Fungal Ecology 62 (2023) 101210

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Habitat matters: The role of spore bank fungi in early seedling establishment of Florida slash pines

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ARTICLE INFO

Corresponding Editor: Kabir Peay

Keywords:
Ectomycorrhizae (ECM)
Habitat restoration
High throughput sequencing (HTS)
Pine rocklands
Pinus
Rhizopogon
Sandhills
Scrub

ABSTRACT

This study investigated broad patterns in communities of ectomycorrhizal fungi from three Florida habitats (sandhills, scrub, and pine rocklands) and the ability of spore bank fungi to associate with *Pinus elliottii* (slash pine) and *Pinus densa* (south Florida slash pine). Efforts to replant pines in the endangered pine rocklands are vital to the persistence of this habitat, yet little is known about the ectomycorrhizal fungi communities or how they may differ from those in other pine-dominated habitats in Florida. We used high-throughput amplicon sequencing (HTS) to assess baseline fungal communities and greenhouse bioassays to bait ectomycorrhizal fungi using seedlings. HTS soil data recovered 188 ectomycorrhizal species but only a few subsequently colonized the bioassay seedlings. We recovered 21 ectomycorrhizal species on pine seedlings including common spore bank fungi such as *Cenococcum*, *Suillus,* and *Tuber,* but *Rhizopogon* species were dominant across all sites and habitats. Habitat type and site were significant variables influencing the community composition of the total soil fungal community, soil ectomycorrhizal community, and the fungi found on seedling root tips. However, we found no significant differences between the ectomycorrhizal communities on seedling roots from the two *Pinus* species.

1. Introduction

Plants in the family Pinaceae are foundational species in major ecosystems worldwide (Ellison et al., 2005, 2016; Harley et al., 2015) and are also some of the most valuable and commonly grown timber species. All Pinaceae species are obligately associated with ectomycorrhizal fungi (ECM) in a symbiotic relationship where the fungal partner provides water and nutrients in exchange for carbon (Smith and Read, 2008). Mycorrhizal fungi provide nutritional benefits to the plant and confer tolerance against abiotic stressors such as drought, heavy metals, and pathogens (Smith and Read, 2008; Gordon and Gehring 2011; Souza et al., 2012). Numerous studies provide examples of how ECM fungi are essential for the establishment and survival of pine seedlings, whether grown in a natural habitat or in a commercial timber setting (Marx, 1980; Kjøller and Bruns, 2003; Bruns et al., 2009; Nguyen et al., 2012; Glassman et al., 2015, 2016). For example, ECM fungi are so critical for pines that attempts to plant pines commercially outside the native ranges of these trees have been unsuccessful when mycorrhizal inoculum was absent (Richardson et al., 1994).

Timber harvested from several pine species (e.g., *Pinus elliottii*, *P. palustris*, *P. taeda*) is the most important economic agricultural commodity in Florida and a major natural product across the southeastern USA (Alavalapati et al., 2002; Hodges et al., 2019). Slash pine, *Pinus elliottii* Engelm, is one of the major economic timber crops in Florida (Barnett and Sheffield, 2004). *Pinus elliottii* is grown for commercial timber production across a broad geographic range within the USA, from the temperate coastal plain of South Carolina to central Florida and west to Louisiana (Little and Dorman, 1954). This species is also commonly found in natural urban and suburban environments throughout the southeastern USA.

A related species, *P. densa* (south Florida slash pine), grows naturally in the southern half of Florida and is the dominant canopy species in the south Florida pine rocklands (Snyder et al., 1990; Weakley et al., 2015). *Pinus densa* is a foundational species in the pine rocklands because their needles provide fuel for fires which are needed to prevent succession into a closed canopy hardwood hammock (Snyder et al., 1990). The

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https://doi.org/10.1016/j.funeco.2022.101210
Received 10 February 2022; Received in revised form 30 October 2022; Accepted 10 November 2022
Available online 30 November 2022

south Florida pine rocklands, endemic to south Florida, is a critically endangered ecosystem, characterized by a canopy of *P. densa* and a dense understory of temperate and tropical plant species growing on oolitic limestone rock beds characteristic of Florida's karst geology (Possley et al., 2008). Less than 2% of the original rocklands habitat outside of the Everglades National Park (USA) still exists, largely because of habitat fragmentation, urbanization, and encroachment by invasive species (Jones and Koptur, 2017). Previous efforts to restore pine rocklands sites by replanting pines have considered factors such as elevation (Harley et al., 2015), hydroperiod (Serra, 2009), and invasive species removal (Smith et al., 2011), but have not explicitly considered ECM fungi as an important factor for facilitating seedling survival.

Spore bank fungi refers to the available pool of metabolically dormant but viable spores or sclerotia present in the soil (Baar et al., 1999). Ectomycorrhizal spore bank fungi are a critical component in facilitating the survival of young pine seedlings by acting as the primary inoculum source for ECM colonization, particularly when young seedlings are establishing away from adult trees (Baar et al., 1999; Taylor and Bruns, 1999; Frank et al., 2009). Pine trees often preferentially associate with certain ECM fungi, even when other taxa are present or more abundant in the soil spore bank. For example, suilloid fungi (primarily species of *Rhizopogon* and *Suillus*), are not always abundant in the spore bank but are often important components of the fungal community associated with Pinaceae (Baar et al., 1999; Bruns et al., 2002). Species of *Rhizopogon* produce spores with a thick wall that allows them to persist over many years in the soil, sometimes becoming more reactive (i.e., germinating easily and colonizing compatible hosts) as they remain longer in the soil (Bruns et al., 2009; Nguyen et al., 2012). Some species such as *R. olivaceotinctus* have spores that can survive and germinate after heat treatments of up to 65 °C (Izzo et al., 2006; Peay et al., 2009, 2010; Bruns et al., 2019). This trait allows *R. olivaceotinctus* to outcompete other, less heat-tolerant fungi. Some *Suillus* and *Rhizopogon* also exhibit a high degree of host specificity, partnering primarily with one species within Pinaceae (Gardes and Bruns, 1996; Bruns et al., 2002). Based on the critical role of ECM fungi in other pine-associated ecosystems (Policelli et al., 2020), suilloid fungi likely facilitate the establishment and persistence of pine species in both the rocklands ecosystems and commercial slash pine forests in the southeastern USA.

The presence of these fungi in the spore bank are therefore considered essential for the establishment and survival of Pinaceae seedlings and may also help to facilitate restoration efforts. Studies from other endangered Pinaceae forests have uncovered unique fungi that are endemic to these habitats (Mujic et al., 2014; Murata et al., 2017; Wen et al., 2018). The identification and preservation of the fungal community in these habitats is therefore an important component to restoration efforts. This is particularly true for restoration efforts targeting the endangered pine rocklands of south Florida, where certain key fungi may be instrumental in the survival of these pines. While a few studies have examined spore banks in the southeastern USA (Bonito et al., 2012; Glassman et al., 2015), none have focused on spore banks in naturally occurring tropical and subtropical pine forests.

Given that the pine rocklands are characterized by shallow soil layers and grow entirely on rocky limestone substrates, there are few areas for seedlings to establish near parent pines compared to other habitat types. Therefore, seedlings in this habitat likely rely more on spore bank fungi or "early-stage" fungi (versus the "late-stage fungi" that colonize the roots of mature trees and may also colonize nearby seedlings during establishment). Despite the critical importance of ECM fungi in the spore bank for the establishment of young pines, almost nothing is known about pine-associated ECM fungi in Florida spore banks. It is possible that spore banks are highly variable across sites and that endangered pine rocklands have unique fungi as compared to other pine-dominated sites across Florida.

This study aims to elucidate the ECM fungal communities present in soils across a gradient of Florida pine habitats. We used high-throughput amplicon sequencing (HTS) to assess the total pool of ECM fungi in the soil followed by greenhouse seedling bioassays to bait for compatible ECM fungi present in the spore bank. We sampled 23 plots from 7 sites across three different habitats in Florida (sandhills, scrub, and pine rocklands). Both *Pinus elliottii* and *P. densa* were planted on soil from each plot and habitat type to assess potential host-specificity and variation in fungal communities across sampling sites. Based on previous studies from temperate pine forests, we predicted that *Rhizopogon* and *Suillus* species would be more dominant in the seedling fungal communities and less frequently detected in the HTS because suilloid fungi are often competitively displaced and replaced by late-successional fungi in the soil and would therefore be less abundant compared to other actively growing fungi as mycelium (Taylor and Bruns, 1999; Policelli et al., 2019). We predicted that seedlings planted in soils collected in northern sites, where at least four species of pine are present and the basal area of Pinaceae is greater, would harbor greater ECM diversity and richness. We also expected that, under the same soil treatments, similar ECM fungal communities would be recovered on root tips of both pine species since the distribution of these pine species are likely a result of environmental pressures and not host-specificity or ECM fungi inoculum availability.

2. MATERIALS and METHODS

2.1. Site characteristics

The northernmost habitat was sandhills, an environment that consists of a wiregrass understory and includes *Pinus elliottii* as well as other ECM pine species (predominantly *P. palustris* - longleaf pine) and ECM species of *Quercus* (Fig. 1A). The central habitat was a Florida scrub habitat along the Lake Wales Ridge that contains ECM hosts such as *P. densa,* and *Quercus*, and non-ECM trees such as saw palmettos (*Serenoa repens*) (Fig. 1B). The southernmost habitat was the pine rocklands habitat which contains *P. densa* and a diverse understory of tropical and temperate non-ECM plant species (Fig. 1C). The National Key Deer Refuge, which is located on Big Pine Key and therefore geographically separated from the rocklands sites on the mainland (Fig. 1E), was the southernmost of the rocklands sites that was sampled (Fig. 1D).

2.2. Soil collection

We sampled a total of 23 plots across Florida, USA, from seven different sites in three habitat types (Table 1; Fig. 1). Each plot was >30 m apart from any other plot. Soils were collected from Ordway-Swisher Biological Station (ORD) (sandhills, 5 plots), Archbold Biological Research Station (ARC) (scrub, 6 plots), University of Florida Tropical Research and Education Center (TREC) (pine rocklands, 2 plots), Larry and Penny Thompson Memorial Park (LPT) (pine rocklands, 3 plots), Navy Wells Park (NWP) (pine rocklands, 3 plots), National Key Deer Refuge (BPK) (pine rocklands, 3 plots), and a private rocklands site (PAB) (pine rocklands, 1 plot).

At each sampling plot we set three 50 m transects starting from a central point and going in three cardinal directions. For each transect, 10 cm soil cores were taken every 10 m (5 cores/transect) and all three transect cores were pooled to represent one plot (15 soil cores total per plot). Soil from each plot was homogenized and passed through a 2 mm sieve that was cleaned with 10% bleach and autoclaved between plots. A small subsample of fresh soil was removed for DNA extraction to characterize the soil fungal communities using HTS (see below). Soil was subsequently dried in paper bags for 2–3 weeks inside a fume hood with constant airflow until there was no detectable moisture. A subsample of the dried soil from each plot (250 g) was sent to the University of Florida/IFAS Analytical Services Laboratory (Gainesville, FL, USA) and analyzed for soil pH, Calcium (Ca), Phosphorus (P), Potassium (K), Magnesium (Mg), total percent organic matter and total percent nitrogen (Supplemental Table 1).

Andrews Nursery (Chiefland, FL, USA) donated *Pinus elliottii* and

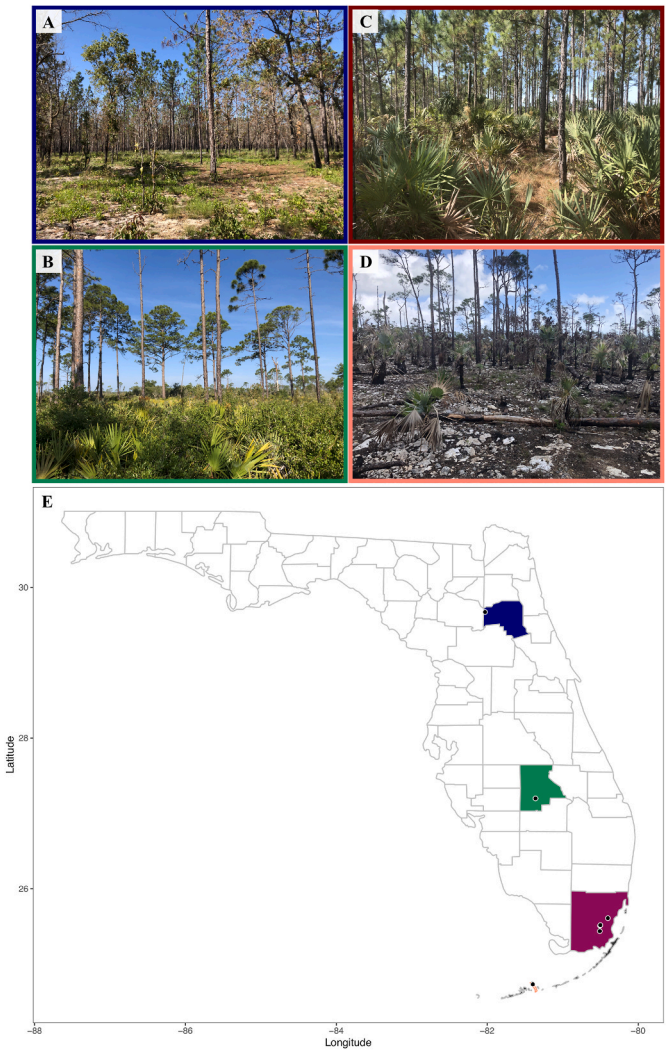

Fig. 1. Map of field site locations representing three different habitat types. Grey points represent the seven sites sampled in this experiment. Habitat types are **(A)** Sandhills, **(B)** Scrub, **(C)** Rocklands-Mainland, **(D)** Rocklands-Island. **(E)** Map of field sites according to each habitat type.

Pinus densa seeds for this study. Seeds were surface sterilized by submerging them in 10% hydrogen peroxide and a drop of Tween® 20 for 15 min. Seeds were then rinsed and soaked overnight on a shaker with autoclaved DI water. The dried soil was mixed in a 2:3 perlite:soil ratio and an aliquot (100 g) was placed in 50 mL cone-tainers that were cleaned in 10% bleach (Stuewe & Sons Inc., Tangent, Oregon, USA). Five replicates of each pine species were planted in soil from all 23 sites. Negative controls consisted of ten replicates of each pine species planted on twice-autoclaved commercial topsoil mixed with perlite. Initially, three seeds were planted in each cone-tainer, and after one month the number of seedlings per cone-tainer was reduced to one, favoring the seedling closest to the center of the cone-tainer. This experiment used a total of 230 treatment seedlings and 20 negative controls. Seedlings were grown inside a greenhouse at the University of Florida in Gainesville, FL, USA from August 2019 to March 2020 with ambient light and temperature (average 27 °C). Plants were watered twice a week for 7 months and fertilized once a month with 1:10 Hoagland's solution (Sigma Aldrich, St. Louis, Missouri, USA) to prevent nutrient deficiencies.

Table 1
Site names, locations, habitat types, and predominant host pine species (*Pinus elliottii* or *Pinus densa*) variety along with additional *Pinus* species found at each of the sampling sites.

Site	Location	Habitat Type	Predominant Slash Pine Variety	Other *Pinus* spp. present
Ordway Swisher Biological Station	Melrose, FL	Sandhills	*P.P. elliottii*	*P. clausa, P. taeda, P. palustris*
Archbold Biological Research Station	Venus, FL	Scrub	*densa*	*P. clausa*
UF Tropical Research and Education Center (TREC)	Homestead, FL	Pine Rocklands (Mainland)	*P. densa*	n/a
Larry and Penny Thompson Memorial Park	Miami, FL	Pine Rocklands (Mainland)	*P. densa*	n/a
Navy Wells Pineland Preserve	Homestead, FL	Pine Rocklands (Mainland)	*P. densa*	n/a
Private Pine Rockland	Homestead, FL	Pine Rocklands (Mainland)	*P. densa*	n/a
National Key Deer Refuge	Big Pine Key, FL	Pine Rocklands (Island)	*P. densa*	n/a

2.3. Root extraction

Pine seedlings were harvested after seven months by cutting the stem at the soil line and rinsing the roots in DI water. Under a dissecting microscope two representatives of each unique ECM morphotype were selected per root system based on color, shape, and branching patterns (Agerer, 1995) and wiped dry with autoclaved paper towels to remove excess water. Intact, fresh ECM-colonized root tips were crushed using autoclaved toothpicks in PCR tubes containing 25 μL of extraction buffer from an Extract-N-Amp PCR kit (Sigma Aldrich, St. Louis, Missouri, USA). Crushed root tips were incubated at 95 °C for 10 min and then 25 μL of dilution solution was added and mixed. PCR amplification of the fungal internal transcribed spacer (ITS) was performed with ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) primers using the following thermocycler conditions: initial denaturation 94 °C for 3 min, 34 cycles of denaturation 94 °C for 1 min, annealing 53 °C 1 min, extension 72 °C 2 min and a final extension of 72 °C for 7 min. To allow for placement of *Rhizopogon* species in a phylogenetic context, the 28S large subunit nuclear ribosomal DNA (LSU) was also amplified from representative ECM roots colonized by *Rhizopogon* species using PCR primer pair LROR and LR5 (Vilgalys and Hester, 1990). Thermocycler conditions were as follows: initial denaturation 95 °C for 2 min, 34 cycles of denaturation 95 °C for 30 s, annealing 55 °C for 45 s, extension 72 °C for 1:30 min and a final extension of 72 °C for 10 min.

PCR products were visualized on a 1.5% agarose gel using SYBR Green (Invitrogen, Carlsbad, CA, USA). Amplicons were sequenced at Eurofins Genomics (Louisville, KY, USA). Sequence quality was assessed and trimmed using Geneious version 2020.1.2 (Biomatters Ltd., Auckland, New Zealand). Operational taxonomic units (OTUs) were determined by clustering ITS rDNA sequences at 97% pairwise similarity using Sequencher version 5.1 (Smith et al., 2007). OTUs were assigned to guilds based on FUNGuild (Nguyen et al., 2016) and tentative taxonomic placements were assigned based on highest informative matches from NCBI BLAST (Table 1). Non-ECM OTUs were excluded from all downstream analyses.

2.4. Soil DNA extraction

To assess the total pool of available fungi in the soil, we used high-throughput amplicon sequencing (HTS). Total soil DNA was extracted from homogenized, composite samples from each of the 23 plots. As detailed above, each composite sample consisted of fifteen homogenized soil cores (3 transects x 5 sampling points/transect). Prior to drying, 250 mg of fresh soil was removed for DNA extraction using the Qiagen PowerSoil DNA kit according to the manufacturer's protocol (Qiagen, Germantown, Maryland, USA). Fungal DNA was amplified using the primer pair ITS1F (Gardes and Bruns, 1993) and ITS2 (White et al., 1990) modified with Illumina Nextera v2 adapters (Illumina, San Diego, CA, USA). PCR was performed in 20 μL reactions containing 4.0 μl 5x GoTaq buffer (Promega Corporation, Madison, WI, USA), 0.4 μl of 10 mM dNTPs (Promega Corporation, Madison, WI, USA), 0.4 μl of each 10 μM primer, 0.16 μl of 20 mg/ml BSA (New England Biolabs, Ipswich, MA, USA), 0.1 μl GoTaq polymerase (Promega, Madison, WI, USA), and 4.0 μl gDNA. The following PCR conditions were used: initial denaturation 95 °C for 1 min, 34 cycles of denaturation 95 °C 30 s, annealing 52 °C, 45 s, extension 72 °C 1 min and a final extension of 72 °C for 7 min. PCR products were visualized on a 1.5% agarose gel with SYBR green before indexing. Amplicons were dual-indexed using Illumina Nextera v2 indices and adapters (Illumina, San Diego, CA, USA) by an eight-cycle PCR reaction. Indexed products were cleaned with Zymo Select-A-Size Clean & Concentrator kits (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Two positive mock community controls were used, a biological mock community composed of seven pine-associated ECM fungi (*Aureoboletus pseudoauriporus, Amanita flavoconia, Lactarius* sp., *Rhizopogon pseudoroseolus, Russula violeipes, Suillus salmonicolor,* and *Xerocomus* sp.) and a non-biological, single-copy, equimolar mock community (SynMock; Palmer et al., 2018). The mock communities were amplified and indexed alongside our samples. Negative PCR and extraction controls were also included. DNA concentration and quality of cleaned products were measured using a Qubit 4.0 Fluorometer (ThermoFisher Scientific, Madison, WI, USA) with the HS-DS-DNA kit (Invitrogen, Carlsbad, CA, USA). Indexed libraries were then equilibrated and pooled. Samples were sequenced on Illumina MiSeq (Illumina, San Diego, CA, USA) using 2x300 paired end v3 chemistry at the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida (Gainesville, FL, USA).

2.5. Bioinformatics

The HTS data were processed using the AMPtk pipeline (version 1.4.2; amptk.readthedocs.io; Palmer et al., 2018). Briefly, ITS1 barcode reads were merged using USEARCH (version 9.2.64) before removing the forward and reverse ITS primers. Reads less than 125 bp were discarded, reads longer than 300 bp were trimmed. Because ITS amplicons are variable in length, reads between 125 and 300 bp were padded with N's to mitigate clustering problems due to length differences (Palmer et al., 2018). To generate operational taxonomic units (OTUs), sequence reads were quality filtered and clustered at 97% similarity and chimeric sequences were removed using UPARSE (Edgar, 2013). All singletons were removed. We used a synthetic mock community, SynMock (Palmer et al., 2018) to determine the rate of index bleed using the filter module in AMPtk. Taxonomic placement of OTUs was assigned using the hybrid taxonomy assignment in AMPtk; this conservative taxonomy assignment method calculates a consensus least common ancestor assignment based off of the results of a reference database and multiple taxonomy classifiers. FUNGuild (version 1.1) was used to assign guilds (Nguyen et al., 2016). FUNGuild generates a confidence ranking for each guild assignment. We performed additional manual NCBI BLAST searches on OTUs with "possible" and "probable" guild assignments to confirm whether the sequences were classified as ECM or non-ECM based on the ECM lineages identified by Tedersoo and Smith (2013).

2.6. Fungal community analyses

All non-fungal reads were removed prior to community analysis. Fungal community analyses were performed using the 'vegan' package (version 2.5–6; Oksanen et al., 2019) of R (version 3.4.2; R Core Team, 2019). To visualize soil fungal community composition based on habitat, we first converted our soil matrix to presence/absence and used the betadiver function to calculate the β-sim dissimilarity matrix (Koleff et al., 2003). Fungal community composition was visualized using non-metric multidimensional scaling (NMDS) with the metaMDS function. To test whether differences in the fungal community composition were attributed to habitat type or site, we used a nonparametic permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) with the "adonis" function and the "betadisper" function was used to test for differences in multivariate dispersion (Anderson, 2006). The same tests were performed on the β-sim dissimilarity matrix calculated from the presence/absence matrix of ECM fungi both in the soil and on seedling root tips to determine if habitat, host species, and site had a significant effect on the ECM fungal community.

2.7. Phylogenetic analysis

Sequences derived from ECM root tips, specimens from the fungarium at the Florida Museum of Natural History (FLAS-F), and sequences from Grubisha et al. (2001) and Grubisha et al. (2002) were included in this analysis. Sequences were aligned with MAFFT version 7.450 and gap regions were excluded when present in less than 50% of sequences (Katoh et al., 2002; Katoh and Standley, 2013). Maximum likelihood (ML) phylogenies based on the ITS + LSU dataset were constructed using RAxML version 8.2.10 (Stamatakis, 2014) under the GTRGAMMA + I evolutionary model. Branch support values were estimated using 1000 bootstrap replicates. All new sequence data were deposited in NCBI (Supplemental Table 3).

3. Results

3.1. Fungal communities from soil

After quality filtering, we generated an average of 66,448 reads per sample from the soil samples (with a range of 49,333 to 82,385 reads and 56 to 225 OTUs per sample). Our synthetic mock community generated 62,757 reads and our biological mock community generated 40,343 reads. The total soil fungal community composition was significantly different between habitat types (adonis $R^2 = 0.43$, *pseudo*-F = 5.1329, $p < 0.001$) and the multivariate dispersion was not significantly different between habitat types (betadisper $p = 0.06$). The total soil fungal community composition was significantly different between sites (adonis $R^2 = 0.53$, *pseudo*-F = 3.2162, $p < 0.001$) and so was the multivariate dispersion (betadisper F = 12.35, $p < 0.001$; Fig. 2A). Overall, HTS of the soil generated 1332 fungal OTUs and 188 OTUs were determined to be ECM fungi. These 188 ECM OTUs were selected and used in separate analyses. The ECM fungi communities from soil were also significantly different between habitat types (adonis $R^2 = 0.41$, *pseudo*-F = 4.28, $p = 0.001$) but multivariate dispersion was not significantly different among habitat types (betadisper $p = 0.419$). Similarly, the ECM fungi communities from the soils were significantly different between sites (adonis $R^2 = 0.57$, *pseudo*-F = 3.32, $p = 0.001$) but multivariate dispersion was not significantly different (betadisper $p = 0.384$) (Fig. 2B). Average fungal richness for the ECM fungi communities from the soils showed that the average fungal richness per site was highest for ORD (sandhills) with 39.4 fungi and lowest for BPK (island-rocklands) with 4 (Supplemental Table 2). From the mainland-rocklands sites LPT had the highest average fungal richness, 12.33, followed by the private rocklands with 8 fungi, NWP with 7 fungi, and TREC with 5 fungi. The ARC site (scrub) had an average of 9.6 fungi. Accumulation curves of soil fungal OTUs reached plateaus, suggesting that sampling

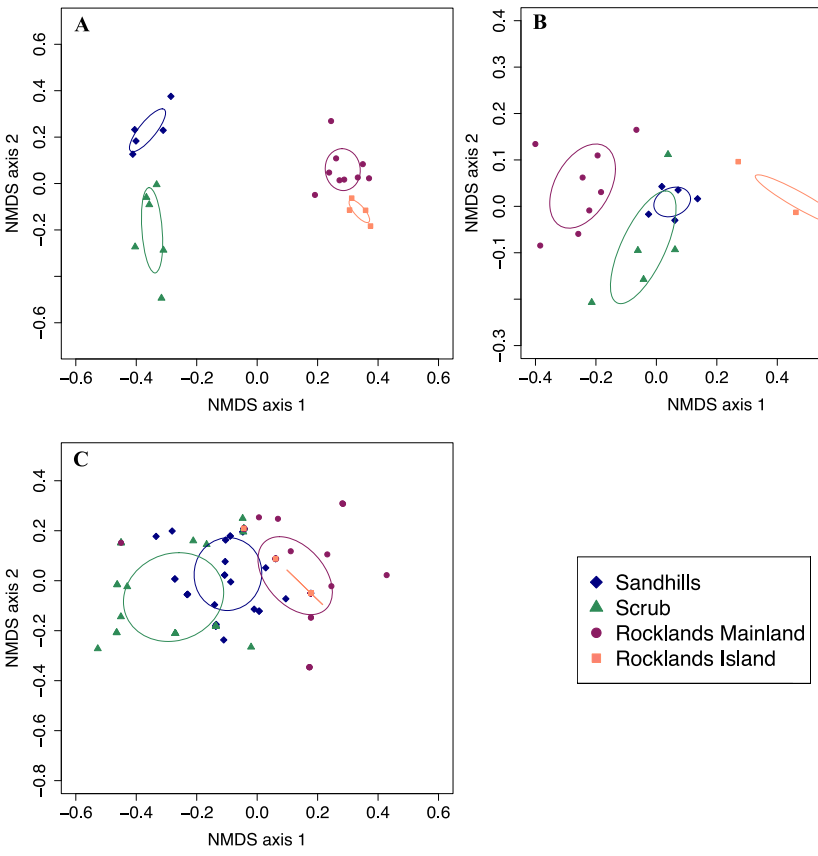

Fig. 2. (A,B) NMDS ordination of the fungal communities from Illumina sequencing of ITS1 from soils collected in pine-dominated scrub, sandhills and rocklands sites across Florida. Each point represents a different plot (**A**) Soil fungal community (stress = 0.127) (**B**) Soil ectomycorrhizal fungal community (stress = 0.056). Color coding by habitat type reveals a strong influence of habitat and geography. (**C**) NMDS ordination of the ectomycorrhizal fungal community from root tips of *Pinus elliottii* and *Pinus densa* seedlings in the greenhouse bioassay experiment (stress = 0.033). Each point represents a different seedling. Note that ectomycorrhizal fungal communities from Rocklands-Island habitat type sites in Big Pine Key (an island in the Florida Keys) are color-coded in salmon because they are unique from all other rocklands sites. Each NMDS is based on two dimensions; both are displayed.

was sufficient to accurately characterize the soil fungal communities (Supplemental Fig. 1A).

3.2. Ectomycorrhizal root tips from seedlings

A total of 203 of 230 treatment seedlings survived the experiment. One of the control seedlings was colonized with a *Suillus* species that was not found on any other seedling in the experiment; all other control seedlings remained uncolonized by ECM fungi. We obtained sequence data of ECM fungi from a total of 139 treatment seedlings (out of 203 surviving treatment seedlings). From these sequences, 21 OTUs were generated and used for downstream analyses (Table 2). The ECM fungal communities from seedling root tips were not significantly different between tree varieties (adonis $p = 0.585$) nor was the multivariate dispersion (betadisper $p = 0.662$). The ECM fungal communities were significantly different between habitat types (adonis $R^2 = 0.31$, *pseudo*-F = 20.341, $p < 0.001$) and the multivariate dispersion was also significantly different (betadisper F = 6.159; $p < 0.001$). The ECM communities were also significantly different between sites (adonis $R^2 = 0.37$, *pseudo*-F = 13.093, $p < 0.001$) and so was the multivariate dispersion (betadisper F = 5.2919; $p < 0.001$; Fig. 2C). Of the 188 OTUs that were detected in the soil using HTS (see above), we identified 15 of these OTUs that were also detected on the ECM roots of seedlings (Fig. 3A). However, six of the OTUs recovered from seedling ECM root tips did not match any OTUs in the soil dataset; these included *Cenococcum geophilum* 2, *Pisolithus tinctorius*, *Rhizopogon truncatus*, *Suillus* aff. *pseudogranulatus*, *Tomentellopsis* sp., and *Tuber* sp. (Fig. 3B). Most of our seedlings were colonized by a small number of dominant taxa with several rare taxa that were less frequent and abundant on the seedlings. The overall average fungal richness per seedling was highest for the private rocklands site, with 2.5 fungi (PAB), followed by sandhills with an average of 2.183 fungi (ORD). Seedlings from the island-rocklands site had the lowest average fungal richness per seedling with 0.79 fungi (BPK). The average fungal richness for the scrub site was 1.09 fungi (ARC), the other mainland rocklands sites had 1.63 fungi (LPT), 1.66 fungi (NWP), and1.08 fungi (TREC) per seedling (Supplemental Table 2). Accumulation curves of the seedling root tips showed that most sites did not reach a plateau and more sampling of these seedlings would likely yield additional rare ECM taxa (Supplemental Fig. 1B).

3.3. Phylogenetic analysis

Rhizopogon was the dominant genus recovered on ECM root tips from seedlings so a phylogenetic analysis of the concatenated ITS-LSU rDNA was constructed to place the Florida pine-associated taxa into a broader context within the genus. ITS-LSU data identified 3 different *Rhizopogon* OTUs that were resolved into 3 major clades that correspond to subgenera *Rhizopogon* (*R. fuscorubens* and *R. truncatus*) and *Roseoli* (*R. pseudoroseolus*) (Supplemental Fig. 2). *Rhizopogon truncatus* was restricted to the scrub habitat and was found on 12 seedlings. *Rhizopogon fuscorubens* and *Rhizopogon pseudoroseolus* were found from several plots in the sandhills, scrub, and rocklands sites.

4. Discussion

Our results demonstrate that habitat type is an important variable that influences the total soil fungal community as well as the community composition of ECM fungi on the roots of *Pinus* seedlings. Overall ECM lineage diversity and richness was highest in the sandhills habitat compared to the scrub and pine rocklands habitat. While habitat type was a significant predictor in fungal community composition, some of the most common fungi on seedlings (e.g., *Rhizopogon pseudoroseolus*, and *Cenococcum geophilum*) were present in all three habitats (sandhills, scrub, and pine rocklands; Fig. 3A). This suggests that while these habitats differ in terms of their soil properties and plant communities, the most common ECM fungi that associate with seedlings in disturbed

Table 2
Phylogenetic placement based on BLAST analysis of ectomycorrhizal fungi detected on the roots of bioassay seedlings based on ITS rDNA sequences. Bold accession numbers indicate sequences generated in this study. The top informative BLAST hit is the highest BLAST hit for which taxonomic information was available in NCBI GenBank.

Contig #	Top Informative Blast Hit	% Identity	Assigned ID	Accession #
EKA514	cf. *Amphinema* sp.	98	*Amphinema* sp.	**OL348349**
EKA335	*Cenococcum geophilum*	100	*Cenococcum geophilum* 1	**OL348336**
EKA487	*Cenococcum geophilum*	99	*Cenococcum geophilum* 2	**OL348345**
EKA473	*Inocybe jacobi*	94	*Inocybe jacobi*	**OL348343**
EKA585	*Laccaria* sp.	99	*Laccaria* sp.	**OL348355**
EKA445	Pezizaceae sp.	97	*Delastria* sp. 1	**OL348342**
EKA509	Pezizaceae sp.	95	*Delastria* sp. 2	**OL348347**
EKA360	*Pisolithus tinctorius*	99	*Pisolithus tinctorius*	**OL348340**
EKA538	*Pulvinula* sp.	96	*Pulvinula* sp.	**OL348350**
EKA340	*Rhizopogon truncatus*	92	*Rhizopogon* cf. *truncatus*	**OL352705**
EKA366	*Rhizopogon fuscorubens*	98	*Rhizopogon fuscorubrens*	**OL348341**
EKA358	*Rhizopogon psuedoroseolus*	98	*Rhizopogon psuedoroseolus* 1	**OL348335**
EKA511	*Russula* sp.	100	*Russula* sp.	**OL348348**
EKA545[a]	*Suillus* aff. *psuedogranulatus*	100	*Suillus* aff. *psuedogranulatus*	**OL348351**
EKA357	*Suillus salmonicolor*	98	*Suillus salmonicolor*	**OL348339**
EKA342	*Thelephora terrestris*	98	*Thelephora terrestris*	**OL348338**
EKA583	Thelephoraceae sp.	97	Thelephoraceae sp. 1	**OL348354**
EKA338	*Tomentella* sp.	93	*Tomentella* sp.1	**OL348337**
EKA506	*Tomentella sublilacina*	97	*Tomentella* sp. 2	**OL348346**
EKA565	*Tomentellopsis echinospora*	99	*Tomentellopsis*	**OL348352**
EKA483	*Tuber oligospermum*	91	*Tuber* sp.	**OL348344**
EKA580	*Wilcoxina mikolae*	99	*Wilcoxina mikolae*	**OL348353**

[a] This OTU was only detected in a single negative control seedling and was not found in any experimental seedlings.

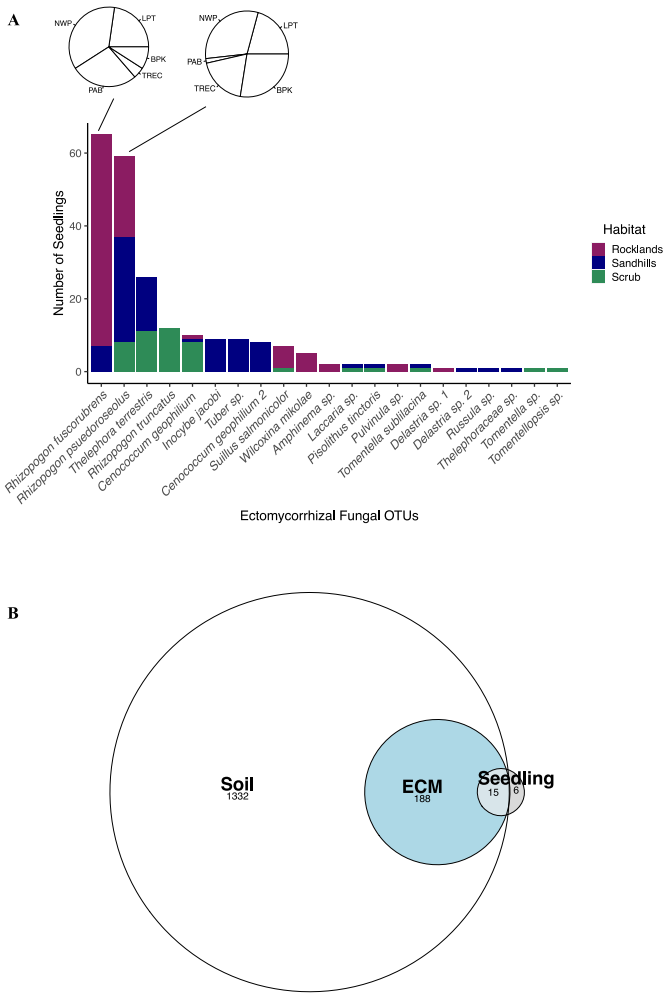

Fig. 3. **(A)** Frequency of ectomycorrhizal OTUs recovered from different habitats on the ectomycorrhizal root tips of *Pinus* bioassay seedlings. Occurrences from seedlings are color coded according to the three different major habitats (sandhills, scrub, and rocklands). Pie charts above *Rhizopogon pseudoroseolus* and *R. fuscorubrens* correspond to the abundance on ectomycorrhizal root tips from the different pine rocklands sites Big Pine Key (BPK), Larry and Penny Thompson (LPT), Navy Wells Preserve (NWP), Tropical Research and Education Center (TREC), and a Private Rocklands (PAB). **(B)** Euler plot showing overlap of OTUs from entire soil fungi community (large white circle), ectomycorrhizal fungi community from soil (medium blue circle) and ectomycorrhizal fungi from bioassay seedlings (small grey circle).

soils are similar across the different habitats and across sites. A similar pattern was documented by Miyamoto and Nara (2016) who found that *Cenococcum geophilum* and suilloid fungi dominated the spore bank communities along an elevation gradient ranging from 850 m to 1850 m in Mt. Ishizuchi, Japan.

Based on previous research, our initial prediction was that suilloid fungi in the genera *Rhizopogon* and *Suillus* would be important "spore bank fungi" and would therefore dominate the ECM fungal communities on seedlings (Baar et al., 1999; Peay et al., 2009; Glassman et al., 2015, 2016; Murata et al., 2017). Several studies have shown that the potential of some suilloid fungi to colonize roots and form ECM associations often increases over time (Bruns et al., 2009; Nguyen et al., 2012) and that some species can survive heating and other stresses (Izzo et al., 2006; Peay et al., 2009; Glassman et al., 2016). While *Rhizopogon* species were common in our dataset, *Suillus* species were rare (Table 2, Fig. 3A). Another study found that sometimes *Suillus* species can be rare in surveys of ECM root tips, even in cases where their above ground fruiting bodies are abundant (Gardes and Bruns 1996). *Suillus* species are generally better than *Rhizopogon* species at long distance dispersal because their spores are spread by both wind and mycophagy (Peay et al., 2012). However, the higher prevalence of *Rhizopogon* taxa in our study may be because *Rhizopogon* species have more durable and reactive spores that allow them to better survive drying and outcompete other ECM fungi (Kennedy et al., 2007, 2011; Bruns et al., 2009; Nguyen et al., 2012). In this study, we homogenized the soil cores prior to the seedling bioassays, which may have favored dominant ECM competitors that rapidly colonize via spores, such as *Rhizopogon* spp. (Kennedy et al., 2009). If we diluted the soils further with other sterile substrates, we may have detected more rare fungi that otherwise were competitively displaced in the bioassay experiments (Taylor and Bruns, 1999; Rusca et al., 2006; Nguyen et al., 2012). Alternatively, since *Rhizopogon* species tend to have thicker spore walls to survive the passage through animal guts (Colgan and Claridge 2002), it is possible that *Rhizopogon* species are better survivors than *Suillus* species in Florida's tropical to subtropical soil environments (Ashkannejhad and Horton, 2006).

Although *Rhizopogon* species were clearly dominant in the ECM fungal spore bank, we also documented other ECM fungi known to have resistant and reactive spores or sclerotia. The community of spore bank fungi was comprised of ECM fungi from 13 lineages, including 5 OTUs from Thelephoraceae and 5 OTUs of Pezizales (*Delastria*, *Pulvinula*, *Tuber,* and *Wilcoxina* species). We also documented two distinct OTUs of *Cenococcum*, a genus that is known to form tough sclerotia with strong survival capabilities under a range of challenging environmental conditions (Obase et al., 2017). Other studies have found variable overlap between the seedlings and the ECM fungi in the soil depending on the ecosystem and the methods that have been used (Taylor and Bruns 1999; Glassman et al., 2015). Our study found that the majority of ECM OTUs

(15 out of 21) found on seedlings were also found in the soil dataset. Six of the ECM seedling OTUs not found in the soil dataset (*C. geophilum 2*, *Pisolithus tinctorius*, *Rhizopogon truncatus*, *Suillus* aff. *pseudogranulatus*, *Tomentellopsis* sp., and *Tuber* sp.) are likely rare in the soil but are nonetheless capable of surviving and colonizing seedlings. Four of these fungi were only found in one of the three habitat types. For example, *R. truncatus* and *Tomentellopsis* sp. were only found on seedlings grown in soil from one site at Archbold Biological Station whereas *C. geophilum* 2 and *Tuber* sp. were only found in seedlings grown in soil from Ordway-Swisher Biological Station. The species accumulation curves suggest that with more sampling, more of these rare taxa would be found on seedling root tips (Supplemental Fig. 1A).

We did not find evidence for an endemic and highly specific ECM community among the early successional fungi on pine seedlings because similar early successional fungi (e.g., *Rhizopogon* spp., *Cenococcum geophilum*) were found across the different habitats and study sites. There were differences in the ECM lineage diversity and fungal richness in the seedling ECM communities with the sandhills harboring the most ECM lineage diversity (9 lineages) and richness compared to the rocklands (6 lineages) and scrub (5 lineages) (Fig. 3A, Supplemental Table 2). These results are consistent with our prediction that compared to the monodominant pine rocklands, the northern sandhills habitat which contains many more ECM hosts (more pine species and nearby oak trees) might contribute to the higher diversity and richness found on seedlings (https://ordway-swisher.ufl.edu/PlantCommunities.aspx) (Supplemental Table 2). While we found unique fungal communities in the soil HTS, most of the dominant taxa on the seedlings were the same across habitats and sites. Those fungi that were only found on rocklands seedlings (i.e., *Amphinema* sp., *Delastria* sp., *Pulvinula* sp., and *Wilcoxina mikolae* – Fig. 3, Table 2) show high sequence homology to fungi that have been found at other sites across the USA. This suggests that the ECM spore bank fungi communities of the pine rocklands were not comprised of unique, endemic taxa. This is different from some other Pinaceae-dominated systems, such as endemic *Pseudotsuga japonica* from China and Japan, which have been shown to host unique taxa that cannot be found from other sites or Pinaceae hosts (Mujic et al., 2014).

Additionally, our results showed that ECM host specificity (colonization of *Pinus elliottii* versus *Pinus densa*) was not a significant variable in determining seedling ECM communities. This is consistent with previous studies where some ECM fungi show host specificity for particular genera of Pinaceae (e.g., some *Rhizopogon* species only occur on *Pseudotsuga* and not on *Pinus* hosts) but rarely exhibit specificity for particular species within a genus (Bruns et al., 2002; Grubisha et al., 2002; Glassman et al., 2015; Ning et al., 2019) (but see Liao et al., 2016). Furthermore, the two species of slash pine (*P. elliottii* and *P. densa*) co-occur in central Florida (Little and Dorman, 1954; Critchfield and Little, 1966), so there is no geographical barrier that might limit the exchange of ECM fungi between the two host species.

In our study, differences in the ECM community between habitats may be attributed to several other variables that we did not measure. These factors include (but are not limited to) differences in the number of different pine host species as well as differences in the basal area of ectomycorrhizal host trees in the different habitats. Differences in fungal richness could also be influenced by tree host age or stand age which might influence the community composition and favor more late-successional taxa (Obase et al., 2009; Kennedy et al., 2018). On the other hand, abiotic factors such as differences in soil types among the habitats, and the subsequent water and nutrient availability to plant hosts, may also drive differences in community composition (Sun et al., 2016). The pine rocklands have notably higher soil pH, and saltier and shallower soils (Supplemental Table 1) whereas both the sandhills and scrub are sandier, well-draining habitats. Temperature differences among the habitat types could also be important factors in the fungal community differences we observed. For example, the sandhills in northern Florida experience winter low temperatures of −6.7 to −3.9 °C whereas the pine rocklands sites experience winter lows of 4.4–7.2 °C and never experience frost or freezing (https://planthardiness.ars.usda.gov/). The soil ECM community in the pine rocklands habitat on Big Pine Key was different from the ones in mainland pine rocklands sites and seedlings grown in these soils had the lowest fungal richness (Supplemental Table 2). This is consistent with the literature showing that isolation by distance plays a role in fungal community composition due to dispersal limitation (Peay et al., 2010). Additionally, salt intrusion from sea level rise and storm activity is already starting to shift the native plant communities in Big Pine Key rocklands towards more salt tolerant plant communities compared to mainland sites (Maschinski et al., 2011). Our data also suggest that similar patterns could also be possible for soil microbial communities.

The pine rocklands are an abiotically stressful environment due to periodic flooding and fire as well as shallow soil layers, high temperatures, salt intrusion, and highly alkaline soils that limit the absorption and availability of nutrients (Snyder et al., 1990). Soil microbial communities are important in mitigating these abiotic stressors (Scharnagl et al., 2018) but few studies have assessed the soil microbial community in the pine rocklands (Kiesewetter and Afkhami, 2021) and no studies have specifically characterized ECM communities. Because ECM fungi reduce plant stress in other harsh environments such as mining sites (Souza et al., 2012), saline environments (Bandou et al., 2006), and serpentine soils (Panaccione et al., 2001), their role in the pine rocklands is likely imperative. Soil factors, particularly pH, are critical drivers of bacterial, fungal, and plant community assemblages (Possley et al., 2008; Ge et al., 2017; Glassman et al., 2017; Barnett et al., 2020). Elevation also plays a critical role in the establishment and survival of young pines in this low-lying habitat (Serra, 2009; Harley et al., 2015), but previous studies have not incorporated mycorrhizae as soil amendments or explored plant-mycorrhizal interactions. Efforts to restore pine rocklands that were previously cleared for agriculture and subsequently invaded by Brazilian pepper (*Schinus terebinthifolia*) have been largely unsuccessful due to high seedling mortality (O'Hare and Dalrymple, 2006; Serra, 2009; Smith et al., 2011). Therefore, a better understanding of the fungi in this habitat, particularly those associated with young pine seedlings, will help inform future restoration efforts that aim to incorporate root-associated mutualistic symbionts.

Furthermore, evidence from other systems show that *Rhizopogon* are important in primary succession (Ashkannejhad and Horton, 2006) and are often competitively displaced by late successional taxa on mature pine roots (Peay et al., 2011). Some of these late successional taxa (such as *Amanita* and *Boletus* species) were detected in our soil samples but not from seedling roots, suggesting these fungi might be important components of the mature pine ECM community in pine rocklands. Future investigations are needed to characterize the ECM fungal communities in mature pine rocklands sites to see whether the early successional taxa detected in our study remain an important part of the community on mature pines, are replaced by generalist ECM fungi from across Florida, or constitute an endemic community of fungi that is specific to these endangered pine rocklands.

Our results have important implications for the preservation and restoration of native soil communities in the pine rocklands. The prevalence of *Cenococcum*, *Tuber* and suilloid fungi found in the spore banks provide promising potential for incorporating these fungi in restoration efforts. These early successional fungi are easily grown in artificial media and have reactive spores and sclerotia, so they could be used to inoculate seedlings in a nursery and improve their survival after planting (Policelli et al., 2020). Since ECM inoculations used in planting have been successful in other harsh environments (e.g., Souza et al., 2012), this approach could be promising for the endangered pine rocklands. This may be especially important for the sites located in low coastal areas since some of the early successional ECM taxa have been shown to tolerate high salt concentrations (Matsuda et al., 2006). Saltwater intrusion from low elevation sites close to the ocean coupled with habitat fragmentation is likely to influence the community composition and diversity of fungi on mature pine roots as well as soil propagules.

However, more comprehensive sampling of sporocarps and mature pine root communities is needed to determine the full scope of diversity and community dynamics in this system.

Data availability

The high through-put amplicon sequencing data will be deposited in the NCBI Sequence Read Archive (SRA), PRJNA789830.

Sanger sequencing GenBank accession numbers are available in Supplemental Table 3.

Declaration of competing interest

The authors have no conflict of interest to declare.

Acknowledgements

We thank the Florida Department of Agriculture and Consumer Services for their support in providing cone-tainers and racks for the bioassay experiment and Jeremy Hardee from Andrews Nursery for providing seeds and knowledge about Florida pine trees. We thank Andrew Rappe for access to Ordway Swisher Biological Research Station, Dr. Stephanie Koontz for access to Archbold Biological Station, Pam Moon and Dr. Bruce Schaffer for access to their pine rocklands, Miami-Dade County Parks and Recreation, and Florida Keys National Wildlife Refuge Complex for research permits. We also thank Dr. Nicole Reynolds and Dr. Marcos Caiafa for their thoughtful comments. We thank Dr. Rosanne Healy and Ben Lemmond for their help with identification of Pezizales sequences. We also thank two anonymous reviewers for their feedback that helped to improve our manuscript. Funding for EKA was provided by the University of Florida graduate research fellowship. This work was supported by the University of Florida and NIFA-USDA award FLA-PLP-005289 (to MES).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2022.101210.

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