

*Full Length Research Paper*

# Non liquid nitrogen-based-method for isolation of DNA from filamentous fungi

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**A simple, efficient, reliable and cost-effective method for isolation of total genomic DNA from fungi, suitable for polymerase chain reaction (PCR) amplification and other molecular applications was described. The main advantages of the method are: (1) does not require the use of liquid nitrogen for preparation of fungi DNA; (2) the mycelium is directly recovered from Petri-dish cultures; (3) the quality and quantity of DNA obtained are suitable for molecular assays; (4) the technique is rapid and relatively easy to perform; (5) it can be applied to filamentous fungi from soil as well as from a fungi from other environmental sources; and (6) it does not require the use of expensive and specialized equipment or hazardous reagents. This method does not require liquid nitrogen for fixation, grinding or storage at -80°C, making it advantageous over other common protocols.**

**Key words:** Genomic DNA extraction, filamentous fungi, polymerase chain reaction (PCR) amplification.

## INTRODUCTION

Molecular methods such as polymerase chain reaction (PCR) are useful analytical tools for evaluating the microbial communities structure and function (Sambrook et al., 1988). PCR have become a common tool for detection, identification and characterization of microbial communities (Madigan et al., 2000; Stefan and Atlas, 1991). DNA extraction procedures are important parts of the investigations. These procedures must provide DNA in sufficient quantity and purity for molecular analyses especially when hundreds of samples need to be analyzed (Sambrook et al., 1988). We lack standardized and specific protocols for the routine molecular biology research of many filamentous fungi. A classic method is to compromise the integrity of the fungal cell wall and membrane, followed by the use of phenol/chloroform to isolate and purify the DNA from fungal cell (Bever et al., 2000). The drawback to this method is that loss of DNA can occur during the purification step, which is particularly important when attempting to isolate DNA from a small number of fungal cells. A similar type of

extraction protocol incorporates the use of isopropanol and ethanol to elute and purify DNA from samples (Elsas et al., 2000). The drawback to this type of assay is that it is time-consuming. Current methods of DNA extraction from filamentous fungi are either time-consuming and require toxic chemicals or are based on expensive technologies (Cheng and Jiang, 2006). They include the use of SDS/CTAB/proteinase K (Wilson, 1990), SDS lysis (Syn and Swarup, 2000), lysozyme/SDS (Flamm et al., 1984), high-speed cell disruption (Muller et al., 1998) and bead-vortexing/SDS lysis (Sambrook and Russel, 2001). Additionally, some give poor yields of DNA, as cell walls or capsules are difficult to lyse (Muller et al., 1998). Many of these methods rely on using a grinder (with or without liquid nitrogen) for initial breaking up of the mycelia. Considerable efforts have been made to facilitate improved DNA preparation from fungi (Liu et al., 1997). The major challenge for isolation of DNA of good quality and quantity from fungi lies in breaking the rigid cell walls, as they are often resistant to traditional DNA extraction procedures (Fredricks et al., 2005). Fungal nucleases and high polysaccharide contents add to the difficulties in isolating DNA from filamentous fungi (Zhang et al., 1996; Muller et al., 1998). Liquid nitrogen can be difficult to

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**Table 1.** List of fungal isolates used in this study.

Isolate number	Species	Source
R	<i>Rhizoctonia</i> sp.	Potato
F	<i>Fusarium solani</i>	Banana
F <sub>1</sub>	<i>Fusarium</i> sp.	Cotton
T <sub>1</sub>	<i>Trichoderma</i> sp.	Soil
A <sub>3</sub>	<i>Aspergillus</i> sp.	Citrus
T <sub>2</sub>	<i>Trichoderma</i> sp.	Soil
U	<i>Ulocladium</i> sp.	Tomato
P <sub>44</sub>	<i>Penicillium</i> sp.	Apple
P <sub>45</sub>	<i>Penicillium verrucosum</i>	Apple
P <sub>42</sub>	<i>Penicillium citrinum</i>	Apple

procure in remote locations; thus, a method not requiring its use would be helpful.

The objective of this study was to develop an easy and rapid protocol that does not require the use of liquid nitrogen for the isolation of good quality total DNA from filamentous fungi and do not require toxic chemicals. Our method is simple, efficient and produces DNA suitable for various molecular biology applications.

## MATERIALS AND METHODS

Ten fungi, listed in Table 1, were cultured on double layer media in 50 mm Petri dishes (Figure 1), one solid and the other liquid. Base media solid, was potato dextrose agar as a film, and the top media, liquid, was peptone yeast glucose (PYG, 1200 µl). Fungi were incubated at 25°C for two days. Fungi mycelia (50 mg) were scraped using slides covers and transferred to sterile Eppendorf tubes (1.5 ml) for DNA isolation.

### DNA isolation

Mycelium was homogenized with sterile extraction buffer using plastic pestle. Homogenized mycelium was handled as follows: mycelium was homogenized by polytron homogenizer in 400 µl of sterile extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Then, 6 µl of 20 mg ml<sup>-1</sup> RNase A (20 mg ml<sup>-1</sup> final concentration) were added and mixed well.

Samples were incubated at 65°C for 10 min; 130 µl of 3 M sodium acetate, pH 5.2 was added to each sample; samples were vortexed for 30 s at maximum speed, and incubated at -20°C for 10 min; the lysate was centrifuged at 13000 rpm and 4°C for 15 min; the supernatant was transferred to fresh tubes, an equal volume of isopropanol was added to each sample, mixed well and samples were incubated at -20°C for 10 min; Samples were then centrifuged for 20 min at 4°C, and 6000 rpm; washing the DNA pellets was performed twice using 700 µl of washing solution (100 and 70% ethanol, respectively); the DNA pellets were subsequently air dried in an oven at 40°C for at least 10 min; the resultant DNA pellet was then resuspended in 100 µl of 1 x TE (10 mM Tris-HCl, 1 mM EDTA) buffer (pH 8.0). All reagents were purchased from Sigma.

### Microsatellite analysis

Microsatellite analysis was done using T3B primer. PCR reaction

was done in a volume of 25 µl reaction mixture containing 10 ng of purified DNA, 20 pmol of primer, 10 mM of each dNTP, 1U Taq polymerase (ABgene, Epsom, UK), 10x PCR buffer and 10 mM of MgCl<sub>2</sub>. PCR amplification was performed in a C1000 Thermal Cycler (Bio-Rad Laboratories, CA, USA) programmed for 3 min at 95°C for denaturation, 40 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C with a final extension of 10 min at 72°C. PCR products were separated by gel-electrophoresis with 1.5% agarose gel in 1x TAE buffer at 150 V with TAE buffer for 50 min. DNA was visualized by UV fluorescence after staining with ethidium bromide. The primer tested is shown in Table 2.

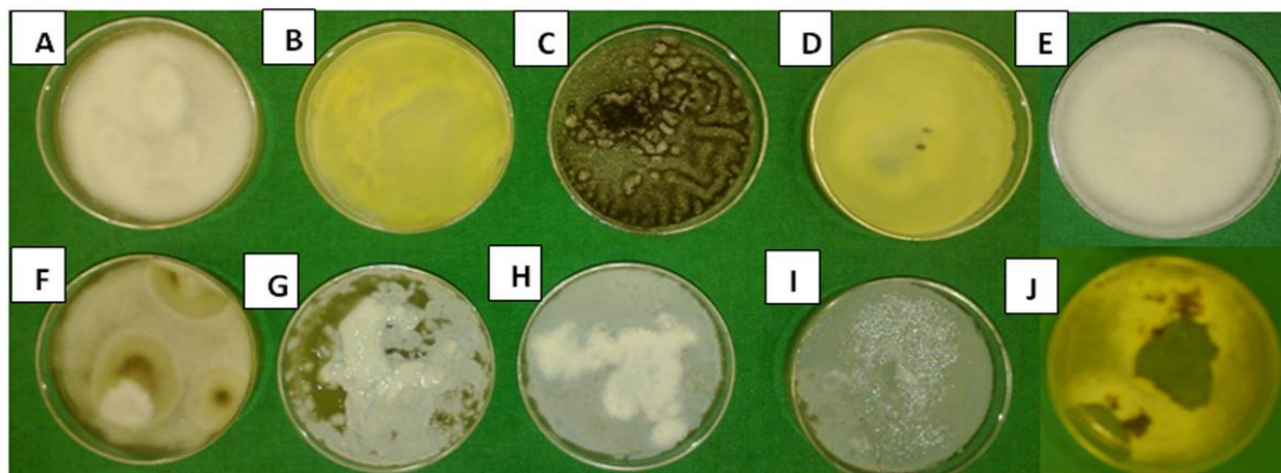
### Universally primed PCR

Amplification reactions were performed in 0.2 ml microcentrifuge tubes in a 25 µl reaction volume containing 10 mM Tris-HCl, at pH 8.8, 50 mM KCl, 0.8 mM NaCl, 3.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 0.4 mM dNTPs, 20 pmol for primer L21, 1.0 U Taq DNA polymerase (Jena Bioscience) and 10 to 15 ng genomic DNA. PCR amplification was performed in a C1000 Thermal Cycler (Bio-Rad Laboratories, CA, USA) programmed for 30 cycles of denaturation at 94°C for 30 s (first denaturation step at 94°C for 3 min), annealing at 56°C for 70 s and polymerization at 72°C for 60 s, with a final extension step of 72°C for 5 min. The reaction tubes were held at 4°C following the final amplification cycle. 2 µl universally primed PCR (UP-PCR) products (1/10 of the total reaction volume) were electrophoresed on 1.5% agarose gel at 150 V with TAE buffer for 50 min. The primer tested is shown in Table 2.

## RESULTS AND DISCUSSION

We report here a simple method for culturing filamentous fungi for isolation of DNA that can be completed within two days from the beginning to end. The growth of mycelia on Petri dishes eliminates the need for still or shaking liquid cultures. Fungal mycelium mats (50 mg) from 2-day-old colonies grown on duplex agar medium was homogenized to DNA extraction. For DNA extraction, typical fungi were grown in liquid shake cultures in Erlenmeyer flasks, Roux bottles or even microtubes (Cenis, 1992).

Since the currently available DNA extraction protocols are rather costly and time-consuming (Wilson, 1990; Syn and Swarup, 2000; Sambrook and Russel, 2001), we



**Figure 1.** Fungi cultured on double layer media in 50 mm Petri dishes. A, *Fusarium solani*; B, *Trichoderma* sp.; C, *Aspergillus* sp.; D, *Trichoderma* sp.; E, *Fusarium* sp.; F, *Ulocladium* sp.; G, *Penicillium* sp.; H, *Penicillium verrucosum*; I, *Penicillium citrinum*; J, *Rhizoctonia* sp.

**Table 2.** Primers used to amplify fungal DNA.

Primer code	Sequence	Amplified region	Reference
T3B	5'-AGG TCG CGG GTT CGA ATC C-3'	Microsatellite repeats	Meyer et al. (2001)
L21	5'-GGA TCC GAG GGT GGC GGT TCT-3'	Universally primed-PCR-derived sequence	Bulat et al. (1998)

adapted a rapid DNA isolation method by combining chemical reagent digestion with mechanical disruption for lysing mycelium of many kinds of filamentous fungi. The whole procedure required approximately about an hour to prepare high quality DNA for any molecular biology application (Figure 2).

The mechanical grinding procedure for isolation of DNA from cells in DNA extracting buffer is very simple and cost effective and it does not need the use of liquid nitrogen, which is often problematic and hazardous or difficult to procure in remote locations, especially when large numbers of samples need to be examined. Motorized pulverization of mycelium generates cell lysates, and often works faster than using cell-wall degrading enzymes or high temperatures (Lugert et al., 2006).

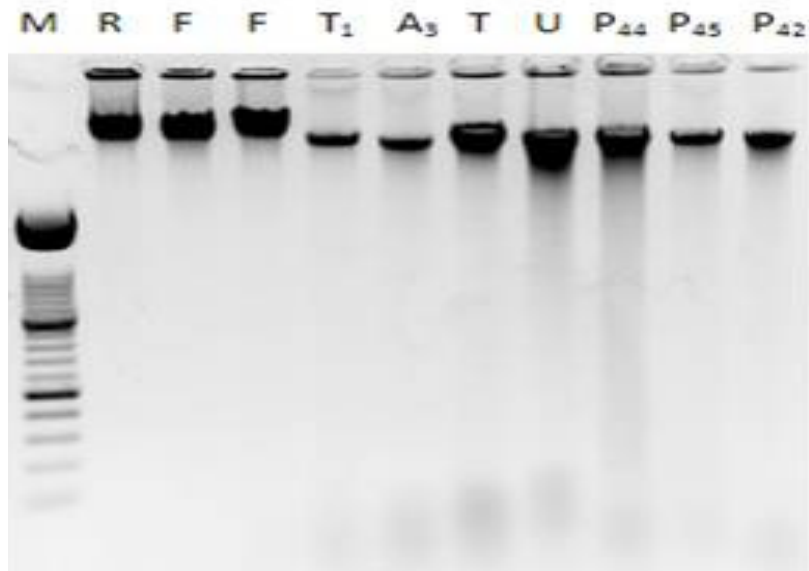
This DNA extraction method has several advantages: a) good yields of high quality genomic DNA; b) there is no need for liquid nitrogen during crushing of the fungal material; c) the number of DNA extraction steps is minimal; d) it is low-cost, as only small amounts of chemicals and little equipment are employed, and e) does not use toxic and potentially hazardous substances such as phenol and chloroform.

In this study, a number of principles were applied to simplify the DNA extraction procedure that did not adversely affect the DNA quality and quantity. The procedure protocol does not include the need for liquid

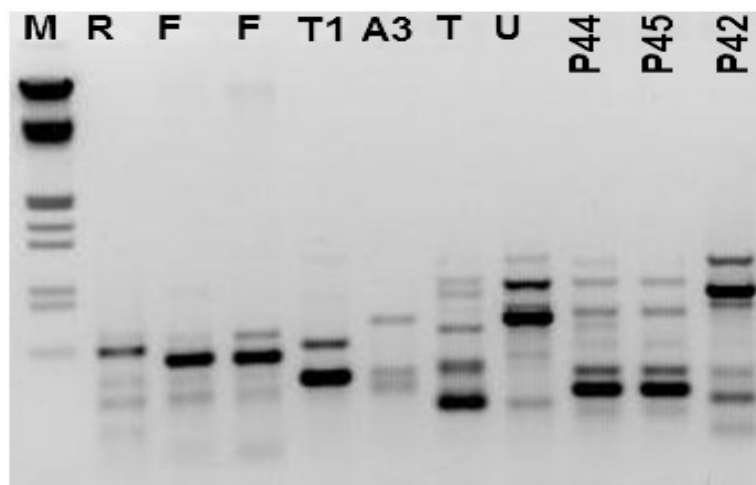
nitrogen for crushing the fungi material. A similar procedure for isolation of DNA from date palms was followed by Ouenzar et al. (1998) and Liu et al. (2000). The development of a rapid DNA extraction method that does not use toxic and potentially hazardous substances, such as phenol and chloroform, should now make practical the large-scale characterization of plant pathogenic fungi which is more easy and safe for researchers using molecular methods based on PCR (Mahuku, 2004). High molecular weight DNA (>40 kb, Figure 2) resulted from the procedure. The  $\lambda_{260}/\lambda_{280}$  ratio was greater than 1.6 (ranging from 1.86 to 2.08), indicating DNA purity and no apparent smear (Sambrook et al., 1989; Henry, 1997). DNA yields were in the range of 20 to 40  $\mu\text{g}$  (in 100- $\mu\text{l}$  elution volumes) from all fungal material tested.

We evaluated the quality of the extracted DNA through two PCR-based techniques: Microsatellite and Universally primed-PCR (Figures 3 and 4). DNA isolated was used for PCR amplification with T3B Microsatellite primer and L21 Universally primed. The DNA isolated produced good banding patterns indicating good quality DNA. The DNA extraction buffer was capable of releasing substantial quantities of DNA from the *Fusarium* mycelium (ranging from 0.5 to 1.6  $\text{mg g}^{-1}$  mycelium), without a separate step for mechanical disruption of cell walls (De-Nijs et al., 1996).

The DNA isolated from all fungi isolates generated



**Figure 2.** Agarose gel electrophoresis of extracted genomic DNA (1.5%) isolated from 10 different filamentous fungi. For each isolate, 10 ng/μl genomic DNA was loaded. R = *Rhizoctonia* sp., F = *Fusarium solani*, F1 = *Fusarium* sp., T1 = *Trichoderma* sp., A3 = *Aspergillus*, T = *Trichoderma*, U = *Ulocladium*, P44 = *Penicillium* sp., P45 = *Penicillium verrucosum*, P42 = *Penicillium citrinum*. M = 100 bp DNA molecular weight ladder.

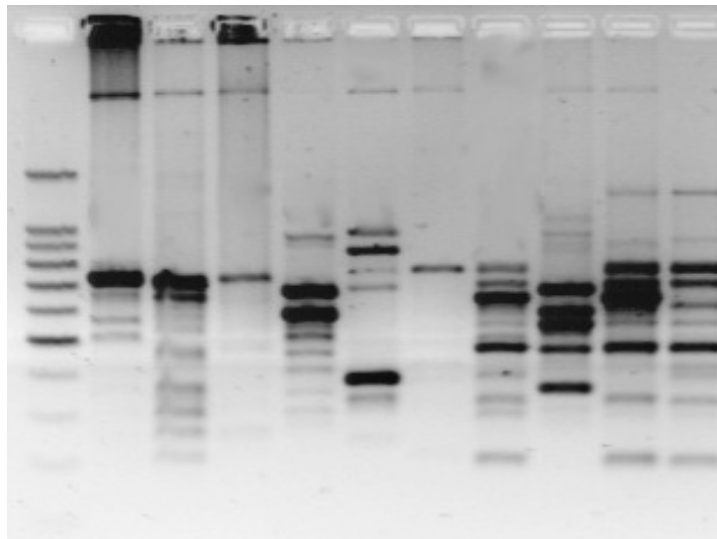


**Figure 3.** Agarose gel (1.5%) electrophoresis of the microsatellite-primed PCR products obtained by using the T3B primer. M = 100 bp DNA molecular weight ladder. For identification of lanes, see legend for Figure 2.

reproducible PCR amplification products that were well resolved on 1.5% agarose gels. This further confirmed that the isolated DNA was free of polysaccharide and polyphenols, which are known to inhibit Taq DNA polymerase and restriction endonucleases (Moyo et al., 2008). Pure DNA eluted from the agarose gels ensures reliable DNA amplification by PCR. Our method does not require liquid nitrogen or expensive commercial DNA extraction kits, which significantly decreases costs and

time for DNA analysis (Moslem et al., 2010).

The growth of the fungus for only two days before DNA extraction also facilitates homogenization and disruption of mycelium as it is not yet melanized. The time required for our DNA extraction method is about 60 min, which is fast when compared with other genomic DNA extraction methods described. Our extraction method generated DNA that can be used in various molecular analyses. The method is especially useful in laboratories that lack the



**Figure 4.** Agarose gel (1.5%) electrophoresis of universally primed-PCR products obtained by using the L 21 primer. M = 100 bp DNA molecular weight ladder. For identification of lanes, see legend for Figure 2.

facilities to work with liquid nitrogen.

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