



Evaluation of Some Potential Protocols to Extract DNA from Paddy Soil

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ABSTRACT

Isolation of DNA from environmental samples is a crucial step in microbial community analyses through molecular methods. The present study was conducted to evaluate a DNA extraction protocol from paddy soil with a comparison on quality, quantity and integrity of the isolated DNA and to determine the suitability of extracted DNA for downstream applications in microbial community analyses. Three protocols (*i.e.* PEG/NaCl, Mannitol/CTAB and Sodium Phosphate Buffer) used for the extraction of DNA from different types of soil were attempted on paddy soil. The quality and quantity of the extracted genomic DNA was quantified spectrophotometrically and integrity was checked by gel electrophoresis. The efficiency of DNA extraction by the three protocols was compared with a commercial soil DNA extraction kit (Norgen's Soil DNA Isolation Plus Kit). Further, quality of the extracted DNA for PCR amplification was assessed using universal primer pairs for bacteria and fungi. DNA extracted using PEG/NaCl method resulted in the highest DNA concentration, while the highest purity was recorded by the DNA extracted by Mannitol/CTAB method ($A_{260}/A_{280} = 1.61$ and $A_{260}/A_{230} = 1.15$). Expected PCR products targeting 16s rDNA and ITS regions were obtained from the DNA samples extracted by Mannitol/CTAB method. Therefore, Mannitol/CTAB method used in the present study is suitable to extract high-quality DNA from paddy soil for molecular microbial studies.

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INTRODUCTION

Soil is an essential non-renewable resource where essential functions needed for life on earth are carried out. Most of these functions depend on microorganisms that inhabit the soil. It is believed that a gram of soil may contain possibly thousands of different species (Knietzsch *et al.*, 2003). Among them, diverse microflora has been identified for its beneficial functions in the soil-plant system. They perform various catabolic activities and are involved in primary production, nutrient recycling, etc. In addition, their interactions with plant root systems play key roles in several other functions, such as decomposition of organic matter, nutrient balancing, and pathogen suppression (Dimitrov *et al.*, 2017). However, the fundamental understanding of the diversity and ecology of these microbial communities has been hampered due to the inability of growing most of the microbes under laboratory conditions (Philippot *et al.*, 2011).

In Sri Lanka, the highest extent of land (1,254,000 ha) is cultivated (both *Yala* and *Maha* season) with Paddy (Department of Census and Statistics, 2015). Hence, paddy soils represent the principal agricultural system in Sri Lanka. Fertile soil provides essential nutrients for crop growth and even more supports for the diverse and active microbial community. Therefore, knowledge of this microbial community structure in paddy soils can improve the understanding of soil processes and microbial functions in rice-based cropping system (Islam *et al.*, 2009).

Introduction of culture-independent techniques, such as analyses of microbial DNA, has upgraded environmental microbiology, resulting in novel information on unculturable microbial populations. Thus, the traditional taxonomy based morpho-physiological and biochemical studies have to be replaced by the DNA based phylogenetic studies of microorganisms (Delmont *et al.*, 2011). Therefore, different methods need to be tested to determine their effectiveness upon soil microbial assessments and developed user-friendly, standardized protocols with reduced time durations (Plassart *et al.*, 2012). As culture-independent approaches are more advantageous, numerous protocols have been developed to extract DNA from different soils in the world. However, the purity of the DNA extracted from the soil often depends on its composition and found unsatisfactory, especially in soils that are rich in humic compounds such as paddy soils. In this context, significant efforts need to be devoted to [optimize](#) soil DNA extraction protocols to obtain

targeted results. However, there is no information published yet on paddy soil metagenomics in Sri Lanka, despite the fact that it has been used as the prime tool in the paddy soil microbiological research. Thus, the present study was carried out to compare and evaluate direct DNA extraction protocols which can be used for metagenomics and other downstream molecular applications.

METHODOLOGY

Sample Collection

Soil samples were collected from a paddy field located at Mihinthale, Anuradhapura, Sri Lanka in September 2019. The sampling was done by collecting soils from nine randomly selected points within the field at 0–10 cm depth using a 1.25 cm diameter soil core. Samples from the field were then combined to form one composite sample and stored at -20 °C until used in analyses. Different soil parameters including pH, electrical conductivity (EC), total nitrogen (N), phosphorus (P), potassium (K), organic carbon (OC) and organic matter (OM) content of the sample were tested (Table 1).

Table 1. Selected properties of the tested paddy soil sample. The values are means of three replicates.

Soil parameter	Value*
pH	6.82±0.062
EC (ds/m)	0.946±0.910
N (g/kg)	0.99±0.034
P (ppm)	18.53±0.270
K (mg/kg)	180.95±4.760
OM (%)	1.70±0.046
OC (%)	0.99±0.027

Extraction of Soil DNA

Three different protocols were tested to extract total DNA from paddy soil and compared with commercially available DNA extraction kit. The protocols are described briefly as follows.

Mannitol/CTAB (Cetyl trimethyl ammonium bromide) method

An extraction protocol described by Fatima *et al.* (2014) was followed with slight modifications. A 1 g of the composite paddy soil sample was ground

with liquid nitrogen (LN₂) using 5 mL of 120 mM phosphate buffer saline at pH 7.4 and shaking at 150 rpm for 10 min at room temperature. The resulted in suspension was centrifuged at 7,000 rpm for 10 min. The pellet was rewashed using phosphate buffer saline and suspended in 10 mL of DNA extraction buffer containing 1 M Tris-HCl (pH 8.0), 5 M NaCl, 0.5 M EDTA (pH 8.0), 10% CTAB, 10% SDS, and 0.5 M mannitol. The suspension was incubated for 1 h at 65 °C with occasional stirring at 250 rpm. The supernatant was collected after centrifuging the suspension at 8000 rpm for 10 min at room temperature. Thereafter, it was extracted with an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) by centrifugation at 12,000 rpm for 10 min at room temperature. The aqueous fraction was taken and mixed with 1/10th volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 70 % chilled ethanol at 4 °C. Finally, the pellet was recovered by centrifugation at 12,000 rpm for 10 min at 65 °C and dissolved in 25 µL TE buffer (10 mM Tris, 1 mM EDTA pH 8) and stored at -20 °C for future use.

Polyethylene Glycol (PEG)/NaCl method

The extraction of DNA was performed using a protocol described in Avinash *et al.* (2016) with little modifications. A 1 g of composite paddy soil sample was mixed with 20 mL of extraction buffer (1 M NaCl, 1% PEG 8000 (w/v) at pH 9.2) and vortexed for 30 s. It was centrifuged at 5,000 rpm for 5 min at room temperature and the supernatant was discarded. A volume of 10 mL of extraction buffer was re-added to the pellet and centrifuged at 15,000 rpm for 20 min at room temperature. The received pellet was re-suspended in 500 µL of suspension buffer (10 mM Tris-HCl, 10% sucrose, 50 mM EDTA, 50 mM NaCl at pH 8.0). 20 µL of freshly prepared lysozyme (20 mg/mL) was added and incubated at room temperature for 45 min. Further, 6 µL of proteinase K was added and incubated at 55 °C for another 45 min. Next, 50 µL of 20% SDS was added and incubated with intermittent mixing at 65 °C for 45 min. The samples were centrifuged at 15,000 rpm for 5 min at 20 °C. A 200 µL of suspension buffer and 50 µL of 20% SDS were added. The samples were subjected to vortexing for 3 min and centrifuged at 15,000 rpm for 5 min at 20 °C. Then, 1/10th volume of 2.5 M potassium acetate (pH 8.0) was added and the tubes were centrifuged at lower (7,000 rpm) and higher speeds (15,000 rpm) respectively, at room temperature for 5 min. The supernatant was taken, an equal volume of isopropanol was added and then it was centrifuged at 15,000 rpm at 4 °C for 20 min. The DNA pellet was washed with 70%

ethanol and air-dried. The pellet was dissolved in 25 µL of TE buffer (pH 8.0) and stored at -20 °C.

Sodium Phosphate Buffer (SPB) method

DNA was extracted using the protocol described in Tsai and Olson (1991) with slight modifications. A 1 g of composite paddy soil was mixed with 2 ml of 120 mM Sodium Phosphate Buffer (SPB) (pH 8.0) and shaken on a platform shaker at 250 rpm for 15 min at room temperature. The samples were centrifuged at 8,000 rpm for 10 min and the supernatant was discarded. The pellet was washed with phosphate buffer. An aliquot of 2 mL of lysis solution I (0.15 M NaCl, 0.1 M disodium EDTA at pH 8.0) containing 20 mg/ml of lysozyme was added to the sample and incubated at 37 °C for 2 h with intermittent mixing. Then, lysis solution II (0.5 M Tris-HCl, 0.1 M NaCl, 10% SDS pH 8.0) was added and freeze-thawed the tubes three times at -20 °C and 65 °C respectively for the complete extraction of DNA from soil microbes. The samples were centrifuged at 8,000 rpm for 15 min, the supernatant was taken and equal volumes of chloroform: isoamyl alcohol (24:1) were added. The samples were centrifuged at 10,000 rpm for 5 min and the aqueous fraction was collected. To that, 0.6% v/v isopropanol was added and incubated at room temperature for 1 h. Then, centrifugation was done at 12,000 rpm for 20 min. The top layer was collected and washed with 70% ethanol and air-dried the pellet at room temperature. The pellet was re-suspended in 25 µL of TE buffer (pH 8.0) and stored at -20 °C.

DNA extraction kit (Norgen's kit)

Commercially available Norgen's Soil DNA Isolation Plus Kit (Biotech Corporation, 3430 Schmon Parkway Thorold, ON, Canada) was used to check the efficiency of DNA extraction. Paddy soil DNA was extracted following manufacturer's instructions.

Assessment of Quantity, Quality, and Integrity of Extracted DNA

Total DNA quantity (ng/µL) and quality (A₂₆₀/A₂₃₀ and A₂₆₀/A₂₈₀ ratios) were measured using a NanoDrop 1000 spectrophotometer (Shimadzu, Japan). Samples of extracted DNA were also examined by agarose gel electrophoresis. The bands were separated on 0.8% (w/v) agarose gel containing ethidium bromide. The gel images were visualized under ultraviolet (UV) light to observe their intact nature.

PCR Amplification

PCR amplification was performed to test their suitability for downstream applications. In the present study, universal bacterial and fungal primer sets were used. Bacterial 16s rDNA regions were amplified using forward primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492r (5'-GGTTACCTTGTTA CGACTT-3'). Amplifications were carried out under the following conditions: 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 53 °C for 30 s, 72 °C for 1.5 min, 72 °C for 7 min. Fungal communities were analyzed using forward primer ITS1f (5'-CTTGGTCATTTAGAGGAAGTAA-3') and reverse primer ITS4r (5'-TCCTCCGCTTATTGATATGC-3') with the following conditions: 95 °C for 5 min; followed by 35 cycles of 95 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min; then a final elongation of 72 °C for 5 min. The amplified products were separated using 1.5% agarose gel under UV illumination using Lambda DNA cut with *EcoRI/HindIII* and the 100 bp size markers for bacteria and fungi respectively.

RESULTS AND DISCUSSION

The efficiency of DNA extraction depends on the biological material used and the protocol followed (Chen *et al.*, 2010). Every DNA extraction protocol consists of a technique to lyse the cells, solubilize the DNA and enzymatic/chemical methods to remove other contaminants. However, a good protocol that can be used to extract DNA should be cost-effective, efficient in terms of time and labor, yield the most DNA possible while limiting the amount of DNA degraded (Chen *et al.*, 2010).

Other than the DNA degradation and incomplete cell lysis, soil DNA extraction procedures also will be influenced by different parameters like sorption of DNA to soil surfaces and extraction of humic contaminants. Thus, proper lysis of microbes and inhibitor-free DNA are major requirements for any soil DNA extraction protocol used for metagenomic studies (Gu *et al.*, 2005). For more effective cell lysis, mechanical treatment should be followed rather than chemical ones (More *et al.*, 1994 and Fatima *et al.*, 2014). According to Frostegard *et al.* (1999), proper grinding of selected sample ruptures the cell wall and thereby facilitates the easy releasing of cellular DNA from the inner compartment of the cell. DNA which is used for downstream application should be free from PCR inhibitors or low in concentration in order to avoid

interferences with the enzymatic reactions (Fatima *et al.*, 2014).

With reference to extraction of DNA from soil, Zhou *et al.* (1996) and Saens *et al.* (2019) reported that soil properties have a significant effect on the efficiency of cell lysis, the amount of DNA loss and the purity of the DNA extract. Moreover, Islam *et al.* (2012) reported that the efficiency of soil microbial DNA extraction often depends on soil qualities, such as soil electrical conductivity, soil texture, inorganic carbon, and nitrogen content but not with pH and organic carbon content. However, microorganisms strongly bind with soil particles through a various binding mechanisms that reduce access to the whole microbial community. Robe *et al.* (2003) reported that organic matter content mainly determined by the organization of micro- and macro-aggregates and microorganisms are heterogeneously distributed inside micro-aggregates and in macro-porosities outside micro-aggregates. Hence, it will be one of the major source of inhibitors that can be co-extracted with soil microbial DNA (Courtois *et al.*, 2001).

The soil used in current study has reported high amount of K and P with low amount of N, OM and OC (Table 1). However, the samples used in present study were taken from one defined location in order to avoid the effect of soil type for DNA extraction.

DNA Yield

In the present study, different extraction protocols were tested to separate microbial community DNA from paddy soils. According to the results (Table 2), DNA extracted by PEG/NaCl method yielded relatively high concentration of DNA than the other tested protocols. The differences of yielded DNA could be due to the differences in protocols followed, especially with the methods used to lyse the cells (Table 3). Several studies have reported that, noticeable difference on DNA yield could be due to differences of the lysis methods used (Zhang *et al.*, 2003; Islam *et al.*, 2012). The current study also suggests that the lysis can be an important factor that can be influenced on DNA yield. In PEG/NaCl method, the enzymatic digestion lysozyme and high concentrated SDS (Li *et al.*, 2011) may effectively break up the cell wall of the microorganism to release DNA easily. On the other hand, SPB method produced a comparatively low quantity of DNA. Islam *et al.* (2012) and Shao *et al.* (2012) reported that repeated freezing-thawing steps can cause a certain degree of damage to nucleic acids. Especially they reported that, DNA larger than.

Table 2. Concentrations and purity of extracted DNA from paddy soils as determined by spectrophotometrically

Protocol	DNA quantity		DNA purity		DNA extraction efficiency (%)*
	g/ μ L	μ g/g of soil	A ₂₆₀ /A ₂₈₀	A ₂₆₀ /A ₂₃₀	
Mannitol/CTAB method	281.47	7.04	1.61	1.15	69.02
PEG/NaCl method	380.51	9.51	0.89	0.67	93.24
SPB method	260.18	6.51	1.11	0.87	63.82
Norgen's kit	408.14	10.20	1.72	1.11	-

* Efficiency of DNA extraction was calculated with reference to the DNA quantity by Norgen's kit; (DNA quantity obtained by a given method / DNA quantity obtained by Norgen's kit) \times 100

Table 3. Differences of major steps involved in three protocols

Step	Mannitol/CTAB method	PEG/NaCl method	SPB method
Cell lysis			
1. Mechanical	-	-	Freezing & thawing
2. Chemical	Liquid N ₂ 10% SDS NaCl, CTAB Mannitol	20% SDS NaCl Sucrose	10% SDS NaCl
3. Enzymatic	-	Lysozyme Proteinase K	Lysozyme
Purification	Phenol Chloroform Isoamyl alcohol	PEG Sodium acetate	Chloroform Isoamyl alcohol
Precipitation	Sodium acetate 70% ethanol	Isopropanol 70% ethanol	Isopropanol 70% ethanol

100 kb in size is highly sensitive to degradation due to freezing and thawing. This may be the reason of having low quantity of DNA extracted by SPB method which involved many freeze/thaw cycles

DNA Purity

The purity of the extracted DNA is important for PCR amplification and other downstream applications such as metagenomic studies, hybridization, denaturing gradient gel electrophoresis, fingerprinting, and sequencing. Therefore, the present study investigated the purity of the extracted DNA. The humic acids, phenol, and other aromatic compounds are absorbed in 230 nm whereas DNA at 260 nm and protein at 280 nm. Therefore, A₂₆₀/A₂₃₀ and A₂₆₀/A₂₈₀ ratios are used as the indicators of the quality DNA. A pure sample of DNA has the A₂₆₀/A₂₈₀ ratio as 1.8 and the A₂₆₀/A₂₃₀ ratio as 2.0 whereas DNA preparation that is contaminated with protein and humic acids will have an A₂₆₀/A₂₈₀

and A₂₆₀/A₂₃₀ ratios lower than 1.8 and 2.0 respectively (Sambrook *et al.*, 1989). In the present study, absorbance ratios of DNA extracted by all three direct DNA extraction protocols were reported below 1.7 (Table 2), which reflects the co-extraction of contaminants such as proteins, phenolic compounds, or humic acid. DNA extracted using Norgen's kit has resulted in a value closest to 1.8 indicating the purity of extracted DNA in comparison to the other three methods. In comparison to the Norgen's kit, Mannitol/CTAB method and SPB method, both ratios given by PEG/NaCl method were surprisingly low, suggesting that PEG/NaCl method was less effective in removing the contaminations such as humic acids, phenols, and proteins. Since humic acid contains the same charge and size characteristics similar to that of DNA, it exhibits absorbance at both 230 and at 260 nm and hence interferes in quantization of DNA (Fatima *et al.*, 2014). Although in PEG/NaCl method PEG is used to remove humic acids, expected results were not obtained. Fatima *et al.*

(2014) also reported that the protocol consists of mannitol and CTAB was the best among tested protocols, which is on par with the findings of the present study. Thereby, grinding soil samples with liquid nitrogen may provide an excellent mechanical force for cell breakup. Moreover, mannitol with sodium chloride within the lysis buffer also promoted disruption of cells and extracted humic acid and other organic contaminants, the presence of which would have otherwise inhibited PCR reaction. Further, CTAB and mannitol also supported to stabilize the enzymes (Fatima *et al.*, 2014). Another advantage of this protocol is the inclusion of Phenol, Chloroform and Isoamyl alcohol to minimize the additional purification steps and remove proteins and humic acids effectively. This has previously been used as a purifying agent in many other studies of soil DNA extraction (Satyanarayana *et al.*, 2017).

DNA integrity

According to Figure 1, it is evident that all three protocols resulted in intact genomic DNA. However, intensity of the genomic DNA band resulted in by SPB method was relatively low and could be due to low amount of DNA yielded by the method (Table 2).

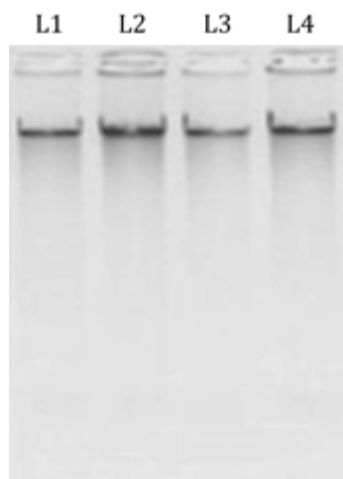


Figure 1: Comparison of genomic DNA extracted by three direct extraction protocols and Norgen's kit; L1 - DNA extracted from Mannitol/CTAB method, L2 - DNA extracted from PEG/NaCl method, L3 - DNA extracted from SPB method, L4 - DNA extracted from Norgen's kit

PCR amplification

Humic acids are one of the most common inhibitors existing in environmental soils. Several

studies revealed that the humic acids can inhibit the PCR at the amplification steps or have adverse effects on DNA extraction via sequestration (Islam *et al.*, 2012; Knauth *et al.*, 2013). Humic substances chelate the Mg^{2+} ions which required for the activity of *Taq* polymerase, restriction enzymes and ligases (Devi *et al.*, 2015).

The present study tested 27f and 1492r; 16s rDNA universal primers which have been widely used for amplifying bacterial ribosomal genes in several studies (Chen *et al.*, 2010 and Devi *et al.*, 2015). According to Figure 2, DNA extracted using Mannitol/CTAB method and by the commercial kit had given expected band (~1500 bp). This could have happened due to low purity of the DNA of the other two methods (Table 2), which ultimately leads to inhibit PCR. Therefore, addition of more purification steps could improve the results. Similarly, as shown in the Figure 2, expected band (~750) was resulted in the DNA sample extracted via Mannitol/CTAB method while others were not (DNA extracted by the commercial kit was not included). This could be due to low purity as previously mentioned. ITS 1 and ITS 4 primer set is broad spectrum. However, some research studies suggested that use of ITS 1 and 2 primers will be more effective in amplifying fungi due to their specificity (Op De Beeck *et al.*, 2014). This may be the reason not having sharp band for DNA extracted using Mannitol/CTAB method. Thus, in addition to adding of more purification steps, use of more effective and specific primer sets also could improve the results. Liu *et al.* (2015) and Yi *et al.* (2019) reported that the abundance of bacterial and fungal community is differing with the depth of paddy soil. Further, they have revealed that bacterial community is higher in surface layers than the fungal community and when increasing the depth of soil fungal community getting lower. Wichern *et al.* (2020) reported that, paddy systems consist with periods of water saturation/ anaerobic and periods of aerobic conditions, which can be both affected by salinization.

However, after flooding the paddy field, water saturation might result in a dilution of accumulated salts and when water is lost from the system, salt accumulation. It is known that soil microorganisms are affected by both, increased soil salinity and reduced oxygen availability. Further, Wichern *et al.* (2020) have reported that fungal ITS1 was significantly affected by water content in soil and there was a tendency of reduced fungal contents at higher water levels and a lower fungi-to-bacteria ratio.

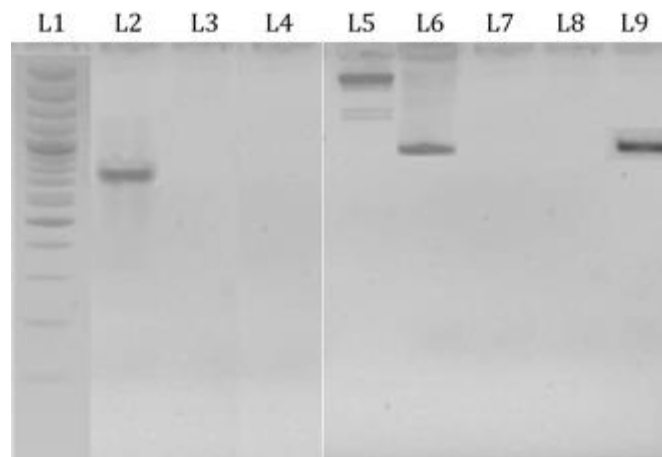


Figure 2. Gel electrophoresis of PCR products amplified with fungal and bacterial universal primers. L1 - 100 bp DNA size marker, L2 – PCR amplification of ITS region with DNA extracted by Mannitol/CTAB method, L3 - PCR amplification of ITS region with DNA extracted by PEG/NaCl method, L4 - PCR amplification of ITS region with DNA extracted by SPB method, L5 - Lambda DNA cut with *EcoRI/HindIII*, L6 - PCR amplification of 16s rDNA with DNA extracted by Mannitol/CTAB method, L7 - PCR amplification of 16s rDNA with DNA extracted by PEG/NaCl method, L8 - PCR amplification of 16s rDNA with DNA extracted by SPB method, L9 – PCR amplification of 16s rDNA with DNA extracted by Norgen's kit

According to the current study, it was proved that the soil DNA isolated by protocol having mannitol was more effective than the other protocols tested. The protocol consists of mannitol in the lysis buffer to isolate DNA from bacterial and fungal mycelia. Moreover, an inclusion of mannitol with NaCl may promote cell disruption and removal of humic acid and other organic contaminants, the presence of which would have otherwise inhibited PCR reaction. Hence, the study confirmed mannitol/CTAB method provides DNA of sufficient quality and integrity to amplify the genetic regions, which may be provided a complete information and understanding of microbial biota. Further, it provides the quality DNA for downstream applications such as DNA cloning, sequencing and metagenomic studies needed for microbial community analysis. Therefore, the research findings would be helpful for soil ecologists and taxonomists to explore the composition and dynamics of microbial communities in paddy soil.

CONCLUSION

The results revealed that all three protocols used in the study are suitable for extraction of DNA

from paddy soil. Comparatively direct DNA extracted using PEG/NaCl method does not provided good quality DNA for downstream application. On the other hand, direct DNA extracted using Mannitol/CTAB method has resulted in higher quality DNA with a considerable yield and amplification of expected size PCR bands proving its suitability for downstream molecular applications. However, the DNA extracted using above three protocols co-existed with contaminants such as proteins and humic acids. Therefore, further optimizations and DNA recovery studies are required to obtain quality DNA with high quantity. Moreover, addition of more DNA purification steps prior to the PCR amplification would result in expected bands via removing of PCR inhibitors effectively.

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