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NOTE

A Rapid PCR-Based Approach for Molecular Identification of Filamentous Fungi

Yuanyuan Chen[1], Bernard A. Prior[2], Guiyang Shi[1], and Zhengxiang Wang[1]*

[1]*The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, P. R. China*
[2]*Department of Microbiology, School of Biological Sciences, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa*

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In this study, a novel rapid and efficient DNA extraction method based on alkaline lysis, which can deal with a large number of filamentous fungal isolates in the same batch, was established. The filamentous fungal genomic DNA required only 20 min to prepare and can be directly used as a template for PCR amplification. The amplified internal transcribed spacer regions were easy to identify by analysis. The extracted DNA also can be used to amplify other protein-coding genes for fungal identification. This method can be used for rapid systematic identification of filamentous fungal isolates.

***Keywords*:** filamentous fungi, genomic DNA extraction, alkaline lysis, internal transcribed spacer regions, identification

Fungi are among the most ecologically important organisms in the world with an estimated 1.5 million fungal species of which less than 5% have been described (Hawksworth, 2001). Fungal identification has traditionally been based on the phenotype of the organism. However, phenotype-based identification can be subjective and molecular comparisons have shown that some earlier identification based on phenotype were inaccurate (Bruns *et al*., 1991; Guarro *et al*., 1999).

With the advances in polymerase chain reaction (PCR) and DNA sequencing technologies, molecular techniques have now become standard approaches when dealing with fungal identification (Bridge, 2002; Blackwell *et al*., 2006). The most relevant molecular methods used in the identification of fungi are based on the variability of the ribosomal genes (White *et al*., 1990; Hibbett, 1992). As the internal transcribed spacer (ITS) regions display the greatest sequence and size variation of the rDNA gene cluster, these regions can be used to identify fungi at the species level via homology searches of sequences in the public domain (Gardes and Bruns, 1993).

The fungal cell wall is a complex structure composed of chitin, glucans, lipids and other polymers which are resistant to enzymatic digestion and chemical breakdown. Therefore the major challenge for isolation of DNA of good quality and quantity from fungi lies in breaking the sturdy cell walls using physical, chemical and enzymatic disruption methods (Karakousisa *et al*., 2006). Current methods for filamentous fungal DNA extraction include using lysis buffer containing sodium dodecyl sulphate (SDS) and proteinase K (Mahuku, 2004), benzyl chloride (Zhu *et al*., 1993), alkaline chemicals (Bir *et al*., 1995), grinding with liquid nitrogen, glass bead milling (Wu *et al*., 2001; Dean *et al*., 2004; Melo *et al*., 2006), microwave treatment (Tendulkar *et al*., 2003) or use of a DNA isolation kit (Fredricks *et al*., 2005). However, most of the methods are designed to obtain very pure DNA, requiring complex extraction and purification protocols, which are time-consuming, with the extraction process taking several hours. Furthermore, some of these methods are expensive and unable to deal with large number of samples (Manian *et al*., 2003; Fredricks *et al*., 2005).

In the present study, a novel method for DNA extraction from bacteria and higher eukaryotic samples (Chomczynski and Rymaszewski, 2006) was further developed for the extraction of filamentous fungal genomic DNA and successfully applied for rapid identification of fungal isolates. Only 20 min were required to prepare the genomic DNA by the alkaline lysis method. The extracted genomic DNAs could be directly used as templates for PCR amplification and the subsequent PCR products were sufficient for sequencing and homology analysis.

Fungal strains, medium, and culture conditions

The 16 fungal strains used in this study are shown in Table 1. All strains were acquired from the Culture & Information Center of Industrial Microorganisms of China Universities (CICIM-CU) or the American Type Culture Collection (ATCC). These strains were grown on potato dextrose agar (PDA; Difco 213400) plates covered with a cellophane disk at 28°C for 1-3 days.

Rapid DNA extraction by alkaline lysis and optimization of extraction conditions

Our method was adopted with modifications from the method of Chomczynski and Rymaszewski (Chomczynski and Rymaszewski, 2006). Fungal mycelia of four species (*Aspergillus niger* CICIM F0313, *Neurospora intermedia* CICIM F0844, *Penicil-*

* For correspondence. E-mail: zxwang@jiangnan.edu.cn; Tel.: +86-510-85918121

Table 1. List of fungal strains used in this study

No.	Strains	Source
1	*Alternaria tenuissima* CICIM F0803	CICIM-CU
2	*Aspergillus flavus* ATCC11498	ATCC
3	*Aspergillus foetidus* ATCC16878	ATCC
4	*Aspergillus niger* CICIM F0313	CICIM-CU
5	*Cladosporium cladosporioides* CICIM F0374	CICIM-CU
6	*Coniochaeta ligniaria* CICIM F0426	CICIM-CU
7	*Fusarium oxysporum* CICIM F0409	CICIM-CU
8	*Mucor circinelloides* CICIM F0362	CICIM-CU
9	*Neurospora intermedia* CICIM F0844	CICIM-CU
10	*Penicillium oxalicum* CICIM F0310	CICIM-CU
11	*Penicillium crustosum* CICIM F0354	CICIM-CU
12	*Pseudeurotium bakeri* CICIM F0378	CICIM-CU
13	*Rhizomucor variabilis* CICIM F0355	CICIM-CU
14	*Rhizopus microsporus* CICIM F0424	CICIM-CU
15	*Talaromyces flavus* CICIM F0884	CICIM-CU
16	*Trichoderma harzianum* CICIM F0357	CICIM-CU

lium crustosum CICIM F0354, and *Trichoderma harzianum* CICIM F0357) were collected from culture plates and 20 mg of each mycelium was added into a 1.5 ml Eppendorf tube with 200 µl of solution I, which contained 2 mM EDTA and 0.01% Tween 20. The mycelium was suspended by vigorous vortexing and the biomass harvested by centrifugation at 10,000×g for 1 min. To each fungal mycelial sample, 100 µl alkaline lysis solution II consisting of 20 mM potassium hydroxide and 60% polyethylene glycol 200, pH 13.4 was added. To improve the fungal DNA extraction, the tube was vortexed and incubated in a heat block (HB100, Bioer, Japan) at different temperatures (room temperature, 50°C, 70°C, and 90°C) for 15 min. The fungal lysates containing extracted fungal genomic DNA were directly used as templates for PCR amplification of the ITS regions.

Microscopic examination

Four standard fungal cultures and the mycelium treated by alkaline lysis at 90°C for 15 min were examined under 400 fold magnification using a light microscope (Axiostar plus, Zeiss, Germany). Images were collected using the Infinity capture application v4.5 software (Lumenera Corporation, Canada).

DNA purification

In order to estimate the yield of DNA (µg) per 20 mg fungal biomass, a 1:1 phenol:$CHCl_3$ mixture was added to an equal volume of the crude DNA extract. After vortexing for 1 min, the mixture was centrifuged at 10,000×g and the aqueous layer was removed. The supernatant was treated with phenol:chloroform:isoamyl alcohol (25:24:1) and the DNA was precipitated with isopropanol. Finally, the DNA was dissolved in 50 µl distilled water. The DNA concentration was obtained by measuring absorbance at 260 nm with an ultraviolet spectrophotometer (U0080D, Hitachi, Japan).

Amplification of ITS region and protein-coding genes

The PCR was carried out in a 50 µl reaction volume in a 0.2 ml tube. The PCR mixture contained 0.2 µl of *Ex Taq* DNA polymerase (TaKaRa, Japan), 5 µl of 10× PCR buffer, 4 µl of dNTPs mixture (2.5 mM of each nucleotide) (TaKaRa), 1 µl of the fungal lysate and 0.5 µl of each universal primer (0.1 µM). The set of primers ITS1 (5′-TCCGTAGGTGAACC TGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) was used for amplifying the ITS region (White *et al*., 1990). The primer pairs for amplifying the elongation factor 1-alpha (*EF-1α*) gene were 983F (5′-GCYCCYGGHCAYCGTGAYT TYAT-3′) and 2218R (5′-ATGACACCRACRGCRACRGTY TG-3′) (Rehner and Buckley, 2005), the primer pairs for amplifying the RNA polymerase II subunit (*RPB2*) gene were fRPB2-5F (5′-GAYGAYMGWGATCAYTTYGG-3′) and bRPB2-7R (5′-GAYTGRTTRTGRTCRGGGAAVGG-3′) (Matheny, 2006). The PCR reaction was performed in an automated thermal cycler (PTC-200, Bio-Rad, USA) with the following steps: an initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 54°C (for ITS1/ITS4) or 53°C (for 983F/2218R) or 46°C (for fRPB2-5F/bRPB2-7R) for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 10 min (White *et al*., 1990; Rehner and Buckley, 2005).

Gel electrophoresis

The PCR products were separated by electrophoresis on 1.0% (w/v) agarose gels (Promega, USA) in 0.5× TBE buffer with DL2000 DNA marker (TaKaRa) using a horizontal electrophoresis system (Bio-Rad) for about 1 h. Gels were stained with ethidium bromide, visualized under UV light and digitized using an Alphaimager EC system (Alpha Innotech, USA).

Sequencing and BLAST analysis

The PCR products were purified using a QIAquick PCR Purification kit (QIAGEN, Germany) according to the product instructions. Selected PCR products were sequenced using an ABI BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and the sequences were read on an Applied Biosystems 3130 genetic analyzer (Applied Biosystems). Sequence analysis was performed using Sequence Scanner v1.0

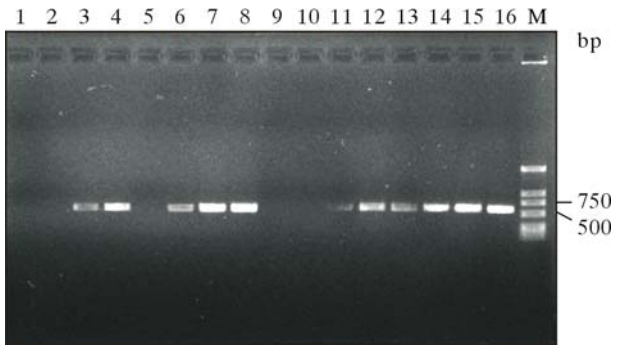

Fig. 1. Agarose gel analysis of ITS region of four fungal strains incubated at different temperatures. Lanes: 1-4, *A. niger* CICIM F0313 incubated at room temperature, 50°C, 70°C, and 90°C; 5-8, *N. intermedia* CICIM F0844 incubated at room temperature, 50°C, 70°C and 90°C; 9-12, *P. crustosum* CICIM F0354 incubated at room temperature, 50°C, 70°C, and 90°C; 13-16, *T. harzianum* CICIM F0357 incubated at room temperature, 50°C, 70°C, and 90°C; M, DL 2000 DNA marker.

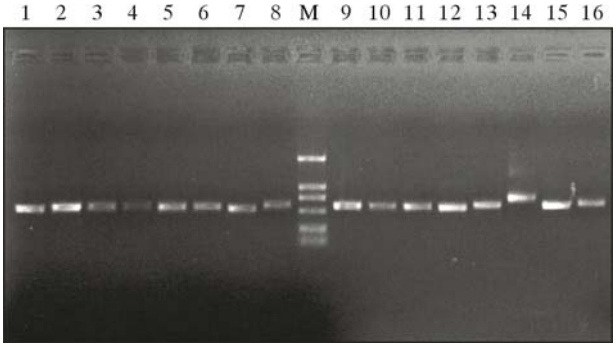

Fig. 2. Agarose gel electrophoresis of ITS-PCR products. Lanes: 1-16, correspond to ITS regions of fungal strains number1-16; M, DL2000 DNA marker.

software, and then compared with sequences in the GenBank databases using the BLAST program (www.ncbi.nlm.nih.gov).

Optimization of DNA extraction conditions at different temperatures

To assess the most efficient condition for extraction of fungal DNA, we selected four different fungal species to determine the best extraction temperature for 15 min. As demonstrated in Fig. 1, all of the ITS regions of the four fungal strains were amplified successfully when the extraction was conducted at 90°C for 15 min. Extraction at 70°C resulted in bands of lower intensity and at 50°C and at room temperature the band intensities were weak or absent.

Genomic DNA yields by alkaline lysis

Extraction of 20 mg of wet fungal biomass from *Aspergillus niger* CICIM F0313, *Neurospora intermedia* CICIM F0844, *Penicillium crustosum* CICIM F0354, and *Trichoderma harzianum* CICIM F0357, respectively yielded (Mean±SD of triplicate extractions) 2.5±0.2, 3.7±0.1, 3.1±0.2 and 5.0±0.2 μg DNA. These concentrations are adequate to obtain a meaningful PCR reaction (Sambrook and Russell, 2001).

Analysis of the quality of PCR products

As demonstrated in Fig. 2, electrophoresis of PCR products in agarose gel revealed clear 550 bp DNA bands. Although some bands are faint in Fig. 2, the ITS regions of all 16 fungal species were amplified efficiently showing that the amount of the DNA obtained by this procedure was adequate for PCR amplification. Although the fungal lysates, which served as templates for PCR, had a pH higher than 13, their pH dropped to 8.8 in the PCR buffer and were within the effective range for PCR (Sambrook and Russell, 2001). The 16 species used in this study belonged to 13 genera and thus these results demonstrate that this new method has wide applicability in filamentous fungal genomic DNA extraction and analysis. The extracted crude DNA also can be used to amplify the *EF-1α* and *RPB2* genes, which are used in fungal identification (Fig. 3).

Microscopic analysis of fungal cell extraction

As demonstrated in Fig. 4, the alkaline treatment at 90°C for 15 min of four fungi resulted in the lysis of the cells. Microscopic analysis indicated that the degree of lysis was less in *A. niger* CICIM F0313 (Fig. 4A) than in the other three fungi yet sufficient genomic DNA was released. In the present study, the mycelium was directly collected from cellophane disks overlaid on solid medium. We found the efficiency of DNA extraction relates closely with culture time of fungi and the use of young mycelia and spores was the most important factor in successful fungal DNA isolation (data not shown).

Analysis of the stability of extracted genomic DNA

The extracted genomic DNA stored at 4°C for 4 weeks was added as template in an ITS-PCR reaction and the results of DNA electrophoresis showed that there were clear target bands in the agarose gel (data not shown). This result supports the conclusion that fungal genomic DNA extracted by this method is stable for at least 4 weeks.

Sequencing results and homology analysis

Sequencing results were analyzed using ABI SeqScanner v1.0 software, which showed that the PCR products were of sufficient quantity for sequencing analysis. The BLAST analysis of sequences using data lodged in the website of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) always corresponded to the original name of the fungus (Table 1).

Using the method described here, the sequence-based alignment and analysis of ITS regions include the following steps: culture of the fungal strain, genomic DNA extraction, PCR assay, DNA sequencing and homology BLAST analysis. The PCR assay and DNA sequencing are already largely automated and therefore fungal genomic DNA extraction has become the bottleneck for identifying large numbers of fungal samples.

In most DNA extraction methods described (Mahuku, 2004), several time-consuming DNA isolation steps are needed to obtain the fungal genomic DNA after breaking the cell walls.

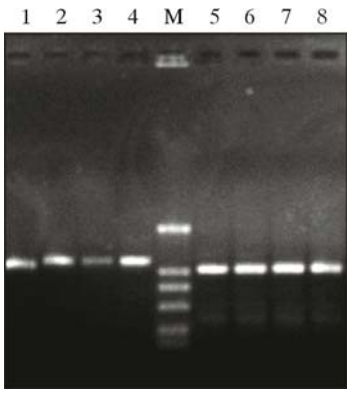

Fig. 3. Amplification of *RPB2* and *EF-1α* genes of four fungal strains. Lanes: 1-4, correspond to *RPB2* gene of *F. oxysporum* CICIM F0409, *N. intermedia* CICIM F0844, *P. oxalicum* CICIM F0310, and *R. variabilis* CICIM F0355; 5-8, correspond to *EF-1α* gene of *A. niger* CICIM F0313, *P. crustosum* CICIM F0354, *R. microspores* CICIM F0424, and *T. harzianum* CICIM F0357; M, DL2000 DNA marker.

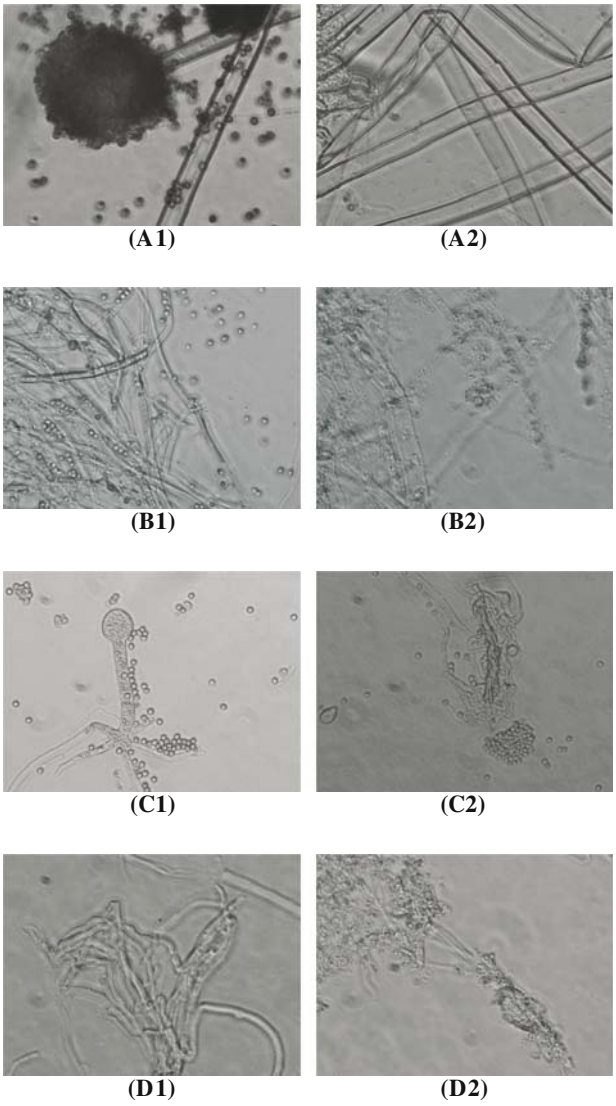

Fig. 4. Microscopic examination (400 fold magnification) of *A. niger* CICIM F0313 (A), *N. intermedia* CICIM F0844 (B), *P. crustosum* CICIM F0354 (C), and *T. harzianum* CICIM F0357 (D). Four fungal species were incubated at 90°C under alkaline conditions. A1, B1, C1, and D1 are fungal mycelia prior of lysis and A2, B2, C2, and D2 are fungal mycelia after incubation at 90°C for 15 min with alkaline solution II.

The extraction protocols take at least 3 h for each sample. However, PCR is such a highly sensitive technique that it is able to amplify very small amounts of DNA, and therefore the extraction protocols do not need to be so extensive for PCR reactions.

A rapid, easy and low-cost method for extraction of filamentous fungal genomic DNA was established in this study. Up to now, we have already successfully identified more than 700 filamentous fungal isolates, which grouped into 89 species belonging to 35 genera, based on this method. Compared to other rapid methods of fungal DNA extraction, the whole DNA extraction procedure in this study can be completed within 20 min in an Eppendorf tube without the need for special equipment, expensive DNA isolation kits, liquid nitrogen, toxic reagents, and long incubation times (Loffler *et al*., 1997; Müller *et al*., 1998; Wu *et al*., 2001; Griffin *et al*., 2002; Manian *et al*., 2003; Fredricks *et al*., 2005). Another advantage of this method is that it can deal with a large number of samples in the same batch. The quantity and quality of the DNA obtained by this method were suitable for ITS-PCR amplification and further DNA analysis. In addition, we found the extracted DNA could be used to amplify other protein-coding genes, such as *EF-1α* and *RPB2*, used in fungal identification.

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