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Laboratory Exercises

A Rapid PCR-RFLP Method for Monitoring Genetic Variation Among Commercial Mushroom Species*

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We report the development of a simplified procedure for restriction fragment length polymorphism (RFLP) analysis of mushrooms. We have adapted standard molecular techniques to be amenable to an undergraduate laboratory setting in order to allow students to explore basic questions about fungal diversity and relatedness among mushroom species. The streamlined protocols allowed students to practice important molecular techniques within the context of self-designed investigative projects. This laboratory experience provided opportunities for students to practice strategies for examining molecular diversity among species.

Keywords: RFLP analysis, biotechnology education, phylogenetic analysis, mushrooms.

Hands-on, investigative laboratories have been widely incorporated as highly successful learning activities in undergraduate molecular biology curricula. In our biotechnology curriculum, we have designed project-based laboratories in which students work collaboratively in international research teams to develop multi-week research projects that provide the context for learning basic molecular technologies. This pedagogical strategy relies on self-motivation to drive student learning and allows students to apply their technical skills toward research questions developed from their own interests. To be successful, the project-based approach requires laboratory methodologies that are dependable, versatile, and easy for students to use. We have developed protocols for molecular phylogenetic analysis that are easily amenable to undergraduate courses in biotechnology or molecular biology. Students learn to apply a standard molecular method toward investigating phylogenetic relationships among fungi by conducting PCR-restriction fragment length polymorphism (RFLP)[1] analysis with commercial mushrooms.

The fungi are a large and diverse group of heterotrophic eukaryotic microorganisms that play an important ecological role as decomposers. Over 100,000 species of fungi have been described, and it is estimated that the total diversity of this group is well over 1 million [1, 2]. Fungi are typically divided into four phyla including Zygomycetes (bread molds), Chytridiomycetes (water molds), Ascomycetes (sac fungi), and Basidiomycetes (club fungi) [3]. The vegetative bodies of fungi consist of a network of thread-like hyphae called a mycelium, which grows throughout a substrate such as soil, rotting wood, or living plant tissue. Fungi reproduce by releasing spores generated either asexually or sexually. Ascomycetes and Basidiomycetes produce specialized macroscopic fruiting bodies called mushrooms in response to various environmental cues.

Several fungi that are pathogenic in humans, *e.g. Candida* sp., have been extensively characterized [4]. Fungi also have economic importance because of their application as nutritional foodstuffs and various medicinal and industrial uses [5, 6]. Over 2,000 edible mushroom species have been characterized, although the number of species that have been commercialized is much smaller (Table I). Edible mushrooms have high nutritional value, being high in certain essential amino acids and low in cholesterol. Various medicinal applications of mushrooms have been documented including managing heart disease, diabetes, obesity, and boosting immune function. There is growing interest in developing the industrial and biotechnological potential of species that produce bioreactive enzymes or secondary products of medicinal or industrial significance.

In exploring potential ecological and economic significance, identification and classification of fungi is an important first step. Fungal biodiversity is poorly understood. Traditionally, fungal classification involves observation of macro- and micro-morphological characteristics, a task often complicated by inconsistencies and convergent morphologies. More recently, molecular methods have been successfully applied to fungal systematics [7]. A common approach involves identification of RFLPs of specific amplified regions of genomic DNA, called PCR-RFLP

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[1] The abbreviations used are: RFLP, restriction fragment length polymorphism; EtBr, ethidium bromide; BME, beta-mercaptoethanol; CTAB, cetyltrimethyl ammonium bromide.

TABLE I
Common and species names for commercial mushrooms

Species name	Common name(s)
Agaricus bispora	Button mushroom, white mushroom, Portabella
Pleurotus flabellatus, P. ostreatus	Oyster mushroom
Flammulina velutipes	Enoki
Auricularia polytricha	Wood ear
Lentinus edodes	Shitake
Boletus edulis	Porcini

analysis. Several reports have documented that RFLP analysis of ribosomal DNA is the most effective method for species classification or distinguishing among various strains [8, 9].

PCR allows amplification of target DNA sequences from a sample containing as little as a single target DNA molecule [10, 11]. In PCR, multiple rounds of *in vitro* replication selectively amplify a specific region of DNA by thermoresistant Taq polymerase. A pair of single-stranded DNA oligonucleotides acts as replication primers and delimits the region of the target molecule that will be amplified. PCR products are linear DNA fragments that can be separated and visualized by gel electrophoresis.

In targeted PCR, the border sequences of the amplified region are known, and the resulting amplified product represents a well-characterized region. In mushroom classification, a common target region is the internal transcribed region of ribosomal DNA, or ITS [12]. While rDNA gene sequences are highly conserved, the intervening ITS regions are polymorphic and thus provide sequence variability that allows distinguishing among different species or strains (Fig. 1). The rDNA ITS region is a useful molecular field mark for fungal taxonomic and phylogenetic studies.

PCR sequences can be directly compared by nucleotide sequence analysis, but this approach is time consuming and expensive. A simpler approach is RFLP mapping, in which the number and location of restriction sites is determined for each PCR-amplified product. RFLP mapping can be performed quickly and inexpensively. Restriction fragment sizes are stable genetic attributes and thus can be used as molecular fingerprints to distinguish individuals or genotypes within or among species.

In this article, we describe RFLP analysis of ITS rDNA isolated from several commercial mushroom species. Genomic DNA extraction methods are often time consuming and involve the use of noxious chemicals and solvents. To facilitate the introduction of this powerful molecular approach in an undergraduate laboratory setting, we developed a rapid DNA extraction protocol that omits the use of BME or phenol. Students isolated DNA from a variety of fresh and dried commercial mushroom species, amplified the rDNA ITS region by standardized PCR, and conducted RFLP analysis to address questions of molecular diversity among species.

EXPERIMENTAL PROCEDURES

Materials

Fresh and dried fruiting bodies of *Agaricus bisporus* and *Pleurotus flabellatus* were obtained from local markets, and were stored refrigerated until use. ITS1 (5′-TCCGTAGGTGAACCT-GCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). ReadyToGo™ PCR beads were obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). PCR Core™ upstream/downstream primers, positive control plasmid DNA, HaeIII, MboI, λHIII, and 100-bp ladder DNA were obtained from Promega (Madison, WI). All buffers and reagents were obtained from Sigma Biochemical Co. (St. Louis, MO).

FIG. 1. **Map of nuclear ribosomal DNA region in fungi.** *Filled boxes* represent coding regions for ribosomal RNAs. Position of ITS1 and ITS4 primers is indicated.

Methods

DNA Extraction—Total DNA was extracted from 50 mg of fresh or dried tissue by a procedure adapted from Gardes and Bruns [8] as follows. Tissue was macerated by hand with polypropylene Kontes micropestle (Fisher Scientific, Pittsburgh, PA) in a 1.5-ml microcentrifuge tube in 500 μl of extraction buffer (100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 2% w/v CTAB), followed by incubation for 10 min at 65 °C. The grindate was clarified by centrifugation for 5 min at 14,000 rpm, and the supernatant was extracted with an equal volume of chloroform. Aqueous and organic phases were separated by centrifugation for 5 min at 14,000 rpm. Nucleic acid was precipitated from the upper aqueous layer by addition of 2 volumes isopropanol at −20 °C for 5 min. DNA was collected by centrifugation at 14,000 rpm, and the pellet was washed twice with 70% ethanol. The final pellet was air dried at room temperature for 10–20 min, then dissolved in 50 μl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

PCR Amplification—For amplification of rDNA ITS region, 1 μl of total DNA was added to a reaction tube containing Amersham ReadyToGo™ PCR bead, 1 μl of 50 nM ITS1 primer (50 pmol total), 1 μl of 50 nM ITS4 primer (50 pmol total), and ddH_2O to 25 μl total. PCR was performed in a PE System 24000 thermocycler with heated lid, or MJ Research Minicycler with 30 μl of mineral oil overlayered in each tube. Cycling parameters were initial denaturation at 95 °C for 3 min, followed by 35 cycles: denaturation step at 95 °C for 1 min 20 s, annealing step at 48 °C for 20 s, primer extension at 72 °C for 1 min 10 s. A final extension step for 10 min at 72 °C was followed by cold storage at 4 °C until use.

Restriction Digestion—Reactions contained 2 μl of amplified DNA and 1 μl of restriction enzyme in appropriate buffer at a volume of 20 μl total. Digestions were incubated 45–60 min at 37 °C and stored at −20 °C until use.

Electrophoresis—Electrophoresis was performed on 1% (total DNA preparations) or 2% (PCR products and RFLP analysis) agarose gels in TBE (45 mM Tris borate, 2 mM EDTA) at 90 V for 30–45 min. Bands were visualized by staining with 5 μg/ml ethidium bromide. Gel results were recorded by one of two methods. Photographs were obtained using Polaroid 667 film and photodocumentation camera and hood from FisherBiotech (Fisher Scientific, Pittsburgh, PA). Digital image files were collected using a Canon digital camera mounted on a homemade photodocumentation box fitted with an orange filter to reduce background illumination from visible light emissions of the transilluminator.

Safety Measures—Safety precautions included handling all ethidium bromide (EtBr) solutions and gels with disposable gloves and wearing ultraviolet-protective goggles when viewing gels on the transilluminator. EtBr was removed from contaminated solutions by filtration prior to disposal [13]. None of the mushrooms species tested was poisonous.

TABLE II
Timeframe for individual steps in RFLP analysis

Procedure	Time required (h)
DNA extraction	1.25
PCR amplification	
Set up	0.5
Run	2[a]
Restriction digestion	
Set up	0.25
Incubation	1
Gel electrophoresis	
Gel set up	0.75[b]
Run	0.75
Gel staining	0.25

[a] Reaction may be set up at the end of the lab period and thermocycler may be programmed to hold samples at 4 °C overnight.
[b] Agarose gel may be poured during restriction digest incubation to save time.

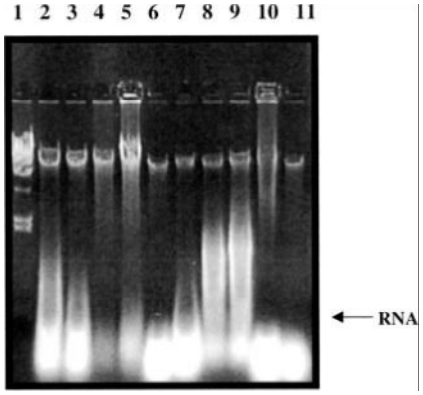

FIG. 2. **Genomic DNA isolated from fresh and dry mushroom specimens.** Genomic DNA was isolated as described from 50 mg fresh or 200 mg of dry tissue, respectively, and 5 μl of each preparation was separated on a 1% agarose gel. *Lane 1*, λHIII marker DNA; *lanes 2* and *3*, 50 and 200 mg fresh *Agaricus*; *lanes 4* and *5*, 50 and 200 mg dry *Agaricus*; *lanes 6* and *7*, 50 and 200 mg fresh *Pleurotus*; *lanes 8* and *9*, 50 and 200 mg *Pleurotus* mycelia; *lanes 10* and *11*, 50 and 200 mg dry *Pleurotus*. *Pleurotus* mycelia were cultured on a sterile nylon cloth overlaid onto nutrient/malt extract agar and harvested directly from the surface of the nylon with sterile forceps.

RESULTS

We modified standard protocols for conducting RFLP analysis to facilitate their application in an undergraduate laboratory setting (Table II). Fungal DNA extraction and incubation times were shortened so that the entire isolation procedure could be conducted in a single 3-h laboratory period. Several noxious reagents, which normally require handling in a chemical fume hood, were successfully omitted from the procedure. BME was not required in the initial extraction buffer in order to obtain sufficient yields of genomic DNA from fresh or dried mushroom tissues (Fig. 2). Similarly, it was possible to omit phenol from the organic extraction step without jeopardizing yield or quality of DNA obtained.

DNA yields varied somewhat among species as did the presence of cellular RNA, visible as broad smears migrating near the bottom of the gel (Fig. 2). In all cases, dry mushroom tissue yielded less DNA as determined by visual inspection of gel lanes. However, mushroom DNA isolated by our protocol, from either fresh or dry specimens, consistently yielded sufficient quantities of genomic DNA for amplification by PCR (Fig. 3). Using ITS1 and ITS4 primers, we consistently observed a single amplification product, corresponding to expected fragment size for this rDNA target region. Specificity was high as reflected by the low background in each of the samples tested.

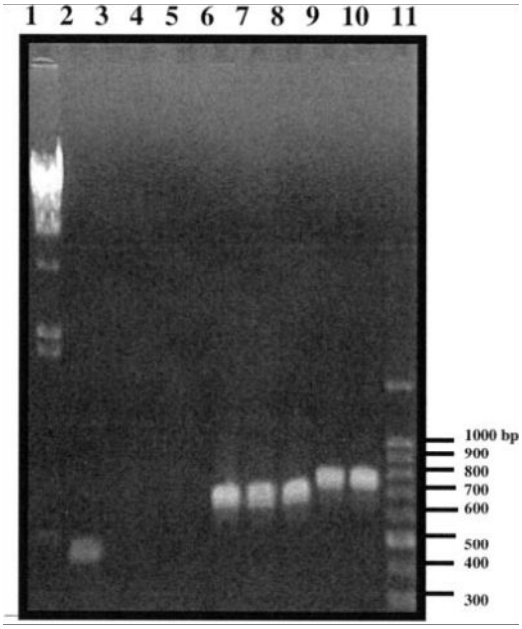

FIG. 3. **PCR amplification of rDNA ITS from fungal DNA.** PCR were conducted according to "Methods," 10 μl was loaded onto a 2% agarose gel, and bands were visualized by EtBr staining. *Lane 1*, λHIII DNA; *lane 2*, positive control plasmid amplicon; *lane 3*, no template DNA control; *lane 4*, ITS1 control; *lane 5*, ITS4 control; *lane 6*, *Pleurotus* (fresh tissue); *lane 7*, *Pleurotus* (dry tissue); *lane 8*, *Pleurotus* (harvested mycelia); *lane 9*, *Agaricus* (fresh tissue); *lane 10*, *Agaricus* (dry tissue); *lane 11*, 100-bp ladder.

RFLP analysis of various mushroom PCR products yielded clear fragment patterns that could be interpreted in light of species variability. *Pleurotus* ITS fragments amplified using genomic DNA from fresh or dried tissue displayed identical restriction fragment patterns when digested with HaeIII (Fig. 4, *lanes 3–5*). HaeIII digestion of PCR products from fresh or dried *Agaricus* tissue yielded identical unique band patterns (*lanes 6* and *7*). Restriction analysis of *Agaricus* ITS using several different endonucleases resulted in distinctly different band patterns for each enzyme tested (Fig. 5*A*). Thus, observed polymorphisms appeared to be species-specific and not due to differences in tissue preparation.

RFLPs were observed upon HaeIII digestion of ITS from various species (Fig. 5*B*). Distinct band patterns were observed between *Agaricus* and *Pleurotus* preparations (compare *lanes 1* and *2*). Interestingly, distinct patterns were observed upon comparison of two distantly related commercial varieties of *Agaricus* (compare *lanes 1* and *3*). Thus, the procedure we describe is useful in distinguishing among diverse species as well as in relatedness studies of various strains of the same species.

DISCUSSION

Molecular analysis has become a powerful strategy for investigating inter- and intra-species relationships, taxa-

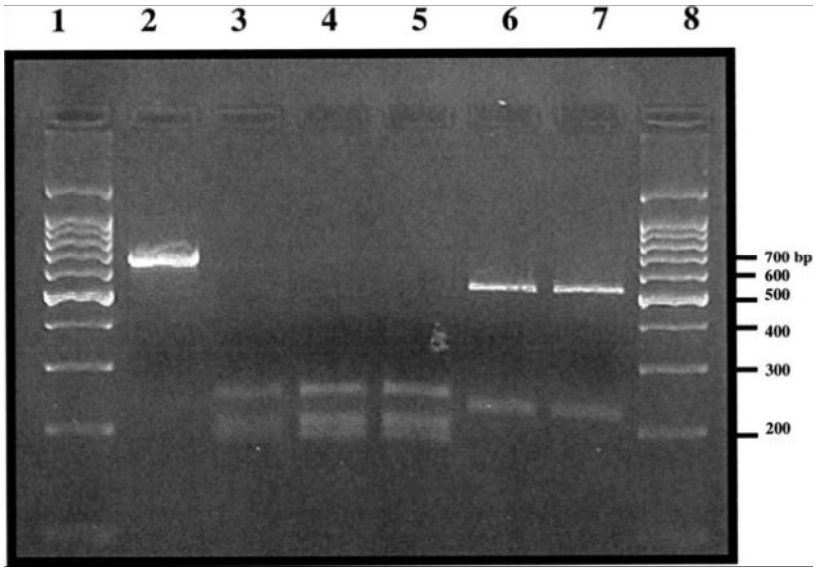

FIG. 4. **Representative RFLP analysis of PCR-amplified ITS region using restriction enzyme HaeIII.** One microliter of each amplified product was digested and separated on a 2% agarose gel. *Lanes 1* and *8*, 100-bp ladder DNA; *lane 2*, undigested PCR product (*Pleurotus*, fresh tissue); *lane 3*, HaeIII-digested *Pleurotus* PCR product (fresh tissue); *lane 4*, HaeIII-digested *Pleurotus* PCR product (dried tissue); *lane 5*, HaeIII-digested *Pleurotus* PCR product (harvested mycelia); *lane 6*, HaeIII-digested *Agaricus* PCR product (fresh tissue); *lane 7*, HaeIII-digested *Agaricus* PCR product (dried tissue).

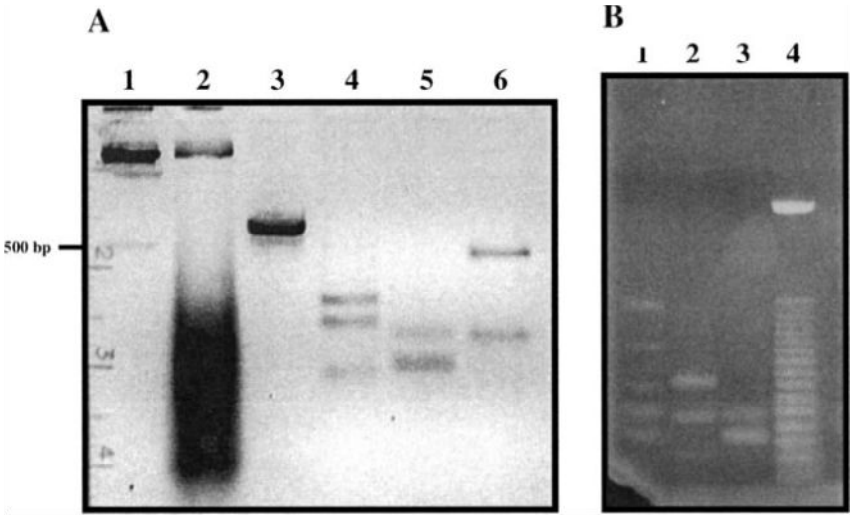

FIG. 5. **PCR-RFLP analysis student results.** *A*, RFLP analysis of *Agaricus* (white mushroom) ITS region. *Lane 1*, λHindIII DNA; *lane 2*, genomic DNA; *lane 3*, PCR-amplified ITS region; *lane 4*, MboI-digested ITS region; *lane 5*, HinfI-digested ITS region; *lane 6*, HaeIII-digested ITS region. *B*, RFLP analysis of ITS region from different species digested with HinfI. *Lane 1*, *Agaricus* (Portabella) ITS; *lane 2*, *Pleurotus* ITS; *lane 3*, *Agaricus* (Button mushroom) ITS; *lane 4*, 50-bp ladder DNA.

nomic classification, and evolutionary diversity among species. The polymorphic ITS region of rDNA is commonly used as a target sequence for molecular classification of fungi. PCR-RFLP analysis is a useful molecular approach that allows careful examination of species diversity at the molecular level and is thus an important technique for biology students to learn.

We designed a molecular strategy for distinguishing commercial mushroom varieties that is easily adapted to investigative projects in an undergraduate molecular laboratory setting [14]. We developed a rapid DNA extraction procedure that eliminates long incubation times and reduces exposure to potentially hazardous reagents. PCR amplification reactions were standardized and simplified by taking advantage of commercially available pre-mixed reaction tubes. Students completed their examination by conducting restriction analysis and gel electrophoresis of PCR products, yielding unambiguous fragment patterns that could be interpreted in terms of the relatedness of different species or strains of mushrooms (see Fig. 5 for representative student results).

In the hands of undergraduate students, the rapid extraction procedure consistently yielded sufficient DNA for PCR-RFLP analysis. Our students successfully isolated DNA from their source mushroom tissue 100% of the time. In addition to the work detailed here with mushrooms, the extraction protocol has also yielded sufficient DNA for PCR-RFLP analysis from other sources. In other courses, our undergraduate students have used the extraction and PCR-RFLP protocols successfully with freshly cultivated bread mold, baker's yeast, fresh tissue of *Drosophila melanogaster*, *Daphnia pulicaria*, and several plant species (not shown).

This project achieved several important learning objectives for molecular biology students. First, students gained facility with basic molecular techniques including DNA isolation, amplification, restriction, and gel electrophoresis. Second, the streamlined protocols enabled students to develop investigative projects that fit into the restricted time frame of the typical undergraduate laboratory. DNA extraction and amplification were achieved in a single 3-h laboratory period, followed by RFLP analysis conducted in a second laboratory period (Table II). Thus students could design investigative projects and obtain interpretable results in a matter of 2–3 weeks on topics such as 1) examining molecular diversity among related varieties of com-

mercial species; 2) characterizing molecular relatedness between the same species obtained from geographically distinct sources; or 3) simple phylogenetic analysis of different species of commercial mushroom.

Student learning objectives included theoretical basis of PCR amplification, evolution of genetic polymorphisms, and molecular phylogenetics. Assessment of student learning was approached in three ways. Individual student comprehension of biological and technological concepts was evaluated by standard written examination. In addition, each research group delivered a PowerPoint presentation of their project. Presentations included detailed analysis of their experimental results in light of their original research question and thus required students to apply their understanding of both the biology and technology in order to interpret their results. Finally, students completed a self-assessment survey in which they reported their learning; all respondents (representing 83% of the class) indicated that their understanding of PCR-RFLP analysis increased significantly. This correlated well with student performance on standard exams and was evident in the depth of analysis included in student presentations. The PCR-RFLP project provided an opportunity for students to examine the basic concepts of molecular variation upon which contemporary taxonomic classification of species is based.

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