



Reliable protocols for DNA extraction from freeze-dried macrofungal samples used in molecular macrofungal systematics studies

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Abstract

Molecular methods to investigate macrofungal communities are faster and more reliable than classical morphological methods. Identification techniques based on total DNA extraction provide excellent identifications. In the present study two techniques of DNA extraction have been tested on four different fungal species. DNA qualities were evaluated, along with DNA suitability for amplification of ITS fragments by polymerase chain reaction (PCR) using a basidiomycete-specific primer pair (ITS1F-ITS4B) to amplify fungal internal transcribed spacer (ITS).

Key words – Cetyl trimethylammonium bromide – DNA – internal transcribed spacer – macrofungi

Introduction

Identification and description of macrofungi is normally based on morphology. However macroscopic identification has certain limitations. One problem would be that morphology cannot always determine if the fungi is associating with nearby plants (Rowe and Pringle, 2005). Fruiting body observations only provide information about the fungi on the surface. In addition, evolutionary relationships cannot be determined accurately through morphology alone. The shortage of molecular based phylogenetic fungal lineages based on large samples sizes shows the need for success molecular methods (Nagy et al. 2011). The evolutionary history and role the fungi plays in the environment can become more evident in careful molecular analyses. For this purpose several molecular PCR-based techniques have been developed over the last 15 years (Bellemain et al. 2010). They offer fast and reliable results. In order to perform a good PCR and reliable sequencing, DNA extraction is so far the first critical step. When samples need to be stored or preserved prior to molecular analysis, this can result in degradation and reduced yield of DNA (Bainard et al. 2010). In this study, we tested two modified DNA extraction protocols with four macrofungi species differing in color and flesh consistency. For amplification of ribosomal DNA the known basidiomycete-specific primers ITS1-F and ITS4-B are generally used (Gardes and Bruns, 1993). The internal transcribed spacer region (ITS) of nuclear DNA (nrDNA) is the preferred DNA barcoding marker both for the identification of single taxa and mixed environmental samples (Bellemain et al. 2010). Internal transcribed spacer (ITS) includes the ITS1 and ITS2 regions, separated by the 5.8S gene (Schoch et al. 2012). These two regions are highly differentiated and provide a good resolution at genus and species level (Nilsson et al. 2008). This region has recently been proposed to be generally used as the official barcoding marker for fungi

(Bellemain et al. 2010). For this study the modification of protocols on prepared samples began with the extraction portion of the molecular analysis. The rationale for modification of generally accepted protocols would be to generate a method that would still yield an ample amount of DNA but with less time and use of materials. Extracting DNA in a time and cost effective way is important for effectively adding to the molecularly identified fungi database. The purpose of this study was to improve and simplify currently available DNA extraction methods for dried macrofungal samples.

Materials and Methods

Fruiting bodies of four macrofungi species (*Amanita rubescens* Pers., *Craterellus cornucopioides* (L.) Pers., *Lactarius vellereus* (Fr.) Fr., *Scleroderma verrucosum* (Bull.) Pers.) (Fig. 1) were harvested in Aïn Draham Northwestern Tunisia (36°46'4.20" N latitude, 8°42'3.59" E longitude). Afterwards these macrofungi were preserved by freeze-drying (Martin Christ Alpha 1-2 LD) for molecular identification.



Fig. 1 – Macrofungi harvested in Aïn Draham, Northwestern Tunisia. **A** *Amanita rubescens*. **B** *Craterellus cornucopioides*. **C** *Lactarius vellereus*. **D** *Scleroderma verrucosum*.

Protocol 1 – DNA extraction from dried mushrooms using a modified MasterPure™ Yeast DNA Purification Kit

DNA extraction was performed with MasterPure-Yeast DNA Purification Kit-Epicentre. Protocol steps were modified from the manufacturer’s original protocol. These changes included dividing by half the volumes of Yeast cell lysis solution, doubling the volumes of MPC Protein precipitation reagent, an increase in the spins at rates to 20 minutes at $\geq 15,000$ rpm and performing one wash of 200 ml 70% ethanol.

Protocol 2 – DNA extraction from dried macrofungi using the C-tab DNA Extraction Protocol

The C-tab (cetyl trimethylammonium bromide) DNA extraction protocol was described in different studies involving DNA extraction from fungi and plants (Devi et al. 2013). We tested this method on our fungal samples and found it necessary to make slight modifications. These changes included doubling the volumes of isopropanol and 70% ethanol, an overnight incubation at -20 °C in 70% ethanol and an increase in the spins at rates to 10 min at $\geq 15,000$ rpm. The DNA pellets were dried on a Vacuum Concentrator for 5 min until water drops on tube wall above the pellet had vanished.

PCR amplification. The conserved primer pair ITS1F-ITS4B (White et al. 1990) was used to amplify the internal transcribed spacer (ITS) sequence of the fungal rDNA region. Localization of the primers to fungal rDNA is presented in figure 2. The PCR reaction was set up using a volume of 22.1 μ l as follows: 2 μ l template DNA, 1.5 μ l of each primer (5 pmol/ μ l), 1.2 μ l MgCl₂ (50 mM), 0.32 μ l dNTP (100 mM), 3.0 μ l 10 \times PCR buffer, 0.12 μ l BSA, 0.2 μ l Prime Taq™ DNA polymerase and 12.26 μ l of Milli-Q water. PCR amplifications were performed in a SensoQuest Thermocycler 48. The PCR program was performed with an initial denaturation at 95 °C for 5 min, followed by 29 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 3 min, and amplification at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis in a 1.5% agarose gel in 0.5 \times TBE buffer, stained by GelRed™ Nucleic Acid Gel Stain, 10,000 \times in water. A MassRuler Express Forward DNA ladder Mix (Thermo Scientific) was used as a size standard.

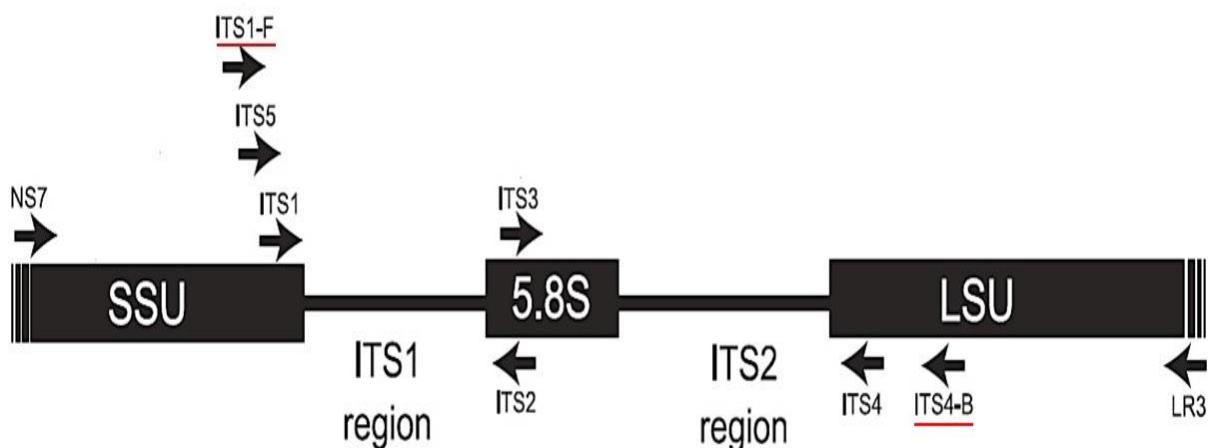


Fig. 2 – Schematic representation of commonly used primers for amplifying parts or the entirety of the ITS region. Underlined: A basidiomycete-specific primer pair (ITS1F-ITS4B) used to amplify fungal internal transcribed spacer (ITS) sequences.

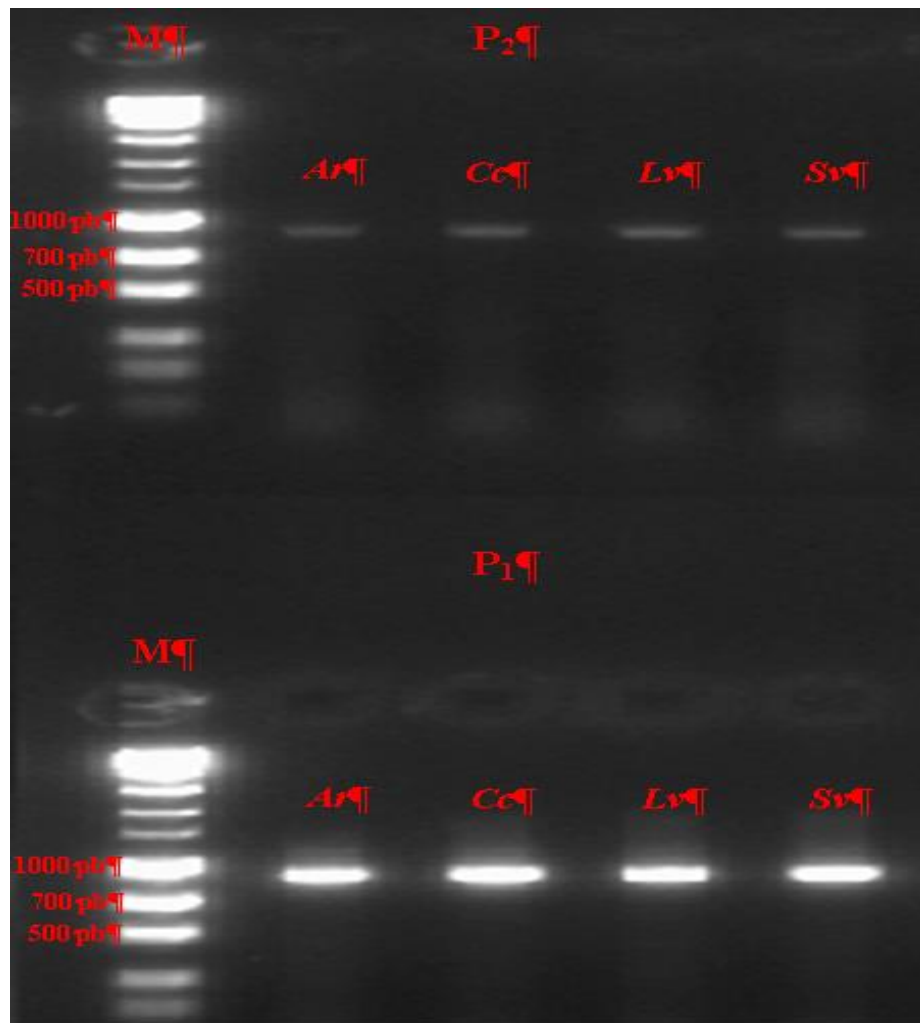


Fig. 3 – ITS amplification products obtained with ITS1-F/ITS4-B primers. **Lane Ar** *Amanita rubescens*. **Lane Cc** *Craterellus cornucopioides*. **Lane Lv** *Lactarius vellereus*. **Lane Sv** *Scleroderma verrucosum*.

M molecular size marker (MassRuler Express Forward DNA ladder Mix).

P1 MasterPure™ Yeast DNA Purification Kit.

P2 C-tab DNA Extraction Protocol.

DNA sequencing. After visualization of positive PCR products on a 1.5% agarose gel, sequencing reactions were carried out in 10 µl volumes containing 3 µl reaction buffer, 1 µl primer (4 pmol/µl), 4 µl Milli-Q water, 1 µl BigDye and 1 µl PCR product. Cycling parameters for sequencing were as follows: initial denaturation at 95 °C for 1 min followed by 30 cycles of denaturation at 95 °C for 10 s, annealing at 50 °C for 5 s and extension at 60 °C for 4 min. Sequences were edited using the software SeqMan-program version 7.1.0 (44.1) and manually corrected before alignment to obtain a consensus sequence. For a DNA-based identification all sequences of the studied species were in-silico compared with the BLAST service of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Results

PCR and sequencing were success using two protocols to extract DNA for dried macrofungal tissue belonging to different specimens and families (Amanitaceae, Cantharellaceae, Russulaceae, Sclerodermataceae). The primers (ITS1F-ITS4B) showed improved performance over universal traditional primers (ITS1-ITS4) in that they produced good PCR product visualization in the electrophoresis gel. With BLAST searches we found that the sequences identified had a 100% match with our morphological identification that were performed with a field book entitled *Guide vigot des*

champignons (Gerhardt 1999).The PCR products produced from the protocol 1 contained a high concentration of DNA than PCR products from protocol 2. Polymerase chain reaction quantities of DNA were correlated with the method of DNA extraction. We used a Nanodrop Spectrophotometer to quantify the DNA content in our samples. For PCR products obtained from protocol 1 we had a wide range of DNA concentrations (184.06-1375.72 ng/ul) compared with DNA concentrations from protocol 2 (3.36-58.64 ng/ul). However this difference didn't have an impact of the PCR-sequencing identification.

Discussion

Molecular identification allows the study of fungi not only to the state of fruiting bodies but also as mycelium (Bellemain et al. 2010). For classical methods more taxonomic expertise is required than for molecular methods. The relative scarcity of trained taxonomists can lengthen the time it takes to identify all collections, and to complete a study (Schmit and Lodge, 2004). In this study we used two molecular techniques to extract DNA. Fragments obtained with the primers ITS1-F/ITS4-B were assayed by gel electrophoresis and good bands were seen on 1.5% agarose (Fig. 3). Both methods yielded high quality DNA from dried macrofungi to perform PCR for amplification of the ITS region. The DNA yields were high and pure enough to be readily amplified by PCR, and the PCR products were suitable for sequencing. For Protocol 1 the number of DNA extraction steps is minimal and avoiding dangerous chemicals (isopropanol, chloroform-isoamylalcohol); this makes the method convenient for molecular studies. Nevertheless, protocol 1 is more expensive than traditional techniques as the C-tab method. This procedure could probably be applied to most other macrofungi species as well. The efficiency and the speed of these methods, together with the use of inexpensive facilities, make them an attractive alternative for DNA extraction from macrofungi. One of the advantages of these procedures is that many samples can be simultaneously processed without any contamination risk and loss of DNA. The DNA extraction procedure can be completed within less than 2 hours. Thus many samples can be simultaneously processed in a short period of time. These two modified protocols for DNA extraction from macrofungi provide fast and reliable methods for generating ITS sequences necessary for systematic studies. These two protocols will enable rapid processing of macrofungi for studies using DNA. The modified protocol presented in this manuscript eliminates much of the laborious and time-consuming steps of most other DNA extraction protocols.

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