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A. H. Harrington
Hendrix College

A. F. Bigott
Hendrix College

B. W. Anderson
Hendrix College

M. J. Boone
Hendrix College

S. M. Brick
Hendrix College

See next page for additional authors

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Department of Biology, Hendrix College, 1600 Washington Avenue, Conway, AR 72032, USA

*Correspondence: willyard@hendrix.edu

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Abstract

Traditional methods for fungal species identification require diagnostic morphological characters and are often limited by the availability of fresh fruiting bodies and local identification resources. DNA barcoding offers an additional method of species identification and is rapidly developing as a critical tool in fungal taxonomy. As an exercise in an undergraduate biology course, we identified 9 specimens collected from the Hendrix College campus in Conway, Arkansas, USA to the genus or species level using morphology. We report that DNA barcoding targeting the internal transcribed spacer (*ITS*) region supported several of our taxonomic determinations and we were able to contribute 5 *ITS* sequences to GenBank that were supported by vouchered collection information. We suggest that small-scale barcoding projects are possible and that they have value for documenting fungal diversity.

Introduction

At present, 70,000 species of fungi have been published (Blackwell 2011); however, estimates suggest there may be as many as 1.5 million extant fungal species, leaving the majority of species to be described (Hawksworth and Rossman 1997). Traditional taxonomy requires diagnosable morphological and/or anatomical characters, but variation within some fungal species and subtle variation between related taxa makes it difficult to rely solely on these characters when identifying an unknown fungal specimen (Hyde et al. 2013). Fungal identifications are greatly complicated because many species do not produce, inconsistently produce, or briefly and seasonally produce the macroscopic reproductive structures (sporocarps) that are used to distinguish between sister taxa, e.g. the gills or pores of mushrooms. Environmental samples, where fungal hyphae are collected without fruiting bodies, are especially challenging to identify using morphological characters as they are likely to represent a mixture of different species (Nilsson et al. 2012). In addition to subterranean fungi, endophytic fungi that grow between living plant cells are very poorly understood taxonomically but are of great interest as a source of antimicrobial metabolites for use in agriculture and human health (Schulz et al. 2002). Small geographic areas have the potential to harbor very high levels of fungal biodiversity that will require extensive surveying, but identifying the fungal specimens from these surveys continues to be a challenge even if sporocarps are available. In order to identify a vascular plant in North America, we could refer to a flora, which lists the known species of each geographic region and provides identification guides and habitat information. Vascular plant flora are incomplete or outdated for many regions of the world. In contrast, a comparable mycoflora is completely absent, even for fungi in North America with large sporocarps. For Arkansas, there is no checklist of fungal species that have been documented to grow here. Promising efforts are underway to create a mycoflora (Bruns 2012) that will rely on well-documented collections with voucher specimens. These comprehensive guides, still in the early stages of compilation, will also integrate genetic relatedness using similarity of DNA sequences (DNA barcoding).

Using Polymerase Chain Reaction (PCR) to amplify a targeted genetic marker and DNA sequencing to determine the content of that sequence, DNA barcoding allows for the comparison of an informative sequence from an unknown fungal sample against a database of identified sequences. This technique will eventually allow us to describe and identify the asexual species and can provide an alternative method of identification for sexual species

when sporocarps are not available. When used in conjunction with morphological identification, DNA barcoding is predicted to provide a more reliable means of species identification and will contribute to the understanding of cryptic species that cannot be reliably distinguished using morphological characters. Although the databases that can be used for reference are limited in several important ways, active efforts are underway to remedy these challenges (Nilsson et al. 2012, Schoch et al. 2014). First, many misidentifications remain that are difficult to correct because the DNA sequence is not paired with a vouchered specimen in a herbarium with adequate geographic collection data. Second, the databases are incomplete. Third, active curation is needed in order to coordinate taxonomic assignments.

A critical shortage of trained mycologists has been noted (Bruns 2012). Given the limited resources that are currently available to train novices on morphological fungal identification and the high likelihood that fungal taxonomy would benefit from local surveys, how might a college undergraduate laboratory best be structured? Novices are unlikely to make substantial contributions to the taxonomic issues. However, our premise was that a small local effort, conducted in a classroom setting, could make use of DNA barcoding to confirm morphological identifications and simultaneously contribute vouchered DNA sequences to a reference database that might help experts document the geographic range of fungal species. These student researchers could apply inquiry-based methods to learn and their results would help others evaluate the effectiveness of DNA barcoding and whether this type of small-scale study can play a role by strengthening geographic distribution information.

We used a protocol for fungal DNA isolation and barcoding (Schoch et al. 2012) that could be adapted by other student researchers or by natural resource managers. Using a limited set of collections, one pair of PCR primers, and one pass of PCR and nucleotide sequencing for each specimen, we evaluated how reliably DNA barcoding can presently be utilized to identify local fungi. In order to accomplish this, we collected fungal fruiting bodies from the Hendrix College campus. We sequenced the *ITS* region because it is taxonomically informative (Schoch et al. 2012) and widely represented in fungal DNA barcoding databases such as GenBank (Benson et al. 2014). DNA barcoding results were compared to our morphological identification to make species determinations. Inconsistencies between molecular and morphological identifications reflect fundamental challenges with the DNA barcoding process, but we suggest that our DNA barcoding project, conducted in an undergraduate laboratory course, has increased the number of vouchered collections in central Arkansas and will support mycologists in their efforts to document our fungi. With improving curation of barcoding databases and mycofloras, we expect these efforts to become increasingly useful in the near future.

Methods

We collected 9 specimens on October 7, 2013 on the Hendrix College campus in Conway, Arkansas (Faulkner County). An edible mushroom (J-M Farms Inc., Miami, OK, USA), purchased from a grocery store, served as a positive control. We photographed each specimen prior to collection, documented the substrate and surrounding environment, and noted morphological characters (Table 1). We removed the fruiting bodies from their substrates close to the base using a knife. We collected spore prints by placing the specimens on herbarium paper with their gills or pores facing the surface of the paper and storing them overnight in a cabinet with limited airflow. Specimens were dried at 28°C for one week then stored in zip-lock bags. Dried specimens along with their spore print and herbarium labels were permanently stored in the Hendrix College Herbarium (HXC) as voucher specimens. We identified each fungal specimen using dichotomous keys and other resources (Arora 1986, Gilbertson and Ryvarden 1988, Lincoff and Nehring 1981, Lincoff and Giovanni 1982, Roody 2003, Jay Justice *pers. comm*.).

To reduce contamination prior to DNA isolation, we scraped off the surface layer of each fruiting body with a razor blade and chopped a 15-20 mg section of the fungal fruiting body into small pieces. We placed these pieces into FastPrep Tubes (MP Biomedicals, Santa Ana, CA, USA) with a ceramic bead, garnet sand, 400 µL AP1 Buffer and 4 µL RNase A from a DNeasy kit (Qiagen, Germantown, MD, USA). We submerged the tubes for 2 minute intervals in alternating dry ice/ethanol and boiling water baths for 6 cycles in order to compromise the fungal cell walls. We processed each sample in a FastPrep homogenizer (MP Biomedicals) using 3 runs of 20 seconds at 6 m/s. We transferred the resulting lysate to a Qiagen DNeasy membrane tube and followed the DNA isolation protocol for the DNeasy kit but eluted DNA

Table 1. Ten fungal samples used in this study.

Sample	Identification	Morphological Characters	Substrate	Latitude and Longitude	Herbarium Accession	GenBank Accession	Consensus Sequence Length
A	*Bjerkandera* sp.	Bracket shape, spongy and fibrous, grey cap, dark grey pores, beige spores, no distinct scent	Stump of unknown hardwood tree species	35.100° N -92.441° W	HXC5819	N/A	N/A
B	*Inonotus dryadeus* (Pers.) Murrill	Indeterminate shape, dimples on cap, spore color unknown, flesh turned black in presence of KOH	Exposed roots of *Quercus* sp.	35.098° N -92.443° W	HXC5812	N/A	N/A
C	*Trichaptum biforme* (Fr.) Ryvarden	Bracket shape, stipe absent, tough and leathery skin, banded green and gray coloration, spore color unknown	Trunk of living *Q. shumardii*	35.098° N -92.443° W	HXC5818	KF986264	720 bps
D	*Russula* sp.	Convex and red cap, white gills, white stipe, brittle stipe and gills, off-white spores, apple-like scent when fresh	Grassy soil near *Q. pagoda* and *Q. phellos*	35.101° N -92.442° W	HXC5817	N/A	N/A
E	*Bondarzewia berkeleyi* (Fr.) Bondartsev & Singer	Overlapping caps with wavy margins, cap upper surface rough and yellow-brown, white flesh, diminished stipe, pore surface decurrent and white, spore color unknown	Trunk of living *Q. phellos* at soil surface	35.099° N -92.441° W	HXC5816	KF986266	444 bps
F	*Ganoderma* sp. (lucidum complex)	Bracket shape, sessile cap, blood-red color, porous surface, tan to brown spores	Trunk of living *Q. palustris*	35.099° N -92.441° W	HXC5820	N/A	N/A
G	*Amanita jacksonii* Pomerl.	Smooth red cap, free gills, peach colored stipe, white and sac-like volva, white spores	Moist soil near roots of *Q. phellos*	35.100° N -92.440° W	HXC5814	KF986265	643 bps
H	*Amanita* sp. (section *Lepidella)*	Dome shape, white with brown scales, brown gills, smooth stipe, partial veil, light brown spores	Mossy, moist soil under *Q. phellos*	35.100° N -92.440° W	HXC5811	N/A	N/A
I	*Boletus bicolor* Raddi	Short with convex brown cap, yellow stipe, olive brown ellipsoidal spores, and no bluing reaction when cap was removed	Grassy soil	35.101° N -92.441° W	HXC5813	KF986268	557 bps
J	*Agaricus bisporus* (J.E. Lange) Imbach	Dome shaped off-white caps, white flesh and stipe, brown gills, brown spores	Commercially cultivated	N/A	HXC5815	KF986267	670 bps

to a final volume of 40 µl. The concentration of the isolated DNA was measured via absorbance using a NanoPhotometer P-Class (Implen, West Lake Village, CA, USA).

We used PCR to amplify about 700 bp of the *ITS* (*ITS1*, *5.8S*, *ITS2*). Primers (IDTdna, Coralville, IA, USA) were designed to be fungal-specific for *18S* (ITS1-F: CTT GCT CAT TTA GAG GAA GTA A; Gardes and Bruns 1993) and *25S* regions (ITS4: TCC TCC GCT TAT TGA TAT GC; White et al. 1990). Each 50 µl PCR reaction contained 1X Bullseye Red Taq DNA Polymerase buffer (Midsci, Valley Park, MO, USA), 0.5mM each primer, and from 30 ng to 150 ng of DNA. The thermocycler settings were as follows: denaturation at 95ºC for 3 minutes; 35 cycles of denaturing at 94ºC for 30 seconds, annealing at 55ºC for 40 seconds, and extension at 72ºC for 50 seconds; and a final extension at 72ºC for 7 minutes.

We ran 3 µl of each PCR product and a ladder designed for approximate quantification (GeneRuler 100 bp, Thermo Fisher Scientific, Pittsburgh, PA, USA) with SYBR green loading dye (1:1000; Life Technologies, Carlsbad, CA, USA) on a 1% agarose gel in sodium borate buffer at 200V for 35 minutes. We photographed gels under UV light to confirm the success of PCR, to estimate amplicon length, and to estimate the quantity of PCR product. We purified PCR products using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions but eluted to a final volume of 32 µl. For one sample with 2 bands, the brightest band was cut from the gel using a razor blade on a UV light table. DNA from the gel slice was purified using the QIAquick Gel Extraction kit (Qiagen). About 20 ng of purified PCR product and 20 pmol of primer were submitted to the DNA Core Facility at the University of Arkansas for Medical Sciences (Little Rock, AR, USA). Each sample was Sanger sequenced twice, once with the forward and once with the reverse PCR primer.

We edited the trace files using Geneious Pro software (vers. 6.1.7; Biomatters Ltd, Auckland, NZ) by trimming low quality ends of the forward and reverse sequences and aligning them to create a consensus sequence of double-stranded, confident reads. A few ambiguous base calls were manually edited to 'N' or the more appropriate base. We used each consensus sequence to search GenBank using MegaBLAST (NCBI 2014) with default parameters. The results of each MegaBLAST search were visualized using a Distance Tree of Results with the default options (Fast Minimum Evolution; Maximum Sequence Difference = 0.75). Trees were downloaded in Nexus format and nodes were collapsed and relabeled using FigTree (vers. 1.4.0; http://tree.bio.ed.ac.uk/software/figtree/). Consensus sequences were submitted to GenBank (Table 1).

Results

We were able to identify 6 collections to the species level (B, C, E, G, I, J) and 4 specimens to the genus level (A, D, F, H) based on morphology (Table 1). DNA isolation failed for sample F when a tube split during homogenization, but PCR was successful for the other 9 samples. Based on agarose gels, amplicons ranged from 550 to 1000 bp and were single-banded except for a faint second band in sample H. We did not submit sample H for sequencing because DNA was not recovered from the gel isolation. The trace files for 3 of 8 samples (A, B, D) showed non-specific amplification that did not support creation of a consensus sequence. Nucleotide sequencing was successful for the remaining 5 specimens (C, E, G, I, J; Table 1), and we created consensus sequences ranging from 444 to 720 bps in length that contained a maximum of 3 unknown base calls per sample.

Pairwise *ITS* similarities supported some of our morphological identifications and raised uncertainty for others. Specimen C, identified morphologically as *Trichaptum biforme*, was confirmed using a query length of 720 bps with 98% identity (95% query cover) to *T. biforme* (AM269815) and the 7 other most similar sequences (90 to 96% identity and 90 to 97% query cover) were also *T. biforme* (Fig. 1A). Specimen E was identified based on morphology as *Bondarzewia berkeleyi* (Arora 1986, Gilbertson and Ryvarden 1988). In contrast, the identity search using a query length of 444 bps found 14 entries from widespread taxonomic groups to all have 89% sequence identity (95 to 100% query cover), none of which were *B. berkeleyi* (Fig. 1B). This consensus sequence was shorter than our others, but only had 0.06% unclear base calls (3 in 444 bps). The morphological, ecological, and distributional features of specimen G suggested *Amanita jacksonii* (Arora 1986). The search using 643 bps found 3 GenBank accessions with 94% sequence identity and 89% query cover - *A. arkansana* H.R. Rosen (JX844674) and 2 species known only from the west coast of the U.S. (*A. calyptroderma* G.F. Atk. & V.G. Ballen (JX844696) and *A. vernicoccora* Bojantchev & R.M. Davis (JX844746)). In addition, there was an incomplete 299 bp *ITS* sequence with 99% sequence identity, *Amanita sp-AR01* (JX844754). Although this match yielded only 67% query cover to our search

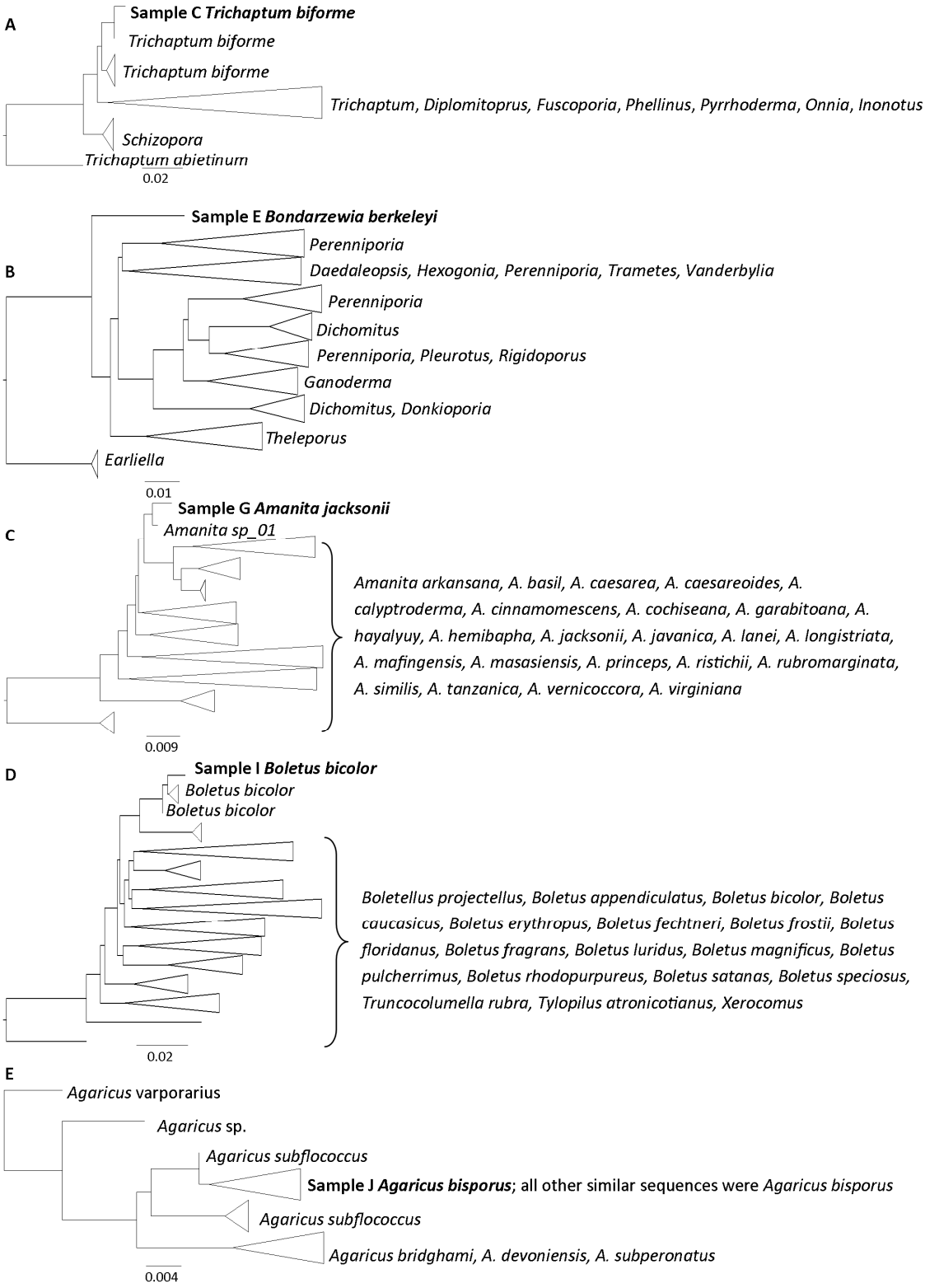

Figure 1. Distance trees showing relationship of each *ITS* nucleotide sequence to similar sequences in GenBank. A, *Trichaptum biforme*; B, *Bondarzewia berkeleyi*; C, *Amanita jacksonii*; D, *Boletus bicolor*; E, *Agaricus bisporus*.

sequence, it covered all of the 299 bp target sequence and our sample was sister to this unpublished taxon in the distance tree (Fig. 1C). Based on morphology, there were 2 candidate species for specimen I with geographic distributions that include central Arkansas - *Boletus bicolor* and *B. luridiformis* Rostk (*B. erythropus* Krombh.). Both species can have some of the colors observed in our specimen, although our sample did not turn blue when the cap was removed (a feature of *B. luridiformis*). The pairwise identity search showed 99% sequence identity (100% query cover) to *Boletus bicolor* (GQ166877) and the distance tree supported the *B. bicolor* accessions as sisters to our specimen (Fig. 1D). For the cultivated mushroom, pairwise identity was in agreement with morphology. For a query length of 670 bps, there was 99% sequence identity (99% query cover) to *Agaricus bisporus,* and this was consistent for the top ten matches.

Discussion

Our method was successfully executed by novice experimenters, suggesting that these are useful DNA isolation, PCR, purification, sequencing, and similarity search techniques. A major limitation in our study was that our experimental design did not budget for repeating failed samples. With only one attempt, 5 out of 10 samples (C, E, G, I, and J) produced nucleotide sequences that we were able to use for DNA barcoding (Table 1). One DNA isolation failure and one gel isolation failure would likely have been overcome by an experimental design that planned for repeating about 20% of the samples. Five of the 8 samples submitted for sequencing yielded a consensus sequence. Failure of nucleotide sequencing in 3 samples that exhibited apparently single-banded PCR products (A, B, D) may be attributed to intra-individual variation in the *ITS* region (Lindner et al. 2013). Because we did not clone our samples, length variation within the individual could create the symptoms we observed in our trace files. Better PCR amplification and sequencing of *ITS* in *Amanita* may be achieved with primer NS7 paired with ITS4 (Lim and Jung 1998). Primers AML1 and ITS4-B tend to produce better amplification in some other taxa (Gardes and Bruns 1993). Alternative DNA barcode markers might also be useful, e.g. the *28S* nuclear ribosomal large subunit rRNA gene (Schoch et al. 2012). We plan for future undergraduate laboratories to repeat 5 of our samples and to test other PCR primer combinations and/or marker combinations for those that fail in a second round.

The high similarity matches to the 5 samples that we obtained indicate that a robust set of *ITS* sequences are presently available in GenBank and that *ITS* currently allows identification to the species level in some cases (Schoch et al. 2012). However, interpretation of these similarity results is not always straightforward. In cases where highly similar database records suggest different species (or even genera), tools such as Distance Trees help visualize the results. We show a distance tree for each of our samples to allow comparison between those with clear results (Fig. 1A, 1D, 1E) with more complicated patterns (Fig. 1B, 1C). For the control sample (Fig. 1E), the assignment to *Agaricus bisporus* was trivial, as all of the most similar nucleotide sequences were assigned to this species. Our morphological determinations for *Trichaptum biforme* (Fig. 1A) and *Boletus bicolor* (Fig. 1D) were also strongly supported by DNA barcoding similarity. However, 2 of our collections raise intriguing questions regarding species identity and also beg the question of what taxonomic determination should be submitted to GenBank. The *ITS* similarities for sample G suggest that we have added another documented location for a putative new species of *Amanita*. Although distance tree relationships (Fig. 1C) and additional analysis of morphological features supported the affinity of our collection for this new species (Tulloss, *pers. comm*.), we used the *Amanita jacksonii* determination for our GenBank submission pending publication of the new species. The polypore (sample E) results are perplexing. Despite 89% identity to 30 samples in GenBank with 95-100% query coverage, our DNA barcode was not grouped within any clade. The morphologically determined *Bondarzewia* was not one of the 11 potential sister genera based on *ITS* similarity. We confirmed that there were *ITS* accessions in GenBank that have been identified as *B. berkeleyi*, but they were not highly similar to ours. Each of the *ITS*-similar genera appear to be highly unlikely candidates based on known geographic distribution or differing diagnostic characters. For example, the sister genus *Theleporus* (Fig. 1B) has only been reported from China; *Daedaleopsis confragosa* in known from Arkansas, but differs in color, spore surface, and substrate of dead wood; *Perenniporia tenuis* var. *pulchella* was reported from Arkansas on an oak, but is described as being bright yellow; *P. robinophila* is reported in Arkansas but mainly grows on dead *Robinia* sp. or on Moraceae stumps (Gilbertson and Ryvarden 1986). A new search in the UNITE database (Koljacg et al. 2013) during review of this paper did not offer any new reference

sequences or new species hypotheses that explain this dilemma. The taxonomy of the polypores is unsettled (Riley et al. 2014), allowing several possible explanations for our results. The morphological characters that we used may not be definitive or may even be misleading, although it is possible that anatomical characters used in the polypores (Gilbertson and Ryvarden 1986) may be helpful. A third possibility is that there are cryptic polypore genera in Arkansas or that our southeastern U.S. species have been assigned to incorrect genera based on their morphology and anatomy. Although not supported by our molecular evidence, we contributed our *ITS* sequence to GenBank with a determination of *Bondarzewia berkelei*. We note that our precise geographic collection location and our voucher provide a means for mycologists to re-examine the specimen.

Broader use of DNA barcoding in fungal identification will be an important tool for the expansion and improvement of quality nucleotide sequence databases (Begerow et al. 2010). Based on the success rate of our study as well as a similar success rate reported by Osmundson et al. (2013) in a larger study, experiments that budgeted for re-runs, included alternative primers for *ITS*, and sequenced additional loci would likely show a successful DNA barcode identity for a majority of the specimens (Schoch et al. 2012). A central assumption is that these databases are already robust and reliable, but Nisson et al. (2006) concluded that not only do public databases not contain representatives of many fungal groups, roughly 20% of accessions are poorly annotated or misidentified. Several competing projects are underway that seek to remedy these problems by actively curating existing sequences and by including DNA barcodes for the type specimens for each fungal taxon (Koljacg et al. 2013, Schoch et al. 2014). Future similarity searches that use UNITE (Koljacg et al. 2013) and RefSeq (Schoch et al. 2014) can expect increasingly reliable DNA barcoding for fungi. However, concurrent morphological identification for fungi with sporocarps is needed in order to expand barcoding databases to include better geographic distributions. It is also important to note that while some literature is currently available for identifying fungi, (e.g. dichotomous keys and field guides), many of these do not include all local species or even all local genera (Arora 1986). Support for continuing development of a North American mycoflora (Bruns 2012) is also vital. Together, well-curated DNA barcode databases and better morphological documentation will facilitate the future identification of environmental samples lacking sporocarps. This process will be useful to analyze the true diversity of fungal species, particularly those that may never produce a fruiting body. Because our vouchered specimens are available in the Hendrix College Herbarium to support the 5 *ITS* sequences we have submitted to GenBank, we suggest that we have made a small contribution to documenting the fungi of central Arkansas and that small scale barcoding projects such as this one are feasible, even in an undergraduate setting.

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