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Full Length Research Paper

Two electrophoreses in different pH buffers to purify forest soil DNA contaminated with humic substances

Weiguo Hou[1,2], Bin Lian[1]* and Sarah Rothenberg[1]

[1]Key State Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang, People's Republic of China.
[2]Graduate University of Chinese Academy of Sciences, Beijing, People's Republic of China.

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Direct extraction of DNA from soils is a useful way to gain genetic information on the soil source. However, DNA extraction from soils, especially forest soils, may be contaminated by humic substances due to their similar physical and chemical characteristics to soil. Even commercial soil DNA extraction kits fail to retrieve DNA from these soils. Using the potential changes of specific charge of DNA and humic substances in a pH solution, we performed two electrophoreses in different pH buffers to eliminate the interfering substances. The method produced high quality soil DNA, which is applicable for PCR amplification.

Key words: DNA extraction, forest soil, humic substances, pH, specific charge, two-step electrophoreses.

INTRODUCTION

More and more attention has been focused on underground microbial ecology as soil ecosystems are gene reservoirs, which contain abundant genetic information indicating the diversity and health state of an ecology or microenvironment (Lian et al., 2007; Martin and Rygiewicz, 2005; Saghirzadeh et al., 2008). Recent soil genetic research included phenoxyacetic acids degrading gene tfdA (Baelum et al., 2008), cry genes resource of *Bacillus thuringiensis* in soil (Zhu et al., 2009), diversity of diazotrophic bacteria in peat soil by cloning of the nifH gene (Zadorina et al., 2009); soil bacterial community composition by 16S rRNA gene clones (Desai et al., 2009; Tsai et al., 2009); nifH gene diversity in soil (Coelho et al., 2009; Sarita et al., 2008; Teng et al., 2009), formyl-CoA-transferase gene diversity in soil (Khammar et al., 2009), and so on.

In the latter studies, conventional culture-dependent methods were used, but researchers were unable to obtain useful genes since most of the microbes were unculturable (Zhang and Xu, 2008). Direct culture-independent extraction of DNA can solve this problem.

Humic substances, which inhibit polymerase chain reaction (PCR) and restrict endonuclease reaction (Wilson, 1997), can be co-extracted with DNA. Furthermore, the DNA quality has a great effect on microbial communities (Thakuria et al., 2008). Humic substances are a series of complicated aromatic compounds with carboxyl groups, negatively charged, significantly different in molecular weight, and may be soluble or insoluble in water. Similar physical-chemical characteristics between humic substances and DNA make it difficult to separate humic substances from DNA (Dong et al., 2006).

Several strategies were used to eliminate or remove humic substances and other contaminants during different stages of DNA extraction. The first strategy was to eliminate the contaminants before cell lysis, e.g. washing soils with PBS (phosphate-buffered saline, pH 8.0) or precipitating humic substances with $CaCl_2$ before extracting (Orsini and Romano-Spica, 2001; Ernst et al., 1996). In this way humic substances could be partially eliminated. Second, humic substances removing agents were added to eliminate contaminants during the stages of cell lysis and DNA extraction. For example, $CaCO_3$ was mixed with soils before DNA extraction (Sagova-Mareckova et al., 2008), hexadecyltrimethylammonium bromide (Cho et al., 1996), polyvinylpolypyrrolidone

Corresponding author. E-mail: bin2368@vip.163.com. Tel: +86 851 5895148. Fax: +86 851 5895148.

Abbreviations: PCR, Polymerase chain reaction; **PBS,** phosphate-buffered saline; **PEG,** polyethylene glycol; **BSA,** bovine serum albumin; **EDTA,** ethylene diamine tetraacetic acid; **dNTP,** deoxyribonucleoside 5'-triphosphate; **rRNA,** ribosomal ribonucleic acid.

(Frostegard et al., 1999; Zhou et al., 1996) were added into the extraction buffer. The third strategy was to remove inhibitants from crude DNA by cesium chloride density centrifugation (Leff et al., 1995; Smalla et al., 1993), gel electrophoresis (Liles et al., 2008; Zhou et al., 1996), PEG precipitation (Krsek and Wellington, 1999), and Sephadex G-200 column (Miller et al., 1999; Tsai and Olson, 1992). The last strategy was to use diluted DNA solution as template DNA or adding BSA (bovine serum albumin) to the PCR system to overcome the inhibiting effects (Cho et al., 1996; Rojas-Herrera et al., 2008). The migrating speeds of DNA and humic substances are determined by their specific charges and voltage strength, and the former are related to pH. In this research, we developed an effective method to purify forest soil DNA contaminated with humic substances by two electrophoreses in different pH buffers.

MATERIALS AND METHODS

Sampling

Forest soils of limestone origin were collected at 5, 10, 15 and 20 cm depth in a planted pine forest about 35-year-old, at Guiyang National Forest Park, Guiyang, China, where the average annual temperature was 15.3°C and total annual precipitation was 1700 mm. Soil pH was 5.8 - 6.5, and increased with soil depth. The soil samples were stored at -80°C after being collected.

Determination of organic matters and humic substances in soil

Firstly, sand and floral particulates were removed from soil, then the soil was air dried and milled into <2 mm powder. The humic substances were extracted according IHSS isolation procedure (Swift and Sparks, 1996). The organic matter in dry soil and the obtained humic substances were determined by combustion method (Sagova-Mareckova et al., 2008).

DNA extraction

Two methods and a commercial kit were used to extract soil DNA in room temperature. 0.25 g soil samples were used to extract DNA with micro-wave method (Orsini and Romano-Spica, 2001) and bead-beating method. The procedures of bead-beating method are as follows: 0.25 g soil samples were added into the 2 ml Eppendorf tubes containing 0.5 g bead about 1 mm in diameter (MO Bio, USA) and 450 µL lysis solution described above. The solution was vortexed at a maximum speed for 10 min; add 500 uL extraction solution described above and vortex for 10 s, incubate in 65°C for 10 min; then centrifuge at 5,700 g for 1 min; transfer supernatant to a new 1.5 ml tube. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added and mixed by inversion. DNA pellet precipitated by isopropyl alcohol was washed with 70% ethanol and resuspended in 100 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Soil DNA was also extracted with UltraClean Soil DNA Kit (Mo Bio, USA) according to the protocol.

Purify the DNA with electrophoresis

50 µL crude DNA was loaded onto 0.7% agarose gel and electrophoresis was carried out at 5 V/cm in pH 8.0 TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA) for 30 min. Agarose strips containing targeting DNA were excised integrally on a portable ultraviolet detector. The strip was put into mold vertically. Cast the second agarose gel in the mold containing the strip. The second electrophoresis was carried out at 5 V/cm for 30 min in pH 8.5 TAE buffer. The procedures of two-step electrophoresis are also showed by Figure 1. The total DNA was recovered with GENECLEAN® *Turbo* Kit (Qbiogene, Canada) low melting point agarose (Zintz and Beebe, 1991). Meanwhile the recovered DNA from the first electrophoresis and that without electrophoresis was carried out as controls.

Determination of purity and amount of purified soil DNA

DNA concentrations and purities were determined by OD260, ratios OD260/230 and OD260/280 (Holben et al., 1988). The concentrations of DNA were also calculated with the Quantity One software (Bio-Rad, California, USA).

PCR amplification assay

Amplifications of bacteria 16S rRNA gene, with primers EubB (27F) (5'-AGAGTTTGATCMTGGCTCAG -3') and EubA (1522R) (5'-AAGGAGGTGATCCANCCRCA -3') (Suzuki and Giovannoni, 1996), and ITS fragment (including ITS1+ITS2+5.8S) of soil fungi, with the primers ITS1F (5'– CTTGGTCATTTAGAGGAAGTAA–3') (Gardes and Bruns, 1993) and ITS4 (5'– TCCTCCGCTTATTG ATATGC– 3') (White et al., 1990) were performed to examine the quality of purified DNA. The PCR amplification mixture (50 µl) contained: 10 ng DNA, 2 µM each primer, 2.5 mM each dNTP, 2 U of Taq DNA polymerase and 5 µl polymerase buffer. The amplifying programs were as follows: 94°C for 5 min; 30 cycles consisting of 94°C for 45 s, 58°C for 30 s and 72°C for 1.5 min; and 72°C for 10 min. 8 µl PCR products were loaded into a 1% ethidium bromide containing agarose gel. After electrifying at 5 V/cm for 30 min, the gel was detected on an ultraviolet detector. Amplifications were performed with DNA from 4 soil depths, and 3 repeats for each depth.

RESULTS AND DISCUSSION

Contents of organic matters and humic substances

Total organic matter and humic substances in soil are always co-extracted during DNA extraction (Dong et al., 2006), and our results showed that total organic matter and humic substances contents of the soil samples we collected were comparatively higher than those reported by some authors (Lakay et al., 2007; Miller 2001; Sagova-Mareckova et al., 2008; Liu et al., 2002; Zhao et al., 2001). At 5 cm depth, maximum values for total organic matter and humic substances were 15.52 and 8.44%, respectively, and values decreased with depths (Figure 2).

DNA extraction

The microwave-based method and bead method both allowed a rapid isolation of high molecular weight DNA,

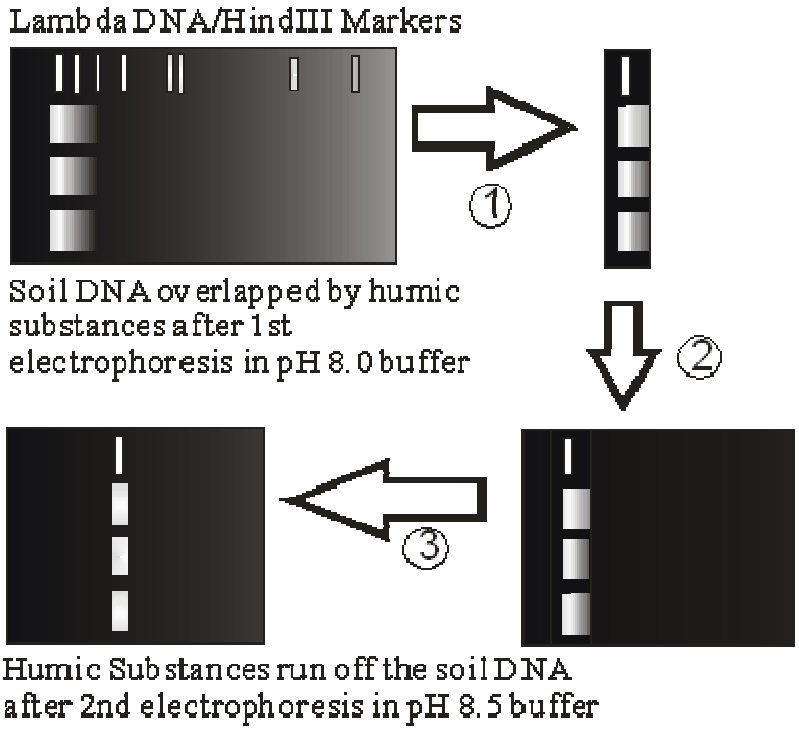

Figure 1. Procedures of two-step to purify the soil DNA containing humic substances (Cut the gel strip containing target DNA after the 1st electrophoresis in pH 8.0; Cast the 2nd gel over the strip; Electrify the gel in pH 8.5 buffer).

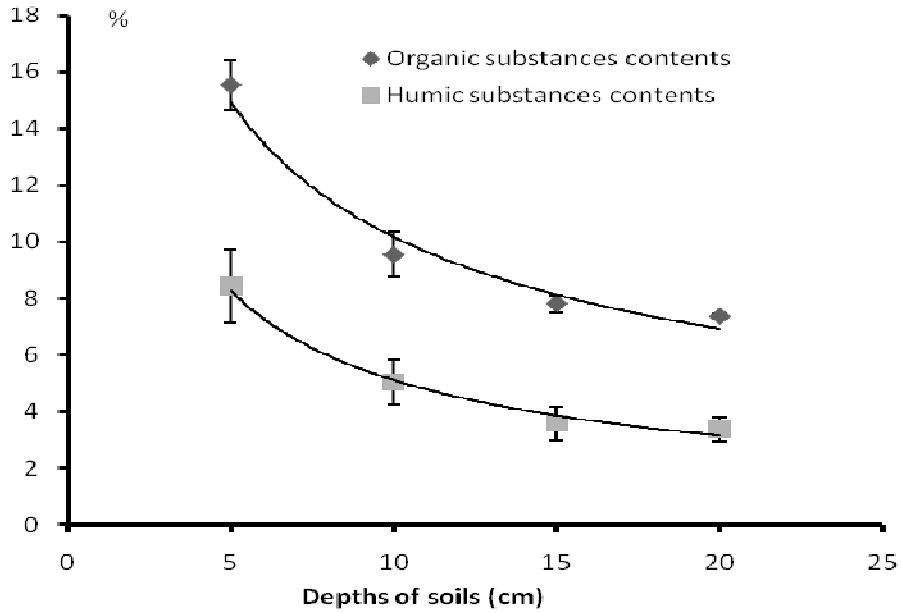

Figure 2. Contents of organic substances and humic substances in dry soils of different soil depths.

about 23 Kb (Figure 3). The crude DNA extracted from soil samples was dark brown, containing substantial pollutants indicated by the values of OD260/230 and OD260/280 (Table 1). A higher quantity of crude DNA, up

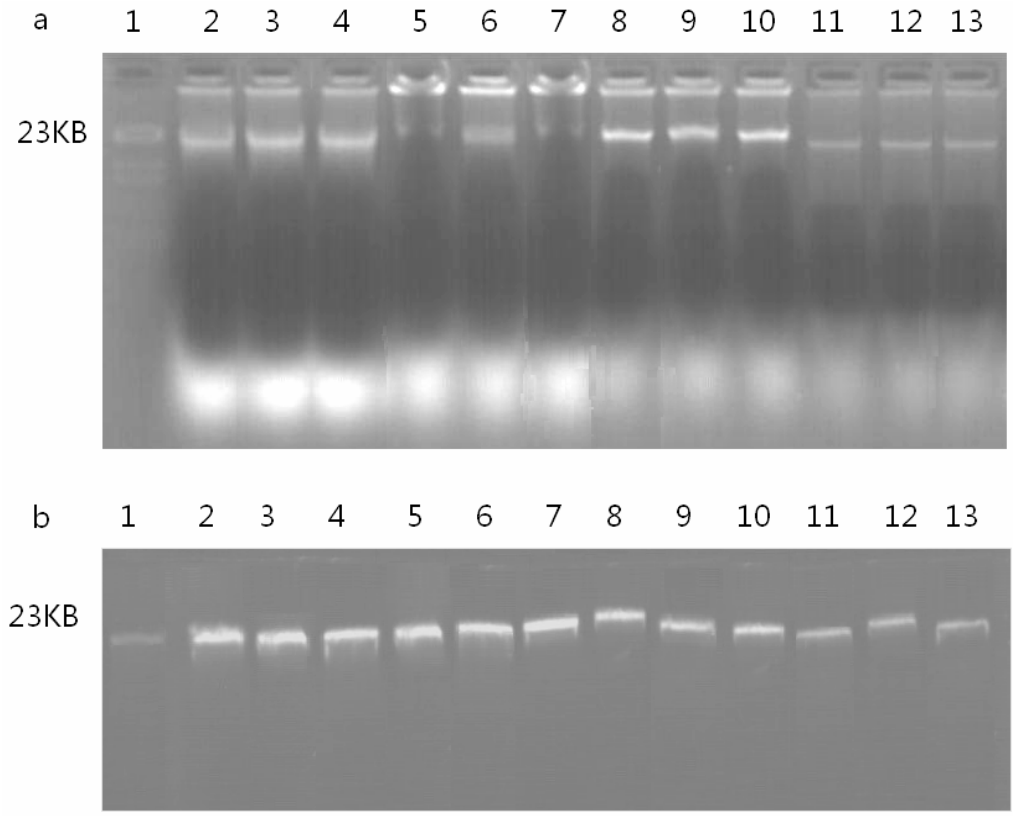

Figure 3. Crude DNA electrophoreses with different pH. (a) Electrophoresis for the first time in pH 8.0; (b) electrophoresis for the second time in pH 8.5. Lane 1: Lambda DNA marker, Lane 2 - 13: crude DNA extracted from soils of 4 depths, with 3 repeats).

Table 1. Quantity, OD260/230 and OD260/280 of Crude DNA extracted from forest soils at 4 depths*.

Soil depths Methods	5 cm DNA (µg/g)	10 cm DNA (µg/g)	15 cm DNA (µg/g)	20 cm DNA (µg/g)	Average OD260/230	Average OD260/280
Microwave	82.4 ± 2.9	68.4 ± 1.4	60.0 ± 1.2	56.4 ± 0.8	1.34 ± 0.3	1.4 ± 0.3
Bead	99.2 ± 4.3	92.7 ± 2.3	74.6 ± 2.2	67.3 ± 0.1	1.2 ± 0.2	1.1 ± 0.2
UltraClean	26.2 ± 1.2	22.7 ± 1.4	21.6 ± 1.2	19.3 ± 1.1	1.7 ± 0.1	1.8 ± 0.02

*Microwave represented microwave method; Bead represented bead-beating method; UltraClean means DNAs were extracted with UltraClean Soil DNA Kit.

to 99.2 µg/g dry soil in 5 cm depth, was obtained with the bead-beating method than that obtained by the other methods (Lakay et al., 2007; Miller et al., 1999; Porteous et al., 1994; Zhou et al., 1996). The quantities of crude soil DNA extracted decreased with soil depth. The qualities of soil DNA extracted with microwave method and bead-beating method were extremely poor indicated by low values of OD260/230 and OD260/280. However, with the same amount of initial soil, the yield of DNA extracted with UltraClean Soil DNA Kit was significantly lower than that with microwave method and bead-beating method. This situation was brought by DNA co-elimination during the progresses of humic substances removement.

Two-step electrophoreses to remove humic substances

Higher amount of humic substances in soil means a larger quantity of DNA can be retrieved. However, this also means more serious contamination with humic substances due to their similar chemical and physical characteristics (Dong et al., 2006; Jackson et al., 1997). After the first electrophoresis, smearing dark brown substances over-lapped the soil DNA (Figure 3a). But after the second electrophoresis in pH 8.5 buffer, the brown substances disappeared around DNA (Figure 3b), which indicated that the second electrophoresis was an effective and

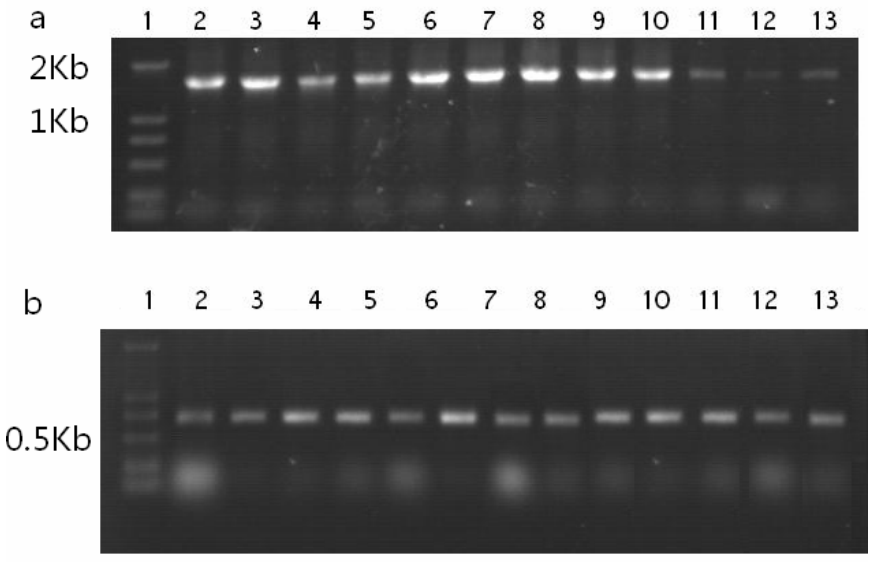

Figure 4. Bacterial 16S rRNA gene and fungal ITS fragment amplifications with two-electrophoreses-purified DNA (Lane 1: DL2000 DNA marker, Lane 2 - 13: crude DNA extracted from soils of 4 depths, with 3 repeats).

Table 2. The PCR amplification efficiency of purified DNA by different methods*.

PCR amplifications	UltraClea Kit	Crude soil DNA	GENE Kit	Electro and GENE	2 Electro and GENE	2 Electro and LMP
16S RNA gene	8 of 12**	None of 12	None of 12	None of 12	12 of 12	12 of 12
ITS fragment	8 of 12**	None of 12	None of 12	None of 12	12 of 12	12 of 12

*UltraClean Kit meant template DNA extracted with UltraClean Soil DNA Kit; Crude DNA meant template DNA extracted by bead-beating methods; GENE Kit meant crude DNA was purified with GENECLEAN® *Turbo* Kit only; Electro and GENE meant template DNA purified with an electrophoresis in pH 8.0 and retrieved with GENECLEAN® *Turbo* Kit; 2 Electro and GENE meant template DNA purified with two electrophoreses and retrieved with GENECLEAN® *Turbo* Kit; 2 Electro and LMP meant template DNA was purified with two electrophoreses and retrieved with low-melting-point agarose gel.
Note**: 8 amplifications of 12 were obtained with soil DNA extracted with UltraClean Soil DNA Kit.

essential step to remove humic substances.

The migrating speed of substances in agarose gel depends on their specific charge, topological structure, as well as the voltage during an electrophoresis. The charges of substances, which are related to the molecular function groups, depend on pH. The molecular weight of humic acids spans from 1,000 to over 300,000 Daltons (Wilson et al., 1999), with a negative charge of carboxyl in neutral pH buffer. DNA and humic substances will overlap in agarose gel during electrophoresis. Due to different functional groups, the specific charges are pH dependent. Our results indicated DNA can be separated from the overlapping substances after a second electrophoresis using different pH buffers.

PCR assays

16S rRNA gene and ITS fragment were amplified successfully with the soil DNAs purified by two electrophoreses regardless of that the DNAs were retrieved with GENECLEAN® *Turbo* Kit or low melting point agarose (Figure 4). However, there were less amplifications with soil DNA extracted by UltraClean Soil DNA Kit, or no amplification obtained with soil DNA purified by an electrophoresis or soil DNA only purified with GENE CLEAN® *Turbo* Kit (Table 2).

Conclusion

The results indicated that two-step electrophoreses was an efficient method to purify crude DNA with high organic contaminant content. During the purifying procedures, soil DNA may be lost in every step, so an efficient DNA extraction method is essential. The bead-beating method produces higher quantity of crude DNA than the microwave method. The agarose strip containing target DNA after the first electrophoresis was buried in a second agarose without extraction. In this way, the loss of DNA was minimized.

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