Microbes Environ. Vol. 19, No. 4, 301–309, 2004
http://wwwsoc.nii.ac.jp/jsme2/

Evaluation of Soil DNA from Arable Land in Japan Using a Modified Direct-extraction Method

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(Received August 31, 2004—Accepted October 30, 2004)

Using a widely used commercial DNA extraction kit and a newly modified direct DNA extraction method proposed in this report, soil DNA was extracted from arable land in diverse geological locations in Japan and the quality and quantity of the DNA were examined. A modified direct DNA extraction method was developed, consisting of one-step extraction and two-step purification using potassium acetate and a DEAE-cellulose column, respectively, and designated as a DSPD (Differential Salt Precipitation and DEAE-cellulose) method. The total time needed to process six soil samples with this method was less than two hours, including the time taken to set up the DEAE-cellulose columns. Other advantages of our DSPD method are the use of non-hazardous reagents and running costs only 5–10% of the commercial kits currently used in soil microbiological research. This DSPD method was tested using 24 soil samples collected from diverse locations in Japan and showed that it is a reliable technique for DNA extraction from a wide range of soil types when compared with commercial DNA extraction kits. This DSPD method will therefore contribute to the molecular and genomic analyses of microbial populations and ecosystems that require numerous samples of soil DNA.

Key words: agricultural ecosystem, DEAE-cellulose column, DSPD method, potassium acetate precipitation, soil DNA

The microbial community plays an indispensable and unique role in both the function and sustainability of diverse ecosystems. The development and application of nucleic acid-based techniques in ecology has made a huge contribution to our understanding of microbial community dynamics and activities in natural environments, as these analyses go beyond culture-dependent methodological approaches[1)]. Recently, environmental genomic approaches have also provided significant insights into the biological properties of individual species within microbial populations[24)]. These molecular and genomic analyses strongly depend on how nucleic acids are extracted and purified from environmental samples. The improvement and optimization of DNA extraction and purification methodology is therefore crucial in the advance of these approaches.

In the last two decades, methods to extract DNA from soil samples have been explored as key techniques to facilitate a better understanding of soil microbiology at the molecular level. To date, two different approaches, direct and indirect extraction, have been proposed for soil DNA preparation. Torsvik and Goksoyr[22)] first described an indirect DNA extraction method that involved the separation of bacterial cells from soil particles by differential centrifugation, followed by cell lysis, DNA recovery and several DNA purification steps. Subsequently, Ogram *et al.*[12)] developed a direct DNA extraction method that involved the release of DNA from cells by physical disruption without separating the cells from the soil matrix, followed by alkaline extraction and a series of purification steps. Holben *et al.*[7)] and other groups have proposed that indirect extraction methods should yield cleaner DNA preparations with higher average molecular weights, whereas direct extraction methods produce higher yields of DNA from a wide range of cell types, including organisms that are important for a better understanding of microbial ecosystems in soils. Furthermore,

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some studies now suggest that by using new purification techniques for nucleic acid extraction, direct extraction methods can yield DNA of equal purity to samples obtained with indirect extraction protocols[9,19]. Although various physical and chemical procedures have been employed in protocols to obtain soil DNA at a constant yield and high purity, most of these studies focused on only a few soil samples. Since natural soils possess both extreme diversity and complexity in their physical, chemical and biological properties, the use of a wide range of soil types may well be needed to properly evaluate these methods for their robustness and reliability.

To date, two major problems have been reported for the molecular analyses of soil microbes, based on direct DNA extraction. One of these is the presence of humic substances in soil, which are derived from various kinds of organic matter, and some of their components, such as humic acids and fulvic acids, are known to act as strong inhibitors of a range of different enzymatic reactions. Significantly, both restriction enzymes and DNA polymerases are key enzymes in the molecular biological analyses of soil microbes and are known to be very sensitive to the presence of humic substances[14,18,23]. Another significant problem with direct DNA extraction is DNA adsorption by the soil matrix[13]. There are some reports that describe such "challenging soils" where the addition of either RNA or of skim milk to the DNA extraction buffer has been recommended[5,8,21,26]. Although these reports indicate the beneficial effects of RNA or skim milk supplements for more effective soil DNA extraction, the conclusions are mainly based on the results of only a few soil samples and PCR experiments. Comprehensive assessments, including PCR and spectrophotometric measurement, using a wide range of soil types, are therefore required to better understand the qualitative and quantitative effects of RNA or skim milk on soil DNA extraction.

The aim of this study was to develop a cost-effective method for extracting DNA directly from arable soils for molecular biological analyses, focusing on the problems described above. We report the evaluation and potential usefulness of a modified direct DNA extraction method, using arable soil samples collected from diverse locations in Japan.

Materials and Methods

Soils and soil analysis

A soil sample taken from an experimental field at the Gene Research Center, University of Tsukuba, Japan, in August 2003 (UT soil in Table 1) was used to determine the optimal conditions for DNA extraction throughout this study. To evaluate the applicability of the new DNA extraction method developed in this study, arable soil samples were collected from diverse locations in Japan (Table 1) in October and November 2003. After sieving the collected soil (2 mm, diameter), 0.5 g aliquots were collected into 2 ml microtubes and stored at −80°C. The soil collection sites and soil properties are shown in Table 1. Soil properties were analyzed by KANKYO ENGINEERING CO., LTD. (Chiyoda-ku Tokyo, Japan) and the pH was determined using a glass electrode in a soil:water ratio of 1:2.5. The determination of electrical conductivity (EC) was made using a conductivity cell to measure the electrical resistance of a 1:5 soil:water suspension. The phosphate adsorption coefficient was determined by a previously described ammonium phosphate method[6]. The quantity of carbon and nitrogen in the soil samples were determined using a vario EL CHNOS Elemental Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). The organic content was determined by the loss on ignition-based method described by Ball[2] and the humic acid content was determined using the Tyurin method[25].

A newly modified direct soil DNA extraction method

The direct DNA extraction method developed in this study was modified from previously established procedures and the extraction buffer was modified from the method of Berthelet *et al.*[3]. Briefly, each soil sample aliquot (0.5 g) was suspended in 0.5 ml of DNA extraction buffer (500 mM Tris (pH 8.0), 100 mM EDTA, 100 mM NaCl, 2% SDS, 8 mg skim milk/g [soil weight]) and 0.5 ml of 300 mM sodium phosphate buffer (pH 8.0) in 2 ml screw-capped tubes. After adding 0.5 g of glass beads (0.1 mm diameter, B. Braun Biotech International, Melsungen, Germany), the tubes were processed in a bead beater (Micro-Dismembrator S, B. Braun Biotech International, Melsungen, Germany) for 1 min at 2,600 rpm. Subsequently, the tubes were centrifuged for 1 min at 16,000×*g* at room temperature. The supernatants were collected and transferred into fresh 1.5 ml microtubes, and mixed by inversion with 0.2 volumes of 8 M potassium acetate. Following incubation for 5 min at room temperature, the microtubes were centrifuged for 5 min at 16,000×*g* at room temperature. The supernatants were collected, transferred into fresh 2 ml microtubes and mixed by inversion with 0.6 volumes of isopropanol. After incubation for 5 min at room temperature, the tubes were centrifuged for 5 min at 16,000×*g*, also at room temperature. The pellets were then washed with 70% ethanol and re-

Table 1. Sampling locations and soil characteristics

Sample name	Sampling location Prefecture/City	FAO soil grouping	pH	EC[a]	PAC[b]	%C[c]	%N[d]	Organic content (%)	Humic acid content (%)
CS[e]	—	—	6.6	7.3	2330	11.0	0.6	27.5	17.0
CS2[e]	—	—	7.0	142.0	2440	5.5	0.3	21.9	4.0
EH	Hokkaido/Eniwa	Andosol	5.4	10.2	1930	3.8	0.7	11.0	8.5
FF	Fukui/Fukui	Planosol	6.6	13.4	1280	3.4	0.8	9.9	7.9
GG	Gifu/Gifu	Andosol	6.0	7.5	544	1.3	0.5	4.1	2.7
HA1	Aomori/Hirosaki	Andosol	5.2	81.7	1880	6.5	0.9	16.6	13.1
HO29	Osaka/Habikino	Andosol	5.2	33.3	470	1.9	0.5	4.6	3.9
IN	Nagasaki/Isahaya	Acrisol	4.2	24.8	1200	1.0	0.6	11.6	2.5
KH1	Hokkaido/Kasai	Andosol	5.6	12.6	1750	2.8	0.6	9.8	7.0
KK	Kagawa/Kagawa	Andosol	6.0	10.7	516	1.6	0.5	3.9	3.9
KN	Nara/Kasihara	Gleysol	5.4	13.9	826	2.0	0.8	5.4	8.9
KO	Okayama/Kurashiki	Andosol	6.4	8.1	540	1.5	0.6	8.5	3.6
MH	Hokkaido/Bibai	Histosol	7.1	16.4	976	3.8	0.6	8.6	6.1
MH2	Hokkaido/Bibai	Histosol	5.6	73.6	1590	17.2	1.4	33.0	39.8
MI	Iwate/Morioka	Andosol	5.8	11.7	2370	9.3	1.0	23.6	21.1
MMO	Miyazaki/Miyakonojo	Andosol	5.8	5.3	1740	5.6	0.9	13.2	11.8
NH	Hokkaido/Yubari	Andosol	6.0	5.2	1830	5.0	0.7	13.2	10.3
OA	Akita/Omagari	Cambisol	5.9	9.0	1120	3.8	0.7	10.4	9.1
SA1	Akita/Senpoku	Andosol	6.5	6.2	1550	3.2	0.5	11.3	7.0
ST	Tochigi/Shioya	Andosol	7.6	12.2	1980	3.0	0.5	15.1	7.2
TI	Ibaraki/Tsukuba	Andosol	5.7	10.2	2290	4.5	0.7	19.2	10.6
UT	Ibaraki/Tsukuba	Andosol	6.1	6.6	1880	4.3	0.6	15.9	10.2
UT2	Ibaraki/Tsukuba	Andosol	5.7	8.8	2200	4.8	0.3	15.5	6.8
YH	Hyogo/Yashiro	Cambisol	5.5	16.7	802	2.8	0.6	6.7	10.3

[a] Electrical conductivity.
[b] Phosphate adsorption coefficient.
[c] Total carbon level.
[d] Total nitrogen level.
[e] Commercial soils.

suspended in 100 µl of TE buffer (pH 7.6).

For the final purification step, the DEAE-cellulose column treatment procedure used was simplified from a method described by Sambrook and Russel[15]. The DEAE-cellulose resin (a grade of column chromatography, Wako Pure Chemical Industries, Ltd., Osaka, Osaka, Japan) was suspended in 20 volumes of TE (pH 7.6) containing 0.6 M NaCl. After settling the resin, the supernatant was discarded. Another 20 volumes of TE (pH 7.6) containing 0.6 M NaCl was then added, and the resin was gently resuspended. After resettling the resin, the supernatant was discarded, and the equilibrated resin was stored at 4°C. The resin (0.8 ml) was poured into the barrel of a 2.5 ml syringe (Terumo Co., Ltd, Shibuya-ku, Tokyo, Japan) plugged with glass wool (10 mg packed in 0.2 ml at the bottom of the barrel), and preconditioned with a series of TE buffers (3 ml of TE buffer (pH 7.6) containing 0.6 M NaCl, 3 ml of TE buffer (pH 7.6) alone and 3 ml of TE buffer (pH 7.6) containing 0.1 M NaCl). The DNA suspension was mixed with an equal volume of TE buffer (pH 7.6) containing 0.2 M NaCl and loaded into a packed DEAE-cellulose column. After washing the column with 3 ml of TE buffer (pH 7.6) containing 0.3 M NaCl, the DNA was eluted with three 0.5-ml washes of TE (pH 7.6) containing 0.6 M NaCl. The second and third 0.5 ml aliquots of the eluted DNA were co-precipitated with 50 µg of glycogen (molecular biology grade, Nacalai tesque Inc., Kyoto, Japan) by adding an equal volume of isopropanol and 1/10 volume of 3M sodium acetate (pH 5.2). The pellets were then washed with 85% ethanol. After air-drying, the pellets were resuspended in 50 µl of sterilized water. This newly modified DNA extraction protocol has been designated the DSPD (Differential Salt Precipitation and

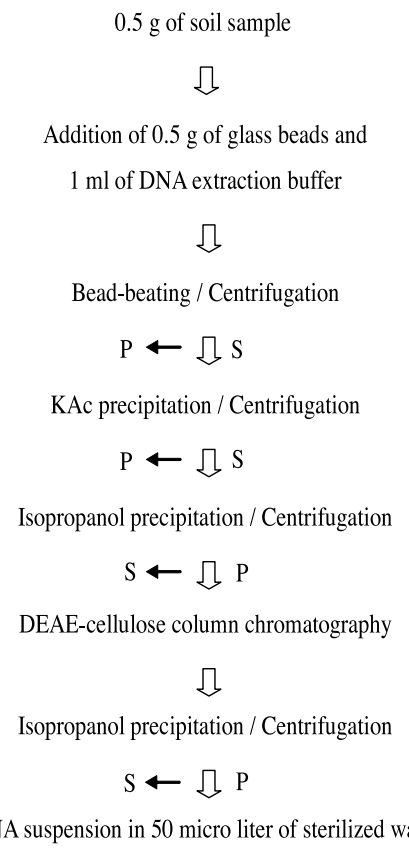

Fig. 1. Outline of DSPD method for soil DNA extraction. KAc, potassium acetate, P, pellet, S, supernatant.

DEAE-cellulose) method and an outline of the procedure is summarized in Figure 1.

Comparison of the DSPD method with other rapid DNA extraction methods

Using a soil sample (UT soil in Table 1), the quality and quantity of soil DNA extracted by the DSPD method were compared with those obtained using either previously published or commercially available DNA extraction methods. In each case, the final volume of the isolated DNA extract was adjusted to 50 µl with sterile water. The protocols assessed against DSPD were:

1. Method of Berthelet *et al.*[3]: DNA extraction from 0.5 g of soil was carried out using buffer supplemented with skim milk (8 mg/g [soil weight]).

2. UltraClean soil DNA isolation kit (MoBio Inc., Solana, CA, USA): DNA extraction was performed according to the manufacturer's protocol using 0.5 g soil samples, with some recommended modifications. Skim milk (8 mg/g [soil weight]) was added to the extraction buffer and the washing step was repeated three times due to the presence of excess humic substances in UT soil.

3. FastDNA SPIN Kit for soil (Qbiogene, Carlsbad, CA, USA): DNA extraction was performed according to the manufacturer's protocol with 0.5 g of soil, except that skim milk (8 mg/g [soil weight]) was added to the extraction buffer and the washing step was repeated three times to compensate for high levels of humic substances.

Qualitative and quantitative evaluation of soil DNA

The purity of soil DNA was assessed spectrophotometrically by calculating both A_{260}/A_{230} and A_{260}/A_{280} ratios. Qualitative evaluation of possible DNA fragmentation was carried out by electrophoresing DNA extracts (5 µl) on a 1% agarose gel. A fluorescence-based assay reported by Kuske *et al.*[10] was employed for the quantification of soil DNA to avoid interference by contaminating substances such as RNA and humic acids. For quantification of the DNA, samples were diluted in 0.1×TAE to an absorbance maximum in the 200- to 300-nm range of less than 0.05. An equal volume of a 200-fold dilution of PicoGreen dye (Molecular Probes, Eugene, OR, USA) was added. Samples were then incubated at room temperature in the dark for 20 min and the fluorescence intensity of the intercalated PicoGreen dye was determined using a Molecular Imager FX (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with band-path filters for Sybr Green I & II. DNA concentrations were then determined relative to a lambda DNA standard curve.

The quality of extracted soil DNA was also examined by PCR amplification with Ex *Taq* DNA polymerase (TAKARA, Kyoto, Kyoto, Japan), according to the manufacturer's protocol, except that BSA was added to a final concentration of 0.2 µg/µl. Universal bacterial primers, targeting bacterial 16S rDNA, 8–27F and 1392–1406R, were used in the reactions[4]. An undiluted 0.5 µl aliquot of each DNA extract was used as template DNA in a 50 µl final reaction volume. Cycling conditions were as follows: initial denaturation for 5 min at 94°C, then 25 cycles consisting of 30 sec at 94°C, 30 sec at 55°C and 2 min at 72°C; and a final extension for 7 min at 72°C. PCR products (2.5 µl) were analyzed by 1% agarose gel electrophoresis in 0.5×TBE.

Comparison of the DSPD method with a commercial kit using arable soil samples collected from diverse geological locations in Japan

Twenty-four soil samples, including UT soil, were collected from diverse geological locations in Japan (Table 1) and subjected to DNA extraction using the DSPD method and the FastDNA SPIN Kit for soil. The soil DNA samples were examined for both quality and quantity as described in

the previous section. Most of these samples were arable soils, except for two commercial soils (CS and CS2) and one grass field soil (UT2).

Results and Discussion

In studies comparing our DSPD method and other rapid DNA extraction methods, using UT soil, the method of Berthelet *et al.*[3] and the UltraClean soil DNA isolation kit failed to yield detectable amounts of DNA, regardless of whether skim milk was added to the extraction buffer. The FastDNA SPIN Kit for soil (FastDNA kit) also failed to yield detectable amounts of DNA with no addition of skim milk to its extraction buffer. Although the addition of skim milk to the extraction buffer using the FastDNA kit yielded a greater quantity of soil DNA than the DSPD method (Table 2), the results of agarose gel electrophoresis indicated the presence of a significantly higher amount of fragmented DNA in the sample obtained with this kit, relative to the levels with DSPD (Fig. 2-A). The cause was deemed to be the use of different types and varying combinations of glass beads as indicated by comparing electrophoresis patterns of DNA samples recovered from the supernatants after the salt precipitation step in both methods (data not shown). The presence of fragmented DNA is undesirable as it is possible that this will generate chimeric amplicons during PCR amplification[11]. Since the soil texture can significantly differ between samples, the types of glass beads and the bead-beating conditions used may need to be adjusted for each one to suppress DNA fragmentation during the extraction step. In addition, the reliability of the yield and the PCR amplification efficiency for soil DNA extracted with the DSPD method was comparable with the FastDNA kit (Fig. 2-B).

In order to evaluate the use of the DSPD method with a wide range of soil types, soil samples were collected from diverse geological locations in Japan, and subjected to DNA extraction by DSPD. Our results showed that high molecular weight DNAs were successfully extracted from each sample and the results of spectrophotometric measurements showed that the preparations were of sufficient quality for molecular biological analyses. Furthermore, our PCR experiments confirmed that all the soil DNA samples investigated could be amplified using universal primers for eubacteria 16S rRNA (data not shown). In comparison, the DNA yields obtained using the FastDNA kit were higher than DSPD in 15 out of 24 samples, and a similar trend was observed with other samples, (Table 2). However, qualitatively, with the exception of the KO and MH samples, the DNA obtained from 10 samples (EH, FF, GG, HA1, HO29, KH1, MMO, NH, SA1 and UT in Table 3) using the DSPD method showed a statistically significant pattern of higher A_{260}/A_{230} ratios compared to the FastDNA kit isolates and this trend was observed with most of the remaining samples. Noticeable differences in the A_{260}/A_{230} ratios between these two methods were observed for some samples (HA1, HO29, KH1, KO, and MMO in Table 3), suggesting that the combination of the choice of DNA extraction method and particular soil type could significantly affect the quality of the extracted DNA.

The high purity of the samples, indicated by the A_{260}/A_{230} ratios, may be important for certain complex applications

Table 2. Comparison of soil DNA yields from local samples

Sample name	DSPD		FastDNA kit[b]
	+[a]	−[a]	
CS	2.90±0.21[c]	ND[d]	ND
CS2	2.48±0.12	ND	ND
EH	1.82±0.02	ND	2.61±0.04*
FF	3.13±0.50	2.57±0.09	3.58±0.55
GG	3.08±0.36	2.59±0.08	3.66±0.26
HA1	2.34±0.07	2.03±0.06*	3.56±0.09*
HO29	2.05±0.09	1.99±0.09	2.53±0.19*
IN	0.41±0.03	ND	0.95±0.02*
KH1	1.99±0.09	ND	2.13±0.11
KK	3.27±0.30	2.47±0.08*	3.23±0.37
KN	3.76±0.09	3.55±0.13	4.11±0.26
KO	2.35±0.11	2.39±0.29	2.65±0.16
MH	2.43±0.10	2.26±0.16	3.38±0.05*
MH2	2.83±0.46	4.69±0.15*	6.27±0.13*
MI	1.87±0.03	ND	2.60±0.12*
MMO	2.92±0.24	2.77±0.27	4.13±0.48*
NH	1.79±0.06	0.34±0.01*	2.54±0.17*
OA	2.50±0.08	2.56±0.11	2.92±0.13*
SA1	1.84±0.01	0.53±0.08*	2.57±0.25*
ST	2.09±0.07	ND	2.87±0.25*
TI	1.88±0.05	ND	2.19±0.10*
UT	1.90±0.21	ND	2.21±0.03*
UT2	2.25±0.06	ND	ND
YH	3.64±0.18	4.05±0.24	4.83±0.34*

[a] The presence (+) or absence (−) of skim milk in DSPD extraction buffer.
[b] FastDNA SPIN Kit for soil.
[c] Mean (μg/g soil)±S.D. of three independent extractions.
[d] Not determined because no DNA band could be detected on agarose gel electrophoresis.
* Statistically significant differences between DSPD preparation with skim milk (+) and other samples at the 0.05 level measured using t-test.

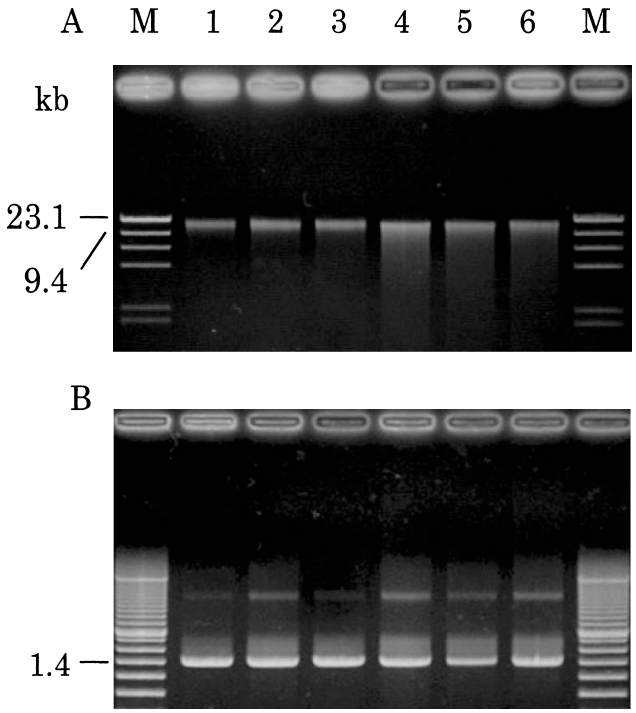

Fig. 2. Comparison of the DSPD method and FastDNA SPIN Kit for soil. Panel A: Agarose gel electrophoresis of soil DNAs. Lanes: M, *Hind*III-cut lambda bacteriophage molecular size markers; 1–3, DNA extracted by DSPD; 4–6, DNA isolated with the FastDNA kit. Panel B: Amplification of 16S rDNA. Lanes: M, 200 bp ladder molecular size Markers; 1–3, Amplicons from DSPD template DNAs; 4–6, Amplicons from FastDNA kit DNAs.

such as microarray analysis, as it is known that the activities of some key enzymes used in molecular studies, such as *Taq* DNA polymerases and restriction enzymes, are inhibited by very low concentrations of humic substances[14,18], and that the presence of humic acids also reduces the efficiency and accuracy of the annealing reaction in PCR[20]. Several DNA samples obtained with the FastDNA kit showed slightly higher A_{260}/A_{280}, ratios compared with the DSPD method using the same soil aliquots. In addition, we found that the FastDNA kit failed to recover soil DNA from three soil samples examined (CS, CS2, and UT2) in either the presence or absence of skim milk in the extraction buffer. The cause is thought to be differences in the extraction buffer, as there was no detectable soil DNA in the supernatants of these samples after bead-beating with the FastDNA kit (data not shown). As the extraction buffer used in the DSPD method contains relatively high concentrations of EDTA and phosphate buffer relative to the levels in other published methods, these factors may also be important for extracting DNA from some soil types. Consequently, the successful recovery of DNA from these soils using DSPD indicates the robustness and usefulness of this technique for DNA extraction from a more diverse range of environmental samples.

Using the same set of soil samples, the effects of skim milk supplements on DNA extraction efficiency were examined using the DSPD method. Based on the comparison of the results of DNA extraction with or without skim milk addition, we found that the presence of skim milk in the extraction buffer was essential to obtain detectable amounts of DNA from 10 of the 24 samples examined (CS, CS2, EH, IN, KH1, MI, ST, TI, UT, and UT2 in Table 2), which was consistent with the results reported previously by Takada-Hoshino and Matsumoto[21]. Interestingly, each of these samples showed very low A_{260}/A_{230} ratios, relative to the DNA quality levels evident in the remaining soil samples. In addition, significant differences between the effects of skim milk on DNA yields were also evident among the soil samples from which DNA could be isolated, regardless of the use of skim milk. For example, DNA yields were increased by the addition of skim milk to the extraction buffer of four soil samples (HA1, KK, NH, and SA1 in Table 2), and decreased in one sample (MH2 in Table 2), indicating that the effects of skim milk on DNA yield need to be examined for each soil sample tested. Moreover, the quality of soil DNA was also affected in a few cases by the presence or absence of skim milk. Negative effects on DNA quality were observed in four samples (GG, KK, MH, and MH2 in Table 3), based on the A_{260}/A_{230} and A_{260}/A_{280} ratios, whereas a positive effect was seen for one sample (NH in Table 3) using the A_{260}/A_{230} measurement.

In this study, one of our concerns in assessing soil properties was the phosphate adsorption coefficient, as the Japanese archipelago was formed by volcanic activity and hence Andosol is widely found throughout the country. Andosol has an extremely high potential for phosphate adsorption (Table 1), which could partially explain the low DNA yields obtained for some soil samples, as polynucleotides can be adsorbed into the soil matrix via the phosphate residues in their backbone structure. This may well explain the positive effects of skim milk on some DNA yields in this study and the findings of a previous report by Takada-Hoshino and Matsumoto[21]. Since skim milk has been widely used as a blocking reagent in molecular biological analyses of nucleic acids and proteins, it may also suppress the adsorption of DNA in the soil matrix. All of the soil specimens that proved difficult to examine in this study also showed very high phosphate adsorption coefficients (CS, CS2, EH, IN,

Table 3. Comparison of soil DNA purity from local samples

Sample name	A_{260}/A_{230}			A_{260}/A_{280}		
	DSPD		FastDNA kit[b]	DSPD		FastDNA kit[b]
	+[a]	−[a]		+[a]	−[a]	
CS	1.17±0.05[c]	ND[d]	ND	1.80±0.01	ND	ND
CS2	1.17±0.05	ND	ND	1.80±0.03	ND	ND
EH	1.51±0.12	ND	1.12±0.06*	1.70±0.04	ND	1.72±0.04
FF	1.89±0.03	1.91±0.02	1.59±0.12*	1.80±0.03	1.85±0.01	1.83±0.01
GG	1.63±0.04	1.76±0.06*	1.46±0.02*	1.75±0.02	1.76±0.01	1.84±0.02*
HA1	1.97±0.01	1.90±0.14	1.33±0.07*	1.84±0.05	1.84±0.07	1.83±0.02
HO29	1.48±0.01	1.50±0.07	0.89±0.21*	1.68±0.07	1.73±0.01	1.82±0.05*
IN	0.62±0.04	ND	0.40±0.36	1.61±0.07	ND	1.68±0.11
KH1	1.61±0.10	ND	0.81±0.14*	1.65±0.03	ND	1.72±0.10
KK	1.36±0.04	1.50±0.02*	1.41±0.10	1.69±0.02	1.73±0.02*	1.84±0.03*
KN	2.04±0.01	2.05±0.02	1.73±0.35	1.80±0.01	1.81±0.03	1.82±0.01*
KO	0.98±0.03	1.02±0.03	1.58±0.15*	1.52±0.02	1.52±0.03	1.84±0.01*
MH	1.22±0.04	1.50±0.07*	1.39±0.09*	1.60±0.03	1.66±0.03	1.83±0.03*
MH2	1.50±0.06	1.77±0.06	1.80±0.19	1.69±0.05	1.79±0.02*	1.82±0.01*
MI	0.89±0.05	ND	0.96±0.08	1.77±0.15	ND	1.68±0.05
MMO	1.93±0.02	1.94±0.02	1.34±0.15*	1.79±0.01	1.80±0.06	1.81±0.03
NH	1.33±0.14	0.78±0.11*	0.92±0.08*	1.71±0.14	1.43±0.22	1.65±0.00
OA	1.69±0.04	1.72±0.11	1.39±0.20	1.75±0.04	1.80±0.03	1.79±0.00
SA1	1.63±0.08	1.59±0.07	1.13±0.15*	1.78±0.08	1.69±0.12	1.76±0.03
ST	1.59±0.05	ND	1.26±0.31	1.76±0.08	ND	1.82±0.01
TI	1.62±0.05	ND	1.85±0.38	1.73±0.06	ND	1.73±0.05
UT	1.40±0.10	ND	0.88±0.06*	1.50±0.06	ND	1.81±0.03*
UT2	1.24±0.06	ND	ND	1.77±0.05	ND	ND
YH	2.02±0.01	2.06±0.01	1.84±0.19	1.81±0.02	1.82±0.01	1.82±0.01

[a] The presence (+) or absence (−) of skim milk in DSPD extraction buffer.
[b] FastDNA SPIN Kit for soil.
[c] Mean±S.D. of three independent extractions.
[d] Not determined because no DNA band could be detected on agarose gel electrophoresis.
* Statistically significant differences between DSPD preparation with skim milk (+) and other samples at the 0.05 level measured using t-test.

KH1, MI, ST, TI, UT, and UT2 in Table 1), and DNA isolated from these samples could only be obtained using skim milk in the extraction buffer. However, we could not conclude that the phosphate adsorption coefficient was the main factor determining the use of skim milk, since we also observed that it was not required in some soils with high phosphate adsorption coefficients for successful DNA preparation (HA1, MH2, MMO, NH, and SA1 in Table 1). This indicated that other soil properties could influence the DNA extraction conditions in certain cases.

In our preliminary experiments, none of the currently published protocols or commercial kits was capable of producing even trace amounts of detectable DNA from UT soil. For such samples, the addition of either skim milk or nucleic acids to the extraction buffer has been previously recommended to overcome this problem. In this study, skim milk was chosen as a DSPD extraction buffer supplement, over the use of RNA, for two reasons. First, the use of skim milk is cost effective, and second, the removal of excess protein during the purification steps is easier than removing foreign RNA from the final DNA sample. Some published methods for soil DNA extraction also recommend excluding EDTA from extraction buffers as humic substances can be co-extracted with the DNA sample in the presence of EDTA[9]. In our preliminary experiments with UT soil, however, EDTA was found to be an essential component in the DSPD extraction buffer as it suppresses DNA fragmentation during extraction. The addition of sodium phosphate to the extrac-

tion buffer was also found to be essential for suppressing DNA fragmentation. Hence, the use of skim milk and EDTA was beneficial for obtaining high molecular weight DNA from UT soil that would be suitable for further molecular and genomic analyses. Although some reports have recommended a combined heat and detergent treatment in the extraction steps[17,27], we found that this caused severe fragmentation and significant reduction in DNA yield (data not shown). We also found that incubating the soil matrix in the DNA extraction buffer at 37°C for one hour caused similar negative effects on DNA yields from UT soil. Furthermore, an initial extraction and purification step was tested in which the UT soil matrix was suspended in DNA extraction buffer with a series of organic solvents (phenol, phenol-chloroform, chloroform, and benzyl chloride) in an attempt to increase DNA yield and purity. However, in contrast to the findings of other reports, this treatment either caused negative effects on DNA preparations, such as severe fragmentation and loss of yield (in the case of phenol or phenol-chloroform extraction) or had no positive effect on either the purity or amount of DNA obtained (in the case of chloroform or benzyl chloride extraction). Furthermore, the positive effects of skim milk in the DSPD extraction buffer could be abrogated by both heat and organic solvent treatment due to de-proteinization effects.

In most published protocols for soil DNA extraction, the purification protocols consist of a series of steps, which can be both time-consuming and costly, particularly when using commercial DNA purification kits. In this study, we have developed a simple and cost effective two-step purification method that can be successfully used to obtain high quality DNA suitable for molecular biological analyses from most of the Japanese arable soil types examined. Although Schneegurt *et al.*[16] have already pointed out the potential usefulness of DEAE-cellulose column treatment in the purification of soil DNA, their protocol consists of a series of purification steps that utilize two commercial DNA purification kits, making it both expensive and time-consuming. Low cost, ease of use and rapidity are now crucial factors for ecological research methodologies in order to process multiple samples and obtain statistically significant results. In addition to simplicity of use, the running costs of our novel DSPD method are only 5–10% of the cost of most commercial DNA extraction kits used in soil microbiology.

In conclusion, our DSPD method is a robust procedure for obtaining DNA from soils with diverse characteristics that is of suitable quality and quantity for molecular biological analyses. This method is simple, fast and cost-effective, and employs only non-hazardous reagents. These advantages of the DSPD method make it suitable for large-scale ecological studies of microbial populations in diverse soils, including Andosol.

Acknowledgements

This work was partially supported by the Japan Society for the Promotion of Sciences, Research Grant JSPS-RFTF00L01602 for the ongoing project “Gene analysis of plant biodiversity and the effects of transgenic plants on the environment”, a biosafety program under the Innovative Development Project.

References

1) Amann, R.I., W. Ludwig and K.H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. **59**: 143–169.
2) Ball, D.F. 1964. Factors affecting the concentrations of lead in British wheat and barley grain. Environ. Pollut. **131**: 461–468.
3) Berthelet, M., L.G. Whyte and C.W. Greer. 1996. Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpolypyrrolidone spin columns. FEMS Microbiol. Lett. **138**: 17–22.
4) Blackwood, C.B., T. Marsh, S.-H. Kim and E.A. Paul. 2003. Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. Appl. Environ. Microbiol. **69**: 926–932.
5) Frostegård, Å., S. Courtois, V. Ramisse, S. Clerc, D. Bernillon, F. Le Gall, P. Jeannin, X. Nesme and P. Simonet. 1999. Quantification of bias related to the extraction of DNA directly from soils. Appl. Environ. Microbiol. **65**: 5409–5420.
6) Hasegawa, S., K. Kamewada, Y. Yamada, S. Hidaka, Y. Ogawa and M. Nanjo. 1997. Physics, and chemistry of soil, p. 21–265. *In* the Committee of Analytical Methods of Soil Environment (ed.), Analytical methods of soil environment. Hakuyusha, Tokyo.
7) Holben, W.E. 1994. Isolation and purification of bacterial DNA from soil. p. 727–751. *In* R.W. Weaver, S. Angle, P. Bottomley, D. Bezdicek, S. Smith, A. Tabatabai and A. Wollum (ed.), Methods of soil analysis. part 2. Microbiological and biochemical properties. Soil Science Society of America, Inc., Madison, WI.
8) Kageyama, K., T. Komatsu and H. Suga. 2003. Refined PCR protocol for detection of plant pathogens in soil. J. Gen. Plant Pathol. **69**: 153–160.
9) Kresk, M. and E.M.H. Wellington. 1999. Comparison of different methods for the isolation and purification of total community DNA from soil. J. Microbiol. Methods **39**: 1–16.
10) Kuske, C.R., K.L. Banton, D.L. Adorada, P.C. Stark, K.K. Hill and P.J. Jackson. 1998. Small-scale DNA preparation method for field PCR detection of microbial cells and spores in soil. Appl. Environ. Microbiol. **64**: 2463–2472.
11) Liesack, W., H. Weyland and E. Stackbrandt. 1991. Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. Microb. Ecol. **21**: 191–198.
12) Ogram, A., G.S. Sayler and T. Barkay. 1987. The extraction and purification of microbial DNA from sediments. J. Microbiol.

Methods **7**: 57–66.
13) Ogram, A., G.S. Sayler, D. Gustin and R.J. Lewis. 1988. DNA adsorption to soils and sediments. Environ. Sci. Technol. **22**: 982–984.
14) Porteous, L.A. and J.L. Armstrong. 1991. Recovery of bulk DNA from soil by a rapid, small-scale extraction method. Curr. Microbiol. **22**: 345–348.
15) Sambrook, J. and D.W. Russel. 2001. Molecular Cloning: A Laboratory Manual, 3rd edn. Vol. 1, 5.26–5.28, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
16) Schneegurt, M.A., S.Y. Dore and C.F. Kulpa Jr. 2003. Direct extraction of DNA from soils for studies in microbial ecology. Curr. Issues Mol. Biol. **5**: 1–8.
17) Selenska, S. and W. Klingmuller. 1991. DNA recovery and direct detection of Tn5 sequences from soil. Lett. Appl. Microbiol. **13**: 21–24.
18) Smalla, K., N. Cresswell, L.C. Mendonca-Hagler, A. Wolters and J.D. van Elsas. 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. J. Appl. Bacteriol. **74**: 78–85.
19) Steffan, R.J., J. Goksoyr, A.K. Bej and R.M. Atlas. 1988. Recovery of DNA from soils and sediments. Appl. Environ. Microbiol. **54**: 2908–2915.
20) Steffan, R.J. and R.M. Atlas. 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. **54**: 2185–2191.
21) Takada-Hoshino, Y. and N. Matsumoto. 2004. An improved DNA extraction method using skim milk from soils that strongly adsorb DNA. Microbes Environ. **19**: 13–19.
22) Torsvik, V.L. and J. Goksoyr. 1978. Determination of bacterial DNA in soil. Soil Biol. Biochem. **10**: 7–12.
23) Tsai, Y.L. and B.H. Olson. 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol. **58**: 754–757.
24) Tyson, G., W.J. Chapman, R. Hugenholtz, E.E. Allen, R.J. Ram, P.M. Richardson, V.V. Solovyev, E.M. Rubin, D.S. Rokhsar and J.F. Banfield. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature **428**: 37–43.
25) Tyurin, I.V. 1951. Analytical procedure for a comparative study of soil humus. Trudy poch. Inst. Dokuchaev. **33**: 5–21.
26) Volossiouk, T., E.J. Robb and R.N. Nazar. 1995. Direct DNA extraction for PCR-mediated assays of soil organisms. Appl. Environ. Microbiol. **61**: 3972–3976.
27) Zhou, J., M.A. Bruns and J.M. Tiedje. 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62**: 316–322.