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A rapid and efficient method of fungal genomic DNA extraction, suitable for PCR based molecular methods

Aamir S, Sutar S, Singh SK, Baghela A

National Fungal Culture Collection of India, Biodiversity and Palaeobiology Group, Agharkar Research Institute, Gopal Ganesh Agarkar Road, Pune, India 411004

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Abstract

We report a rapid and efficient method of genomic DNA extraction from filamentous fungi with high-throughput potential. The method involves disruption of fungal cell by bead beating in a homogenizer, followed by RNase treatment, phenol: chloroform: Isoamyl alcohol extraction and precipitation with isopropanol. The method does not involve any enzymatic digestion and it can be completed within 2.5 hours. The method yielded good quality and quantity (60 µg – 230 µg/200 mg of wet fungal mass) of the DNA. Being a closed system of gDNA extraction, our method has been found to be useful in avoiding the laboratory borne contamination during DNA extraction. The extracted DNA was found to be suitable for PCR based molecular methods like single and multi-copy gene amplification and RAPD analysis.

Key words – β-tubulin – DNA isolation – Fungi – ITS-rDNA – RAPD

Introduction

The development of PCR-based molecular techniques has become the method of choice for identification & characterization of fungi. It becomes important to isolate genomic DNA (gDNA) of superior quality and quantity for analyzing through PCR based applications. The major challenge for isolation of such DNA from fungi lies in breaking the rigid cell walls, as, they are often resistant to traditional DNA extraction procedures (Fredricks et al. 2005). Generally there are two major steps involved in fungal genomic DNA extraction, which are disruption of cell wall and extraction followed by purification of genomic DNA. The extraction of genomic DNA is usually done with cetyl trimethyl ammonium bromide (CTAB) extraction buffer (Doyle & Doyle 1987) followed by purification through phenol/chloroform extraction and precipitation with isopropanol or ethanol (Ashktorab & Cohen 1992). Out of several methods applied for breaking cell walls, one of the most common methods is grinding of mycelia using glass rods or liquid nitrogen (Lee et al. 1988, Wu et al. 2001). Magnetic beads (Faggi et al. 2005), microwave exposure (Goodwin & Lee 1993), dry ice (Griffin et al. 2002), benzyl chloride (Xue et al. 2006), enzyme digestion (Li et al. 2002), bead–vortexing/SDS lysis and high speed cell disruption (Sambrook & Russel 2001, Muller et al. 1998) and sometimes in combination (Zhang et al. 2008) have been used by some researchers. However,

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Corresponding Author: Abhishek Baghela – e-mail –abhishekbaghela@aripune.org

most of these protocols and techniques results in variable quality and quantity of DNA and are time consuming as well.

In recent years, studies on fungal communities and fungal diversity studies have increased rapidly (Dighton et al. 2005). The large scale community studies often require the identification of a large number of fungal species and strains, and for molecular identification of such a large numbers of fungal isolates, the genomic DNA also needs to be isolated in a high-throughput manner. Barring few commercially available DNA extraction kits, many of the available fungal gDNA extraction methods are time consuming, and are not suitable for a large number of samples. In addition, for a fungal culture collection or a fungal molecular identification service provider like us (National Fungal Culture Collection of India [NFCCI], Pune, India), the timely identification also depends upon, how fast one can get the fungal gDNA. A rapid method of gDNA extraction would effectively reduce the time for DNA sequencing based molecular identification of fungi. Therefore, we developed a rapid method of fungal gDNA extraction with high throughput potential.

Materials & Methods

Fungal strains

A total number of fifteen fungal strains available at National Fungal Culture Collection of India (NFCCI) – Agharkar Research Institute, Pune, India were included in the study (Table 1).

Fungal genomic DNA extraction

The genomic DNA was extracted from five to seven days old fungal cultures grown either in liquid broth or culture plates. The fungal mass from the culture plate was scraped out with the help of a fine spatula and fungal mass from the culture broth was obtained by filtering the culture broth through a 10 ml syringes containing glass wool that will allow the broth to pass through, while retaining the fungal mass. The fungal mass obtained from the culture plate or broth was placed in a 2ml tube containing a ceramic pestle, 60–80 mg sterile glass beads (425–600 µM, Sigma) and lysis buffer (100 mM Tris HCl [pH8.0], 50mM EDTA, 3% SDS). Homogenization of fungal mass was done twice in a FastPrep®-24 tissue homogenizer (MP Biomedicals, USA) at 6 M/S for 60 sec. The resulting fungal tissue homogenate was centrifuge at 13,000 rpm for 10 min and supernatant was transferred to a fresh microcentrifuge tube. To the supernatant, 2 of RNase A (10mg/ml) was added and incubated at 37°C for 15 min. After the RNase A treatment, equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1) was added and mixed well, followed by centrifugation at 13,000 rpm for 10 min (Note: this step can be repeated once more to completely get rid of proteins/cell debris). The upper aqueous layer was taken in a fresh micro centrifuge tube and then equal volume of 100% ethanol was added. Following precipitation at -20°C for 30 min, the whole content was centrifuged at 12,000 rpm for 10 min to pellet down the DNA. The DNA pellet was washed with 70% ethanol and centrifuged at 12,000 rpm for 5 min. The DNA pellets were air dried and dissolved in 1× TE buffer.

Quantity and quality determination

The quantity of the extracted gDNA was determined by measuring the absorbance at 260 nm using Thermo Scientific Nano Drop 1000 spectrophotometer. The quality of extracted gDNA was accessed by subjecting them on 0.8% agarose gel electrophoresis and suitability for downstream application in RAPD (Random Amplified polymorphic DNA) analysis, single copy gene and multi-copy gene amplification. The PCR reactions for RAPD analysis were performed in a 25µl reaction volume containing 16 µl PCR grade water (Sigma), 100ng of genomic DNA, 2.5 µl of 10 × reaction buffers, 2.5 µl of 10mM dNTPs mix (Sigma-Aldrich), 2µl (10 pmol/µl) of random decamer oligonucleotide primer OPA–1 (5′-CAGGCCCTTC-3′), and 1 µl (5 U/µl) of Taq DNA polymerase (Sigma-Aldrich). Amplification was performed in an Eppendorf Master Cycler (Eppendorf, Hamburg). The PCR cycling conditions consisted of an initial denatuartion step of

94°C for 2 min and subjected to 40 cycles of the following program 94°C for 30 s, 37°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min.

Table 1 Fungal strains used in the study, their NFCCI numbers, source and place of isolation and DNA yield

S. No.	Fungal strains	NFCCI* accession numbers	Source & place	DNA yield µg/200mg of fungal mass
1	*Cladosporium tenuissimum*	3497	Bat guano, Uttar Pradesh (U.P.), India	110
2	*Penicillium concentricum*	3564	Bat guano, U.P., India	115
3	*Emericella variecolor*	3496	Bat guano, U.P., India	80
4	*Aspergillus oryzae*	3495	Bat guano, U.P., India	190
5	*Aspergillus flavus*	3499	Bat guano, U.P., India	195
6	*Aspergillus terreus*	3547	Soil, Kerala, India	112
7	*Curvularia affinis*	3523	Rotten bamboo, Assam, India	110
8	*Rigidoporus vinctus*	3522	Rotten bamboo, Assam, India	120
9	*Inonotus pachyphloeus*	3571	Rotten bark, Assam, India	155
10	*Curvularia lunata*	3548	Turmeric leaf, Tamil Nadu, India	60
11	*Earliella scabrosa*	3584	Tamil Nadu, India	220
12	*Penicillium oxalicum*	3582	West Bengal, India	220
13	*Fusarium incarnatum*	3598	Crossandra, Tamil Nadu, India	225
14	*Fusarium phaseoli*	3238	Papaya root, Bihar, India	230
15	*Fusarium sp.*	3634	*Terminalia arjuna*, Tripura India	225

***** NFCCI – National Fungal Culture Collection of India, Agharkar Research Institute, Pune, India.

A single copy β-tubulin gene was amplified from the extracted gDNA using the primers described by Chauhan et al. 2007. The PCRs were performed in a 25µl reaction volume containing 16µl PCR grade water (Sigma), 2µl (10 pmol/µl) each of β-tubulin gene specific forward (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and reverse (5'-ACCCTCAGTGTAGTGACCCTT GGC-3') primers, 2.5 µl of 10 × reaction buffers, 2.5 µl of 10mM dNTPs mix (Sigma-Aldrich), 1µl (5 U/µl) Taq DNA polymerase (Sigma-Aldrich), and approximately 20–50 ng of gDNA. PCR was performed in an Eppendorf Master Cycler (Eppendorf, Hamburg) following the conditions described by Chauhan et al. 2007.

The multi-copy ITS-rDNA gene amplification was performed with universal fungal primer pairs ITS4/ITS5 (White et al. 1990). The PCRs were performed in a 25µl reaction volume containing 16µl PCR grade water (Sigma), 2.5 µl PCR buffer (10×), 2.5 µl of 10mM dNTPs mix (Sigma-Aldrich), 1µl of each primer (20 pmol/µl), 1 µl (5 U/µl) of Taq polymerase (Sigma-Aldrich) along with 20–50ng of template DNA. PCR was performed in an Eppendorf Master Cycler (Eppendorf, Hamburg). The amplification program consisted of an initial denaturation step at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing for 30 seconds at 55 °C and extension for 1 min at 72 °C. A final extension step at 72 °C for 7 min was included at the end of the amplification. All PCR products were electrophoresed, imaged and analyzed in a Gel Documentation System (Syngene Inc. Cambridge).

Results & discussion

The proposed fungal gDNA extraction method yielded good quality high molecular weight DNA from filamentous fungi, as evident upon agarose gel electrophoresis (Fig. 1A). The total genomic DNA yield was found to be in range of 60 µg – 230 µg/200 mg of wet fungal mass (Table1). The DNA yield was found to be comparable with the previously described methods (Abd-elsalam et al. 2003, Al-Samarrai et al. 2000, Amer et al. 2011, Faggi et al. 2005, Mishra et al. 2014, Griffin et al. 2002, Griffiths et al. 2006, Karakousis et al. 2006, Karthikeyan et al. 2010, Lee et al. 1988, Liu et. al. 2000, Mendoza et al. 2010, Mishra et al. 2008, Moller et al. 1992, Nawrot et al. 2010, Płaza et al. 2004, Prabha et al. 2013, Van Burik et al. 1998, Zhang et al. 2010) (Table 2). The gDNA from yeasts could also be isolated by using the developed protocol (data not shown). The isolated DNA was found to be suitable for various downstream PCR based molecular biology applications. We could get successful amplification by using random decamer primer OPA-1 in RAPD analysis for all the fifteen samples tested (Fig. 1B). The ITS (internal transcribed spacer) region of rDNA has been considered as a de facto barcode for most of the fungi (Schoch et al. 2012). Our developed method could successfully amplify a multi-copy (ITS-rDNA) gene, which confirmed that the extracted DNA could be used for molecular identification as well as phylogenetic studies (Fig. 1C). Protein-coding single–copy genes are also widely used in mycology, for both higher-level phylogenetic studies and species level identification, for example translation elongation factor 1-α has been used for Fusarium (O'Donnell et al. 2010) and β-tubulin for Penicillium (Frisvad & Samson 2004). The DNA isolated by the proposed method could be used to amplify a portion (400–450bp) of a single copy gene like β-tubulin from the tested fungi, which shows that the quality and quantity of the isolated DNA is suitable even for single copy protein coding gene amplification as well (Fig. 1D).

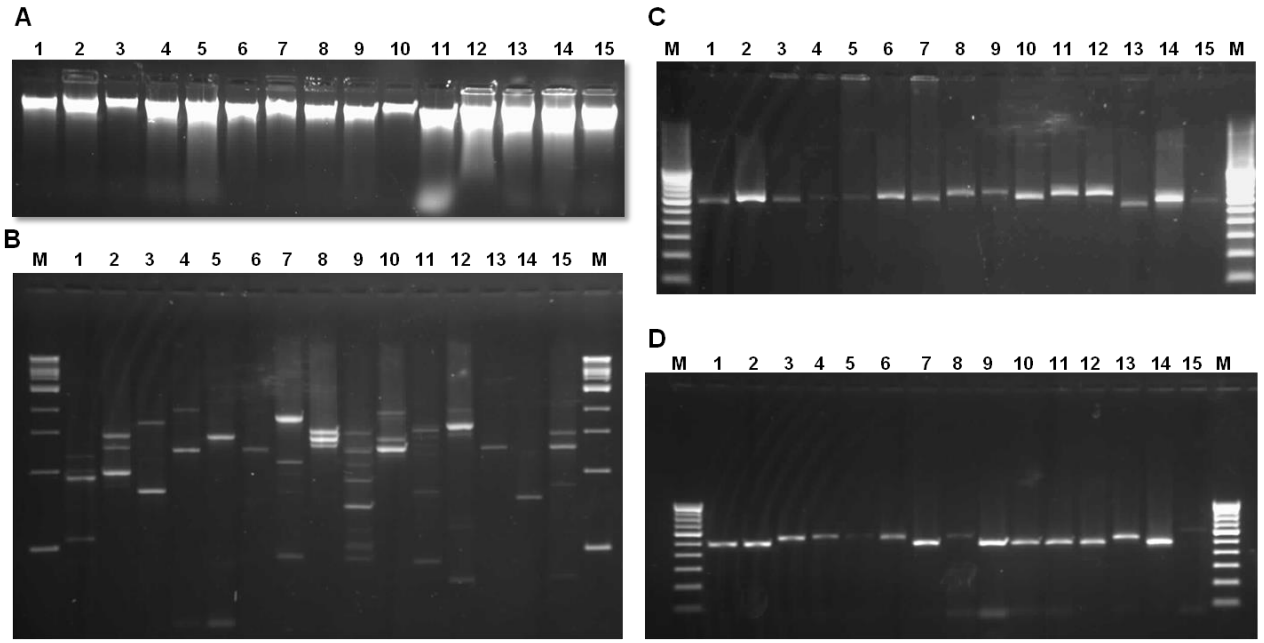

Fig 1 – A: Agarose gel electrophoresis of gDNA, B: The RAPD profile with the primer OPA01 of the fungal strains studied, C: Agarose gel electrophoresis of multi-copy gene (ITS-rDNA) amplicons of the fungal strains studied, D: Agarose gel electrophoresis of single copy gene (β-tubulin) amplicons of the fungal strains studied. Lane 1 to 15; *Cladosporium tenuissimum, Penicillium concentricum, Emericella variecolor, Aspergillus oryzae, Aspergillus flavus, Aspergillus terreus, Curvularia affinis, Rigidoporus vinctus, Inonotus pachyphloeus, Curvularia lunata, Earliella scabrosa, Penicillium oxalicum, Fusarium incarnatum, Fusarium phaseoli*, *Fusarium* sp., and M- 100bp DNA ladder & M- Supermix DNA ladder (Merck-Genei).

Table 2 Comparison of proposed method of fungal genomic DNA extraction with previously described methods*

Authors	Method type	Duration	Starting material	Yield	Fungal coverage
Proposed method	Manual	2h 30 min	200 mg	60 µg – 230 µg/200 mg of fungal mass	Multiple fungi
Abd-elsalam et al., 2003	Manual	4 h	N.A.	4-6 µg	*Fusarium* species
Al-Samarrai et al 2000	Manual	1h	30 mg	0·34–0·87 µg DNA/mg freeze-dried mycelium	*A. niger, A. flavus, F. graminarum, Neotyphodium lolii, Penicillium citrinum* and *Rhizopus nigricanes*
Amer et al., 2011	Manual	1h 45min	50 mg	20-40µg/100µl	*Rhizoctonia* sp., *Fusarium* sp., *Trichoderma* sp., *Aspergillus* sp., *Ulocladium* sp., *Penicillium* sp.
Faggi et al., 2005	Manual + kit	N.A.	N.A.	223 - 5684 µg/ml	*C. albicans, C. neoformans* and dermatophytes
Mishra et al., 2014	Manual	N.A.	200 mg	310 – 1879 µg/g dried mycelium	*Aspergillus fumigatus, A. niger, A. flavus, A. terreus, A. awamori, A. ficcum.*
Griffin et al., 2002	Manual + kit	N.A.	< 3 mg	N.A.	*Fusarium* sp., *Cladosporium* sp., *Curvularia* sp., *Sclerotium* sp., *Aspergillus* sp., *Coccodinium bartschii*
Griffiths et al 2006	Manual + kit	3 h	N.A.	10 conidia	*A. fumigatus* conidia
Karakousis et al., 2006	Manual + kit	N.A.	10 - 15 mg	2.5-10 µg	Multiple fungi
Karthikeyan et al. 2010		3h	50mg	5-10ng	*Fusarium* sp., *Bipolaris* sp., *Verticillium* sp., *Aspergillus*
Lee et al., 1988	Manual	N.A.	60-100 mg dry, or 0.5-1.0 g wet	200 ug DNA/0.1 g lyophilized mycelium	Multiple fungi
Liu et. al., 2000	Manual	1h	N.A.	N.A.	Multiple fungi
Mendoza et al., 2010	Manual	1h 30min	10 mg	8.6-9.3 µg/mg fresh weight	*Fusarium oxysporum, Pyrenochaeta terrestris*
Mishra et al., 2008	Manual	N.A.	50 mg	0.55- 0.92 µg DNA/ mg freeze dried mycelia	*F. solani, Colletotrichum capsici Rhizactonia solani, Phytophthora colocasiae, Pythium aphanidermatum*
Moller et al., 1992	Manual	N.A.	30-60 mg	(100 µg/30 mg dried mycelium)	Multiple fungi
Muller et al., 1998	Manual + kit	N.A.	10^7 - 10^8 conidia/cells	1 – 49 µg/ml	*C. albicans, C. neoformans, T. beigelii, A. fumigatus, F. solani, S. cerevisiae, P. boydii, R. arrhizus*
Nawrot et al., 2010	Kits	N.A.	N.A.	10^1 – 10^2 spores	*A. fumigatus* spores
Płaza et al., 2004	Manual	2h	200-500 mg	N.A.	Multiple fungi
Prabha et. al., 2013	Manual	N.A.	N.A.	20 mg/g of mycelium	*Saprolegnia* sp., *Aphanomyces* sp., *Aspergillus flavus* and *Pythium* sp.
Van Burik et al., 1998	Manual	N.A.	30 – 100 mg	3.2 – 81.8 µg	Multiple fungi
Zhang et al., 2010	Manual + kit	40–50 min	N.A.	N.A.	*Ophiocordyceps sinensis* specimens

*Note: Some of the fungal DNA extraction methods could not be listed in the table because of the space limitations. N.A.: Not available.

The developed method utilizes bead beating in a tissue homogenizer, which has been found to be very efficient in breaking the fungal cells. There is an obvious one time instrument set up cost involved with the method; however considering the advantages of a tissue homogenizer like FastPrep®-24, the instrument cost is justified. Fungal spores, such as conidia from Aspergillus spp. and other molds, might be present in the air. Such airborne spore inoculation during the DNA extraction process could potentially lead to false-positive results, especially if panfungal primers are applied (Löffler et al. 1999). The advantage of a tissue homogenizer is that it is a closed system, wherein, there are no chances of laboratory borne contamination. Therefore, our method will be very useful in avoiding the laboratory borne contamination during DNA extraction. In the current scenario, the numbers of biodiversity studies have increased, wherein; a large number of fungal isolates need to be processed for molecular identification or phylogenetics (Dighton et al. 2005). Our developed protocol would be of great help particularly dealing with the large number of fungal samples, as, at a given time 24 different fungal samples can be homogenized in a FastPrep®- 24 tissue homogenizer. This method with the high-throughput potential can significantly speed up the molecular identification and phylogenetics work that would result in enrichment of biodiversity inventory in future. Lastly, the entire procedure of gDNA extraction could be finished within 2.5 hours that will effectively reduce the total time taken for a PCR based downstream applications. The developed protocol would enable us to speed-up the fungal molecular identification services, being offered by our culture collection NFCCI, Pune, India.

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