Kit-Based, Low-Toxicity Method for Extracting and Purifying Fungal DNA from Ectomycorrhizal Roots

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Ectomycorrhizal fungi are ecologically and economically important because when they form associations with roots of species such as pines, oaks, and eucalypts, they may increase nutrient and water uptake (15). Functional and taxonomic diversity among the ectomycorrhizal fungi is great and thus, in many studies, it is important to identify individual fungal species. During the past few years, researchers have perfected DNA-based methods for the identification of ectomycorrhizal fungi. In most cases, this involves the extraction and purification of DNA from colonized roots, amplification of fungal genomic DNA, and either analysis of restriction fragments or sequencing (1, 3–5,7,9,10).

The extraction of DNA from ectomycorrhizal roots, as commonly published, involves some combination of manual grinding, freezing and thawing, and incubation at elevated temperatures, while purification may involve chloroform or phenol/chloroform extraction of impurities and precipitation of DNA by alcohol (3,5,7,10). The process may take hours to complete, and it requires the use of a fume hood to vent chloroform and phenol vapors. We desired a simple, inexpensive, rapid, and less hazardous method for extracting and purifying readily amplifiable fungal DNA from ectomycorrhizal roots, and a kit-based method seemed ideal. However, as far as we are aware, there are no kits specifically designed to extract and purify fungal DNA from ectomycorrhizal roots. Here, we report on the successful use of a commercially available kit to do this without manual grinding, specialized shaking apparatus, or phenol/chloroform.

The three extraction/purification methods reported here use the UltraClean™ Microbial DNA Kit (Mo Bio Laboratories, Solana Beach, CA, USA). The kit was originally designed to extract DNA from solution-cultured microbial cell pellets. It consists of 1.9-mL microcentrifuge extraction tubes containing garnet beads, MicroBead buffer solution, a detergent-based extraction solution (M1), an acetate-based solution to precipitate impurities (M2), a salt solution to cause the DNA to bind to the supplied silica column (M3), an ethanol washing solution (M4), and 10 mM Tris (M5) for DNA elution.

For Method 1, clusters of ectomycorrhizal roots comprising 2–4 root tips were sampled by hand from the fermentation layer of the forest floor from an approximately 65-year-old red pine (*Pinus resinosa*) plantation located in State College, Centre County, PA, USA. The ectomycorrhizal root clusters were viewed using a dissecting microscope (10–40×) with reflected light to separate them according to morphotype. The clusters of a single morphotype, morphotype G (unpublished data), were selected. All root clusters were frozen at -20°C before use. Two days later, DNA from two replicate ectomycorrhizal root clusters was extracted and purified. Each root cluster was placed in its own extraction tube. Then, 300 μL MicroBead solution were added, followed by 50 μL M1 and 50 μL inhibitor removal solution (IRS) (Mo Bio Laboratories). IRS is included in the UltraClean Soil DNA Kit to inactivate the phenolic components of soil organic matter that can inhibit DNA amplification (16). Phenolic compounds are also present in the roots of many ectomycorrhizal trees (8,13,14, 17), so it was used here. Each extraction tube was heated to 65°C for 15 min in an oven to aid in the extraction. The tubes were then clipped to the vortex adapter (Mo Bio Laboratories) attached to a Vortex Genie 2 Mixer® (Fisher Scientific, Pittsburgh, PA, USA) and agitated at the highest speed for 15 min. The adapter consists of a 14.5-cm diameter plastic plate with 12 plastic clips around the perimeter to hold the tubes. We attached the adapter to the mixer with a strong adhesive, not with the loop and pile tape supplied by the manufacturer. This transferred more of the energy from the mixer to the tubes. A newer version of the adapter that does not require this adhesive step is
now available from the manufacturer. The extraction tubes were heated a second time for 30 min at 65°C and then centrifuged at 10 000×g for 30 s. Approximately 300 µL supernatant from each extraction tube were transferred to new microcentrifuge tubes (supplied with the kit). To each tube, we added 100 µL M2, followed by brief mixing. The tubes were then held at -20°C for 10 min to precipitate contaminants and centrifuged at 10 000×g for 1 min. Supernatant (450 µL) from each tube was transferred to a new microcentrifuge tube to which 900 µL M3 were added, and the mixture was shaken briefly. From each tube, 700 µL were added to silica columns (supplied with the kit), which were centrifuged at 10 000×g for 30 s, and the liquid was discarded. This process was repeated. The DNA adhering to each column was washed once with 300 µL M4, followed by centrifugation at 10 000×g for 30 s. The wash solution was discarded, and the silica columns were again centrifuged at 10 000×g for 1 min to remove all ethanol. The DNA was then eluted from each silica column into new microcentrifuge tubes by the addition of 50 µL M5 and centrifugation at 10 000×g for 30 s.

We tested the utility of the IRS and heating in the extraction by omitting each of these steps from Method 1 (i.e., No IRS and No Heat methods, respectively). Four days later, clusters of ectomycorrhizal roots comprising 2–3 tips each of morphotype G were sampled from the forest floor of the red pine plantation described earlier. Root clusters were kept frozen at -20°C for subsequent use. Two days later, DNA from individual clusters was extracted using the three methods, each with two replications.

The No Heat method was also applied to clusters of ectomycorrhizal roots (2–6 tips/cluster) representing several different morphotypes, including those tentatively categorized as A (Russula brevipes), B (Cenococcum geophilum), C (Lactarius chrysorheus), C’ and D (Tylopilus felleus), and F, G, H, and I (Scleroderma citrinum).

For the three extraction/purification methods, 5 µL DNA extract were used in 50-µL amplification reactions similar to those described elsewhere (3–5,7,9,10) but with some modifications. The DNA concentration was variable among the replicates of a single morphotype and among morphotypes. Despite this, all extractions yielded amplifiable DNA (see below). We used the primers ITS1F (3) and ITS4 (7) (Operon Technologies, Alameda, CA, USA) at a concentration of 30 pmol/reaction (0.6 µM). We used 1.25 U Taq DNA polymerase (Gene Choice; PGC Scientific, Frederick, MD, USA) in each reaction. The total MgCl2 concentration (including that contained in the proprietary PCR buffer) was 3.0 mM. The concentration of BSA was 0.4 µg/µL. BSA is used to improve the efficiency of the DNA amplification in the presence of various amplification inhibitors (11). The dATP, dGTP, dCTP, and dTTP were each added at 20 nmol/reaction (0.4 mM each). The DNA amplifications were completed in a Primus 96 Plus peltier thermal cycler (MWG-Biotech, Ebersberg, Germany). The lid temperature was 102°C. The thermal...
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cycling program included an initial denaturation step of 95°C for 2 min, followed by 40 cycles of denaturation, annealing, and elongation. All denaturations during the 40 cycles occurred at 94°C for 40 s, and all elongations occurred at 72°C for 40 s. Annealings initially occurred at 62°C and dropped 0.5°C each cycle until finally reaching 50°C. This “touchdown” PCR is recommended as a means to reduce nonspecific annealing of primers and to increase yield (2,6). The final elongation occurred at 72°C for 7 min, followed by an indefinite holding period at 8°C.

Negative controls (no DNA template) were used in every experiment to test for the presence of contaminating DNA in the PCR reagents. In no case were PCR products observed in the negative controls (data not shown).

PCR products were electrophoresed in horizontal 0.8% agarose gels (4 mm thick × 12 cm × 14 cm) (model B-2; Owl Separation Systems, Portsmouth, NH, USA) with approximately 6 V/cm applied between electrodes. The 100-bp DNA ladder was purchased from New England Biolabs (Beverly, MA, USA). DNA was visualized following staining with ethidium bromide with the EDAS® 290 electrophoresis documentation system (Eastman Kodak, Rochester, NY, USA) employing 1-D imaging software, version 3.5.3 (Eastman Kodak).

Method 1 applied to morphotype G ectomycorrhizal root clusters yielded PCR products of just fewer than 800 bp (Figure 1A, lanes 2 and 3). The IRS appeared to be important for the amplification of fungal DNA extracted from the red pine roots [compare the No IRS method (Figure 1B, lanes 2 and 3) with Method 1 (Figure 1B, lanes 4 and 5)]. The roots of pine and other conifers contain condensed tannins and other phenolic compounds (8,13,14,17), some of which are capable of interfering with the PCR (16). BSA added to the PCR mixture may also help to improve the DNA amplification in the presence of such phenolic inhibitors (11).

The heating steps during the extraction process did not appear to be necessary because the No Heat method had a similar yield as Method 1 [compare Method 1 (Figure 1B, lanes 4 and 5) with the No Heat method (Figure 1B, lanes 6 and 7)]. Again, each method resulted in a single product of just fewer than 800 bp, but the No Heat method is significantly more rapid than the other methods, requiring approximately 45 min less per set of extractions. For each of the nine root morphotypes extracted with the No Heat method, a single product of variable length was produced (Figure 1C).

We cannot be sure that in every case we extracted and amplified the DNA from ectomycorrhizal fungi. DNA from saprotrophic fungi growing on the surface of ectomycorrhizal roots could also have been extracted. However, if that did happen, a different saprotroph would have to have been amplified from each of the different ectomycorrhiza morphotypes shown in Figure 1C, which seems unlikely. Moreover, four of the nine species shown in Figure 1C have been identified as ectomycorrhizal fungi by terminal restriction fragment length polymorphism (T-RFLP) analysis (12) of both root-tip fungal DNA and DNA from sporocarps of known ectomycorrhizal fungal species (data not shown). Further matches between the root tips and sporocarps will probably be made as we analyze T-RFLP data from sporocarps of more ectomycorrhizal fungal species. Finally, all PCR products from morphotype G ectomycorrhizal roots (Figure 1, A and B) are of identical length, suggesting further that the chance amplification of DNA from contaminating saprotrophic fungi was not likely.

Jonsson (9) and T. Horton (personal communication) have found that the commonly used method of DNA extraction of Gardes et al. (3) involving freeze/thaw cycles and micropestle grinding may result in the successful amplification of fungal DNA from root tips in 50%–95% of cases. Using the No Heat method on previously frozen roots, we have successfully amplified fungal DNA from ectomycorrhizal roots in 38 of 41 attempts. However, we have failed to extract DNA from un-

Figure 1. PCR products amplified from fungal DNA extracted from ectomycorrhizal pine roots. (A) Lane 1, 100-bp ladder (from bottom to top, 600, 700, 800, 900, and 1000 bp) and lanes 2 and 3, Method 1 applied to morphotype G. (B) Lane 1, 100-bp ladder; lanes 2 and 3, No IRS method applied to morphotype G; lanes 4 and 5, Method 1 applied to morphotype G; lanes 6 and 7, No Heat method applied to morphotype G; and lane 8, 100-bp ladder. (C) Lane 1, 100-bp ladder; lanes 2–10, No Heat method applied to morphotypes A, B, C, D, F, G, H, and I, respectively; and lane 11, 100-bp ladder. According to T-RFLP analysis (data not shown), morphotype A corresponds to R. brevipes; C. L. chryosphaer; D. T. felleus; and I. S. citrinum which are all known to be ectomycorrhizal. Based on morphotype analysis, B is C. geophilum.
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frozen roots on numerous occasions.

Our results suggest that the No Heat method is adequate for the routine extraction of fungal DNA from colonized roots. With a single mixture, 12 samples can be easily processed in under 2 h. In contrast, Method 1 would require nearly 3 h. The rapidity and enhanced safety of extraction and purification using the No Heat method over previously published methods stem from three innovations. First, heating, freezing and thawing, and grinding are not necessary. Apparently, the combination of prior freezing (for storage) and the physical disruption of the fungal cells with garnet beads during the extraction appears to efficiently extract fungal DNA. A common laboratory vortex mixer is sufficient to generate the force for the physical disruption of fungal cells. Specialized shaking apparatus is unnecessary. Second, the use of the IRS in the extraction mixture effectively counteracts the inhibition of PCR by phenolic compounds that are inherent in the roots of many ectomycorrhizal trees. This permits a simpler and safer purification process. Third, purification is further simplified by using silica columns for binding and washing the DNA. Thus, potentially hazardous chloroform or phenol/chloroform extractions of impurities and precipitation of DNA by alcohol are unnecessary.

REFERENCES


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Rescue of Completed but Unread HIV-1 Antibody ELISA Microplates by Freezing

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Freezing and thawing (FT) of biological specimens before or after the analysis of various analytes is a common laboratory practice. In general, biological analytes present in specimens are usually quite refractory to FT. For example, in the area of infectious disease testing, studies describing multiple FT cycles indicated no deleterious consequences on subsequent analytical analysis for nucleic acid of hepatitis C virus (10) or of HIV-1 (5–7), for infectivity of varicella-zoster (2) or of HIV-1 (3), or for HIV-1 antibody reactivity in ELISAs (4,11).

Routine laboratory testing for the detection of HIV-1 antibodies is performed with ELISAs (9). This type of testing is generally completed within 3–4 h and is conducive to efficiently testing large numbers of specimens at one time. However, an interruption in any step of the ELISA due to such circumstances as power outage would typically require repeating the entire group of specimens from the start of the ELISA procedure. In such unusual circumstances, several steps of the ELISA procedure, including washing, could be performed manually without the aid of automated equipment. The mainte-