



# Relationships among wood-boring beetles, fungi, and the decomposition of forest biomass

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## Abstract

A prevailing paradigm in forest ecology is that wood-boring beetles facilitate wood decay and carbon cycling, but empirical tests have yielded mixed results. We experimentally determined the effects of wood borers on fungal community assembly and wood decay within pine trunks in the southeastern United States. Pine trunks were made either beetle-accessible or inaccessible. Fungal communities were compared using culturing and high-throughput amplicon sequencing (HTAS) of DNA and RNA. Prior to beetle infestation, living pines had diverse fungal endophyte communities. Endophytes were displaced by beetle-associated fungi in beetle-accessible trees, whereas some endophytes persisted as saprotrophs in beetle-excluded trees. Beetles increased fungal diversity several fold. Over forty taxa of Ascomycota were significantly associated with beetles, but beetles were not consistently associated with any known wood-decaying fungi. Instead, increasing ambrosia beetle infestations caused reduced decay, consistent with previous *in vitro* experiments that showed beetle-associated fungi reduce decay rates by competing with decay fungi. No effect of bark-inhabiting beetles on decay was detected. Platypodines carried significantly more fungal taxa than scolytines. Molecular results were validated by synthetic and biological mock communities and were consistent across methodologies. RNA sequencing confirmed that beetle-associated fungi were biologically active in the wood. Metabarcoding of the LSU/28S marker recovered important fungal symbionts that were missed by ITS2, though community-level effects were similar between markers. In contrast to the current paradigm, our results indicate ambrosia beetles introduce diverse fungal communities that do not extensively decay wood, but instead reduce decay rates by competing with wood decay fungi.

## KEYWORDS

carbon sequestration, mutualism, Ophiostomatales, *Pinus*, Platypodinae, Scolytinae

## 1 | INTRODUCTION

Living trees are among the largest terrestrial sinks for atmospheric carbon dioxide. Following the death of a tree, however, much of the

carbon stored in its tissues is released to the atmosphere as the metabolic waste of fungal decomposers (Chambers, Schimel, & Nobre, 2001). Consequently, wood decomposition rates have a major impact on global carbon cycles (Floudas et al., 2012; Rayner & Boddy, 1988). Wood is composed of lignified cellulose and hemicellulose, and although many saprotrophic organisms can degrade cellulose

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and hemicellulose, lignin presents a formidable chemical and biological barrier to these polysaccharides in wood. Degrading lignified polysaccharides requires highly specialized enzymatic and nonenzymatic processes possessed by few organisms, mostly fungi belonging to the phylum Basidiomycota (Blanchette, 1991; Floudas et al., 2012). The rate at which these fungi decompose wood under natural conditions is influenced by ecological interactions including animal-facilitated dispersal of fungi (Jusino, Lindner, Banik, Rose, & Walters, 2016; Ulyshen, 2016), competition and combative interactions among wood decaying organisms and nondecayers (Boddy, 2000; Fukami et al., 2010), interactions with plant defence systems (Boddy, 1992), and the presence of fungal endophytes that become saprotrophs after plant host death (Cline, Schilling, Menke, Groenhof, & Kennedy, 2018; Parfitt, Hunt, Dockrell, Rogers, & Boddy, 2010). One of the most important determinants appears to be which fungi are first to colonize freshly dead wood (Dickie, Fukami, Wilkie, Allen, & Buchanan, 2012; Hiscox et al., 2015; Lindner et al., 2011).

Bark and ambrosia beetles (Curculionidae; Scolytinae and Platypodinae) are among the first insects to colonize the wood of dead and dying trees, and they bring communities of symbiotic fungi. These beetles comprise more than 7,000 species in the weevil subfamilies Scolytinae and Platypodinae and occur abundantly on every continent except Antarctica (Kirkendall, Biedermann, & Jordal, 2015). They are attracted en masse to tree-stress-related chemicals such as ethanol and terpenes, as well as aggregation pheromones released by other beetles and volatile organic compounds released by their symbiotic fungi (Hulcr, Mann, & Stelinski, 2011; Kandasamy, Gershenzon, Andersson, & Hammerbacher, 2019; Wood, 1982). The beetles have relationships with fungi that range from incidental commensalism to co-evolved, reciprocally obligate nutritional mutualisms (Skelton, Johnson, et al., 2019). Bark and ambrosia beetles differ by their feeding ecology. Most bark beetles species infest the inner bark of trees (phloem) and feed primarily on the plant tissues, though their diet is supplemented to varying degrees by symbiotic fungi (Harrington, 2005). In contrast, ambrosia beetles bore tunnels into the sapwood (xylem) where they cultivate a garden of symbiotic fungus which produces nutritional structures that comprise the majority or entirety of the beetle diet (Batra, 1963; Hulcr & Stelinski, 2017). Fungus-farming ambrosia beetles have evolved at least 14 times (Johnson et al., 2018), and ambrosia fungi have evolved at least seven times (Hulcr & Stelinski, 2017; Mayers et al., 2015; Vanderpool, Bracewell, & McCutcheon, 2018). Ambrosia beetles, and some bark beetles, possess pit- or pouch-like organs termed “mycangia” (singular mycangium) used to isolate and carry specific fungal symbionts (Batra, 1963; Francke-Grosman, 1956; Skelton, Johnson, et al., 2019). Other fungi produce sticky spores that adhere to the external surfaces of beetles, while yet other fungi are carried in the beetle gut.

Bark and ambrosia beetles are generally thought to accelerate wood decomposition because they facilitate fungal colonization of wood and increase fungal diversity in early stages of saprotrophic fungal community assembly (Müller, Varama, Heinonen, & Hallaksela, 2002; Persson, Ihrmark, & Stenlid, 2011; Strid, Schroeder, Lindahl,

Ihrmark, & Stenlid, 2014; Ulyshen, 2016). However, the majority of beetle-associated fungi are not known to decompose lignified cellulose (Kasson et al., 2016; Skelton, Loyd, et al., 2019). Instead, these fungi consume labile nonstructural compounds such as sugars (Huang, Skelton, & Hulcr, 2018; Wang, Lu, Cheng, Salcedo, & Sun, 2013). True decay fungi also utilize these labile resources in addition to more recalcitrant lignocellulose, and competition between nondecay and decay fungi for labile compounds can reduce decay rates (Hulme & Shields, 1970). Ophiostomatalean fungal symbionts of pine-infesting bark and ambrosia beetles can compete with wood decaying fungi, excluding them from portions of the wood and limiting wood degradation (Skelton, Loyd, et al., 2019). Thus, we hypothesized that bark and ambrosia beetles suppress decay of pine sapwood by introducing nondecay fungi during early stages of saprotrophic community assembly, which subsequently compete with wood decay fungi.

To test our hypothesis, we conducted a survey of the fungi associated with bark and ambrosia beetles, combined with a beetle exclusion experiment. To establish baseline data on fungi found in the southern pine ecosystem, we utilized DNA and culture-based surveys to identify fungal associates of seven common genera of pine-infesting bark and ambrosia beetles in northern Florida, USA. Concurrently, a beetle-exclusion experiment determined the effects the beetles had on fungal community assembly and wood decomposition of mature pine trunks. To determine the portion of the fungi carried into logs by beetles that is biologically active in wood, as opposed to dormant spores or dead cells, we compared DNA-based and RNA-based metabarcoding community characterizations (Baldrian et al., 2012). We also compared results from the same samples obtained from two commonly used marker genes for DNA barcode studies of fungi, Internal transcribed spacer II (ITS2) and the 28S ribosomal large subunit (LSU), to examine primer-specific biases and limitations.

## 2 | MATERIALS AND METHODS

### 2.1 | Study site

Field work was conducted at the University of Florida Austin Cary Experimental Forest from autumn of 2016 to spring 2017. The field site (29.721489, -82.225136) was dominated by planted loblolly pine (*Pinus taeda*) approximately 20–30 cm diameter at breast height (DBH), with a mixed understory of sweetgum (*Liquidambar styraciflua*) and oak species (*Quercus* spp.).

### 2.2 | Experimental setup

We conducted a beetle exclusion experiment to determine the effects of bark and ambrosia beetles on fungal community assembly and wood decay. Following published best practices for studies of arthropod effects on wood decay (Ulyshen & Wagner, 2013), the experiment used largely intact and naturally occurring wood substrate, minimally disruptive exclusion cages, and a sampling strategy that

measured decay throughout wood substrate rather than targeting only insect-colonized portions. We felled four healthy loblolly pines (25 cm DBH) at evenly spaced intervals along a 150 m transect. The bottom portion of each tree was cut into two logs, each 1.7 m in length. A disc of 20 cm thickness was taken from the bottom of each log to determine initial wood density (dry mass divided by volume). To minimize environmental contaminants and excessive drying introduced by cutting, the ends of each log were sanitized by misting with 95% ethanol until dripping and then sealed with Spectracide pruning seal (United Industry Corp.). One log from each tree was assigned to the enclosure treatment, and one to the exposed treatment, alternating the top and bottom sections for each treatment. Each log was set vertically within a wooden frame to simulate a standing dead trunk, the most common condition in the system. Enclosure treatment frames were covered in 0.5 mm steel mesh on all sides. Frames assigned to the insect-exposed treatment were sham cages, covered by mesh on only the southeast and southwest facing sides to provide shading similar to the enclosure treatment, while allowing insects to access to the log from the northeast and northwest sides. Temperature and humidity loggers (ThermoPro TP50, iTronics) were placed within enclosure and exposed treatments for a period of 5 days to confirm similar microclimate in enclosures and sham cages and thus avoid conflating microclimate effects and insect effects (Figure S1).

The experiment was concluded after 124 days. A disc 20 cm thick was taken from the bottom, middle, and top third of each log to determine final dry mass density by taking four evenly spaced 50 cc cubical subsamples of sapwood from each disc. All bark was removed from each log section to collect and identify macroinvertebrates present and record the number of beetle galleries that penetrated the bark and xylem (i.e., ambrosia beetle galleries) or bark only (i.e., bark beetle galleries). Logs were then extensively and carefully dissected to collect and identify all beetles still present in galleries that penetrated the xylem.

### 2.3 | Survey of beetle symbionts

During the experimental period, dispersing bark and ambrosia beetles were trapped in-flight for analysis of symbiotic fungal communities using a combination of Lindgren Funnel traps baited with 95% ethanol and a racemic mixture of alpha and beta-pinene (Synergy Semiochemicals Corporation; item no. 3076) and light trapping on a lighted sheet. Trap cups were filled with moistened clean tissue paper, and holes were drilled in the cup bottoms to prevent pooling of water that could cause cross-contamination among trapped beetles. Traps were checked at 1–2 day intervals. All beetles from traps and light sheets used for molecular analysis were handled with flamed forceps and immediately and individually placed in DNA-sterile cell lysis solution (CLS) for DNA extraction (Lindner & Banik, 2009) and then frozen. The entire beetle was crushed with a sterile micro-pestle in CLS prior to DNA extraction. Beetles used for culture-based analysis were kept alive on clean moistened tissue and sampled as described in Skelton et al. (2018) within 24 hr of capture.

### 2.4 | Fungal sample collection from wood

We sampled the sapwood of each log for fungi at three time points; at the initiation of the experiment when the trees were alive, i.e., fungal endophyte samples (1 November 2016), at 88 days after felling (28 January 2017), and again at 124 days after felling (5 March 2017). Samples were taken at the middle, at 20 cm from the bottom, and at 20 cm from the top. At each location, four evenly spaced subsamples were taken around the circumference. For each subsample, a flame-sterilized chisel was used to remove a 1 cm<sup>2</sup> section of bark to expose the xylem. A DNA-sterile 8 mm drill bit was used to excavate 10 cc of xylem shavings which were collected by placing a DNA-sterile funnel around the drill. To limit the introduction of fungi to the wood while sampling, resulting holes were immediately plugged with autoclave-sterilized sections of pine dowel, and sealed with pruning seal. Subsamples were homogenized and split three ways; one portion was stored cool (approximately 5°C) until processing for fungal culturing within 24 hr, one was submerged in DNA-sterile CLS, transported to the laboratory cool, and then stored at –20°C until processing for DNA sampling, and the remaining portion was flash frozen in the field by submersing sample tubes in a slurry of crushed dry ice and ethanol, transported to the laboratory on dry ice, and then stored at –80°C until RNA extraction.

### 2.5 | Culture-based sampling

Beetles were sampled by dilution plating of body washes and mycangium dissections following published methods (Skelton et al., 2018). Xylem and beetle samples were cultured on two media; standard potato dextrose agar (PDA) and a medium that is selective for ophiostomatalean fungi – malt extract agar amended with streptomycin and cycloheximide. The selective agar excludes most fungi in Microascales and Hypocreales. For each xylem sample, 12 wood shavings were separately embedded in each medium and incubated in the dark at 25°C until colony formation, purified by subculturing, and assigned to morphotypes. DNA from representative isolates of each morphotype from each sample were extracted and the 28S/Large Ribosomal Subunit (LSU) was sequenced for identification following published methods using the primers LR0R and LR3 (Bateman, Šigut, Skelton, Smith, & Hulcr, 2016; Vilgalys & Hester, 1990). All sequences from fungal cultures were manually trimmed and aligned using Sequencher v4.9, and OTUs were clustered at 99% similarity. The longest sequence from each cluster (cluster centers) were used as a reference library in a local BLASTn search to match OTUs from high throughput metabarcoding to sequences obtained from culture.

### 2.6 | DNA and RNA extraction

DNA extraction from pine sapwood and whole crushed beetles was performed at the Center for Mycological Research (CFMR) at the USFS Northern Research Station, Madison WI. DNA extraction from pine sapwood followed Lindner and Banik (2009), with the

modifications used by Jusino et al., (2014), and extraction from beetles was performed the same way with one modification; 40  $\mu$ l of CLS was added to the beetles prior to crushing, and the extraction proceeded with 40  $\mu$ l CLS. RNA extraction was conducted at the University of Florida, School of Forest Resources and Conservation, Gainesville FL. A total of 15 ml of 65°C CTAB extraction buffer was added to each 10 cc sample of frozen xylem shavings. The mixture was allowed to thaw at 65°C for 30 min with intermittent mixing. RNA was extracted using a previously published pine RNA extraction method (Chang, Puryear, & Cairney, 1993). Next, the RNA was cleaned and concentrated using the RNA Clean & Concentrator-5 kit (Zymo Research) and RNA concentration was determined using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized starting with 3 ng of RNA and using the Verso cDNA synthesis Kit (Thermo Fisher Scientific). Each cDNA sample was treated with RNase H (catalog# En0201, Thermo Fisher Scientific) at 37°C for 20 min. PCR was performed using Phusion high-fidelity DNA polymerase (New England BioLabs) and ribosomal large subunit primers: LROR and JH-LS-369rc (Li et al., 2015; Vilgalys & Hester, 1990).

## 2.7 | Mock community spike-in controls

Estimates of community composition and alpha diversity can be skewed in metabarcoding pipelines due to PCR mismatches and amplification biases, chimera formation, index bleed (aka “tag switching”), and inappropriate sequence clustering parameters (Palmer, Jusino, Banik, & Lindner, 2018). Thus, we analyzed mock community samples of known and relevant composition in parallel with experimental samples to inform bioinformatics decisions and validate our results. We included an equimolar synthetic mock community (SynMock; Palmer et al., 2018) of single-copy nonbiological sequences to parameterize our bioinformatics. Because SynMock sequences do not occur in nature, they are not confounded by biological sequences present in a sequencing run, providing an independent internal standard. We also included a biological mock community composed of equimolar genomic DNA from four common genera of bark and ambrosia beetle-associated fungi; *Flavodon ambrosius* (JH9633) isolated from *Ambrosiodmus minor* in Gainesville FL, *Ambrosiella* sp. isolated from *Cnestus* sp. in Tam Dao Vietnam, *Sporothrix* sp. (isolate LL195; KX590863.1) and *Raffaelea* sp. (LL188; KR018423.1) isolated from *Euwallacea interjectus* in China. Genomic DNA was extracted from pure cultured hyphae preserved in CLS using the methods above, quantified using a Qubit 2.0 fluorometer with the high-sensitivity DNA quantification kit (ThermoFisher Scientific), and combined in equimolar concentration prior to PCR amplification and metabarcoding.

## 2.8 | Metabarcoding amplicon sequencing

While ITS is generally the preferred DNA barcode for fungal amplicon studies and therefore offers the largest reference databases (Schoch et al., 2012), PCR amplification of this region is difficult

and inconsistent for many fungal taxa associated with bark beetles (Dreaden et al., 2014). LSU may provide a suitable substitute for metabarcoding studies of fungal communities. We sequenced both regions for all wood samples to compare results. A single-step PCR protocol was used to generate Ion Torrent compatible sequencing libraries using primers designed according to the manufacturer's recommendations. Primers used for ITS barcoding targeted the fITS7 (Ihrmark et al., 2012) and ITS4 (White, Bruns, Lee, & Taylor, 1990) priming sites and were modified for sequencing as in Palmer et al. (2018). In short, the forward primers included the Ion A adapter sequence, the Ion key signal sequence, followed by a unique Ion Xpress barcode sequence (10–12 bp), and a single base-pair linker (A), and lastly the fITS7 primer (Ihrmark et al., 2012). The reverse primer included the Ion trP1 adapter and the ITS4 primer (White et al., 1990). Primers used for LSU (28S) barcoding targeted the LROR (Vilgalys & Hester, 1990) and JH-LSU-369rc sites (Li et al., 2015) and were modified similarly to our ITS primers for Ion Torrent compatibility. PCR products were individually cleaned using Zymo Select-a-Size spin columns (Zymo Research), quantified using a Qubit 2.0 fluorometer with the high-sensitivity DNA quantification kit (ThermoFisher Scientific), and combined in equimolar concentration prior to sequencing at the CFMR on two chips on an Ion Torrent PGM according to manufacturer's recommendations using Ion PGM Hi-Q Sequencing Kits, and Ion PGM sequencing chips (316v2). Raw data were processed via the Ion Torrent Suite v5.0.3 with the “--disable-all-filters” flag given to the BaseCaller.

## 2.9 | Bioinformatics

We processed our ion torrent PGM sequencing data using AMP<sub>TK</sub> v0.10.0 (Palmer et al., 2018). We preprocessed our individually barcoded ion reads using USEARCH (version 9.2.64), then removed the forward and reverse primers. We discarded any ITS reads shorter than 125 bp, any reads longer than 300 bp were trimmed to 300, and any reads between 125 and 300 bp were padded with N's to help improve sequence clustering (Palmer et al., 2018). We discarded any LSU reads shorter than 250 bp, and any reads longer than 350 bp were trimmed to 350. The sequences from each PGM run were preprocessed separately, then concatenated before clustering. Samples with fewer than 10,000 reads were dropped before clustering to avoid clustering errors, resulting in one LSU sample dropped. ITS sequence reads were quality filtered with expected errors less than 1.0 (Edgar & Flyvbjerg, 2015), de-replicated, and clustered at 97% similarity to generate operational taxonomic units (OTUs) using UPARSE (Edgar, 2013). Following clustering, any padded N's were removed, and the processed ITS sequences were mapped to the OTUs. LSU sequence reads were de-noised and quality filtered using expected error trimming by the DADA2 algorithm (Callahan et al., 2016) in the DADA2 module of AMP<sub>TK</sub>. We clustered the resulting inferred sequences (iSEQs) into traditional OTUs using UCLUST and 97% similarity, and the processed sequences were then mapped back to the OTUs. We used the synthetic mock community to account for observed rates of

index bleed using the filter module in AMP<sub>TK</sub> following Palmer et al. (2018). Finally, the OTUs were assigned taxonomy using the hybrid taxonomy algorithm in AMP<sub>TK</sub>, and compared to sequences from cultured fungi using a local BLASTn search. All nonfungal OTUs were removed prior to statistical analysis.

## 2.10 | Data analysis

All analyses were conducted in R version 3.4.2 (2017-09-28) (R Development Core Team, 2010). The effect of beetle exclusion on fungal richness was determined using generalized linear mixed models (GLMMs) implemented by the `glmer()` function of the package LME4 (Bates, Maechler, Bolker, & Walker, 2014) specifying the Poisson family, with beetles (presence or absence), sampling day (as a factor), and their interaction as fixed effects, and random intercepts for the source tree. Bark beetles were detected in two replicates of the enclosure group which were subsequently treated as beetle infested. ITS and LSU data sets were analyzed separately. The effects of beetles on the composition of fungal communities were determined for sampling days 88 and 124 using permutations-based multivariate ANOVA (PERMANOVA; Anderson, 2001), implemented by the `adonis()` function of the VEGAN package (Oksanen et al., 2013) on a Raup Crick distance matrix. Beetle presence/absence, log section (top, middle, or bottom), and sampling day were included as factors in that order, with permutations constrained within tree to account for block design. We also tested for differences in multivariate dispersion among treatments using the `betadisper` function (Anderson, 2006). PERMANOVA and `betadisper` were used to compare DNA and RNA-based results on day 88 of the experiment, and a paired *t* test was used to compare fungal richness. Effects were visualized in two dimensions by nonmetric multidimensional scaling (NMDS), implemented by the `metaMDS()` function in the VEGAN package, using Raup Crick distances.

We used detection frequency to quantitatively classify fungal OTUs to three different fungal assemblages; beetle associates, nonassociated saprotrophs, and endophytes. Classifications were made using multilevel pattern analysis (De Cáceres, Legendre, & Moretti, 2010), implemented by the `multipatt()` function of the INDICESPECIES package for R (De Cáceres & Jansen, 2012), using the “r.g” statistic. This is a permutations-based indicator species analysis that tests for significant associations between each taxon (i.e., OTUs) and multiple combinations of sample grouping factors. OTUs that were significant indicators of only infested logs were classified as beetle associates. OTUs that were significant indicators of only dead logs (infested or not), were classified as non-associated saprotrophs. Significant indicators of only living tree samples were classified as endophytes. Results for common OTUs were visualized as a heat map using the `pheatmap()` function of the PHEATMAP R package (Kolde & Kolde, 2015).

Endophytes are sometimes considered to be important determinants of saprotroph community assembly and decay processes. Therefore, we used variation partitioning (Peres-Neto, Legendre,

Dray, & Borcard, 2006) to determine the amount of variation among saprotroph communities that was explained by the composition of fungal endophyte communities at the start of the experiment, the amount explained by infestation by beetles (binary), and the section of log being sampled (ends vs. middle as binary). Separate analyses were conducted for sampling days 88 and 124, and ITS and LSU data sets. In each analysis, principal coordinates analysis (pcoa()); APE package: Paradis, Claude, & Strimmer, 2004) was used to decompose the fungal endophyte community matrix from day 0 (living trees) into eigenvectors to be used as predictors for subsequent saprotroph community composition. To avoid overfitting the final model, we conducted automated forward model selection (Blanchet, Legendre, & Borcard, 2008) to retain only significant eigenvectors. Model selection was implemented using the `forward.sel()` function of the PACKFOR package for R (Dray, Legendre, & Blanchet, 2009), with  $\alpha = 0.05$ . Variation partitioning was implemented on Raup Crick distance matrices using the `varpart()` function in the VEGAN package. Statistical significances of the testable fractions were determined by distance-based redundancy analysis (`dbrda()` in the VEGAN package). Significant fractions from variation partitioning analysis were visualized as Euler diagrams using the EULERR package (Larsson, 2019).

Linear mixed models were used to determine the effects of bark and ambrosia beetle infestation, log section (top, middle or bottom), and water content (wet mass divided by dry mass at conclusion of the experiment) on the decay of pine sapwood. Decay was measured as the percent loss in dry mass divided by volume of 50 cm<sup>3</sup> subsamples. We choose to use log transformed number of bark and ambrosia beetle galleries instead of the experimental treatment (enclosure vs. exclosure) to account for the orders-of-magnitude variation in infestation densities observed among logs and log sections. The best models were chosen and validated using a top down approach following Zuur, Ieno, Walker, Saveliev, and Smith (2009) section 5.8.2.4. Models were fitted using the `lmer` function of the R package LME4 (Bates et al., 2014) for decay. Fungal richness was modelled using the generalized linear model for Poisson data via maximum likelihood implemented by the `glmer()` function in the LME4 package (Bates et al., 2014). Richness estimates from each marker gene (LSU and ITS) were analyzed separately. Tables were generated using the `tab_model()` function in the sjPlot package (Lüdtke, 2018).

To determine which fungi were probably brought into the wood of experimental logs by bark and ambrosia beetles, we sampled the most common pine trunk-infesting bark and ambrosia beetles in our area which included four genera of scolytine bark beetles (*Ips*, *Hylastes*, *Dendroctonus*, and *Orthotomicus*), two genera of scolytine ambrosia beetles (*Xyleborus* and *Gnathotrichus*), and one platypodine ambrosia beetle species (*Myoplatypus flavicornis*). We used ANOVA and PERMANOVA to compare diversity and composition of fungi associated with these three groups of beetles recovered from metabarcoding of ITS2. We used the multilevel pattern analysis described above to determine OTUs that had nonrandom associations with either scolytine

bark beetles, scolytine ambrosia beetles, platypodines, or any combination of those groupings.

### 3 | RESULTS

#### 3.1 | Effects on decay

Ambrosia beetles suppressed early decay of pine sapwood. The best GLMM of loss in wood density showed a negative relationship between decay and the number of ambrosia beetle galleries per log section, and a positive relationship with the percent water content on the final day of the experiment (Table 1). Thus, decay decreased with the number of ambrosia beetle galleries per log section and increased with water content (Figure 1). Water content and the number of ambrosia beetle galleries were not significantly correlated. Log section (bottom, middle, or top) and number of bark beetle holes were not significant predictors of wood density loss and were dropped out during model selection.

#### 3.2 | Effects of beetles on fungal communities in wood

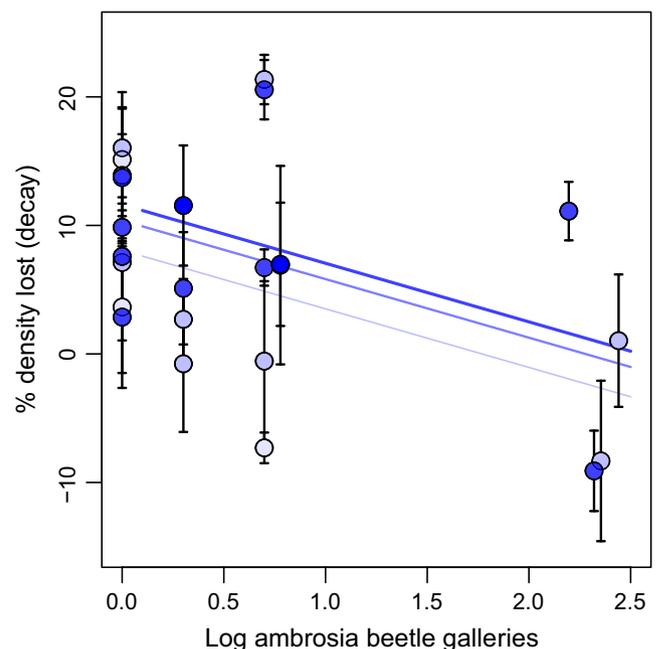
Beetle infestations increased fungal richness several fold and extensively altered the taxonomic composition of fungi in pine sapwood. GLMMs of fungal richness for the LSU and ITS data sets revealed consistent results among marker genes (Table 2). Prior to the experiment, the living pine trunks had surprisingly high endophyte diversity. After 88 days post-cutting, fungal diversity decreased in trunks where beetles were excluded. In contrast, trunks infested with beetles maintained fungal richness similar to live trees, which then increased from day 88 to 124 (Figure 2: top panels). Beetles, sampling date, and trunk section had significant effects on saprotroph community composition, with beetle presence explaining the majority of variation among samples in both the ITS and LSU data sets (Table 3). Trunks with and without beetles were most divergent

in fungal community composition in the middle sections (Figure 2: bottom panels), as the intrusion of environmental saprotrophs from the cut ends of the trunks had a homogenizing effect across the treatments.

There was little overlap in fungal community membership inferred from LSU DNA sequencing among living trees, dead trees without beetles, and dead trees infested by beetles (Figure 3). Virtually none of the fungal endophytes present in the live trees were detected 88 days after tree death, though some reappeared at 124 days in beetle-excluded logs. Only three fungi were prevalent in beetle-excluded logs, OTU2 - *Diplodia seriata*, OTU6 - *Trichoderma* sp., and OTU 191 - *Lasiodiplodia* sp. These taxa were classified by multilevel pattern analysis as nonassociated saprotrophs. In contrast, numerous fungi were prevalent among beetle-infested logs, many of which had statistically significant associations with beetle infestation. In the LSU data set, 46 OTUs were classified as significant beetle associates (Table S1). Sixteen beetle associated OTUs (35%) were yeasts in Saccharomycetales, nine (19%) were Ophiostomataleans, two were *Graphium* species, and one the pervasive pine pathogen *Heterobasidion*. Living trees also had diverse and distinct fungal communities; 41 OTUs were classified as fungal endophytes of living pines. Similar results were obtained from the ITS data set (Table S2) with a few notable differences. Reflecting the larger overall number of OTUs in the ITS data set, a larger number of significant indicator species were identified (119), though the proportion of OTUs that were

**TABLE 1** Mixed effects model for the effects of ambrosia beetle galleries and water content on decay, measured as loss of dry mass density

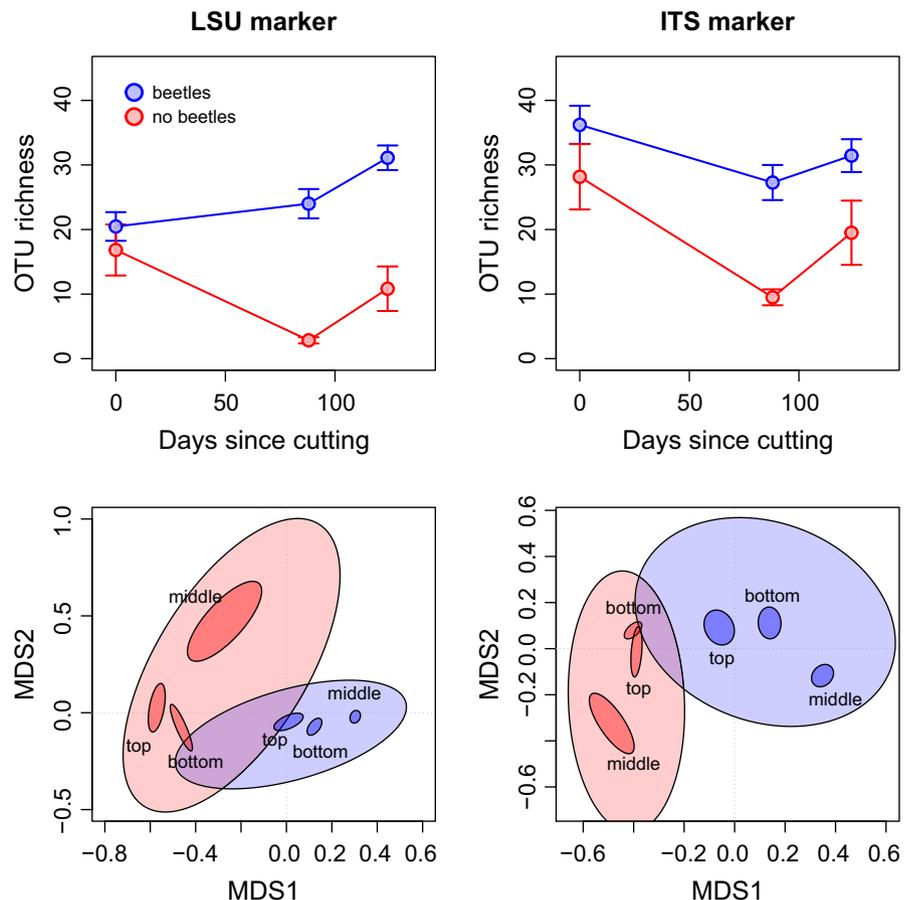
Predictors	Percent density lost		
	Estimates	CI	p
(Intercept)	-6.22	-18.58 to 6.13	.324
Log(1 + ambrosia beetle galleries)	-4.56	-8.15 to -0.97	.013
Percent water	0.33	0.08 to 0.57	.009
Random effects			
$\sigma^2$	76.60		
$\tau_{00}$ factor(log section)	33.25		
ICC <sub>factor(log section)</sub>	0.30		
Observations	95		
Marginal $R^2$ /Conditional $R^2$	.166/.419		



**FIGURE 1** Increasing numbers of ambrosia beetle galleries and lower water content led to decreased decay of pine sapwood. Symbols with error bars show mean loss of wood density ( $\pm$ SE) of log sections estimated from four subsamples each. Shading represents relative water content, lighter is drier. Lines show mixed-effects model fits for wood with moisture content reflecting the first (thinnest), second, and third quartiles (thickest) of observed values [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

**TABLE 2** Generalized linear mixed-effects model for the effects of beetle infestation and time on the fungal richness estimates from large ribosomal subunit (LSU) and internal transcribed spacer 2 (ITS2) amplicons from experimental pine trunks

Predictors	LSU marker			ITS2 marker		
	Incidence rate ratios	CI	<i>p</i>	Incidence rate ratios	CI	<i>p</i>
(Intercept)	16.29	12.80–20.73	<.001	26.55	21.53–32.73	<.001
Beetles	1.26	1.00–1.58	.053	1.36	1.13–1.64	.001
Day 124	0.64	0.47–0.88	.006	0.69	0.55–0.88	.002
Day 88	0.17	0.10–0.28	<.001	0.34	0.25–0.46	<.001
Beetles × Day 124	2.36	1.68–3.31	<.001	1.26	0.97–1.65	.083
Beetles × Day 88	6.96	4.09–11.86	<.001	2.25	1.63–3.11	<.001
Random effects						
$\sigma^2$	0.05			0.04		
$\tau_{00}$	0.02 <sub>Tree</sub>			0.02 <sub>Tree</sub>		
ICC	0.28 <sub>Tree</sub>			0.35 <sub>Tree</sub>		
Observations	71			68		
Marginal $R^2$ /Conditional $R^2$	.870/.906			.728/.824		

**FIGURE 2** Exclusion experiment shows that beetles drive fungal diversity (top row; mean richness  $\pm$  1 SE) and community composition (bottom row) in pine sapwood, measured by high-throughput metabarcoding of two marker genes, large ribosomal subunit (28S; left) and internal transcribed spacer 2 (right). Composition is shown as nonmetric multidimensional scaling (NMDS). Large ellipses show total range for samples from logs with and without beetles, smaller ellipses show 1 SE around centroid for log sections within each treatment [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

significant indicator species were similar among ITS and LSU data sets, 27% and 25%, respectively. Notably, the species of the Ophiostomatalean genus *Raffaelea*, which are commonly farmed by many ambrosia beetles, were represented by three OTUs in the LSU data set but not detected in the ITS data set.

### 3.3 | Fungal endophytes and beetles

The effects of beetles on saprotroph community composition superseded the effects of fungal endophyte community composition. On both day 88 and 124, beetle infestation explained the largest

Predictors	LSU marker			ITS2 marker		
	F	R <sup>2</sup>	p	F	R <sup>2</sup>	p
Beetles (yes, no)	1,065.68	.67	.001	1,300.96	.60	.001
Section (bottom, mid, top)	214.12	.27	.001	357.61	.33	.001
Day (88, 124)	48.62	.03	.001	118.98	.05	.001
Residuals		.03			.02	

Note: Data obtained from LSU (left) and ITS2 (right) were analyzed separately. In both models, all three factors were significant, with beetles explaining the majority of variation among samples.

fraction of variation in fungal community composition identified by metabarcoding of LSU, with the section of the log explaining a significant but smaller fraction. Endophytes had a latent effect on saprotroph composition that was nullified when beetles were present. Several endophyte OTUs that were present in live trees, disappeared after 88 days, and reappeared on day 124, but only in the beetle-excluded logs (Figure 3). Fungal endophyte community composition was a significant predictor only on day 124 (Figure 4).

### 3.4 | Culture-based results

Results of culture-based sampling were qualitatively similar to DNA and RNA approaches, showing highly significant effects of beetles and no significant effects of endophytes on pine saprotroph communities. The overall observed richness was much lower in the culture-based data set than the culture-independent data sets. We cultured significantly higher diversity in logs exposed to beetles (GLMM;  $z = 2.55$ ,  $p = .010$ ), with samples from exposed logs yielding an average of  $3.91 \pm 1.56$  OTUs per log section (mean  $\pm$  SD) and enclosure logs yielding  $2.08 \pm 1.73$  OTUs. There was a significant effect of beetles on the community composition of cultured fungi (PERMANOVA;  $F = 4.01$ ,  $R^2 = .16$ ,  $p = .017$ ), but no significant effect of log section ( $F = 1.637$ ,  $p = .164$ ). We cultured 30 fungal OTUs from the trees while they were alive and 22 OTUs at day 88. Only three fungi were recovered at both time points: *Fusarium solani* (ref\_002), *Penicillium citrinum* (ref\_007), and a *Cladosporium* sp. (ref\_009). There was no significant correlation between the endophyte communities cultured at the beginning of the experiment and saprotroph communities sampled from the same logs after 88 days after killing the trees (Mantel test of Raup-Crick distance matrices; Mantel's  $r = -.068$ ,  $p = .69$ ). Counts and GenBank accession numbers for all isolates cultured and sequenced from the experimental logs are provided in the supplemental material.

### 3.5 | Active and inactive community components – RNA and DNA

Similar to total community inferences from DNA-based results, the active fungal community inferred using RNA was much richer in trunks with beetle infestation than in caged trunks, and was composed of a significantly different assemblage. Community composition and richness were similar between DNA and RNA-based data

**TABLE 3** Permutations-based multivariate ANOVA models for effects of Beetles, log section, and sampling day on fungal community composition of pine sap wood

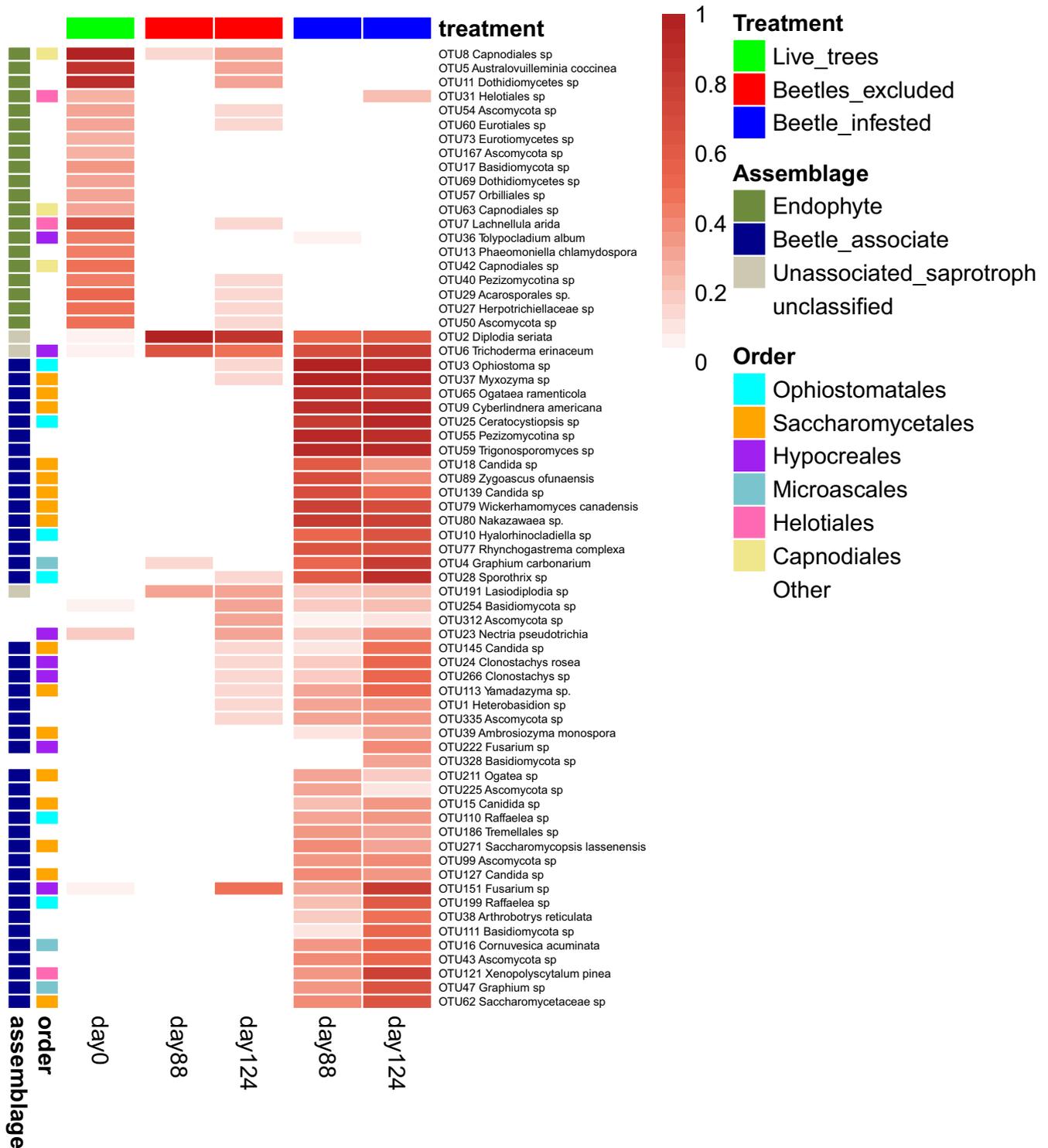
sets; however the variance among samples was significantly higher in the RNA data set (Figure 5). Similar to DNA, RNA revealed a significant difference in fungal richness between trunks with and without beetles ( $26.94 \pm 14.36$  SD vs.  $7.25 \pm 6.50$  SD OTUs per sample;  $t = -2.639$ ,  $p = .016$ ), and a significant difference in fungal composition (Table 4).

### 3.6 | Fungi on flying beetles

Sequencing of in-flight beetles revealed that platypodine ambrosia beetle *Myoplatypus flavicornis* harboured fungal communities that were more diverse and compositionally distinct from the scolytine genera (Figure 6). Within the Scolytinae, bark beetles appear to transport a slightly greater diversity of fungi than the ambrosia beetles, although the difference was not statistically significant. Multilevel pattern analysis recovered only one significant OTU for scolytine bark beetles, and one for scolytine ambrosia beetles. In contrast to scolytines, platypodines had nonrandom associations with 26 OTUs, including nine yeasts in Saccharomycetales (Table S3). Estimated colony forming units and GenBank accession numbers for all isolates cultured and sequenced from dispersing beetles are available in the supplemental material.

### 3.7 | Insects present in experimental logs

Enclosure treatments effectively reduced insect colonization. The average number of wood-borer holes in the xylem of each log was  $263 \pm 37.2$  in exposed logs, versus  $8.75 \pm 4.78$  in enclosures, with 93.6% of holes occurring in the bottom sections of the logs. From the exposed logs, we recovered one species of platypodine ambrosia beetle (*M. flavicornis* - 34%) and five species of scolytine ambrosia beetles (*Xyleborus ferrugineus* - 21%, *Gnathotrichus materiarius* - 21%, *Xyleborus pubescens* - 9%, *Xyleborus affinis* - 7%, and *A. minor* - 3%; in order of relative abundances), whereas no ambrosia beetles were recovered from enclosure logs. The phloem was predominantly colonized by bark beetles (25.7%) and dipteran larvae (20.8%) and pupae (28.8%), other Curculionidae (5.0%), Cerambycidae (3.2%), and less than one percent each of Staphylinidae, Histeridae, Dermaptera, Hymenoptera, and Collembola. Adult bark beetles consisted of *Dendroctonus terebrans* (43.6%), *Ips* spp. (34.4%), and *Orthotomicus caelatus* (22.0%).



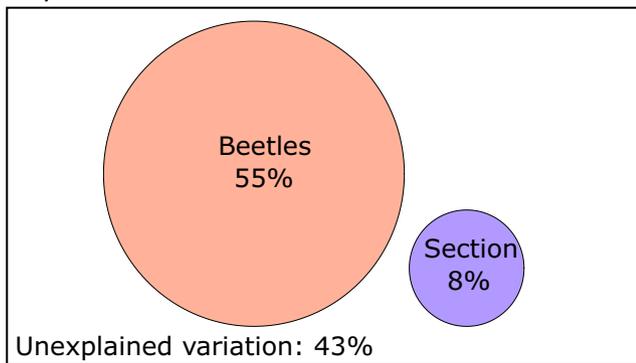
**FIGURE 3** Frequency of common fungal OTUs (LSU marker) detected in sapwood of living pine trees, and the same logs 88 and 124 days after felling, with and without wood-boring beetles. Heat map shows proportion of replicates for which each OTU was detected. Coloured annotations on left indicate fungal taxonomic order and fungal assemblage membership determined by multilevel pattern analysis for each OTU. Only OTUs that occurred in at least 25% of samples for any one treatment at any one time are shown. Complete lists of fungal assemblages are available in Tables S1 and S2. Putative identifications are given to lowest taxonomic rank based on AMPTK taxonomy assignment and BLASTn search of NCBI database. All OTU nucleotide sequences are available in Supporting Information [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

### 3.8 | Sequencing standards and controls

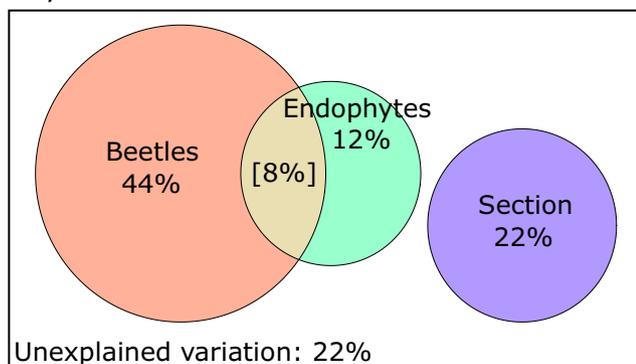
Results from our biological and synthetic mock communities showed that number of reads do not reflect the relative abundances of taxa

in a sample, and our biological mock demonstrated that the ITS and LSU primer sets have different biases. Although biological mock members were combined in equal molar amounts, the number of reads among taxa varied by four orders of magnitude and one

Day 88



Day 124



**FIGURE 4** Beetle infestations had the largest effect on fungal community composition in experimental pine logs at day 88 and 124. Circles represent the proportions of variation in community composition explained by the presence/absence of beetles, the fungal composition of logs when they were alive (endophytes), and the section of the log being sampled (ends vs. middle). Circles size and the size of overlap represents the independent and overlapping fractions of variation in fungal community that is explained by each factor according to variation partitioning analysis of DNA-based LSU data set. Only statistically significant fractions are shown. Note that fractions do not sum to 100% because of nonsignificant and negative fractions (not shown) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

important taxon (*Raffaelea*) was not recovered by metabarcoding of ITS2. The number of reads recovered from ITS2 of the mock community sample was 56,875 for *Ambrosiella*, 15,963 for *Flavodon*, 417 for *Sporothrix*, and 0 for *Raffaelea*. For LSU we recovered 33,240 from *Ambrosiella*, 11,478 from *Sporothrix*, 629 from *Raffaelea*, and 77 reads from *Flavodon*. After quality filtering, no spurious OTUs were detected in the mock community samples, indicating that our sequencing and bioinformatics pipeline does not inflate estimates of alpha diversity and no laboratory contamination was detected.

## 4 | DISCUSSION

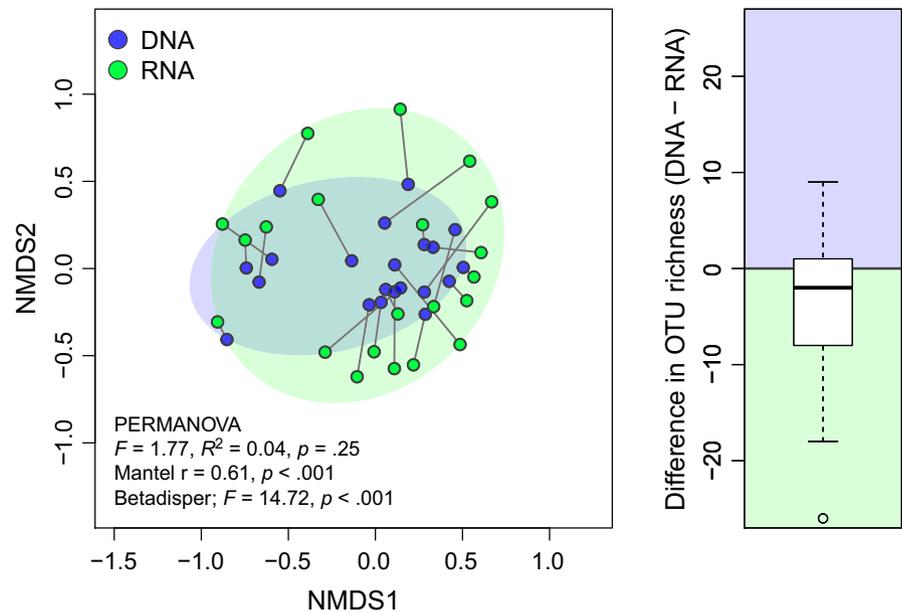
How many of the fungi in dead wood are brought by bark and ambrosia beetles? Using high throughput amplicon sequencing, we found that each individual beetle carries dozens of fungal taxa or more. This result is congruent with other recent metabarcoding studies

of bark beetles (Kostovcik et al., 2015; Miller, Hopkins, Inward, & Vogler, 2016; Miller, Inward, Gomez-Rodriguez, Baselga, & Vogler, 2019). In this study, exposed trunks were colonized by hundreds of beetles which resulted in two- to four-fold higher fungal richness than insect-excluded trunks, and others have reported similar increases (Müller et al., 2002; Persson et al., 2011; Strid et al., 2014). It is possible that the holes created by insects alone could facilitate the establishment of windblown spores, however, experimental tests did not find a significant contribution of this mechanism to fungal diversity in wood (Strid et al., 2014), indicating that phoresy is the main mechanism by which insects facilitate fungal colonization. Although other insects were present in our experimental trunks, with the exception of dipteran larvae and pupae, all other taxa were rare when compared to the hundreds of bark and ambrosia beetle galleries present in each exposed trunk. Furthermore, the majority (74%) of the fungal OTUs identified as significantly associated with beetle infestation of wood were also detected by metabarcoding on flying beetles. Our culture-based survey of flying beetles also recovered many fungal isolates with DNA sequences that matched significant OTUs, including eight of the nine ophiostomatalean OTUs recovered from experimental logs that were significantly associated with beetle infestation. Thus, it appears that the majority of fungi present in the sapwood of pines during early stages of decay are brought into the wood by bark and ambrosia beetles.

Are the fungi introduced by beetles active members of the saprotroph community? Soil and leaf litter saprotroph communities contain a large portion of inactive taxa present as dormant spores, dead cells, or extracellular DNA (Baldrian et al., 2012). In contrast, our results from wood in the early stages of decay showed no significant difference between RNA-based and DNA-based community assessments, suggesting that the large majority of taxa detected in our study by DNA-based methods were metabolically active. Therefore, the increased diversity we inferred from sequences from beetle-infested logs versus enclosure logs cannot be explained by inactive spores or dead fungal biomass, and the fungi introduced by beetles appear to be active members of the wood saprotroph community.

Bark and ambrosia beetles are widely thought to facilitate wood decay by introducing fungi (Ulyshen, 2016). In contrast, our results show they can actually suppress decay, despite increasing fungal diversity. This result is consistent with work in other systems showing decreased wood decay with increased fungal diversity as a result of antagonism among fungi (Fukami et al., 2010). The majority of beetle species in our system do not vector fungi capable of decaying wood, as our culture and DNA-based sampling suggest, with the unusual exception of the invasive *A. minor* vectoring *F. ambrosius*; (Kasson et al., 2016). The pathogenic wood-decayer *Heterobasidion* was detected significantly more frequently in exposed logs, but it was not detected on dispersing beetles by either metabarcoding or culture. Another recent study also noted significant increases in *Heterobasidion* in exposed logs, but not on dispersing bark beetles (Strid et al., 2014). These results suggest that *Heterobasidion* is either vectored by other insects, or simply benefits from disturbance to the

**FIGURE 5** Similar fungal communities were observed using DNA and RNA-based methods, suggesting that most detected community members are active in the wood. Nonmetric multidimensional scaling ordination shows average fungal community composition was not significantly different between methods. Lines connect observations from the same sample, each characterized by RNA-based (green) and DNA-based (blue) methods. RNA based results had significantly higher variability among replicates visible as a larger sample cloud. Boxplot shows there was no significant difference in OTU richness between methods ( $t = -0.91$ ,  $p > .05$ ) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



outer bark caused by boring insects. In contrast to decay fungi, nine species of ophiostomatalean fungi were significantly associated with beetle infestation. Eight of them were cultured from dispersing beetles, and ophiostomatalean fungi were recovered from 80% (19/24) of beetles sequenced by metabarcoding.

Several lines of evidence indicate that resource and/or interference competition between beetle-associated fungi and wood decay fungi caused the reduced decay observed in experimental logs heavily infested with ambrosia beetles. It is well established that several species of Ascomycota reduce wood decay by competing with wood-decaying Basidiomycota, some of which are used commercially for this purpose (Behrendt, Blanchette, & Farrell, 1995; Bruce, Srinivasan, Staines, & Highley, 1995; Hulme & Shields, 1970; Schubert, Fink, & Schwarze, 2008). It is also established that beetle-associated ophiostomataleans quickly consume and degrade available labile carbohydrates and other extractives in fresh wood (Blanchette et al., 1992; Schirp, Farrell, Kreber, & Singh, 2007; Wang et al., 2013). Most importantly, recent laboratory microcosm experiments using co-inoculations of beetle-associated fungi and wood decay fungi demonstrated that ophiostomatalean fungal symbionts of bark and ambrosia beetles reduce decay rates of pine sapwood

by excluding and/or competing with a common brown rot and a common white rot decay fungus (Skelton, Loyd, et al., 2019). The in situ study presented here indicates that this symbiont-mediated suppression of early decay is significant in the field, under conditions of natural complexity.

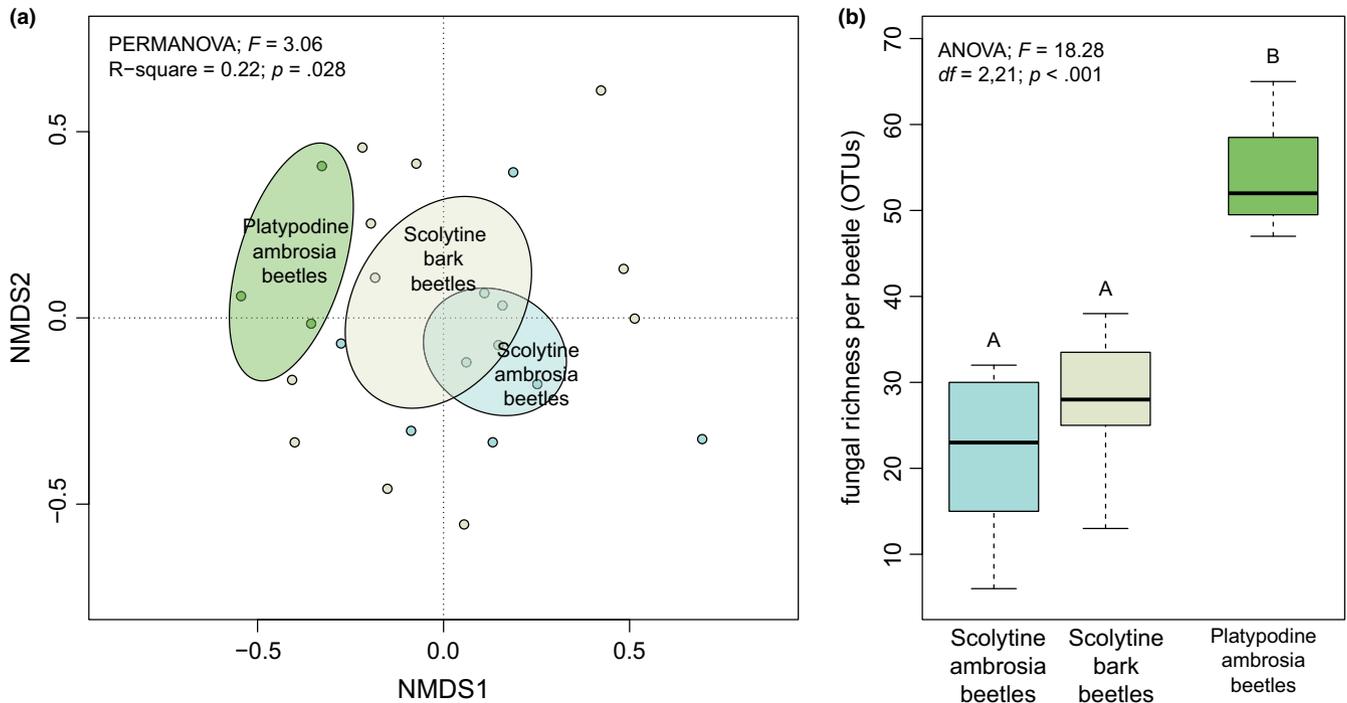
The competition-based suppression of decay in sapwood most likely depends on localized heavy propagule loading of symbiotic fungi introduced directly into xylem from ambrosia beetle galleries. This inference is supported by the observed density-dependent relationship between ambrosia beetle galleries and decay, and a lack of a significant relationship between decay and bark beetle galleries. This result is consistent with the localized effects of beetle symbionts observed in vitro (Skelton, Loyd, et al., 2019). It also provides an explanation for the discrepancies between this study and similar studies which found positive or no significant effects of wood borers on decay in northern temperate forests (Jacobsen, Sverdrup-Thygeson, Kausarud, Mundra, & Birkemoe, 2018; Müller et al., 2002) where ambrosia beetles represent a much smaller fraction of the wood-borer communities. Future efforts should examine the effects of ambrosia beetles on decay in tropical forest ecosystems where ambrosia beetle assemblages are at their most diverse and abundant (Beaver, 1979; Hulcr, Mogia, Isua, & Novotny, 2007).

Our results may help explain the counterintuitive results of previous insect enclosure experiments conducted on pines in the southeastern United States. Ulyshen, Wagner, and Mulrooney (2014) found that logs exposed to insects lost significantly more wood volume than insect-excluded logs, due to maceration from termite chewing. However, the remaining wood had significantly higher density when insects were present. They speculated that the insects may have employed antibiotics that slowed microbial decomposition, resulting in less loss of wood density in the presence of insects. In contrast, our results suggest that it is the fungi that insects carry into logs that slow decomposition by competing with wood decay fungi.

**TABLE 4** Permutations-based multivariate ANOVA models for effects of Beetles, log section, fungal community composition of pine sap wood on one sampling date (day 88)

Predictors	DNA			RNA		
	F	R <sup>2</sup>	p	F	R <sup>2</sup>	p
Beetles (yes, no)	126.61	.68	.001	15.64	.44	.001
Section (bot- tom, mid, top)	21.38	.23	.005	1.47	.08	.322
Residuals		.09			.47	

Note: Data obtained LSU amplicon sequencing of DNA (left) and cDNA generated from RNA (right) were analyzed separately.



**FIGURE 6** Platypodine and scolytine beetles carry different fungal communities, but scolytine bark and ambrosia beetles are similar. (a) NMDS ordination of fungal communities inferred from metabarcoding sequencing of ITS2 of entire beetles caught while dispersing showing differences between the platypodine *Myoplatypus* and scolytine beetles. Ellipses represent 95% CI around group centroids. (b) Box and whisker plot showing differences in ITS2 OTU richness. Letters indicate significant differences according to Tukey's HSD post hoc test [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Fungal endophytes have functional significance to live plants such as herbivore defence and disease resistance (Carroll, 1988), and it has been suggested that some endophytes persist after a tree dies and influence saprotroph community assembly and wood decay (Cline et al., 2018; Osés, Valenzuela, Freer, Sanfuentes, & Rodriguez, 2008; Parfitt et al., 2010). We found that fungal endophyte communities of loblolly pines had taxonomic richness that was comparable to saprotroph communities of beetle-infested dead wood, but that the two groups shared very few taxa as a result of nearly complete species turnover after tree death. Many of the taxa recovered from both culture and metabarcoding methods of live trees could not be matched to sequences from vouchered specimens available on public databases at even coarse taxonomic levels, though many matched sequences from other culture-independent methods such as cloning/sequencing of environmental DNA and living pine sapwood (Jusino et al., 2015). These findings reiterate the diversity of common, widespread, and undescribed endophytic taxa in living pines that await discovery. Few endophytes persisted as saprotrophs in subsequent samples, and most of those only persisted in the absence of beetle infestation, indicating that beetle-vectored fungal communities supersede pine endophyte communities after tree death. This conclusion is supported by (a) a several-fold decrease in fungal richness observed in exclusion treatments after tree death, (b) the near complete absence of endophyte taxa in beetle-infested trees, (c) and results of variation partitioning analysis that showed relatively weak and/or insignificant predictive power of endophyte community composition

for explaining saprotroph composition after tree death. Furthermore, on day 124 when endophyte composition was a significant predictor of saprotroph composition, most of the variation explained by endophytes was also explained by beetle treatment suggesting that the effect of endophytes was contingent on the absence of beetles. This was because endophyte taxa were mostly observed in dead trees only when beetles were excluded. Endophytes communities may be a more important component of saprotroph communities and decay processes in tree species and geographic locations in which bark and ambrosia beetles are less prevalent and abundant.

Sequencing-based results showed that fungal communities of flight dispersing beetles were similar in composition and richness between scolytine bark and ambrosia beetles, whereas the platypodine *M. flavicornis* carried significantly richer fungal communities that were also significantly different in taxonomic composition. This result suggests that coarse-scale phylogenetic differences among vectors (i.e., Platypodinae vs. Scolytinae) are more important than ecological differences (i.e., bark vs. ambrosia beetles) in determining the diversity and community composition of associated fungi.

The pine-infesting beetles examined in this study were primarily associated with fungi in the Ophiostomatales and Saccharomycetales (Figure 3). Common bark and ambrosia beetles from other tree types are also associated with fungi in these orders, as well as diverse other fungal lineages such as Hypocreales (*Fusarium* and *Geosmithia*), Microascales (*Ceratocystis*, *Graphium*, *Phialophoropsis*, *Meredithiella*, and *Ambrosiella*), and Polyporales

(*Flavodon*) (Harrington, 2005; Hulcr & Stelinski, 2017). Continued work is needed to determine if other beetle-associated fungal lineages have effects on decomposition that are similar to those observed in this study of pine-infesting beetles. This continued effort is particularly important for beetles that have been widely introduced, attained high abundances in their introduced ranges, and vector fungi that are novel to their new environments. For example, several Asian species of the ambrosia beetle genera *Ambrosiodmus* and *Ambrosiophilus* have been introduced to multiple continents where they have thrived. Unlike all other ambrosia beetles known, these beetles farm an ambrosia fungus that is an aggressive wood-decayer which can exclude native fungi, and thus these introduced beetles could potentially offset the effects of native beetles on decay rates (Hulcr, Skelton, Johnson, Li, & Jusino, 2018; Kasson et al., 2016; Skelton, Loyd, et al., 2019). Continued studies of how beetle-associated fungi interact with free-living fungi in decaying wood will help clarify the total impact of bark and ambrosia beetle introductions on ecosystem processes in native forests.

Integrating multiple methodologies and experimental controls in this study highlighted important sampling biases, and also helped to mitigate them. The culture-independent sequencing approach compensated for culturing bias and increase sampling depth by orders of magnitude, while live cultures provided vouchered organisms and longer reference sequences for improved taxonomy of OTU sequences. Cultures also verified that many of the DNA sequences recovered from metabarcoding were from present, viable fungi. But like culturing, metabarcoding methods also impose biases. The results from synthetic and biological mock community samples confirmed that read numbers cannot be used to infer abundance or relative abundance of taxa because of orders of magnitude differences in PCR amplification among taxa, similar to other recent studies using mock communities (Jusino et al., 2019; Palmer et al., 2018). The use of a synthetic mock community allowed us to directly measure and account for index bleed that occurs in metabarcoding studies. This is important because failing to account for even low levels of index bleed can lead to erroneous assignment of OTUs to experimental treatments and artificially inflated estimates of diversity in each sample (Palmer et al., 2018). Additionally, primer choice imposes taxonomic biases by amplifying only some community members and failing to amplify others. While the higher variability in ITS2 resulted in the recovery of more OTUs per sample than LSU, it failed to detect *Raffaelea* species in our biological mock community positive control, and in environmental samples in which they were detected by LSU. This result highlights the need for question-specific positive controls in metabarcoding studies and is congruent with previous reports of difficulty amplifying and sequencing ITS in *Raffaelea* (Dreaden et al., 2014). Because fungi in *Raffaelea* are known to be important fungal mutualists of many genera of ambrosia beetles worldwide (Hulcr & Stelinski, 2017), LSU offers an alternative marker for improved metabarcoding studies of ambrosia beetles and their fungi.

In conclusion, bark and ambrosia beetles have comprehensive effects on the fungal communities in decaying wood. They introduce a

diverse and biologically active consortium of fungi that replaces the comparably diverse endophyte communities present in living woody tissues. The beetle-associated consortium generally lacks fungi capable of decomposing wood, and instead competes with wood decay fungi. This competition slows down early stages of decay, challenging the long-standing paradigm in forest ecology that bark and ambrosia beetles facilitate wood decay. Our combined methodology showed that metabarcoding approaches are valuable for studies of insect-associated microbial communities, but appropriate positive and negative controls must be used, care must be taken in primer choice, and read counts should not be interpreted as measures of relative or absolute abundance of microbial taxa. Future experimental work should examine the effects of ambrosia beetles in tropical hardwood forests where ambrosia beetles are most abundant and diverse, and should determine the effects of other widespread native and introduced beetle-associated fungal lineages to achieve a more complete understanding of the impacts of these beetles and their fungi on forest ecosystems and global carbon dynamics.

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## AUTHOR CONTRIBUTIONS

J.S., M.A.J., P.M.C., K.S., M.T.B., D.L.L., J.M.P., and J.H. designed the research. J.S., M.A.J., P.M.C., K.S., and J.M.P. performed the research. D.L.L., and J.H. contributed reagents/tools. J.S., M.A.J., and J.M.P. analyzed the data. J.S. wrote the paper.

## DATA AVAILABILITY STATEMENT

Raw Ion Torrent sequence data are available in the Short Read Archive of the National Center for Biotechnology Information (accession PRJNA575777). For convenience, sequences and taxonomy from post-processing OTUs are available as electronic supplements. Sanger DNA sequences from representative fungal cultures are available on GenBank (accessions MN384583–MN384665). All other data are available on Dryad (<https://doi.org/10.5061/dryad.sbcc2fr27>).

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### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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