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Diversity and specificity of orchid mycorrhizal fungi in a leafless epiphytic orchid, *Dendrophylax lindenii* and the potential role of fungi in shaping its fine-scale distribution

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Orchids grow in diverse habitats worldwide with most (approximately 69%) growing on trees as epiphytes. Although orchid mycorrhizal fungi have been identified as potential drivers for terrestrial orchid distribution, the influence of these fungi on the fine-scale distribution of epiphytic orchids is poorly understood. In this study, we investigated the mycorrhizal fungal community and fine-scale distribution of *Dendrophylax lindenii*, a rare and endangered epiphytic orchid that is leafless when mature. We used amplicon sequencing to investigate the composition of orchid mycorrhizal fungi in the roots of 39 *D. lindenii* individuals in their natural habitat, the swamps of Florida. We compared the orchid mycorrhizal fungi of *D. lindenii* to those of co-occurring epiphytic orchids, as well as to the orchid mycorrhizal fungal communities of bark from potential host trees, with and without *D. lindenii*. Our results show that *D. lindenii* has a high specificity for a single *Ceratobasidium* species, which is widely distributed on phorophytes and detected in both wet and dry periods in the orchid's habitat. This *Ceratobasidium* species was mostly absent or only recorded in low frequency in the roots of co-occurring epiphytic orchids. Phylogenetic analysis documented that this *Ceratobasidium* was conspecific with the strain that is used to germinate *D. lindenii* *ex-situ*. However, our findings suggest that laboratory germinated adult *D. lindenii* transplanted into the field had lower read abundances of this *Ceratobasidium* compared to naturally occurring plants. These findings suggest that this orchid mycorrhizal fungus may play a significant role in the fine-scale distribution of naturally occurring *D. lindenii*.

KEYWORDS

conservation, *Ceratobasidium*, host tree specificity, amplicon sequencing, ghost orchid

Introduction

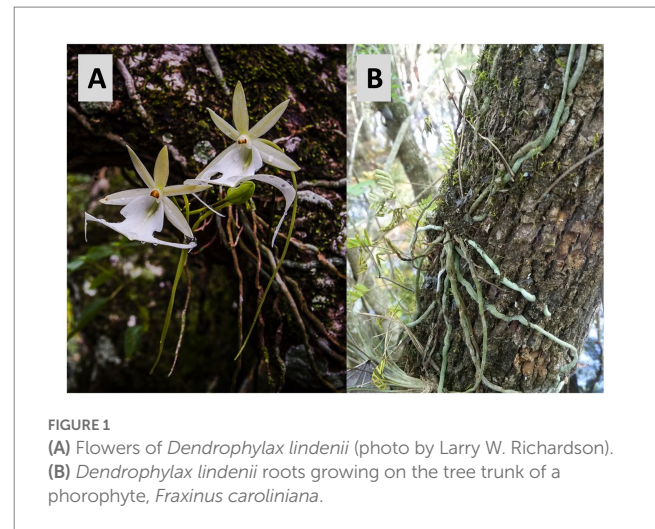
Mycorrhizal fungi are well known mutualists that are essential for their plant partners' abundance and spatial distribution (Smith and Read, 2010; McCormick and Jacquemyn, 2014). While ca. 69% of orchid species are tropical epiphytes (Zotz, 2016), little is known about the orchid mycorrhizal fungi (OMF) they associate with compared to temperate terrestrial orchids.

Epiphytic orchids, like their terrestrial counterparts, enlist OMF to facilitate seed germination and seedling development, but it remains unclear to what degree epiphytes continue to utilize OMF into maturity (Dearnaley et al., 2012; Rasmussen et al., 2015; Selosse et al., 2022). Stable isotope work by Gebauer et al. (2016) revealed that a greater number than previously thought of orchids are likely reliant on OMF, and are functioning as myco-heterotrophs even though they are photosynthetic as adults. This finding of likely orchid dependence on OMF as adults, especially epiphytic orchids, raises the question of the potential role that OMF play in driving their fine-scale spatial distribution.

The drivers of fine scale epiphyte spatial distribution and host tree (phorophyte) specificity have been debated within the literature for over a century since the writings of Schimper (1888) see review by Wagner et al. (2015). Debate has focused on the role of various abiotic factors (e.g., microclimate and host bark characteristics) and biotic factors (e.g., symbiotic fungi and co-occurrence with moss). Research by McCormick et al. (2018) has demonstrated that while OMF may restrict terrestrial orchid distributions at local scales, at broad geographic scales terrestrial orchids are not constrained by OMF. Most of these findings were established for terrestrial orchids, with investigations of epiphytic orchids still pending (Li et al., 2021). Recently, studies have investigated fungal communities in the bark of phorophytes of epiphytic orchids, which providing insights into phorophyte specificity and spatial distribution of epiphytic orchids (Izuddin et al., 2019; Eskov et al., 2020; Pecoraro et al., 2021; Petrolli et al., 2021, 2022). Eskov et al. (2020) further explored OMF and revealed that fungi colonizing epiphytic orchid roots were significantly different from the phorophytes' branches. Pecoraro et al. (2021) studied the phorophyte specificity of two epiphytic orchid species, as well as the environmental factors influencing the relationship between the orchids and their phorophytes. They concluded that the orchid phorophyte associations were influenced by the phorophyte bark's OMF communities and potentially its pH and water holding capacity. Recent studies have also revealed examples of a strong fungal specificity of epiphytic orchids associated with a single OMF species, *Ceratobasidium* or Tulasnellaceae species (Rammitsu et al., 2019, 2020) despite inconclusive early studies (Gowland et al., 2013; Wang et al., 2017).

Amplicon sequencing, a type of environmental sequencing, is a cost-effective advancement for investigating fungal communities compared to traditional culture-based methods (McCormick and Jacquemyn, 2014; McCormick et al., 2018). Ectomycorrhizal (ECM) fungi as well as some OMF are known to be recalcitrant to being cultured and recent studies utilizing amplicon sequencing have detected a diversity of ECM fungi in the roots of both epiphytic and terrestrial orchids (Selosse et al., 2022). Additionally, amplicon sequencing can increase the detection of potential OMF in epiphytic orchid roots compared to Sanger Sequencing as Sanger Sequencing is often limited by the need for first culturing the fungi (Waud et al., 2016; Jacquemyn et al., 2017; Novotná et al., 2018; Johnson et al., 2021).

We chose the rare leafless epiphytic orchid *Dendrophylax lindenii* (Lindl.) Bentham ex Rolfe (Figure 1) as our study taxon to further document OMF communities of rare tropical epiphytic orchids and to examine the potential role of OMF as drivers of their phorophyte specificity. In addition to sampling the roots of *D. lindenii* we sampled co-occurring epiphytic orchids and the bark of potential phorophytes with and without *D. lindenii* to uncover evidence of OMF specificity



during two periods, flooded and not flooded in the area of its natural distribution in the United States (Supplementary Figures S1, S2).

Dendrophylax lindenii, also known as the Ghost Orchid, is restricted to southwestern Florida and the western tip of Cuba (Brown, 2002) where it remains vulnerable to poaching and environmental changes (Mújica et al., 2018, 2021). In Florida, less than 1,500 individuals are thought to remain (Haaland et al., 2022), and in Cuba, the number is even fewer [<500; (Mújica et al., 2018)]. In the Florida Panther National Wildlife Refuge where about 1/3rd of the state's Ghost Orchids are found, Mújica et al. (2018) calculated that *D. lindenii* numbers will decline by 20% during the next decade. Consequently, the species is now a candidate for U.S. Federal protection under the Endangered Species Act (Haaland et al., 2022). The Florida habitats of *D. lindenii* consist of cypress domes and strand swamps in the Big Cypress Basin. According to a study by Mújica et al. (2018) in 2015, 69% of the growth of *D. lindenii* in Florida is found on the trunks of *Fraxinus caroliniana* Mill., while occurring less frequently (36%) on *Annona glabra* L. These trees are typically located in the lower canopy under *Taxodium distichum* (L.) Rich. (Brown, 2002; Stewart and Richardson, 2008). Although *D. lindenii* grows in a moist habitat, it experiences dry periods during the region's dry season which lacks any standing water (Mújica et al., 2018).

Like all orchids, *D. lindenii* requires OMF for germination (Hoang et al., 2017). Early seedling stages of *D. lindenii* have a rudimentary ephemeral leaf. As an adult, the orchid lacks leaves and shoots and photosynthesizes predominantly via its roots (Benzing and Ott, 1981; Benzing et al., 1983; Hoang et al., 2017). Benzing and Ott (1981), have shown that the mature roots of *D. lindenii* utilizes CAM photosynthesis, and Chomicki et al. (2014) using microscopy hypothesized that it forms a mutualism with an OMF (*Ceratobasidiaceae*) to obtain carbon to supplement its photosynthesis. Furthermore, seed germination experiments by Hoang et al. (2017) and Mújica et al. (2018) have confirmed that *D. lindenii* associates with a *Ceratobasidium* and that this fungus is present in mature roots.

Our primary aim for this study was to identify the OMF associated with *D. lindenii* and to investigate the potential role of OMF in influencing its fine-scale distribution within naturally occurring populations (i.e., why it was found on some potential phorophytes and not on others). We tested two hypotheses: (1) *D. lindenii* has a specific community of OMF compared to co-occurring epiphytic orchids; and

(2) the OMF colonizing *D. lindenii* are found in the bark of *D. lindenii* phorophytes in higher abundances than in the bark of trees without *D. lindenii*. Given that *D. lindenii* is currently state-listed as endangered, we primarily restricted our sampling to root tips to minimize damage to the plant. To investigate if additional OMF were missed with this sampling method we also investigated the fungal community of four whole roots.

Materials and methods

Study sites, tree bark and orchid root sampling

During 2016 and 2018 we collected >100 root and bark samples from five sites at the Florida Panther National Wildlife Refuge (FPNWR) a 10,684 ha area (Supplementary Table S1). Four of the sites were natural habitats for *D. lindenii*. The fifth site lacked naturally occurring plants but had *D. lindenii* explants that were micropropagated under axenic conditions in the lab and subsequently transplanted (attached) on appropriate species of trees. The site with explants we identified as Site 4 in our study. Most of the sites were dominated (over 90%) by *F. caroliniana* as the main phorophyte. Sites were either sloughs or strand swamps and were separated by about 1 km from each other. When we collected samples in 2016 (March), FPNWR sites all had standing water in sloughs and swamps (Supplementary Figure S1), but all sites were dry (not flooded) when we sampled in 2018 (April) (Supplementary Figure S2). This sampling period in 2018 was unusually dry. The precise sites at the FPNWR are not disclosed herein because *D. lindenii* and several co-occurring orchids are state-listed as endangered and remain highly vulnerable to poaching. For each site, Special Use collecting permits were obtained (USFWS, OMB Control # 1018–0102), and permission to access and sample *D. lindenii* populations was subsequently granted.

In March 2016, root samples were collected from four sites at the FPNWR Sites 1–4 (Supplementary Table S1). Root samples were collected from the leafless epiphytic orchid species *D. lindenii* ($n=9$) and several co-occurring epiphytic orchids: *Campylocentrum pachyrrhizum* (Rchb.f.) Rolfe ($n=3$), *Dendrophylax porrectus* (Rchb.f.) Carlswald & Whitten ($n=6$), *Epidendrum amphistomum* A. Rich. ($n=4$), *Epidendrum nocturnum* Jacq. ($n=1$) and *Prosthechea cochleata* (L.) W. E. Higgins ($n=3$). Simultaneous with the collection of root samples, bark samples were collected from phorophytes adjacent of all epiphytic orchids (Supplementary Table S1).

In April 2018, sampling of the roots of an additional 27 *D. lindenii* plants was carried out at the original four sites plus one additional site (Site 5). Concurrent with the root tissue collection, bark samples ($n=57$) were collected from phorophytes of *D. lindenii* and trees without *D. lindenii* (Supplementary Table S1). Five trees with and five trees without *D. lindenii* individuals were sampled at each of the five sites. The sampling design considered the position of *D. lindenii* on the tree and bark samples were collected from (1) the base of the tree trunk, (2) above *D. lindenii*, (3) the side of roots of *D. lindenii* root; and (4) the opposite side of the tree trunk (Supplementary Figure S3). In instances where *D. lindenii* was not present bark samples were collected from the base of the tree and three additional samples were taken at a height of at breast height (1.5M) from base, where *D. lindenii* would typically grow.

Additionally, we conducted a pilot study to assess the success of amplicon sequencing of root tips for revealing the OMF community of *D. lindenii*. We obtained 50 mm root samples collected from three mature individuals of *D. lindenii* at the FPNWR. A root of a *D. lindenii* that was home cultivated from Redlands, Florida was also sampled. For this pilot study, roots were cut into 5 mm long segments starting from the tip and labelled alphabetically (i.e., A, B, C, etc. see Supplementary Figure S4).

DNA extraction, PCR amplification, and amplicon sequencing

Approximately 5 mm of root tip and bark tissue was collected and stored in cetyltrimethylammonium bromide (CTAB) buffer. Root and bark samples were surface sterilized with 70% ethanol, and 50% Clorox® (2.6% sodium hypochlorite) using the method outlined in Bayman et al. (1997). Next, genomic DNA was extracted from root samples using the Qiagen DNeasy extraction kits (Qiagen, Valencia, CA, United States) following the manufacturer's instructions. DNA from bark samples was extracted with the modified CTAB method of Murray and Thompson (1980), and for difficult to extract samples the MOBIO Power Soil DNA Extraction kit (MOBIO Laboratories, Carlsbad, CA, United States) was used following the manufacturer's instruction.

The extracted genomic DNA from the 2016 root samples was amplified using the primers: ITS86f (5'-GTGAATCATCGAA TCTTTGAA-3'; Turenne et al., 1999) and ITS4 (5'-TCCTCCGCT TATTGATATGC-3'; White et al., 1990). These fungal primers (ITS86F/ITS4) amplify the internal transcribed region ITS, the standard fungal barcode, for ITS subregion 2 which is shown to be effective for delimiting OMF such as those in the Cantharellales.

Next, amplicons from the PCR products were produced using a three step PCR sequencing protocol (see Johnson et al., 2021 materials and methods). This included PCR steps that used modified primers with indices from the Nextera XT kit for 96 indices to sequence 2×250 bp. The final amplicon libraries generated for root and bark samples were quantified using a Qubit dsDNA HS kit (Invitrogen) and a Bioanalyzer-Agilent 2100 (Agilent Technologies, Santa Clara, CA, United States). Final amplicon libraries for root and bark samples were pooled together in equimolar concentrations and the final pool was then sequenced on an Illumina MiSeq at the Pritzker Lab at the Field Museum (Chicago, IL).

The root sections from the pilot study, root tips, and bark samples from 2018 were PCR amplified using modified fungal primers ITS86F/ITS4 with barcodes supplied from Novogene Bioinformatics Institute (Beijing, China) following the protocol applied in 2016. The generated final amplicon libraries were pooled to equimolar concentrations then shipped to Novogene and sequenced on an Illumina HiSeq. Sequences generated from this study were submitted to NCBI's Sequence Read Archive under the BioProject PRJNA948888.

Bioinformatics and statistical analyses

Initially, bioinformatics analyses were performed on roots and bark collected in 2016 separately. Subsequently, the sequences obtained from the bark, root, and root sections of the 2018

dataset were integrated, and bioinformatics analyses were conducted on these samples collectively to determine patterns of similar Operational Taxonomic Units (OTUs) between sample types.

To conduct bioinformatic analyses, the sequences were first quality filtered, followed by OTU clustering utilizing the PIPITS pipeline (version 1.4.0) default settings as described by Gweon et al. (2015). Briefly, PIPITS joined reads and quality filtered short reads (<50 bp), extracted non ITS fungal reads with the script ITSx (Bengtsson-Palme et al., 2013), then clustered OTUs at 95% sequence similarity. Additional PIPITS scripts assigned taxonomy to OTUs with the Ribosomal Database Project Classifier [a Naïve Bayesian Classifier (Wang et al., 2007)] and the UNITE database (Nilsson et al., 2019). Sequences for the HiSeq dataset was analyzed separately from the 2016 MiSeq dataset. The single difference between analysis of the MiSeq data analyses and HiSeq data analyses was omitting the ITSx step for the HiSeq data.

To further investigate differences between fungal communities we filtered rare OTUs that were less than 1,000 sequences, and the raw read abundances were then normalized with Cumulative Sum Scaling in the R package metagenomeSeq (Paulson et al., 2013). All statistical analyses were conducted within R (R Core Team, 2022). The visualization of abundance of sequences was first accomplished using Krona charts, which were generated using Krona-2.8.1 within R (Ondov et al., 2011). Bar graphs showing relative and read abundances were produced with the R package ggplot2. To better visualize differences between read abundances, the y-axis was truncated using the R package ggbreak (Xu et al., 2021).

Principal coordinate analysis (PCoA) was generated using Bray-Curtis distances with the R package vegan and visualized with ggplot2 (Wickham, 2016). Significance between fungal communities of *D. lindenii* and epiphytic orchid roots, phorophyte and the bark of trees without *D. lindenii* present, and location of sites were determined with “permutational manova” (Anderson, 2001) in R package vegan (adonis2 function) by first permuting the raw data with 9,999 permutations (Oksanen et al., 2022). Prior to executing adonis2 for the permutational multivariate analysis of variance (PERMANOVA) we also investigated the dispersion for groups using another vegan function betadisper. In addition, pairwise comparisons were completed for the PERMANOVA using the pairwiseAdonis R package with Bonferroni corrections (Martinez Arbizu, 2017). *p*-values that are <0.05 were considered significant.

Phylogenetic analyses were undertaken to investigate relationships among the community of recovered Ceratobasidiaceae sequences from 2016 and 2018 root and bark fungal samples. The phylogenetic tree incorporated *Ceratobasidium* sequences from NCBI GenBank. A sequence of *Tulasnella* from the UNITE fungal database was used as an outgroup. We used MUSCLE (Edgar, 2004) in AliView version 1.27 (Larsson, 2014) for multiple sequence alignments and also used AliView to generate a Maximum Likelihood tree using the default settings of the program FastTree version 2.1.10 (Price et al., 2009). The final tree was rooted and visualized using FigTree version 1.4.4.¹

Results

Sequence analyses of root and bark samples

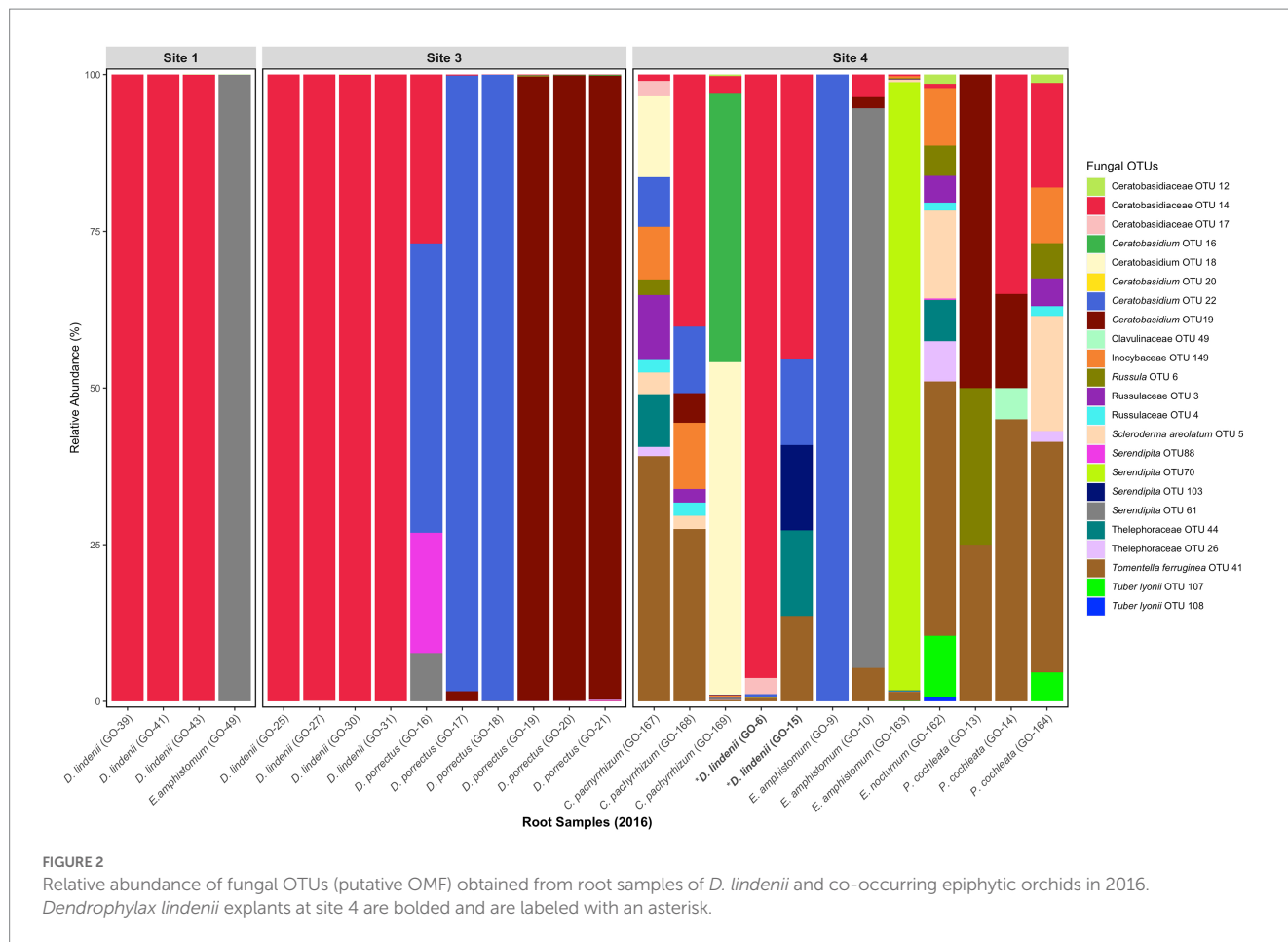
Fungal sequence data were obtained from roots of *D. lindenii* and other co-occurring epiphytic orchids collected in the field in 2016 and 2018. Root samples in 2016 and 2018 yielded 537,371 ($n=26$) and 1,691,086 reads ($n=30$), respectively, resulting in the identification of 526 and 1,077 Operational Taxonomic Units (OTUs) at the 95% sequence identity level. Sequences generated from sections of whole roots yielded 3,205,959 reads ($n=37$ root section samples) and resulted in the identification of 1,372 OTUs. In 2016, we collected 30 bark samples and sequencing yielded 693,482 reads with a total of 550 OTUs. Most phorophytes sampled in 2016 were from *F. caroliniana* (over 90%) with a small proportion of *A. glabra* also being sampled (Supplementary Table S1). Additionally, a bark sample was collected from a *Taxodium distichum* that had an explant affixed to it (Supplementary Table S1). In 2018 we successfully sequenced 57 bark samples mostly from *F. caroliniana*, from trees with *D. lindenii* ($n=43$) and trees without *D. lindenii* ($n=14$), yielding 7,245,995 reads with a total of 1,141 OTUs resolved.

The increase in read and OTU counts in 2018 can be attributed to the use of the HiSeq platform instead of the MiSeq platform, as well as the greater sampling intensity of root samples. The average OTU richness observed in root and bark samples for 2016 was 67 and 55 respectively, whereas the OTU richness for 2018 samples for roots was 233 and 304 for bark (Supplementary Table S1). Unfortunately, no amplicon libraries were generated for root samples collected at Site 2 in 2016 as the library preps were unsuccessful resulting in sequence data that was unsuitable for data analysis. The analysis of the pilot study examining potential differences in fungal communities in different sections of entire roots of both cultivated and wild collected *D. lindenii* documented that fungal communities were similar across all sections but were different between cultivated vs. wild collected plants (Supplementary Figure S5).

Ceratobasidiaceae is the dominant OMF associated with *Dendrophylax lindenii*

The fungal communities of naturally occurring *D. lindenii* roots across all sites were observed to be similar and dominated by several Ceratobasidiaceae, even during the flooded (2016) (Figure 2; Supplementary Figure S6A) and not flooded (2018) periods (Supplementary Figure S6B). The dominant Ceratobasidiaceae OTUs associated with *D. lindenii* were OTU 11 (recovered from bark samples), OTU 14 (recovered from 2016 root samples); and OTU 76 (recovered from both 2018 root and bark samples). Phylogenetic analysis resolved each of these dominant *Ceratobasidium* OTUs as part of a well-supported monophyletic clade, Clade 2, and are considered conspecific (Figure 3). A visual analysis of sequence alignments further supports this finding, as Clade 2 OTUs exhibit a sequence similarity of more than 98%. *Ceratobasidium* Clade 2 includes other individuals that were previously recovered from mature roots of *D. lindenii*. Included in Clade 2 is Dlin-394, which was derived from cultures isolated from mature roots of *D. lindenii* and has been used to germinate seeds of *D. lindenii* (Hoang et al., 2017).

¹ <http://tree.bio.ed.ac.uk/software/figtree/>



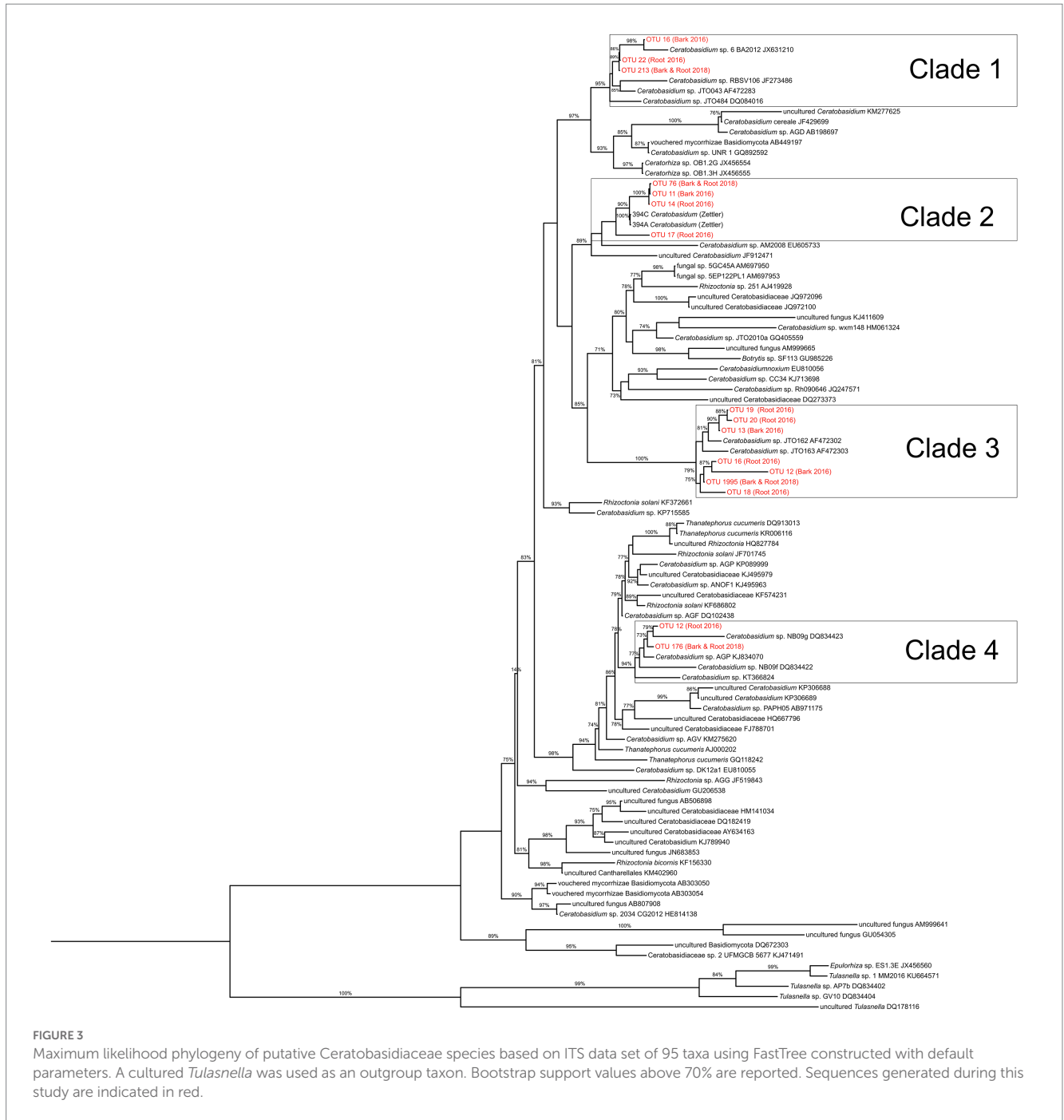
The pilot study undertaken to assess if the OMF community recovered from root tips of *D. lindenii* provided was reflective of the full root OMF community provided further evidence of the dominance of *Ceratobasidium* Clade 2 in naturally occurring *D. lindenii*. The majority of root sections from naturally occurring *D. lindenii*, including the root tips, were dominated by *Ceratobasidium* Clade 2 (Supplementary Figure S5). Of note, root section samples of the home-cultivated *D. lindenii* lacked *Ceratobasidiaceae* OTUs. Instead, an abundance of Ascomycota OTU reads (*Lasiodiplodia* OTU 1174 and *Diaportheales* OTU 627) were recovered (Supplementary Figure S5).

Ceratobasidium Clade 2 was present in most samples of *D. lindenii* (Supplementary Figure S6C); however, it was absent or only had very low read numbers from the roots of co-occurring epiphytic orchids (Figure 2). Other taxa of *Ceratobasidiaceae* were recovered from these orchids, e.g., *Ceratobasidiaceae* OTUs 19 and 22 were abundant in root samples of *D. porrectus* (Figure 2). These OTUs belonged to different clades (Clade 1 and 3, Figure 3). Other OMF taxa that were recovered from co-occurring epiphytic orchid roots collected in 2016 (Figure 2; Supplementary Figures S6A,B) included taxa of putative OMF Serendipitaceae and ECM fungi such as *Inocybaceae*, *Russulaceae*, *Scleroderma*, *Thelephoraceae*, *Tomentella*, and *Tuber* species. While present in lower proportions (<1%) we did not detect a widespread presence of *Tulasnellaceae* OTUs, a traditional OMF.

Diversity of OMF in bark of *Dendrophylax lindenii* phorophytes and trees without *Dendrophylax lindenii*

Unlike the root fungal community, the bark fungal community was not dominated by *Ceratobasidium* Clade 2 or other *Ceratobasidiaceae* OTUs. *Ceratobasidiaceae* OTUs accounted for less than 5% of the total reads (Supplementary Figures S7A,B). Nonetheless, *Ceratobasidium* Clade 2 was present in most bark samples (Figure 4). Other putative OMF detected in bark samples were also rare and included a few Serendipitaceae and *Tulasnella* OTUs. Additional rare OTUs also included ECM fungi including *Mycena*, *Russula*, *Thelephoraceae*, and *Tomentella*.

The principal coordinate analysis (PCoA) and subsequent PERMANOVA tests on root samples collected in both 2016 and 2018 showed significant differences between sites and orchid species (Supplementary Figures S8A,B). Specifically, the 2016 root samples reveal potentially significant differences in both orchid species (PERMANOVA: $F_{5,25} = 2.11$, $R^2 = 0.29$, $p < 0.05$, betadisper: $F = 2.08$, $p = 0.11$) and location (PERMANOVA: $F_{2,25} = 3.20$, $R^2 = 0.18$, $p < 0.05$, betadisper: $F = 5.09$, $p = 0.01$). Furthermore, pairwise comparisons showed significant differences between Site 4 (site with only introduced *D. lindenii* lab grown explants) and the two other sites, Site 1 (adjusted $p = 0.009$) and Site 3 (adjusted $p = 0.003$). In addition, Site 4 also differed from Site 3 (adjusted



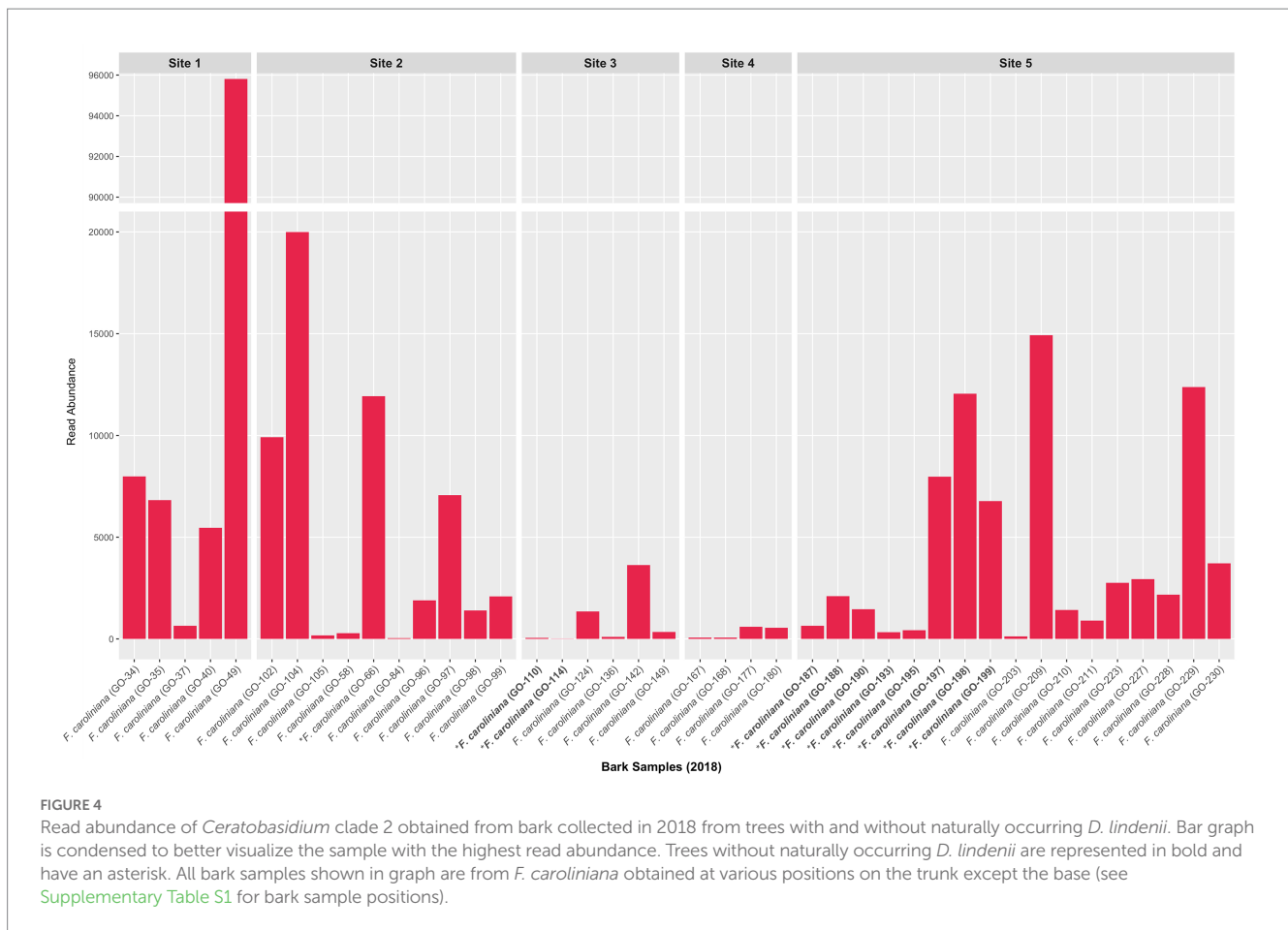
$p = 0.03$). Similarly, the *D. lindenii* root samples collected in 2018 revealed significant differences by site (PERMANOVA: $F_{4,24} = 1.26$, $R^2 = 0.20$, $p = 0.036$, betadisper: $F = 1.24$, $p > 0.5$). However, pairwise comparisons revealed no significant differences between sites when adjusted p values were generated.

PCoA of bark data collected for 2016 (Supplementary Figure S9A) revealed significant differences for both location (PERMANOVA: $F_{4,34} = 1.82$, $R^2 = 0.16$, $p < 0.05$, betadisper: $F = 1.47$, $p > 0.05$) and the presence of *D. lindenii* (PERMANOVA: $F_{4,34} = 1.50$, $R^2 = 0.03$, $p = 0.028$, betadisper: $F = 0.69$, $p = 0.4$). Although sites were not different during the flooded period of 2016, pairwise comparisons of the 2018 bark

data (corresponding PCoA is Supplementary Figure S9B) revealed differences between Site 1 and Site 3 (adjusted $p = 0.01$); differences between Site 2 and Site 5 (adjusted $p = 0.03$); and differences between Site 2 and Site 4 (adjusted $p = 0.05$).

Discussion

Our study provides strong evidence that *D. lindenii* may have a high specificity for a single Ceratobasidiaceae OTU (*Ceratobasidium* Clade 2) in its natural habitat at the Florida



Panther National Wildlife Refuge (FPNWR). This OTU was found to be abundant in *D. lindenii* roots, and rare (<1% of total reads) in other co-occurring epiphytic orchids at the FPNWR. *Ceratobasidium* Clade 2 was also widespread at all sites in the bark of phorophytes with *D. lindenii* and potential phorophyte trees without *D. lindenii* during both flooded (2016) and not flooded periods (2018).

This apparent extreme fungal specificity for one OMF, *Ceratobasidium* Clade 2, is similar to that reported for mycoheterotrophic orchids (McKendrick et al., 2002; Selosse et al., 2002), terrestrial orchids (Thixton et al., 2020), and some epiphytic orchids (Otero et al., 2002, 2004; Graham and Dearnaley, 2012; Rammitsu et al., 2019, 2021a,b). Our findings of potential high specificity with *Ceratobasidium* Clade 2 aligns with previous studies demonstrating the importance of *Ceratobasidium* taxa supporting healthy populations of other epiphytic orchids. For instance, Qin et al. (2021) and Rammitsu et al. (2019) reported on other leafless epiphytic orchids that have a high specificity for single *Ceratobasidium* species. Furthermore, *Ceratobasidium* Clade 2 is conspecific (>99% similar) with *Ceratobasidium* (Dlin-394) that was isolated and brought into culture from roots of *D. lindenii* that was used to germinate *D. lindenii* seeds (Hoang et al., 2017).

We also observed evidence of possible specificity in some of the other co-occurring epiphytic orchids, but the sample size was small for many of these epiphytic orchids and clear hypotheses could not

be tested. Nevertheless, these orchids associated with different *Ceratobasidium*. For example, *Ceratobasidium* OTU 19 and 22 were detected primarily in *D. porrectus*, another leafless epiphytic orchid. We hypothesize, with a caveat of small sample size, that mature roots of leafless epiphytic orchids are dominated by a single OMF unique to that species.

In addition to traditional OMF, we detected low read abundances of ECM fungi in the roots of the epiphytic orchids examined. This is in contrast to aerial roots of *V. planifolia* which were heavily colonized by ECM fungi (Johnson et al., 2021). *Vanilla planifolia* is a hemiepiphytic orchid and it is possible that the ECM fungi in the aerial roots are from systemic colonization emanating from the terrestrial roots. ECM fungi have been commonly reported from terrestrial orchids, but except for those detected by Johnson et al. (2021) an abundance of ECM fungi has not been reported colonizing arial/epiphytic orchid roots.

Foliar orchids exhibited lower read abundances relative to *D. lindenii* and other leafless epiphytic orchids (data not shown) was observed in our study. We hypothesize that the greater photosynthetic capacity of foliar orchids provided by their leaves reduces their dependence on OMF for supplemental fungal carbon.

Amplicon sequencing enabled us to document the presence of ECM fungi that are resistant to culturing in the orchid root communities. However, we were not successful in recovering species

of Tulasnellaceae. Whether this was actually due to very low abundance of these species is not clear. Some primer bias of the primer pair ITS86F/ITS4 for *Tulasnella* species has been reported and this primer pair is likely poor for detecting *Tulasnella* spp. (Tederloo et al., 2015; Vogt-Schilb et al., 2020; Johnson et al., 2021; Rammitsu et al., 2021a). Thus, future work using primers that are not biased towards Tulasnellaceae is needed.

While bark is not a carbon source for orchids (Eskov et al., 2020), it is the likely source of the OMF that epiphytic orchids need for establishment including seed germination and seedling growth (Rasmussen et al., 2015). Pellitier et al. (2019) documented that tree bark can serve as an environmental filter for the fungal communities available to epiphytic orchids. Thus, the distribution of OMF in tree bark throughout an orchid's range could influence its fine-scale distribution. *Ceratobasidium* OTU Clade 2 was recovered from all trees with *D. lindenii*, the fungus was also recovered in low abundance from many potential phorophytes without the orchid, indicating that additional studies are necessary to comprehend the factors that contribute to the fine-scale distribution of *D. lindenii* beyond the presence of the required OMF. Although several *A. glabra* trees, the other phorophyte of *D. lindenii* in Florida, were sampled, we were not successful in obtaining sequences from those samples. Thus, attempts should be made to sample sufficient numbers of *A. glabra* to better understand the situation in Florida. Additionally, *D. lindenii* in Cuba is found on several phorophyte species in comparison to the two primary phorophyte species associated with *D. lindenii* in Florida. Therefore, a fuller understanding of factors influencing the fine-scale distribution of *D. lindenii* needs to include an analysis of Cuban phorophytes.

When present, *Ceratobasidium* Clade 2 in bark was recovered at low read abundances, i.e., <5% relative abundance even from bark samples collected adjacent to actively growing root tips of *D. lindenii*. If *Ceratobasidium* Clade 2 is functioning as a saprobe in bark, then it is likely an inefficient saprobe and being outcompeted by more efficient saprotrophic fungi in the bark fungal community.

Although some *F. caroliniana* in Site 4 had *Ceratobasidium* Clade 2 it may be below the threshold of abundance to facilitate establishment and support the growth of naturally occurring plants (McCormick et al., 2016). Understanding site differences in terms of the presence/abundance of *Ceratobasidium* Clade 2 and other factors influencing establishment is crucial to sustaining populations of *D. lindenii* and preventing 'senile' populations, an ageing orchid population that lacks seedling recruitment (Rasmussen et al., 2015).

The findings of this study indicate that *D. lindenii*, has high specificity for a specific taxon of *Ceratobasidium*, Clade 2. While this study provides data that suggest that the presence/absence (or very low abundance) of the required OMF influences which tree *D. lindenii* is likely to establish and persist, the fungus is probably not the sole factor driving fine-scale distribution. Future studies should focus on the role of abiotic factors, such as bark characteristics like pH and phenolics, on *Ceratobasidium* growth, as well as the ability of the orchid to establish on the tree surface. Pellitier et al. (2019) demonstrated that fungal communities are likely affected by pH and total phenolic content, therefore experiments to test this hypothesis should consider other abiotic factors.

This study establishes the usefulness of amplicon sequencing as a method to examine fungal communities in the roots of endangered orchids such as *D. lindenii*. Sampling the actively growing root tips provides a non-destructive sampling method for future studies of this and other threatened and endangered orchids. Naturally occurring *D. lindenii* appears to partner with a specific undescribed species of *Ceratobasidium*. However, lab-grown explants of *D. lindenii* have low abundance so long-term survival and successful reintroduction to natural habitats should account for potential phorophytes with abundant *Ceratobasidium* present for a viable conservation method. Additionally, while the presence of the required fungus is necessary for establishment of the orchid on a particular tree, it is likely that other factors which impact its fine-scale distribution, are also involved. Understanding how the difference in OMF abundance between naturally occurring plants and explants and the factors influencing successful establishment on phorophytes are needed to enhance the success of efforts to augment the population of the Ghost Orchid and refine conservation actions.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found at: NCBI; PRJNA948888.

Author contributions

LJ and MC contributed to the study conception, performed sample preparation, and data collection. LJ and GM wrote the first draft of the manuscript with an initial review by LZ. All authors contributed with comments on the later versions of the manuscript and approved of the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2023.1057940/full#supplementary-material>

References

- Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 26, 32–46. doi: 10.1111/j.1442-9993.2001.01070.pp.x
- Bayman, P., Lebrun, L. L., Tremblay, R. L., and Lodge, D. J. (1997). Variation in endophytic fungi from roots and leaves of *Lepanthes* (Orchidaceae). *New Phytol.* 135, 143–149. doi: 10.1046/j.1469-8137.1997.00618.x
- Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A., et al. (2013). Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. *Methods Ecol. Evol.* 4, 914–919. doi: 10.1111/2041-210X.12073
- Benzing, D. H., Friedman, W. E., Peterson, G., and Renfrow, A. (1983). Shootlessness, velamentous roots, and the pre-eminence of Orchidaceae in the epiphytic biotope. *Am. J. Bot.* 70, 121–133. doi: 10.2307/2443212
- Benzing, D. H., and Ott, D. W. (1981). Vegetative reduction in epiphytic Bromeliaceae and Orchidaceae: its origin and significance. *Biotropica* 13:131. doi: 10.2307/2387715
- Brown, P. M. (2002). *Wild Orchids of Florida, with References to the Atlantic and Gulf Coastal Plains*. Gainesville, Florida: University Press of Florida.
- Chomicki, G., Bidel, L. P. R., and Jay-Allemand, C. (2014). Exodermis structure controls fungal invasion in the leafless epiphytic orchid *Dendrophylax lindenii* (Lindl.) Benth. Ex Rolfe. *Flora-Morphol. Distrib. Funct. Ecol. Plants* 209, 88–94. doi: 10.1016/j.flora.2014.01.001
- Dearnaley, J. D. W., Martos, F., and Selosse, M.-A. (2012). “12 orchid Mycorrhizas: molecular ecology, physiology, evolution and conservation aspects” in *Fungal Associations*. ed. B. Hock (Berlin, Heidelberg: Springer Berlin Heidelberg), 207–230.
- Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340
- Eskov, A. K., Voronina, E. Y., Tedersoo, L., Tiunov, A. V., Manh, V., Prilepsky, N. G., et al. (2020). Orchid epiphytes do not receive organic substances from living trees through fungi. *Mycorrhiza* 30, 697–704. doi: 10.1007/s00572-020-00980-w
- Gebauer, G., Preiss, K., and Gebauer, A. C. (2016). Partial mycoheterotrophy is more widespread among orchids than previously assumed. *New Phytol.* 211, 11–15. doi: 10.1111/nph.13865
- Gowland, K. M., van der Merwe, M. M., Linde, C. C., Clements, M. A., and Nicotra, A. B. (2013). The host bias of three epiphytic Aeridinae orchid species is reflected, but not explained, by mycorrhizal fungal associations. *Am. J. Bot.* 100, 764–777. doi: 10.3732/ajb.1200411
- Graham, R. R., and Dearnaley, J. D. W. (2012). The rare Australian epiphytic orchid *Sarcochilus weinthalii* associates with a single species of *Ceratobasidium*. *Fungal Divers.* 54, 31–37. doi: 10.1007/s13225-011-0106-0
- Gweon, H. S., Oliver, A., Taylor, J., Booth, T., Gibbs, M., Read, D. S., et al. (2015). PIPITS: an automated pipeline for analyses of fungal internal transcribed spacer sequences from the Illumina sequencing platform. *Methods Ecol. Evol.* 6, 973–980. doi: 10.1111/2041-210X.12399
- Haaland, D., Estenoz, S., Williams, M., Director, P. D., and Williams, L. (2022). Petition to List the Ghost Orchid (*Dendrophylax lindenii*), as Threatened or Endangered Under the Endangered Species Act, and to Designate Critical Habitat.
- Hoang, N. H., Kane, M. E., Radcliffe, E. N., Zettler, L. W., and Richardson, L. W. (2017). Comparative seed germination and seedling development of the ghost orchid, *Dendrophylax lindenii* (Orchidaceae), and molecular identification of its mycorrhizal fungus from South Florida. *Ann. Bot.* 119, 379–393. doi: 10.1093/aob/mcw220
- Izuddin, M., Srivathsan, A., Lee, A. L., Yam, T. W., and Webb, E. L. (2019). Availability of orchid mycorrhizal fungi on roadside trees in a tropical urban landscape. *Sci. Rep.* 9:19528. doi: 10.1038/s41598-019-56049-y
- Jacquemyn, H., Waud, M., Brys, R., Lallemand, F., Courty, P. E., Robionek, A., et al. (2017). Mycorrhizal associations and trophic modes in coexisting orchids: an ecological continuum between auto- and mixotrophy. *Front. Plant Sci.* 8:1497. doi: 10.3389/fpls.2017.01497
- Johnson, L. J. A. N., del González-Chávez, M., Carrillo-González, R., Porras-Alfaro, A., and Mueller, G. M. (2021). *Vanilla* aerial and terrestrial roots host rich communities of orchid mycorrhizal and ectomycorrhizal fungi. *Plants People Planet* 3, 541–552. doi: 10.1002/ppp3.10171
- Larsson, A. (2014). AliView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics* 30, 3276–3278. doi: 10.1093/bioinformatics/btu531
- Li, T., Wu, S., Yang, W., Selosse, M.-A., and Gao, J. (2021). How mycorrhizal associations influence orchid distribution and population dynamics. *Front. Plant Sci.* 12:647114. doi: 10.3389/fpls.2021.647114
- Martinez Arbizu, P. (2017). PairwiseAdonis: Pairwise Multilevel Comparison Using Adonis. R Package Version 0.4.1.
- McCormick, M. K., and Jacquemyn, H. (2014). What constrains the distribution of orchid populations? *New Phytol.* 202, 392–400. doi: 10.1111/nph.12639
- McCormick, M. K., Taylor, D. L., Whigham, D. F., and Burnett, R. K. (2016). Germination patterns in three terrestrial orchids relate to abundance of mycorrhizal fungi. *J. Ecol.* 104, 744–754. doi: 10.1111/1365-2745.12556
- McCormick, M. K., Whigham, D. F., and Canchani-Viruet, A. (2018). Mycorrhizal fungi affect orchid distribution and population dynamics. *New Phytol.* 219, 1207–1215. doi: 10.1111/nph.15223
- McKendrick, S. L., Leake, J. R., Lee Taylor, D., and Read, D. J. (2002). Symbiotic germination and development of the myco-heterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp. *New Phytol.* 154, 233–247. doi: 10.1046/j.1469-8137.2002.00372.x
- Mújica, E. B., Herdman, A. R., Danaher, M. W., González, E. H., and Zettler, L. W. (2021). Projected status of the ghost orchid (*Dendrophylax lindenii*) in Florida during the next decade based on temporal dynamic studies spanning six years. *Plants*. 10:1579. doi: 10.3390/plants10081579
- Mújica, E. B., Mably, J. J., Skarha, S. M., Corey, L. L., Richardson, L. W., Danaher, M. W., et al. (2018). A comparison of ghost orchid (*Dendrophylax lindenii*) habitats in Florida and Cuba, with particular reference to seedling recruitment and mycorrhizal fungi. *Bot. J. Linn. Soc.* 186, 572–586. doi: 10.1093/botlinnean/box106
- Murray, M. G., and Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8, 4321–4326. doi: 10.1093/nar/8.19.4321
- Nilsson, R. H., Larsson, K.-H., Taylor, A. F. S., Bengtsson-Palme, J., Jeppesen, T. S., Schigel, D., et al. (2019). The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* 47, D259–D264. doi: 10.1093/nar/gky1022
- Novotná, A., Benítez, Á., Herrera, P., Cruz, D., Filipczyk, E., and Suárez, J. P. (2018). High diversity of root-associated fungi isolated from three epiphytic orchids in southern Ecuador. *Mycoscience* 59, 24–32. doi: 10.1016/j.myc.2017.07.007
- Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., et al. (2022). Vegan: Community Ecology Package. R Package Version 2.6-4.
- Ondov, B. D., Bergman, N. H., and Phillippy, A. M. (2011). Interactive metagenomic visualization in a web browser. *BMC Bioinformatics* 12:385. doi: 10.1186/1471-2105-12-385
- Otero, J. T., Ackerman, J. D., and Bayman, P. (2002). Diversity and host specificity of endophytic *Rhizoctonia*-like fungi from tropical orchids. *Am. J. Bot.* 89, 1852–1858. doi: 10.3732/ajb.89.11.1852
- Otero, J. T., Ackerman, J. D., and Bayman, P. (2004). Differences in mycorrhizal preferences between two tropical orchids. *Mol. Ecol.* 13, 2393–2404. doi: 10.1111/j.1365-294x.2004.02223.x
- Paulson, J. N., Colin Stine, O., Bravo, H. C., and Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nat. Methods* 10, 1200–1202. doi: 10.1038/nmeth.2658

- Pecoraro, L., Rasmussen, H. N., Gomes, S. I. F., Wang, X., Merckx, V. S. F. T., Cai, L., et al. (2021). Fungal diversity driven by bark features affects phorophyte preference in epiphytic orchids from southern China. *Sci. Rep.* 11:11287. doi: 10.1038/s41598-021-90877-1
- Pellitier, P. T., Zak, D. R., and Salley, S. O. (2019). Environmental filtering structures fungal endophyte communities in tree bark. *Mol. Ecol.* 28, 5188–5198. doi: 10.1111/mec.15237
- Petrolli, R., Augusto Vieira, C., Jakalski, M., Bocayuva, M. F., Vallé, C., Cruz, E. D. S., et al. (2021). A fine-scale spatial analysis of fungal communities on tropical tree bark unveils the epiphytic rhizosphere in orchids. *New Phytol.* 231, 2002–2014. doi: 10.1111/nph.17459
- Petrolli, R., Zinger, L., Perez-Lamarque, B., Collobert, G., Griveau, C., Pailler, T., et al. (2022). Spatial turnover of fungi and partner choice shape mycorrhizal networks in epiphytic orchids. *J. Ecol.* 110, 2568–2584. doi: 10.1111/1365-2745.13986
- Price, M. N., Dehal, P. S., and Arkin, A. P. (2009). Fasttree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* 26, 1641–1650. doi: 10.1093/molbev/msp077
- Qin, J., Zhang, W., Feng, J.-Q., and Zhang, S.-B. (2021). Leafless epiphytic orchids share Ceratobasidiaceae mycorrhizal fungi. *Mycorrhiza* 31, 625–635. doi: 10.1007/s00572-021-01043-4
- R Core Team (2022). R: A Language and Environment for Statistical Computing. Available at: <https://www.R-project.org/>.
- Rammitsu, K., Abe, S., Abe, T., Kotaka, N., Kudaka, M., Kudaka, N., et al. (2021a). The endangered epiphytic orchid *Dendrobium okinawense* has a highly specific mycorrhizal association with a single Tulasnellaceae fungus. *J. For. Res.* 26, 215–221. doi: 10.1080/13416979.2021.1876587
- Rammitsu, K., Kajita, T., Imai, R., and Ogura-Tsujita, Y. (2021b). Strong primer bias for Tulasnellaceae fungi in metabarcoding: specific primers improve the characterization of the mycorrhizal communities of epiphytic orchids. *Mycoscience* 62, 356–363. doi: 10.47371/mycosci.2021.06.005
- Rammitsu, K., Yagame, T., Yamashita, Y., Yukawa, T., Isshiki, S., and Ogura-Tsujita, Y. (2019). A leafless epiphytic orchid, *Taeniophyllum glandulosum* Blume (Orchidaceae), is specifically associated with the Ceratobasidiaceae family of basidiomycetous fungi. *Mycorrhiza* 29, 159–166. doi: 10.1007/s00572-019-00881-7
- Rammitsu, K., Yukawa, T., Yamashita, Y., Isshiki, S., and Ogura-Tsujita, Y. (2020). The mycorrhizal community of the epiphytic orchid *Thrixspermum japonicum* is strongly biased toward a single Ceratobasidiaceae fungus, despite a wide range of fungal partners. *Am. J. Bot.* 107, 1654–1662. doi: 10.1002/ajb2.1575
- Rasmussen, H. N., Dixon, K. W., Jersáková, J., and Těšitelová, T. (2015). Germination and seedling establishment in orchids: a complex of requirements. *Ann. Bot.* 116, 391–402. doi: 10.1093/aob/mcv087
- Schimper, A. F. W. (1888). *Die Epiphytische Vegetation Amerikas*. Jena, Germany: Gustav Fischer Verlag.
- Selosse, M.-A., Petrolli, R., Mujica, M. I., Laurent, L., Perez-Lamarque, B., Figura, T., et al. (2022). The waiting room hypothesis revisited by orchids: were orchid mycorrhizal fungi recruited among root endophytes? *Ann. Bot.* 129, 259–270. doi: 10.1093/aob/mcab134
- Selosse, M.-A., WEIss, M., Jany, J. L., and Tillier, A. (2002). Communities and populations of sebacoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) L.C.M. rich. And neighbouring tree ectomycorrhizae. *Mol. Ecol.* 11, 1831–1844. doi: 10.1046/j.1365-294x.2002.01553.x
- Smith, S. E., and Read, D. J. (2010). *Mycorrhizal Symbiosis*. 3rd edn New York, NY: Academic Press.
- Stewart, S., and Richardson, L. (2008). Orchid flora of the Florida Panther National Wildlife Refuge. *North Am. Native Orchid J.* 14, 70–104.
- Tedersoo, L., Anslan, S., Bahram, M., Pöhlme, S., Riit, T., Liiv, I., et al. (2015). Shotgun metagenomes and multiple primer pair-barcode combinations of amplicons reveal biases in metabarcoding analyses of fungi. *MycKeys* 10, 1–43. doi: 10.3897/mycokeys.10.4852
- Thixton, H. L., Esselman, E. J., Corey, L. L., and Zettler, L. W. (2020). Further evidence of *Ceratobasidium* D.P. Rogers (Basidiomycota) serving as the ubiquitous fungal associate of *Platanthera leucophaea* (Orchidaceae) in the north American tallgrass prairie. *Bot. Stud.* 61:12. doi: 10.1186/s40529-020-00289-z
- Turenne, C. Y., Sanche, S. E., Hoban, D. J., Karlowicz, J. A., and Kabani, A. M. (1999). Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. *J. Clin. Microbiol.* 37, 1846–1851. doi: 10.1128/JCM.37.6.1846-1851.1999
- Vogt-Schilb, H., Těšitelová, T., Kotilinek, M., Sucháček, P., Kohout, P., and Jersáková, J. (2020). Altered rhizocytia assemblages in grasslands on ex-arable land support germination of mycorrhizal generalist, not specialist orchids. *New Phytol.* 227, 1200–1212. doi: 10.1111/nph.16604
- Wagner, K., Mendieta-Leiva, G., and Zotz, G. (2015). Host specificity in vascular epiphytes: a review of methodology, empirical evidence and potential mechanisms. *AoB Plants* 7:plu092. doi: 10.1093/aobpla/plu092
- Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naïve bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07
- Wang, X., Li, Y., Song, X., Meng, Q., Zhu, J., Zhao, Y., et al. (2017). Influence of host tree species on isolation and communities of mycorrhizal and endophytic fungi from roots of a tropical epiphytic orchid, *Dendrobium sinense* (Orchidaceae). *Mycorrhiza* 27, 709–718. doi: 10.1007/s00572-017-0787-7
- Waud, M., Busschaert, P., Lievens, B., and Jacquemyn, H. (2016). Specificity and localised distribution of mycorrhizal fungi in the soil may contribute to co-existence of orchid species. *Fungal Ecol.* 20, 155–165. doi: 10.1016/j.funeco.2015.12.008
- White, T. J., Bruns, T., Lee, S., and Taylor, J. W. (1990). “Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics” in *PCR Protocols: A Guide to Methods and Applications*. eds. M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White (New York: Academic Press, Inc), 315–322.
- Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag.
- Xu, S., Chen, M., Feng, T., Zhan, L., Zhou, L., and Yu, G. (2021). Use ggbreak to effectively utilize plotting space to deal with large datasets and outliers. *Front. Genet.* 12:774846. doi: 10.3389/fgene.2021.774846
- Zotz, G. (2016). “The role of vascular epiphytes in the ecosystem” in *Plants on Plants—the Biology of Vascular Epiphytes*. (Springer Cham: Springer International Publishing), 229–243. doi: 10.1007/978-3-319-39237-0