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Evaluation of simple and rapid DNA extraction methods for molecular identification of fungi using the internal transcribed spacer regions

Lakkhana K. Wingfield[1,*] and Jirawalun Atcharawiriyakul[1]

[1]Division of Biological Science, Faculty of Science, Prince of Songkla University, Songkla, Thailand
[*]Corresponding author: Lakkhana.k@psu.ac.th

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Abstract

Routine DNA extraction method previously used in our laboratory usually involved cetyl trimethyl ammonium bromide and phenol/chloroform extraction which was entirely time-consuming and cost-ineffective especially when performed large-scale species identification. The aim of the study was thus to evaluate alternative, simple, and cost-effective methods for extracting DNA from filamentous fungi and mushrooms isolated from various environmental samples. Five simple and rapid methods for extracting DNA were evaluated for molecular identification. Each method was assessed based on polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) regions, measurement of yield and purity of extracted DNA including time required in the procedures. Method previously developed by the International Rice Research Institute (IRRI) was the best method for extracting fungal DNA. Meanwhile the IRRI and the sodium hydroxide-Tris (NaOH-Tris) methods were the best two methods that produced identical PCR amplification results from most samples (PCR efficiency of 85% and 89%, respectively). However, the NaOH-Tris method was superior to the IRRI method as it required short extraction procedure, reduced the need of using hazardous chemicals and was cost-effective. In contrast, ultra-simple, Tris-EDTA (TE) buffer and water methods were not effective for most fungal genera. We thus propose that the IRRI and NaOH-Tris methods were effective for rapid DNA isolation of large numbers of fungal cultures, specially replacing the use of conventional DNA extraction procedures. In addition, these methods can be applied to diverse fungal genera isolated from various environmental sources for fungal population and molecular studies.

Keywords: DNA extraction, Internal transcribed spacer (ITS) region, PCR amplification

1. Introduction

Fungi are ubiquitous eukaryotic organisms found in diverse natural environment and are constituents of ecosystems as saprotrophs, mutualists, commensal symbionts and pathogens. They compose of large diversity of taxa with various life cycles and morphologies ranging from unicellular yeasts to multicellular mushrooms. Fungi usually grow on a variety of substrates such as soil and plant tissues, although many of these fungi may grow without producing reproductive spores or other distinguishing features when cultured. Therefore, molecular techniques have become the standard approaches of identifying fungal samples in many circumstances.

Together with the studies on fungal morphology, studies of the internal transcribed spacer (ITS) regions of the ribosomal RNA have become a useful tool in fungal identification. The ITS genes have been widely used to analyze the diversity of fungal community because it helps differentiate between different species within a genus level. As polymerase chain reaction (PCR) based methods have currently become a common approach for fungal identification, detection and diagnosis as well as characterization of fungal communities [1], a step of DNA extraction is thus crucial and inevitable especially for the identification of large numbers of fungi through PCR-based analysis.

Extraction of fungal DNA generally involves two main steps which includes: (1) breaking of fungal cell walls and (2) extraction and purification of fungal DNA. A typical method usually performed for fungal DNA

extraction is using Cetyl trimethyl ammonium bromide (CTAB) extraction buffer [2] and then DNA purification through phenol/chloroform extraction is performed followed by isopropanol or ethanol precipitation [3]. Other protocols have also been developed for DNA isolation which include high-speed cell disruption [4], sodium dodecyl sulfate (SDS) lysis [5], lysozyme/SDS extraction [6], CTAB/proteinase K method [7,8], bead-vortexing/SDS extraction [9] and SDS/phenol/chloroform extraction [10-12]. Although these methods generally provide DNA of satisfactory quantity and quality, many of these methods are entirely time consuming and cost ineffective. Additionally, some methods require hazardous reagents and special equipment whereas some are not versatile and not suitable for extracting DNA from diverse groups of fungi [11-14]. Commercial DNA isolation kit provides rapid procedure and high-quality DNA. Conversely, the method is expensive and it is not suitable for extensively use in the laboratories that perform large-scale species identification, particularly in the laboratories with minimal resources. The aim of this study was thus to evaluate alternative, rapid, simple and cost-effective methods for extracting DNA from filamentous fungi and mushrooms isolated from various environmental samples. The evaluation criteria were in the aspect of DNA yield and purity, efficiency for PCR amplification including time and cost required.

2. Materials and methods

2.1 Fungal materials

The filamentous fungi and mushroom samples were collected and isolated from soil and air samples as well as the plant materials. These fungal isolates were cultured on potato dextrose agar (PDA). The plates were inoculated at 25 °C for 3 to 14 d. The mycelium from pure fungal colonies were transferred into a 100 mL potato dextrose broth (PDB) and incubated at 25 °C for 7 d under shaking condition at 150 rpm. The mycelium was harvested by filtration through a sterile Whatman® filter paper (no.1), washed with sterile distilled water and stored at -20 °C.

2.2 DNA extraction methods

Frozen mycelium was ground to a fine powder using liquid nitrogen. Then, 0.1 g of ground fungal mycelium was transferred to a sterile 2.0 mL microcentrifuge tube and performed DNA extraction as follows. Five rapid and simple DNA extraction methods were performed with minor modification. Fruiting bodies were directly used for DNA extraction of the mushroom samples.

Modified 'NaOH-Tris method' (adapted from Wang et al. [15]): A volume of 100 µL of 0.5 M sodium hydroxide (NaOH) was added to a microcentrifuge tube containing 0.1 g of mycelium and the sample was mixed by inversion several times. A volume of 1.4 mL of 0.1 M Tris solution (pH 8.0) was added and the mixture was centrifuged at 13,000 rpm for 2 minutes. The supernatant was transferred to a new sterile microcentrifuge tube and stored at -20 °C.

Modified 'Ultra-simple method' (adapted from Ikeda et al. [16]): A volume of 300 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) was added to a microcentrifuge tube containing 0.1 g of mycelium and the sample was mixed by inversion several times. The mixture was then place in a water bath (70 °C) for 15 minutes and a 1.2 mL of modified TE buffer (10 mM Tris-HCl, 0.1 mM EDTA pH 8.0) was added. The mixture was then centrifuged at 13,000 rpm for 2 minutes. The supernatant was transferred to a new sterile microcentrifuge tube and stored at -20 °C.

Modified 'TE buffer method' (adapted from Ikeda et al. [16]): A volume of 1 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) was added to a microcentrifuge tube containing 0.1 g of mycelium and the sample was mixed by inversion several times. The mixture was then centrifuged at 13,000 rpm for 2 minutes. The supernatant was transferred to a new sterile microcentrifuge tube and stored at -20 °C.

Modified 'Water method' (adapted from Collard et al. [17]): A volume of 1 mL of sterile H_2O was added to a microcentrifuge tube containing 0.1 g of mycelium and the sample was mixed by inversion several times. The mixture was then place in a water bath (70 °C) for 10 minutes and then centrifuged at 13,000 rpm for 2 minutes. The supernatant was transferred to a new sterile microcentrifuge tube and stored at -20 °C.

Modified 'IRRI method' (adapted from Zheng et al. [18]): A volume of 1 mL of extraction buffer [50 mM Tris-HCl (pH 8), 25 mM EDTA, 300 mM NaCl, 1% SDS] was added to a microcentrifuge tube containing 0.1 g of mycelium and the sample was mixed by inversion several times. Proteins were subsequently removed by adding 500 µL chloroform/isoamyl alcohol (24:1) and mixed by inversion. Then, a mixture was centrifuged at 13,000 rpm for 1 minutes. A 750 µL supernatant was transferred to a new 2.0 mL microcentrifuge tube and an equal volume of cold absolute ethanol was added to precipitate the DNA. The mixture was gently mixed by inversion and centrifuged at 13,000 rpm for 3 minutes. The supernatant was discarded and the resulting pellet was washed with 500 µL of 70% ethanol. After discarding the ethanol, the pellet was dried for at least 20 min at room temperature. The extracted DNA was resuspended in 100 µL of TE buffer (10 mM Tris-HCl, 1 mM

EDTA pH 8.0) and stored at -20 °C. The three replicate samples from each fungal culture were selected randomLy to DNA extraction by the modified NaOH-Tris, ultra-simple, TE buffer, water and the IRRI methods.

2.3 Evaluation of DNA yield and purity

DNA quality and intensity were estimated by mean of electrophoresis in 1% agarose gels, followed by staining with ethidium bromide (10 mg/mL). The purity of DNA was measured through the absorbance of A_{260}/A_{280} ratio, whereas the DNA yield was measured using MaestroNano spectrophotometer (USA). Each of the sample tested for each method was read in triplicate. ANOVA was used to compare mean values of A_{260}/A_{280} nm ratios. Turkey's honestly significance difference test was used for making multiple comparisons between means.

2.1 PCR amplification

PCR amplification of fungal ITS regions was carried out using three ITS primer sets; ITS1F/ITS4N, ITS1/ITS4N and ITS5/ITS4N which amplified *ca*. 600-800 bp section of the ITS and had the following sequences: ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3'), ITS4N (5'-TCCTCCGCTTATTGATATGC-3'), ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3').

PCR amplification was performed in a 50 µL reaction mixture containing: 0.2 µL *Taq* DNA polymerase (5 U/ µL, Invitrogen, USA) along with 5.0 µL 10X PCR buffer, 1.0 µL dNTP mixture (10 mM), 1.5 µL $MgCl_2$ (50 mM) and 2.5 µL primer mix (10 µM each). One microliters of each DNA sample extracted from different methods were used directly for PCR. The PCR conditions were as follows: initial denaturation step at 94 °C for 5 minutes, 35 amplification cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 90 sec, followed by a final extension at 72 °C for 10 minutes. All reactions were performed using a Thermocycler (USA). Aliquots of PCR products (5 µL) were resolved in 1% agarose gels in 1XTAE buffer and visualized by UV light after staining with ethidium bromide (10 mg/mL).

For ITS analysis, PCR efficiency using the templates from different methods was assessed by calculating the number of successful PCR amplifications divided by the total number of PCR reactions. PCR intensity was visually estimated from the PCR products of each method relative to the PCR products produced by the IRRI method using a three-class scale.

3. Results and discussion

3.1 DNA yield and purity

Five simple and rapid DNA extraction methods were determined whether they could be used to extract DNA from different fungal genera and from different environmental sources. With these methods, genomic DNA of 58 fungal strains including zygomycetes, ascomycetes and basidiomycetes isolated from various natural sources were subjected to each extraction method. Efficiency of the methods were evaluated by means of DNA quality and intensity through electrophoresis analysis and measurement of absorbance of A_{260}/A_{280} ratio. Comparison of five different DNA extraction methods revealed the significantly differences between mean DNA yields and differences between purity of the DNA samples isolated from difference methods (Table 1 and Table S1). The spectrophotometer measurement readings demonstrated that the NaOH-Tris method yielded the highest DNA concentration, followed by the TE buffer method, the IRRI method, the water method and the ultra-simple method, respectively. Nucleic acid absorbance ratios were determined to evaluate quality of extracted DNA. Absorbance ratio (A_{260}/A_{280}) of extracted DNA ranged 1.4-1.7, which indicated the presence of contaminating proteins in the DNA samples.

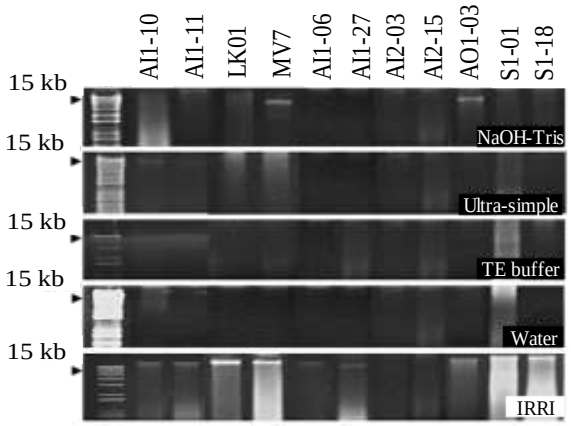
Table 1 Comparison of five different rapid DNA extraction methods.

Methods	NaOH-Tris	Ultra-simple	TE buffer	Water	IRRI
Mean yield (ng/µL) [1]	1435.96[a]	863.38[b]	1302.47[c]	904.24[d]	1260.76[e]
Visibility on gel (%)	18/58 (31)	6/58(10)	0/58 (0)	0/58 (0)	48/58 (83)
Purity (A_{260}:A_{280} ratio) [1]	1.434[d]	1.631[b]	1.561[c]	1.690[a]	1.404[d]
PCR efficiency (%)	52/58 (89)	23/58 (40)	11/58 (19)	7/58 (12)	49/58 (85)
PCR product intensity[2]	+++	++	-	-	+++

[1]Means followed by the same letter (in row) are not significantly different from each other based on Turkey's test.

[2]PCR product intensity: -, no band detected; +, faint bands; ++ adequate PCR amplification for scoring; +++, good PCR amplification.

The detection of extracted DNA using gel electrophoresis was not consistent with the results from the spectrophotometer reading, except for the IRRI method (Figure 1 and Figure S1). For the IRRI method, 48 out of 58 fungal samples (83%) revealed the bands of extracted DNA on agarose gels and the DNA bandings were clear and intact. Meanwhile the NaOH-Tris method, which gave the highest yield of DNA from the spectrophotometer reading, showed detected DNA bands of only 31% (18 detected bands out of 58 fungal samples). The TE buffer and water methods showed no visible band on the gels even though the DNA concentration could be read.



Isolate code	Source	Genus	Phylum
AI1-06	Air	*Curvularia* sp.	Ascomycota
AI1-10	Air	*Aspergillus* sp.	Ascomycota
AI1-11	Air	*Penicillium* sp.	Ascomycota
AI1-27	Air	*Cladosporium* sp.	Ascomycota
AI2-03	Air	*Alternaria* sp.	Ascomycota
AI2-15	Air	*Fusarium* sp.	Ascomycota
AO1-03	Air	*Mycelia sterilia*	Unidentified
S1-01	Soil	Mitosporic fungi	Unidentified
S1-18	Soil	*Absidia* sp.	Zygomycota
MV7	Plant leaf	*Diaporthe* sp.	Ascomycota
LK01	Mushroom	*Lignosus* sp.	Basidiomycota

Figure 1 Partial results of agarose gel electrophoresis of the genomic DNA extracted from different DNA extraction methods. M=1 kb Plus DNA Ladder (Invitrogen); AI1-10=*Aspergillus* sp. AI1-10; AI1-1 =*Penicillium* sp. AI1-11; LK01=*Lignosus* sp. LK01; MV7=*Diaporthe* sp. MV7; AI1-06=*Curvularia* sp. AI1-06; AI1-27=*Cladosporium* sp. AI1-27; AI2-03=*Alternaria* sp. AI2-03; AI2-15=*Fusarium* sp. AI2-05; AO1-03=*Mycelia sterilia* AO1-03; S1-01=Mitosporic fungi S1-01; S1-18=*Absidia* sp. S1-18.

3.2 PCR amplification of ITS regions

A PCR based technique was performed to check the quality and suitability of the DNA isolated from each extraction method for molecular analysis. PCR amplification of ITS regions using specific primers were performed and a desired band of approximately 600-800 bp was obtained from the DNA samples as shown in Figure 2. This ITS analysis showed that PCR products can be generated using crude DNA samples. However, no PCR product was generated from initial amplification using crude DNA samples for most strains. In this instance, dilution of the extracted DNA as a template was applied to solve the problem (data not shown). Furthermore, PCR amplification was successful even though no visible band of the genomic DNA is observed on agarose gels in some fungal samples.

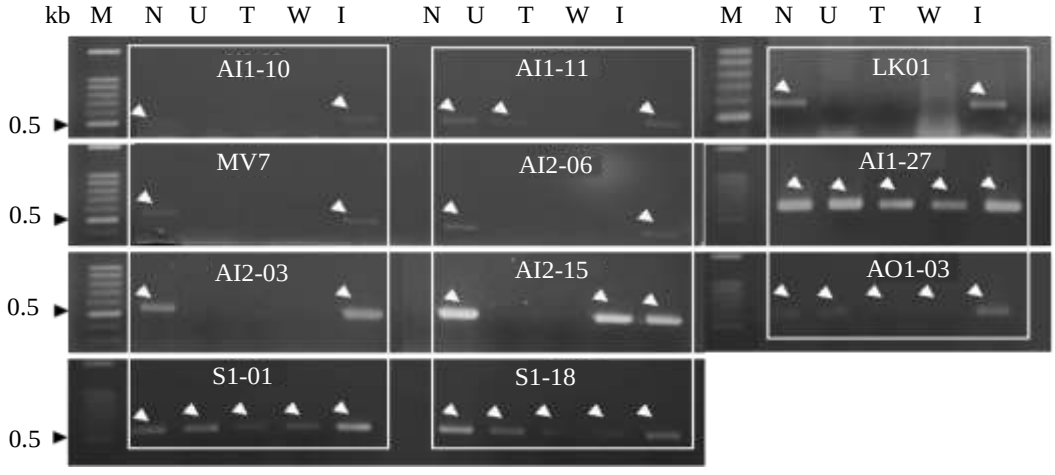


Figure 2 Partial results of PCR amplification profiles of ITS region using DNA samples extracted from different.

DNA extraction methods, showing 600-800 bp PCR fragments. M=1 kb Plus DNA Ladder (Invitrogen); N=NaOH-Tris method; U=Ultra-simple method; T=TE buffer method; W=Water method and I=IRRI method; AI1-10=Aspergillussp. AI1-10; AI1-11=Penicillium sp. AI1-11; LK01=Lignosus sp. LK01; MV7=Diaporthe sp. MV7; AI1-06=Curvularia sp. AI1-06; AI1-27=Cladosporium sp. AI1-27; AI2-03=Alternaria sp. AI2-03; AI2-15=Fusarium sp. AI2-05; AO1-03=Mycelia sterilia AO1-03; S1-01=Mitosporic fungi S1-01; S1-18=Absidia sp. S1-18. White arrows indicated PCR products of ITS regions.

The best amplification results were obtained using the IRRI and NaOH-Tris methods which demonstrated comparable PCR efficiency of 85% and 89%, respectively. PCR intensity was also depending on the methods of extraction, since IRRI and NaOH-Tris methods generated clearer and more intense PCR products compared to the other three methods evaluated in the present study (Figure 2). Although the ultra-simple method showed poor release of fungal DNA, weak bands of PCR products could be generated in some fungal strains (40% PCR efficiency). In addition, PCR amplification using DNA samples extracted from the water method was usually unsuccessful (12% PCR efficiency) and only very faint bands of the PCR products were detected in few fungal DNA samples. However, a clear band of PCR product using DNA extracted from water method extraction method was detected in the fungal isolate AI2-15. The results indicated that PCR efficiency was depend on the extraction methods used and fungal species. In addition, PCR efficiency and intensity were also depending on the quality of the ITS primers, since some ITS primers generated clearer and more intense PCR products compared to other primers (data not shown).

3.3 Estimation of time required

Time required for each DNA extraction method is varied from 37 to 62 min when evaluated from a set of 10 samples (assuming that all solution and buffer are prepared in advance). As shown in Table 2, the TE buffer method required the least time followed by the NaOH-Tris, the water, the ultra-simple and the IRRI methods, respectively. Although the IRRI method offered the best DNA quality as visualized on agarose gels, it required the longest time of extraction because the precipitation step is included.

Table 2 Times required for five different rapid DNA extraction methods.

Methods	No. of samples	Duration for different stages (min)[1]				
		Break[2]/wash	Incubation/lysis	Purification	Precipitation	Total
NaOH-Tris	10	30	10	-	-	40
Ultra-simple	10	30	24	-	-	54
TE buffer	10	30	7	-	-	37
Water	10	30	15	-	-	45
IRRI	10	30	7	-	25	62
CTAB[3]	10	50	30-60	50-60	30	165-200

[1]The time for each stage is estimated according to our experience. Dash (-) means the lack of a stage.
[2]Frozen mycelium was ground to a fine powder using liquid nitrogen.
[3]Genomic DNA extraction method of fungal samples described by Zhang et al. [19].

For the identification of fungi using ITS regions, factors used for selection of DNA extraction methods are crucial. This particularly applies to some laboratories dealing with the large number of fungal samples and encountering limited financial resources. Practically, DNA extraction method must be selected not only based on reliability but also simplicity, time-saving and cost-effectiveness. Five different DNA extraction methods were selected for this study based on the aforementioned factors. The NaOH-Tris method described by Wang et al. [15] has initially been used for simple preparation of the plant DNA samples for PCR. Likewise, the IRRI method developed by the International Rice Research Institute (IRRI) has been used for extracting DNA from rice seedlings for marker-assisted selection [17]. The ultra-simple, TE buffer and water methods are the fastest and simplest methods that have been described and evaluated for PCR analysis in plants [17]. However, these simple methods have not been employed for extracting fungal DNA except for the NaOH-Tris method. Here, we determined that rapid DNA extraction methods developed for plant samples were also applicable to fungi.

Gel electrophoresis results indicated that the detection of extracted DNA samples was not consistent with the results from the spectrophotometer reading except for the IRRI method. It can be suggested that impurities of the DNA samples may have caused an overestimation of the DNA concentrations for most of the extraction methods. On the other hand, chromosomal proteins that have not been completely removed from the samples were still bound to the DNA, resulting in the prevention of DNA migration. These results also suggested that chloroform extraction used in the IRRI method was effective as some proteins were eliminated, however, boiling was not. Phenol/chloroform-based extraction has been used to extract fungal DNA when high DNA

yield and purity are required because the methods usually remove protein and co-precipitated polysaccharides [11,12]. However, as reviewed, these methods require harmful organic solvents.

Regarding the DNA yield, the NaOH-Tris method gave the highest yield which was unexpected because the method did not include the SDS lysis in the process while the IRRI method did. Possible explanation for the high DNA yield obtained from the NaOH-Tris method is that the samples may be contaminated by proteins, causing impurity of the samples. Similarly, degradation of the DNA may have caused inaccurate spectrophotometer reading [20]. Absorbance ratio (A_{260}/A_{280}) of extracted DNA ranged 1.4-1.7, which indicated the presence of some contaminating proteins. According to data analysis of DNA purity, the water method demonstrated the purest samples. The highest A_{260}/A_{280} ratios detected for the water method, and to a lesser extend the ultra-simple method, was not expected. Similar explanation regarding the inaccuracy of spectrophotometer measurement caused by impurities may be applied to this ambiguity. Regarding time required for rapid DNA extraction, the operation time was reduced greatly in each extraction procedure when compared with other previously described methods. Although the IRRI method was the best protocol for DNA extraction tested in this study, the NaOH-Tris method required less than half of the time as a single transfer of material between tubes was performed. To our knowledge, the most rapid method for extracting fungal DNA was a mini preparation of fungal DNA described by Saitoh et al. [21] which did not require preculture in liquid medium, harmful chemicals, and maceration in liquid nitrogen. However, the method required much time for preparation of lysis and TE buffers and some chemicals needed might not be available in some laboratories. On the other hand, the modified NaOH-Tris method only required NaOH and Tris solution and no final precipitation step was needed.

For PCR amplification of ITS regions, the DNA extracted from fungal isolates showed reproducible PCR products that were well resolved in 1% agarose gels, nevertheless these methods performed good amplification for most, but not all, of fungal genera. The analysis revealed that the best amplification results were observed when DNA samples extracted with the NaOH-Tris and IRRI methods were used, the methods of which produced PCR products that were comparable in intensity. This signified that the amount and quality of extracted DNAs obtained from the NaOH-Tris and IRRI methods were suitable for PCR amplification. In addition, the production of good DNA banding also indicated sufficient good quality of DNA. In contrast, the other three DNA extraction methods evaluated in the present study did not generate good DNA quality that would be suitable for ITS identification in practice. This could be contributed to the presence of some polysaccharides and polyphenols which are known to inhibit *Taq* DNA polymerase in the samples [22]. However, successful PCR amplification of some fungal samples extracted from each method could be explained that the amount of PCR inhibitors may vary with fungal species, also chemicals used in each extraction method might exhibit different effect on DNA yield and protein function. Wang et al. [15] suggested that nuclear DNA might be optimally extracted with NaOH, which the alkaline pH might also inactivate nuclease during extraction. It can also be noted that visualization of extracted DNA templates by electrophoresis is not always necessary because PCR products could be generated even though no visible band of the DNA was detected.

The fungal DNA extraction methods evaluated in the present study could eliminate the laborious steps and time consuming described in previous protocols [2-4,8,10,11]. Interestingly, the IRRI and NaOH-Tris methods also worked well with some other macrofungi tested, in this case *Lignosus* and *Coprinus*. Regardless of the presence of extracted genomic DNA on agarose gels, the NaOH-Tris method seemed to be superior to the IRRI method because it required shorter extraction procedure, lacked additional wash steps and final ethanol precipitation step, generated less wastes and suppressed the need of using hazardous chemicals, making it simpler and cost-effective over the IRRI method. We thus favour the modified NaOH-Tris method when the work necessitates rapid DNA extraction for further study. On the other hand, the IRRI method is favoured when higher DNA yield and purity are required for molecular identification of fungi. This method offered comparable DNA yield and purity to the extraction method described by Vazquez-Angulo et al. [12], however, the former method only required chloroform whereas the latter required both chloroform and phenol for DNA extraction.

4. Conclusion

Here, we proposed the efficiency, reliability of PCR amplification and time of the DNA extraction methods with the conclusions that the NaOH-Tris and IRRI methods were effective and simple and allowed simultaneous DNA extraction from large numbers of fungal isolates. In addition, the methods were suitable for extracting the DNA from the diverse fungal species from various environmental sources to be used in molecular analysis. This suggested that the two rapid DNA extraction methods were broadly useful across the fungal taxa.

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