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

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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 congener species, 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 congener species (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.

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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 globalized, 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 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.
Tropical Botanical Garden (XTBG) and a school-yard 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 mycorrhizal (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 μm/L), 0.25 μL Reverse Primer (10 μm/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–MT611097, 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) and manually trimmed and optimized. All new ITS sequences from this study were deposited in GenBank (MT610978–MT611097, MT610999–MT611020, and MT611021–MT611054).

### 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 conger (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 OTUs 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 (*Neoantherapanus*) 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
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.

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.
**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

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**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      |       |           |
|               | FSS    | 3             | 3                  | 3               | 1.10  | 1.00      |       |           |
|               | 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      |       |           |
|               | CT     | 2             | 1                  | 1               | 0.00  | 0.00      |       |           |
| *E. graminea* | FTBG   | 3             | 3                  | 3               | 1.10  | 1.00      |       |           |
|               | 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.*
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

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

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 ($F_{1,4} = 8.513, P = 0.043; SE = 0.28 and 0.024$).

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.
Pine rocklands are pyrogenic communities, and frequently region has extreme seasonality, and sites were flooded for extended periods of time. The extremely low germination rates were observed for the invasive *E. alta* failed to germinate in situ. The highest germination rates overall, as compared to ex situ trials, (+/-) indicate seed germination without advanced protocorm development, and (--) no seed germination.

For native orchids, mean pairwise distances (MPD) for NTB and OMF were signiﬁcantly lower than invasive 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.

### 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.

| Fungus source | Fungal ID | OTU     | Taxonomic group | Habitat | Native orchids | Invasive orchids |
|---------------|-----------|---------|-----------------|---------|----------------|------------------|
| *C. punctatum* adult FTBG | F-3       | Ceratobasidium | live oak | +/-     | -              | -                |
| *C. punctatum* adult FTBG | F-11      | Ceratobasidium | live oak | -       | -              | -                |
| *C. punctatum* seedling FTBG | F-10      | Fomes     | slough         | -       | -              | -                |
| *E. alta* seedling BT       | F-16      | *Neonothopanus* | pinelands  | -       | +              | +                |
| *C. punctatum* seedling FTBG | F-5       | Phlebia   | live oak      | -       | -              | -                |
| *E. alta* adult CSS        | F-21      | NTB_OTU1  | Oxyporus      | -       | -              | -                |
| *C. flavum* ZOO            | OM-32     | NTB_OTU1  | Oxyporus      | -       | +              | -                |
| *C. flavum* GC             | 851-B     | NTB_OTU1  | Oxyporus      | -       | -              | +/-              |
| *C. flavum* seedling GC    | OM-24     | NTB_OTU3  | *Phanerochaete* | - | +/-     | +              |
| *C. flavum* seedling GC    | 851-A     | NTB_OTU3  | *Phanerochaete* | - | +/-     | +              |
| *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. punctatum* adult FSS   | F-4       | *Tulasnella* | pinelands  | -       | +/-            | +                |
| *E. graminea* seedling BT  | F-16      | *Tulasnella* | pinelands  | -       | +              | +                |
| *C. flavum* protocorm BT   | F-9       | *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-
*Tulasia* 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, Poly-
porales 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 for-
est), and saprobes may form beneficial associa-
tions 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 grow-
ing primarily in locations where their appropri-
ate 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 specializa-
tion on fungi that are also locally abundant,
rather than any one type. For terrestrial orchids,
some of the same OTUs were detected over geo-
graphically 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 sup-
port 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 *Tricho-
derma virens*, a known plant pathogen, we did
not detect any ascomycetes in association with
the invasive *E. graminea*; instead, it only associ-
ated with presumably beneficial fungi belonging
to known OMF groups. Furthermore, when indi-
viduals 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-re-
lated taxon. A possible explanation is that poten-
tially pathogenic ascomycetes may dominate in
native ranges while OMF are much less abun-
dant. 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 habi-
tats. 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 orchids 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.

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**Supporting Information**

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