

Rapid procedure for the extraction of DNA from fungal spores and mycelia.

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A method is described for the reliable preparation of DNA from fungal spores and mycelia and from plant tissues. A number of fungal and plant species were used in the study to indicate the generality of the method. The DNA prepared by this protocol was digested by restriction endonucleases and served as template using standard polymerase chain reaction conditions.

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 (Saghai-Marooof, *et. al.* 1984 Proc. Natl. Acad. Sci. USA **81**:8014-8018) or employ direct sample extraction with organic solvent as the principal means of denaturing and eliminating contaminating protein (Blin and Stafford 1976 Nucl. Acids. Res. **3**:2303-2308). Whereas the CTAB method is considered superior at 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 intractable to extraction by these procedures.

The method outlined below was designed to produce a rapid, inexpensive and reliable procedure for the extraction of total nucleic acids from plant and fungal tissue, including fungal spores. White quartz sand (-50 + 70 mesh; Sigma Co. product # S-9887) is used as a pulverizing agent in the protocol, obviating the need for freezing the sample with liquid nitrogen or for germinating spores prior to DNA extraction. The method is similar to that described by Chow and Kafer (Fungal Genet. Newsl. **40**:25-27) but is applicable to a wider range of tissue types. Fungal species tested here include the rust fungi *Puccinia graminis* (wheat stem rust), *Uromyces appendiculatus* (bean rust), *Puccinia helianthi* (sunflower rust), and the mildews *Plasmopara halstedii* (sunflower downy mildew) and *Erysiphe graminis* (barley powdery mildew). Other fungi include the basidiomycete *Rhizoctonia solani*, the ascomycete *Pyrenophora teres*, the deuteromycetes *Phoma betae* and *Cercospora beticola*, and the oomycetes *Aphanomyces cochlioides*, and *Pythium ultimum*. Plant species included in the study were *Beta vulgaris*, *Solanum tuberosum*, *Helianthus annua*, and *Hordeum vulgare*. Recipes for the phenol/CHCl₃/isoamyl alcohol (25:24:1) mixture and the electrophoresis buffers can be found in Maniatis, *et al.* (1982, "Molecular Cloning- A laboratory manual" Cold Spring Harbor Press)

Fresh plant leaf material was weighed directly (for grass species) or after removal of the petiole and midrib sections (for dicot species). Fungal mycelia were either scraped directly from the surface of an agar culture or were trapped on Miracloth (Calbiochem) by filtration of a liquid culture. The Miracloth was placed mycelia side up onto a large absorbent paper towel to wick away as much liquid as possible. The final mycelial mat was weighed before placing it into a mortar. Dry rust and mildew spores were weighed and added directly to the mortar. NOTE:

Doubling of the liquid volumes and mass amounts of sand is advised as a starting point for application of the procedure to dry rust and mildew spores.

White quartz sand was added to the tissue at a ratio of 3 g of sand per gram of tissue. Sufficient extraction buffer [100 mM Tris- HCl (pH 8.0), 20 mM Na₂ EDTA, 0.5 M NaCl and 1% sodium dodecylsulfate] was added to the mortar so that the sand/mycelia mixture became saturated, but no excess liquid was present (~0.4 ml of buffer per gram of sand). A mixture of buffer saturated phenol/CHCl₃/isoamyl alcohol was added at a ratio of 0.5 ml per gram of tissue.

The mixture was ground vigorously for ~30 sec with a pestle to form a thick paste. Two milliliters of extraction buffer and 1 ml of buffered phenol/CHCl₃/ isoamyl alcohol per 0.5 g of starting tissue were then added and the solution was mixed thoroughly. A 1 ml plastic micro-pipette tip was cut with scissors about 1 cm from the tip and the mixture was transferred into several microfuge tubes using this tip and a micropipettor. The microfuge tubes were capped and centrifuged at 16,000 x g for 5 min at room temperature; tissue debris and the sand pelleted to the bottom of the tube. The aqueous phase was transferred to a new tube and was mixed with 0.6 vol of isopropanol. (If needed, the sample can be extracted with phenol/CHCl₃/ isoamyl alcohol one more time followed by centrifugation as above before precipitation with isopropanol.) Samples were incubated at room temperature for 10 min and centrifuged at 4 ° C for 15 min at 16,000 x g to recover the precipitate. After rinsing the pellets with 95% EtOH, the pellets were allowed to air dry briefly and were subsequently resuspended in 340 ul of TE (10 mM Tris-HCl, 1 mM Na₂EDTA, pH 8.0) containing ribonuclease A at 20 ug/ml. Samples were incubated at 37 ° C for 30 min were then extracted with 0.3 ml of phenol/CHCl₃/ isoamyl alcohol. The aqueous phase (~300 ul) was transferred to a new tube and 1/2 volume of 7.5 M ammonium acetate and 2.5 vol of EtOH were added and mixed, and the samples were incubated for 30 min at -20 ° C. The samples were then centrifuged at 4 ° C for 15 min at 16,000 x g. The pelleted DNA was rinsed with 95% EtOH, air dried and resuspended in 100 ul of TE. Quality and quantity of the DNA obtained were determined by subjecting a portion of the preparation to agarose gel electrophoresis and UV spectrophotometry, respectively. Yields varied from ~10 ug to 50 ug of DNA recovered from 1 g of plant tissue to ~100 ug of DNA obtained from 1 g of rust or mildew spores. A fraction of the DNA preparations were subjected to gel electrophoresis before (Figure 1) and after (Figure 2) digestion with restriction endonucleases. A subset of the samples was also tested as template in polymerase chain reaction experiments (Figure 3).

The method described is reliable, inexpensive and efficient for extracting DNA from fungal and plant samples. With the inclusion of diatomaceous earth (bentonite) in the extraction buffer, high quality RNA also can be prepared by the method (not shown). The protocol adds to the repertoire of nucleic acid preparations that can be applied to diverse tissue sources.

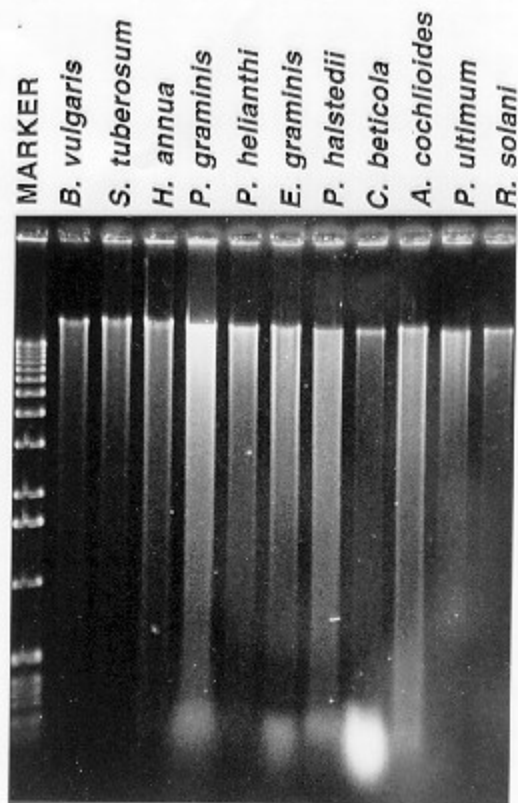


Figure 1. Agarose gel electrophoresis of a subset of the DNA preparations. The lanes are labelled according to the tissue source. After spectrophotometric quantification, samples were adjusted to a DNA concentration of ~50 ug/ml and 10 ul of each sample was loaded onto a 1% agarose gel (1X Tris- acetate electrophoresis buffer). One- half microgram of the 1 kb ladder(BRL- Life Sciences) was loaded into the "Marker" lane. The gel was stained with ethidium bromide before photographing with Polaroid 667 film.

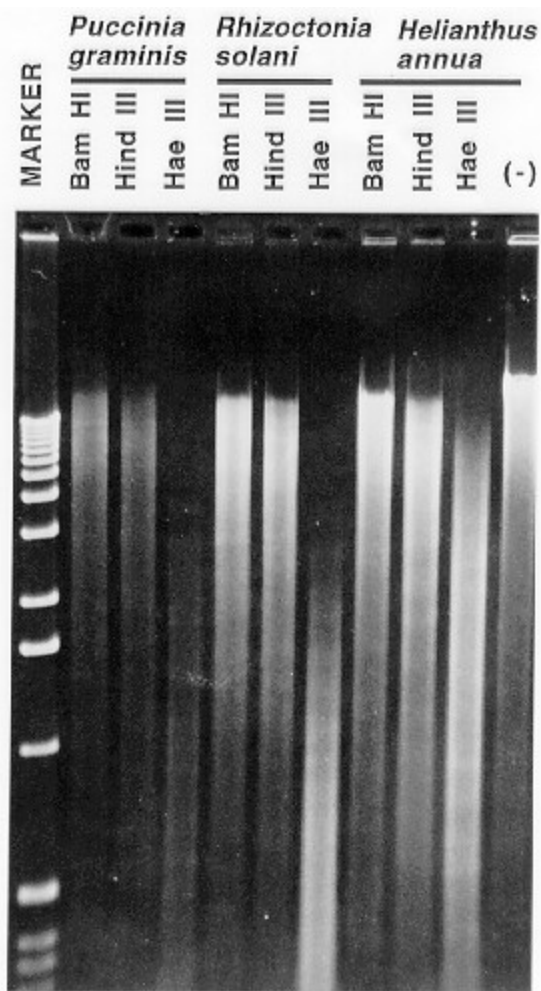


Figure 2. Restriction enzyme digestion of DNA prepared by the described method. Approximately 1 ug of DNA from *Puccinia graminis*, *Rhizoctonia solani*, and *Helianthus annua* was digested with the restriction endonucleases *Bam*HI (six base recognition site, GC-rich), *Hind*III (six base recognition site; AT- rich), and *Hae*III (four base recognition site). Incubation of the DNA prepared from *Helianthus annua* in restriction enzyme buffer in the absence of added endonuclease (-) indicates the lack of endogenous deoxyribonucleases contaminating the preparations. The 1 kb ladder is also shown (Marker).

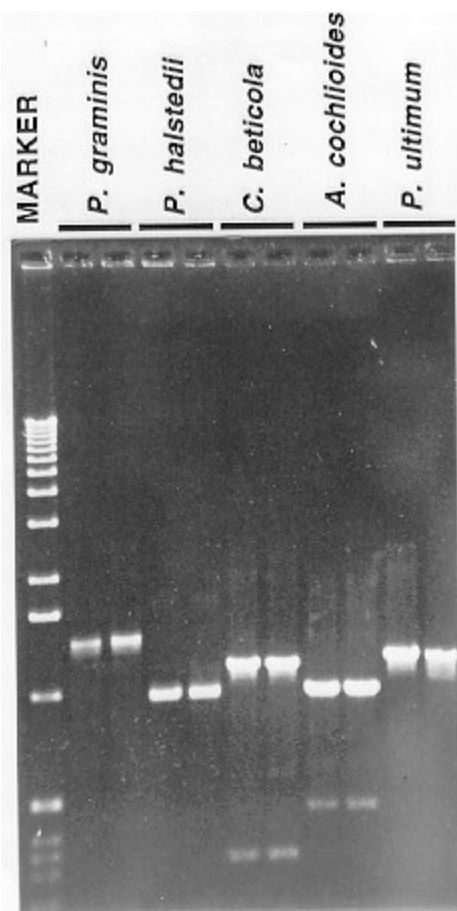


Figure 3. Polymerase chain reaction (PCR) amplification of DNA using fungal DNA prepared by the described method as template. Reactions were performed in duplicate using genomic DNA of *Puccinia graminis*, *Plasmopara halstedii*, *Cercospora beticola*, *Aphanomyces cochlioides*, and *Pythium ultimum*. Opposing oligonucleotide primers were designed based on consensus sequences derived from the comparison of several fungal actin genes. Each PCR reaction had a volume of 50 μ l and contained 50 mM KCl, 10 mM TRIS-HCl (pH 9.0), 0.1% TRITON- X100, 1.5 mM MgCl₂, 0.1 mM each d(G,A,T,C)TP, 50 ng each of the oligonucleotide primers, 10 ng of fungal DNA template and 2 U of Taq DNA polymerase (Promega). Samples were overlaid with mineral oil and were incubated in an MJR PTC- 100V thermocycler (94° C, 1 min; 55° C, 1 min; 72° C, 3 min : 40 cycles). A portion (7 μ l) of each reaction was electrophoresed in a 1% agarose gel (1X Tris- borate buffer) in parallel with the 1 kb ladder (Marker).

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