A simple method for total genomic DNA extraction from water moulds

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Water moulds grow on submerged and floating leaves, twigs and wood in well-aerated waters. The morphological descriptions of many rare fungal species have been increasingly supplemented by their molecular characteristics. The isolation of genomic DNA is a prerequisite for their molecular characterization. The DNA isolation procedure developed herein is based on the sodium dodecyl sulphate/phenol method, without the addition of β-mercaptoethanol and proteinase K; instead it uses phenol/chloroform extraction. This protocol resulted in good quality DNA which was further validated using polymerase chain reaction amplification, cloning and sequencing and other downstream processes. The whole procedure can be done in a relatively short period of time without the need to employ β-mercaptoethanol, CTAB, lysozyme digestion, proteinase K treatment, etc., thereby reducing the overall costs involved.

Keywords: Extraction, genomic DNA, molecular characterization, water moulds.

THE biota of aquatic habitats includes water moulds that can be isolated easily from submerged and floating organic matter in well-aerated waters. Most of these water moulds belong to Oomycota that were historically classified as fungi[1], but ultra-structural, biochemical and molecular sequence analyses strongly indicated that they are close to algae than to fungi and hence classified within 'Stramenopiles'[2].

Along with the studies on morphology, physiology and pathogenicity, comparative studies of the internal transcribed spacer (ITS) regions of the ribosomal RNA have also become a useful tool in fungal taxonomy, as these regions evolve rapidly and it helps distinguish different species within a genus level[3]. The isolation of pure DNA is crucial in these filamentous fungi because it is a prerequisite for several molecular biology techniques, including gene isolation by polymerase chain reaction (PCR), Southern blotting and the construction of genomic DNA libraries. However, DNA extraction from these filamentous fungi has been described as being rather complicated, because most of the available protocols include additional lysis steps, such as mechanical disruption or sonication, enzymatic digestion or use of toxic chemicals[4,5]. A number of protocols have been established for fungal DNA. Though many of these protocols are apparently suitable for certain groups or morphological forms of fungi, they may not be versatile and efficient for extracting nucleic acids from diverse groups of filamentous fungi[6,7].

Recently, researchers have started using kits to extract DNA[8–11]; these kits are costly and their use restricts the number of samples that can be extracted per day. Numerous reports have described procedures for the extraction and purification of fungal DNA. Many of these are modifications of the CTAB method originally developed for plant tissue extraction[12–14] or protocols employing direct sample extraction with organic solvent as the principal means of denaturing and eliminating contaminating protein[15]. While the CTAB method is considered superior for removing unwanted carbohydrate from DNA preparations, procedures that use organic solvents directly in the extraction buffer, often can be performed more rapidly. Despite the range of techniques available for the preparation of fungal DNA, some fungal mycelia and most fungal spore samples remain inextractable by these procedures. Therefore, the objective of this study was to develop an easy and rapid protocol for the isolation of good quality total DNA from fungi such as *Saprolegnia* sp., *Aphanomyces* sp., *Aspergillus flavus* and *Pythium* sp.

Samples of brown decaying twigs, leaves and wood of the local dominant vegetation were collected from Vaigai River, Madurai, Tamil Nadu and were brought to the laboratory in separate sterile polyethylene bags. Oomycetes were isolated from these samples by the usual baiting techniques as described elsewhere[16–18]. These samples were then placed in sterilized petri dishes containing sterile distilled water and several hemp seed halves (*Cannabis sativa*) and were incubated at room temperature (15–20°C). Once the seeds were colonized, a single hypha or sporangium was isolated under the microscope and transferred to the plate containing potato carrot agar (PCA) medium. After 3–4 days, a block of agar containing purified colonies of oomycetes fungus was cut off and grown in potato dextrose broth (PDB).

The following solution and reagents were used: Liquid nitrogen; extraction buffer solution (200 mM Tris-HCl, pH 7.5; 25 mM EDTA and 250 mM NaCl and 0.5% SDS); cold phenol : chloroform (1 : 1); chloroform; cold iso-propanol and cold 70% ethanol.

The DNA extraction procedure consisted of the following steps.

1. Purified colonies of water moulds were grown in PDB broth for 3 days in a shaker at 30°C.
2. The 3-day-old mycelium was allowed to drain for about 3 min after being placed on a filter paper. The mycelium was then ground separately with mortar and pestle in liquid nitrogen to a fine powder.

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3. The frozen powder was then transferred to a 2 ml centrifuge tube and 500 µl of extraction buffer solution was added.
4. The reaction mixture was vortexed for 5 sec and kept at room temperature for 30 min.
5. The reaction mixture was centrifuged at 13,000 rpm for 1 min.
6. The supernatant was then transferred to a new centrifuge tube and equal volume of cold phenol : chloroform was added to it.
7. The reaction mixture was vortexed briefly and centrifuged again at 13,000 rpm for 2 min.
8. The supernatant was again transferred to a new centrifuge tube and re-extracted twice with 300 µl of chloroform and centrifuged again as in step 6.
9. The final supernatant was then transferred to a new centrifuge tube; 300 µl of cold iso-propanol was added to it and gently mixed by inverting the tubes twice or thrice.
10. The reaction mixture was incubated for 30 min at –80°C or –20°C.
11. The nucleic acids were then recovered by centrifugation at 13,000 rpm for 5 min.
12. The supernatant was discarded and pellet washed with 70% cold ethanol and dried for 15 min at 37°C.
13. Finally, the isolated DNA was resuspended in 50 µl of sterile water and stored at –20°C for further use.
14. Ten microlitres of total DNA solution was loaded onto a 1% agarose gel and electrophoresed to separate DNA.

A PCR was performed in a total volume of 20 µl containing 10× assay buffer (1× contains 10 mmol/l Tris-HCl, pH 8.8 at 25°C, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$). ITS1, ITS2 regions and the intermediate 5.8S ribosomal gene were amplified using the primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) at a concentration of 50 nmol (HPLC purified, Operon Technologies), 40 ng template DNA, 80 µmol of each of the four dNTPs and 1 unit of *Taq* polymerase (Bangalore Genei). PCR conditions were as follows: denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 sec, primer-specific annealing temperature at 55°C for 30 sec and extension at 72°C for 30 sec, and a final extension at 72°C for 7 min. The reaction was carried out in the Gene Amp® PCR system 9700 (Applied Biosystems). The PCR products were resolved by electrophoresis in a 1% agarose gel in 0.5 × TBE buffer. The gels were pre-stained with 10 mg/ml ethidium bromide.

Since the currently available DNA extraction protocols are comparatively costly and/or time-consuming[19], we attempted the development of a rapid DNA isolation method with the exclusion of most of the toxic chemicals, high-speed cell disruption, bead-vortexing and mechanical (glass beads) shearing for lysing the hyphae of the fungal cells used during the fungal DNA isolation procedure. The isopropanol and ethanol step allowed effective precipitation of DNA, rendering it more stable and resulting in good yields of high-quality genomic DNA (Figure 1). The protocol also worked well with some other fungal species tested, e.g. *Aspergillus flavus* and *Penicillium* sp. (Figure 1; lanes 11 and 12). The nucleic acid absorbance ratios of A260/280 were determined to evaluate quantity, quality and integrity of isolated DNA. The A260/280 averaged 1.9, suggesting that the DNA fraction was pure and may be used for further analysis. The quality of DNA was assessed by PCR amplification with universal primers ITS1 and ITS4. Amplification produced bands of approximately 800 bp to 1 kb (Figure 2). The same was successfully applied to other water moulds and filamentous fungal species (data not shown).

The key steps of the modified protocol are: (1) Exclusion of CTAB buffer. (2) Exclusion of harmful chemicals like β-mercaptoethanol. (3) Exclusion of costly chemicals like urea and lysozyme and other enzymes usually used for the isolation of DNA[20]. (4) The number of steps in DNA isolation is minimal. (5) It is efficient because as little as 0.05 g of mycelium gives large amount of pure, intact DNA, amenable for restriction digestion, PCR and Southern hybridization analysis compared to the DNA extracted using conventional method.

Important features of this protocol are: (1) It works well with other species of fungus where extraction of pure genomic DNA is a constraint and also works well in parasitoid insects like *Trichogramma* sp., the most widely studied agents of biological control in the field of entomology (data not shown).

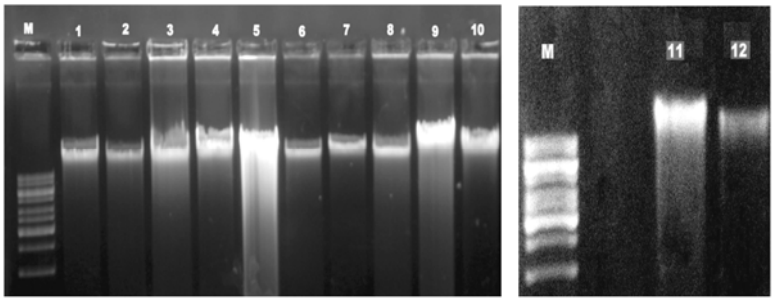

Figure 1. Gel electrophoresis of the total DNA extracted from oomycetes fungus and other filamentous fungi. Lanes 1–10, *Pythium* and *Saprolegnia* sp.; lanes 11, 12, *Aspergillus niger* sp. and *Penicillium* sp.; Lane M, Ladder.

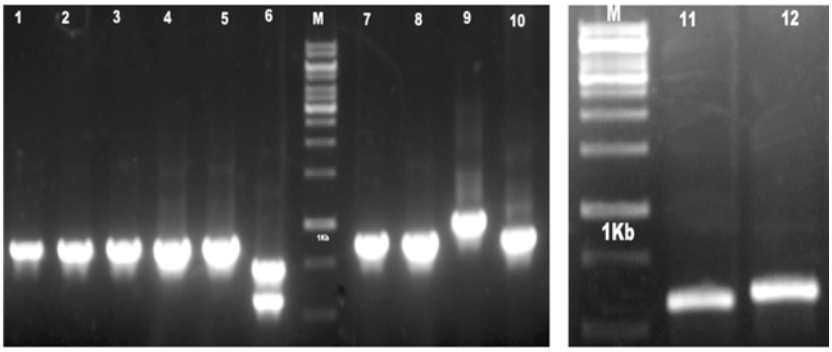

Figure 2. Amplification profile of oomycetes fungus and other filamentous fungi, showing 800 bp–1 kb fragments. Lanes 1–10, *Pythium* and *Saprolegnia* sp.; lanes 11, 12, *Aspergillus niger* sp. and *Penicillium* sp.; Lane M, DNA ladder.

(2) It yielded high-quality DNA, more than 20 mg/g of mycelium, whereas the protocol described by Ke *et al.*[20] yielded 45 μg/ml when extracted with the improved CTAB methodology.

(3) The method is simple, not labour-intensive, and economical compared to other methods and readymade kits which are costly.

In this study, we also found that grinding the biomass in liquid nitrogen produced higher yield of DNA, contrary to earlier reports. Nevertheless, the quantity as well as quality of the extracted genomic DNA was high enough to perform hundreds of PCR-based reactions (Figure 2) and could also be used for other DNA manipulation techniques like Southern blot analysis and DNA library construction (data not shown).

Our aim was to design a protocol that is inexpensive, quick and efficient for DNA isolation with the capability of obtaining good quality of total nucleic acids from water moulds. Additionally, this protocol provides a rapid, reliable (good quality DNA) and low-cost alternative to the existing DNA purification protocols used in research and clinical laboratories. The DNA extraction protocol described here is rapid and technically easy for preparing nucleic acid that is useful for further molecular studies.

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