and second axes were 0.675 and 0.046.

Diagrams given by the Biolog method differed among the AWCD values. At an AWCD of 0.2, soil moisture content and C/N were significant environmental factors related to the Biolog profiles of the bacterial communities (Fig. 5c). Eigenvalues for the first and second axes were 0.590 and 0.078, respectively. At an AWCD of 0.6, soil moisture and TN contents were significantly related to the profiles of the bacterial communities at $p=0.05$, and BD was a marginally significant environmental factor ($0.05 < p < 0.10$, Fig. 5d). Eigenvalues for the first and second RDA axes were 0.540 and 0.072, respectively. At an AWCD of 1.0, pH was the significant environmental factor related to the profiles at $p=0.05$ (Fig. 5e). Eigenvalues for the first RDA axis decreased to 0.369, and that for the second axis was 0.085. At an AWCD of 1.4, soil moisture and TN contents were significantly related to the bacterial community profiles (Fig. 5f). The eigenvalue for the first RDA axis increased to 0.411, and that for the second RDA axis was 0.068. The BG samples were far from each other in the ordination diagram at an AWCD of 0.2 (Fig. 5c), and the distance gradually decreased as the color development proceeded, while the DEF samples showed the reverse trend.

In each diagram, the degradation gradient was explained by the first axis, which scored an eigenvalue at least 4 times larger than the second axis. This indicates that deforestation was the most significant source of the variation of soil bacterial community profiles. Soil moisture content, pH, TN and BD related to soil bacterial community profiles, reflecting the degradation gradient. For the data set with an AWCD of 0.2, the C/N ratio was a significant source of variation of soil bacterial community profiles, not along with the degradation gradient (Table 1).

DISCUSSION

Anthropologic land degradation may affect single or multiple soil environmental factor(s). Heavy metal contamination is relatively well documented as a single primary cause of changes as reflected by antibiotic resistance profiles (e.g., Roane & Kellogg 1996), sole carbon source utilization profiles (e.g., Kelly & Tate 1998) or biotic molecular profiles (Frostegård *et al.* 1993) of soil microbial communities. The soil degradation following deforestation also involves changes in multiple soil environmental factors (Table 1). Deforestation resulting in multiple soil environmental changes also resulted in changes in bacterial population structure (Doi & Sakurai, 2003), pattern of sole carbon source utilization by soil microbes (Staddon *et al.*

1997) and microbial molecular distribution pattern (Borneman & Triperett, 1997). In these cases, the relationships between the altered soil environmental factors and the changes in soil microbial profiles are complicated.

It has been recognized that particular environmental changes alter the multivariate profiles of soil microbial communities. Some soil environmental changes involved in deforestation and soil degradation are thought to stress living things (Giuffre *et al.* 2001; Sakurai *et al.* 1998). In the SERS, the drier condition in the BG soil was suggested to be the most significant factor reducing number of bacterial cells (Table 1) and diversity of the soil bacterial community (Doi & Sakurai 2003). The drier soil condition in the BG soil may make it hard for some soil bacteria to survive, while may not effect others (Kilbertus & Proth 1979). The difference in soil moisture content was likely to be the main soil environmental factor differentiating the profiles (Fig. 5, Doi & Sakurai 2003).

A selective force was perhaps a source of variation in the soil bacterial community profile in the SERS. The low number of soil bacterial cells in the BG soil (Table 1) suggests the importance of a selective force. Another possibly important factor is the change inside of bacterial cells in response to environmental changes. Some environmental changes have been shown to alter bacterial phenotypes such as culturability (Arana *et al.* 1992) and antibiotic resistance (McInroy *et al.,* 1996) and genotypes such as carbon source utilization (Velkov, 1999) and antibiotic resistance (Pote *et al.* 2003). These possibly related mechanisms need to be investigated.

Land degradation has various aspects (Mausbach & Seybold, 1998). It is thought to be worth analyzing these different aspects with a multivariate strategy (Sena *et al.,* 2000). The aspects investigated in this research are thought to reflect independently involved events in the degradation (Fig. 5). The resistance of bacteria to particular antibiotics was shown to have linkages to the possible environmental stresses in the BG soil such as acidity (Ramos *et al.* 1987) or high temperature (Pillai and Pepper, 1991). A difference in above ground vegetation resulted in a variation of sole carbon source utilization patterns revealed by characterizing the soil bacterial isolates (Westover *et al.,* 1998). The sole carbon source utilization profile of bacterial isolates may be altered when the cell encounters particular environmental stresses (Velkov, 1999). Such linkages between environmental factors and cells are thought to occur independently in the degradation.

The Biolog method profiled the bacterial communities based on the sole carbon source utilization patterns (Zak *et al.,* 1994), while methods 1 and 2 measured the patterns of antibiotic resistance and susceptibility, respectively. Different profiled aspects have implied the multidimensionality of soil discrimination (Insam & Rangger, 1997). The discrimination patterns shown in the PC score plots differed among the methods and the Biolog color development stages (Fig. 4), suggesting multidimensionality. By revealing different ordination planes, Fig. 5 shows differences among the aspects in relation to the bacterial profiles and the environmental factors. RDA integrates environmental and biotic data sets, and then, directly relates the biotic profiles to the environmental factors. The computation found significant environmental factors based on variation of plant (e.g., Bubier, 1995; Hutto *et al.,* 1999; Lyon & Sagers, 2002; Stohlgren & Bachand, 1997; Wali, 1999) and other communities (e.g., Bird *et al.,* 2000; Hemerik & Brassaard, 2002; van den Brink *et al.,* 1996) that reflected various environmental changes.

On the other hand, few soil microbial community profiles have been analyzed for this purpose (Bossio & Scow, 1995; Pankhurst *et al.,* 2001). Soil microbial community profiling may be less labor intensive than plant species surveying. With respect to conservation, rehabilitation or agricultural production, community profile analysis may suggest a favorable management practice in the ordination diagram (Pankhurst *et al.,* 2001). Furthermore, given pieces of information may differ among communities in the same space as shown by Hermerik & Brussaard (2002). Thus, profiling soil microbial communities is another informative alternative.

The empirical approach adopted by Bossio & Scow (1995) could be powered by investigating possible cause and effect relationships as pointed out by Kourtev *et al.* (1998). Any of the significant soil environmental factors indicated in Fig. 5 may be the cause of the poor productivity of the BG soil, while the environmental changes summarized in Table 1 are results of deforestation. The altered Biolog profile of the BG soil likely reflected altered soil bacterial functions (Zak *et al.* 1994). Benefical soil bacteria are known to contribute to plant growth through mineral solubilization (Derylo & Skorupska, 1992), nitrogen fixation (Albrecht *et al.* 1981), plant growth hormone production (Neitko & Frankenberg, 1989) and plant pathogen suppression (Handman *et al.* 1991). Investigating relationships among environmental changes, plant growth and the benefical soil bacterial functions will help us develop methods to rehabilitate degraded soils.

As described in a previous report (Doi & Sakurai, 2003), soil moisture content was thought to be the most decisive environmental factor on the variation in soil bacterial community profiles (Fig. 5). Other soil environmental changes involved in the degradation were also the possible causes and/or the effects of the changes in soil bacterial community profiles. The

change in TN (Palmer & Young, 2000; Yang *et al.,* 2001), pH (Flis *et al.* 1993) or BD (Li *et al.* 2002) may affect the multivariate soil microbial community profile. Some soil bacteria fix nitrogen (Albrecht *et al.* 1981), and others may contribute to variations of soil pH, which leads to changes in other soil characteristics (Brown *et al.* 1994). BD is known to be altered by macrofauna such as earthworms (Beare *et al.,* 1995), while the contribution of microbes to the decrease in BD has not been shown. The altered soil bacterial community profile in BG is an effect of deforestation and degradation, and at the same time, the changes in community structure (Fig. 2) and sole carbon source utilization patterns (Fig. 4c to f) may be the cause of the poor productivity of this soil.

As the contrast between pH and TN shows (Fig. 5), soil microbial variables are not necessarily related to the soil environmental factors that most significantly reflect degradation, but may be related to ones that less significantly reflecting the impact (e.g., Jha *et al.* 1992). TC did not relate to soil bacterial community profiles, though deforestation comparably affected TC to TN (Table 1), which related to the profiles. While multiple soil biotic variables relate differently to the same soil environmental gradient (Jha *et al.* 1992), each soil biotic variable may relate differently to comparably significant environmental gradients (e.g., Donaldson & Henderson, 1990). Such complicated relationships lead to the multidimensionality of any gradient of our interest (Oline & Grant, 2002). The difference in the relationship between the first RDA axis and the soil environmental factors again shows the multidimensionality as previously reported (Pankhurst *et al.* 2001; Hemerik & Brussaard, 2002). The ordination axes given by each method or AWCD value exist in the ordination space in different locations (Fig. 5). The second RDA axis, derived from the data set on an AWCD of 0.2, as well as the first RDA axis from that on an AWCD of 1.0, uniquely exits in the ordination space.

In most cases, the criterion for the efficacy of a method for multivariate soil microbial profiling was the separation of soil samples in the ordination diagram (e.g., Choi & Dobbs, 1999; Garland, 1996). In such a diagram, the most significant variation is shown, while the residual variation is ignored. On the other hand, the Wilk's lambda computation involves all the variation. The Biolog data set, at an AWCD of 1.0, apparently showed successful discrimination of the sample groups in the PC score plot (Fig. 4e), but the rest of the variation led to ambiguous discrimination according to the Wilk's lambda statistic (Table 2). Minor variation of a multivariate data set often gives useful information such as the integrated measure of occurrence of plant disease (Flancl, 1993) or above ground productivity (MacMillan, 1991). Thus, it is advantageous to find minor variations, as well as the most significant ones. The low values of Wilk's lambda showing the high significance for the antibiotic methods indicate that the aspects observed by these methods could find even minor variations.

Culturing techniques, as those used in this research, are sometimes criticized, because only a subset of soil bacterial population can be observed (Gray 1990). However, the subset has reflected soil environmental changes with patterns of antibiotic resistance (Brønstad *et al.* 1996, Doyle & Stotzky 1993) and sole carbon source utilization (Insam & Rangger 1997). Thus, the subset provides information on how the communities respond to changes in their soil environments (Kennedy 1994). A possibly more important limitation of the methods used in this research is that a portion of the bacterial community may contribute to plural variables. It will be worth comparing the methods applied in this research with another that is free from this latent limitation (e.g. Fujie *et al.,* 1998).

The antibiotic methods are cost-effective and easy to perform, and the experimental conditions can be changed, e.g., by the addition/substitution of antibiotics depending on availability. In this research, mixed antibiotics were shown to have a role different from the individual antibiotics in the profiling and discrimination. Thus an additional variable, with an independent meaning, may be obtained by mixing antibiotics, allowing the use of a smaller number of antibiotics. Though preparation of the microtiter plate or the agar plate is more labor intensive than the Biolog method, which uses the factory-made product and gives several types of data (Lindstrom *et al.,* 1998), the antibiotic methods do not need sophisticated equipment, e.g., an ELISA plate reader which is a prerequisite for the Biolog method. Therefore, the antibiotic approach is an inexpensive alternative for laboratories. Antibiotic resistance and/or susceptibility patterns of soil bacterial communities may be directly linked to ecological conditions of the communities (Gottlieb, 1976; Stevenson, 1954). The Biolog method provided different RDA ordination planes and AWCD values. This indicates that we should consider the meaning of patterns of discrimination at some AWCD stages.

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