were digested with the appropriate restriction enzyme (Enz 3 in Figure 1) to locate the mutation. The first time through the procedure for a new mutagenic primer, it was helpful to do a plasmid preparation of the entire pool of transformed cells. A digestion with Enz 3 would quickly determine if the mutagenesis procedure was successful. If the plasmid was at least partially converted to the linear form, then the mutation was successful, and it was easy to find a mutated plasmid in a colony when colonies were screened.

This procedure was successfully performed using three different mutagenic primers with 16–18 bases on each of three different double-stranded plasmids. The entire procedure could be completed within 3 days. In one instance, a simultaneous deletion of 75 bases was accomplished. Two of the three mutations were part of a gene for each of two different proteins; in these instances, each protein was expressed and shown to be the correct size by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), providing evidence that an unwanted deletion or insertion had not occurred as a result of the mutagenesis.

Of the colonies tested, the mutation frequency averaged 50%. This frequency was determined by randomly picking colonies and digesting with the enzyme that would cut at the new restriction site on the inserted DNA (Enz 3).

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A Simplified Method for the Preparation of Fungal Genomic DNA for PCR and RAPD Analysis

The use of the polymerase chain reaction (PCR) for rapid analysis of genomic DNA is well documented and has simplified genotypic analysis (6). Analysis of fungal genomic DNA by PCR has proved to be an expensive and time-consuming process because of the DNA extraction methods used. Most procedures include multiple phenol/chloroform extractions, ethanol precipitation and cesium chloride centrifugation (2–5). We have developed a simple and rapid method for extraction of fungal genomic DNA. The DNA is suitable for PCR and random-amplified polymorphic DNA (RAPD) (7) analysis. The method eliminates the need for cesium chloride centrifugation and uses only one chloroform extraction followed by ethanol precipitation. This method can be applied to a variety of fungi with the advantage of produc-

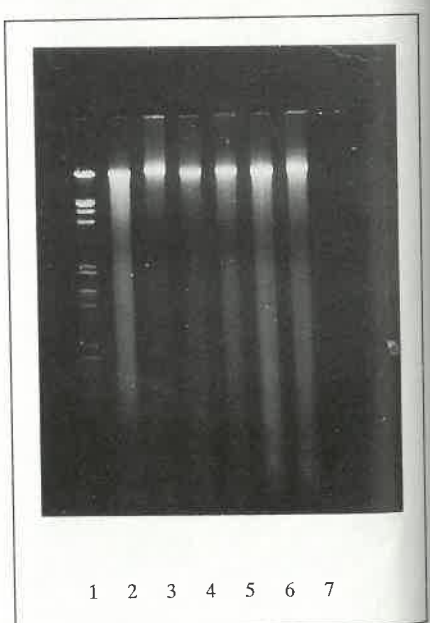

Figure 1. Genomic DNA isolated from fungal sporangiophore. Lane 1, lambda *Hin*dIII/*Eco*RI marker DNA (Boehringer Mannheim, Melbourne, Australia); Lanes 2–7 genomic DNA from isolates of *Pseudocercospora.* Resolution was on a 1.0% agarose gel and the DNA visualized with ethidium bromide.

ing clean DNA of high molecular weight in a very short time. The method is potentially useful in large-scale screening of field samples and does not require expensive equipment or special skills.

Samples of the fungi to be analyzed were cultured in an appropriate culture medium, collected by sedimenting the mycelia and removed with a spatula to a pre-cooled mortar (-2°C). The samples were ground to a fine powder in liquid nitrogen. The tissue was weighed before DNA extraction and 1 ml of extraction buffer (2% [wt/vol] hexadecyltrimethylammonium bromide [CTAB; Sigma Aldrich, Castle Hill, New South Wales, Australia], 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA) was added per gram of sample and mixed by gentle inversion. The samples were then incubated at 55°C for 20 min. After incubation the samples were centrifuged for 5 min at 15000× *g* in a microcentrifuge. The supernatant was collected, and 1 volume of chloroform:isoamyl alcohol (24:1) was added and mixed by gentle inversion for 2 min before centrifugation at 15000× *g* for 20 s. The upper aqueous phase was collected, and 1/10 volume of 7.5 M ammonium acetate and 2 volumes of ice-cold absolute ethanol were added and mixed by gentle inversion. The samples were placed into a freezer at -20°C for 60 min to precipitate the genomic DNA. The samples were then centrifuged at 1500× *g* for 1 min and the supernatant discarded. The DNA was washed twice with 70% (vol/vol) ethanol, mixing gently by inversion. Finally, the DNA was dried in a desiccator and dissolved in approximately 200 µl of 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA was then analyzed on a 1% (wt/vol) agarose gel in 1× TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) (Figure 1). Spectrophotometric determination of the quantity and quality of the DNA was performed to help in preparation of PCR and RAPD assays. This method allowed high molecular weight genomic DNA with an A_{260}/A_{280} ratio between 1.85 and 1.92 to be simply extracted from fungi. The DNA remains stable at 4°C for at least 6 months.

To demonstrate the suitability of fungal DNA prepared in this way for PCR and RAPD analysis, the genomic DNA from 6 isolates of *Pseudocercospora* was used. PCR 10-mer primers of arbitrary nucleotide sequence were obtained from Operon Technologies (Alameda, CA, USA) and used in the following reaction mixture to a total volume of 50 µl: reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2.0 mM $MgCl_2$, 200 µM each of dATP, dTTP, dCTP and dGTP, 0.2 µM of a single 10-mer oligonucleotide, 1.0 unit *Taq* DNA Polymerase (Perkin-Elmer, Milton, Queensland, Australia) and 0.1 µg of template DNA. The solutions were overlaid with 30 µl of paraffin oil. The thermal cycling program was run on a Perkin-Elmer 480 DNA Thermal Cycler. All programs had 1 initial cycle with melting at 95°C for 3 min, annealing at 36°C for 2 min and extension at 72°C for 2 min. The subsequent 40 cycles were melting at 95°C for 1 min, annealing at 40°C for 2 min and extension at 72°C for 2 min. The RAPD products were visualized on a 2% (wt/vol) agarose gel in 1× TBE buffer after staining with ethidium bromide for 40 min and destaining for 10 min in 1× TBE buffer. The RAPD analysis of the genomic DNA was successful and showed clear DNA marker profiles (Figure 2).

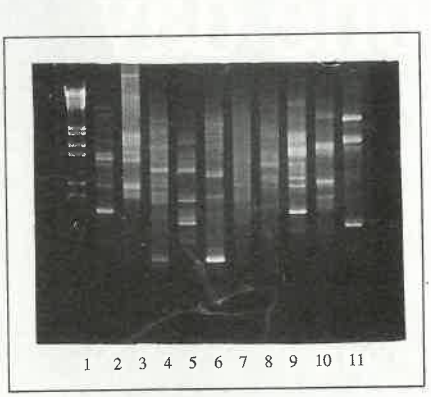

Figure 2. RAPD profiles of a fungal DNA isolate. Lane 1, lambda *Hin*dIII/*Eco*RI marker DNA (Boehringer Mannheim); Lanes 2–11, PCR products from a *Pseudocercospora* DNA isolate with each of nine primers Operon No. OPA-01–OPA-10. Resolution was on a 2.0% agarose gel and the DNA visualized with ethidium bromide.

Recently Ashktorab and Cohen (1) reported a method for fungal DNA extraction involving repeated phenol extraction. In our opinion this is a more laborious protocol with associated dangerous chemical considerations than the protocol presented here. With the simplified method proposed here, we have shown that it is possible to process up to 40 fungal samples in a day.

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