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A Simple Evaluation System for Microbial Property in Soil and Manure

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Abstract

Analyses of microbial properties in soil and manure had always included the problem that there was no available standard method to evaluate microbial property. The one of the major problems was the vast diversity and the enormous population of soil microorganisms [1], the other was an existence of numerically dominant unculturable microorganisms which comprise 99% of soil habitat [2]. We evaluated whether our newly developed method, by which taxonomies and their number of each bacterial groups were estimated, could be used as evaluation method of microbial properties of soils and manures. In the forest soil, *β*-Proteobacteria, which included *Burkholderia* sp., *Ralstonia* sp., and *Alcaligenes* sp., was numerically dominant bacteria (3.64×10^6 MPN g^{-1} dry soil), followed by *γ*-Proteobacteria (1.32×10^6 MPN), *δ*-Proteobacteria (0.006×10^6 MPN), and the other gram negative bacteria (0.006×10^6 MPN). In the commercial manure, Actinobacteria, which included *Streptoverticillium salmonis*, *Mycrococcus* sp., *Streptomyces bikiniensis*, and *Microbacterium ulmi*, was numerically dominant bacterial group (30.8×10^6 MPN), followed by *α*-Proteobacteria (26.0×10^6 MPN), *β*-Proteobacteria (17.1×10^6 MPN), *δ*-Proteobacteria (11.2×10^6 MPN), the other Firmicutes (1.71×10^6 MPN), *γ*-Proteobacteria (0.5×10^6 MPN), and the other gram negative bacteria (0.05×10^6 MPN). In the upland field, the other Firmicutes, which included *Paenibacillus* sp., was numerically dominant bacteria (4.41×10^6 MPN), followed by Actinobacteria (2.14×10^6 MPN), *Bacillus* sp. (2.14×10^6 MPN), and *γ*-Proteobacteria (0.35×10^6 MPN). Although the precision of the affiliations became lower because of higher diversity of samples and the number of some Antinobacteria and Firmicutes might be underestimated by the used PCR condition, the method was found suitable as a candidate of a new evaluation system of soil and manure.

Keywords

Evaluation System, Microbial Property, Soil and Manure, Multiple Enzyme Restriction Fragment

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1. Introduction

To establish sustainable agricultural system, by which crops and vegetables had been produced stably, maintaining soil fertility was primary important. In order to utilize the soil microorganisms effectively for soil management, property of soil microorganisms had to be evaluated as similarly as those of physical and chemical properties; while analyses of microbial properties in soil and manure had always included the problem that there was no available standard method to evaluate microbial property. The one of the major problems was the vast diversity and the enormous population of soil microorganisms [1], the other was an existence of numerically dominant unculturable microorganisms which comprise 99% of soil habitat [2].

Although denaturing gradient gel electrophoresis (DGGE), by which microbial flora could be analyzed without any culture steps [3], had widely been used within this decade for environmental microbiological researches, the method was found to have the following weak points: 1) PCR bias always disturbed to provide the exact information not only of microbial number but also ratio of each microbial groups by changing a ratio of the amplified DNAs from that of the original genomes [4]-[7]. 2) Microorganisms, affiliated by this method, were not a representative of whole microorganism but one part, which produced discrete bands in the DGGE gel [8]-[11], and the large portion in background smear remained unclear as "interband" region [12]. All of the PCR products could only been affiliated when the selective primer for the specific groups were used [13]-[15], which resulted in another selection bias caused by PCR primers. 3) The migration rates of each bands varied depending on the prepared gel condition, which inhibited to use the past data as reference database. 4) It required a lot of time and labor not only to remove completely PCR inhibiting substances, such as humic substance contained in the sample [16], but also to prepare and run DGGE gel, and affiliation of each band. These weak points disturbed the method to come into wide use. Especially as evaluation system, there was no method available by which numbers and contained microorganisms could systemically be analyzed without any preliminary information of microorganisms included in the sample.

Until now, we had found a new affiliation method of microorganisms based on restriction fragment polymorphism analysis, and developed a system and method by which bacterial affiliations could be completed systematically [17]. By using isolated environmental bacteria, precision of bacterial affiliation have been evaluated [18] [19]. Its combined use of the most probable number method (MPN) was found useful to provide numbers and taxonomies of each bacterial group without isolation in the former papers [20] [21].

As the method also seemed suitable as simple evaluation method of microbial properties in soils and manures, we presented our evaluation results whether the method could be used as systematic analyses method of soils and manure in this manuscript.

2. Materials and Methods

2.1. Samples of Soils and Manure

Soil samples were obtained from surface of upland field (U; Gleysol) at Itoshima, Fukuoka, Japan, where vegetable had been cultivated under conventional field management, and from the surface horizon of forest soil (F; brown forest soil) at Wajiro-Hgashi, Fukuoka, Japan. Commercial manure (M) made from rice straw and cattle feces was used in this study. To test vials (5 replicates) including Biolog Universal Growth Medium (BUGM; BIOLOG Hayworth, CA, USA) broth [22], serial 10-fold dilutions (10^{-4} to 10^{-10}) prepared from samples (1 g fresh wt.) were inoculated. After 3 days incubation at 30˚C, bacterial DNA in each vial was extracted described previously [20] [21] and purified by the conventional methods.

2.2. PCR Amplification and Restriction Digestion

Using the V2 forward primer (41f), and the V6 reverse primer (1066r) [23], 16S rDNA was amplified according to the former study [18] [19]. After restriction digestion of the PCR product (10 µl) by each of 10 units of the restriction enzyme, *Hae* III or *Hha* I or *Rsa* I or *Scr* F1 (Takara Bio Co. Ltd., Shiga, Japan) in Low salt buffer so-

lution (10xLow salt buffer, Takara Bio Co. Ltd.) and 5 folds dilution by de-ionized water (for Low salt buffer), restriction fragment lengths were measured by microchip electrophoresis system (MCE-202 MultiNA; Shimadzu Co., Ltd., Kyoto, Japan).

2.3. Used Reference MERFL Database for Phylogenetic Estimation

The newly constructed database was used for this research, which was edited using the method of Watanabe and Okuda [17] described previously [19]. For 41f/1066r primers, 30,844 post-amplification sequence files, which were consisted from 1379 bacterial genera, including uncultured and unidentified bacteria, were mainly re-edited using small subunit rRNA files in RDP II release 9_61 [24] under 5-bases mismatches in the both in primer annealing sites.

2.4. Selection of the Measured MERFLP Originated from the Homogeneous 16S rDNA and Phylogenetic Estimation

As the reference MERFL database was edited from the homogeneous 16S rDNA sequences, the measured MERFL digested from the homogeneous 16S rDNA had to be used for phylogenetic estimation.

The major RFs, which had the highest relative mole concentration (ratio of fluorescent intensity to fragment size) and represented as H in **Table 1**, were selected among the mixed heterogeneous FRs as described previously. The 2nd major RFs, represented as M in **Table 1**, were similarly selected among the remained mixed heterogeneous FRs after subtraction of the major RFs. The 3rd major gene, represented as L in **Table 1**, were similarly selected using the remained mixed heterogeneous FRs after subtraction of the 2nd major RFs.

The pairwise distance (D_{AB}) between the measured RFLP (A) and the theoretical RFLP (B) was calculated according to Nei and Li [25]. For similarity search, the theoretical MERFLP (B) having the smallest pairwise distance (D_{ABME}), which was an average of all the D_{ABs} for used restriction enzymes, to the measured MERFLP (A) were searched in the reference database as described previously (Watanabe *et al.*, 2008). Similarity (%) in **Table 1** was calculated as the following equation; $(1\text{-}D_{ABME}) \times 100$.

In phylogenetic estimation, identical theoretical MERFL (100%) was searched preferentially by using all the 4 measured MERFL data at first. When the completely identical theoretical MERFL was not found, combinations of 3 restriction enzymes were used for the next searches (**Table 1**). When the completely identical theoretical MERFL (100%) was not found, combinations of 2 restriction enzymes were used for the next searches (**Table 1**). When the completely identical theoretical MERFL (100%) was not found using 2 restriction enzymes, the theoretical MERFL having the highest similarity to the measured MERFL was indicated in **Table 1** [17] [19].

2.5. Estimation of Numbers of Each Bacterial Group by MPN

After differentiation of the measured MERFLs into 8 groups (A~J) based on the phylogenetic estimation. Numbers of each group were estimated by MPN for five-tube, three-decimal-dilution experiment (**Table 2**). Confidence limits shown in **Table 2** were obtained using FDA's Bacterial Analytical Manual [26].

3. Results and Discussion

3.1. Grouping and Affiliation of Bacteria Based on MERFLP

Affiliations of fifty MERFLs were summarized in **Table 1**. The MERFLs in this study was found to have a higher bacterial diversity than those in the former studies as the followings; all of the 50 MERFLs were divided into 47 OTUs, then ratio of total number of the OTUs to that of MERFLs was 94%, which was higher than that of upland field using selective medium (62.2%) [20], that of manures during composting (60.4%) [21] and that of commercial food products (34.6%; unpublished results). The higher diversity of MERFLs was caused from higher bacterial diversity of samples and non- selectivity of the used incubation medium. They were divided into 8 groups for the MPN calculation as the followings; Actinobacteria (Group A, 10 MERFLPs), *Bacillus* spp. (Group B, 5 MERFLs), the other Firmicutes (Group C, 11 MERFLs), α-Proteobacteria (Group D, 4 MERFLs), β-Proteobacteria (Group E, 8 MERFLs), γ-Proteobacteria (Group F, 7 MERFLs), δ-Proteobacteria (Group G, 3 MERFLs), and the other gram negative bacterial group (Group H, 2 MERFLs) (**Table 1**).

Table 1. Affiliation of bacteria grown in serially diluted BUGM medium by MERFL[a].

	Vial No.[b]	Restriction enzymes[c]	Similarity (%)	Name (Accession number)[d]
A	M10^{-7}1M	R, Sc	100	*Streptoverticillium salmonis* (X53169)
	M10^{-7}4H	Ha, R, Hh	90.5	*Micrococcus lylae* (X80750), *Agrococcus jenensis* (AJ717350)
	M10^{-7}5H	Ha, R, Hh	95.2	*Streptomyces bikiniensis* (AB208713)
	M10^{-8}1H	Ha, Hh	100	*Microbacterium ulmi* (AY06021)
	U10^{-5}1M	Ha, Hh	90	rubrobacteridae bacterium (AB245333)
	U10^{-5}2M	R, Hh	87.5	*Mycobacterium* sp.S19 (AB355701), *M. mucogenicum* (AY457073)
	U10^{-5}3L	R, Hh	92.5	uncultured Actinobactereria (AY921946)
	U10^{-6}2M	R, Sc, Hh	89	*Actinomadura pelletieri* (AF163119), *Microtetraspora pusilla* (D85491), *Excellospora viridulutea* (D86943)
	U10^{-6}4M	R, Hh	93	*Corynebacterium genitalium* (U87820)
	U10^{-7}5H	Ha, R, Sc	95	*Arthrobacter citeus* (Arb.citrus)
B	U10^{-5}1H	Ha, R, Hh	100	*B.cereus* (AY907828)
	U10^{-5}4H	Ha, R, Hh	100	*B. firmus* (DQ173158), *B. smithii* (X60643), *B. azotoformans* (B.axzotofos)
	U10^{-6}4H	R, Sc, Hh	100	*B. fusiformis* (L14013), *B. spaericus* (L15015)
	U10^{-6}5H	R, Sc, Hh	100	
	U10^{-7}5H	R, Sc, Hh	89	
C	M10^{-6}4H	Ha, R, Sc	91.7	*Paenibacillus gluconolyticus* (D78470)
	M10^{-7}2M	R, Sc	92.7	*Eubacterium cylindoides* (Eub.cylin2)
	M10^{-7}3M	Sc, Hh	82.9	*Staphylococcus arlettae* (AB009933), *S. cohnii* (AB009936), *S. delphini* (AB009938), *Macrococcus carouselicus* (X15713)
	U10^{-5}3M	Ha, Hh	87.5	*Paenibacillus* sp. (DQ112248), *P. azotofixans* (Pae.azofix), *P. glucanolyticus* (Pae.glulyt)
	U10^{-5}5M	Ha, R, Hh	84.1	uncultured Clostridiaceae (AY684073, AY684096, AY684098)
	U10^{-6}1H	Ha, R, Hh	100	*Paenibacillus azoreducens* (AJ27229), *P. rhizoshaerae* (AY751754), *Paenibacillus* sp. (B518; AY839866, 2S3; DQ243814)
	U10^{-6}2H	Ha, R, Hh	100	*Paenibacillus turicensis* (AF378699), *P. marquariensis* (Pae.macqr), Paenibacillus sp. (CWBI-B; DQ112248, Tibet-IB15; DQ177465)
	U10^{-6}5M	Ha, Hh	100	*Weissella paramesenteroides* (AB362621)
	U10^{-7}1H	Ha, R, Hh	100	*Paenibacillus pocheonensis* (AB245386), *P. ginsengarvi* (AB271057)
	U10^{-7}3H	Ha, R, Hh	100	*Paenibacillus* sp. (GT05-08; AM162296, YT0011; AB362822), *P. agaridevorans* (AJ345023, D84023)
	U10^{-6}1M	Ha, Hh	89	uncultured gram positive bacteria (AY177762)
D	M10^{-7}1H[e]	Ha, R	92.9	*Agrobacterim* sp. (AB006037)
	M10^{-7}4M	R, Hh	90	*Sphingomonas* sp. (BHC-A; AY973169, HI-D4; DQ205302), *S. yanoikuyae* (Spg.yano10), *Blastomonas natatoria* (X73043)
	M10^{-8}3H[f]	Ha, R, Sc	90.5	
	M10^{-8}3H[f]	R, Sc, Hh	90.5	*Erythrobacter citreus* (AF118020), *Sphingomonas terrae* (Spg.terrae)
	M10^{-8}3M	Ha, R	100	*Erlichia caffeensis* (CP000236,U60476), *E. ruminantium* (CR925677, CR925678), *E. ewingii* (M73227)

Continued

E	F10^{-5}5M	Ha, Hh	82.9	*Burkholderia* sp. (SFA1; AB232333, AK-5; AB103080)
	F10^{-6}4H	Ha, R, Sc	90.5	*Hydeogenophaga pseudoflave* (AF078770), *Streptoverticillium abikoense* (X53168)
	F10^{-6}4L	Ha, Hh	80	*Burkholderia koreensis* (AB201286), *Halomonas venusta* (L42618)
	F10^{-6}5H[f]	Sc, Hh	92.9	*Alcaligenes latus* (D88007), *Dactylosporangium roseum* (Dct.roseu2)
	F10^{-6}5H[f]	R, Hh	92.9	beta proteobacteria (AB076863)
	F10^{-6}5H[f]	Ha, Hh	92.9	*Pandoraea* sp. (AF247691, AF247696)
	F10^{-7}1H	R, Sc, Hh	95.2	*Ralstonia eutropha* (AF027407), *Burkholderia cepacia* (Bur.cepaci), *Streptomyces* sp. (U93336, U93338), *Streptverticillium baldaccii* (X53164)
	M10^{-7}2H	Ha, Rs, Hh	91.7	*Alcaligenes* ap. H (AJ412685)
	M10^{-8}2H	Rs, Hh	89	
	M10^{-8}4H	Ha, Rs, Hh	91.7	
F	F10^{-6}4M	Ha, Sc	87.5	*Haemophilus haemolyticus* (H.haemolyt), *H. paraomfluenzae* (H.parainfl), *Pasteurella mairii* (Pas.mair89, Pas.mairii)
	F10^{-7}2H	Ha, R, Hh	100	gamma proteobacterium FI1 (AY139001)
	M10^{-7}3H	Ha, R, Hh	100	uncultured gamma proteobacteria (AJ318204)
	U10^{-5}2H[f]	R, Sc, Hh	93.7	*Pseudomonas alcaligenes* (D84006), *P. fulva* (D84015)
	U10^{-5}2H[f]	Ha, R, Hh	93.7	*P. alcaligenes* (D84006), *P. putida* (DQ229317), *P. straminea* (D84023)
	U10^{-5}3H	Ha, R, Hh	93.7	*P. aeruginosa* (AY771716), *Pseudomonas* sp. (DY-A; AJ544239, SF1; AJ135269)
	U10^{-5}5H[f]	R, Sc, Hh	93.3	*P. fulva* (D84015)
	U10^{-5}5H[f]	Ha, R, Hh	93.3	*Pseudomonas* sp. (FP1-3; DQ118952, F25; DQ1275322, BWDY-5; DQ2008562, H; DQ205301)
	U10^{-6}3H	Ha, R, Hh	100	*Pseudomonas graminis* (DQ59301), *Pseudomonas* sp. BWDY-29 (DQ200851)
G	F10^{-5}5H	R, Hh	90	*Desulforegula conservatrix* (AF243334), *Emiliania huxleyi* (AY741371)
	M10^{-7}1H[e]	R, Sc	92.9	*Desulfobacterrium cetonicum* (AJ237603), *Desulfosarcina variabilis* (M34407), *Desulfonega mgnum* (U45989), *Syntrophus buswellii* (Syt.buswel)
	M10^{-7}2M	R, Sc	85.7	*Desulfovibrio fructosavorans* (AF050101), *Micrococcus luteus* (AF057289), *Pedomicrobium manganicum* (X97691)
	M10^{-8}4M	Sc, Hh	83.7	*Chondromyces robustus* (AJ233941)
H	F10^{-5}2H	Sc, R	100	*Leptospira interrogans* (Lps.interK)
	M10^{-6}4M	R, Hh	87.5	*Kouleothrix aurantiace* (AB079638, AB079639), *Polyangium cellulosum* (AF387627)

[a]Grouping was based on affiliation by MERFL; Actinobacteria (Group A), Bacillus spp. (Group B), the other Firmicutes (Group C), *α*-Proteobacteria (Group D), *β*-Proteobacteria (Group E), *γ*-Proteobacteria (Group F), *δ*-Proteobacteria (Group G), and the other gram negative bacterial group (Group H); [b]The 1st letter in vial indicates samples; "F" stands for the sample from forest soil, "M" stands for the sample from commercial manure, and "U" stands for the sample from upland field soil . Exponential of vial number represents the decimal dilution of the vial. The 2nd number of vial number (1 - 5) represents number in 5 replicates for the each decimal dilution. H of last letter represents MERFL originating from the major 16S rDNA, M represents from the 2nd major 16S rDNA, and L represents from the 3rd major 16S rDNA; [c]Restriction enzymes used for similarity search; "Ha", "R", "Sc", and "Hh" stand for *Hae* III, *Rsa* I, *Scr* F1, and *Hha* I. For the measured MERFLP which had no completely identical theoretical MERFLP, the theoretical MERFLP having the highest similarity using all the RFLPs was presented with the similarity as described in the materials and method; [d]Species name (accession number) of the theoretical MERFL having the highest similarity with the measured MERFL; [e]Additional name (accession number) of the theoretical MERFL using the different restriction enzymes; [f]Different accession number of the theoretical MERFL in the same group using the different restriction enzymes.

Table 2. Most probable numbers of each groups (A~H) and 5% confidence limits obtained using FDA's Bacterial Analytical Manual [26].

a	Forest soil				Manure				Upland field			
	Three dilutions	Score	$\times 10^6$ MPN g^{-1} dry soil	5% limits Low/High	Three dilutions	Score	$\times 10^6$ MPN g^{-1} dry soil	5% limits Low/High	Three dilutions	Score	$\times 10^6$ MPN g^{-1} dry soil	5% limits Low/High
A					$10^{-7}10^{-8}10^{-9}$	3-1-0	30.8	9.79/72.7	$10^{-6}10^{-7}10^{-8}$	2-1-0	2.14	0.57/5.35
B									$10^{-6}10^{-7}10^{-8}$	2-1-0	2.14	0.57/5.35
C					$10^{-6}10^{-7}10^{-8}$	1-2-0	1.71	0.5/4.2	$10^{-6}10^{-7}10^{-8}$	3-2-0	4.41	1.79/11.3
D					$10^{-7}10^{-8}10^{-9}$	2-2-0	26.0	9.51/61.5				
E	$10^{-6}10^{-7}10^{-8}$	3-1-0	3.64	1.16/6.94	$10^{-7}10^{-8}10^{-9}$	1-2-0	17.1	5.03/42				
F	$10^{-6}10^{-7}10^{-8}$	1-1-0	1.32	0.23/3.97	$10^{-6}10^{-7}10^{-8}$	0-1-0	0.5	0.03/1.93	$10^{-5}10^{-6}10^{-7}$	3-1-0	0.35	0.11/0.66
G	$10^{-4}10^{-5}10^{-6}$	0-1-0	0.006	0.0003/0.023	$10^{-7}10^{-8}10^{-9}$	1-1-0	11.2	1.96/33.6				
H	$10^{-4}10^{-5}10^{-6}$	0-1-0	0.006	0.0003/0.023	$10^{-5}10^{-6}10^{-7}$	0-1-0	0.05	0.003/0.19				
b	$10^{-6}10^{-7}10^{-8}$	2-2-0	3.07	1.12/7.27	$10^{-7}10^{-8}10^{-9}$	5-4-0	364	101/1119	$10^{-8}10^{-9}10^{-10}$	5-4-0	4048	1132/12579

[a]Groups: A: *B. cereus*, B: *Bacillus* spp., C: *Clostridium*, D: The other Fumicutes, E: Actinobacteria, F: Proteobacteria, G: Prevotella, H: Cytophagales, I: Gram negative bacteria; [b]Total number of bacteria.

The precision of the affiliations of each MERFLs was lower than that of the former studies. With respect to the major MERFL, ratio of the MERFLs with 100% similarity to the corresponding theoretical MERFLs (43.3%) was lower than that of field soils using selective medium (90.5%) [20], that of the manures during composting (62.9%) [21] [23], and that of the commercial food products (59.6%). The lower precision of the affiliations was caused from higher bacterial diversity of sample. As the diversity of sample became higher, the each MPN vial included many kinds of bacteria, which made it difficult to select MERFLs originated from homogenous 16S rDNA.

3.2. Estimation of Numbers of Each Bacterial Groups by MPN

There was a large difference in microbial properties among the three samples as the followings. In the forest soil (F), Group E, which included *Burkholderia* sp., *Ralstonia* sp., and *Alcaligenes* sp., was numerically dominant bacterial group (3.64×10^6 MPN g^{-1} dry soil), followed by Group F (1.32×10^6 MPN g^{-1}), Group G (0.006×10^6 MPN g^{-1}), and Group H (0.006×10^6 MPN g^{-1}) (**Table 2**, **Figure 1**). The some bacterial group detected here, e.g., *Burkholderia* spp., was reported to be detected using clone library sequencing [27] except for phyla Acidobacteria which could not be detected by the culture based method [28].

In the commercial manure (M), Group A, which included *Streptoverticillium salmonis*, *Mycrococcus* sp., *Streptomyces bikiniensis*, and *Microbacterium ulmi*, was numerically dominant bacterial group (30.8×10^6 MPN g^{-1}), followed by Group D (26.0×10^6 MPN g^{-1}), which included *Agrobacterim* sp., *Sphingomonas* sp., *Erythrobacter citreus* and *Erlichia* sp., Group E (17.1×10^6 MPN g^{-1}), which included *Alcaligenes* sp., and *Ralstonia* sp., Group G (11.2×10^6 MPN g^{-1}), which included various sulfate reducing bacteria and *Chondromyces robustus*, Group C (1.71×10^6 MPN g^{-1}), which included *Paenibacillus gluconolyticus*, *Eubacterium cylindoides*, and *Staphylococcus* sp., Group F (0.5×10^6 MPN g^{-1}), and the Group H (0.05×10^6 MPN g^{-1}) (**Table 2**, **Figure 1**).

The microbial property of M was different from those of the manures during composting in the former paper as the followings [21]: Total bacterial number (3.64×10^8 MPN g^{-1}) was lower than that of the manure after thermophilic phase (7.89×10^{10} MPN g^{-1}), and that after maturing phase (14.83×10^{10} MPN g^{-1}). The reason of the lower number was attributed to an absence of *Bacillus* spp., which was the dominant bacteria in thermophilic phase, and the decrease of the other Firmicutes, sulfate reducing bacteria and the other gram negative bacterial group, which were dominant bacteria in maturing phase [21]. While α and β-Proteobacteria, which once disappeared after maturing phase, recovered in considerable number, and Actinobacteria, which increased during maturing phase, was remained [21].

In the upland field (U), Group C, which included *Paenibacillus* sp., and *Weissella paramesenteroides*, was numerically dominant bacterial group (4.41×10^6 MPN g^{-1}), followed by Group A (2.14×10^6 MPN g^{-1}), which

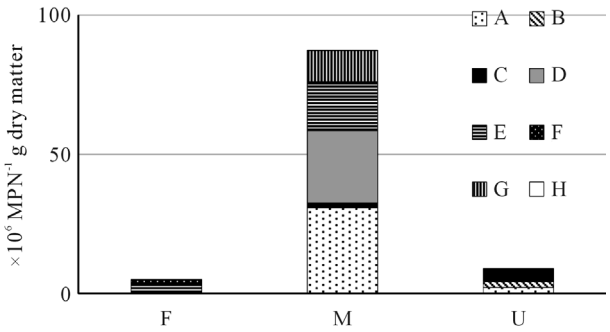

Figure 1. Numbers of bacterial groups estimated by MPN and MERFLP in forest soil (F), commercial manure (M), and upland field (U). Number of Actinobacteria (Group A; ⬚), the other Bacillus spp. (Group B; ▨), the other Firmicutes (Group C; ■), α-Proteobacteria (Group D; ▢), β-Proteobacteria (Group E; ▤), γ-Proteobacteria (Group F; ▩), δ-Proteobacteria (Group G; ▥), t and the other gram negative bacterial group (Group H; □) were presented.

included *Mycobacterium* sp., *Corynebacterium genitalium*, and *Arthrobacter citeus*, Group B (2.14×10^6 MPN g^{-1}), which included *B.cereus*, *B.fusiformis*/*B.spaericus* and *B.firmus*/*B.smithii*/*B.azotoformans*, and Group F (0.35×10^6 MPN g^{-1}), which included *Pseudomonas* sp. (**Table 2**, **Figure 1**). Number of *Bacillus* spp. was similar to that of the upland Andosol field in the former study using selective medium for *Bacillus* spp. (1.58×10^6 MPN g^{-1}), and those estimated by dilution plate method (2.30×10^6 CFU g^{-1}) [20]. The most bacterial groups detected here were reported to be detected using culture-based method [19], or DGGE [9], or clone library sequencing [29] except for phyla Acidobacteria [28].

There was a difference between the total bacterial number estimated by MPN using all the amplified vials and those of the sum of the each bacterial MPN (**Table 2**). In the forest soil (F), the sum of the each MPN (4.7×10^6 MPN g^{-1}) was higher than that of the total bacterial MPN (2.9×10^6 MPN g^{-1}). The over estimation was caused from the reason that some positive vial was repeatedly counted not only as the major MERFLs, but also as the 2nd major, and the 3rd major MERFLs. In the forest soil, two 2nd major MERFLs and one 3rd major MERFL were additionally counted as the positive results to the 6 major MERFL. In the manure (M), the sum of the each MPN (74.9×10^6 MPN g^{-1}) was lower than the total bacterial MPN (312×10^6 MPN g^{-1}). Differentiation of the whole MERFLs into the 6 sub-groups was the major factor of the underestimation (**Table 2**). Because not all bacteria in each vial were detected by the method due to the PCR bias, the MPN scores of each group were lower than the true MPN scores. This under estimation could be decreased by conjugating the small sub-groups into the larger group with higher MPN score. In upland field soil (U), the sum of the each MPN (9.0×10^6 MPN g^{-1}) was much lower than that of the total bacterial MPN (4088×10^6 MPN g^{-1}). The under estimation was also caused from low PCR amplification rate of the numerically dominant bacteria, for which bands detected in the highest dilutions (10^{-8} and 10^{-9}) were too weak to afford visible fragments after restriction digestion. The other our research indicated that amplification rates of some Antinobacteria and Firmicutes were low, and the other Actinobacteria was not amplified by the used PCR condition (unpublished results). The analysis by a new PCR condition including newly designed PCR primer for these bacteria will be presented in the following manuscripts.

4. Conclusions

In this method PCR inhibiting substances included in manure and soils had no serious effect on the results in spite of the used DNA extraction method, which included no extra purification step, because the effect of humic substances was decreased by using DNA extracted after proliferation in the growth medium, especially in higher decimal dilution vials of MPN, where the numerically dominant microorganisms were detected, concentration of the inhibiting substance was minimized. Only in forest soil, the inhibiting substance might cause the under estimation of some microbial group, because amplification band was observed until under 10^{-7} dilution vials and PCR inhibition was observed until $10^{-5} \sim 10^{-6}$ dilution vails, which afforded 6 positive vials. The under estimation might be avoided by using the conventional extraction method for environmental DNA, which included purification step.

Although the present method was culture based method, which eliminated unculturable microorganisms, we thought that the method was suitable as evaluation system of soil and manure in aim to maintain soil fertility. Because one of the unculturable microorganism, which couldn't proliferate without the other microorganisms, was detected by this method [30] [31], and the other unculturable microorganism, which lost proliferation ability and had no effect on soil fertility, was eliminated. As to another type of unculturable microorganism, which could proliferate, but no-one knew how to proliferate them such as phyla Acidobacteria [28], we already developed an unculture-based new method (unpublished result), which was found useful for unculturable microbial analysis in activated sludge. The difference of the results between culture based and unculture based method will be presented in the following manuscripts.

Classification and affiliation in species or genus level was possible by this method [18] [19]. However, enumeration of each differentiated sub-groups by MPN depended on a diversity of samples; e.g., in order to enumerate in species or genus level, three-tube, three-decimal-dilution experiment was sufficient for a sample having lower microbial diversity such as food (unpublished results), while five-tube, three-decimal-dilution experiment was insufficient for a sample having higher microbial diversity such as soil or manure and ten-tube, four-decimal-dilution experiment would improve accuracy of enumeration. Because differentiation of whole MERFLs into detailed sub-groups with lower number of MERFLs caused underestimation due to PCR bias which preferentially amplified specific DNA in MPN vials [20] [21].

As the system required lower cost for instrument and running and RFLP data was automatically obtained by MultiNA, the method was suitable as evaluation system of soil and manure. Although some data processing was manually processed at this moment, the method was the versatile system used not only as evaluation system of environmental microorganisms, but also inspection method of food microorganisms (unpublished results). Compared to the next-generation method such as pyro-sequencing, reliable affiliations of all the bacteria might be difficult by our method, our method might not be suitable for pure research purpose, but suitable as inspection method due to its lower running cost and simplicity. A difference of the results obtained by this culture-based technique and by the unculture-based technique, such as DGGE, will be presented in the next manuscripts.

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References

[1] Torsvik, V., Goksoyr, J. and Daae, F.L. (1990) High Diversity in DNA of Soil Bacteria. *Applied and Environmental Microbiology*, **56**, 782-787.

[2] Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic Identification and *in Situ* Detection of Individual Microbial Cells without Cultivation. *Microbiological Reviews*, **59**, 143-169.

[3] Muyzer, G., Waal, E.C.D. and Uitterlinden, A.G. (1993) Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA. *Applied and Environmental Microbiology*, **59**, 695-700.

[4] Suzuki, M.T. and Giovannoni, S.J. (1996) Bias Caused by Template Annealing in the Amplification of Mixtures of 16S rRNA Genes by PCR. *Applied and Environmental Microbiology*, **62**, 625-630.

[5] Ishii, K. and Fukui, M. (2001) Optimization of Annealing Temperature to Reduce Bias Caused by a Primer Mismatch in Multitemplate PCR. *Applied and Environmental Microbiology*, **67**, 3753-3755. http://dx.doi.org/10.1128/AEM.67.8.3753-3755.2001

[6] Neilson, J.W., Jordan, F.L. and Maier, R.M. (2013) Analysis of Artifacts Suggests DGGE Should Not Be Used for Quantitative Diversity Analysis. *Journal of Microbiological Method*, **92**, 256-263. http://dx.doi.org/10.1016/j.mimet.2012.12.021

[7] Direito, S.O.L., Zaura, E., Little, M., Ehrenfreund, P. and Röling, W.F.M. (2014) Systematic Evaluation of Bias in Microbial Community Profiles Induced by Whole Genome Amplification. *Environmental Microbiology*, **16**, 643-657. http://dx.doi.org/10.1111/1462-2920.12365

[8] Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G. and Bailey, M.J. (2000) Rapid Method for Coextraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNA- and rRNA-Based Microbial Community Composition. *Applied and Environmental Microbiology*, **66**, 5488-5491. http://dx.doi.org/10.1128/AEM.66.12.5488-5491.2000

[9] Duineveld, B.M., Kowalchuk, G.A., Keijzer, A., van Elsas, J.D. and van Veen, J.A. (2001) Analysis of Bacterial Communities in the Rhizosphere of Chrysanthemum via Denaturing Gradient Gel Electrophoresis of PCR-Amplified 16S rRNA as Well as DNA Fragments Coding for 16S rRNA. *Applied and Environmental Microbiology*, **67**, 172-178. http://dx.doi.org/10.1128/AEM.67.1.172-178.2001

[10] Sun, H.Y., Deng, S.P. and Raun, W.R. (2004) Bacterial Community Structure and Diversity in a Century-Old Manure-Treated Agroecosystem. *Applied and Environmental Microbiology*, **70**, 5868-5874. http://dx.doi.org/10.1128/AEM.70.10.5868-5874.2004

[11] Huang, J., Sheng, X., He, L., Huang, Z., Wang, Q. and Zhang, Z. (2013) Characterization of Depth-Related Changes in Bacterial Community Compositions and Functions of a Paddy Soil Profile. *FEMS Microbiology Letters*, **347**, 33-42. http://dx.doi.org/10.1111/1574-6968.12218

[12] Nikolausz, M., Sipos, R., Revesz, S., Szekely, A. and Marialigeti, K. (2005) Observation of Bias Associated with Re-Amplification of DNA Isolated from Denaturing Gradient Gels. *FEMS Microbiological Letters*, **244**, 385-390. http://dx.doi.org/10.1016/j.femsle.2005.02.013

[13] Santegoeds, C.M., Ferdelman, T.G., Muyzer, G. and Beer, D.D. (1998) Structural and Functional Dynamics of Sulfate-Reducing Populations in Bacterial Biofilms. *Applied and Environmental Microbiology*, **64**, 3731-3739.

[14] Watanabe, T., Asakawa, S., Nakamura, A., Nagaoka, K. and Kimura, M. (2004) DGGE Method for Analyzing 16S rDNA of Methanogenic Archaeal Community in Paddy Field Soil. *FEMS Microbiology Letters*, **232**, 153-163. http://dx.doi.org/10.1016/S0378-1097(04)00045-X

[15] Chu, H., Fujii, T., Morimoto, S., Lin, X., Yagi, K., Hu, J. and Jiabao, Z. (2007) Community Structure of Ammonia-Oxidizing Bacteria under Long-Term Application of Mineral Fertilizer and Organic Manure in a Sandy Loam Soil. *Applied and Environmental Microbiology*, **73**, 485-491. http://dx.doi.org/10.1128/AEM.01536-06

[16] Tsai, Y.L. and Olson, B.H. (1991) Rapid Method for Direct Extraction of DNA from Soil and Sediments. *Applied and Environmental Microbiology*, **57**, 1070-1074.

[17] Watanabe, K. and Okuda, M. (2003) Method and System for Searching for Relationships between Base Sequences in Genes. Japanese Patent 3431135, (2006) US Patent 7,006,924.

[18] Watanabe, K. and Koga, N. (2009) Use of a Microchip Electrophoresis System for Estimation of Bacterial Phylogeny and Analysis of NO_3^- Reducing Bacterial Flora in Field Soils. *Bioscience Biotechnology and Biochemistry*, **73**, 479-488. http://dx.doi.org/10.1271/bbb.70712

[19] Watanabe, K., Okuda, M. and Koga, N. (2008) A Newly Developed System Based on Multiple Enzyme Restriction Fragment Length Polymorphism—An Application to Proteolytic Bacterial Flora Analysis. *Soil Science and Plant Nutrition*, **54**, 204-215. http://dx.doi.org/10.1111/j.1747-0765.2007.00230.x

[20] Watanabe, K., Horinishi, N. and Matumoto, K. (2015) Antibiotic-Resistant Bacterial Group in Field Soil Evaluated by a Newly Developed Method Based on Restriction Fragment Length Polymorphism Analysis. *Advances in Microbiology*, **5**, 807-816. http://dx.doi.org/10.4236/aim.2015.512085

[21] Watanabe, K., Horinishi, N., Matumoto, K., Tanaka, A. and Yakushido, K. (2015) Bacterial Groups Concerned with Maturing Process in Manure Production Analyzed by a Method Based on Restriction Fragment Length Polymorphism Analysis. *Advances in Microbiology*, **5**, 832-841. http://dx.doi.org/10.4236/aim.2015.513088

[22] Bochner, B. (1989) "Breathprints" at the Microbial Level. *ASM News*, **55**, 536-539.

[23] Weidner, S., Arnold, W. and Puhler, A. (1996) Diversity of Uncultured Microorganisms Associated with the Seagrass *Halophila stipulacea* Estimated by Restriction Fragment Length Polymorphism Analysis of PCR-Amplified 16S rRNA Genes. *Applied and Environmental Microbiology*, **62**, 766-771.

[24] Cole, J.R., Chai, B., Farris, R., Wang, Q., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Bandela, A.M., Cardenas, E., Garrity, G.M. and Tiedje, J.M. (2007) The Ribosomal Database Project (RDP-II): Introducing myRDP Space and Quality Controlled Public Data. *Nucleic Acids Research*, **35**, D169-D172. http://dx.doi.org/10.1093/nar/gkl889

[25] Nei, M. and Li, W.H. (1979) Mathematical Model for Studying Genetic Variation in Terms of Restriction Endonucleases. *Proceedings of the National Academy of Sciences of the United States of America*, **76**, 5269-5273. http://dx.doi.org/10.1073/pnas.76.10.5269

[26] Blodgett, R. (2010) FDA, Bacterial Analytical Manual, Appendix 2 Most Probable Number from Serial Dilutions. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ ucm109656.htm

[27] Tsai, S.H., Selvam, A., Chang, Y.P. and Yang, S.S. (2009) Soil Bacterial Community Composition across Different Topographic Sites Characterized by 16S rRNA Gene Clones in the Fushan Forest of Taiwan. *Botanical Studies*, **50**, 57-68.

[28] Naether, A., Foesel, B.U., Naegele, V., Wust, P.K., Weinert, J., Bonkowski, M., Alt, F., Oelmann, Y., Polle, A., Lohaus, G., Gockel, S., Hemp, A., Kalko, E.K.V., Linsenmair, K.E., Pfeiffer, S., Renner, S., Schoning, I., Weisser, W.W., Wells, K., Fischer, M., Overmann, J. and Friedricha, M.W. (2012) Environmental Factors Affect Acidobacterial

Communities below the Subgroup Level in Grassland and Forest Soils. *Applied and Environmental Microbiology*, **78**, 7398-7406.

[29] Gardener, B.B.M. (2004) Ecology of *Bacillus* and *Paenibacillus* spp. in Agricultural Systems. *Phytopathology*, **94**, 1252-1258. http://dx.doi.org/10.1094/PHYTO.2004.94.11.1252

[30] Tang, H., Xiao, C., Ma, J., Yu, M., Li, Y., Wang, G. and Zhang, L. (2009) Prokaryotic Diversity in Continuous Cropping and Rotational Cropping Soybean Soil. *FEMS Microbiology Letters*, **298**, 267-273. http://dx.doi.org/10.1111/j.1574-6968.2009.01730.x

[31] Bae, J.W., Kim, J.J., Jeon, C.O., Kim, K., Song, J.J., Lee, S.G., Poo, H., Jung, C.M., Park, Y.H. and Sung, M.H. (2003) Application of Denaturing Gradient Gel Electrophoresis to Estimate the Diversity of Commensal Thermophiles. *Journal of Microbiology and Biotechnology*, **13**, 1008-1012.