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RESEARCH

Ectomycorrhizal fungal communities associated with *Crocanthemum* and *Lechea* (Cistaceae) in subtropical Florida sandhill habitats

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Abstract

Cistaceae are shrubs, subshrubs and herbs that often occur in stressful, fire-prone or disturbed environments and form ectomycorrhizal (ECM) associations with symbiotic fungi. Although some Cistaceae are long-lived shrubs that grow to significant size, others are herbaceous annuals or short-lived plants. Thus, Cistaceae are atypical ECM hosts that are fundamentally different in their biology from trees that are the more typically studied ECM hosts. The Mediterranean region is the center of diversity for Cistaceae and the ectomycorrhizal fungi associated with Cistaceae hosts have primarily been studied in Europe, North Africa, and the Middle East. Mediterranean Cistaceae often host diverse communities of ECM fungi, but they also act as hosts for some ECM fungi that putatively show host-specificity or strong host preference for Cistaceae (including species of *Delastria, Hebeloma, Terfezia*, and *Tirmania*). The ECM associations of Cistaceae in North America, however, remain highly understudied. Here we use fungal DNA metabarcoding to document the ectomycorrhizal fungal communities associated with *Crocanthemum* and *Lechea* (Cistaceae) in open, fire-prone sandhill habitats in north Florida. At each site we also sampled nearby *Pinus* to determine whether small, herbaceous Cistaceae have specialized ECM fungi or whether they share their ECM fungal community with nearby pines. The ECM communities of Florida Cistaceae are dominated by *Cenococcum* (Ascomycota) and *Russula* (Basidiomycota) species but were also significantly associated with *Delastria*, an understudied genus of mostly truffle-like Pezizales (Ascomycota). Although many Cistaceae ECM fungi were shared with neighboring pines, the ECM communities with Cistaceae were nonetheless significantly different than those of pines.

Keywords ECM · Rock-rose family · Mutualism · Fire-prone habitats

Introduction

Cistaceae is a family of heliophyte shrubs, subshrubs and herbs that typically occur in open, sunny areas on poor soils. There are nine genera and approximately 180 species of Cistaceae distributed mostly in temperate and subtropical regions of the Northern Hemisphere (Guzmán and Vargas 2009; Byng et al. 2016). Cistaceae species diversity is highest in Mediterranean areas of Europe, North Africa, and the Middle East where members of this family are important colonizers during early successional stages in disturbed habitats (Guzmán and Vargas 2005). All known members of the family form ectomycorrhizal (ECM) associations with symbiotic ECM fungi, including both woody perennial species and herbaceous annual species (Brundrett 2009). Cistaceae species in the Mediterranean region host a wide diversity of ECM fungi (Comandini et al. 2006; Leonardi et al. 2020) although some ECM fungi appear to be specifically associated with

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Cistaceae hosts (Díez et al. 2002; Comandini et al. 2006; Eberhardt et al. 2009). For example, several species of *Hebeloma* are putatively specialist fungi that exclusively form ECM with *Cistus* species (Comandini et al. 2006; Eberhardt et al. 2009). Similarly, species of the charismatic desert truffle genera *Terfezia* and *Tirmania* are primarily or exclusively associated with Cistaceae hosts, mostly in the genus *Helianthemum* (Montecchi and Sarasini 2000; Díez et al. 2002). In contrast, the majority of the ECM fungi associated with Cistaceae hosts can also colonize co-occurring trees (Dickie et al. 2004; Buscardo et al. 2012; Leonardi et al. 2020). Due to their ruderal strategy, Cistaceae hosts can often serve as a reservoir of ECM fungi and may facilitate the establishment of dominant trees, particularly in stressful habitats (Dickie et al. 2004; Martín-Pinto et al. 2006).

Three genera of Cistaceae are native to North America: *Crocanthemum*, *Hudsonia* and *Lechea* (Brizicky 1964). *Crocanthemum* has a disjunct distribution with all but one species located in North America, Central America and the Caribbean. One *Crocanthemum* species (*C. brasiliense*) is found in Uruguay, northern Argentina and southern Brazil (Govaerts et al. 2021). *Hudsonia* is distributed in subarctic and temperate regions across most of Canada and in the eastern United States; *Lechea* is distributed across most of North America, Central America and the Caribbean (Govaerts et al. 2021). Previous studies on the ECM communities in *Crocanthemum*, *Hudsonia* and *Lechea* in North America have reported that species in these genera form ectomycorrhizal associations with generalist fungi in the genera *Cenococcum* (Ascomycota), *Cortinarius, Laccaria* and *Russula* (Basidiomycota) (Malloch and Thorn 1985; Dickie et al. 2004; Massicotte et al. 2010; Byers et al. 2021). However, these studies of North American Cistaceae have generally examined a small number of host plants and have been geographically limited to temperate forests in eastern Canada and the Midwestern United States. Though Cistaceae are distributed throughout tropical and subtropical habitats in North America, Central America and the Caribbean, the fungal symbionts in these habitats remain completely unstudied.

Cistaceae are generally widespread and relatively common in some habitats across the Southeastern USA (Brizicky 1964). In Florida, there are six species of *Crocanthemum* and nine species of *Lechea* (Wunderlin et al. 2023) and these plants typically occur in sandhill ecosystems (Spaulding 2013). Sandhill habitats are subtropical to temperate forest-savannah habitats characterized by sandy soils and a xeric environment with minimal understory vegetation and an open canopy that is maintained by frequent, low intensity fires (Myers 1985; Sorrie and Weakley 2006). Sandhill habitats dominated by *Pinus palustris* (Longleaf Pine) are also considered endangered habitats because they occupy only ca. 3% of their previously widespread range in the Southeastern United States and are increasingly endangered by urbanization and changes in the fire regime (Frost 1993; Costanza et al. 2015). These ecosystems also serve as the only habitat for endangered species such as the red-cockaded woodpecker (*Dryobates borealis*) and the eastern indigo snake (*Drymarchon couperi*) (Walters et al. 1988; Hyslop et al. 2014).

Ectomycorrhizal fungal communities have rarely been studied in sandhill ecosystems even though the dominant trees and some common understory plants rely on ECM fungi for their growth (Rasmussen et al. 2018). While some ECM have specific associations with Cistaceae hosts in the Mediterranean basin, host-specificity of mycobionts associated with Cistaceae have not been evaluated in subtropical ecosystems in the Americas. The goal of this study was to characterize the ECM fungal symbionts associated with *Crocanthemum* and *Lechea* species in northern Florida, USA. We hypothesized that: 1) Cistaceae host plants share the majority of their ectomycorrhizal symbionts with co-occurring trees but that 2) deeper sampling will detect some specialist ECM fungi that preferentially or specifically associate with Cistaceae hosts. Characterizing the ECM fungal communities on these common and widespread Cistaceae hosts is a critical first step towards understanding the dynamics of ECM fungi in sandhill ecosystems and in determining whether Cistaceae hosts may facilitate the survival of ECM fungi and promote colonization by ECM trees such as *Pinus palustris*.

Material and methods

Study area and sampling design

Sampling was conducted in four locations in northern Florida. The study areas included Cedar Key Scrub State Reserve, Etoniah Creek State Forest, Ordway-Swisher Biological Station and Twin Rivers State Forest (Fig. 1a, Table 1). The climate of the region is humid subtropical (Kottek et al. 2006). Although these sites are hot and humid throughout much of the year, rain is concentrated in the summer months and there is typically a cool period during November-January (Florida Climate Center 2024). Temperatures may reach 0 ˚C a few times per year, but these sites may also periodically experience "hard freeze" events where temperatures as low as -12 ˚C have been recorded (Osland et al. 2021).

At each location, we selected 3–5 local sites in sandhill habitats (Fig. 1b) within a 4 km^2 area based on the presence of common species of *Crocanthemum* (Fig. 1c) and *Lechea* (Fig. 1d) (Cistaceae). Between September 2018 and March 2019, we opportunistically collected whole specimens of *Crocanthemum* spp. and *Lechea* spp. as well as 10–50 g of roots from nearby *Pinus* species (within 1–5 m from the Cistaceae plant) at each site. *Pinus palustris* was the dominant pine species at each site, but at least four other *Pinus* species were present across the sampling sites (*Pinus*

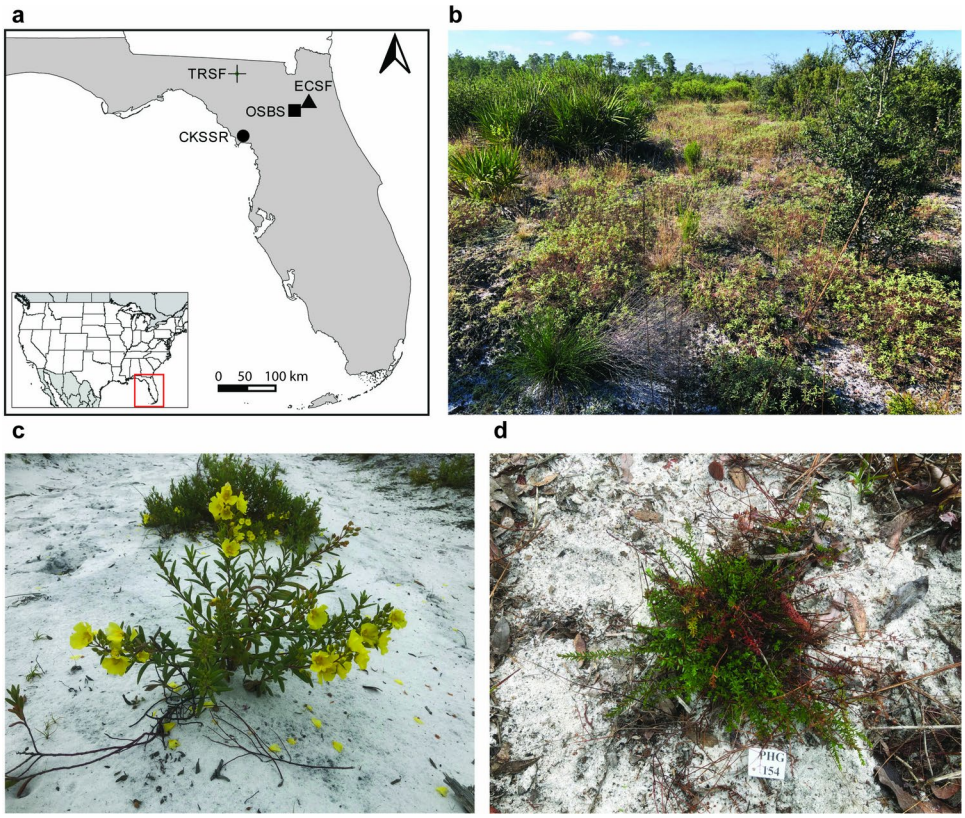

Fig. 1 **a** Map of the study area in north Florida, USA. Cedar Key Scrub State Reserve (CKSSR), Etoniah Creek State Forest (ECSF), Ordway-Swisher Biological Station (OSBS) and Twin Rivers State Forest (TRSF), **b** Sandhill ecosystem where Cistaceae specimens were collected, **c.** Specimen of *Crocanthemum nashii,* **d** Specimen of *Lechea torreyi*

Table 1 Host presence, average concentration of P, K, Ca, Mg and Al and pH in the soil 0–10 cm layer at the four locations of the study area

Study Site	Host Plants Present	Soil Variables (mg kg^{-1})					
		P[1]	K	Ca	Mg	Al	pH[2]
Cedar Key Scrub State Reserve	*Crocanthemum* sp., *C. nashi*, *C. corymbosum*, *Lechea sp*., *L. torreyi*, *Pinus palustris,* and *P. clausa*	1.4	8.9	124	20.4	13	4.78
Etoniah Creek State Forest	*Crocanthemum* sp., *C. corymbosum*, *L. sessiflora*, *L. minor*, *Pinus palustris, P. clausa, P. elliotti, P. glabra,* and *P. serotina*	6.9	9.6	85	12.1	49	4.94
Ordway-Swisher Biological Station	*Crocanthemum* sp., *C. corymbosum*, *L. sessiflora*, *L. minor*, and *Pinus palustris*	11.3	9.6	82	11.9	133	5.31
Twin Rivers State Forest	*C. corymbosum*, *L. sessiflora*, *L. minor*, *L. torreyi,* and *Pinus palustris*	8.8	12.4	143	17.8	122	5.30

[1] Following EPA 200.7 and the P was extracted with Melich 1

[2] Following EPA 150.1

clausa, *P. elliottii*, *P. glabra*, and *P. serotina*). All plants were actively growing and had green leaves at the time they were sampled. Because our sampling was destructive to individual Cistaceae host plants, we only sampled plants in areas where there were sufficient populations of many individuals – small or highly isolated plants were not sampled. All plant material was transported on ice to the lab, stored at 4 ˚C, and processed within 2 days. To characterize soils, two samples from each local site were collected, air-dried, and passed through a 2-mm sieve. Soil samples were then analyzed at the University of Florida Extension Soil Testing Laboratory.

Processing of plant samples

Aboveground Cistaceae plant parts were cut and deposited as plant herbarium specimens in the Florida Museum of Natural History Herbarium (FLAS). When they were present, we preserved flowers to facilitate plant identification. However, it was not always possible to sample flowering individuals, so some samples could only be accurately identified to the genus level. Roots were rinsed in running tap water to remove adhering soil. Cleaned roots were stored up to 48 h in water at 4 ˚C. Roots were then inspected under a dissecting microscope

and healthy ECM root tips were collected with clean forceps. We removed all actively growing and healthy ECM root tips from each plant and compiled them into a clean petri dish filled with water. The ECM roots from each individual plant were placed into a 1.5 ml tube to sample ECM fungal communities using amplicon sequencing (see below). Samples for amplicon sequencing were placed in cetyltrimethylammonium bromide (CTAB) buffer (Gardes and Bruns 1993) and kept at -20 ˚C prior until processing. However, some individual plants of *Crocanthemum* and *Lechea* with abundant ECM root tips were examined to further characterize the morphology of dominant ECM morphotypes (see below).

For morphological characterization of representative morphotypes, we selected plants with high ECM colonization and sampled eight individual ECM root tips from a dominant morphotype. Six of the eight root tips per morphotype were stored in FAA solution (45:5:5:45 volume per volume ratio of 95% ethanol:formaldehyde:acetic acid:distilled water) for visualization by light microscopy. The remaining two ECM root tips were cleaned and subjected to DNA extraction using the Extract-N-Amp Plant Kit (Sigma-Aldrich, St. Louis, Missouri) for rapid DNA extraction and amplification. We amplified and Sanger sequenced the ITS region using ITS1F and ITS4 primers (White et al. 1990) and following the protocols in Karlsen-Ayala et al. (2023). DNA sequences from these individual morphotypes are available in GenBank (Table S1).

Microscopy

Root tips fixed in FAA were gradually dehydrated in an ethanol series (50%, 70%, 95%) to absolute ethanol (several changes in 100%) followed by 1:1 absolute ethanol:xylene and then several changes of xylene, with a minimum of 12 h in each solution along with gentle agitation on a shaker. The ECM root tips were gradually infiltrated with melted Paraffin (mp of 56˚C) in a 60˚C oven, with occasional agitation over the course of seven days until the paraffin had completely displaced the xylene. Root tips were perpendicularly oriented in fresh, melted paraffin in an embedding tray and allowed to harden overnight. Wax blocks of embedded root tips were trimmed and 8 µm thick cross sections were cut with a rotary microtome. These were mounted on Probe on Plus slides (Thermo Fisher Scientific, Fairlawn, NJ, USA) in a drop of water and allowed to solidify overnight on a warming tray set to the lowest heat. Sections were deparaffinized to water in a graded series of xylene, ethanol and water solutions, and stained in Toluidine Blue O (Sigma-Aldrich, St. Louis, MO USA) 0.05% in water. Stained sections were dehydrated in a graded series of water, ethanol, and xylene solutions, and the xylene-soaked sections mounted in a drop of permount (Thermo Fisher Scientific, Fairlawn, NJ USA) with a coverslip applied on top. Mounted sections were viewed with a Zeiss Axio Imager A2 compound microscope (Carl Zeiss, Oberkochen, Germany).

DNA extraction, amplification and sequencing

Before DNA extraction, ECM root tips sampled for amplicon sequencing were ground using a drill press fitted with a sterile plastic pestle. Genomic DNA was then extracted using a modified glass milk extraction protocol described by Lindner and Banik (2009) with the modifications of Jusino et al. (2014). We amplified the fungal rDNA internal transcribed spacer region 1 (ITS1) using the primers ITS1F (Gardes and Bruns 1993) and ITS2 (White et al. 1990). Libraries were prepared using a dual-index sequencing strategy (Kozich et al. 2013) with the first PCR step used to amplify the ITS1 region using modified primers with Illumina Nextera v2 adapters (Illumina, San Diego, CA, USA) and the second PCR step used to ligate the barcodes from the Illumina Nextera v2 Kit. The first PCR conditions were 94 °C (3 min) followed by 35 cycles at 94 °C (30 s), 52 °C (30 s), 68 °C (30 s) and a final extension at 68 °C (7 min) using 5– 10 ng of DNA, DNA-free water, 5X Green GoTaq buffer (Promega, Madison, WI, USA), 10 mM of each primer, 20 mg/mL BSA (New England BioLabs, Ipswich, MA, USA), 10 mM dNTPs (Promega, Madison, WI, USA) and 5 units/ml GoTaq® Polymerase (Promega, Madison, WI, USA). Thermocycler conditions for the second PCR were 95 °C (3 min) followed by 8 cycles at 95 °C (30 s), 52 °C (30 s), 72 °C (1 min) and a final extension at 72 °C (7 min). Positive and negative controls were used during extraction, PCR, and sequencing. Positive controls included a biological mock community consisting of known ectomycorrhizal fungi (*Amanita* sp., *Cortinarius iodes, Inocybe pallidicremea, Lactarius scrobiculatus, Russula vesca*, *Suillus* sp., and *Tuber lyonii*). A single-copy mock community (SynMock) consisting of single-copy, non-biological ITS sequences combined in equimolar amounts (Palmer et al. 2018) was also used as a positive sequencing and bioinformatics control. Barcoded samples were then cleaned and size-selected at ≥ 300 bp with Zymo Select-A-Size Clean & Concentrator kits (Zymo Research, Irvine, CA, USA). Resultant products were then quantified using an Invitrogen Qubit 4.0 Fluorometer and Qubit 1X dsDNA HS Assay kit (Invitrogen, Eugene, OR, USA). Samples were then equilibrated and combined. The final library was sequenced in both directions using Illumina MiSeq v3 2 × 300 bp at the Interdisciplinary Center for Biotechnology Research at the University of Florida.

Bioinformatics

Illumina MiSeq sequencing data were processed using AMPtk v1.5.3 (Palmer et al. 2018). FastQ files were demultiplexed and the forward and reverse primers were stripped using VSEARCH v2.10.4. (Rognes et al. 2016). Sequences < 125 bp were discarded and reads > 450 bp were trimmed to 450 bp. Sequences were quality-filtered

with expected errors less than one (Edgar and Flyvbjerg 2015), denoised and clustered at 97% similarity into operational taxonomic units (OTUs) using UPARSE (Edgar 2013). The 97% cutoff is commonly used to approximate fungal species (Smith et al. 2007a; Kõljalg et al. 2013). After clustering, we used SynMock (Palmer et al. 2018) to account for observed rates of index bleed using the filtering module of AMPtk. We assigned OTU-level taxonomy by using the taxonomic algorithm in AMPtk and by performing BLAST searches via the National Center of Biotechnology Information (NCBI). Finally, we identified ECM fungal OTUs using FUNGuild (Nguyen et al. 2016). Further analysis of fungal guilds retained OTUs in taxa with confidence levels of "probable" and "highly probable". When some OTUs could not be easily evaluated for their ECM status with FUNGuild (e.g. they belong to families or genera that contain both ECM and non-ECM fungi and their identity was ambiguous), we evaluated them using methods in Karlsen-Ayala et al. (2023). Specifically, we used BLAST searches to carefully evaluate ECM status. We considered these OTUs as ectomycorrhizal if the closest BLAST hit matched an ECM species hypothesis with > 90% similarity and > 90% coverage. ECM status was also compared with the ECM lineages identified by Tedersoo and Smith (2013). The identification of OTUs in Pezizales was conducted by phylogenetic approaches using the ITS datasets from (Healy et al. 2022). If an OTU could not unambiguously be determined to be ECM, we excluded that OTU from the ECM guild for the purposes of our analysis. All sequences were deposited at NCBI Sequence Read Archive under BioProject accession number PRJNA1073980.

Data analysis

All analysis were performed using R version 4.1.1 (R Core Team 2021). To account for uneven sequencing depth, we took a conservative approach and rarefied the OTU table to a depth of 4,370 sequences/sample. This approach enabled us to retain the largest number of samples and sequencing depth within the dataset. We inspected the extraction and PCR controls and all samples suspected of lab contamination were excluded from the analysis. To reduce issues due to potential sequencing artifacts, we removed rare OTUs that had less than 100 sequences across all samples.

To visualize the ECM fungal communities associated with different host genera and geographic locations in ordination space, we used nonparametric multidimensional scaling (NMDS) with the vegan package (Oksanen et al. 2019). To test whether different host plants harbor different ECM fungal communities and how these are affected by site, we performed a nonparametric permutational multivariate ANOVA (PERMANOVA) (Anderson 2001) using the adonis2 function in the vegan package (Oksanen et al. 2019) with the Raup-Crick dissimilarity metric, which has been shown to be appropriate for zero-weighted datasets (Chase et al. 2011). We tested differences in multivariate dispersion among host genera using the function betadisper (Anderson 2006). Bipartite networks were generated using the ggalluvial package (Brunson 2020). To determine host preference of different ECM fungi, we performed an indicator species analysis based on the indicator value index (IndVal) using the indicspecies package (De Cáceres and Legendre 2009).

Results

Plant collection and morphological characterization of ectomycorrhizal root tips

We collected at total of 51 specimens of *Crocanthemum*, 49 specimens of *Lechea* and 53 root samples from nearby *Pinus*. *Crocanthemum* specimens included *C. corymbosum* and *C. nashi* whereas *Lechea* specimens included *L. sessiliflora*, *L. deckertii*, *L. minor*, and *L. torreyi*.

We observed ECM colonization on all host plant species. Colonized root tips exhibited a swollen appearance with a wide degree of morphological variability in the fungal mantle and emanating hyphae that was variable based on the identity of the ECM fungal species (Fig. 2 a-d, m-p). Microscopy of root tip cross-sections of the most common ECM morphotypes on *Crocanthemum* spp. (Fig. 2 e–h) and *Lechea* spp. (Fig. 2 i–l) confirmed the presence of a mantle and Hartig net, indicating functional ECM structures. We also obtained sequences of diverse communities of ECM fungi using both Sanger sequencing on individual root tips (Table S1) and ITS1 amplicon sequencing on pooled ECM root tips with multiple morphotypes from a single plant (Table S2).

In general, the ectomycorrhizae were ca. 0.5–3 mm long and 0.5 mm thick and included yellow, light brown, dark brown and black morphotypes. For example, there was frequent branching in *C. corymbosum* + *Delastria* sp. (Fig. 2e) and *C. corymbosum* + *Xerocomus hypoxanthus* (Fig. 2h). Some ECM roots were short and small as in *L. sessiliflora* + *C. geophilum* (0.5 mm; Fig. 2n) or were elongated as in *C. corymbosum* + *Delastria* sp. (2 mm; Fig. 2e) and in *L. sessiliflora* + *Delastria* sp. (3 mm; Fig. 2p). In most ECM root tips cross-sections, the Hartig net was confined to the outer layer of radially elongated epidermal cells, but in some cases the fungus penetrated to the second row of plant cortical cells, as in *C. corymbosum* + *Xerocomus hypoxanthus* sp. (Fig. 2h) and *L. sessiliflora* + *Pisolithus arrhizus* (Fig. 2o).

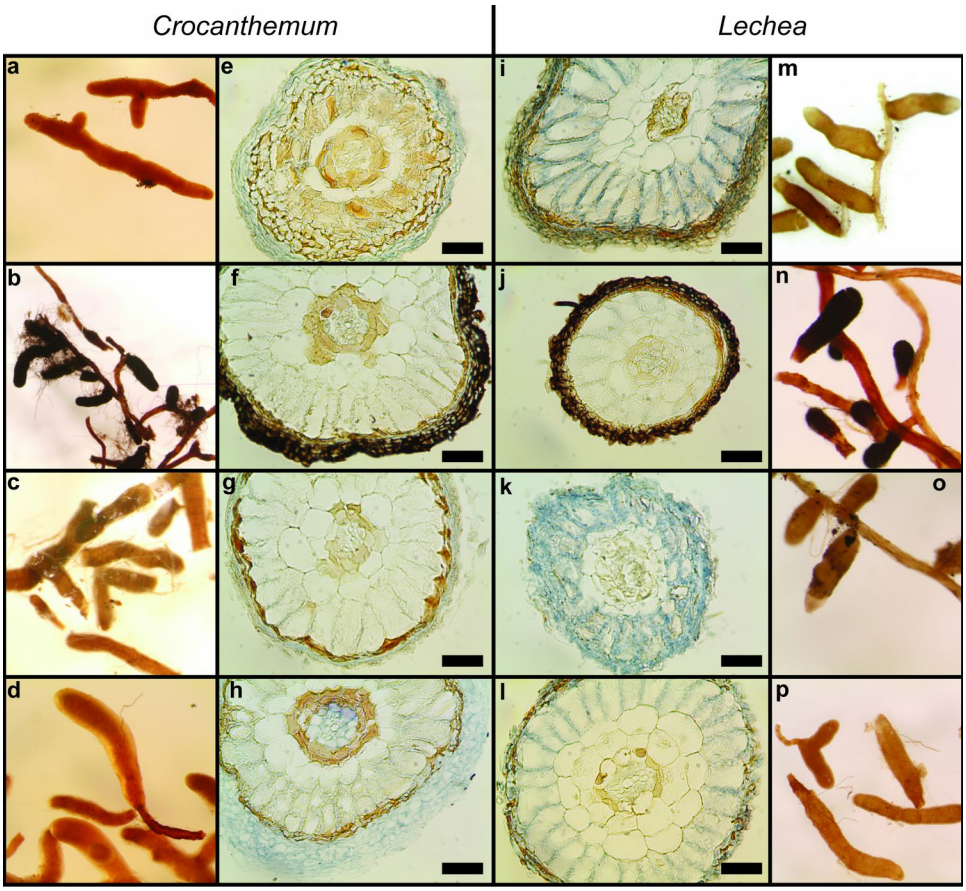

Fig. 2 Ectomycorrhizal root tips of representative morphotypes from specimens of *Crocanthemum* spp. (**a–h**) and *Lechea* spp. (**i–p**). **a**. Photograph of root tips and Hartig net (**e**) of ectomycorrhizal root colonized by *Delastria* sp., **b**. Photograph of root tips and Hartig net (**f**) of ectomycorrhizal root colonized by *Cenococcum geophilum,* **c**. Photograph of root tips and Hartig net (**g**) of ectomycorrhizal root colonized by *Tricholoma equestre,* **d**. Photograph of root tips and Hartig net (**h**) of ectomycorrhizal root colonized by *Xerocomus hypoxanthus*., **i.** Photograph of root tips and Hartig net (**m**) of ectomycorrhizal root colonized by *Russula* sp., **j.** Photograph of root tips and Hartig net (**n**) of ectomycorrhizal root colonized by *Cenococcum geophilum,* **k.** Photograph of root tips and Hartig net (**o**) of ectomycorrhizal root colonized by *Pisolithus arrhizus*, **l** Photograph of root tips and Hartig net (**p**) of ectomycorrhizal root colonized by *Delastria* sp. Scale bars = 20 µm

Ectomycorrhizal fungi associated with Cistaceae hosts based on amplicon sequencing

The Illumina MiSeq run resulted in 3,482,863 fungal ITS reads with an average of 21,632 reads/sample and a total of 1,473 OTUs. We examined extraction and PCR negative controls and found two samples suspected of lab contamination that were excluded from further analyses. After rarefaction and excluding rare OTUs (those with a total of 100 read or less), the total number of OTUs was 985. From those 985 OTUs, 204 were identified as ECM fungi taxa and 168 were associated with Cistaceae plants. Overall, there was a high overlap among ECM fungal communities associated with Cistaceae hosts and *Pinus* species (Fig. 3). Ectomycorrhizal fungal communities from all hosts were significantly affected by location (adonis, $R^2 = 0.11$, pseudo-F = 6.30, $p < 0.001$). Ectomycorrhizal fungal communities associated with *Crocanthemum* were slightly but significantly different than ECM fungi associated with *Pinus* species (adonis, $R^2 = 0.047$, pseudo-F = 4.84, $p < 0.001$; Fig. 3) and multivariate dispersion of ECM communities was significantly different between the two hosts (betadisper, F = 6.23, $p < 0.05$). Similarly, ECM fungal communities associated with *Lechea* were slightly but significantly different than ECM fungi associated with *Pinus* species (adonis, $R^2 = 0.05$, pseudo-F = 5.11, $p < 0.001$; Fig. 3) and had a significant difference in multivariate dispersion (betadisper, F = 5.43, $p < 0.05$). In contrast, ECM fungal communities did not differ between Cistaceae host genera (adonis, $R^2 = 0.017$, pseudo-F = 1.67, $p = 0.063$; Fig. 3) and had a similar multivariate dispersion (F = 0.54, $p = 0.46$). The results above are based on frequency-based measures (i.e. Raup-Crick) but we also found that PERMANOVA and multivariate dispersion with abundance-based (i.e. Bray–Curtis) metrics gave similar results (data not shown).

Perhaps unsurprisingly due to the small sizes of their root systems, both *Crocanthemum* and *Lechea* species harbored lower ECM species richness than *Pinus* (Fig. 4a). Species accumulation curves also suggest that there is likely additional, undetected diversity for all three genera (Fig. 4a). From the 204 ECM OTUs we detected in the roots of Cistaceae hosts and nearby pines, 83 OTUs were shared among the three genera, whereas 11 OTUs were only associated with *Crocanthemum,* 26 OTUs were only associated with *Lechea*, and 36 OTUs were only associated with *Pinus* (Fig. 4b). The ECM OTUs that were most frequently associated with both the Cistaceae and pine hosts included *Cenococcum geophilum*, *Lactifluus hygrophoroides, Pisolithus arrhizus* and *Russula* spp. (Fig. 4c, Table S2). In the case of Cistaceae hosts,

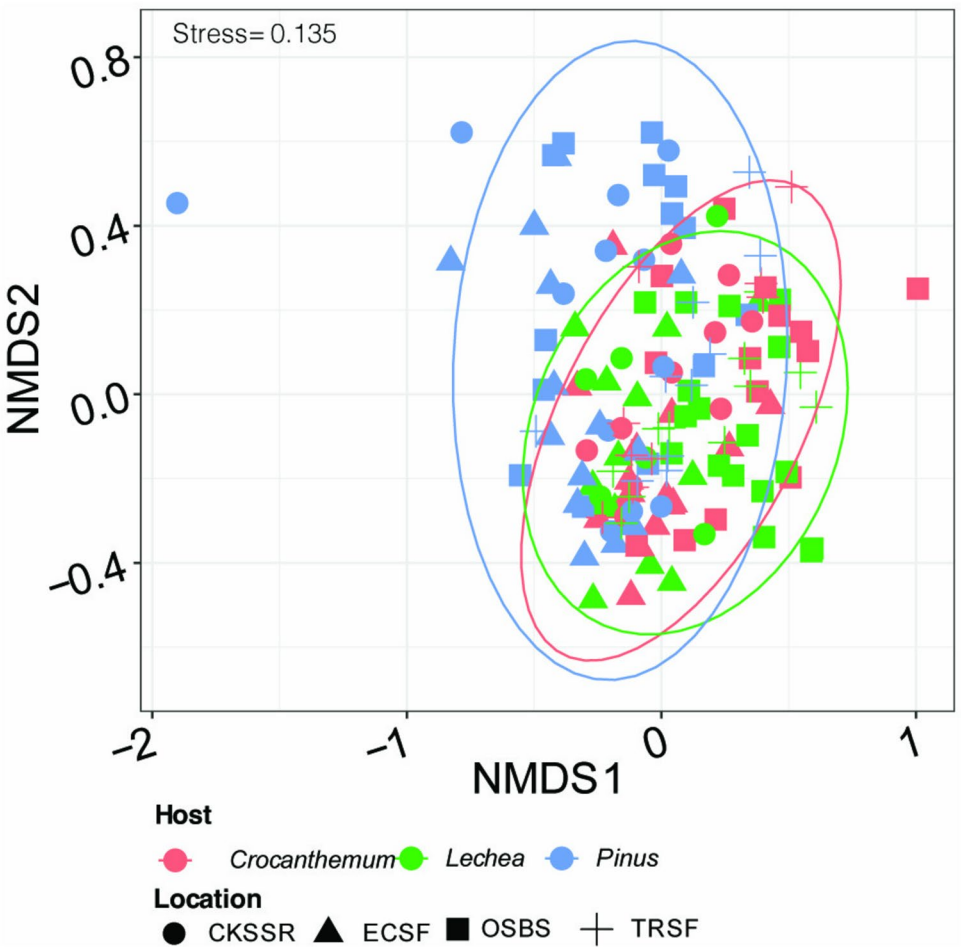

Fig. 3 Non-metric multi-dimensional scaling (NMDS) ordination of ECM fungal communities from *Crocanthemum*, *Lechea* and *Pinus* root tips using a modified Raup-Crick dissimilarity metric. Cedar Key Scrub State Reserve (CKSSR), Etoniah Creek State Forest (ECSF), Ordway-Swisher Biological Station (OSBS) and Twin Rivers State Forest (TRSF)

the most frequent ECM fungi also included *Delastria* spp. (Fig. 4c, Table S2). This preferential association between Cistaceae hosts and *Delastria* spp. was corroborated by our indicator species analysis (Table S3). It is notable that 13 out of 14 OTUs determined by the indicator species analysis to be preferentially associated with Cistaceae belonged to Ascomycota. In contrast, 7 out of 8 OTUs that were preferentially associated with *Pinus* were Basidiomycota.

Discussion

We explored the ECM fungal community associated with two Cistaceae genera, *Crocanthemum* and *Lechea,* in sandhill ecosystems in Florida. As far as we know this is the first study to document the ECM fungal communities in subtropical Cistaceae in the Americas. We found that plants of *Crocanthemum* and *Lechea* were consistently colonized by morphologically differentiated ECM fungi that formed typical fungal mantels and Hartig nets (Fig. 2). Using amplicon sequencing based on fungal ITS1, we detected a diverse community of ECM fungi on Cistaceae host plants (168 ECM OTUs). It is important to note that while OTU number is strongly correlated with the number of species, it does not represent an exact number of species because some OTUs might represent ITS1 sequence variants of the same species (Lindahl et al. 2013). Similar to previous studies that reported that Cistaceae host plants share a large number of ECM fungi with nearby trees (Dickie et al. 2004; Buscardo et al. 2012; Leonardi et al. 2020), we detected a high overlap among ECM communities between *Crocanthemum*, *Lechea* and co-occurring *Pinus* species. Despite this large overlap in the ECM fungal communities, the ECM fungal communities associated with Cistaceae hosts nonetheless differed significantly from those associated with *Pinus* (Fig. 3). The ECM fungal communities from *Crocanthemum* and *Lechea*, however, were not significantly different from each other. This is an expected result because, in addition to being members of the same plant family, species of *Crocanthemum* and *Lechea* are frequently found in the same sunny, sandy, fire-prone habitats (Fig. 1a) and often co-occur at the same sites. Some fungi in the Mediterranean region have specific associations with certain Cistaceae genera, but others are generalists and colonize different Cistaceae genera as well as co-occuring trees (Comandini et al. 2006; Eberhardt et al. 2009; Albuquerque-Martins et al. 2019; Leonardi et al. 2020).

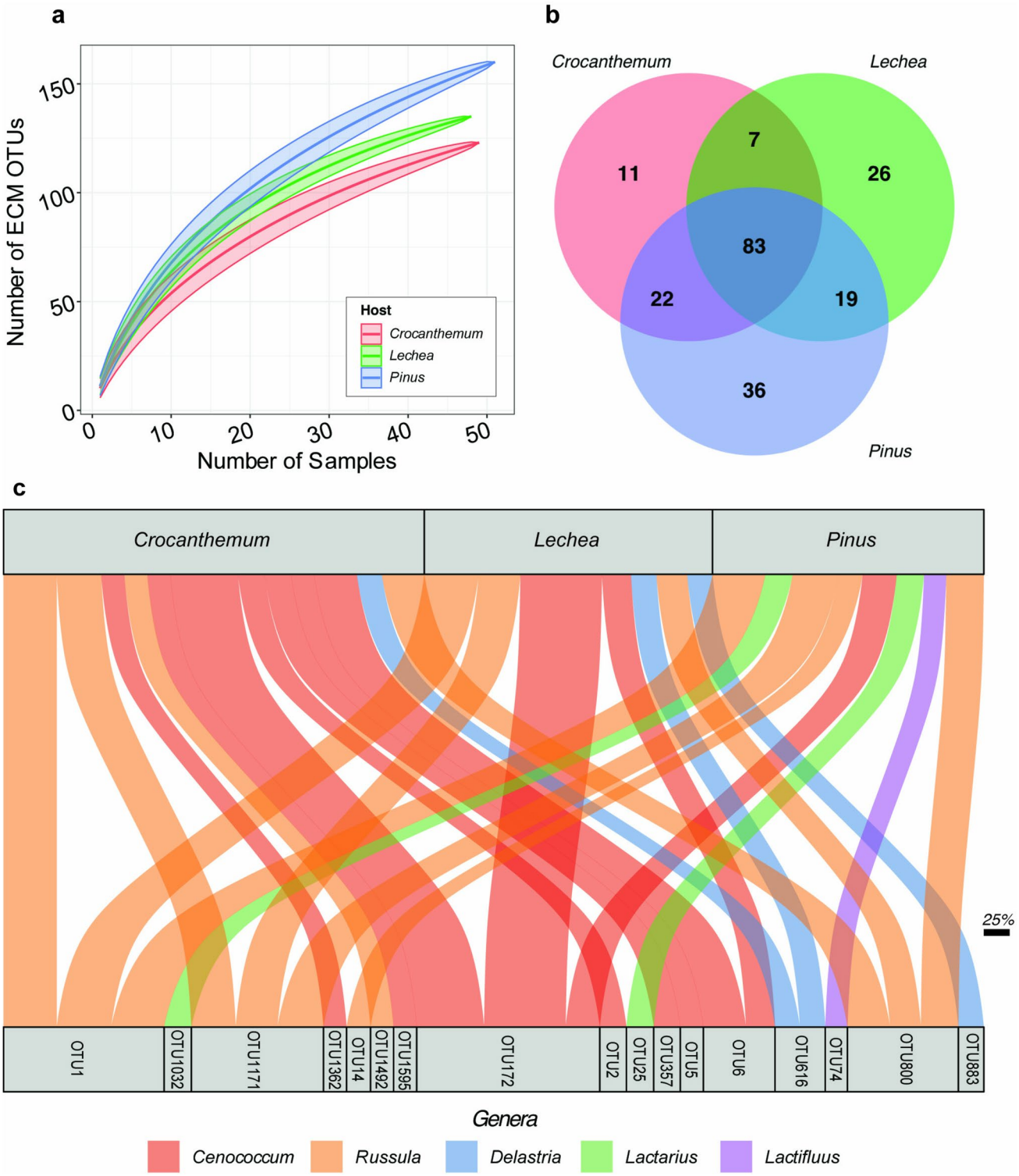

Fig. 4 **a** Species accumulation curve for the ectomycorrhizal fungi with *Crocanthemum*, *Lechea* and *Pinus* showing the lower and upper 95% confidence intervals (shaded region), **b** Venn diagram showing the number of ectomycorrhizal fungi operational taxonomic units (OTUs) associated with each host plant genus and the number of OTUs shared among them, **c** Bipartite network of the three plant genera (*Crocanthemum*, *Lechea* and *Pinus*) (upper level) and the ectomycorrhizal fungi OTUs found in at least 25% of samples from each genus (lower level)

Our results also show that ECM fungal communities varied significantly across locations. This pattern is partially explained in part by large distances between some of our sampling sites (> 150 km) as well as differences in soil properties across sites, with notable variation in soil P, Ca, and Al (Table 1). While our experimental design did not explore spatial patterns at a fine scale within individual field sites, future work might consider manipulative experiments to determine the strength of host preferences and whether they are influenced by both physical and phylogenetic distances between host plants.

The majority of the Cistaceae ECM community was comprised of generalist ECM fungi, many of which were also found on nearby pines (Figs. 3 and 4). The most frequently occurring fungi, *Cenococcum geophilum* and *Russula* species, were also found previously on the ECM roots of *Crocanthemum* and *Hudsonia* species in eastern Canada and the midwestern United States (Malloch and Thorn 1985; Dickie et al. 2004; Massicotte et al. 2010). *Cenococcum geophilum* and *Russula* species frequently dominate ECM fungal communities with a wide diversity of gymnosperms and angiosperms, including *Pinus* and *Quercus* species (Trappe 1962; Dickie et al. 2004; Miyamoto and Nara 2016; Rasmussen et al. 2018; Karlsen-Ayala et al. 2023), and these taxa were also common on the roots of the pines that we sampled (Fig. 4c). *Cenococcum* is often an important component of the ECM communities in abiotically stressful environments such as serpentine soils (Gonçalves et al. 2009) and xeric habitats (Smith et al. 2007b). It is worth noting that *Cenococcum geophilum* is represented by more than seven different OTUs in our dataset (Fig. 4, Tables S1, S2 and S3). Previous studies in northern Florida revealed high phylogenetic diversity of *Cenococcum*, with multi-locus sequencing that resolved six major clades that likely represent phylogenetically distinct taxa (Obase et al. 2016).

Although Cistaceae ECM communities were dominated by host generalists, we nonetheless found evidence for specialization or host preference among some of the ECM fungi on *Crocanthemum* and *Lechea*. In particular, our indicator species analysis revealed 14 OTUs that were preferentially associated with the Cistaceae hosts (Table S3). This group of Cistaceae-associated fungi was dominated by Ascomycota (13 out of 14 OTUs), six of which belonged to the genus *Delastria* (Pezizales). Although *Delastria* spp. have been found to form ectomycorrhizas with Florida *Pinus* seedlings (Karlsen-Ayala et al. 2023), the majority of the *Delastria* observations in our dataset were on Cistaceae hosts and these OTUs were the most strongly Cistaceae-associated taxa that we recovered. Interestingly, most of the *Delastria* species that have been described are associated with Mediterranean Cistaceae, including species of *Cistus*, *Halimium*, and *Tuberaria* (Paz et al. 2018), although these fungi also sometimes form ECM on nearby *Quercus* and *Pinus* trees. It is also notable that the type species of the genus, *Delastria rosea*, is often associated with *Cistus ladanifer*, another member of Cistaceae (Alvarado et al. 2011). While *Delastria* species are common ECM symbionts associated with Florida Cistaceae, there are no described species in this genus from North America. The preferential association of *Delastria* and other Ascomycota ECM fungi with North American Cistasceae is consistent with the observations of ECM communities with Cistaceae in Europe, North Africa and the Middle East. Across this range, Cistaceae are consistently associated with *Delastria* and with other Pezizales such as the desert truffle genera *Terfezia* and *Tirmania* (Montecchi and Sarasini 2000; Díez et al. 2002; Paz et al. 2018). Interestingly, many of the ECM fungi in Pezizales have either contact or short-distance exploration types and they often retain some saprotrophic capabilities (Tedersoo and Smith 2013; Hughes et al. 2020; Healy et al. 2022). Species of *Terfezia* and *Tirmania* as well as all but one species of *Delastria* produce truffle-like fruiting bodies with tough, durable spores that are likely able to withstand hot, disturbance-prone environments (Montecchi and Sarasini 2000). All these traits are probably advantageous for *Delastria, Terfezia* and *Tirmania* when colonizing small, herbaceous hosts in stressful environments, both in the Mediterranean region and Florida.

This is the first study that explores the ECM fungal communities associated with Cistaceae in the subtropical Americas. The only tree in Cistaceae, *Pakaraimaea dipterocarpaceae* – a tropical tree occurring in the Guyana Shield in South America – is associated with a wide diversity of ECM fungi (Smith et al. 2013). It is important to note that, similar to Cistaceae in the Mediterranean basin (Martín-Pinto et al. 2006; Guzmán and Vargas 2009), Cistaceae in the southeastern United States are subject to frequent disturbances. Low severity fires are a common disturbance that is critical for the sandhill ecosystem and for the dominant pine species, *P. palustris* (Myers 1985). The sandy soils within these habitats often have low water availability and minimal organic material, making them stressful environments for plant communities (Foster and Brooks 2001). Because both *Crocanthemum* and *Lechea* species in sandhill ecosystems shared a large proportion of their mycobionts with *Pinus*, it is possible that these Cistaceae hosts serve as an ECM fungi reservoir that might facilitate pine establishment (Martín-Pinto et al. 2006). Thus, future research should explore the importance of these Cistaceae host plants and their potential impacts and ecological connections in these important endangered ecosystems.

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Author contributions MES and PHG conceived the study; PHG, EKA, WMW and MES performed fieldwork; WMW and PHG identified host plants, PHG, MAJ, EKA and NKR performed lab work; PHG and RH performed microscopy observations and captured images; MVC and MAJ processed and analyzed the data; MVC, MES and PHG wrote the first draft; All authors contributed critically to the drafts and gave final approval for publication.

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Data availability Illumina MiSeq sequencing data was deposited at NCBI Sequence Read Archive under BioProject accession number PRJNA1073980.

Declarations

Competing interests The authors declare no competing interests.

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