DNA RECOVERY FROM SOIL IN RESTORATION AREA AND INDICATED BIODIVERSITY

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Abstract

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A simple, rapid and cheaper method for direct extraction of DNA from damaged soil where the vegetation was being restored since the 1970s was developed. The soil was difficult to extract DNA from because of low TN (0.162 $mg.g^{-1}$) and TP (0.227 $mg.g^{-1}$), but higher humus content (0.469 $mg.g^{-1}$). The method was based on lysis with a high-salt extraction buffer (0.15 M NaCl, 0.1M Na2EDTA) and extended heating (2h) of the soil suspension in the presence of proteinase K and sodium dodecyl sulfate (SDS). The extraction method required 4h and was tested on the special restoration soil from which DNA extraction is comparatively difficult. Several other methods were tried but failed to yield DNA. This method was much cheaper than commercially available kits. The DNA fragment size in the crude extracts was about 20kb. Crude DNA was purified by improved gel extraction. The results showed that the recovery of DNA from different communities varied greatly. The chemical properties of the soil also showed significant difference among different communities and indicated a great spatial heterogeneity. Spatial heterogeneity could reflect biodiversity. DNA recovery was used as the direct index for biodiversity instead of soil properties. The F-test predicts that the restoration area can and will accommodate high biodiversity.

Key words: DNA extraction, DNA recovery, restoration area, damaged soil, biodiversity

Introduction

Total bacterial counts from environmental samples are usually more than one order of magnitude greater than plate counts, and most (99.5 to 99.9%) of the soil bacteria observed with fluorescent microscopy cannot be isolated or cultured on laboratory media (Torsvik et al., 1990; Roszak, Colwell, 1987). Consequently, they will not be included in estima-

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tions of phenotypic diversity (Torsvik et al., 1990), but isolation of bacterial nucleic acids from natural environments has become a useful tool for detecting these bacteria (Liesack, Stackebrandt, 1992; Torsvik et al., 1990). To apply such molecular techniques to ecological studies, a new method for extracting DNA from the natural environment is a potentially very important advance (Jae-Chang Cho et al., 1996).

Most DNA extraction methods have been tested on a limited number of soil types, such as loam and sandy loam (Zhou et al., 1996), so their general applicability for comparative ecological studies is unknown. The soil in this study was from granite parent rock and the vegetation was subject to severe disturbances for many years. No one has previously studied this type of soil before. Over the past 30 years, the landscape was gradually being restored. The objectives of this study were to evaluate and improve DNA extraction and purification methods in terms of cost, speed, simplicity, DNA yields, DNA fragment size, applicability to a broader variety of soils. In this study a direct method for DNA extraction from this type of soil was developed using a lysozyme and freezing-thawing procedure, followed by purification of crude DNA and an improved agarose gel electrophoresis step, which yields high purity DNA. The described procedure for extracting DNA from soil environments could be a useful tool for molecular ecological study in ecosystems undergoing restoration.

Spatial heterogeneity of soil properties is caused by the presence of plants in many types of terrestrial ecosystems (Boettcher, Kalisz, 1990; Charley, West, 1975; Hirose, Tateno, 1984; Matson, 1990). Knowledge of the spatial heterogeneity of soil properties is also important for understanding the ecosystem processes and microbial diversity (Stoyan et al., 2000). Diversity maybe enhanced by spatial and temporal heterogeneity caused by abiotic factors (such as human distribution). In this study, total nitrogen and total phosphorus were analyzed and the spatial heterogeneity of soil properties was measured in this restoration area. The spatial heterogeneity of soil nutrients produced a high level of biodiversity, while we also found a huge difference in the recovery of DNA from different communities in this area. In our analysis we linked DNA recovery with related biodiversity, which will allow us to omit measurements of soil nutrients in future studies. Thus, by using the necessary microcosmic processes in modern ecological research, we can simultaneously obtain more important macroscopical information. No previous studies have integrated this information.

Materials and methods

Soil. Samples were collected in September, 2004 from the top 10cm of soil in 9 different communities after clearing the surface litter.

1. *Robinia pseudoacacia* L. [facing the sun] community;
2. *Robinia pseudoacacia* L. [opposing to the sun] community;
3. pine community [facing and opposing the sun];
4. pine community [opposing to the sun];
5. *Platycladus rientalis* (L.) Franco and pine community (facing the sun);
6. *Quercus acutissima* Carruth. Community (facing the sun);
7. *Platycladus rientalis* (L.) Franco and *Robinia pseudoacacia* L. community (facing the sun);
8. *Robinia pseudoacacia* L. and pine community (facing the sun);
9. farmland.

All communities were located in the Fanggan mountainous area at the center of Shandong Province, China, where the vegetation is being restored. Six samples were selected randomly from each community. Parts of soil samples were stored at -70 °C for DNA extraction and others were analyzed for soil chemicals.

Chemical analysis of the soil. Part of each soil sample was sieved (< 2mm) and analyzed for total nitrogen (TN), total phosphorus (TP) and humus content. The TN content was determined using micro-Kjeldahl digestion (Nelson, Sommers, 1980). For TP analyses, soil samples were solubilized in Bray's P-l and then subjected to flow injection analysis (Robertson et al., 1993). For humus analyses, the soil was extracted with sodium pyrosulfurate and NaOH (Edaphon Study, 1985).

DNA extraction.

Protocol 1. The soil samples (5g) were initially suspended in 10ml of lysis solution (0.15 M NaCl, 0.1M Na2EDTA [pH 8.0]) containing 15 mg of lysozyme · ml-1 and incubated at 37 °C for 1hour. Then 10ml of SDS solution (0.1M NaCl, 0.5 M Tris-HCl [pH 8.0], 10% sodium dodecyl sulfate) was added. Four cycles of freezing at –70 °C (10 min) and thawing at 65 °C (10 min) were conducted to release the DNA from the bacterial cells in the soil. Then 10-ml of phenol saturated with 0.1 M Tris-HCl (pH 8.0) was added and the sample was gently vortexed to obtain an emulsion. The mixture was centrifuged at 12 000 × g for 10 min (Beckman coulter TM, USA, JA-20 rotator). The top aqueous layer was collected and extracted once with an equal volume of a phenol-chloroform mixture and twice with a chloroform mixture. Nucleic acids in the aqueous phase were precipitated with an equal volume of ice-cold isopropanol at –20 °C for 30 min. Nucleic acids were pelleted by centrifugation at 12 000 × g for 15 min. The nucleic acid pellet was resuspended in 300-µl TE buffer (20 mM Tris-HCl [pH 8.0], 1 mM Na2EDTA [pH 8.0]) containing heat-treated RNase A (0.2 µg·µl^{-1}) and incubated at 37 °C for 1hour. The resulting DNA extracts were stored at –20 °C.

Protocol 2. Crude DNA extraction was according to the method of Moré et al. (1994).

Protocol 3. Crude DNA extraction was according to the method of Yang (2000).

Gel electrophoresis and DNA purification.

Protocol 1. The DNA extracts were loaded onto a 1% agarose gel containing 0.5 µg of ethidium bromide · ml^{-1} and 2% PVP and then subjected to electrophoresis (4 V/cm) for 60 min in Tris-acetate-EDTA (TAE) buffer according to a standard protocol (Ausubel et al., 1995). Escherichia coli digest (Takara) was used for molecular weight standard markers. The resulting DNA bands were cut out of the gel under UV radiation and purified with an improved standard protocol.

a. Preparation of spin columns: an inhibitor removal resin (500µl) was added to 500µl centrifuge tubes. The very top of the tube was cut after 2 minutes, and the tube was placed in a 1.5 ml centrifuge tube without a cap. This was centrifuged for 1 min. at 2000 × g to pack the sample column, the 1.5 ml tube was discarded, and the column was moved to a new one.
b. The supernatant (100µl) obtained from the 9th step of the standard gel purification protocol (Sambrook, Russell, 2001) was transferred directly onto the prepared spin column (from section a).
c. The column was centrifuged for 2 min. at 2000 × g.
d. Discard the column and then add 16µl acetic amine (10M) and 160µl ethanol (100%, 4 °C) to the remaining 1.5 ml tube. Incubate at room temperature for 10'.
e. Centrifuge for 20' at 5000 × g and remove the supernatant.
f. Rinse the precipitant with 70% ethanol and resuspend in TE (pH 8.0)

Protocol 2. The DNA extracts were purified with the modified steps of UNIQ-10 Column Genomic DNA Isolation Kit (Sangon).

Determination of the purity of DNA. To quantify the amount of DNA, the concentration of DNA was determined by an Eppendorf Biophotometer (Gene Company Limited, Germany). The purity of the DNA was assessed spectrophotometrically by calculating A260/A230 and A260/A280 ratios for humic acid contamination and protein impurities, respectively.

Note: This method was selected for determining the purity of the DNA because the method was convenient, relatively exact, and the results were only used to compare one sample with another.

Results

Chemical properties of soil. The mean values of total N (TN) and total P (TP) in the 9 communities were 0.162 mg×g^{-1} and 0.227 mg×g^{-1}, respectively. The mean humus content was 0.469 mg×g^{-1}. There were significant differences in the total N (TN) among the different

forest types (F (8, 45) = 342.7229, P < 0.001). The same was true for the total P (F (8, 45) = 198.6883, P < 0.001).

Evaluation of DNA extraction from challenging soil. Protocol 1 for the preparation of DNA samples from a restoration area was developed as part of this study so that further ecological studies on soil bacterial communities and aboveground biodiversity would be easier. The following figure (Fig. 1) shows an example of the ethidium bromide-stained agarose gels used to visualize the DNA extracts after RNase and other treatments. The largest DNA fragment size was about 20kb. Most of the DNA had a high molecular weight and there was only a little DNA shearing. With protocols 2 and 3 for crude DNA extraction, little or no DNA was isolated from our soil samples so that the DNA was not easily visible in EB-stained gels viewed under UV radiation (figures unlisted).

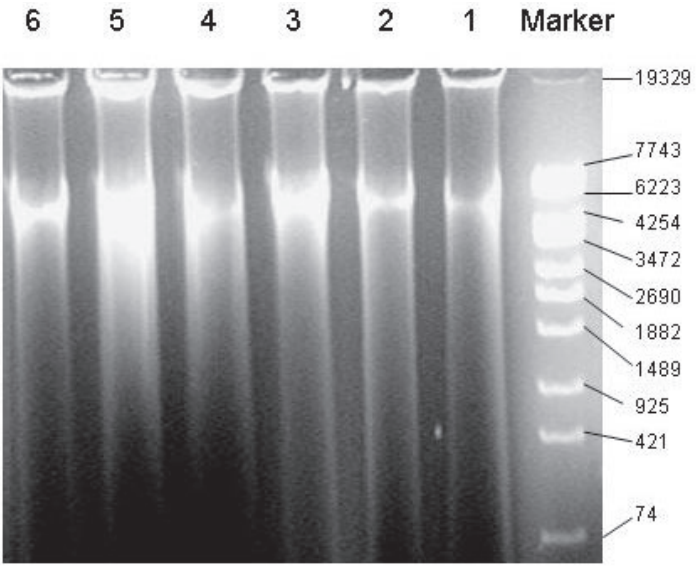

Fig. 1. Agarose gel electrophoresis of crude DNA extracted from *Robinia pseudoacacia* L. (facing sun) communities.
* The tracks of 1–6 are from the 6 samples of the community

Comparison of DNA purification methods

To evaluate the purity of the DNA for further application, 2 methods were compared using portions of the crude extracts from different communities. Because of the small capacity of the minicolumn and the loading slot used in these methods, only about 1/30 of the crude DNA extract from 5 g of soil was purified at a time. DNA purification (Protocol 1) resulted

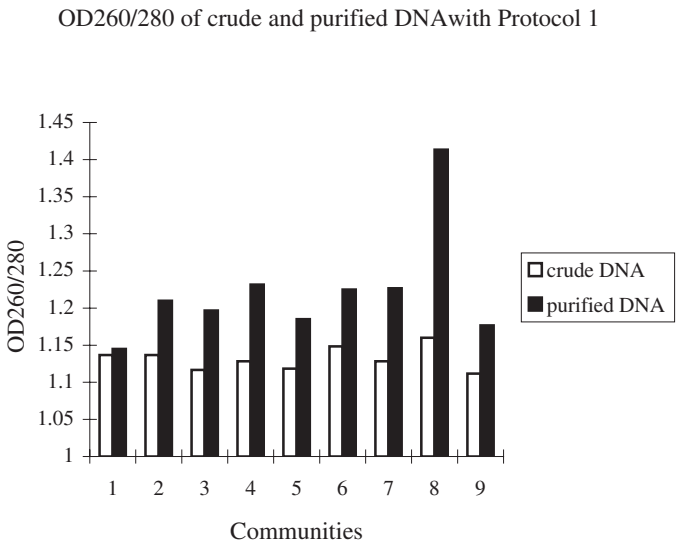

Fig. 2. 1. *Robinia pseudoacacia* L. (facing sun) 2. *Robinia pseudoacacia* L. (opposing to sun) 3. pines (facing sun) 4. pines (opposing to sun) 5. *Quercus acutissima* C a r r u t h. 6. pines and *Robinia pseudoacacia* L. 7. *Platycladus rientalis* (L.) F r a n c o and pines 8. *Platycladus rientalis* (L.) F r a n c o and *Robinia pseudoacacia* L. 9. farmland.

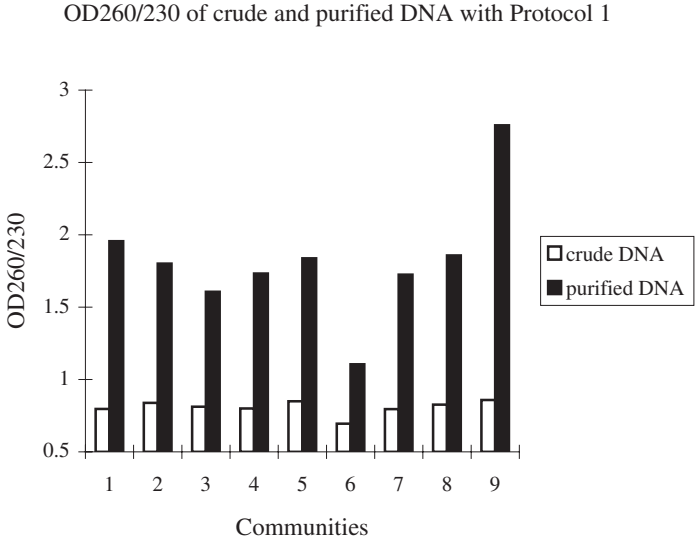

Fig. 3. Refer to Fig. 2. for details of community types.

in complete removal of the brown color from the crude DNA solutions. Fig. 2 shows the increase in the mean of the OD260/280 from the crude extracts from nine different communities. Moreover, the OD260/230 increased greatly (Fig. 3). Protocol 2 was applied to randomly selected communities (as described in the figure legend) (Fig. 4). The results showed no obvious effects.

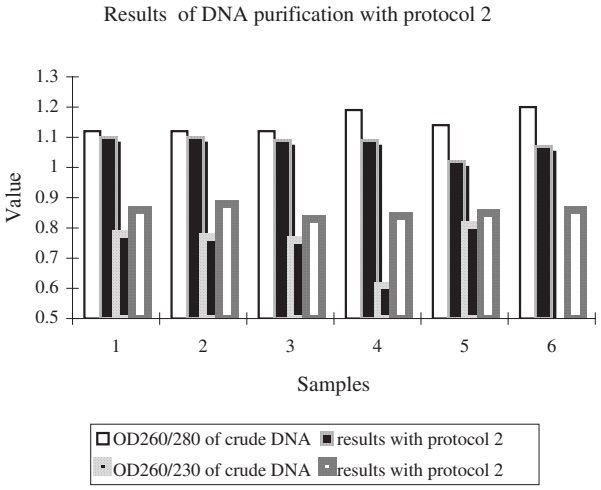

Fig. 4. The six samples selected at random from the Robinia pseudoacacia L. and pines community (facing the sun).

DNA recovery after agarose gel purification. DNA recovery varied with different purification methods. Higher recovery was obtained with improved gel purification than with minicolumn protocol. The recovery following gel purification was similar among different samples from the same community, while the recovery varied greatly (Fig. 5) within the nine different communities ($F(8, 45) = 5.97722$, $P = 0.00003 < 0.01$).

Discussion

In the initial stage of this study, various methods for extracting DNA from soil were considered after referring to the literature. The methods of Moré et al. (1994) and Yang (2000) failed in this study. The methods of Tsai and Olson (1991), Erb and Wagner-Döbler (1993), and Jae-Chang Cho et al. (1996) were combined and largely modified to produce a new

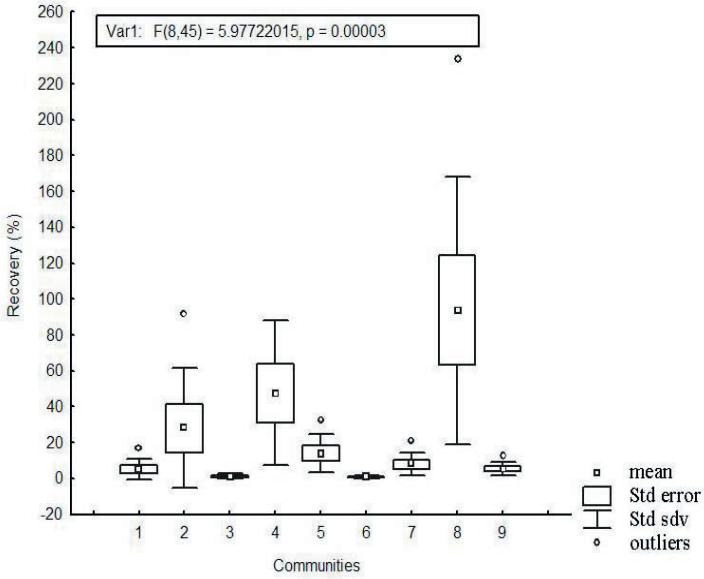

Fig. 5. Refer to Fig. 3. for definitions of community types.

method, which increased purity and convenience, while decreasing the cost. Importantly, we focused on soil that was difficult to extract DNA from as indicated by the low mean values of TN, TP and high humus content. No other study has attempted to extract DNA from this kind of soil.

Most previous studies used approximately 1 g of soil for DNA extraction. However, in this study the bacterial number in the restoration area was low. The yield of DNA might be severely decreased due to minor technical problems in sample handling. Therefore, 5 g of soil were used for extraction and four freezing-thawing cycles were employed for releasing DNA from cells. The incubation time required for lysozyme and RNase treatments was also shortened (from 6 to 4 hours). Previous studies have attempted to increase DNA yields from soil by using severe physical treatments such as mechanical grinding with beads and sonication to lyse indigenous microbial cells. Such treatments can shear DNA to sizes of 5 to 10 kb or less (Liesack, Stackebrandt, 1992; Ogram et al., 1987), and in at least one study, the average fragment size was 100 to 500 bp (Simonet et al., 1991). In our study, we completely omitted such procedures and obtained high yields of high-molecular-weight DNA from the soil.

During the steps of DNA purification, cutting DNA bands from gels was better than column purification. The band at about 19kb was useful for molecular studies such as PCR, while the other bands were not. The methodology used in this work was for a specific environment. It was applied to soil of the Yellow River Delta, a well-known wetland many times,

with no successful DNA extraction. Importantly, we found that the high salt buffer was not deleterious to microorganisms in the Delta because they have already been conditioned to an environment where the salt content (3%) was higher than other habitats.

Most ecosystems are heterogeneous and in many systems spatial heterogeneity is positively correlated with microhabitat and species richness (Brown, 2003). In this study, 54 samples were randomly selected from nine different communities in this restoration area. The soil TN and TP among these different communities were significantly different. In other words, we obtained our results from macroscopical studies, which showed that there were a number of microhabitats, indicating the area could and would accommodate high biodiversity embodied in species richness. In the same experiment, we simultaneously obtained DNA from microorganisms for further study, which is more important for modern ecological developments. There were also significant differences among the recoveries of DNA from different communities ($F(8, 45) = 5.97722$, $P < 0.001$). The same methods were used for DNA recovery in all communities, so the differences were due to different microhabitats, which were also revealed by significantly different soil chemical properties. Normally determining the chemical properties of soil is very boring and fussy. Therefore the determination could be omitted and DNA recovery could be used as an index for indigenous microhabitats and biodiversity. The results of significantly different DNA recovery clearly showed that there was a great spatial heterogeneity in Fanggan mountainous area. This area could and would accommodate high biodiversity, which will be very significant for the success of restoration of these typical examples of warm temperate ecosystems in China.

All of the protocols described here could be completed within 4 hours, yielding good results within the time taken by conventional techniques. The SDS-based freezing-thawing and improved gel purification procedure was particularly suited to the study of microorganisms in challenging soils. It provides a short cut for extracting DNA from challenging soils. In this study, we tested two methods of purification (improved band cutting and commercial kits), but they represented two aspects of purification methods in present study field, so they were meaningful in practice also. Particularly we put forward the recovery index and linked microcosmic methods with macroscopical results to illustrate ecological problems, expanding the new foreground of ecological study. Fanggan country demonstrated high spatial heterogeneity and thus, should be able to accommodate high biodiversity, which could direct the restoration process and was very meaningful for environment projects.

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