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COMPARISON OF METHODS FOR TOTAL COMMUNITY DNA EXTRACTION FROM TROPICAL PEATLAND

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SUMMARY

Soil DNA extraction is usually complicated by the co-purified contaminants especially humic substances, which subsequently inhibit post-extraction analyses such as PCR-DGGE. Conventional methods employing sodium dodecyl sulphate (SDS) and lysozyme-based treatment yield better DNA quantity but higher amount of humic substances are also being co-extracted together with the total DNA. Nucleospin (Macherey-Nagel, Germany) obtained PCR-amplifiable DNA with purity of A_{260nm}/A_{230nm} ratio of 1.70 and humic acid level measuring at 0.0041 (A_{320nm}). PCR amplification was also improved by using skimmed milk and *Pfu* DNA polymerase.

KEY WORDS: Tropical peat soil, DNA extraction, humic substances

INTRODUCTION

Microorganisms are the main constituent of the soil biota (Schimel, 2007). The rapid development of molecular techniques has allowed direct access to microbial diversity in the soil via direct extraction of soil DNA. Various DNA extraction techniques have been developed or modified for soil studies with SDS being the common extractant (Tsai and Olson, 1992; Zhou *et al*., 1996; Yeates *et al*., 1998; Frostegard *et al*., 1999; Sharma *et al*., 2007; Pang *et al*., 2008). Variations in DNA extraction methods can significantly influence the findings on microbial diversity and community structure (Thakuria *et al*., 2008).

DNA extraction from soil is usually complicated by the presence of organic matter and clay particles that can bind to nucleic acids and inhibit their purification (Moran *et al*., 1993). The humic substances in the organic matter comprise of both humic and fulvic acids that will inhibit post-extraction enzymatic analyses, i.e. PCR amplification (Tebbe & Vahjen, 1993) and restriction endonucleases (Wilson, 1997). Humic substances can produce two types of inhibition; inhibition of DNA polymerase enzymatic activity and inhibition of the DNA template due to the binding of humic substances to the template (Opel *et al*., 2010).

Various methods have been developed to remove humic substances from soil DNA, including the use of cesium chloride density centrifugation (Leff *et al*., 1995), aluminium sulphate (Dong *et al*., 2006), hexadecyltrimethylammonium or CTAB (Cho *et al*., 1996), polyvinylpolypyrrolidone or PVPP (Frostegard *et al*., 1999), gel electrophoresis (Zhou *et al*., 1995) and Sephadex G-200 column (Miller *et al*., 1999). To reduce inhibition problems of humic substances include the use of NaOH treatment (Bourke, 1999), Bovine Serum Albumin (BSA) (Yang *et al*., 2007), skimmed milk (Arbeli & Fuentes, 2007) and *Pfu* DNA polymerase (Matheson *et al*., 2010). Most of these methods are time-consuming or expensive, and also result in a decrease in DNA recovery and can even eliminate some of the DNA templates (e.g. microbes found at low levels of abundance) (Zhou *et al*., 1995; Kuske *et al*., 1998; Dong *et al*., 2006).

Thakuria *et al*. (2008) reported that contamination of soil DNA extracts with PCR inhibitory substances is highly dependent on soil types. Tropical peat soils contain more than 65% soil organic matter, with significantly higher content of humic substances in comparison to mineral soils. This creates the great need to develop suitable DNA extraction protocol that can successfully overcome the issue of interferences by humic substances to PCR-based microbial community analysis.

MATERIAL AND METHODS

Soil sampling

Peat soil samples from an oil palm plantation in Sibu, Sarawak, Malaysia (02°12’N 111°50’E) were taken using a peat auger. By using a sterile spoon, approximately 50 g of the peat soil samples were placed in the polyethylene bags with fasteners. The bags were immediately placed in a cooler box packed with ice to be transported back to the laboratory and stored at -20°C.

DNA extraction

In this study, the four (4) different methods used to extract total soil DNA were SDS-based:

1. *Conventional method 1*
   Soil (2 g) was suspended in 3 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 25% SDS and lysozyme) and vigorously shaken for 30 min before incubation at 37°C for 1 h. The samples were then further incubated with 30 µl proteinase K (10 $mgml^{-1}$) at 60°C for 1 h. Then 500 µl of phenol:chloroform (25:24) was added to each sample. The samples were then shaken until a milky emulsion was formed. After centrifugation at 19,280 g for 10 min, 300 µl of supernatant from each sample was transferred into a new tube with 300 µl chloroform:isoamyl alcohol (24:1) added before shaking until a milky emulsion was produced. The mixture was again centrifuged at 19,280 g for 10 min prior to transferring 200 µl into a new tube. One tenth volume of 3 M Potassium Acetate (KAc) and 400 µl of absolute ethanol were added to the samples. Samples were inverted slowly and left to stand at room temperature for 45 min before being centrifuged at 19,280 g for 15 min to produce DNA pellets. These pellets were washed with cold 70% ethanol, dried at room temperature and dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

2. *Conventional method 2*
   Freeze-crushed soil (2 g) was suspended in 5 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0 and 1.5 M NaCl) and 0.1 ml lysozyme (10 $mgml^{-1}$). Each sample was incubated at 37°C for 1 h and further incubated at 65°C with 0.2 ml SDS (20 % w/v) and 1.5 µl proteinase K (20 $mgml^{-1}$) for 2 h shaking at 15 min intervals. The samples were then centrifuged at 8,000 g for 10 min to remove soil residues. The supernatant from each sample was transferred into a clean tube with 1 ml extraction buffer and 0.1 ml SDS added. Each sample was again incubated at 65°C for 10 min before centrifugation at 8,000 g for 10 min. Equal volume of phenol:chloroform (25:24) were added to the supernatant and the samples were centrifuged again at 8,000 g for 10 min. The aqueous layer from each sample was separated and equal volume of chloroform:isoamyl alcohol (24:1) was added. After centrifugation at 8,000 g for 10 min, 1 volume of isopropanol was added followed by incubation at room temperature for 1 h. To pellet DNA, samples were centrifuged at 8,000 g for 30 min and washed with cold 70% ethanol. These DNA pellets were dried at room temperature and dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

3. *ISOIL Large for Beads ver.2 DNA Isolation Kit*
   Extraction was carried out according to the manufacturer's instructions (Nippon Gene, Japan).

4. *NucleoSpin Soil Nucleic Acid and Protein Purification Kit*
   Extraction was carried out according to the manufacturer's instructions (Macherey-Nagel, Germany).

PCR amplification

PCR amplification was performed by adding 3 µl soil DNA extract to a PCR tube pre-filled with 3 µl of PCR Buffer A (10 x buffer, Vivantis), 3 µl $MgCl_2$ (50 mM), 0.6 µl dNTPs (10

mM), 0.5 µl of *Pfu* DNA polymerase (5 unitsμl^{-1}, Vivantis), 0.3 µl of each 20 mM primer (341F and 907R), 0.3% (w/v) dry skimmed milk (Nestlé, Switzerland) and distilled deionized water up to 30 µl of the total volume. Stock solution of 30% (w/v) skimmed milk was prepared in sterile distilled deionized water. Hot-start PCR amplifications with *Pfu* DNA polymerase involved initial denaturing at 95°C for 2 min followed by 30 cycles of 95°C for 1 min, 56°C for 30 sec and 72°C for 40 sec, and a final extension at 72°C for 5 min. Amplifications were performed with a MyCycler (Biorad, USA).

Agarose gel electrophoresis

DNA was visualized by electrophoresis with 5 µl aliquots of extracts through 1% (w/v) agarose gels in 1 x TAE for PCR product, stained in 1 $\mu g ml^{-1}$ ethidium bromide bath. Gels were visualized and images captured using Molecular Imager® GelDoc^{TM} XR System (BioRad, USA).

Spectrophotometry

Absorption spectrum of DNA extracts at 230 to 320 nm was determined using Lambda 25 spectrophotometer (Perkin Elmer, USA) according to the manufacturer's instructions. The DNA was quantified based on the absorption at 260 nm and expressed as $ng\mu l^{-1}$. A decrease in absorption ratios at 260/230 and 260/280 nm was used as indicator of humic acid, polysaccharide and protein impurities (Zipper *et al*., 2003; Thakuria *et al*., 2008).

RESULTS AND DISCUSSION

Comparison of DNA quantity and quality extracted from the four methods are shown in Table 1. Both conventional extraction methods 1 and 2 yielded higher concentration of DNA (303.60 and 722.40 $ng\mu l^{-1}$) respectively compared to extraction by ISOIL (58.95 $ng\mu l^{-1}$) and Nucleospin Kit (93.60 $ng\mu l^{-1}$). But when the DNA extracts were run on 1% w/v agarose gel, both conventional methods 1 and 2 did not show higher DNA concentration as compared to the ISOIL and Nucleospin Kit as shown in Fig. 1. This is because RNA was co-purified with total DNA as both the conventional methods used were without RNAse treatment. Spectrophotometric measurements also do not differentiate between DNA and RNA. Therefore, in this study RNA contamination can lead to an over-estimation of DNA concentration in which the presence of RNA would also inhibit downstream application but not PCR.

Table 1. Effect of extraction method on DNA quantity and quality.

Method	DNA ($ng\mu l^{-1}$)	Humic acids (A_{320nm})	A_{260nm}/A_{280nm}	A_{260nm}/A_{230nm}	Successful PCR amplification
Conventional 1	303.60	0.0615	1.16	0.66	Nil
Conventional 2 (+Crushing)	722.40	0.2569	0.97	1.04	Nil
ISOIL Kit	58.95	0.0053	1.44	0.91	Nil
Nucleospin Kit	93.60	0.0041	1.77	1.70	Positive detection

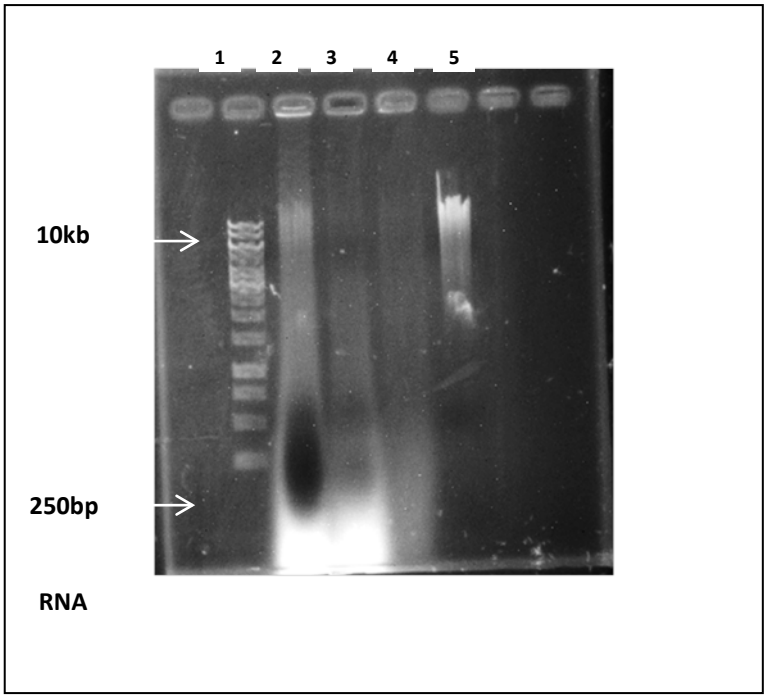

Fig. 1. Agarose gel electrophoresis (1% w/v) of soil genomic DNA extracted by different methods. Lane 1: 1kb molecular marker (1µg); lane 2: conventional method 1; lane 3: conventional method 2; lane 4: ISOIL Kit; lane 5: Nucleospin Kit.

To obtain a better representation of original microbial community structure, integrated methods (chemical and physical) with high DNA yield and phylogenetic diversity were used (Jiang *et al.*, 2011). Therefore, addition of a physical lysis step (i.e. freeze-crushing) to conventional method 2 was aimed to further lyse fungi and actinomycete cells which are more difficult to lyse than bacterial cells. However, it was not seen as a successful measure in increasing DNA concentration (Fig.1, lane 2). There were other studies that showed DNA yield was not correlated with bacterial diversity (Martin-Laurent *et al.*, 2001; Luna *et al.*, 2006; Jiang *et al.*, 2011).

Since all the four methods were SDS-based, the variation in the DNA yield from all the methods were reliant on the precipitation step of each method. Precipitation of DNA with potassium acetate and ethanol was used in conventional method 1, while isopropanol in coventional method 2. Precipitation of DNA was commonly achieved with isopropanol (Zhou *et al.*, 1996; Miller *et al.*, 1999; Martin-Laurent *et al.*, 2001) but at the same time, significant amount of humic substances were reported to be co-precipitated with DNA (Arbeli and Fuentes, 2007). Ethanol and polyethylene glycol are also used for precipitation of DNA. LaMontagne *et al.* (2002) reported that using 10% polyethylene glycol 8000 (PEG) instead of isopropanol resulted in fourfold reduction in humic substance content, without decreasing DNA yield.

In terms of DNA purity, extraction by Nucleospin Kit yielded the highest purity with A_{260nm}/A_{230nm} ratio of 1.70 showing lower contamination from impurities. The other three

methods showed the value in the range of 0.66 to 1.04. Ratio of less than 1.0 shows significant amount of impurities i.e. proteins and saccharides. Ratio of acceptable levels are from 1.5 to 2.0, with high purity expressed as more than 2.0. None of the four methods showed the A_{260nm}/A_{280nm} ratio between 1.8 to 1.9. The A_{260nm}/A_{280nm} ratio for Nucleospin kit was only 1.77 and the ratio of less than 1.8 indicated protein contamination.

Humic substances are one of the biggest problem encountered when extracting DNA from biological materials recovered from soil. Conventional method 2 with additional crushing yielded the highest amount of humic acid with A_{320nm} value of 0.2569 followed by conventional method 1 with A_{320nm} value of 0.0615. Both ISOIL and Nucleospin kits displayed low amount of humic acid with A_{320nm} reading of 0.0053 and 0.0041 respectively. Employment of SDS and lysozyme in the chemical treatment of soil samples were reported to co-extract the highest amount of humic substances (Jiang *et al*., 2011). Both precipitation by potassium acetate (conventional method 1) and isopropanol (conventional method 2) yielded highest amount of humic substances. Therefore, *Pfu* DNA polymerase was used in this study because *Pfu* DNA polymerase was previously found to be least affected by inhibition from humic and fulvic acids suggesting that there might be a greater tolerance in the DNA polymerases of this species (Matheson *et al*., 2010).

PCR amplification of bacterial 16S rRNA gene was used as a final evaluation test of the various extraction methods. All methods except extraction by Nucleospin Kit showed negative results for PCR amplification. PCR reactions also incorporated skimmed milk, previously reported to improve PCR amplification (Fujii *et al*.,, 2010). The mechanism by which skimmed milk improves PCR amplification is likely to be similar to BSA (Yang *et al*., 2007). Without the addition of skimmed milk and 5 mM $MgCl_2$, PCR amplification of the DNA obtained from Nucleospin Kit showed negative results. Magnesium chloride concentration should normally be between 1 mM and 4 mM but higher concentration (5 mM) of magnesium was used in this study to lower the fidelity of *Pfu* DNA polymerase. Generally, *Pfu* DNA polymerase will give lower yield of PCR product because of its high fidelity with proof-reading capabilities but it is used in this study because it is least affected by humic substances (Matheson *et al*., 2010).

CONCLUSION

Nucleospin (Macherey-Nagel, Germany) obtained PCR-amplifiable DNA with purity of A_{260nm}/A_{230nm} ratio of 1.70 and humic acid level measuring at 0.0041 (A_{320nm}). PCR amplification was improved by using skimmed milk and *Pfu* DNA polymerase. However, further studies to improve purification of total DNA by employing polyethylene glycol (PEG) and sodium chloride (NaCl) as precipitation agent to substitute isopropanol are still required. This will enable optimization of the conventional method that can be used for total DNA extraction from tropical peat that is of better quality, cost effective and time-saving for soil microbial community structure analyses.

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