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A minimally invasive method for sampling nest and roost cavities for fungi: a novel approach to identify the fungi associated with cavity-nesting birds

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Abstract. Relationships among cavity-nesting birds, trees, and wood decay fungi pose interesting management challenges and research questions in many systems. Ornithologists need to understand the relationships between cavity-nesting birds and fungi in order to understand the habitat requirements of these birds. Typically, researchers rely on fruiting body surveys to identify the fungal players in these relationships. Fruiting body surveys enable nondestructive sampling, but vastly underestimate fungal presence and diversity and may miss species of critical importance to cavity-nesting birds; thus new methods for such analyses are necessary. Here we present a novel technique to nondestructively sample the wood inside tree cavities, which produces samples that can be processed using DNA-based methods to identify fungi. We tested our method on Red-cockaded Woodpecker *Picoides borealis* excavations, half of which were from trees with *Porodaedalea pini* fruiting bodies. Using our new approach, we detected *P. pini* in 90% of the excavations in trees with fruiting bodies, but also in 60% of the excavations in trees without fruiting bodies and identified nine additional taxa of wood decay fungi that did not have fruiting bodies present. Our approach offers improved detection of fungi through non-destructive sampling of excavated cavities and we developed an improved primer specific to the fungal phylum that contains most wood decay fungi (Basidiomycota), thus providing managers and researchers a critical tool to better determine which fungi are important to cavity-nesting birds.

Key words: Basidiomycota specific primer, cavity-excavating birds, cavity-nesting birds, excavators, fungi, hole-nesting birds, ITS4b-21, *Picoides borealis*, *Porodaedalea pini*, Red-cockaded Woodpecker, wood decay fungi

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INTRODUCTION

Primary and secondary cavity-nesting birds rely heavily upon tree cavities. Heartwood infecting fungi help create suitable excavation habitat for cavity-excavating birds by softening the wood surrounding excavation sites (Conner et al. 1976, Jackson & Jackson 2004, Witt 2010, Cockle et al. 2012, Zahner et al. 2012). Fungi also create non-excavated or "naturally formed" cavities which are used by cavity-nesters in many systems (Cornelius et al. 2008, Cockle et al. 2011, Wesołowski 2012). However, very little is known about which fungi actually inhabit the wood surrounding excavated and non-excavated cavities. A better understanding of the fungal inhabitants of excavation sites and sites of naturally occurring cavities is necessary in order to better understand cavity formation processes and the dynamics of cavity-nesting communities and to inform forest management affecting these species.

Some studies have implicated specific wood decay fungi to be associated with cavity-excavating birds. For example, Red-cockaded

Woodpeckers *Picoides borealis* have long been thought to have an association with *Porodaedalea pini* (previously *Phellinus pini*), a fungus that infects the heartwood of the living pine trees in which the birds excavate (Ligon 1970, Conner et al. 1976, Jackson & Jackson 2004). Cockle et al. (2012) recently suggested that fungi in the family Polyporaceae facilitate cavity excavation for woodpeckers in Argentina. *Fomitopsis pinicola* has been linked to Picid excavations in northwestern USA (Huss et al. 2002). Many others have noted that *Phellinus tremulae*, one of the most common causes of decay in live aspen, may be important for cavity-excavating birds in aspen-dominated systems (Hart & Hart 2001, Savignac & Machtans 2006, Witt 2010).

Traditionally, correlations between cavity-excavating birds and wood decay fungi have been based on surveys of external fruiting bodies observed on trees that birds choose for excavation. However, fruiting body surveys are biased to the fungal species fruiting at the time of sampling and thus, a limited fungal community is observable with this method (Blanc & Martin 2012, Cockle et al. 2012). Moreover, reliance on visual surveys could cause important fungal species that rarely produce fruiting bodies or produce microscopic or cryptic fruiting bodies to be overlooked completely. Even among those species producing visible fruiting bodies, while the presence of a fruiting body indicates a tree is infected by a fungal species, the absence thereof is not indicative of the absence of fungi. Fungal mycelia can be present within the heartwood of a tree for years, even decades before producing a fruiting body and hence, fruiting body surveys drastically underestimate the prevalence of fungi (Lindner et al. 2011b), as well as the diversity of fungi inhabiting individual trees. Rather, fruiting bodies provide information about which of the fungi present in a tree happen to be fruiting at the time of the survey (Boddy 2001). Furthermore, the presence of a fruiting body on the trunk of a tree may represent a localized fungal infection and does not demonstrate that the entire tree, including the cavity excavation location, has been affected. The columns of decay above and below fruiting bodies are highly variable across host and fungal species (Silverborg 1954). Some heartwood decaying fungi can be responsible for decay in other areas of a tree (e.g. the sapwood) and thus are not necessarily affecting the excavation site. Similarly, fruiting bodies of fungi that are known as agents of heart rot are not always reliable indicators of the presence of heart rot — for example, *Ganoderma applanatum* can be an agent of both heart and butt rot (Ostry et al. 2011). Thus, fruiting body surveys tell us little about which fungal species are actually in the wood surrounding a cavity that has been excavated.

Reliance on fruiting body surveys to examine the relationships between cavity-nesting birds and wood decay fungi results in management guidelines that promote the preservation of trees with existing cavities and visible signs of decay. While it is important to preserve trees with existing cavities and visible symptoms of decay, infected trees that do not have visible signs of decay (i.e. apparently sound trees) may be equally or even more important to cavity-nesting birds (Blanc & Martin 2012). Some bird species, including Red-cockaded Woodpeckers, make their excavations in living trees and continue to use their excavations for many years. For these species, living trees without visible signs of decay may be more suitable for excavation than living trees with visible signs of decay because cavities in the former will endure longer, this may hold true for dead trees in some systems as well. While most cavity excavators complete excavation in dead trees in a relatively short amount of time and only utilize their cavities for one breeding season, it is important that they select trees that are sound and will withstand natural stochastic events, trees with some internal decay but without fruiting bodies fit this description. Furthermore, when a tree with a cavity remains on the landscape for multiple years, it is beneficial to the non-excavating community of cavity users as well as excavating species (Blanc & Walters 2008). Birds often select trees without visible signs of decay for excavation, perhaps purposefully selecting trees that do not have fruiting bodies and thus have not yet entered an advanced stage of decay.

Currently, several methods exist to detect the presence of internal decay in living trees, such as increment coring, destructive sampling (large-scale dissection), the use of Resistographs and sonic and electrical impedance tomography (Brazee et al. 2011). In most cases, these methods only measure the soundness of wood, either visually (coring and destructive sampling) or via resistance (Resistographs). None of these methods allow researchers to determine the identities of the actual agents of decay, and thus like fruiting body surveys, are limited in their utility in documenting fungal communities. Culture-based work is one method that does allow for accurate species

identification, under the proper conditions, but the time and effort required frequently makes large-scale culture-based community studies impractical for managers and researchers alike.

The Red-cockaded Woodpecker is an endangered, cooperatively breeding bird that is an important cavity excavator in the longleaf pine ecosystem of the southeastern United States (Ligon 1970, Walters et al. 1988). Red-cockaded Woodpeckers may have a particularly interesting relationship with heartwood infecting fungi due to their excavation behavior. Uniquely, Red-cockaded Woodpeckers solely excavate their cavities through the sapwood and into the heartwood of living pine trees, and the time to complete this process can range from less than one year to over 15 years (Harding & Walters 2004). Populations of these birds can be limited by the availability of cavities and therefore understanding their excavation behavior is an aspect that continues to be critical to successful management efforts of these endangered birds (Walters 1991).

In an effort to better understand Red-cockaded Woodpecker habitat requirements and help inform forest management decisions based on these requirements, we designed a tool to collect samples of fungi from the wood surrounding Red-cockaded Woodpecker cavities in a non-destructive, minimally invasive manner. These samples were processed with molecular methods using a known fungal specific forward primer (ITS1F; Gardes & Bruns, 1993) and a newly designed reverse primer (ITS4b-21; described herein) specific for the phylum Basidiomycota, the group that contains most wood decay fungi. Our new, non-destructive sampling tool, primer, and method not only aid in determining whether or not a tree selected for excavation is decayed, but also allow for the identification of the main agents of decay within the excavation site. This novel approach can be used to uncover ecological linkages between excavators and fungi and to help guide future management decisions that focus on the preservation of trees suitable for excavation, not only for Red-cockaded Woodpeckers but also for other avian species in other systems.

METHODS

Study site

We conducted this research on Marine Corps Base Camp Lejeune (MCBCL), in Onslow County, on the central coastline of North Carolina, USA. This military installation consists of 110,000 acres of land and 26,000 acres of water. Of the pine stands on MCBCL that are not heavily mixed with hardwoods, 64% are dominated by Loblolly Pine *Pinus taeda*, 24% by Longleaf Pine *Pinus palustris* and 11% by Pond Pine *Pinus serotina* (Walters 2004). The only heart rot fungus we observed fruiting on living pine trees on MCBCL was *Porodaedalea pini* s.l. (Southeastern clade), hereafter referred to as *P. pini* SE. Fruiting body surveys of this close relative of *Porodaedalea pini* s.s. (see description of *Porodaedalea* sp. 1 in Brazee & Lindner 2013) would suggest that this species is not found in abundance on MCBCL. Over 4 years of intermittent but intense visual surveying of our study site, we located only 24 *P. pini* SE fruiting bodies in Red-cockaded Woodpecker habitat on MCBCL. Approximately 2% of the trees housing complete or incomplete, human-made (drilled) or Red-cockaded Woodpecker-made excavations had *P. pini* SE fruiting bodies. The proportion of pine trees in similar habitat with *P. pini* SE fruiting bodies and without excavations is less than 1% (M. A. Jusino unpublished data).

Sampling tool

To collect wood from the interior of Red-cockaded Woodpecker cavities, we designed a specialized tool that can scrape and capture wood shavings from within a cavity without causing destruction of the cavity itself. This tool can be sterilized and easily used at cavity height. Our sampling tool, hereafter called "the sharpened spoon", was designed with ease of sampling in mind. By design, the spoon is less arduous to use for wood collection at cavity height than other tools such as increment borers, chisels or drills. The sharpened spoon enables the collection of wood samples from the interior of cavities or cavity starts and causes much less damage to the tree or the interior of the cavity than drills, chisels, and increment borers.

The prototype of our sampling tool consisted of a 20 gauge stainless steel ½ teaspoon measuring spoon, a 0.33 meter long M6 x 1.0 (¼"-20) threaded steel rod and a M6 x 1.0 steel hex nut (Fig. 1A). We cut the handle of the spoon to a length of 5 centimeters. A 5 centimeter long slot was created in one end of the rod with a grinding wheel affixed to a rotary Dremel tool. The spoon was test fitted into the rod and its width adjusted to allow fitting of the M6 x 1.0 hex nut over the spoon handle. After test fitting, the pieces were disassembled and a sufficient amount of paste flux was applied

to the slot within the rod and the spoon handle to allow for a clean solder connection. The pieces were re-assembled and the M6 x 1.0 nut was threaded onto the rod, locking the spoon handle into place. To ensure the pieces would not loosen over time, we soldered the spoon handle into the rod with a flux-core solder and a propane torch. Finally, we sharpened the outer rim and the inside edge of the spoon with a small grinding stone affixed to the Dremel tool and metal files. Sharpening the inside edge allowed the spoon to scrape the target wood more efficiently (Fig. 1B). Field trials suggest the spoon may need to be re-sharpened after sampling about 100 trees. A 0.3 centimeter hole was drilled through the rod opposite the spoon end, allowing a 2.5 centimeter metal key ring to be added for attaching the tool to a belt for field use (Fig. 2).

Sample collection

We tested our collection tool against two other methods of decay detection, visual fruiting body surveys and core samples at cavity height, using 20 trees on MCBCL with Red-cockaded Woodpecker excavations. Of the 20 trees selected, 10 trees had fruiting bodies of *P. pini* SE and 10 trees did not have fruiting bodies. We photographed and aseptically sampled fruiting bodies, but did not collect them because they are very rare on our study site. One of us (MAJ) climbed each selected tree with Swedish climbing ladders, a climbing harness and a tree strap. Once cavity height was reached, the sharpened spoon was flame sterilized with 70% ethanol. We collected samples from two to three locations within the cavity

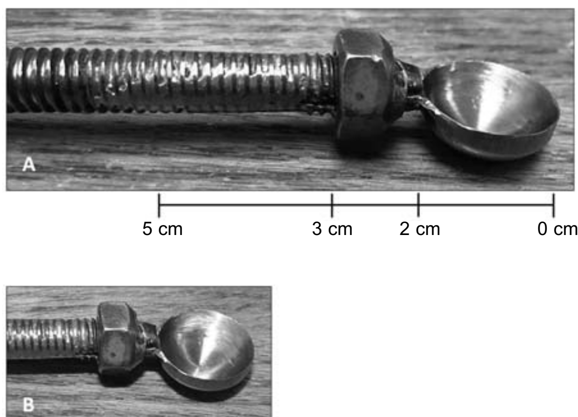

Fig. 1. The design of our sampling tool, which is made of a sharpened measuring spoon, a steel rod and a hex nut. A — side view, with a size reference, B — top view of the inside of the spoon.

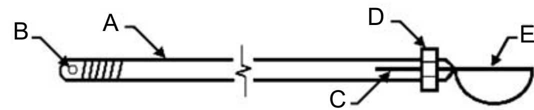

Fig. 2. An outline of our sampling tool with the components labeled. A — the threaded steel rod; B — key ring hold; C — five centimeter long slot; D — hex nut; E — sharpened measuring spoon.

depending on whether the tree housed a fully excavated cavity or a cavity start (i.e. incomplete excavation; Fig. 3). We flame sterilized the spoon between each sampling location.

The sampling locations within the cavity were determined by how accessible they were with the spoon (Fig. 3). Sampling location A refers to the entrance tunnel of the cavity, and represents the sapwood of the tree. Location B refers to the dome (or roof) of the cavity and consists of heartwood. Location C refers to the back wall of the cavity, and consists of heartwood. Location E refers to the front of a cavity start and is sapwood whereas location D refers to the back of a cavity start and is typically heartwood. If a start was not advanced enough to have reached the heartwood, we collected E, but not D.

We used the sharpened spoon to scrape wood shavings from each sampling location. Some force was used to effectively scrape the wood and gather the shavings in the bottom of the spoon. After each scrape, the sharpened spoon was extracted from the cavity and the sample was transferred from the spoon into a sterile 1.5 mL tube. Though each scrape could be considered a separate sample, our data are pooled for this analysis because our goal is to show that the sharpened spoon can be used to identify agents of decay in the wood surrounding Red-cockaded Woodpecker excavations. After attaining cavity height, the total time to sterilely sample all locations in one excavation with the spoon ranged from 5 to 10 minutes.

Additionally, the cavity starts in our sample were aseptically cored approximately 20 cm above the cavity entrance using a sterilized borer (Fig. 3F). The heartwood of these cores was kept, the sapwood portion was re-inserted into the core site to prevent the introduction of pathogenic organisms. Completed cavities were not cored at cavity height because it is possible to introduce a fissure in the dome of a cavity when coring, which would allow resin to drip into the body of the cavity and cause harm to the cavity occupant(s). There was

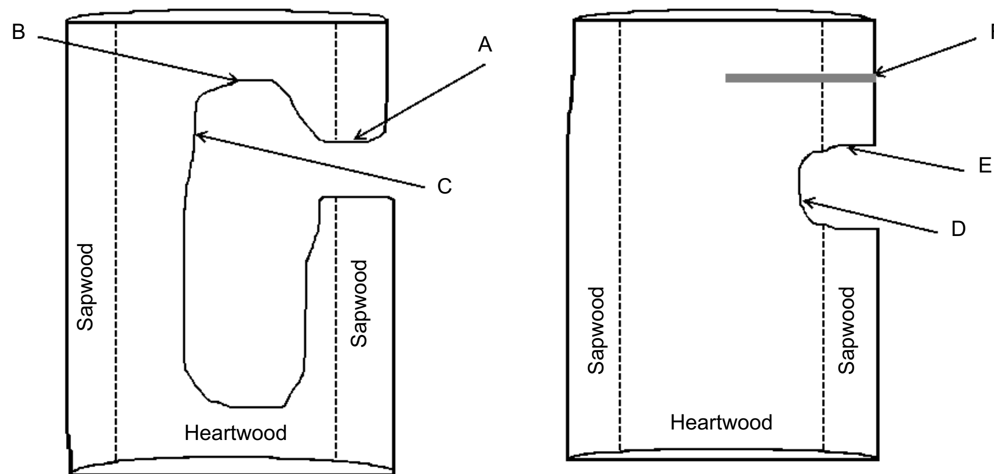

Fig. 3. Cross sections of a complete cavity (left), and a cavity start (right), showing approximate sampling locations as well as approximate heartwood and sapwood dimensions. A-E — scrape samples: A & E — in the sapwood, B, C & D — in the heartwood, F — the approximate coring site.

no such danger for cavity starts. The total time to properly clean a borer and collect a core sample at cavity height ranged from 30 to 40 minutes, not including climbing time.

DNA extraction, PCR, cloning and sequencing
Scraped wood samples and fruiting body samples were stored in 200 μL of filter-sterilized CTAB cell lysis solution (see Lindner and Banik 2009 for solution protocol) and frozen at -80 °C. DNA extraction followed the protocol described by Brazee & Lindner (2013). Increment core samples were placed in 30 mL of filter-sterilized CTAB cell lysis solution (Lindner & Banik 2009) and ground using a sterilized immersion hand blender. After the cores were ground, they were frozen at -80 °C for at least 24 hours. Subsequent extraction of DNA from cores followed a modified version of the protocol described by Brazee & Lindner (2013). Frozen samples were placed in a 65 °C water bath for 3 hours. Then one mL of supernatant was transferred to a 1.5 mL tube and centrifuged at 10,000 rcf for 10 minutes and 100 μL of supernatant was transferred to a new strip tube. Negative controls were included for both extraction protocols, and these extractions served as our negative controls for each downstream step.

We tested standard PCR, cloning and sequencing protocols on *Porodaedalea pini* SE fruiting body DNA using a primer pair that is reportedly specific for fungi in the phylum Basidiomycota, ITS1F and ITS4b (Gardes & Bruns 1993). We had limited success directly sequencing or cloning and sequencing *Porodaedalea pini* SE DNA using this primer pair, even after significant manipulation of variables such as annealing temperature, number of PCR cycles, etc. To ensure we successfully extracted *Porodaedalea pini* SE DNA, we performed PCR, cloning and sequencing using the general fungal primer pair ITS1F and ITS4 (Gardes & Bruns 1993), and had no issues obtaining sequences using these primers. Because we wanted to ensure detection of fungi from the phylum Basidiomycota, and especially fungi in the Hymenochaetoid clade, we performed an analysis of mismatches between known sequences from the Hymenochaetoid clade and the primer ITS4b. Based on this analysis, we designed a new primer specific for Basidiomycota, ITS4b-21 (CAGGAGACTTGTACACGGTCC), which is derived from ITS4b (Gardes & Bruns 1993) and which had fewer mismatches for members of the Hymenochaetoid clade. Preliminary testing determined that ITS4b-21 amplifies members of the Hymenochaetoid clade of fungi, including *Porodaedalea pini* SE, that are not amplified by ITS4b (M. A. Jusino and D. L. Lindner own data). Many important white-rot fungi belong to the Hymenochaetoid clade, and therefore their detection is vital to studies of fungi associated with cavity excavation.

We performed PCR on all samples taken with the sharpened spoon and the increment borer using ITS1F and ITS4b-21. Our PCR protocol was similar to the protocol for ITS1F and ITS4 used by Lindner & Banik (2009), however, the thermocycler conditions differed (see below). We diluted the 5x green GoTaq reaction buffer to a 1x working

concentration and we used 0.04 units of GoTaq DNA Polymerase per microliter of reaction volume. We also added 0.13 μL of 3% polyvinylpyrrollidone (PVP) per microliter of each PCR reaction. All other PCR component concentrations can be found in Lindner and Banik (2009). Our thermocycler conditions were as follows: initial denaturing at 95 °C for 5 minutes; 15 cycles of denaturing at 94 °C for 30 seconds, annealing at 55 °C for 45 seconds and extension at 72 °C for 1 minute; 25 cycles of denaturing at 94 °C for 30 seconds, annealing at 52 °C for 45 seconds and extension at 72 °C for 1 minute; a final extension at 72 °C for 10 minutes.

After amplification, we ran the PCR products on a 1.5% agarose gel stained with ethidium bromide (hereafter, gel). Reactions with visible amplification products were cloned and sequenced following the protocol described by Lindner & Banik (2009) and the sequencing protocols described by Lindner et al. (2011a). We did not sequence in both directions, ITS4 was used for all sequencing reactions. We edited our sequences with Sequencher 4.9 (GeneCodes Corp.). Sequences were assigned identities based on BLAST comparisons to GenBank (NCBI) sequences.

RESULTS

Fungal prevalence was determined by successful PCR amplification which was confirmed via gel electrophoresis for 18 of the 20 trees sampled (90%), with varying rates of success achieved by sampling method (scrapes or cores) and presence of fungal fruiting bodies. Specifically, 18 of the 20 trees (90%) sampled with the sharpened spoon (scrapes) and 6 of the 13 (46%) that were cored produced PCR products that could be visualized on a gel. Of the 10 trees that had fruiting bodies, 9 of the 10 (90%) that were scraped and 5 of the 7 (71%) that were cored produced PCR products that could be visualized on a gel. Of the ten trees without fruiting bodies, 8 of the 10 (80%) that were scraped and 1 of the 6 (17%) that were cored produced PCR products that could be visualized on a gel. None of the negative controls produced PCR products that could be visualized on a gel.

PCR products were ligated and cloned regardless of whether they could be visualized on a gel, although the only samples that produced viable bacterial colonies were those that could be visualized. The most prevalent wood decay fungus we identified in our samples was *P. pini* SE, which we successfully cloned and sequenced from 16 of the 20 (80%) excavations that we sampled with spoon scrapes, and in 6 of the 13 (46%) of the cores (Table 1). We also identified 9 other wood decay fungi from spoon scrapes from 8 of the 20 (40%) trees sampled: *Agaricomycetes* sp. 1, *Atheliaceae* sp. 2, *Atheliales* sp. 3, *Peniophora cinerea*, *Peniophora* sp. 4, *Phlebia brevispora*, *Postia sericeomollis*, *Stereum* sp. 4, and *Trametes versicolor* (Table 1). *Porodaedalea pini* SE was the only fungus detected from the core samples.

Among trees with *P. pini* SE fruiting bodies (i.e. trees that are known to be infected with a wood decay fungus), we were able to detect *P. pini* SE in 9 of the 10 (90%) excavations that were sampled in such trees with the sharpened spoon and in 5 of the 7 (71%) core samples. We detected *P. pini* SE in 1 of the 6 (17%) core samples from above excavations in trees without fruiting bodies. However, when these same excavations were scraped, we detected wood decay fungi in 8 out of 10 (80%) excavations, including 6 detections of *P. pini* SE. We did not detect any fungi in cores or scrapes from 2 of the 10 (20%) trees with excavations but without fruiting bodies (Table 1).

DISCUSSION

Our novel protocol provides a considerable improvement over traditional methods for detecting fungi associated with excavating and non-excavating cavity-nesting birds. Our method can be used to identify fungi that would otherwise be undocumented because of the absence of fruiting bodies and the failure of other methods to detect these fungi. When combined with molecular methods and the primer we designed (ITS4b-21), our collection tool can be used to help determine the incidence and identity of decay fungi as well as the other fungal species (lacking fruiting bodies) present within the wood surrounding an excavation site. By identifying the fungi present we can determine which fungi are associated with excavating and non-excavating cavity-nesters. This information can be used to inform habitat management for cavity-nesting species.

We found the sharpened spoon to be more effective in detecting fungi than the increment borer in both complete and incomplete Red-cockaded Woodpecker excavations (Table 1). We also found that molecular methods can be used to detect fungi from both types of samples. However, we detected far less Basidiomycota diversity in the

Table 1. Cavity type (complete cavity or cavity start), presence of a *Porodaedalea pini* SE fruiting body, the total number of fungal taxa cloned from the scrape and core samples, and the identities of the wood decay fungi detected from scrape and core samples collected from Red-cockaded Woodpecker excavations, and the closest Genbank BLAST hits of the decay fungi detected, with accession numbers and % identity match to those hits. NA denotes not applicable as not all completed cavities used in this analysis were cored. Taxa indicated with (*) may be associated with processes other than the decay of heartwood.

P. pini SE fruiting body	Excavation type	Scrapes		Nearest GenBank match (accession number, identity match)	Cores	
		# fungal taxa	Decay fungi		# fungal taxa	Decay fungi
Present	Complete	6	*Porodaedalea pini* SE	JX110039.1; 100%	1	*Porodaedalea pini* SE
			Atheliales sp. 3*	JN943909.1; 92%		
	Complete	5	*Agaricomycetes* sp. 1*	EF694649.1; 99%	NA	
			Porodaedalea pini SE	JX110039.1; 100%		
			Peniophora sp. 4	EF672293.1; 99%		
	Complete	3	*Porodaedalea pini* SE	JX110039.1; 100%	NA	
	Complete	2	*Porodaedalea pini* SE	JX110039.1; 100%	NA	
	Start	5	*Porodaedalea pini* SE	JX110039.1; 100%	1	*Porodaedalea pini* SE
	Start	2	*Phlebia brevispora*	AB084616.1; 99%	0	
			Porodaedalea pini SE	JX110039.1; 100%		
	Start	2	*Porodaedalea pini* SE	JX110039.1; 100%	1	*Porodaedalea pini* SE
	Start	2	*Porodaedalea pini* SE	JX110039.1; 100%	0	
			Trametes versicolor	KC176344.1; 99%		
	Start	1	*Porodaedalea pini* SE	JX110039.1; 100%	1	*Porodaedalea pini* SE
	Start	0			1	*Porodaedalea pini* SE
Absent	Complete	6	*Phlebia brevispora*	AB084616.1; 99%	NA	
			Postia sericeomollis	KC585367.1; 98%		
	Complete	5	*Phlebia brevispora*	AB084616.1; 99%	NA	
	Complete	4	*Atheliaceae* sp. 2	GU187502.1; 96%	NA	
			Peniophora cinerea	GU062269.1; 99%		
			Porodaedalea pini SE	JX110039.1; 100%		
			Trametes versicolor	KC176344.1; 99%		
	Complete	1	*Porodaedalea pini* SE	JX110039.1; 100%	NA	
	Start	2	*Porodaedalea pini* SE	JX110039.1; 100%	0	
			Stereum sp. 4	JX460856.1; 98%		
	Start	1	*Porodaedalea pini* SE	JX110039.1; 100%	0	
	Start	1	*Porodaedalea pini* SE	JX110039.1; 100%	1	*Porodaedalea pini* SE
	Start	1	*Porodaedalea pini* SE	JX110039.1; 100%	0	
	Start	0			0	
	Start	0			0	

cores (1 taxon overall) than in the samples taken with the sharpened spoon (24 taxa overall, 10 of which were decay fungi). Analysis of samples taken with the sharpened spoon confirmed the presence of multiple species of fungi that were not externally apparent and that were not observed with the core samples. Furthermore, the sharpened spoon can be used to collect samples from within an excavation, and thus can be used to examine the fungi associated with the cavity occupant.

We were able to sample within the actual cavity or cavity start with the spoon and safely sample the heartwood of completed cavities without the risk of creating a fissure in the top of the cavity, which is one potential problem with heartwood cores at cavity height. Creating a fissure in the dome of a cavity would allow resin to leak into the cavity, thus introducing a possible risk to any bird using the cavity. Additionally, samples taken with the sharpened spoon require much less time for collection and processing compared to samples taken with the increment borer. Based on this study, we found that an experienced climber can sample more than sixteen excavations in an eight-hour field day with the sharpened spoon, twice as many as when an increment borer is used. Sampling with the sharpened spoon also required less lab processing time than cores. Unlike the fine scrapings collected with the sharpened spoon, cores collected by an increment borer require a vigorous sterile grinding step prior to DNA extraction. Even though our implementation of an immersion blender required less time than traditional core grinding methods, processing cores was still much more laborious and time consuming than processing spoon-collected samples.

For this study, our principal objective was accurate detection and species identification of the fungi collected using various sampling techniques. Therefore, we choose traditional Sanger sequencing methods over newer next-generation techniques because Sanger methods provide longer read lengths and more accurate sequence identification than most cost-comparable next-generation sequencing methods. We detected multiple fungal taxa in 12 of the 20 trees we tested and may have detected even more taxa if we had used a more general primer pair capable of capturing more than one fungal phylum, such as ITS1F and ITS4 (Gardes & Bruns 1993). We did not use a more general fungal primer pair for our PCR reactions because we were specifically interested in determining if the samples collected with the sharpened spoon could be used to detect wood decay fungi. Fungi from the phylum Ascomycota are very common and tend to dominate samples (based on preliminary data using general primers; Jusino et al. unpublished data), and while some fungi from this phylum can be important contributors to wood decay, most known decay fungi are in the phylum Basidiomycota. Wood decay fungi from the phylum Basidiomycota would have been amplified with a more general primer pair, but we increased our chances of successfully detecting such species by using a more specific primer pair. By using our modified primer, ITS4b-21, we were able to detect wood decay fungi in the Hymenochaetoid clade that otherwise would have been missed.

While the presence of a fungal fruiting body indicates that a fungus is present in a tree, it does not indicate that the fungus has spread throughout the entire stem of a tree. Thus, when sampling Red-cockaded Woodpecker excavations in trees with *P. pini* SE fruiting bodies, one should not always expect to find *P. pini* SE in the wood surrounding the cavity. This may explain the instances in which either the sharpened spoon or increment borer failed to detect *P. pini* SE in trees with fruiting bodies. However, that *P. pini* SE was detected in the core sample taken near the cavity in one of these instances, and in the spoon sample but not the core sample in the other two instances, suggests that we did not always detect every heart rot species present in the trees we sampled with our methods. We note that all of these detection failures were associated with incomplete rather than complete excavations. Nevertheless, we have demonstrated that the absence of a fruiting body does not imply the absence of a fungus given that we successfully detected *P. pini* SE from 6 of the 10 (60%) trees without fruiting bodies as well as six other wood decay fungi, from four such trees.

Recently, it has been suggested that cavity excavators use fungal fruiting bodies as visual cues to select trees suitable for excavation (Hart & Hart 2001, Witt 2010). This hypothesis has only been tested once, by Rudolph et al. (1995), who found no support for this idea related to Red-cockaded Woodpeckers. Rudolph et al. (1995) attached *Porodaedalea pini* fruiting bodies to 40 trees within 10 active Red-cockaded Woodpecker territories, using 40 other trees within those territories as controls. After three years, none of the trees with fruiting bodies were used by Red-cockaded Woodpeckers, though the birds excavated a cavity start in one control tree.

Some researchers have suggested that cavity excavators use acoustic cues to detect decay in a tree (e.g. Zahner et al. 2012). Specifically, some birds may assess the density of wood acoustically, although this would be difficult to test for most cavity excavators. There are multiple other cues and cue combinations that primary cavity excavators can use to determine trees and locations on trees suitable for excavation, including olfactory signals. The use of our new method will help researchers determine if cavity excavators select excavation sites based on the presence or absence of certain fungal species. If they do, the mechanism behind excavation site selection can be tested.

Our results indicate that trees without visible signs of decay may harbor wood-decaying fungi and thus be a potentially important resource for cavity-nesting birds. For researchers, internal detection of rot via acoustic methods is an improvement over visual surveys, however the information gained from these methods is based on the density of wood. One does not learn the identity of important hidden players in a fungal community by simply examining a substrate visually or acoustically. To understand whether specific fungi or specific groups of fungi are important to cavity-excavating and cavity-nesting birds, it is necessary to determine which fungal groups are associated with the nest and roost cavities of these birds. Our novel approach allows us to more robustly determine the fungi present in the wood surrounding excavations, a critical first step in resolving the relationships between cavity users and fungi so that forests can be managed effectively for cavity-nesters and cavity excavators.

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STRESZCZENIE

[Nowa metoda pobierania prób w dziuplach w celu identyfikacji występujących w nich grzybów]

Zależności pomiędzy dziuplakami, drzewami i grzybami rozkładającymi drewno stanowią ważne zagadnienie zarówno z punktu widzenia ochrony przyrody, jak i interesujące pytanie badawcze. W tym drugim przypadku poznanie zależności między dziuplakami i występującymi w dziuplach grzybami może mieć istotne znaczenie dla zrozumienia wymagań siedliskowych wielu gatunków ptaków.

Dotychczas w celu określenia obecności i gatunków grzybów występujących w danym drzewie analizowano owocniki tych grzybów. Badanie owocników należy do nieinwazyjnych sposobów pobierania prób, jednak wiąże się z dużym niedoszacowaniem częstości występowania i różnorodności grzybów, a także pomijaniem gatunków, które mogą mieć kluczowe znaczenie dla gatunków gnieżdżących się w dziuplach. Dlatego rozwój metod mających na celu identyfikację grzybów rozkładających drewno, szczególnie tych obecnych w miejscach powstania lub wykuwania dziupli, jest bardzo ważny.

W pracy przedstawiono nową, nieinwazyjną technikę pobierania z wnętrza dziupli prób drewna, które następnie wykorzystywane są do określania obecności i taksonów grzybów na podstawie metod molekularnych. Do pobierania prób z wnętrza dziupli zastosowano nowe narzędzie — stalową „łyżeczkę" umieszczoną na metalowym pręcie (Fig. 1, 2). Krawędzie tej łyżeczki zostały zaostrzone tak, aby możliwe było zdrapywanie drewna z wnętrza dziupli. Taka konstrukcja jest wygodna do używania w przypadku pobierania prób na wysokości, pozwala także na łatwą sterylizację narzędzia przed każdym pobraniem próby. Zebrane wióry analizowane były następnie metodami molekularnymi — autorzy zaprojektowali nowy starter specyficzny dla podstawczaków z grupy zawierającej grzyby rozkładające drewno.

Nową metodę pobierania prób oraz analiz molekularnych przetestowano na dziuplach dzięcioła skromnego. Wybrano drzewa, z których połowa miała widoczne owocniki grzyba z grupy *Porodaedalea pini*. Wióry z wnętrza dziupli pobierano w trzech miejscach, tak, aby zebrać próby dla obu rodzajów drewna — zarówno bieli, jak i twardzieli (Fig. 3). W przypadku zaczątków dziupli dodatkowo wykonywano nawiercenie w celu pobrania do badań rdzenia (Fig. 3).

Używając nowej metody pobierania prób z wnętrza dziupli oraz nowego protokołu do analiz molekularnych stwierdzono obecność grzybów w 90% dziupli w drzewach z widocznymi owocnikami, oraz w 60% dziupli w drzewach, na których nie było owocników (Tab. 1). Dodatkowo, w próbach zebranych przy pomocy nowego narzędzia stwierdzono 9 dodatkowych taksonów grzybów rozkładających drewno, żaden z nich nie został stwierdzony w próbach pobranych z wywierconych rdzeni (Tab. 1).

Wypracowana metoda pobierania prób i ich analizy pozwala na dokładniejsze identyfikowanie grzybów występujących dokładnie w miejscu wykucia dziupli przez dzięcioły, co pozwala na dalsze badania dotyczące powiązań pomiędzy grzybami rozkładającymi drewno a dziuplakami.