Research Paper

Fungal communities associated with acorn woodpeckers and their excavations

Michelle A. Jusino [a,b,*], Natasha D.G. Hagemeyer [c], Mark T. Banik [a], Jonathan M. Palmer [a], Daniel L. Lindner [a], Matthew E. Smith [b], Walter D. Koenig [d,e], Eric L. Walters [c]

[a] Center for Forest Mycology Research, United States Department of Agriculture, Forest Service, Northern Research Station, One Gifford Pinchot Drive, Madison, WI, 53726, USA
[b] Department of Plant Pathology, University of Florida, Gainesville, FL, 32611, USA
[c] Department of Biological Sciences, Old Dominion University, Norfolk, VA, 23529, USA
[d] Cornell Lab of Ornithology, 159 Sapsucker Woods Road, Ithaca, NY, 14850, USA
[e] Hastings Reservation, University of California Berkeley, 38601 E. Carmel Valley Rd., Carmel Valley, CA, 93924, USA

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ABSTRACT

Wood-decay fungi soften wood, putatively providing opportunities for woodpeckers to excavate an otherwise hard substrate, yet the fungal community composition in tree cavities and the specificity of these relationships is largely unknown. We used high-throughput amplicon sequencing of the fungal ITS2 region to examine the fungal communities associated with acorn woodpeckers (*Melanerpes formicivorus*) and their cavities in mature valley oak (*Quercus lobata*) and blue oak (*Q. douglasii*) trees in an oak savannah of central coastal California, USA. Acorn woodpeckers and their excavations harbored over 1500 fungal taxa, including more than 100 putative wood-decay fungi. The fungal communities found on the birds were more similar to those found in excavated cavities than those found in trees without excavated holes. These results suggest that symbiotic associations between acorn woodpeckers and fungi are highly diverse, with low specificity. Symbiotic associations between cavity-excavators and fungi are likely more common and widespread than previously thought.

1. Introduction

Tree cavity excavators such as woodpeckers, along with wood decay fungi, act as important ecosystem engineers by creating cavities and facilitating natural cavity formation. Woodpeckers and other cavity excavators may depend on partnerships formed with fungi that soften the wood surrounding excavation sites. Simultaneously, those fungi depend on excavators for dispersal (Jusino et al., 2015, 2016). A comprehensive review of the ecological relationships between birds and fungi reported case studies of 30 cavity-excavating bird species that are likely to have associations with wood-decaying fungi (Elliott et al., 2019). Thus, there is an emerging realization that symbioses between cavity excavators and fungi may be important for the maintenance of forest biodiversity as well as for the retention of important ecosystem components such as carbon and nutrient cycling driven by saprotrophic microbes.

Past studies of woodpeckers and fungi have generally relied upon fruit body surveys or measures of wood density to detect decay and understand woodpecker excavation site selection (Conner et al., 1976; Jackson and Jackson 2004; Cockle et al., 2012; Zahner et al., 2012). These approaches provide limited information and have led to a "one excavator, one fungus" paradigm based on limited data (Jusino et al., 2015). Recent work using molecular detection and identification of fungi via cloning and Sanger sequencing has demonstrated that complex fungal community interactions, involving many fungal taxa, occur within tree cavities excavated by red-cockaded woodpeckers (*Dryobates borealis*) and that the woodpeckers directly influence these interactions (Jusino et al., 2015, 2016).

These findings indicate that the one excavator, one fungus paradigm is insufficient for understanding the interactions between cavity excavators and fungi. Jusino et al. (2015) suggested two alternative hypotheses: the "bird facilitation hypothesis", whereby cavity-excavating birds select trees without any evidence of decay and facilitate fungal colonization of the wood during the process of excavating their nesting

cavities, and the "tree selection hypothesis", whereby cavity-excavating birds selectively excavate trees with specific communities of fungi. However, red-cockaded woodpeckers are unique in their habit of excavating through the sapwood and into the heartwood of living pine trees (*Pinus* spp.), so it is possible that this particular system represents a unique case. It is more likely, however, that many other cavity excavators are also associated with communities of fungi. Symbioses between cavity excavators and fungi may indeed be common, but the only well-documented evidence so far is from red-cockaded woodpeckers and their fungal communities (Jusino et al., 2016). Insight into these putative interactions between excavators and fungi has important implications for understanding ecological processes in forest ecosystems.

Acorn woodpeckers (*Melanerpes formicivorus*) are geographically and phylogenetically distinct from red-cockaded woodpeckers, but both species are primary cavity excavators, cooperative breeders who live in family groups, and well-studied taxa with associated long-term data sets (Koenig and Mumme, 1987; Walters 1991). Acorn woodpeckers are found in association with oak (*Quercus* spp.) savannah habitat (Koenig et al., 2020) where they build specialized granaries for acorn storage (MacRoberts and MacRoberts 1976). These woodpeckers are strong primary excavators, creating cavities for both roosting and nesting (Koenig et al., 2020). Cavity excavation is a communal activity that can occur at any time of year but has been generally observed during the winter and spring (Koenig and Mumme, 1987). Cavities tend to be excavated in relatively large trees when available, including both live trees and snags (standing dead trees) (Koenig et al., 2020). Cavities located in live trees are warmer and have less temperature variation than those located in dead limbs or snags (Hooge 1989; Hooge et al., 1999). A single acorn woodpecker territory may contain multiple cavities, several of which may be used for roosting and any one of which may be used for nesting (Koenig et al., 2020).

Both coniferous and angiosperm trees, including oaks, sycamores (*Platanus racemosa*), and pines, are used for cavity excavation by acorn woodpeckers (Hooge et al., 1999; Arsenault 2004; Koenig et al., 2020). Acorn woodpecker nest site selection, however, may be associated with demographic parameters. In one study in coastal central California, nests in sycamores had significantly higher fledging rates and were associated with larger groups, as were nests located in live limbs of any tree species (Hooge et al., 1999).

Red-cockaded woodpecker cavities are associated with distinct fungal communities (Jusino et al., 2015) and the birds themselves play a role in fungal transmission from tree to tree (Jusino et al., 2016). Here we test these associations in acorn woodpeckers, a species for which relatively little is known about excavation site selection (Gutiérrez and Koenig, 1978; Hooge et al., 1999; Koenig et al., 2021) and nothing is known about fungal associations, despite over five decades of ecological and behavioral study (Koenig and Mumme, 1987; Koenig et al., 2020). Long-term patterns of nest reuse suggest that acorn woodpeckers are cavity limited (Koenig et al., 2021). Cavity excavation is thus a potentially important component of this species' social organization. To understand the ecology of cavity creation, it is therefore critical to determine whether their complete and incomplete excavations harbor different fungal communities than non-excavated trees. While it is widely known that birds are important seed dispersers (Howe and Smallwood 1982; Pesendorfer et al., 2016), and recent work has shown that some birds are important for the dispersal of mycorrhizal fungi, including truffles (Caiafa et al., 2021), we are only just beginning to realize the importance of birds for dispersing wood-inhabiting fungi (Elliott et al., 2019).

We used molecular methods to examine the fungal communities associated with acorn woodpeckers and their nesting and roosting cavities. We tested whether acorn woodpeckers associate with a specific community of fungi and whether the fungi from established nesting

cavities are distinct from the fungi in non-excavated trees. We also examined whether birds disperse fungi and whether at least some of these fungi are wood decay specialists that are also found in excavated cavities. To address these questions, we sampled 37 acorn woodpecker excavations in living branches of old-growth valley (*Quercus lobata*) and blue (*Q. douglasii*) oaks in California and used high-throughput amplicon sequencing (HTAS) of the fungal internal transcribed spacer (ITS) region to examine the fungal communities and elucidate patterns of symbiosis and degree of specificity.

2. Materials and methods

2.1. Study site and field methods

Field work was conducted during 2 weeks in April–May 2015 at Hastings Natural History Reservation in Carmel Valley, California (36.387 N, 121.550 W). The acorn woodpecker population at Hastings has been continuously and intensively studied since 1968 (MacRoberts and MacRoberts 1976; Koenig and Mumme, 1987; Koenig et al., 2016). We compared the fungi associated with (1) fully excavated cavities that had been used for nesting by acorn woodpeckers; (2) incomplete excavations, termed "cavity starts", that were excavated by acorn woodpeckers; (3) drilled samples from non-excavated control trees of similar size and species to excavated trees; and (4) the bills and wings of captured acorn woodpeckers.

We sampled 18 complete and 19 incomplete acorn woodpecker excavations in living branches of valley and blue oaks by climbing to excavation height (range 1.8–19 m) and aseptically collecting wood shavings from within the excavations with a sterilized, sharpened collection device following the protocol of Jusino et al. (2014). We sampled only excavations that were made by and currently in use by acorn woodpeckers. For comparison, we also sampled the stems of 10 non-excavated living *Q. lobata* and *Q. douglasii* trees located near (mean = 2.5 m) the excavated trees that were sampled.

Non-excavated trees were sampled using a handheld battery-powered drill with sterilized drill bits (bit size: 6–8 mm), and wood shavings were collected in sterile modified funnels constructed from sterile 50 mL tubes. Samples were transferred from the funnels into sterile 50 mL tubes. We also swabbed acorn woodpecker bills and wing feathers with sterile cotton swabs following protocols of Jusino et al. (2016). Bill and wing swabs were collected aseptically and separately to allow for comparisons of fungi from bills and wings from each bird. Birds were either captured at their roosting cavity (n = 4) following the protocol of Stanback and Koenig (1994) or via mist nets (n = 5). Intermittent playback of agonistic vocalizations was used to lure woodpeckers towards the decoy and into the net. Nets were monitored continuously, and woodpeckers were removed from the net immediately upon entering. Hands were washed between captures to prevent cross-contamination. Negative field controls were also collected for all sample types and processed similarly to the samples. For example, negative controls for drill samples consisted of handling the drill, drill bits, funnels, and collection tubes at a collection site using similar technique to that used to collect samples, but instead of drilling into the tree, the drill was simply spun in the air. Following collection, all samples (including controls) were stored in filtered cell lysis solution (CLS; Lindner and Banik, 2009) and frozen at −20 °C to await DNA extraction, PCR, and HTAS.

2.2. Molecular methods

We extracted DNA from the wood samples following the CLS/glass milk extraction protocol described in Lindner and Banik (2009). Swab samples were processed using the modifications for swabs described in

Jusino et al. (2016). Following DNA extraction, we amplified the fungal DNA present in the samples to prepare HTAS libraries using individually barcoded Ion Torrent compatible fungal-specific primers fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990), according to manufacturer's recommendations. Briefly, the forward primer was composed of the Ion A adapter sequence, followed by the Ion key signal sequence, a unique Ion Xpress Barcode sequence (10–12 bp), a single base-pair linker (A), followed by the fITS7 primer (Ihrmark et al., 2012). The reverse primer was composed of the Ion trP1 adapter sequence followed by the conserved ITS4 primer (White et al., 1990).

PCR was performed using the following reagents: 7.88 μl DNA-free molecular grade water, 1.5 μl 10x Pfx50 amplification buffer (Thermo-Fisher Scientific, Madison, WI, USA) for a final concentration 1x, 0.3 μl of 10 mM dNTPs (Promega Corporation, Madison, WI, USA) for a final concentration 0.2 mM of each dNTP, 0.3 μl of each 10 μM primer for a final concentration of 0.2 μM of each primer, 0.12 μl of 20 mg/ml BSA (New England BioLabs, Ipswich, MA, USA), and 0.2 μl (1 unit) of Pfx50 DNA polymerase (ThermoFisher Scientific, Madison, WI, USA). Thermocycler conditions were as follows: initial denaturation of 94 °C for 3 min, followed by 11 cycles of (94 °C for 30 s, 60 °C for 30 s (drop 0.5 °C per cycle), 68 °C for 1 min), then 26 cycles of (94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min), with a final extension of 68 °C for 7 min. PCR products were cleaned using Zymo Select-a-size spin columns (Zymo Research, Irvine, CA, USA). All libraries were quantified using a Qubit® 2.0 Fluorometer with the high-sensitivity DNA quantification kit (ThermoFisher Scientific, Madison, WI, USA), then equilibrated to 2500 pM, and combined after equilibration.

We sequenced barcoded libraries using an Ion Torrent semi-conductor sequencing platform. Our sequencing run included SynMock (Palmer et al., 2018), an equimolar spiked-in mock community control consisting of non-biological synthetic ITS sequences, and this control was used to parameterize our bioinformatics pipeline. The mock community allowed us to cluster and estimate our operational taxonomic units (OTUs) based on realistic parameters, an important and necessary component to any HTAS study of environmental samples (Nguyen et al., 2015; Palmer et al., 2018; Jusino et al., 2019). The synthetic mock community allows reliable detection of index bleed/barcode crossover, a documented problem across HTAS platforms (Schnell et al., 2015; Palmer et al., 2018).

2.3. Bioinformatics

We bioinformatically processed our Ion Torrent data using AMPtk version 1.0.0 (Palmer et al., 2018; amptk.readthedocs.io). We pre-processed our merged, individually barcoded reads using USEARCH (version 9.2.64) and then removed the forward and reverse ITS primers. We discarded any reads shorter than 100 bp. Reads longer than 300 bp were trimmed to 300 and any reads shorter than 300 bp were padded with Ns to help improve the clustering step (Palmer et al., 2018). One sample had fewer than 5000 reads and was dropped before clustering to avoid clustering errors. Sequence reads were then quality-filtered with expected errors less than 1.0 (Edgar and Flyvbjerg 2015), de-replicated, and clustered at 97% similarity to generate OTUs using UPARSE (Edgar 2013). Following clustering, any padded Ns were removed, and the processed sequences were mapped to the OTUs. We used SynMock (Palmer et al., 2018) to account for observed rates of barcode crossover using the filter module in AMPtk. Finally, the OTUs were assigned taxonomy using the hybrid taxonomy algorithm in AMPtk. Taxonomic guilds, including putative wood decay fungi, were assigned using a combination of FUNGuild (Nguyen et al., 2016) and our own taxonomic expertise.

2.4. Community analyses

Analyses were performed in R (R Core Team 2018). Community analyses were performed with the *vegan* package (Oksanen et al., 2019) using a β-sim dissimilarity matrix, calculated from our untransformed presence/absence data using the *betadiver* function (Koleff et al., 2003). To visualize fungal communities in ordination space, we performed nonparametric multidimensional scaling (NMDS) using the *metaMDS* function on our β-sim dissimilarity matrix. We used a nonparametric permutational multivariate ANOVA (PERMANOVA) test (Anderson 2001) performed by the *adonis* function to test for significant differences in fungal community composition among the sample types (i.e., bird swabs, complete excavations, incomplete excavations, or non-excavated trees). We then performed pairwise PERMANOVA tests using the *adonis. pair* function in the *EcolUtils* package (Salazar 2019). To test for differences in multivariate dispersion (Anderson 2006), we used the *betadisper* function. We visualized the number of shared OTUs between sample types with Euler diagrams using the *euler* function in the *eulerr* package (Larsson 2019). Additionally, to determine whether the capture location had an effect on the observed fungal community, we conducted another PERMANOVA using only bird swabs with capture location (i.e., roost cavity versus mist net) as the predictor variable.

3. Results

Acorn woodpeckers and their excavations, and non-excavated trees harbored over 1500 fungal OTUs, including more than 100 putative wood-decay fungi. Samples taken from woodpeckers were particularly diverse, with as many as 617 fungal taxa observed on a single bird (range 298–617; median 452; mean 455; n = 9), whereas we detected as many as 174 fungal OTUs in a single complete cavity (range 24–174; median 76; mean 82; n = 18), 138 in a single incomplete cavity (range 41–138; median 67; mean 79; n = 19), and 132 in a single non-excavated tree (range 33–132; median 88; mean 85; n = 9). We were unable to recover sequences from 1 of the 10 samples in the non-excavated, control tree group.

The fungal communities found in complete and incomplete acorn woodpecker excavations were similar to each other but not to those found in non-excavated trees (Fig. 1; Table 1). Moreover, although the fungal communities found on the birds were more diverse than those found in excavated and non-excavated trees, many fungal taxa were shared between the communities from acorn woodpeckers and those from their excavations (Fig. 2). The fungal communities found on acorn woodpeckers were far more similar to those found in complete and incomplete excavations than to fungal communities found in non-excavated trees (Fig. 1; Table 1). Wood decay fungi were common in our dataset. They were common in bird swabs, complete excavations, and incomplete excavations but were relatively rare among non-excavated trees (Table 2).

Fungal community composition differed significantly among sample types (i.e., bird swabs, complete excavations, incomplete excavations, or non-excavated trees; $r^2 = 0.21$, *pseudo* $F_{3,51} = 4.46$, $p < 0.0001$; Fig. 1), indicating that these communities were distinct. Multivariate dispersion did not differ among sample types ($F_{3,51} = 1.89$, $p = 0.14$; Fig. 1), indicating that the differences in fungal communities detected by the PERMANOVA test were due to multivariate centroid location (i.e., differences in community composition) rather than multivariate dispersion (i.e., differences in the dispersion of the communities in ordination space).

Fungal communities on acorn woodpeckers captured in their cavities differed significantly from those captured in mist nets ($r^2 = 0.23$, *pseudo* $F_{1,7} = 2.04$, $p = 0.012$; Fig. S1), with no statistical differences in multivariate dispersion ($p = 0.88$).
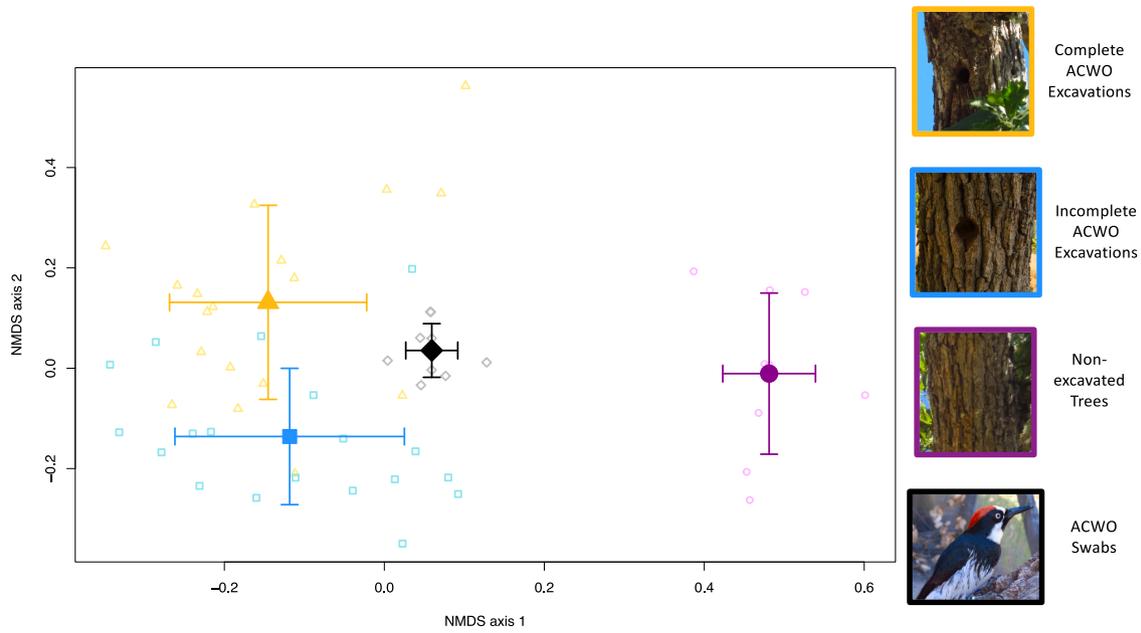
Fig. 1. Non-metric multidimensional scaling (NMDS) ordination of the fungal communities found in complete and incomplete (starts) acorn woodpecker (ACWO) excavations, non-excavated trees, and on ACWO swabs. NMDS is based on a β-sim matrix. NMDS stress is 0.19, k = 3, dimensions 1 and 2 are displayed.

Table 1
Pairwise PERMANOVA tests for each sample type combination, based on the β-sim matrix used to generate Fig. 1. Combinations that were statistically significant after the alpha correction was applied are in bold.

Comparison	pseudo F	r^2	p-value	Corrected p-value
Complete excavations – Non-excavated trees	11.7	0.32	<0.0001	**0.0002**
Complete excavations – Incomplete excavations	3.11	0.08	<0.0001	**0.0002**
Complete excavations – ACWO swabs			1.00	1.00
Non-excavated trees – Incomplete excavations	11.1	0.3	<0.0001	**0.0002**
Non-excavated trees – ACWO swabs	2.6	0.14	0.0044	**0.0066**
Incomplete excavations – ACWO swabs			1.00	1.00

4. Discussion

Acorn woodpeckers are associated with diverse communities of fungi. We recovered >1500 fungal OTUs from the birds and their excavations. We found that acorn woodpecker excavations were inhabited by a different suite of fungi than non-excavated trees (Fig. 1), indicating that excavation by acorn woodpeckers likely changed the fungal communities present in living oaks. Furthermore, several species of fungi that were associated with woodpeckers and their excavations, as opposed to non-excavated trees, were wood decay fungi (Table 2). In combination with prior results in red-cockaded woodpeckers, these results suggest that symbiotic associations between woodpeckers and diverse communities of fungi are more common than previously thought.

Our results support the "bird facilitation hypothesis" in that the fungal community composition on acorn woodpeckers was similar to the fungal community composition of their complete and incomplete excavations, but not to non-excavated trees (Table 1). Because complete and incomplete acorn woodpecker excavations were inhabited by similar communities of fungi, this pattern could be the result of both bird facilitation and tree selection, where the birds select trees associated with certain fungal communities for excavation and also help facilitate the transmission of fungi found in their excavations. Interestingly, we observed a significant difference in fungal community composition between birds captured from their roost cavities versus those captured in mist nets (Fig. S1). This raises the possibility that fungal propagules are

rapidly shed in flight. Larger sample sizes are needed to validate this potentially interesting result, however.

Many woodpecker species have previously been thought to be associated primarily with fungi found fruiting on their cavity trees, leading to the one excavator, one fungus paradigm, by which the fungi fruiting near the excavation sites help the birds by preparing the excavation sites through wood decay. This paradigm has been previously refuted with red-cockaded woodpeckers, a species with an extraordinarily prolonged excavation time, in which excavations are teeming with a diverse array of fungi that formed communities distinct from non-excavated trees (Jusino et al., 2015). Moreover, previous work demonstrated that red-cockaded woodpeckers facilitated fungal colonization through their excavation behavior (Jusino et al., 2016). Few, if any, of the fungi associated with either red-cockaded woodpeckers nor acorn woodpeckers are regularly observed fruiting on or near excavation sites, illustrating the need for alternative field methods that do not depend on fructifications for the detection of fungal taxa.

The intact bark of living trees typically makes them well-defended against colonization by wood-inhabiting fungi; this is particularly true in arid Mediterranean climates such as the California oak woodlands where acorn woodpeckers are common. Consequently, animals that excavate holes in bark are excellent dispersal agents for fungi in living trees because of their propensity to create potential infection courts during excavation and foraging activities. There are many examples of wood-boring insects that can facilitate the spread of fungi, including bark and ambrosia beetles (Curculionidae; Scolytinae and
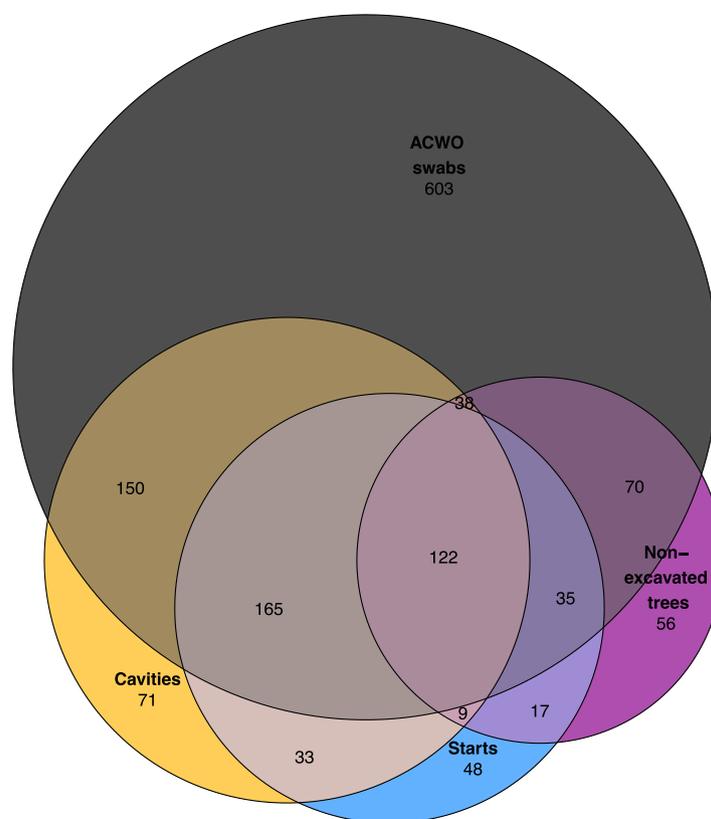
Fig. 2. Euler diagram of fungal operational taxonomic units (OTUs) found in complete (cavities) and incomplete (starts) acorn woodpecker (ACWO) excavations, non-excavated trees, and ACWO swabs. OTU counts are displayed for each group, and shaded areas are approximately proportional to the counts.

Table 2
Frequency and identity of the 12 most commonly encountered wood decay fungi in the dataset. These wood decay fungi were often associated with completely excavated cavities, incomplete cavities (starts), and swabs taken from acorn woodpeckers (ACWO) but rarely associated with non-excavated trees.

OTU	Total samples	ACWO cavities	Unexcavated trees	ACWO starts	ACWO swabs	
OTU86	35	15	0	16	4	*Coniophora* sp. (Boletales)
OTU460	21	5	2	9	5	*Hyphodontia* sp. (Hymenochaetales)
OTU774	16	6	0	9	1	Phallales sp. (Phallales)
OTU154	13	2	1	3	7	*Peniophora* sp. (Boletales)
OTU1585	12	4	0	5	3	Lachnocladiaceae sp. (Russulales)
OTU198	12	4	5	0	3	*Phlebiella* sp. (Polyporales)
OTU301	10	4	0	4	2	*Sistotrema* sp. (Cantharellales)
OTU104	10	2	0	1	7	*Stereum* sp. (Russulales)
OTU265	9	0	0	4	5	*Stereum hirsutum* (Russulales)
OTU841	9	1	0	2	6	Polyporales sp. (Polyporales)
OTU149	9	1	1	1	6	*Athelia* sp. (Atheliales)
OTU185	9	1	0	0	8	*Exidia glandulosa* (Auriculariales)

Platypodinae), longhorn beetles (Cerambycidae), and wood wasps (Siricidae) (Ulyshen 2016).

High-throughput amplicon sequencing presents new opportunities for work on cavity excavators and fungi. Previous molecular-based studies in this field, though informative, were limited in their ability to estimate total community diversity because they were accomplished with cloning and sequencing, yielding far fewer DNA sequences per sample per unit effort (Amend et al., 2010; Tedersoo et al., 2010). In this HTAS study of woodpecker-associated fungi, we recovered ten-fold more fungal OTUs from each bird relative to a previous study using cloning and sequencing approaches (Jusino et al., 2016).

Although employing HTAS provides much greater sampling depth and economy, it also has limitations and caveats. Caution must be taken in preparing and analyzing environmental data from all next-generation sequencing platforms (Lindahl et al., 2013; Nilsson et al., 2019). Issues such as biases introduced by primer selection, PCR amplification, and sequencing can skew estimates of taxon presence, frequency, and relative abundance. Additional issues such as chimera formation, index/-barcode bleed, and lab contamination can also inflate estimates of diversity. Therefore, proper controls need to be taken in the field and laboratory, and positive mock community controls should be used to help parameterize downstream bioinformatics (Nguyen et al., 2015; Palmer et al., 2018; Jusino et al., 2019).

Here we used an equilibrated, single-copy, synthetic (i.e., non-biological) ITS mock community as a positive sequencing and bioinformatics control. Combined with the AMPtk bioinformatics pipeline

([Palmer et al., 2018](#)), this mock community can be used to help detect and mitigate PCR, sequencing, and bioinformatics errors. We were also able to validate our estimates of alpha diversity with the mock community. Thus, we are confident that our estimates of high fungal OTU diversity on the birds and in the excavations in this system are not inflated by sequencing or bioinformatics issues.

Further experimental work will be needed to determine the extent to which acorn woodpeckers facilitate fungal infection in their excavations and whether they seek out trees already infected with certain fungi for excavation. Additional experimental work is also needed to determine if the fungi found in acorn woodpecker excavations decrease excavation time or help maintain excavated cavities, for example by excluding fungi that may be detrimental to the birds.

Data availability statement

Raw Ion Torrent sequence data are deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (accession PRJNA775654). Our OTU table is provided in the supplemental information.

Declaration of competing interest

The authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2022.101154.

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