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Generalized mycorrhizal interactions and fungal enemy release drive range expansion of orchids in southern Florida

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Citation: Downing, J. L., H. Liu, M. K. McCormick, J. Arce, D. Alonso, and J. Lopez-Perez. 2020. Generalized mycorrhizal interactions and fungal enemy release drive range expansion of orchids in southern Florida. Ecosphere 11(8):e03228. 10.1002/ecs2.3228

Abstract. A species' ability to establish and spread is influenced by different types of biotic interactions encountered in a new range. Species with high dependency on biotic interactions, such as orchids, are believed to have low ability to expand ranges. Using a comparative approach, we addressed the role of below-ground biotic interactions in the naturalization and spread of two introduced orchids. Using fungus-specific DNA primers and symbiotic germination trials, we identified the types of fungal taxa associated with *Cyrtopodium flavum* and *Eulophia graminea*, two invasive orchids in southern Florida, with that of two native congeners, *C. punctatum* and *E. alta*. We quantified the degrees of mycorrhizal specificity as well as associations with pathogenic fungi. We identified a total of 57 distinct fungal taxa, collected from a total of 104 root samples (67 adults and 37 seedlings), and 111 protocorms derived from fungal baits in southern Florida and southwest China. We found that invasive orchids were capable of associating with a broader range of mycorrhizal fungi than co-occurring native congeners (i.e., generalist strategy). Concurrently, invasive orchid species were less likely to harbor pathogenic fungal groups (Ascomycete) than native congeners, suggesting enemy release played a role as well in these orchids' naturalization and spread. These findings provided insights into the complex roles of mycorrhizal symbioses in range expansions.

Key words: invasive species; mutualism; orchid ecology; plant–fungal interactions.

Received 16 January 2020; revised 1 May 2020; accepted 5 May 2020; final version received 16 June 2020. Corresponding Editor: Michael F. Allen.

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Introduction

Understanding the environmental factors, both positive and negative, that influence plant range expansions has been an ongoing research interest in ecology. This is particularly relevant in the context of rapid globalization, which has increased the movement of species well beyond their natural ranges. Successful establishment of a new introductions is rare and depends on the suitability of abiotic conditions as well as the number and types of biotic interactions a species encounters upon arrival (Pringle et al. 2009, Canavan et al. 2019, Frost et al. 2019). For plants, the ability to acquire compatible mycorrhizal fungi in the recipient environment can facilitate the success of colonization and the lack of this ability can completely inhibit establishment as in obligate ectomycorrhizal trees (Nunez et al. 2009, Harrison et al. 2018). However, the plant–fungi network is dynamic and highly variable. This poses challenges to how we identify the mechanisms driving establishment, particularly regarding the role of plant specificity (obligate to facultative) which may be as important as the availability of compatible fungal symbionts

(Dickie et al. 2017, Séne et al. 2018). On the other hand, a plant's ability to resist antagonistic or pathogenic fungal interactions, as suggested by the enemy-release hypothesis (ERH), has likely contributed to the successful invasion of plants in a new range (Mitchell and Power 2003, Liu and Stiling 2006, Mangla and Callaway 2008, Diez et al. 2010, Kruse et al. 2016). No study, however, has explored the role of these two contrasting types of biotic interactions simultaneously in species range expansion.

Orchids as a plant family are seen as exceptions in global patterns of plant invasion because the family has much lower than average naturalization and invasion rates (Pemberton and Liu 2008). The dependency for obligate biotic interactions was proposed as a possible explanation for this low tendency or ability to expand range. Yet, below-ground orchid–fungal interactions are extremely diverse, and their ever increasing complexity is now appreciated as a result of major advancements in molecular taxonomy and electron microscopy, which allow deeper understanding in the topic (Dearnaley et al. 2012). All species of orchids derive the nutrients necessary for seed germination from orchid mycorrhizal fungi (OMF) in nature (Rasmussen 1995, Dearnaley et al. 2016). The type and degree of specificity in these interactions can regulate species' abundance and distribution and thus likely play a major role in both orchid rarity and invasiveness (McCormick et al. 2009, 2012, Swarts et al. 2010, Nomura et al. 2013, but see Pandey et al. 2013 and Phillips et al. 2011 for exceptions). In adult orchids, the relationships can be more complicated and may encompass the full range of associations with endophytic root fungi: from parasitic to mutualistic, pathogenic to mycorrhizal, and from generalist to specialist in their associations (Taylor 2004, McCormick et al. 2006, Dearnaley et al. 2016). The most documented orchid mycorrhizal associations are those with of basidiomycete fungi in the Class Agaricomycetes and particularly with members of the Sebacinales, Ceratobasidiaceae, and Tulasnellaceae. However, some fungi within the Ascomycetes, and the Class Pezizomycotina, have been found to be mycorrhizal in many tropical autotrophic orchids, important genera include *Tuber, Tricharina, and Peziza* (see review paper Dearnaley et al. 2012). Outside of the orchids, these fungi can also encompass the full spectrum of ecological roles including parasitic, saprotrophic, and endo- and ectomycorrhizal in other plants (Martos et al. 2009, Smith and Read 2010). De Long et al. (2013) examined the effect of orchid–fungus specificity on species range expansions by comparing terrestrial native orchids in Australia. It was found that the rapidly expanding, weedy species had a greater breadth of mycorrhizal associations and habitat types than less abundant co-occurring species. These results also provide important insights into how fungal specificity enables some orchid species to spread into new ranges.

In this study, we sought to understand how some orchids were able to beat the odds to become invasive in a new range. The variety of mycobiont interactions obtained by orchids allows the simultaneous testing of two hypotheses to explain the role of mutualistic vs. antagonistic biotic interactions in species expansion, that is (1) orchids that are able to invade new ranges are more generalized in their mycorrhizal associations than native congeners (i.e., greater species richness and phylogenetic breadth), and (2) invasive orchids will be less infected with pathogenic fungi in a new range than their native congeners (i.e., the enemy-release hypothesis or ERH; Keane and Crawley 2002). Our first hypothesis follows the patterns detected by Harrison et al. (2018), where legumes that were able to associate with a broad range of Rhizobia had more extensive introduced ranges than those that were only able to associate with specific Rhizobia. Species were able to expand their ranges where they encountered appropriate Rhizobia symbionts and species with specific associations were less likely to encounter an appropriate symbiont than more general ones. Our second hypothesis was formulated following the stipulation of the popular enemy-release hypothesis in that an introduced species become a successful invader because it leaves its specialist antagonistic partners behind (Liu and Stiling 2006), but with a flipped outcome when one considers mutualist partners. To test these two hypotheses, we employed a natural experiment in which two exotic orchids, *Cyrtopodium flavum* Link & Otto ex Rchb, and *Eulophia graminea* Lindl., recently naturalized and spread into natural areas in southern Florida, where they co-occur with two native congeners, *C. punctatum* (L.)Lindl. and *E.*

alta (L.) Fawc. & Rendle. Comparisons conducted within a phylogenetic context can limit confounding differences that may be intrinsic to taxonomic groups, providing greater power to distinguish characteristics specifically related to invasiveness.

Methods

Study species

The Florida cowhorn orchid (*C. punctatum*) is an epiphytic orchid that was thought to be historically abundant throughout southern Florida (Luer 1972; Fig. 1a, b). However, due to habitat loss and ongoing poaching, it is now rare and legally endangered in the state of Florida (Wunderlin and Hansen 2012). It is largely restricted to a small number of cypress domes and freshwater sloughs in southwestern Florida. In southeastern Florida, a small population persists near coastal areas in the Everglades National Park, and a semi-natural population also occurs at Fairchild Tropical Botanic Garden (FTBG). The Brazilian yellow cowhorn *C. flavum* (formerly *C. polyphyllum*) is a showy terrestrial orchid native to the southeastern coast of Brazil (Pansarin et al. 2008; Fig. 1c, d). Since the 1970s, it has volunteered in mulched areas and residential yards throughout central and southern Florida and has invaded at least three pine rockland forests in Miami-Dade County, Florida (Liu and Pemberton 2010). Wild coco (*E. alta*) is a terrestrial orchid that is native to southern Florida, but has a large natural distribution that spans North, Central, and South America, as well as West Africa (Luer 1972; Fig 1e, f). In Florida, *E. alta* is uncommon and restricted to the southwestern region with a patchy distribution (Stewart and Richardson 2008). It occupies a range of habitats from damp and semi-aquatic swamps and roadside ditches, to sunlit pastures. The Chinese ground orchid (*E. graminea*) is a terrestrial species that is native to Southeast Asia. (Fig 1g, h). The species has recently naturalized in Australia and North America. In Florida, it is currently listed as a Category II invasive plant (Florida Exotic Pest Plant Council List 2019). Pemberton et al. (2008) first reported this species in the Miami area in 2007, sporadically appearing in residential mulch piles throughout Miami-Dade County of Florida. Now, it can be readily found throughout urban landscapes, as well as in natural areas.

In situ tissue collection and fungal baiting

Protocorms and roots were collected during the rainy season (May–November) each year between the years 2013–2018. We obtained protocorm material from young seedlings and from fungus baits (Fig 2a, b). When we were unable to obtain living protocorms, we attempted to isolate the fungi and extract fungal DNA. Protocorms derived from fungal baits were then used to estimate germination rates at each site. For native species, we occasionally collected naturally germinated seedlings that still had protocorm bodies (Fig. 2c). For each species, we collected at least five root sections (>5 cm) from each adult individual sampled. We only selected roots that were in direct contact with the substrate for the best chance of obtaining fungi (Fig. 2d). Roots were removed using a clean razor blade and were stored at 4°C for up to one week before fungal isolation and DNA extraction.

For *C. punctatum,* we collected roots from 35 plants at two sites in southern Florida; Fairchild Tropical Botanic Garden (FTBG) and Fakahatchee Strand State Park (FSS). At FTBG, we sampled 22 plants (14 adults and 8 seedlings) that grow sporadically throughout trees in this 83-acre public garden. At FSS, we sampled a total of 13 plants (12 adults and one seedling). Within FSS, only two *C. punctatum* seedlings were observed, and due to the vulnerability of this population, we chose to collect only one root sample. For the native *E. alta*, we sampled a total of 9 plants at Corkscrew Swamp Sanctuary (CSS) site (6 adults and 3 seedlings). For the non-native *C. flavum*, we randomly sampled 32 plants at two newly invaded pine rocklands: ZOOMiami/ Gulf Coast Railroad Museum (ZOO) and the heavily infested Boystown Pineland (BT). At the ZOO site, we sampled 20 plants (7 adult and 13 seedlings), and at BT site, we sampled 12 plants (7 adults and 5 seedlings). For non-native *E. graminea*, we sampled a total of 26 plants (18 adults and 8 seedlings) from the FTBG and BT sites. At FTBG, we sampled 20 plants (13 adults and 7 seedlings), and at BT site, we sampled 6 plants (4 adults and 2 seedlings). We also collected roots from two adult plants in the native range of *E. graminea* in China, one each from Xishuangbanna

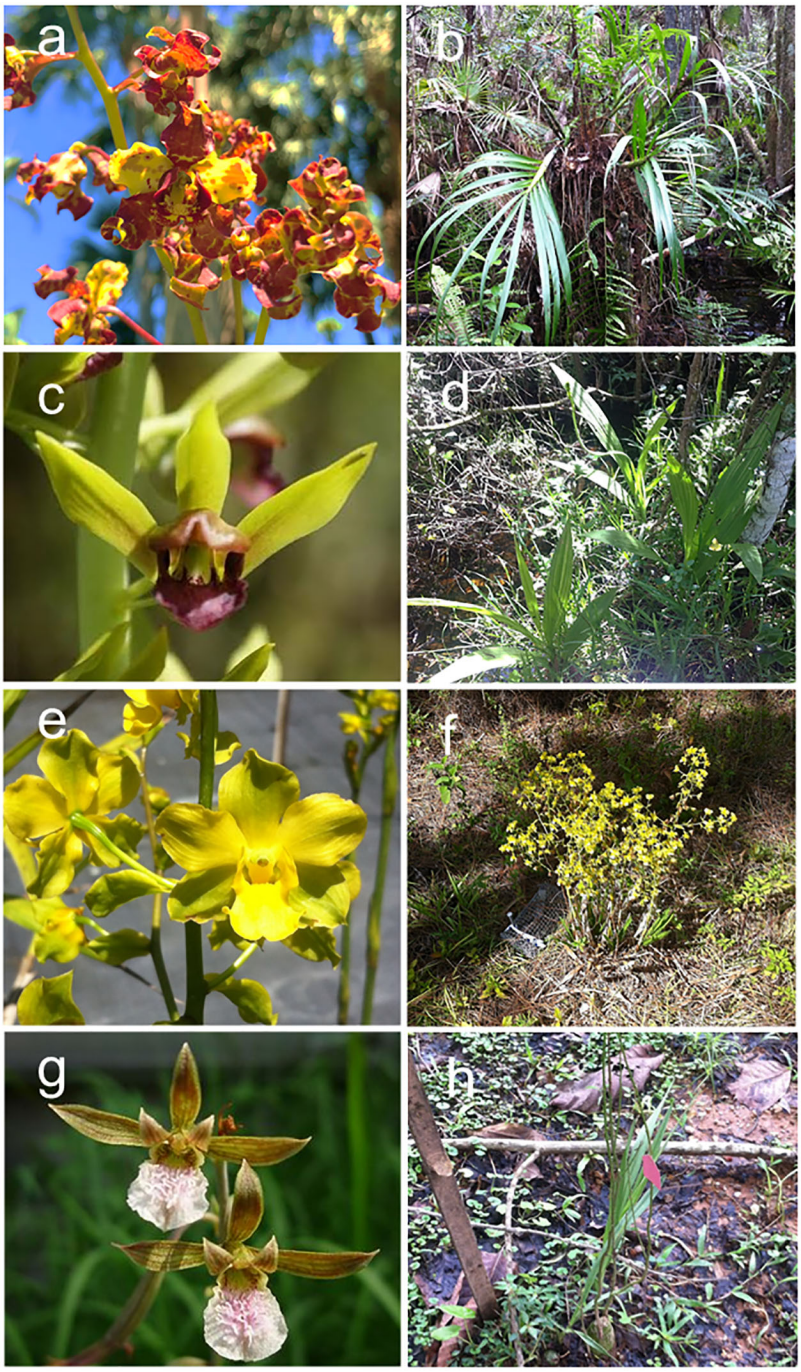


Fig. 1. Study species; Florida native *Cyrtopodium punctatum* flower (a), adult plant at Fakahatchee Strand State Park (b). *Eulophia alta* flower (c; photo: Christine Cook), adult plant (d) All plants were located at Corkscrew Swamp Sanctuary, the invasive *Cyrtopodium flavum* flower (e), adult plant (f) at Boystown Pineland, Miami-Dade County, Florida, and *Eulophia graminea* flowers (g; photos: Christine Cook), and adult plant (h) growing at Xishuangbanna Tropical Botanic Garden, Yunnan Province, China.

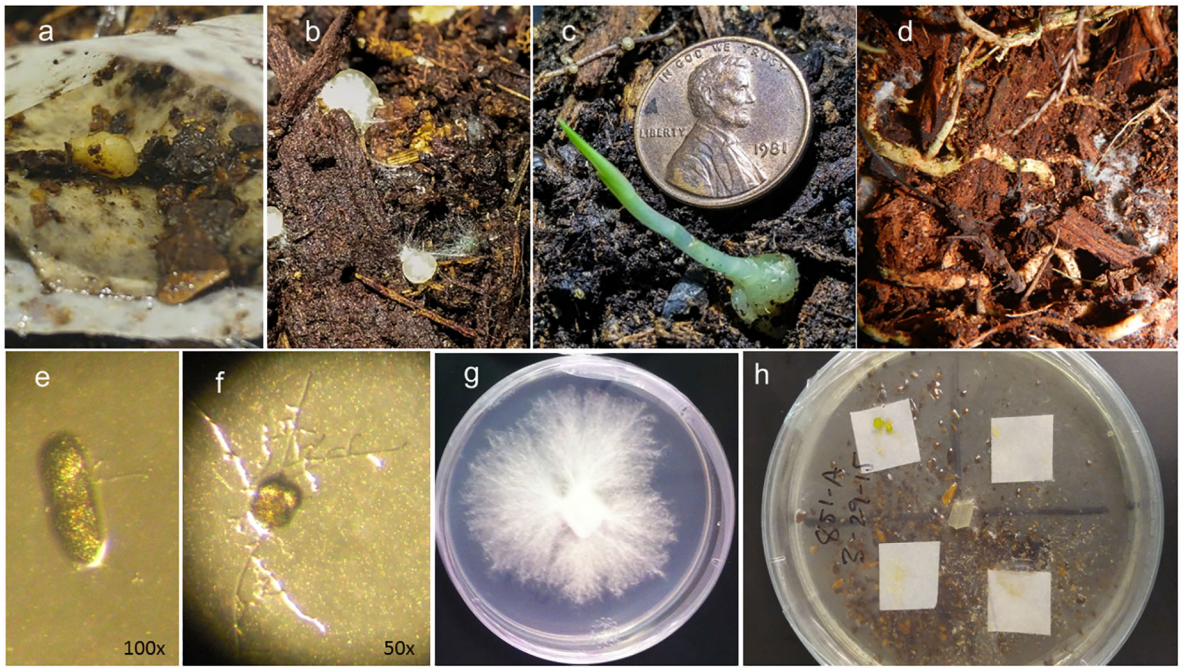


Fig. 2. Protocorm of *Cyrtopodium flavum* recovered from fungal baits at the invaded Boystown Pineland site (a), protocorms of *C. flavum* (b) showing hyphal penetration recovered in situ in mulch near Zoo Miami (b), Miami-Dade County, Florida. *Cyrtopodium punctatum* seedling with apical meristem and protocorm body (c), and *C. punctatum* roots with fungal hyphae at Fakahatchee Strand State Park (d; photo: Dennis Giardina), (e–g) cultures of Polyporales fungus (F-7) taken from *C. flavum* protocorms at Boystown Pineland; (e) isolated peloton after 24 h incubation, (f) peloton after 96 h incubation, (g) subculture of fungus after 2 weeks, and (h) cross germination plate showing symbiotic germination of all four study species using lean wood media and fungus 851-B isolated from *C. flavum* at Boystown Pineland.

Tropical Botanical Garden (XTBG) and a schoolyard lawn in Nanning, Guangxi, China (SYN).

A total of 165 nylon mesh fungus baits containing ~123,750 orchid seeds were deployed in situ to sample the fungi necessary for germination. Using protocols adapted from Brundrett et al. (2003), fungus baits were constructed from 30-cm strips of 80-μm plankton netting that was heat impulse sealed to create a single row of fifteen equally sized (2.5 × 2.5 cm) square compartments. Fifty fresh seeds were placed in each compartment on a 1.5 × 1.5 cm square of sterile moistened Whatman filter paper and sealed closed. Fungal baits were spread throughout each study site often several meters apart, and when available, baits were also placed near conspecific adults and seedlings. For terrestrial orchids, baits were buried 1–5 cm into the substrate and covered with a thin layer of humus, while epiphyte fungal baits were attached to different host trees and directly to bark using small staples, in moist branch crotches, and/or within pockets of moss or detritus (Appendix S1: Fig. S1). For *C. punctatum*, 30 fungus baits were deployed at the FTBG and FSS sites (2 sites × 30 baits × 15 compartments × 50 seeds = 45,000 seeds total). The first 15 baits were put out at each site in May of 2013 and scored in November 2013. For the second deployment, 15 more baits were placed out in March of 2014. Fifteen fungus baits for *C. flavum* were deployed in November–December of 2013, and in February of 2014, at the ZOO and BT sites (2 sites × 30 baits × 15 compartments × 50 seeds = 45,000 seeds total). After the first year of baiting, we observed very little germination, so we added 0.5 g of wood that had been ground and sterilized to remaining baits. For both *E. alta* and *E. graminea*, we

deployed one set of fungus baits at each site. The *E. alta* fungus baits were deployed once in March of 2014 at CSS (1 site × 15 baits × 15 compartments × 50 seeds = 22,500 seeds total), and *E. graminea* in February of 2014 and September of 2015 (1 site × 30 baits × 15 compartments × 50 seeds = 45,000 seeds total). Fungus baits were checked for protocorm development every six months, and un-germinated seeds were left in the field for up to two years. Baits for *C. punctatum* and *C. flavum* were retrieved in December of 2015, and baits for *E. alta* and *E. graminea* were retrieved in 2016–2017. A total of 111 protocorms were observed and/or retrieved from baits for fungal isolation and DNA extraction.

Isolation of mycorrhizal pelotons

We obtained pure fungal cultures from tissue samples for DNA identification and symbiotic seed germination trials by surface sterilizing protocorms or roots, then isolating, and culturing fungi from pelotons (coils of fungal hyphae within orchid cells) under sterile conditions (Fig 2e, f) as per McCormick et al. (2006). For each sample, the remnant pieces of the roots were frozen and used for DNA identification. We used isolates to conduct germination assays to determine whether these fungi formed functional mycorrhizae (i.e., capable of supporting germination or enhancing growth and survivorship).

Ex situ germination trials

Germination trials were conducted during and following the field sampling and between years 2015 and 2017. A total of 19 fungal isolates were tested for the ability to germinate each of the four study species. We only included isolates identified to be within Phylum Basidiomycota, since all fungi within Ascomycota were identified to taxa consistent with endophytic pathogens and not from any previously described ascomycete OMF groups (see Dearnaley et al. 2012); furthermore, isolates showed no ability to germinate seeds in pilot studies. Seeds were surface sterilized using 1% bleach solution, a small drop of liquid soap, and sterile water. A Petri dish containing 2% lean wood agar was divided into four sections, a 1 × 1 cm block of fungi was inoculated in the center, and 5–20 seeds from each species were placed on individual sterile filter paper squares using an inoculating loop. Lean wood agar media was selected because of its low nutrient and sugar content that allows for slow fungal growth without unwanted asymbiotic germination of the seeds. Plates were incubated at 25°C in the dark for 4–16 weeks. Plates were scored for protocorm development monthly (Fig 2h), and mean germination rates were calculated as the number of protocorms/ total number of seeds. Differences in mean germination rates were arcsine transformed to normalize the residual distribution. We used one-way ANOVAs to compare mean differences, and post hoc pairwise treatment comparisons were carried out using Tukey–Kramer. All statistical tests were performed in SPSS 17.0 for Windows.

DNA extraction, PCR, and sequencing

Genomic DNA was extracted from the lyophilized plant tissues and fungal isolates grown in liquid media using the DNeasy Mini Plant kit (QIAGEN, Venlo Limburg, The Netherlands) at FTBG laboratories Amplification of DNA from the first and second internal transcribed spacers, and the 5.8s subunit of the nuclear ribosomal repeat (hereafter ITS) was accomplished using three fungal primer pairs: ITS 1F/4 (universal fungus primer), ITS1OF/4OF (orchid basidiomycete fungi primer excluding *Tulasnella*), and ITS 5/ITS4-Tul (*Tulasnella*-specific primer pair). Primers were chosen to amplify DNA from the full range of orchid endophytic fungi in tissues and cultures (Gardes and Bruns 1993, Taylor and McCormick 2008). A total of 4.5-μL DNA template was added to the PCR master mix to form 10-μL total PCR mixture. For all reactions, the PCR Master Mix consisted of 0.25 μL Forward Primer (10 μmol/L), 0.25 μL Reverse Primer (10 μmol/L), and 5 μL Redmix HI fidelity TAQ (Applied Biosystems, Waltham, Massachusetts, USA). PCR reactions were performed using a thermocycler and the following program: 96°C for 1 min; 35 cycles of 94°C for 30 s, 54°C (ITS 1F/4 and 5/4Tul) or 60°C (ITS 1OF/4OF) for 30 s, 72°C for 30 s; and 72°C for 10 min. Gel electrophoresis (2% agarose gel) was completed in order to verify successful PCR amplification, visible as a single band of the expected size. All PCR products were stored at −20°C until DNA sequencing. The PCR reactions were conducted at FTBG and the Smithsonian Environmental Research Center (SERC; Edgewater, Maryland,

USA). Post-PCR cleanup was conducted using ExoSAP-IT reagent (Affymetrix, Santa Clara, California, USA) and/or Sephadex G-50 (fine) Centri-Sep spin columns (Princeton Separations, Adelphia, New Jersey, USA). Treated PCR products were stored at −20°C until sequencing reaction. Sequencing reactions were completed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) and consisted of 1 μL (3.75 μM) of forward and reverse primers, 1 μL PCR product, 2 μL 5 × Buffer (Big Dye), 2 μL Big Dye Terminator, and 4 μL sterile ddH2O. The samples were stored at −20°C for up to 24 h until Sanger sequencing. Samples were sent to Florida International University, Core DNA Facility (Miami, Florida, USA), and to the Smithsonian Institution's Laboratory of Analytical Biology (Suitland, Maryland, USA) for Sanger sequencing. All sequences were aligned using the MAFFT alignment plugin implemented in Geneious version 8.1.5 (Kearse et al. 2012, Biomatters, Auckland, New Zealand) and manually trimmed and optimized. All new ITS sequences from this study were deposited in GenBank (MT610978–MT610997, MT610999–MT611020, and MT611021–MT611054).

Molecular analysis

Resulting sequences were compared to known fungal sequences in GenBank through a nucleotide BLAST search. Estimations of phylogenies, and comparisons of taxonomic breadth, were conducted by generating consensus trees using neighbor-joining, and maximum likelihood (ML) with 500 random addition replicates implemented in Geneious (Biomatters, Auckland, New Zealand). Tree topologies were qualitatively similar for both estimations. Because of difficulties aligning the highly variable ITS sequences among distantly related taxa, we designated all taxa as belonging to either ascomycetes, non-*Tulasnella* basidiomycetes (NTB), or *Tulasnella*, and generated separate consensus trees for each taxonomic grouping. Using the classification criteria from Dearnaley et al. 2012, fungi designated as (NTB) and *Tulasnella* were considered orchid mycorrhizal fungi (OMF), but were designated putative OMF if there was no evidence of seed germination or if they were non-culturable. For this study, all ascomycete fungi were classified as pathogens since we did not detect any fungi in the Order Pezizales, a known OMF group. Operational taxonomic units (OTUs) were grouped by calculating the pairwise sequence distances from ML consensus trees and were represented as the tips of the phylogenetic trees. We considered sequences with greater than 97% similarity for the ascomycetes and NTB to be the same OTU. Because of the rapid evolution in *Tulasnella*, we set the cut off value at 95% similarity for these taxa (see Downing et al. 2017). For each orchid species, the total number of OTUs, the minimum and maximum, and the average number of OTUs were calculated for each population (site). To compare the diversity of each fungal group for each orchid species, the Shannon–Weiner Index (H') was calculated using counts of distinct OTUs detected at each site. The indices were then compared using Student's t tests. Rarefied accumulation curves were created by plotting the number of OMF OTUs as a function of the number of samples (number of individual sequences recovered) over different sites. Index values and rarified curves were obtained using R project and vegan package (Oksanen et al. 2019, version 3.0.1; R Development Core Team 2017).

To compare differences in phylogenetic breadth of fungal association between the two orchid types (native and invasive), we calculated Mean Pairwise Distance (MPD; Faith 1992) and phylogenetic distance (PD) using the picante Package of the R program (Kembel et al. 2010) for all species, and across all sampling sites. To correct for small sample sizes, we also calculated the standardized effect sizes (SES) for each site. A t test was performed to determine differences in the mean number of distinct fungal taxa, and MPD values between each native and invasive orchid species.

Results

A total of 89 fungal ITS sequences were successfully amplified from 104 root samples and 111 protocorm samples. We identified 53 distinct fungal OTUs, six OTUs were shared among at least two species, and four OTUs were shared between native and invasive species (Asco_OTU 1 (*Fusarium oxysporum*), NTB _OTU 1 (Polyporales), and TUL_OTU4, TUL_OTU6 (*Tulasnella*). Fungal sequences were identified and grouped

based on taxonomic classification and putative ecology (ML trees; Appendix S1: Figs. S2–S4). Approximately 52% (46/89) of the fungal sequences identified were classified as OMF (NTB or *Tulasnella*), and 48% (43/89) identified as plant pathogens (Ascomycetes). A total of 29 fungal OTUs were recovered from the two native species, and 27 OTUs for the two invasive species (Fig. 3a, b). Native orchid species harbored fewer OMF taxa than invasive orchids with 52% of the total OTUs identified as Ascomycete and classified as putative pathogens (Fig. 3a). Conversely, for invasive orchids OMF groups were the dominant fungal associates comprising nearly 75% of the total fungi identified, with 20 OMF and 7 pathogen OTUs identified (Fig. 3b).

Among the two orchid genera, the genus *Cyrtopodium* associated with a greater number of fungal taxa. A total of 35 OTUs (one shared between species) were recovered, with 18 OTUs detected for each *Cyrtopodium* species (Fig. 3c, d). Half of the OTUs associated with the native *C. punctatum* was classified as pathogenic fungi (Fig. 3c), which was significantly greater than the number of pathogens associated with the invasive *C. flavum* (~17% of total OTUs detected; Fig. 3d). For orchids in the genus *Eulophia*, 22 OTUs (three shared; 2 *Tulasnella* and 1 NTB) were recovered (Fig 3e, f). We detected similar patterns of pathogen association in the native species, with *E. alta*, harboring more pathogenic fungi (46% of total OTUs detected; Fig. 3e) than the invasive congener (36% of total OTUs detected; Fig. 3f).

Within fungi classified as the OMF community, diversity differed as well (Fig. 3g, h). Native species associated predominantly with *Tulasnella* fungi (50% of the total OMF OTU richness) but also formed minor associations with five other Basidiomycete fungi: *Ceratobasidium* (14%), Polyporales (14%), Russulales (7%), *Fomes* sp. (7%), and *Phlebia* sp. (7%) (Fig. 3g), and only three OMF taxa were shared between native and invasive species (NTB_OTU1, Tul_OTU4, Tul_OTU6). The invasive orchids also associated with six mycorrhizal groups but showed more preference for non-*Tulasnella* fungi, with the greatest proportion of OTUs belonging to the order Polyporales (45%), followed by *Tulasnella* (25%), other non-related basidiomycetes (25%; *Fomes. Phlebia, and Phanerochaete spp*.), and a single Agaricales fungus (5%; Fig. 3h). A greater proportion of OMF were also shared between the two different invasive species, with ten individuals associating with the same OTU across multiple sites (see Appendix S1: Figs. S2 and S3 for complete OMF phylogenies).

Phylogenetic analyses

Maximum-likelihood tree topology suggested a diverse assemblage of ascomycete taxa, with most sequences related to known endophytes and plant root pathogens. We obtained a total of 23 ITS sequences, comprising 21 distinct fungal OTUs (Appendix S1: Fig. S2; ML consensus tree). Ascomycete richness in native orchids was more than double that in invasive orchids across all sites. Only two fungi, uncultured Asco_OTU 1_*Fusarium oxysporum* and uncultured_Asco_OTU2_*Plectosphaerella cucumerina*, were shared among individual plants. The pathogenic fungus *F. oxysporum* was detected in all species except for native *E. alta*. We detected two different OTUs of the plant pathogen *Trichoderma* (100% bootstrap support) in two *E. alta* at FSS. The remaining 12 ascomycete OTUs were largely unrelated and were not shared among individuals or species.

The NTB phylogeny included 31 sequences, and we identified 22 distinct OTUs (Appendix S1: Fig. S3; ML consensus tree). Of the NTB fungi identified, ~71% (22 of the 31 sequences) belonged to Agaricales, Russulales, and Polyporales. Within these orders, we distinguished two distinct OTUs that were shared among the orchid species. Fungus NTB_OTU1 belonged to Polyporales and was detected in protocorms of both invasive orchids at the BT and ZOO sites and in one adult *E. alta* at CSS. Only invasive orchids associated with the Agaricales fungus NTB_OTU2 (*Neonothopanus*) at BT and ZOO sites.

Fungi belonging to the genus *Tulasnella* were the second most abundant OMF detected. The *Tulasnella* tree included 35 sequences that belonged to 11 distinct OTUs (Appendix S1: Fig. S4; ML consensus tree). The tree topology supported two distinct *Tulasnella* clades, reported here as A and B (100% bootstrap support). Clade A consisted of Tul_OTU1 and Tul_OTU2. Tul_OTU1 was the most frequently detected and was associated with only the invasive species (nine plants in total). Tul_OTU2 was found in four *C. punctatum* seedlings only at the FTBG site. Clade B was comprised of four distinct OTUs (Tul_OTUs 3–6). The Tul_OTU3 only associated

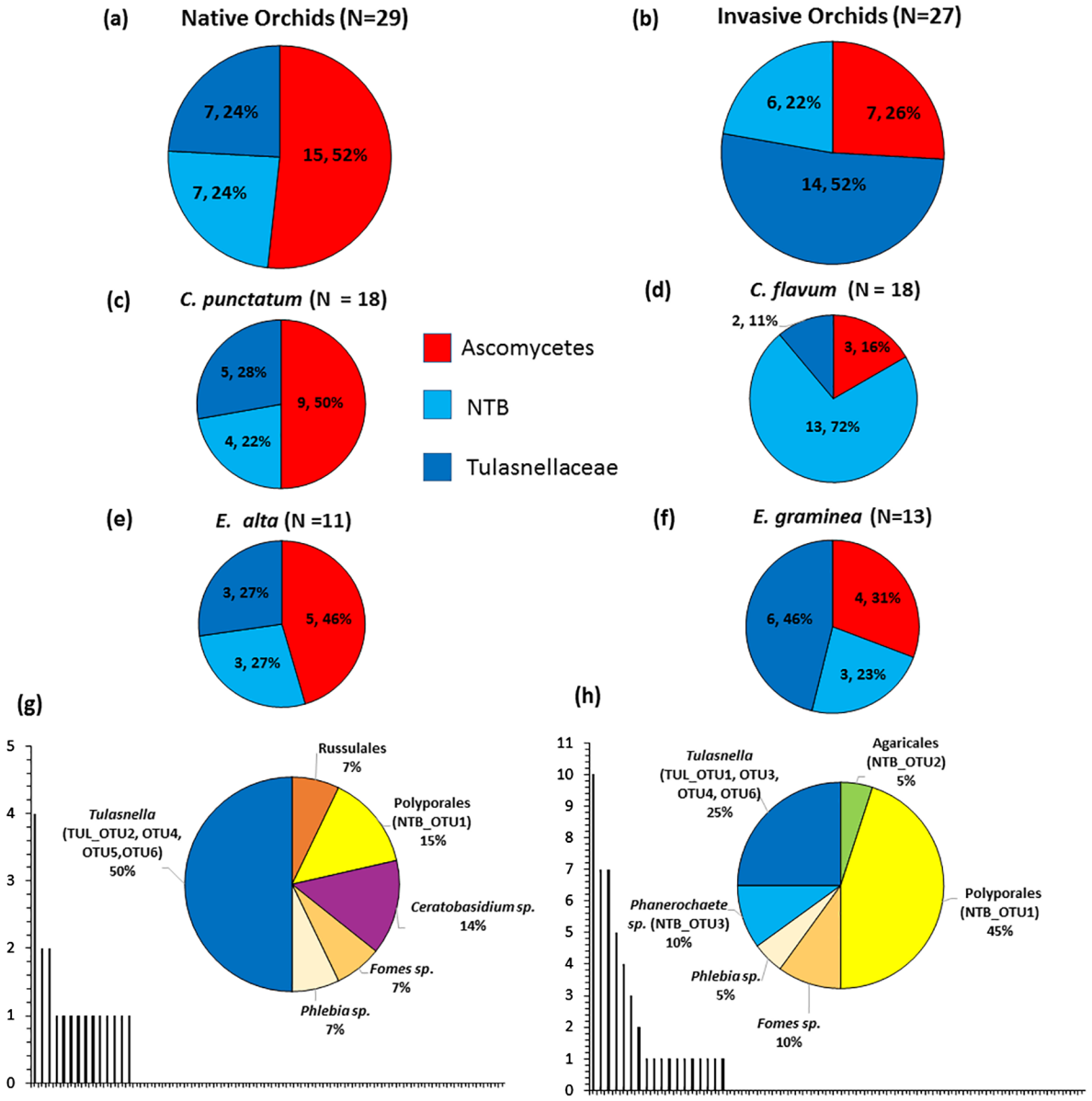


Fig. 3. Pie charts showing the breakdown of distinct OTUs for different fungal groups identified between the (a) native species and (b) invasive species (N = total no. of OTUs). NTB = non-*Tulasnella* basidiomycete. Pie charts showing the number and diversity of fungal OTUs identified in four congeneric orchid species in southern Florida; (c) and (e) are native species, and (d) and (f) are invasive species. Comparison of mycorrhizal communities associating with native and invasive orchid congeners. Native orchid sites included FTBG, FSS, and CSS (g). Invasive orchid sites included FTBG, BT, ZOO, and CSS (h). Numbers in the pie charts represent the number of OTUs, and the percentage of total OTUs detected for each fungal grouping.

with *E. graminea* plants in a small patch of pine rockland at the FTBG site. Tul_OTU4 was identified in one protocorm of *C. flavum* at the ZOO site and one seedling of *E. graminea* at BT. Tul_OTU5 was only detected in two adult *E. alta* plants at FSS. Tul_OTU6 had the most host species, forming associations with native and invasive orchid species in three sites.

Fungal diversity estimates

We found differences in site-level total species richness, Shannon diversity (H'), and evenness (J'), for ascomycete OTUs between native and invasive orchid sites. Overall, ascomycete richness in native orchids (fifteen total OTUs, ranging from 1 to 6 OTUs from orchids at each site) was more than double that in the invasive species (six total OTUs, min. of one and max. of three OTUs, from each site). Diversity for ascomycete OTUs was higher for the native orchids (H' = 1.11, SE = 0.22) than for their invasive congeners (H' = 0.28, SE = 0.36; $F_{1,4}$ = 8.014, P = 0.031). There was also a difference in evenness at the site level between native and invasive orchids; J' = 0.74, SE = 0.18, and J' = 0.25, SE = 0.22, respectively (Table 1).

Species richness for the potential OMF (NTB and *Tulasnella* combined) was higher at sites with the invasive orchids (12 total OTUs, min. of three and max. of five OTUs, from each site) than at native orchid sites (seven total OTUs, min. of one and max. of two OTUs, from each site). Mean diversity index (H') for OMF OTUs was also higher at the invasive orchid sites (H' = 1.2, SE = 0.14) than at the native sites (H' = 0.61, SE = 0.38). There was a significant difference in total evenness indices between invasive and native orchid sites; J' = 0.88, SE = 0.024, and J' = 0.56, SE = 0.28, respectively ($F_{1,4}$ = 8.515, P = 0.043; Table 2; Appendix S1: Fig. S5).

Phylogenetic breadth estimates

Invasive orchids associated with a greater phylogenetic breadth of potential OMF taxa, reflected by the mean pairwise distances, NTB MPD = 0.881 and *Tulasnella* MPD = 0.68, than their native congeners, NTB MPD = 0.783 and *Tulasnella* MPD = 0.434. Within the native species, MPD values between NTBs for *C. punctatum* were 0.800, and for *E. alta* were 0.767 (mean = 0.78, SE = 0.07). For the invasive species, within-group MPD values were 0.895 for *C. flavum*, and 0.867 for *E. graminea* (mean = 0.881, SE = 0.067). For native species, MPD values between *Tulasnella* taxa for *C. punctatum* were 0.785, and for *E. alta* values were the lowest of all species at 0.083 (mean = 0.43, SE = 0.07). For the invasive species, within-group MPD values of *Tulasnella* were 0.469 for *C. flavum* and 0.893 for *E. graminea* (mean = 0.68, SE = 0.13; Appendix S1: Table S1).

In situ and ex situ germination rates

Fungus baiting resulted in extremely low in situ germination rates (<1% mean germination rate for highest species). However, germination rates differed between the four orchids species ($F_{3,\ 161}$ = 3.684, P = 0.013). The highest germination rates were observed for the invasive *E. graminea* (0.435%), with a total of 59 protocorms recovered (22 out of 30 baits total at FTBG and BT sites), followed by *C. flavum* (0.2%) with a total of 36 protocorms (10 out of 60 baits total at

Table 1. Diversity of pathogenic fungi (ascomycete OTUs) identified in the roots of native and invasive orchids at three native sites in southern Florida, one native site in Yunnan Province China (XTBG), and four invasive sites in Miami-Dade County, Florida.

Species	Site	No. of plants	Ascomycetes (OTUs)	Species richness	H'	SE (H')	J'	SE (J')
Native								
C. punctatum	FTBG	7	7	6	1.75		0.98	
C. punctatum	FSS	3	3	3	1.10		1.00	
E. alta	CSS	5	5	5	1.61		1.00	
E. graminea	XTBG	1	1	1	0.00		0.00	
Total		16	16	**15**	**1.11**	**0.39**	**0.74**	**0.18**
Invasive								
C. flavum	ZOO	1	1	1	0.00		0.00	
C. flavum	BT	2	1	1	0.00		0.00	
E. graminea	FTBG	3	3	3	1.10		1.00	
E. graminea	BT	1	1	1	0.00		0.00	
Total		7	6	**6**	**0.28**	**0.27**	**0.25**	**0.22**

Notes: H' indicates Shannon–Weiner diversity index; J', the evenness index; and SE (x′), standard error. Numbers in bold represent mean diversity indices for each group.

Table 2. Diversity of orchid mycorrhiza (basidiomycete OTUs) identified from native orchids at three sites (FTBG, FSS, and CSS), and from invasive orchids at three sites (FTBG, ZOO, and BT) in southern Florida.

		NTB			*Tulasnella*											
Site	No. of plants	OTU1	OTU2	OTU3	OTU1	OTU2	OTU3	OTU4	OTU5	OTU6	Total	OTU richness	***H′***	SE *(H′)*	***J′***	SE (*J′*)
Native																
FTBG	5				1	4					5	2	0.50		0.72	
FSS	1									1	1	1	0.00		0.00	
CSS	5	1						1	2	1	5	4	1.33		0.96	
Sub total	11	1			1	4		1	2	2	11	7	**0.61**	**0.38**	**0.56†**	**0.28**
Invaded																
FTBG	13	1			2		7			3	13	4	1.16		0.83	
ZOO	10	5	2	2	4			1			14	5	1.47		0.91	
BT			3		4			1			8	3	0.97		0.89	
Sub total	23	6	5	2	10			2		3	35	12	**1.2**	**0.14**	**0.88†**	**0.024**

Notes: *H′* indicates Shannon–Weiner diversity index; *J′*, the evenness index; and SE (x′), standard error. Numbers in bold represent mean diversity indices for each group.

† Significantly different ($F_{1,4} = 8.515$, $P = 0.043$; SE = 0.28 and 0.024).

FTBG and BT sites), and the lowest rates were for *C. punctatum* (0.013%) with only 4 protocorms recovered (four out 60 baits total at FTBG and FSS sites). For *E. alta,* no seeds germinated in baits. Tukey–Kramer pairwise comparisons showed there was a significant difference in germination between *C. punctatum* and *E. graminea* fungi baits ($P = 0.012$), but not between any other pairwise comparisons.

Of the 53 distinct OTUs, we successfully cultured 25 OMF isolates, of which 19 were used in ex situ germination trials. Fungi varied in their ability to stimulate germination in other species and support protocorm development (Table 3). Most notably, the Polyporales isolates NTB_OTU F-7 and F-8, obtained from the invasive *C. flavum* protocorms, were capable of germinating all four of the study species, while all of the OMF isolated from the endangered *C. punctatum* lacked interspecies compatibility.

Discussion

This study attempted to understand how contrasting plant–fungus interactions may influence orchid species abundances and range distributions. Specifically, we found that invasive orchids associated with a greater diversity and breadth of OMF than co-occurring native congeners. Simultaneously, those invasive orchids harbored fewer pathogenic fungi than their native counterparts.

Are mycorrhizal associations of invasive species more generalized than natives?

Estimating degrees of mycorrhizal specificity remains difficult, since our understanding of the relative abundance and distribution of fungi compared to what is sampled in the roots within a particular environment is limited or completely unknown. As a result of these obstacles, using molecular surveys of the root endophytes to estimate diversity values and phylogenetic breadth of OMF remains among the most efficient methods to estimate diversity and degrees of specialization. In this study, the OMF diversity (OTU richness, MPD, *H′*), germination rates, and compatibility were significantly higher for the invasive congeners and may represent less specialization as compared to native congeners. This suggests two possible scenarios to consider: (1) native orchids have narrower OMF associations than the invasive congeners (i.e., compatible with fewer taxa), and (2) specificity here is related to the diversity and availability of compatible OMF found in the habitat.

Scenario 1.—We observed narrower OMF associations for native orchids. This was especially true for the native *C. punctatum*, which had lower diversity values (*H′*), ranging from 0 to 0.5 across different habitat types (garden vs. cypress slough), as compared to its congener *C. flavum*, with (*H′*) > 1.87 which was restricted to pine

Table 3. Results of ex situ cross germination trials for fungi cultured across all taxa include incipient species, fungal taxonomic group, and habitat type or host tree type.

					Germination			
					Native orchids		Invasive orchids	
Fungus source	Fungal ID	OTU	Taxonomic group	Habitat	*C. punctatum*	*E. alta*	*C. flavum*	*E. graminea*
C. punctatum adult FTBG	F-3		*Ceratobasidium*	live oak	+/−	−	−	−
C. punctatum adult FTBG	F-11		*Ceratobasidium*	live oak	−	−	−	−
E. graminea seedling CSS	F-14		*Fomes*	slough	−	−	−	−
C. punctatum seedling FTBG	F-10		*Fomes*	bottonwood	+/-	−	−	−
E. graminea seedling BT	F-16	NTB_OTU2	*Neonothopanus*	pinelands	−	−	+	+
C. punctatum seedling FTBG	F-5		*Phlebia*	live oak	−	−	−	−
E. alta adult CSS	F-21	NTB_OTU1	*Oxyporyus*	slough	−	−	−	−
C. flavum ZOO	OM-32	NTB_OTU1	*Oxyporyus*	pinelands	−	−	+	-
C. flavum GC	851-B	NTB_OTU1	*Oxyporyus*	pinelands	−	−	+	+/-
C. flavum adult GC	OM-24	NTB_OTU3	*Phanerochaete*	pinelands	−	+/−	+	+
C. flavum seedling GC	851-A	NTB_OTU3	*Phanerochaete*	pinelands	+	+	+	+
C. flavum protocorm BT	F-6a		Polyporales	pinelands	−	−	+	+
E. alta adult CSS	F-13		Polyporales	slough	−	+	−	−
C. flavum seedling ZOO	F-60		Polyporales	pinelands	+	+	+	+
C. flavum protocorm BT	F-7		Polyporales	pinelands	+	+	+	+
C. flavum protocorm BT	F-8	TUL_OTU1	*Tulasnella*	pinelands	−	+/−	+	+
C. punctatum adult FSS	F-4		*Tulasnella*	bald cypress	+	-	-	-
E. graminea seedling BT	F-16	TUL_OTU1	*Tulasnella*	pinelands	−	−	+	+
C. flavum protocorm BT	F-9	TUL_OTU2	*Tulasnella*	pinelands	+/−	+	+	+

Note: The symbols (+) indicate seed germination with advanced protocorm development (primordial roots and leaf development), (+/−) indicate seed germination without advanced protocorm development, and (−) no seed germination.

rockland habitat. Native species also associated with a narrower phylogenetic breadth of OMF. For native orchids, mean pairwise distances (MPD) for NTB and *Tulasnella* OTUs (OMFs) were significantly lower than invasive species regardless of site type. The differences in MPD values indicated that native species associated with closely related OMF communities within and among study sites.

Both in situ and ex situ germination trials supported lower germination rates for the native species. In situ germination rates for native species were extremely low, less than 0.1% combined germination rates (total of 4 protocorms recovered out of ~3000 seeds deployed), and *E. alta* failed to germinate in situ. The highest germination rates were observed for the invasive *E. graminea* (0.435%). The extremely low germination rates overall, as compared to ex situ trials, could be attributed to pronounced inundation, fires, and droughts in the field. The south Florida region has extreme seasonality, and sites were frequently flooded for extended periods of time. Pine rocklands are pyrogenic communities, and at least one site burned within the study period, preventing recovery of seed baits that season.

Tulasnella fungi comprised 50% of the total OMF detected in both native species, suggesting strong specialization toward this fungal group, regardless of epiphytic or terrestrial life histories. Generalist interactions with *Tulasnella* mycobionts across co-occurring orchid taxa have been widely demonstrated, particularly in the tropics (Dearnaley et al. 2012, Oberwinkler et al. 2017, Herrera et al. 2018). However, on a larger, regional scale, generalist orchids may be more appropriately identified by their ability to exploit multiple and diverse groups of fungi that are also locally abundant.

Scenario 2.—Studies have shown that OMF associations can be influenced by ecosystem type and growth habit (epiphytic or terrestrial). We did see differences in the OTU richness and diversity between the different site types, and pine rockland (dry forest) sites were more diverse overall than the pine flatwood (wet forest) and botanic garden/disturbed sites (semi-natural). Regardless of habitat type, invasive

orchids were more likely to associate with non-*Tulasnella* fungi (representing 88% of total OMF OTUs detected) than did the natives. Most belonged to known saprophytes and OMF (demonstrated here in germination trials) in the Polyporales and were mostly restricted to pine rockland habitats. Yet the native terrestrial *E. alta* also associated with Polyporales fungi, despite being geographically separated and in a different habitat type (pine flatwoods). Interestingly, Polyporales isolates obtained from pine rockland sites were capable of germinating all four of the study species in ex situ germination trials. Invasive orchids also formed strong associations with fungi in the Agaricales at pine rockland sites. Although not previously reported to occur in the new world, one OMF that was detected in both invasive species and was capable of germinating both was closely related to *Neonothopanus sp*. (a bioluminescent saprotrophic fungus known from Africa and Asia). Research has demonstrated that fungal associations may be more influenced by fungus ecological function (saprobe and tree pathogens; Volk 2000, Reinprecht 2016) than by a specific habitat condition (wet forest or dry forest), and saprobes may form beneficial associations with other orchid species in a variety of habitats when available.

It is also possible that compatible NTB fungi are particularly widely dispersed and abundant in the region. Therefore, the observed preference for NTB particularly in pine rocklands may be driven by the high densities of these fungi in that community. Previous studies have shown that the highest densities of orchids are often in areas that support high densities of their mycorrhizal fungi (McCormick et al. 2009, 2012). Similarly, Jacquemyn et al. (2014) found that co-occurring orchid species that used distinctive mycorrhizal communities were spatially segregated by growing primarily in locations where their appropriate fungi were abundant.

Differences between epiphytic and terrestrial rhizosphere are also considered to be a primary factor influencing mycorrhizal associations (Xing et al. 2019). Unfortunately, a strictly epiphytic comparison could not be made here, because there are no other purely epiphytic *Cyrtopodium* species that are naturalized in southern Florida. Interestingly, *C. punctatum* did not share any OTUs between garden and natural sites where it was sampled. This suggests possible specialization on fungi that are also locally abundant, rather than any one type. For terrestrial orchids, some of the same OTUs were detected over geographically distant sites (i.e., SE and SW Florida), and in different habitat types. Although there has been a recent surge in studies examining the relationship between habitat type variation and mycorrhizal associations, overall patterns remain unclear (Jacquemyn et al. 2016, 2017, Xing et al. 2019).

Do invasive orchids have fewer pathogenic fungi?

Our results showed that the native orchids we studied had more associations with ascomycete fungi, which may be pathogens and did not support seed germination, than did their recently introduced congeners, even at the same sites. This suggests that the enemy-release hypothesis may be another mechanism enabling the spread of invasive orchids. For example, within 50m of the native *E. alta* individuals infected with *Trichoderma virens*, a known plant pathogen, we did not detect any ascomycetes in association with the invasive *E. graminea*; instead, it only associated with presumably beneficial fungi belonging to known OMF groups. Furthermore, when individuals of *E. graminea* were sampled in their native range in China, only ascomycetes were detected in DNA surveys and resulting fungal isolations, while the majority of plants sampled in Florida harbored at least one type of OMF-related taxon. A possible explanation is that potentially pathogenic ascomycetes may dominate in native ranges while OMF are much less abundant. However, a co-evolution of orchids and their native pathogens might best explain the observed results presented here. We also found that the number of ascomycetes detected was similar for both native species even though one species is epiphytic and the other is terrestrial. This suggests that differences in pathogenic infection were not solely related to ascomycete abundance among terrestrial and epiphytic habitats. Rather, these native species may be more susceptible to co-evolved pathogens. In this study, all of the ascomycete OTUs we identified belonged to predominantly pathogenic taxa. We identified one OTU as *Fusarium oxysporum*, a known plant pathogen, but members of genus have been found to assist in plant seed

germination (Vujanovic et al. 2000) and to be endophytic in some orchids (Těšitelová et al. 2012).

If invasive orchids have a release from pathogens in a new site, it may increase overall fecundity through the reallocation of resources from defense to growth and reproduction. Pathogen absence can also provide more vacancies for colonization of OMF in the cortical root cells. Understanding to what extent root pathogens influence mycorrhizal associations remains a highly evolving area of plant research, and the future study of this tangled bank of plant–fungus interactions promises to reveal much about the population and community ecology of orchids.

Conclusions

Compared to co-occurring native congeners, invasive orchids had greater diversity and showed less preference for any one type of OMF and at the same time harbored fewer pathogenic fungal taxa. The invasive species also had higher in situ and ex situ germination rates and compatibility. Moreover, native orchids were twice as likely as invasive orchids to harbor antagonistic fungal taxa. This means that both the ability to form generalized and positive (from the plant perspective) below-ground biotic interactions and resisting antagonistic ones (enemy-release hypothesis) likely contributed to invasive orchid to range expansion in South Florida.

The results of this project also have broader implications for orchid conservation and biology. Our study supports the notion that fungal interactions can at least partially explain differences in orchid population abundances and where they can occur (Suarez et al. 2006, McCormick et al. 2012, Waud et al. 2017). In addition, orchids can be sensitive to changes in environmental conditions that may influence fungal communities (McCormick et al. 2012). With climate change, understanding these relationships becomes more urgent, because global changes may have the most impact on taxa that are heavily dependent on other taxa (Fitter et al. 2000, Compant et al. 2010). On the other hand, those plant species that can adopt more flexible fungal requirements, or avoid pathogens, in the face of environmental changes may become more widespread in the future.

Acknowledgments

The National Science Foundation's East Asian Pacific Islands Summer Institute Grant Award #1414633 to J.L. Downing helped sampling efforts in China. H. Liu and M. K. McCormick contributed equally to the work reported here. The research was also supported through resources and funds provided by Fairchild Tropical Botanic Garden, Fairchild Graduate Fellowship Grant, Fairchild Challenge Education Program, The American Orchid Society, Smithsonian Environmental Research Center, Smithsonian Laboratory of Analytical Biology, Atlanta Botanical Garden, Xishuangbanna Tropical Botanic Garden, Guangxi University, Kelly Foundation Tropical Botany Grant, and the McKnight Dissertation Fellowship Program. Special thanks to the biologists Mike Owen, Dennis Giardina at the Fakahatchee State Park Reserve and Shawn Clem at the Corkscrew Sanctuary in assisting our sampling efforts, and gratitude to Miami-Dade County Parks and Recreation for access to sampling site. We thank Christine Cook for her photographs. We thank Marc-André Selosse and an anonymous reviewer for comments that greatly improved the manuscript.

Literature Cited

Brundrett, M. C., A. Scade, A. L. Batty, K. W. Dixon, and K. Sivasithamparam. 2003. Development of in situ and ex situ seed baiting techniques to detect mycorrhizal fungi from terrestrial orchid habitats. Mycological Research 107:1210–1220.

Canavan, K., I. D. Paterson, M. P. Hill, and T. L. Dudley. 2019. Testing the Enemy Release hypothesis on tall-statured grasses in South Africa, using *Arundo donax, Phragmites australis,* and *Phragmites mauritianus* as models. Bulletin of Entomological Research 109:287–299.

Compant, S., M. G. van der Heijden, and A. Sessitsch. 2010. Climate change effects on beneficial plant–microorganism interactions. FEMS Microbiology Ecology 73:197–214.

De Long, R., N. D. Jonathan, K. W. Swarts, and L. M. Egerton-Warburton. 2013. Mycorrhizal preference promotes habitat invasion by a native Australian orchid: *Microtis media*. Annals of Botany 111:409–418.

Dearnaley, J. D., F. Martos, and M. A. Selosse. 2012. Twelve orchid mycorrhizas: molecular ecology, physiology, evolution and conservation aspects. In Fungal associations, Springer, Berlin, Heidelberg, Germany.

Dearnaley, J., S. Perotto, and M. A. Selosse. 2016. Structure and development of orchid mycorrhizas. Molecular Mycorrhizal Symbiosis 2016:63–86.

Dickie, I. A., J. L. Bufford, R. C. Cobb, M. L. Desprez-Loustau, G. Grelet, P. E. Hulme, and P. H. Thrall. 2017. The emerging science of linked plant–fungal invasions. New Phytologist 215:1314–1332.

Diez, J. M., I. Dickie, G. Edwards, P. E. Hulme, J. J. Sullivan, and R. P. Duncan. 2010. Negative soil feedbacks accumulate over time for non-native plant species. Ecology Letters 13:803–809.

Downing, J. L., H. Liu, S. Shao, X. Wang, M. McCormick, R. Deng, and J. Gao. 2017. Contrasting changes in biotic interactions of orchid populations subject to conservation introduction vs. conventional translocation in tropical China. Biological Conservation 212:29–38.

Faith, D. P. 1992. Conservation evaluation and phylogenetic diversity. Biological Conservation 61:1–10.

Fitter, A. H., A. Heinemeyer, and P. L. Staddon. 2000. The impact of elevated CO_2 and global climate change on arbuscular mycorrhizas: a mycocentric approach. New Phytologist 147:179–187.

Florida Exotic Pest Plant Council List. 2019. http://bugwoodcloud.org/CDN/fleppc/plantlists/2019/2019_Plant_List_ABSOLUTE_FINAL.pdf

Frost, C. M., W. J. Allen, F. Courchamp, J. M. Jeschke, W. C. Saul, and D. A. Wardle. 2019. Using network theory to understand and predict biological invasions. Trends in Ecology and Evolution 34:831–843.

Gardes, M., and T. D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular Ecology 2:113–118.

Harrison, T. L., A. K. Simonsen, J. R. Stinchcombe, and M. E. Frederickson. 2018. More partners, more ranges: Generalist legumes spread more easily around the globe. Biology Letters 14:20180616.

Herrera, P., I. Kottke, M. C. Molina, M. Méndez, and J. P. Suárez. 2018. Generalism in the interaction of *Tulasnella* mycobionts with orchids characterizes a biodiversity hotspot in the tropical Andes of Southern Ecuador. Mycoscience 59:38–48.

Jacquemyn, H., R. Brys, V. S. Merckx, M. Waud, B. Lievens, and T. Wiegand. 2014. Coexisting orchid species have distinct mycorrhizal communities and display strong spatial segregation. New Phytologist 202:616–627.

Jacquemyn, H., K. J. Duffy, and M. A. Selosse. 2017. Biogeography of orchid mycorrhizas. *In* L. Tedersoo, editor. Biogeography of mycorrhizal symbiosis. Springer, Cham, Switzerland.

Jacquemyn, H., M. Waud, V. S. Merckx, R. Brys, D. Tyteca, M. Hedrén, and B. Lievens. 2016. Habitat-driven variation in mycorrhizal communities in the terrestrial orchid genus *Dactylorhiza*. Scientific Reports 6:37182.

Keane, R. M., and M. J. Crawley. 2002. Exotic plant invasions and the enemy release hypothesis. Trends in Ecology & Evolution 17:164–170.

Kearse, M., et al. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649.

Kembel, S. W., P. D. Cowan, M. R. Helmus, W. K. Cornwell, H. Morlon, D. D. Ackerly, and C. O. Webb. 2010. Picante: R tools for integrating phylogenies and ecology. Bioinformatics 26:1463–1464.

Kruse, J., M. Pautasso, and G. Aas. 2016. A test of the enemy release hypothesis for plants in the Ecological-Botanical Gardens, Bayreuth, using data on plant parasitic microfungi. Nova Hedwigia 10:239–249.

Liu, H., and R. Pemberton. 2010. Pollination of an invasive orchid, *Cyrtopodium polyphyllum* (Orchidaceae), by an invasive oil-collecting bee, *Centris nitida*, in southern Florida. Botany-Botanique 88:290–295.

Liu, H., and P. Stiling. 2006. Testing the enemy release hypothesis: a review and meta-analysis. Biological Invasions 8:1535–1545.

Luer, C. A. 1972. The Native Orchids of Florida. Botanical Garden, New York, New York, USA.

Mangla, S., and R. M. Callaway. 2008. Exotic invasive plant accumulates native soil pathogens which inhibit native plants. Journal of Ecology 96:58–67.

Martos, F., M. Dulormne, T. Pailler, P. Bonfante, A. Faccio, J. Fournel, and M. A. Selosse. 2009. Independent recruitment of saprotrophic fungi as mycorrhizal partners by tropical achlorophyllous orchids. New Phytologist 184:668–681.

McCormick, M. K., D. L. Taylor, K. Juhaszova, R. K. Jr Burnett, D. F. Whigham, and J. P. O'Neill. 2012. Limitations on orchid recruitment: not a simple picture. Molecular Ecology 21:1511–1523.

McCormick, M. K., D. F. Whigham, J. P. O'Neill, J. A. Becker, S. Werner, H. N. Rasmussen, T. D. Bruns, and D. L. Taylor. 2009. Abundance and distribution of *Corallorhiza odontorhiza* reflect variations in climate and ectomycorrhizae. Ecological Monographs 79:19–635.

McCormick, M. K., D. F. Whigham, D. Sloan, K. O'Malley, and B. Hodkinson. 2006. Orchid-fungus fidelity: A marriage meant to last? Ecology 87:903–911.

Mitchell, C. E., and A. G. Power. 2003. Release of invasive plants from fungal and viral pathogens. Nature 421:625–627.

Nomura, N., Y. Ogura-Tsujita, S. W. Gale, A. Maeda, H. Umata, K. Hosaka, and T. Yukawa. 2013. The rare terrestrial orchid Nervilia nipponica consistently associates with a single group of novel mycobionts. Journal of plant research 126:613–623.

Nunez, M. A., T. R. Horton, and D. Simberloff. 2009. Lack of belowground mutualisms hinders Pinaceae invasions. Ecology 90:2352–2359.

Oberwinkler, F., D. Cruz, and J. P. Suárez. 2017. Biogeography and ecology of Tulasnellaceae. Ecological Studies 230:237–271.

Oksanen, J. F., et al. 2019. vegan: community ecology package. R package version 2.5-6. https://CRAN.R-project.org/package=vegan

Pandey, M., J. Sharma, D. Taylor, and V. L. Yadon. 2013. A narrowly endemic photosynthetic orchid is non-specific in its mycorrhizal associations. Molecular Ecology 22:2341–2354.

Pansarin, L. M., E. R. Pansarin, and M. Sazima. 2008. Reproductive biology of *Cyrtopodium polyphyllum* (Orchidaceae): a Cyrtopodiinae pollinated by deceit. Plant Biology 10:650–659.

Pemberton, R. W., T. M. Collins, and S. Koptur. 2008. An Asian orchid, *Eulophia graminea* (Orchidaceae: Cymbidieae), naturalizes in Florida. Lankesteriana 8:5–14.

Pemberton, R. W., and H. Liu. 2008. Potential of invasive and native solitary specialist bee pollinators to help restore the rare cowhorn orchid (*Cyrtopodium punctatum*) in Florida. Biological Conservation 141:1758–1764.

Phillips, R. D., M. D. Barrett, K. W. Dixon, and S. D. Hopper. 2011. Do mycorrhizal symbioses cause rarity in orchids? Journal of Ecology 99:858–869.

Pringle, A., J. D. Bever, M. Gardes, J. L. Parrent, M. C. Rillig, and J. N. Klironomos. 2009. Mycorrhizal symbioses and plant invasions. Annual Review of Ecology, Evolution and Systematics 40:699–715.

R Development Core Team. 2017. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rasmussen, H. N. 1995. Terrestrial Orchids: from Seed to Mycotrophic Plant. Cambridge University Press, Cambridge, UK.

Reinprecht, L. 2016. Wood deterioration, protection and maintenance. John Wiley & Sons, Hoboken, New Jersey, USA.

Séne, S., M. A. Selosse, M. Forget, J. Lambourdière, K. Cissé, A. G. Diédhiou, and L. Vincenot. 2018. A pantropically introduced tree is followed by specific ectomycorrhizal symbionts due to pseudo-vertical transmission. The ISME Journal 12:1806–1816.

Smith, S. E., and D. J. Read. 2010. Mycorrhizal symbiosis. Academic press, Cambridge, Massachusetts, USA.

Stewart, S. L., and L. W. Richardson. 2008. Orchid flora of the Florida Panther National Wildlife Refuge. North American Native Orchid Journal 14:70–104.

Suarez, J. P., M. Weiss, A. Abele, S. Garnica, F. Oberwinkler, and I. Kottke. 2006. Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. Mycological Research 110:1257–1270.

Swarts, N. D., E. A. Sinclair, A. Francis, and K. W. Dixon. 2010. Ecological specialization in mycorrhizal symbiosis leads to rarity in an endangered orchid. Molecular Ecology 19:3226–3242.

Taylor, D. L. 2004. Myco-heterotroph-fungus marriages - is fidelity over-rated? New Phytologist 163:217–221.

Taylor, D. L., and M. K. McCormick. 2008. Internal transcribed spacer primers and sequences for improved characterization of Basidiomycetous orchid mycorrhizas. New Phytologist 177:1020–1033.

Těšitelová, T., J. Těšitel, J. Jersáková, G. Řihová, and M.-A. Selosse. 2012. Symbiotic germination capability of four *Epipactis* species (Orchidaceae) is broader than expected from adult ecology. American Journal of Botany 99:1020–1032.

Volk, T. 2000. An introduction to the characters used to identify poroid wood decay fungi. McIlvainea 14:74–82.

Vujanovic, V., M. St-Arnaud, D. Barabé, and G. Thibeault. 2000. Viability testing of orchid seed and the promotion of colouration and germination. Annals of Botany 86:79–86.

Waud, M., R. Brys, W. Van Landuyt, B. Lievens, and H. Jacquemyn. 2017. Mycorrhizal specificity does not limit the distribution of an endangered orchid species. Molecular Ecology 26:1687–1701.

Wunderlin, R. P., and B. F. Hansen. 2012. Atlas of Florida Vascular Plants (http://www.plantatlas.usf.edu/). [SM Landry and KN Campbell (application development). Florida Center for Community Design and Research.]. Institute for Systematic Botany, University of South Florida, Tampa, Florida, USA.

Xing, X., H. Jacquemyn, X. Gai, Y. Gao, Q. Liu, Z. Zhao, and S. Guo. 2019. The impact of life form on the architecture of orchid mycorrhizal networks in tropical forest. Oikos 128:1254–1264.

Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.3228/full