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DNA BARCODING

Rapid and reliable high-throughput methods of DNA extraction for use in barcoding and molecular systematics of mushrooms

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Abstract

We present two methods for DNA extraction from fresh and dried mushrooms that are adaptable to high-throughput sequencing initiatives, such as DNA barcoding. Our results show that these protocols yield ~85% sequencing success from recently collected materials. Tests with both recent (<2 year) and older (>100 years) specimens reveal that older collections have low success rates and may be an inefficient resource for populating a barcode database. However, our method of extracting DNA from herbarium samples using small amount of tissue is reliable and could be used for important historical specimens. The application of these protocols greatly reduces time, and therefore cost, of generating DNA sequences from mushrooms and other fungi vs. traditional extraction methods. The efficiency of these methods illustrates that standardization and streamlining of sample processing should be shifted from the laboratory to the field.

Keywords: FTA, fungi, internal transcribed spacer, internal transcribed spacer, proteinase K, rDNA, ribosomal RNA, Whatman

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Introduction

The push for DNA barcoding in all organisms (Hebert *et al.* 2003, Hajibabaei *et al.* 2007; Kress & Erickson 2008; Janzen *et al.* 2009; Seifert 2009) and the appearance of new high-throughput sequencing technologies (Ivanova *et al.* 2006, 2009) also require the development of high-throughput methods of DNA extraction from fungi. Current DNA extraction methods from fungi rely on protocols that are either inconsistent and unreliable (various commercial kits designed for DNA extraction from other groups of organisms) or that have a long history of application to fungi but that are time-consuming, labour-intensive and require the use of toxic chemicals (Zolan & Pukkila 1986; Rogers *et al.*1989; Bruns *et al.* 1990; Haines & Cooper 1993; Rogers 1994). Although high-throughput methods have been developed for other groups of eukaryotes (Ivanova *et al.* 2006, 2009; Whitlock *et al.* 2008), most methods developed for plants and animals cannot be directly applied to fungi and it is not clear whether they will work for these or other organisms.

In this study, we provide two DNA extraction protocols that have been evaluated by their success in generating ribosomal RNA internal transcribed spacer (ITS) DNA barcodes from freshly collected or dried herbarium specimens of mushrooms (filamentous fungi). The first method combines DNA-absorbing filter paper (Whatman FTA® cards) with a commercial DNA extraction kit (Sigma Extract-N-Amp™ Plant PCR Kit) that reliably provides PCR-ready DNA in 10 min from fresh mushrooms. This method enables processing of newly collected tissues prior to specimen preservation, thereby maximizing the quality of isolated DNA. The second method, essentially that developed by Ivanova *et al.* (2006) for vertebrate animal tissues, relies on enzymatic extraction of dried samples without requiring extensive

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grinding of the material beforehand, simplifying the extraction process and making it adaptable to a 96-well plate format. We had to modify the original protocol to tailor it specifically to fungi, whose unique biochemical and structural features typically require alternative methods to those developed for plants and animals. For convenience, we have provided a step-by-step version of our modified protocol of Ivanova *et al.* (2006) for fungi in Document S1.

We encourage users to adopt the first protocol here for standard processing of new collections for DNA work. However, we recommend the second method for processing preserved tissues that might otherwise be difficult or impossible to recollect: our results demonstrate that this second method works for specimens that are over 100 years old, indicating that it could be used for historically important collections including types. With either of these two methods, hundreds of sequences can easily be generated in a single day by one technician and even more sequences if these methods are incorporated in an automated workflow such as at the Canadian Centre for DNA Barcoding facility (http://www.dnabarcoding.ca/). These modified protocols can be adopted as standard methods for processing fungal tissue for DNA analysis for both documenting biodiversity and molecular systematic studies.

Materials and methods

Protocol 1 (FTA) – DNA extraction from fresh mushrooms using Whatman FTA® cards

Taking samples in the field. Small sections of fresh tissue (~1 cm^2 × ~0.5–1 mm thick, preferably containing the hymenium) were excised from the mushroom with flame-sterilized forceps, scalpel, or scissors and laid broadside down in the centre of a quadrant of a Whatman FTA® PlantSaver card. After closing the plastic-reinforced flap over the tissue, a rubber mallet or similar blunt instrument was used to pound the tissue with moderate force. A few smart whaps were usually sufficient to squash the tissue. A video demonstration of this technique can be viewed on YouTube (http://www.youtube.com/watch?v=Gir56iYspTE). It is important to open the flap and observe the liquid contents of the tissue soaking through the card completely. If this is not seen, then there may be insufficient tissue used, insufficient force was applied when hammering or the tissue source is too dry to yield reliable DNA for PCR. We successfully used this method with young, fresh specimens from a wide range of taxa across the Agaricomycetidae, including polypores, e.g. *Ganoderma* spp., and other taxa with little water content, e.g. *Pterula* spp., but we did not thoroughly test this method with drier fruit body types like corticioids and trimitic polypores. The cards were then either allowed to dry on a tabletop or, such as when sampling in the humid tropics, placed in freezer-quality sealable plastic bags that contain a small amount of indicating desiccant. The desiccant should be replaced with a fresh supply whenever necessary to maintain low humidity in the bags.

Lab processing of card samples. The first step is to remove a sample of the Whatman card that is saturated with the fungal DNA. We used the recommended Harris MicroPunch with a 2.0-mm tip and mat, but sharp, clean razors or scalpels worked as well. The punched-out 2.0-mm disc was placed in a well of a 96-well PCR plate and the Harris MicroPunch was then cleaned by punching through several layers of sterile filter paper. We experimented with rinsing the MicroPunch once in ELIMINase® (Decon Labs, Inc.) and twice in two batches of sterile water, but fluid accumulation in the corer became problematic after a few rinses, even though the MicroPunch was dried by punching discs from sterile Whatman filter paper and/or by wiping with a clean KimWipe before punching a disc from the next collection sample. Despite our initial concerns about cross-contamination by sequential uses of the MicroPunch, we did not detect carry-over between samples, confirming Whatman's own testing (Whatman Application Note 8143). Nonetheless, added precaution is encouraged for users who may be dealing with samples where it may be difficult to detect contamination.

After the discs have been transferred to the 96-well plates, we extracted DNA using commercially prepared reagents. Initially, we experimented with the Whatman purification protocol supplied with the FTA cards, but our PCR and sequencing results were inferior to those obtained when using the Sigma Extract-N-Amp™ Plant PCR Kit (Product Code XNAR). In addition, by using the Sigma kit, we were able to rapidly extract reliable, PCR-ready DNA in 10 min rather than the >1 h required for the Whatman protocol. Using the Sigma reagents, we added 25 μL of extraction solution to each well and then incubated the plate in a thermal cycler set at 95 °C for 10 min. After incubation, an equal volume of Dilution solution was added to the reaction to terminate the extraction reaction. From this, a 1:9 to 1:49 dilution in water was used for PCR (should be determined empirically, but 1:29 generally works well). The proprietary reagents used in the extraction kit inhibited standard PCR reactions unless they were diluted beforehand.

We note that Whatman has recently endorsed the GenSolve™ Extraction kit (GenVault) for use with FTA cards (http://www.whatman.com/GenSolve.aspx), but this protocol is still more laborious and time-consuming (1 h) than the Sigma protocol presented in this study.

Protocol 2 (EDGF) – DNA extraction from dried mushrooms using enzymatic digestion and glass-fibre filtration

Ivanova *et al.* (2006) described a protocol for extracting DNA from mammals in 96-well plates using glass fibre (silica) filters. In their protocol, tissues are incubated overnight in a lysis buffer containing proteinase K. Then the DNA is bound to glass fibre filters in 96-well plates and contaminating proteins and other cellular debris are washed away before releasing the silica-bound DNA for downstream applications. We tested this method on our fungal samples and discovered that it was necessary to make slight, but necessary modifications to the original protocol of Ivanova *et al.* (2006) for fungi. These changes included doubling the volumes of Lysis Buffer and proteinase K in the initial incubation, an increase in the Protein Wash Buffer volume to 250 μL and performing two washes each of 300 μL Wash Buffer. We also provided an improved, *optional* protocol where we replaced the centrifugation steps with vacuum-assisted washes using a Promega Vac-Man® Laboratory Vacuum Manifold as in Whitlock *et al.* (2008): the vacuum manifold was much easier to use and greatly reduced the time involved in carrying out the extraction procedure. We typically used a 1:49 dilution in water of extracts from this method for PCR, but this should be determined empirically depending on the amount of tissue used for the extraction. Our modified protocol is available in Document S1.

PCR and sequencing

PCR amplification. Based on an alignment of selected Agaricomycetidae and Ascomycota, we designed two new primers (Fig. S1): the forward primer 'ITS8-F' (5'-AGTCGTAACAAGGTTTCCGTAGGTG-3') and the reverse primer 'ITS6-R' (5'-TTCCCGCTTCACTCG-CAGT-3'), which provided improved amplification and sequencing success of mushrooms over traditional primer pairs (White *et al.* 1990) when a quick enzyme-based PCR clean-up method was used (see below). This primer combination produced strong PCR bands, had very little homo- and hetero-dimer formation, and provided enough flanking reads in the sequencing reactions to consistently identify the CATTA– and –GACCT motifs (sometimes with slight modifications) that represented the 3'- and 5'-termini of the 18S and 25S ribosomal RNA subunits adjacent to either end of the ITS 1 and 2 regions respectively. Amplification using PCR was carried out in 10-μL reactions containing 1.0 μL of 10× PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM potassium chloride, 2.5 mM magnesium chloride, 0.1% gelatin, 1.6 mg/mL bovine serum albumin), 1.6 μL dNTPs (1.25 mM of each dNTP), 0.2 μL of each primer (10 μM), 0.1 μL of Invitrogen Platinum *Taq*, 2 μL of DNA and 4.9 μL of sterile water. We also successfully used the Sigma JumpStart *Taq* ReadyMix for PCR amplification, a component of the Plant DNA Extraction Kit, after increasing the $MgCl_2$ concentration in the ReadyMix to a final concentration of 2.5 mM. Thermal cycling was executed on an Eppendorf MasterCycler (model 5345) with the following parameters: initial denaturation at 95 °C for 2 min, five cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min; followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min; a final extension at 72 °C for 10 min and a final step of indefinite refrigeration at 4 °C.

DNA sequencing. After visualization of positive PCR products on a 1% agarose gel, PCR samples were cleaned prior to sequencing using a combination of exonuclease I and shrimp alkaline phosphatase. To each sample was added 0.4 volumes of a master mix containing 1% exonuclease I (10 units/μL), 10% shrimp alkaline phosphatase (1 unit/μL) and water. The solutions were mixed and incubated at 37 °C for 15 min, then at 80 °C for 15 min in a thermal cycler. Alternatively, bands were isolated by excising them from gels and placing them in the top of a cut Progene® UltraClear universal fit 200 μL filter barrier tip (catalogue no. 24-TF200-RS) set in a 1.5-mL microfuge tube and the DNA was collected in the flow-through after spinning the tubes at 10 000 *g* for 10 min. Typically, 2 μL of clean PCR was used in the sequencing reaction. The sequencing reactions were carried out in 10-μL volumes containing 2 μL reaction buffer (ABI), 1 μL primer (10 μM), 2 μL betaine (5 M), 2.75 μL water, 0.25 μL BigDye (ABI) and 2 μL PCR product. Cycling parameters for sequencing were as follows: initial denaturation at 96 °C for 1 min followed by 35 cycles of denaturation at 96 °C for 10 s, annealing at 55 °C for 10 s and extension at 60 °C for 4 min. Sequencing reactions were precipitated using ethanol precipitation as described by the manufacturer (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040995.pdf) and resuspended in 15-μL Hi–Di formamide prior to loading on an ABI capillary sequencer (Prism 3100 or 3730).

Results

Based on ~600 samples, we achieved ~95% PCR success and ~86% sequencing success using the FTA protocol for fresh tissue described above. The EDGF protocol for herbarium tissue yielded PCR and sequencing success from recently (<2 years old) collected specimens that was similar to that from FTA cards blotted with fresh tissue

(~95% PCR success, ~85% sequencing success; based on 192 samples), whereas samples older than *c*. 10 years showed lower and less-consistent success in both PCR (~40–70%) and sequencing (~25–50%). Our new primers showed improved performance over traditional primers in that they did not produce dimers during the PCR reaction, allowing us to use the quick enzymatic PCR clean up for cheap and rapid presequencing sample preparation.

Discussion

Our application of Whatman FTA® cards to sampling DNA from fresh mushrooms in the field is very reliable and DNA extraction from tissue blotted on card discs is extremely quick (10 min) when used in combination with the Sigma Extract-N-Amp™ Plant PCR Kit. New robotic systems capable of rapidly processed FTA card samples (http://www.whatman.co.uk/UserFiles/File/Protocols/Bioscience/Preparing%20FTA%20Punches%20using%20a%20Xyril%20Liquid%20Handling%20System.pdf) make the FTA card protocol an extremely attractive method for rapid processing of fresh fungal samples. With our combination of FTA® cards and the Sigma kit (FTA protocol), our success rate in mushrooms is comparable with that of Ivanova *et al*.'s (2009) recently reported study for animal tissues. Surprisingly, the Whatman protocol supplied with the cards was the least reliable method we tested. We suspect that the poor success of the manufacturer's protocol is due to inefficient extraction of DNA from the cards' fibres compared with the other two methods we assayed, a conclusion that is supported by the high success rate from combining a traditional DNA extraction kit for plant tissue with the filter paper discs.

Although the up-front cost of FTA® cards may be prohibitive for some labs (USD $1.25/sample when using each quadrant of a FTA PlantSaver card for a different sample, not including the cost of the Sigma Extract-N-Amp™ Plant PCR Kit), the long-term savings in terms of cost of labour (~1 h for 96 samples vs. traditional methods that can take 2 days for an equivalent number of samples) no doubt exceed the initial investment. The addition of the Sigma Extract-N-Amp™ Plant PCR Kit only adds USD$0.36 and this includes a PCR ReadyMix with thermostable *Taq* polymerase. Furthermore, this protocol also offers additional advantages over traditional protocols that are implemented after specimens have been processed and preserved in the field. First, Whatman FTA® cards are easy to travel with because they are compact and designed to protect DNA from damaging conditions such as humidity, heat and degradation by specimen-colonizing microorganisms, making them suitable to all types of fieldwork. Moreover, according to the manufacturer, the DNA preserved on the cards is stable for years under standard tabletop conditions, unlike traditional DNA extractions that need to be frozen at −80 °C or colder to avoid degradation. This ease of storage of DNA on FTA® cards will also greatly reduce the cost of DNA archival because they do not require refrigeration. In fact, these cards are the ideal complement to herbarium vouchers and could easily be included with the specimen in herbarium containers.

The second method (EDGF protocol), a modification of Ivanova *et al*. (2006), has greatly simplified and reduced common extraction procedures from fungi so that they can be used in a 96-well plate format. We have employed this protocol for over 1000 tissue samples and have found it to be a robust method for extracting high-quality DNA from herbarium specimens, with varying PCR and sequencing success depending on the age of the samples. To examine if this method was capable of extracting PCR-ready DNA from very old material, similar to the ages of many type specimens, we extracted DNA from 118 herbarium samples from a broad taxonomic sampling of Agaricomycetes, which were collected between 1890 and 1942. Using primers ITS8-F and ITS6-R, we could only amplify 6% of samples, but by using the primer pair ITS8-F and 5.8S (Vilgalys & Hester 1990) to amplify the shorter ITS1 region (~250 bp), we increased our success rate to 35%, indicating that not only were we able to obtain DNA but also that it was in enough quantity and of high enough quality (albeit in a degraded state) to amplify one-third of all specimens without laborious troubleshooting. These amplifications were verified with sequencing. The oldest specimens from which we were able to obtain ITS1 sequences were a 119-year-old collection of *Hygrophorus pustulatus* (collected in 1890) and a 112-year-old collection of *Schizophyllum commune* (collected in 1872). However, the sequencing trace files were not always clean, indicating that adequate DNA extraction does not guarantee adequate sequences from old material. The older samples may exhibit DNA degradation because of the time since collection, but other confounding factors, such as inadequate preservation and specimen processing, make it difficult to determine what the true cause is of the lower success with older tissues. In any case, older specimens may not be worth mining for populating a DNA barcode database or for large systematic studies except in special circumstances, such as when data from type specimens are needed to resolve taxonomic ambiguities.

Polymerase chain reaction success is not necessarily correlated with the quantity of DNA in the extracts. We used a NanoVue™ Spectrophotometer (GE Healthcare) to quantify the DNA content of our enzyme/glass filter extractions. From 285 samples in three plates, representing a broad sampling of taxa across the Agaricomycetidae

that were selected from two herbaria and representing collections from 1981 to 2006 (median year = 1992), we had a wide range of DNA concentrations (6.6–5784 ng/μL) and an average of 460.70 ± 36.61 ng/μL (mean ± standard error). The FTA card protocol could not be compared because the solutions used in the Sigma extraction kit are incompatible with the spectrophotometer. To evaluate whether spore-bearing (SB; i.e. lamellae) or non-spore-bearing (NSB; i.e. pileus context) tissue is a more reliable source of DNA, we selected 95 samples containing a broad sampling of taxa (Russulales, Phallales, Agaricales, Polyporales, Boletales, Cantharellales, Hymenochaetales) collected from 1981 to 1990 and extracted them in parallel using the enzymatic/glass filter method. Our quantification of these extracts shows that they are quite variable in DNA content (SB: 6.6–1154 ng/μL, NSB: 6.2–496 ng/μL) but that, on average, SB tissue provided twice as much DNA than NSB tissue (214.6 ± 21.88 ng/μL vs. 100.3 ± 10.79 ng/μL respectively; mean ± standard error). Thus, SB tissue is preferred for the enzyme/glass filter method. We suspect that the higher DNA concentrations from SB tissue are due to increased hyphal density and the presence of large numbers of spores.

Although the modest success achieved with older material illustrates the importance of new collections for populating a DNA barcode database or for large systematics studies, our EDGF protocol could be adopted by herbaria around the world to acquire DNA sequence data from important historical specimens because only a very small amount of tissue is required. The amount of destructive sampling for this method causes no more damage to the specimens than is caused by traditional micromorphological examination. These minute amounts of tissue also make this extraction method attractive for quick processing from other sources of DNA, such as ectomycorrhizal root tips, which could be processed in large batches.

In conclusion, we have developed two high-throughput protocols for DNA extraction from mushrooms that provide rapid and reliable methods for generating ITS barcodes and for systematic studies where large sequencing efforts are needed. Because mushrooms are filamentous fungi, these methods will enable rapid processing of most fungi for studies using DNA. However, rapid processing of material is only useful if the preprocessing of specimens from many collectors is funnelled through a single DNA barcoding facility because the time required for specimen collection and preprocessing will exceed that of identification through DNA barcodes (Borisenko *et al.* 2009). Importantly, a preprocessing protocol for DNA barcoding of macrofungi (mushrooms and allied fungi) should be universally applied and include several basic criteria, including a high-quality photo of the fresh collection showing important diagnostic features (preferably with a tag containing a 1-cm scale bar and collection ID in the photo), GPS coordinates of the collection in degree-decimal format and preservation of the specimen using dry heat (~45 °C). Proper specimen preparation is *sine qua non* to DNA barcoding because the success of DNA barcoding relies on the proper curation of a DNA sequence database representing vouchered specimens. These vouchered specimens, the traditional currency of natural history collections, will remain the key resource for DNA-based identifications and enable taxonomic re-examination in the future (Ruedas *et al.* 2000, Borisenko *et al.* 2009).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Document S1. Enzymatic/glass filter plate extraction for fungi.

Fig. S1 Sequence alignment of the 3′-18S and 5′-25S termini from selected Ascomycota (*Eupenicillium* through *Acremonium*) and Agaricomycetidae (*Hydnellum* through *Cantharellus*) used to design new primers for ITS rDNA amplification in mushrooms.

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