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Research article

Assessing DNA extraction methods for metagenomic analysis from crop soil in Thailand

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Abstract

Importance of the work: Metagenomic analysis has been used to study environmental microbial diversity. However, isolating soil DNA for metagenomic analysis can be problematic.

Objectives: To evaluate six methods for extracting DNA from soil growing major crops in Thailand.

Materials & Methods: DNA from soil growing rice, maize, oil palm or cassava was manually extracted and analyzed using agarose gel electrophoresis and spectroscopy. The presence of polymerase chain reaction (PCR)-inhibiting contaminants was assessed based on 16s rRNA amplification. Then, agarose gel purification was evaluated for its efficiency in removing the impurities.

Results: The Tanveer and Gupta D protocols yielded the highest amount of DNA on average, while the Gupta C and Gupta E protocols produced clear genomic DNA bands. However, all DNA samples were contaminated with PCR inhibitors. Therefore, additional purification was needed. Agarose gel purification was performed only on DNA from the Gupta C and Gupta E protocols, with genomic bands detected. The purification removed sufficient contaminants with an average recovery rate of 50%, with Gupta C having a higher recovery rate variation. The statistical analyses applied to the concentration and absorbance ratios of purified DNA showed that the Gupta E protocol was suitable for soil growing oil palm and maize, whereas the Gupta C protocol was appropriate for soil growing rice and cassava.

Main finding: An appropriate DNA extraction method with an additional purification process should be selected for different soil types to obtain qualified samples for metagenome analysis.

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Introduction

Microorganisms can be very diverse in the environment and play critical roles in the ecosystem (Schulz et al., 2013). In recent years, there have been substantial changes in approaches to studying soil microbial communities, with new methods and techniques becoming available for soil microbiologists to gain access to more of the microorganisms in the soil, allowing for better assessment of the microbial community (Hill et al., 2000). Metagenomic analysis is a culture-independent method that utilizes recently developed next-generation sequencing and bioinformatics tools (Nakamura et al., 2016). However, the extraction of DNA for metagenomic analysis is often a limiting step in identifying microbes directly from the environment. Extracting DNA from soil often encounters problems, including incomplete cell lysis, DNA sorption to soil surfaces, loss of DNA, damage of DNA and the presence of contaminants (Miller et al., 1999). Common contaminants from the soil are humic acid and co-purified contaminants, which can inhibit DNA amplification by interfering with the function of *Taq* DNA polymerase, an enzyme critical for polymerase chain reaction (PCR; Yeates et al., 1998; Ning et al., 2009). The agarose gel purification techniques can eliminate these contaminants from DNA samples (Miller et al., 1999). Nowadays, many methods are available for extracting DNA from soil, including commercially available soil DNA extraction kits (such as DNeasy Powersoil Pro Kits [Qiagen] and Soil DNA Isolation Kit [Norgen Biotek]) and various manual DNA extraction methods (such as methods used in Tanveer et al., 2016; Gupta et al., 2017; Osman et al., 2017; Verma et al., 2017) that differ in the chemicals used and the incubation steps. Manual DNA extraction methods have yielded higher levels of nucleic acid concentration and purity than DNA extraction kits (Tanase et al., 2015). However, the chosen manual methods also depend on the type of soil in the experiment (Tanveer et al., 2016; Gupta et al., 2017; Osman et al., 2017; Verma et al., 2017).

Therefore, the current study aimed to evaluate and identify appropriate methods for extracting DNA for soil metagenomic analysis from agricultural fields growing major crops in Thailand: *Oryza sativa* (rice), *Zea mays* (maize), *Elaeis guineensis* (oil palm) and *Manihot esculenta* (cassava). Altogether, this study would provide a guide for selecting an appropriate DNA extraction and purification method for preparing Thailand's primary crop soil DNA samples for metagenomic analysis.

Materials and Methods

Soil sample collection

Soil samples were collected from four types of agricultural fields growing *O. sativa* (Os1 and Os2) and *Z. mays* (Zm1 and Zm2) from northern Thailand, as well as *E. guineensis* (Eg1, Eg2 and Eg3) and *M. esculenta* (Me1, Me2 and Me3) from central Thailand. The soil samples were collected from the plant rhizosphere at a depth of 5–10 cm. Samples (each approximately 20 g) were collected from three locations in each field before mixing and pooling. All the soil samples were transferred to the laboratory and kept at -80 °C.

Soil chemical analysis

The chemical properties of all soil samples were analyzed: soil pH, electrical conductivity and the soil organic and soil nitrogen percentages, as described in Beck (1999).

Extraction of metagenomic DNA

The study selected six published methods for DNA extraction: Tanveer (Tanveer et al., 2016), Gupta C, Gupta D, Gupta E (Gupta et al., 2017), Verma (Verma et al., 2017) and Osman (Osman et al., 2017). These protocols were modified by using 1 g of soil, instead of 5 g for practical purposes and the amount of buffer used was scaled, accordingly. DNA was resuspended in 50 µLTris-EDTA buffer.

Quality and quantity determination of DNA samples

To assess the quality of DNA using gel electrophoresis, 3 µL of soil DNA was resolved in 1% (weight per volume) agarose gel before being stained with ethidium bromide and visualized under ultraviolet light using the Alpha Innotech gel documentation system (BIO-RAD). A260, A260/A280 and A260/A230 values indicating the DNA concentration and contamination were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Elimination of polymerase chain reaction contaminant using gel purification

Samples (200 ng) of crude DNA extracted from each protocol (unless otherwise noted) were separated from contaminants in

0.7% low melting agarose gel. The genomic DNA band with a size larger than 10kb was excised and subjected to purification using a FavorPrep™ GEL/PCR Purification Kit (Favorgen Biotech Corp). The quality and quantity of purified DNA were measured using the spectrophotometer.

DNA polymerase chain reaction

Extracted DNA was assessed for the presence of PCR inhibitors by amplifying partial 16s rRNA genes using the primers Bac27F (5'-AGAGTTTGATCMTGGCTCAG-3') and Univ1492R (5'-GGYTACCTTGTTACGACTT-3'). The positive control used the genomic DNA of the *Escherichia coli* strain DH5alpha. The predicted product size was 1,500 bp (Gupta et al., 2017). The PCR product was analyzed using agarose gel electrophoresis.

Statistical analysis

The Kruskal-Wallis rank sum test and the pairwise Wilcoxon rank sum test were applied to examine whether different extraction protocols provided a significantly different amount of non-purified DNA for each agricultural soil type. All statistical analyses were done using the R software package (R Core Team, 2013) version 4.1.0. The linear mixed model and analysis of variance (ANOVA) were applied to identify any significant factors determining DNA quality. The linear mixed model defined the protocol (P_i), the type of agricultural soil (S_j) and their interaction ($P_i \times S_j$) as fixed effects. The biological and technical repeat measurements were defined as random effects (a_{ij}). The model in which Y_{ij}, μ and ε represented the measurements, the overall mean and residue, respectively, is shown in Equation 1. Type III ANOVA with Satterthwaite's method was applied to identify which fixed effects were significant factors. Finally, Student's t test was applied to evaluate whether the quality measurement was significantly different from the expected value (1.8 for both the absorbance ratios) and the measurement differences caused by the factor were statistically significant.

$$Y_{ij} = \mu + P_i + S_j + \ + (P_i \times S_j) + a_i + \varepsilon \quad (1)$$

Results and Discussion

Quality and quantity assessment of unpurified DNA samples

Because the soil samples used in this work were from fields growing four different plant species with varying preferences of soil and agricultural practices, six extraction methods were selected, namely Tanveer (Tanveer et al., 2016), Gupta C, Gupta D, Gupta E (Gupta et al., 2017), Osman (Osman et al., 2017) and Verma (Verma et al., 2017). From each publication, the method yielding the best quality and quantity of extracted DNA was chosen. Except for Gupta et al. (2017), three protocols were chosen that provided similar quality and quantity values of the extracted DNA. These selected protocols differed in pre-extraction, extraction buffer used, methods for cell lysis, DNA purification and DNA precipitation steps; therefore, they would have different levels of efficiency in extracting DNA from the various crop soils included in this study. This study aimed to provide a guide for selecting an appropriate protocol for a specific crop soil.

The four crops selected for this work (rice, maize, oil palm and cassava) were grown in different types of soil (clay, silt, peat and sandy soil, respectively). Basic chemical properties of these soil samples were measured, consisting of: organic matter percentage, nitrogen content percentage, pH and electroconductivity (Table S1). Notably, there were no significant differences in these properties among the four groups of crop soil. However, there was a large fluctuation in these properties among the soil samples collected from oil palm plantation, indicating high variability in soil that might affect DNA isolation efficiency.

After extracting DNA using the six different protocols, the average crude DNA yields were calculated based on the total volume of 50 µL obtained per 1 g of soil. The results showed that Gupta D provided the significantly highest yield of DNA extracted from the crop soil of *E. guineensis* and *Z. mays*, while Gupta D and Tanveer provided the highest amount of DNA extracted from the crop soil of *M. esculenta* and *O. sativa*. In addition, Tanveer and Gupta D gave, on average, the highest yield of DNA extracted (7.77–29.55 µg/g soil), followed by Gupta C and Gupta E (2.53–4.98 µg/g soil) and Verma and Osman (0.02–0.82 µg/g soil), respectively (Table 1). These yields were within the range observed among other reports: 0.2–2.5 µg/g of soil used for growing maize (Laurent et al., 2001), 48.6 µg/g of wet compost (Howeler et al., 2003), 2.0–13.0 µg/g of Irish soil (Carrigg et al., 2007) and 7.9–33.8 µg/g of soil from cornfields and forests (Schneegurt et al., 2003).

Table 1 Average yield (± SD; µg/g soil) of unpurified DNA obtained from selected protocols

Method	O. sativa	Z. mays	E. guineensis	M. esculenta
Gupta C	3.50±0.63^{c}	3.25±0.03^{d}	2.97±0.45^{c}	2.53±0.51^{b}
Gupta D	9.70±6.79^{a,b}	21.55±3.61^{a}	29.55±5.03^{a}	26.00±3.67^{a}
Gupta E	4.98±2.23^{b,c}	4.55±0.92^{c}	2.78±0.62^{c}	2.58±0.54^{b}
Osman	0.22±0.03^{d}	0.82±0.18^{e}	0.22±0.05^{d}	0.22±0.08^{c}
Tanveer	12.12±5.03^{a}	11.07±3.64^{b}	7.77±1.32^{b}	19.70±20.82^{a}
Verma	0.04±0.01^{e}	0.02±0.00^{f}	0.06±0.04^{e}	0.09±0.01^{d}

Mean±SD in the same column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

As Tanveer and Gupta C used similar extractions and cell lysis buffers but had different yields, the high yield of Tanveer was likely due to pre-extraction incubation with skimmed milk, shown previously to increase the efficiency of soil DNA extraction, potentially by blocking the DNA adsorption capacity of clay particles (Takada-Hoshino and Matsumoto, 2004; Ugawa et al., 2012). Compared to Gupta E, Gupta D used Cetyltrimethyl ammonium bromide (CTAB) with 5% Sodium dodecyl sulfate (SDS) for extraction and isopropanol instead of Polyethylene glycol (PEG) for precipitation. In addition, Gupta D differed from Osman in the SDS concentration used. As CTAB itself could not increase the yield of DNA extracted from soil (Zhou et al., 1996) but high concentration of SDS did (Natarajan et al., 2016), the high DNA yield of Gupta D was likely the effects of SDS itself as an anionic detergent, or in combination with CTAB (a cationic detergent) that could help release DNA from charged clay particles (Dias et al., 2004). In addition, PEG was previously reported to reduce the DNA yield compared to isopropanol, despite its high efficiency in removing humic acid (Blanc et al., 1999; LaMontagne et al., 2002). In this case, if the subsequent purification to remove humic acid had been performed, it might be better to use isopropanol to achieve a higher yield of crude DNA.

Only the Gupta C and Gupta E protocols consistently extracted intact genomic DNA from the various types of soil (Fig. 1). The differences between these protocols to Gupta D and Tanveer, which yielded the highest DNA concentration in all soil types, were using 0.3% SDS without CTAB for lysing the cells and using phenol/chloroform/isoamylalcohol (PCI) instead of phenol for purification step. However, as CTAB and SDS were more likely to relate to releasing DNA from clay particles in soil as described above, the key to maintaining the genomic integrity might be using PCI for purification, since the presence of chloroform could reduce the interphase and efficiently eliminate contaminants. As soil contaminants, including phenolic compounds and humic acid, could interfere with A260 absorbance, the DNA concentration measured using spectroscopy might not accurately reflect the amount of DNA extracted. In addition, contaminating enzymes within the soil sample, if not efficiently removed, could degrade genomic DNA, leading to the lack of an intact genomic DNA band in samples extracted using the Gupta D and Tanveer protocols.

Because all the DNA samples failed to produce any PCR products when used as templates for 16s *rRNA* gene amplification (Fig. 2A), these samples must have been contaminated with PCR inhibitors, indicating that a purification step was required for preparing DNA samples for metagenomic library construction.

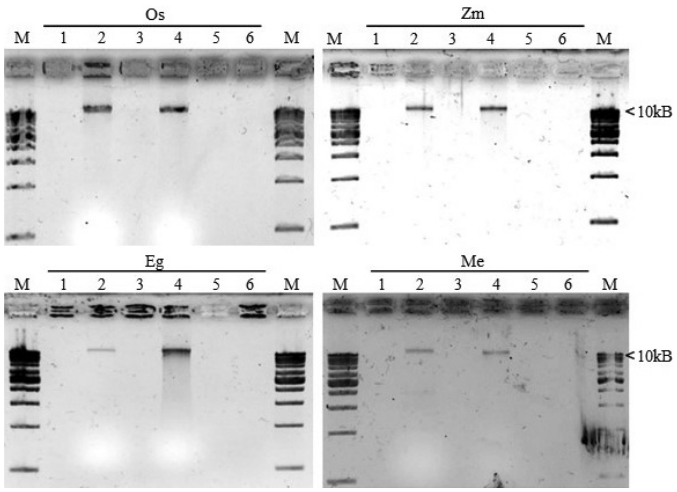

Fig. 1 Agarose gel electrophoresis of genomic DNA before purification, where lanes 1–6 represent DNA extracted using protocols of Tanveer, Gupta C, Gupta D, Gupta E, Verma and Osmen, respectively, M = size marker, with total amount of 200 ng loaded into each well, except for lanes 5 and 6 that had 7 ng and 40 ng, respectively, (Os); lane 5 had 149 ng (Zm); lane 3 had 36 ng (Eg); and lane 14 had 61 ng (Me) loaded due to low concentration of DNA obtained

Elimination of contaminants using gel purification

Several purification methods have been reported, including the Sephadex G-200 column, SpinBind column and agarose gel purification (Miller et al., 1999). Despite lower efficiency than other methods (Miller et al., 1999), agarose gel purification was selected based on its low cost and wide availability in molecular laboratories. Because only Gupta C and E could provide intact genomic DNA (Fig. 1), the purification step was performed on detectable bands obtained from these methods. The other methods were not analyzed further due to the lack of an intact genomic band on agarose gel, which is a crucial requirement for gel purification. The partial 16s *rRNA* gene was amplified from the purified DNA samples to confirm that the purification step could effectively remove PCR inhibitors. Strong visual bands of PCR products with a size of 1,500 bp were detected (Fig. 2B) despite using a much lower amount of DNA template (as low as 1 ng) compared to the PCR products shown in Fig. 2A (with 40 ng DNA template). The PCR inhibitor eliminated in this process was likely humic acid, an abundant contaminant in soil with similar solubility to DNA that make it hard to remove during the extraction process (Moreira, 1998). Thus, the separation of this contaminant from genomic DNA in agarose gel results in purified DNA free of PCR contaminants (Miller et al., 1999). Successful PCR amplification here confirmed the necessity of and efficiency in eliminating PCR inhibitors using agarose gel purification.

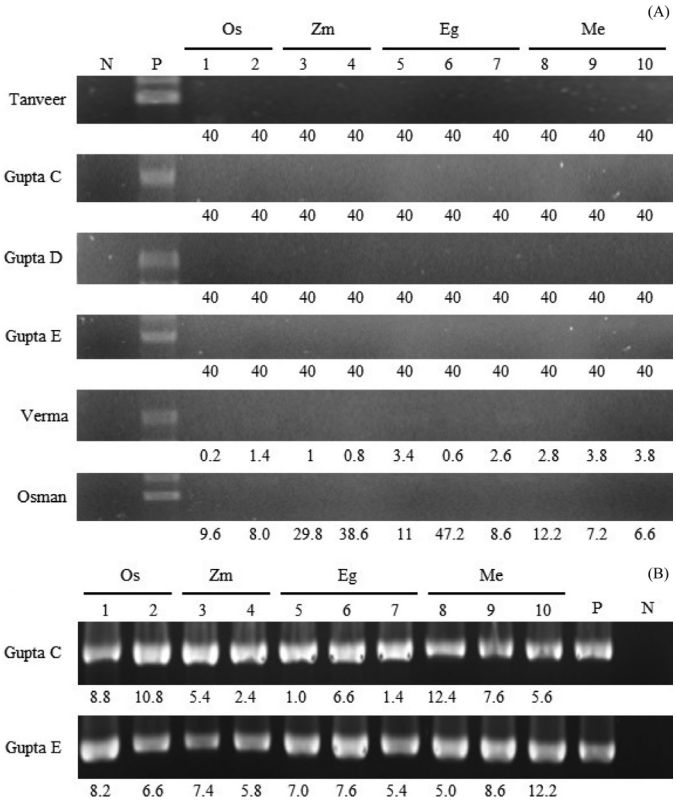

Fig. 2 Agarose gel electrophoresis of polymerase chain reaction (PCR) amplification of *16S rRNA* gene: (A) non-purified DNA; (B) purified DNA, where lanes 1–10 represent PCR products using DNA from Os1, Os2, Zm1, Zm2, Eg1, Eg2, Eg3, Me1, Me2 and Me3 as templates, lane P represents positive control and lane N represents negative control using water as template and numbers at bottom of gel specify amounts in nanograms of DNA used as template for each PCR reaction

Quantity assessment on the quality of purified DNA samples

The concentration, A260/280 and A260/230 ratios of purified DNA samples were measured using spectrophotometry. The linear mixed model and ANOVA were applied to identify factors influencing these values (Table 2). The ANOVA p values suggested that the type of crop soils (Sj) significantly affected these three measurements. In addition, an appropriate protocol for a specific crop soil would be required for retrieving a higher concentration and a better A260/A280 ratio.

Table 2 Analysis of variance performed on fitted linear mixed model to examine influence of fixed effect on purified DNA quality measurements of concentration, A260/A280 ratio and A260/A230 ratio

		Sum of squares	Mean square	Degrees of freedom	F value	*p* value
Purified DNA concentration	Protocol	3.422	3.422	1	2.825	0.099
	Sample	49.053	16.351	3	13.499	< 0.001
	Protocol×Sample	20.344	6.781	3	5.598	0.002
A260/280	Protocol	0.003	0.003	1	3.920	0.053
	Sample	0.020	0.007	3	7.401	< 0.001
	Protocol×Sample	0.020	0.007	3	7.372	< 0.001
A260/A230	Protocol	0.001	0.001	1	0.592	0.446
	Sample	0.065	0.02	3	10.265	< 0.001
	Protocol×Sample	0.008	0.003	3	1.323	0.278

The tests were considered significant at $p < 0.05$.

Because the interaction between the protocol and the type of agricultural soil ($P_i \times S_j$) significantly affected the concentration and A260/A280 ratio (Table 2), the concentration and absorbance ratios obtained from the Gupta C and Gupta E methods were compared for each type of crop soil (Fig. 3). Regarding the DNA concentration, soil growing *M. esculenta* (cassava) yielded approximately 4 ng/µL of purified DNA, regardless of which protocol was used. Therefore, either protocol (Gupta C or Gupta E) could be used for soil from growing cassava in this aspect. On the contrary, Gupta E provided a significantly higher concentration of purified DNA from soil growing *E. guinnensis* (oil palm) and *Z. mays* (maize). In comparison, Gupta C yielded a significantly higher concentration when used to extract DNA from soil growing *O. sativa* (rice). Therefore, these results supported the ANOVA results (Table 2) and suggested that a specific type of crop soil should be extracted using a suitable protocol, to achieve the highest amount of DNA sample.

Absorbance ratios can be used to evaluate the contamination of proteins, other organic matters or phenolic compounds. For protein contamination, the comparison of A260/A280 ratios showed no significant difference between purified DNA obtained from Gupta C and the ones obtained from Gupta E in the samples of soil growing *E. guinnensis, O. sativa* and *Z. mays*. However, this ratio of the DNA obtained from soil growing *M. esculenta* using Gupta C was significantly higher than that obtained from Gupta E. The comparison of the A260/230 ratios used to evaluate the organic matter and phenolic contamination showed no significant difference between the two protocols in every soil type. Notably, the A260/A280 ratios of all DNA samples, except the cassava soil DNA provided by Gupta C, were significantly lower than 1.8. This low A260/A280 ratio suggested that the DNA sample may contain proteins; however, because the values were around 1.7, the degree of impurity would not be high. On the contrary, the A260/A230 ratio of every DNA sample was significantly higher than 1.8, suggesting low amounts of other organic matter and phenolic contaminants in purified DNA. Comparing these absorbance ratios to the values measured from crude DNA extract, these values of the purified DNA were higher in all samples (data not shown). Therefore, these results supported the ANOVA results (Table 2) and suggested that different protocols slightly affected the A260/A280 ratio. The results also confirmed that agarose gel purification could eliminate the contaminants that might act as PCR inhibitors.

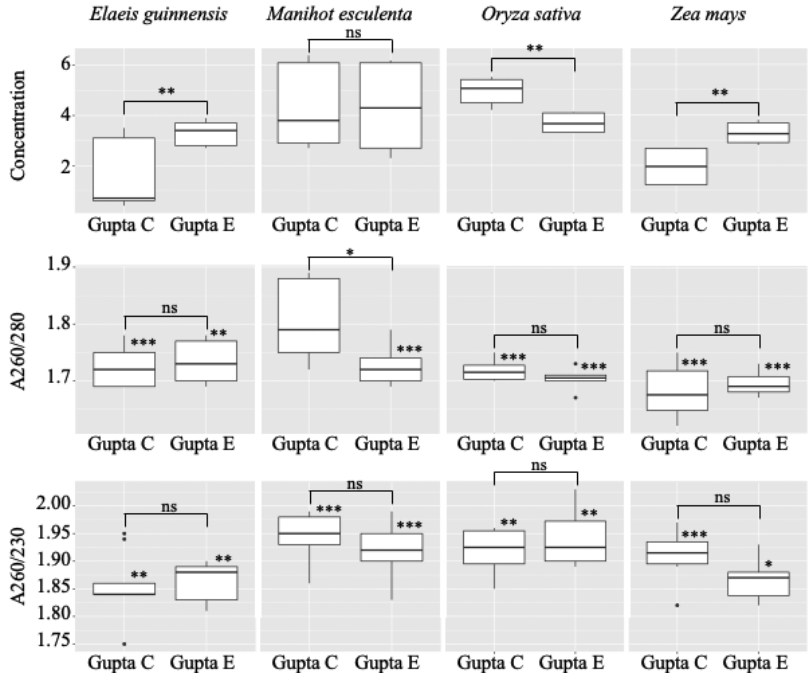

Fig. 3 Comparison of DNA quality levels obtained from Gupta C to those obtained from Gupta E: (A) concentration; (B) A260/A280 ratio; (C) A260/A230 ratio, where results are shown as box and whisker plots, the two-sample t test (Welch two-sample t test) results are shown above the top square brackets for every measurement, the one-sample t test results showing differences between mean and standard value of 1.8 are shown only for the two absorbance ratios, ns = not significant ($p > 0.05$) difference, *, ** and *** denote significant differences for $p < 0.05$, < 0.01 and < 0.001, respectively, and black dots denote outliers in measurements

Taking all three ratio parameters into account (concentration, A260/A280 and A260/A230) Gupta C (Fig. 3) should be used for extracting DNA from soil growing *M. esculenta* and *O. sativa*, whereas Gupta E should be used for extracting DNA from soil growing *E. guinnensis* and *Z. mays*. Since there was no difference in terms of the DNA concentration obtained between the two protocols for cassava soil, Gupta C was selected based on the higher DNA purity indicated by the significantly higher A260/A280 ratio. The selection of Gupta C for rice soil and Gupta E for both oil palm soil and maize soil was mainly based on the significantly higher DNA concentrations.

It is noteworthy that despite no clear differences in the organic matter and nitrogen percentages, pH and electroconductivity among the four groups of crop soil (Table S1), the different types of soil had different suitable protocols for extraction. Even a large fluctuation in these properties among the oil palm soil samples did not show any correlation to the concentration of DNA extracted. It remained to be assessed which soil properties other than the four properties determined here affected the DNA extraction efficiency.

In addition, the percent recovery after the gel purification step was calculated. The total amount of purified DNA was calculated based on the 30 µL elution volume and the percentage recovery was based on the 200 ng of pre-purified DNA loaded in each well. The recovery rates varied depending on the protocol and soil type, with an average of 50%; however, the Gupta C protocol had a higher variation for this rate than Gupta E (Table S2). Therefore, Gupta E generally had higher stability in recovering the amount of extracted DNA than Gupta C.

In conclusion, this work demonstrated that an appropriate manual DNA extraction and an extra purification step using agarose gel purification could be used to prepare DNA from crop soil for metagenomic library construction. However, different DNA extraction methods were suitable for different soil samples. The DNA of soil growing *E. guinnensis* and *Z. mays* should be extracted using the Gupta E protocol, whereas soil growing *O. sativa* should be extracted using the Gupta C protocol to obtain purified DNA with high concentration. The DNA of soil growing *M. esculenta* should be extracted using the Gupta C protocol to get purified DNA with high concentration and low protein contamination. Despite providing sufficient amounts of DNA that can be used successfully for PCR amplification, these methods should be further assessed for their effectiveness in recovering microbiome from soil samples, as that aspect was beyond the scope of this paper. Because the current study focused on obtaining DNA samples from four major economic crops in Thailand (rice, maize, oil palm and cassava), the results should provide the first step in facilitating metagenomic analysis of crops soil in Thailand, which would help gain insight into the interactions among plants, agricultural practice and microbe, leading to devising best practice to increase crop yield.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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