A restriction factor of direct DNA extraction from volcanic ash soils

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Abstract
The methods of direct DNA extraction from soils made dramatic improvements in analysis of soil microbial communities. Andosol, the volcanic ash soils, are very important soils which account for approximately 50 % of the field crop in Japan. Andosol contains a large amount of humic substances up to 10 % w/w. We tried to extract DNA from these volcanic ash soils directly, but it was impossible to extract DNA using earlier methods. Andosols have special characteristics such as high phosphorus adsorption and humic substances because the soils contain an amorphous aluminium in a silicate called allophane. In our research, the amorphous Al adsorbed DNA contains phosphorus bases in nucleotides. We tried to eliminate DNA adsorption using a high concentration of EDTA as chelator and phosphoric acids as masking material. In this paper, we show the effects of EDTA addition. By the removal of Al from soils in the extraction stage, the DNA also could be extracted. It was a proof that the restriction factor of the difficulty of DNA extraction from Andosol was amorphous Al. We improved the extraction buffer composition and combined with an easy purification step for acquiring purified DNA at high yield.

Key Words
Direct extraction, soil DNA, volcanic ash soils, amorphous aluminium, humic substances.

Introduction

Various microbes inhabit in soils and the diversity of the soil microbes is great. However, it is considered the 90 % or more of the soil microbes are unable to be cultivated at present. Consequently the cultivation methods of soil microbes, limit in the analysis of microbial community structure and it is clear that gene analysis of the un-culturable microbes in soil cannot be performed.

As a recent approach instead of the culture-dependent analysis of microbial community structures, PCR amplification products of crude DNA which was extracted from environmental samples is used for denaturing gradient-gel electrophoresis (DGGE), temperature gradient-gel electrophoresis (TGGE) or cloning techniques.

If the soil DNA can be extracted and analysed directly, it will become possible to know what microbes are living in soil and to obtain the information on new genes of them as DNA sequences whether the soil microbes are culturable or not. It is thought that the potential utility value of soil DNA including the information on new genes of these microbes is very high. The trial of extracting DNA from soils was performed for the first time by Torsvik and Goksoyr (1978). They carried out separation and recovery of the fraction which contained soil microbes with using pyrophosphate buffer, etc from soils, and then DNA was extracted from the fraction including microbes washed out from soil. They used the term "soil DNA" for DNA obtained using the method described above. This method once collects fraction of microbes derived from soils, and so it is called indirect extraction method now. By this method, the DNA of the unrecoverable microbes even after washing soil particles with buffer solution could not be obtained. There were microbes attached to soil particles such as actinomyces, which were living in the dead plant body, and inhabiting the inside of soil particles, whose DNA could not be extracted by this method.

Then, the direct extraction method was developed successively by Ogram *et al.* (1996), Tsai and Olson (1987) and Zhou *et al.* (1991), etc. Without separating microbes from soils, in these methods, soils were treated directly with alkaline solution containing enzyme such as lysozyme and proteinase K and surfactant denaturing protein such as SDS. Microbes were lysed in extraction solution and DNA was extracted in the presence of soil substances (sand, silt, clay, humic-substances, etc) as matrix. This method is considered to reflect more the actual microbial community structure than the indirect extraction methods, and to obtain in good yield. However, by this method, because of heat-treatment of soils in an alkaline solution for a long time, the contamination of humic-substances cannot be ignored.

1 – 6 August 2010, Brisbane, Australia. Published on DVD.

On the other hand, the method using a beads beater for the purpose of extracting DNA from more soils microbes was newly developed. It destroys microbial cells with beads mechanically (Kuske *et al.* 1998). Conditions of beads-beating were considered in detail by Burgmann *et al.* (2001). By this method, the microbial cells are mechanically crushed even if gram positive bacteria whose cells cannot be easily destroyed by surfactant such as SDS are included, because of the polysaccaride membranes surrounding their cells. Therefore the DNA can be extracted from such bacteria with extremely high yield by this method. Moreover, since the extraction procedure is for a short time, a crude soil DNA can be obtained with less contamination of humic-substances compared to heating methods.

There are some kits which prepare DNA from soils by the original methods over a short time, such as Bio101 Fast DNA spin kit (Qbio USA) and UltraClean Soil DNA kit (MoBio USA), produced commercially in this decade. These kits required a beads beater and the soil DNA extraction operation is finished in a short time.

We tried to extract DNA directly from volcanic ash soils in the Kanto region in Japan but it was impossible to extract DNA using earlier methods. Volcanic ash soils have special characterics such as high phosphorus adsorption and much humic substances because the soils contain an amorphous aluminium silicate called allophane. We tried to improve the DNA extraction method for volcanic ash soils, especially examining the composition of the extraction buffer. We also tested an easier purification step for acquiring purified DNA.

Material and Methods

Chemical analysis of sample soils

We sampled 31 soils in Kanto, Kinki and Tohoku regions which represented typical soil types in Japan. Especially, we sampled volcanic ash soils (Andosol), which are very important soils which account for approximately 50 % of crop area in Japan. Chemical analysis of soil pH (H_2O), pH (KCl), total nitrogen and carbon determined by N/C analyzer (NC-90A, Sumika Analytical Center) were carried out. We measured the amounts of amorphous aluminium and iron contained in Andosols and which distinguish Andosols from other soils, by a selective dissolution method (Blackmore *et al.* 1981). The results of chemical analysis of the soils which were used for the detailed experiments are shown in Table 1.

Soil DNA extraction with conventional extraction buffer

We tried to extract DNA from 31 soil samples with a conventional buffer which consisted of 1 % SDS, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA. Extraction buffer was added to 0.5 g soil and beads for beating for 30 sec (at 5 m / sec). After bead beating, the test tubes were centrifuged at 10000 x g for 10 min and the supernatants were collected. Denaturing proteins were removed by chloroform and the DNA was precipitated with 2-propanl.The DNA was subjected to electrophoresis in 1 % agarose gel and stained with ethidium bromide.

The effects of EDTA concentration in extraction buffer

EDTA has ability to chelate metal cations strongly. We tested the improvement of the extracted DNA yield by adding EDTA to extraction buffer especially from volcanic ash soils. The extraction solution consisted of 1 % SDS, 100 mM Tris-HCl (pH 8.3) and EDTA. The concentration of EDTA ranged between 0 ~ 300 mM. The extraction buffer was added to 0.5 g soil, beating beads and denaturing proteins were removed with chloroform and the DNA was precipitated with 2-propanol. The agarose gel was stained with SYBR Green I and quantitative analysis of the DNA concentration used fluorescent intensity by reference to the λ Hind III digest markers signal.

The relationship between soil DNA yields and quantities extracted metal ions from soils.

The extraction solution consisted of 1 % SDS, 100 mM Tris-HCl (pH 8.3) and EDTA. The concentration of EDTA ranged between 0 ~ 400 mM. After bead beating and centrifugation, a part of the supernatants was collected for quantitative analysis of metal cations. The metal cations (Al, Fe, Ca, Mg) in extracted supernatants were determined by ICP-AES (SPS-6000 Seiko).

The comparison of the original method with the other methods and soil DNA extraction kits

Five methods (the original, heating extraction (Zhou *et al*. 1996), beads beating extraction (Cullen *et al.* 1998), and two commercial kits (UltraClean Soil DNA kit and Bio101 Fast DNA spin kit) were tested for soil DNA extraction from 7 volcanic ash soils, 5 non-volcanic ash soils (one of them, Souti res. pasture was contaminated with a little volcanic ash by volcanic eruption)

The original method was carried out in the procedure. 0.5 g fresh soil and 1 g of silica-zirconia beads (0.1 mm : 0.5 mm =3 : 1 mix) were added to 2 ml screw-capped tubes and 1200 µl of lysis buffer was added (1 % SDS, 100 mM Tris-HCl, 200 mM EDTA, 500 mM Na_2HPO_4 (pH 8.6)). The high concentration of EDTA and sodium phosphoric acid could chelate and mask amorphous aluminium. After beating with beads at 5 m / sec for 30 sec the supernatants were collected by centrifugation at 12,000 x g for 5 min. 750 µl of supernatant was transferred to a new 2 ml tube and add 250 µl of 5 M NaCl and 250 µl of 10 % CTAB (cetyltrimethylammonium bromide) added and incubated at 65 °C for 5 min. After mixing vigorously with a vortex mixer for 15 sec, the tube was centrifuged at 15,000 x g for 10 min. 1,000 µl of the aqueous layer was transferred to a new 2 ml tube and an equal volume of 12 % PEG was added. The DNA was precipitated by centrifugation at 20,000 x g for 20 min at 4 °C. The DNA pellet was washed with 70 % cold ethanol and dissolved in 100 µl TE buffer.

Results

Soil DNA adsorption to volcanic ash soils.

We show the result of DNA extraction from several soils (volcanic and non-volcanic soils) with low EDTA concentration buffer in Figure 1. The soil DNA could be extracted from non-volcanic soils but little or no soil DNA could be extracted from volcanic ash soils and the soil contaminated with volcanic ash. It showed the volcanic ash soils strongly interfered in the extraction of DNA. The effects of EDTA for DNA extraction from soils is shown in Figure 2. From Osaka crop field soil (non-volcanic soil), the DNA could be extracted easily with 30 mM EDTA at least. However, from Tochigi forest soil, the DNA could not be extracted with 100 mM EDTA. The addition of 300 mM EDTA made the extraction of soil DNA possible. The concentration of EDTA was higher than usual for in molecular biological experiments.

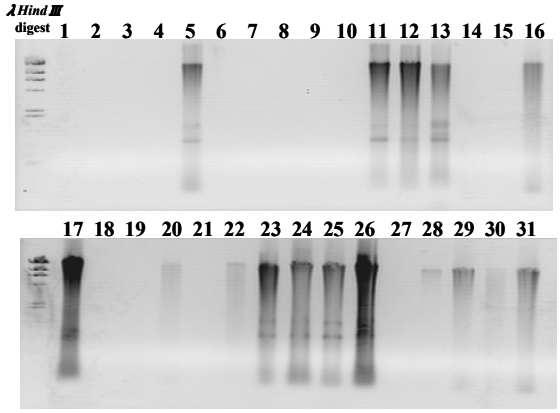

Figure 1. The result of DNA extraction from several soils (volcanic and non-volcanic soils). The extracted DNA were measured by electrophoresis in agarose gel with *λ Hind III* digest marker (ethidium bromide stained). (Volcanic ash soil: 1~4,6~7,9~10,14~16,18~22,27~31; Non-volcanic soils: 5,11~13,17,23~26; Non-volcanic soil contaminated with a little volcanic ash: 8).

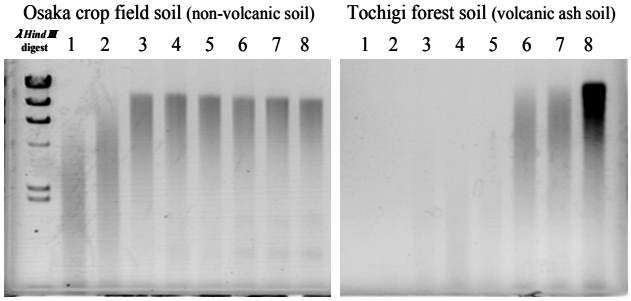

Figure 2. The effects of EDTA for DNA extraction form soils. The extraction solution was consisted of 1% SDS/ 100mM Tris-HCl (pH 8.3) and EDTA. The concentration of EDTA was changed. (1;10 mM, 2;10 mM, 3;20 mM, 4; 30mM, 5;50 mM, 7;200 mM, 8; 300mM).

A restriction factor of soil DNA extraction from volcanic ash soils.

The effects of the concentration of EDTA in extraction buffer for soil DNA yield and extracted metal ions are shown in Figure 3. It was thought that the high concentration of EDTA released soil DNA from adsorption by the soil particles. The volcanic ash soils contain high amounts of active aluminium and iron (amorphous mineral called allophane). To measure amorphous aluminium and iron adsorbed DNA, we determined the quantities of the extracted aluminium and iron in the supernatants after beads beating by ICP-AES.

In Figure 3, the removal of aluminium from soils by chelating reaction of EDTA made soil DNA recovery higher. It was suggested that amorphous aluminium in volcanic ash soils adsorbed DNA in the extraction buffer and made the direct soil DNA extraction difficult.

Comparison between the original direct soil DNA extraction method and the other methods

The DNA yields were showed in Figure 4. The earlier methods and two commercial kits could extract the soil DNA from non-volcanic soils, but not extract from volcanic ash soils at all. The original method could extract soil DNA at high yield from not only non-volcanic soils but also volcanic ash soils.

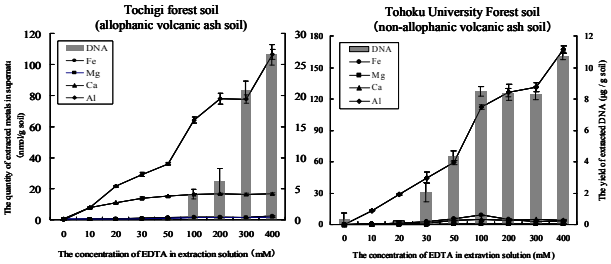

Figure 3. The effects of the concentration of EDTA in extraction buffer on soil DNA yield and extracted metal ions. The DNA yields were determined by stained agarose gel. The metal ions in extracted suspensions were determined by ICP-AES.

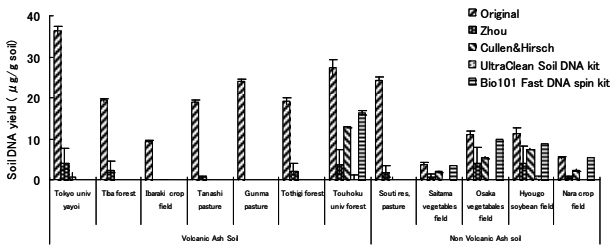

Figure 4. A result comparing the original method in this research with earlier two methods and two extraction kits. The DNA yields were determined by SYBR-Green I stained agarose gel electrophoresis.

Table 1. The results of soil analysis. Total N, C were determined by N/C analyzer. The selective dissolution extracts were determined by ICP-AES. Allophanic Al was calculated (Acidic oxalate extractive Al – Pyrophosphate extractive Al).

soils	Soil Taxnomy	pH (H_2O)	pH (KCl)	Total N (%)	Total C (%)	Acidic oxalate extraction (mg/g soil)			Pyrophosphate extraction (mg/g soil)		Allophanic Al (mg/g soil)
						Si	Fe	Al	Fe	Al	Al
Tokyo univ yayoi	Andisol (Allophanic)	6.98	5.58	0.27	3.12	26.17	25.45	47.52	1.01	2.38	45.14
Tiba forest	Andisol (Allophanic)	6.2	5.11	0.43	5.40	15.77	26.75	62.88	1.57	6.03	56.85
Ibaraki crop field	Andisol (Allophanic)	6.3	5.69	0.34	4.64	10.41	19.79	50.56	1.33	5.41	45.15
Tanashi pasture	Andisol (Allophanic)	4.96	4.3	0.41	4.90	14.98	27.12	62.58	2.00	7.15	55.43
Gunma pasture	Andisol (Allophanic)	5.82	5.45	0.37	3.97	9.67	8.61	39.07	1.08	3.56	35.51
Tothigi forest	Andisol (Allophanic)	5.28	4.38	0.68	8.76	6.25	12.42	38.35	5.00	14.22	24.13
Touhoku univ, forest	Andisol (un-Allophanic)	4.97	4.24	0.45	9.19	4.07	16.39	38.62	8.13	17.80	20.82
Souti res, pasture	Inceptisol	6.11	5.35	0.27	3.64	1.44	7.91	10.99	3.77	5.17	5.83
Saitama vegetables field	Inceptisol	5.01	3.98	0.13	1.18	0.45	4.10	2.09	1.79	0.87	1.22
Osaka vegetables field	Inceptisol	6.9	6.37	0.15	1.05	0.21	3.22	0.90	2.53	0.30	0.59
Hyougo soybean field	Inceptisol	7.81	7.4	0.16	1.44	0.42	2.11	0.89	1.51	0.32	0.56
Nara crop field	Inceptisol	5.11	3.98	0.22	1.47	0.45	6.25	1.52	2.50	0.62	0.89

Conclusion

We developed new direct DNA extraction method which extracts soil DNA from volcanic ash soils. The quantity of soil DNA in volcanic ash soils was higher than non-volcanic soils. The soil DNA was up to 20～40 μg / g soil and it suggested the population of microbial communities of volcanic ash soils were 10^9 orders as bacteria (calculated at 10 fg DNA / one bacteria). We expect the new method could reveal the microbial community structure in volcanic ash soils and be applied to measuring the biomass in soils through the quantitative analysis of extracted soil DNA.

Acknowledgement

This research was supported by JSPS KAKENHI (20880007) and Sumitomo Foundation (Environmental Research Projects No, 083228).

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