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WIDESPREAD MYCORRHIZAL SPECIFICITY CORRELATES TO MYCORRHIZAL FUNCTION IN THE NEOTROPICAL, EPIPHYTIC ORCHID *IONOPSIS UTRICULARIOIDES* (ORCHIDACEAE)[1]

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Tropical orchids constitute the greater part of orchid diversity, but little is known about their obligate mycorrhizal relationships. The specificity of these interactions and associated fungal distributions could influence orchid distributions and diversity. We investigated the mycorrhizal specificity of the tropical epiphytic orchid *Ionopsis utricularioides* across an extensive geographical range. DNA ITS sequence variation was surveyed in both plants and mycorrhizal fungi. Phylogeographic relationships were estimated for the mycorrhizal fungi. Orchid functional outcomes were determined through in vitro seed germination and seedling growth with a broad phylogenetic representation of fungi. Most fungal isolates derived from one clade of *Ceratobasidium* (anamorphs assignable to *Ceratorhiza*), with 78% within a narrower phylogenetic group, clade B. No correlation was found between the distributions of orchid and fungal genotypes. All fungal isolates significantly enhanced seed germination, while fungi in clade B significantly enhanced seedling growth. These results show that *I. utricularioides* associates with a phylogenetically narrow, effective fungal clade over a broad distribution. This preference for a widespread mycorrhizae may partly explain the ample distribution and abundance of *I. utricularioides* and contrasts with local mycorrhizal diversification seen in some nonphotosynthetic orchids. Enhanced orchid function with a particular fungal subclade suggests mycorrhizal specificity can increase orchid fitness.

Key words: *Ceratobasidium*; functional outcomes; *Ionopsis utricularioides*; neotropics; Orchidaceae; orchid mycorrhizae; *Rhizoctonia*; specificity.

The Orchidaceae is one of the most species-rich plant families, and many orchid species are characterized by limited geographic distributions (Tremblay et al., 2004). The availability of suitable mycorrhizal fungi is probably a key factor constraining the distribution of orchid species. Orchid seeds, lacking endosperm, have minimal stored resources for germination and seedling growth, relying instead upon mycorrhizal associations to provide both fixed carbon and mineral nutrients (Alexander et al., 1984; Hadley, 1984; Alexander and Hadley, 1985). In some orchids, this mycoheterotrophic state continues into adulthood, with the orchid never acquiring photosynthetic abilities and depending exclusively on their mycorrhizal fungi for nutrition. While most orchid species do develop photosynthetic ability after germination, mycorrhizal associations generally persist in adult photosynthetic plants, although changes in fungal associations between juvenile and adult plants have been documented in at least one orchid species (Rasmussen, 2002).

The specificity of orchid mycorrhizal (OM) interactions is of crucial importance to orchid ecology and conservation. Broadly distributed orchids might be expected to be either general in their preferences for mycorrhizal fungi or specific and associated with a broadly distributed fungus, as in many mutualistic relationships (Bascompte et al., 2003; Vazquez and Aizen, 2003). In contrast, a narrow OM specificity could be a reason for rarity and vulnerability of the plant species.

Recently, molecular phylogenetic approaches have facilitated the study of OM specificity. In this context, specificity may be defined as the phylogenetic breadth of associations between different taxa (Thompson, 1994). An orchid species may be considered specific if it interacts with fungal taxa contained within a single, restricted phylogenetic clade. Studies to date show that orchid mycorrhizal interactions are, in general, more specific than other mycorrhizal systems. The level of dependence of orchids on their mycorrhizae has been invoked as a major determinant of the levels of specificity in OM associations. Thus, nonphotosynthetic orchids have been hypothesized to be more specific because of their heightened nutritional needs (Taylor et al., 2002; McCormick et al., 2004; Selosse et al., 2004). Studies in temperate mycoheterotrophic species have revealed a high degree of specificity, with different orchid species associated with distinct phylogenetic clades of fungal partners (Taylor and Bruns, 1997; Selosse et al., 2002). However, certain photosynthetic orchids, both tropical epiphytes (Otero et al., 2002) and temperate terrestrials

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(McCormick et al., 2004), have been shown to be at least as specific as these nonphotosynthetic species.

The degree of specificity in OM associations will ultimately depend upon the ecological coincidence of the orchid and fungi (ecological specificity), the physiological compatibility between the fungus and orchid, and the relative functional outcomes (fitness) of each interaction. Ecological specificity is determined by the range of potential fungal partners available (Perkins and McGee, 1995). The mechanisms underlying physiological compatibility are still unknown, but the functional outcomes of OM interactions, at least for the orchid, are more tractable to investigators.

Not all mycorrhizal fungi are functionally equivalent in their interactions with orchids (Zelmer and Currah, 1997; McCormick et al., 2004; Otero et al., 2004, 2005). An orchid species may have a broad phylogenetic specificity but function significantly better with a restricted proportion of its fungal partners (e.g., Schemske and Horvitz, 1984). Zelmer et al. (1996) found that orchid seedlings associated with a wider range of fungi than older plants, possibly reflecting fairly low specificity at the germination stage. However, only those plants with optimum compatibility developed to adulthood. Thus, in addition to studying phylogeny, researchers must also measure the functional outcomes of OM interactions to accurately assess specificity.

Although the majority of orchid taxonomic diversity is found in the tropical epiphytes, the mycorrhizal fungi of these species are least known (Richardson et al., 1993; Zettler et al., 1999; Otero et al., 2002, 2004, 2005; Ma et al., 2003; Pereira et al., 2005a, b, c). Studies of a number of epiphytic orchid species in Puerto Rico revealed differing levels of mycorrhizal specificity amongst species (Otero et al., 2002, 2004, 2005), but variation in mycorrhizal specificity of tropical orchids over a broader geographical distribution has not been explored.

One of the most fungal-specific orchids identified in Puerto Rico is *Ionopsis utricularioides* (Swartz) Lindley (Otero et al., 2002, 2004). This species is a photosynthetic, twig epiphyte in the subtribe Oncidiinae (Williams et al., 2001) and grows on small trees or shrubs such as guava (*Psidium guajava* L.) and tintillo (*Randia aculeata* L.). It is broadly distributed across the neotropics, from Florida, USA, to tropical South America, including the Caribbean and Galapagos Islands. It is locally abundant in Puerto Rico and parts of Central America (Ackerman, 1995).

In Puerto Rico, *I. utricularioides* has mycorrhizal associations almost exclusively with one of four clades of *Ceratobasidium* (clade B) isolated from the nine orchid species studied (Otero et al., 2002). *Ceratobasidium* is a teleomorphic (sexual stage) genus associated with strains of the anamorphic genus *Ceratorhiza*. These fungi, in common with most orchid mycorrhizal fungi, are classified as *Rhizoctonia*-like fungi (Moore, 1987). *Ionopsis utricularioides* also had a higher functional outcome with those *Ceratobasidium* fungi isolated from the same species than with those isolated from another related, sympatric species (Otero et al., 2004).

In the present study we further investigate the mycorrhizal specificity of *I. utricularioides*, addressing three specific questions: (1) Do the fungal associations of *I. utricularioides* vary over a broad geographic range? (2) Is there a correlation between genetic structure of the orchid and its mycorrhizal partner across this broad distribution? (3) Are there functional differences among different fungal isolates from *I. utricularioides*? To answer these questions, we isolated mycorrhizal fungi from *I. utricularioides* collected over a broad geographic distribution. Using a molecular phylogeny based on nuclear ribosomal internal transcribed spacer (ITS) sequences, we compared the fungal isolates with those isolated previously in Puerto Rico. Genetic variation at the ITS locus was also surveyed within *I. utricularioides*. Finally, to investigate variation in mycorrhizal function, we performed in vitro experiments to test seed germination and seedling growth of *I. utricularioides* with a range of mycorrhizal fungi isolated from adult plants of *I. utricularioides*.

MATERIALS AND METHODS

Sampling material—Roots of *I. utricularioides* were collected from the following locations across the distribution of the species (Table 1): La Chorrera, Panama; La Selva and Puerto Viejo, Costa Rica; on the Arima-Blanchisseuse road, Trinidad; Soroa (Pinar del Río), Cuba; and Chiclayo, Peru. Fungi isolated from *I. utricularioides* at four sites in Puerto Rico (Cambalache, Dorado, San Cristóbal, and Tortuguero) were previously characterized (Otero et al., 2002, 2004).

Potential mycorrhizal fungi were isolated from the orchid plants and identified from pure culture. *Ionopsis utricularioides*, like many other tropical epiphytic species, has few active pelotons (the coils of fungal hyphae that form within root cortex cells) suitable for isolation (Otero et al., 2002); thus, endophytic fungi were isolated from single hyphal tips emerging from sterilized root portions. Four roots per plant were surface sterilized by a 2-min wash with tap water and 0.01% Tween 20 or Triton X; a 1-min and 0.5-min wash with 70% ethanol and 2.5% sodium hypochlorite (2.5% sodium hypochlorite), respectively; and a 1-min rinse in 70% ethanol (Otero et al., 2002). Three pieces (0.5–1.0 cm each) of each root were incubated in a petri dish with potato dextrose agar (Difco, Lawrence, Kansas, US) and malt extract agar (Difco). Petri dishes were incubated at 25°C with 12-h light–12-h dark cycles for 1–2 wk. *Rhizoctonia*-like colonies identified at 400x magnification (Sneh et al., 1991) were transferred to pure culture.

The orchid plants from which the fungi were isolated were also sampled for population genetic variation. These plants were sampled across sites in Puerto Rico, Costa Rica, and Trinidad. Unfortunately, it was not possible to amplify plant ITS sequences from samples collected in Panama and Peru.

Sequencing of ITS region and phylogenetic analysis—The DNA was extracted from pure cultures (for fungi) and from leaves (for plants) using a minipreparation (Lee and Taylor, 1990). In a few cases, fungi were identified through direct PCR from pelotons following the methods of Kristiansen et al. (2001). The ITS region of nuclear ribosomal DNA was amplified by PCR with primer pairs ITS1/ITS4 for fungi and ITS5/ITS4 for plants (White et al., 1990). The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, California, USA) and sequenced using the BigDye cycle sequencing kit on an ABI Prism 377 (Applied Biosystems, Foster City, California, USA). Sequences were edited using the program Sequencher 3.0 (Gene Codes, Ann Arbor, Michigan, USA).

Sequences from 10 fungal isolates were identical to those of other isolates from the same plant and therefore may have come from the same fungal individual. These sequences were excluded from subsequent analyses. The ITS sequences obtained in the present study were aligned with 18 previously identified from Puerto Rico (Otero et al., 2002, 2004) for phylogenetic analysis. Those GenBank accessions most similar to these sequences were identified using the BLASTn algorithm (http://www.ncbi.nlm.nih.gov/) (see Table 1 for these accessions), and alignments were performed using Clustal W with the program Bioedit 5.0.9 (Hall, 1999) and checked visually.

The ITS fungal alignment had 614 bases. The most likely model of evolution was estimated as the Hasegawa, Kishino, Yano model with a proportion of invariable sites P(I), and gamma distribution shape parameter, G (HKY+I+G (P(I) = 0.3728; G = 0.5583) using the program Modeltest 3.06 (Posada and Crandall, 1998). Phylogenetic relationships were estimated using both maximum parsimony (MP) and maximum likelihood (ML) with the heuristic search option and the tree bisection-reconnection (TBR) branch-swapping routine in the program PAUP* (version 4.0b10; Swofford, 2003). The MP analysis utilized 106 informative characters resulting in 36 equally parsimonious trees with score of 402. The most likely tree had a score of –

TABLE 1. Isolates of *Rhizoctonia*-like fungi used in this study.

Isolate	Country	Site	GenBank no.	Reference
JTO-010	Puerto Rico	Tortuguero	AF472280	Otero et al., 2002
JTO-024	Puerto Rico	Tortuguero	AF472281	Otero et al., 2002
JTO-032	Puerto Rico	Cambalache	AF472282	Otero et al., 2002
JTO-047	Puerto Rico	Tortuguero	AF472284	Otero et al., 2002
JTO-060	Puerto Rico	San Cristobal	AF503978	Otero et al., 2004
JTO-063	Puerto Rico	San Cristobal	AF503979	Otero et al., 2004
JTO-078	Puerto Rico	San Cristobal	AF472293	Otero et al., 2002
JTO-092	Puerto Rico	Tortuguero	AF503981	Otero et al., 2004
JTO-093	Puerto Rico	Tortuguero	AF472296	Otero et al., 2002
JTO-112	Puerto Rico	San Cristobal	AF503982	Otero et al., 2004
JTO-116[a]	Puerto Rico	San Cristobal	AF472299	Otero et al., 2002
JTO-127	Puerto Rico	Dorado	AF503985	Otero et al., 2004
JTO-131	Puerto Rico	Dorado	AF503988	Otero et al., 2004
JTO-132	Puerto Rico	Dorado	AF503989	Otero et al., 2004
JTO-133	Puerto Rico	Dorado	AF503990	Otero et al., 2004
JTO-155	Puerto Rico	Dorado	AF504004	Otero et al., 2004
JTO-158	Puerto Rico	Dorado	AF504003	Otero et al., 2004
JTO-160	Puerto Rico	Dorado	AF504007	Otero et al., 2004
JTO-201	Panamá	La Chorrera	DQ083999	This study
JTO-203	Panamá	La Chorrera	DQ084001	This study
JTO-209	Panamá	La Chorrera	DQ084003	This study
JTO-212	Panamá	La Chorrera	DQ084004	This study
JTO-215	Panamá	La Chorrera	DQ084005	This study
JTO-217	Trinidad	Blanchisseuse road	DQ084007	This study
JTO-218	Costa Rica	La Selva	DQ084006	This study
JTO-219	Costa Rica	La Selva	DQ084008	This study
JTO-222	Costa Rica	La Selva	DQ084009	This study
JTO-226	Costa Rica	La Selva	DQ084010	This study
JTO-230	Costa Rica	La Selva	DQ084011	This study
JTO-231	Costa Rica	La Selva	DQ084012	This study
JTO-232	Costa Rica	La Selva	DQ084013	This study
JTO-235	Costa Rica	Puerto Viejo	DQ084014	This study
JTO-236	Costa Rica	Puerto Viejo	DQ084015	This study
JTO-484	Perú	Chiclayo	DQ084016	This study
JTO-485[b]	Cuba	Soroa	DQ084017	This study
JTO-486[b]	Cuba	Soroa	DQ084018	This study
JTO-487[b]	Cuba	Soroa	DQ084019	This study
Ceratobasidium AG-A[c]	Japan	soil	AF354092	González et al., 2001
Ceratobasidium AG-Q[c]	Japan	soil	AF354095	González et al., 2001
Ceratobasidium AG-S[c]	Florida, USA	*Pittosporum*	AJ427400	V. González, unpublished data
Rhizoctonia Oss2-2[c]	Singapore	*Oncidium* sp.	AJ318421	M. Ma et al., unpublished data
Rhizoctonia Onv4B2[c]	Singapore	*Oncidium* sp.	AJ318437	M. Ma et al., unpublished data
Ceratobasidium AG-O[c]	Japan	soil	AF354094	González et al., 2001
Ceratobasidium AG-L[c]	Japan	soil	AF354093	González et al., 2001
Thanatephorus cucumensis[d]	Japan	soybean	AF153778	Pope and Carter, 2001

[a] Fungi isolated from *Ionopsis satyrioides*, not *I. utricularioides*.
[b] ITS sequences obtained by direct PCR from pelotons.
[c] ITS sequences in GenBank identified as most closely related to the fungal isolates from *I. utricularioides* by a search using Blastn.
[d] ITS sequence used as outgroup for the phylogenetic inference.

2856.65 (Fig. 1). Bootstrap support was calculated using MP and Neighbor-Joining (NJ) analysis. *Thanatephorus cucumeris* (GenBank AF153778 from soybean in Japan; Pope and Carter, 2001) was used as the outgroup for the fungal sequences (Otero et al., 2002, 2004).

Seed germination and growth—Seed germination experiments exclusively used seeds and fungi from Puerto Rico because of restrictions on the import and export of organisms between countries. However, the fungi chosen were representative of the phylogenetic diversity found across the distribution sampled (see Results).

Ten fruits (one per plant; $N = 10$ plants) of *I. utricularioides* were collected in the Tortuguero Natural Reserve (Manatí, PR) on 12 August 2001, when capsules were mature (the fruits were swollen and changing color from green to yellow). Fruits were transported to the laboratory inside plastic zip-closure bags, and fruits were surface-sterilized as previously described for the roots. Seeds were transferred to sterile water, and viability was estimated with triphenyl tetrazolium chloride (TTC) staining after 24 h (Vujanovic et al., 2000). Seed viability was 50–96%. Seeds of the five fruits with highest viability were pooled for germination experiments. Seed were sown within 48 h after collecting.

Thirteen fungi, including representatives of all clades found in this study, were inoculated on to different petri plates containing cellulose agar, a modification of Clements' isolation medium (Clements, 1988), with sucrose replaced by 10 g cellulose powder/L (Otero et al., 2004, 2005). Cellulose is a carbon source available to fungi but not plants, and orchid seedlings do not grow on culture media without either sugars or mycorrhizal fungi (Rasmussen, 1995). The pH of the agar was slightly acidic (5.5–6.5). Three days after fungal inoculation, a suspension of pooled seeds (249 ± 103 seeds, mean + SD) in 100 μL of sterile water was added to each plate. Each fungus–orchid combination was replicated three times. The plates were sealed with parafilm and incubated in the laboratory with a natural light regime at 24°C. Controls were established with cellulose agar plates inoculated with seeds but not with *Ceratobasidium* fungi. A long incubation (3 months) allowed endophytic fungi to grow on control plates (without *Ceratobasidium* fungi), but such fungi were

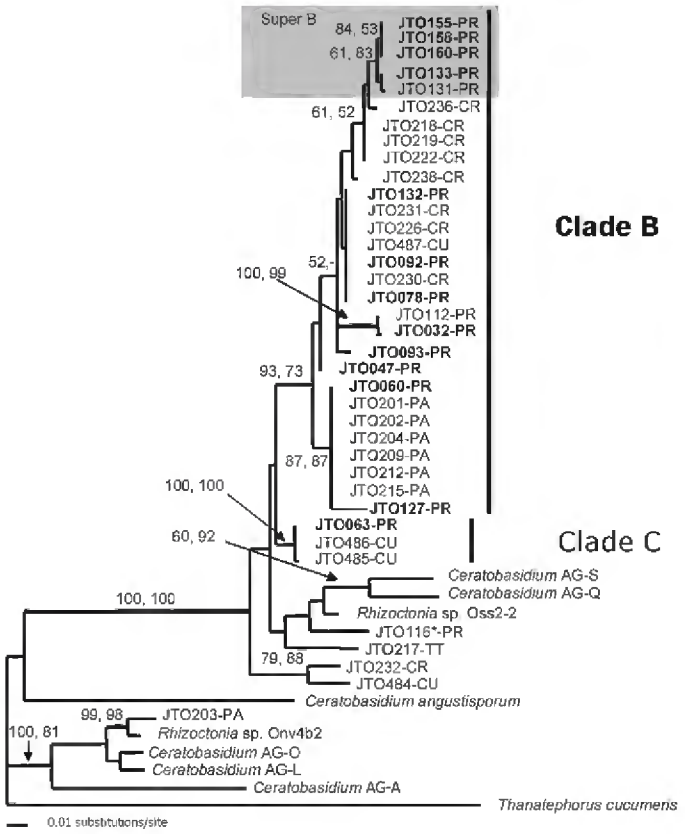

Fig. 1. Maximum likelihood tree of ITS sequences of *Ceratobasidium* fungi isolated from *Ionopsis utricularioides* showing clade B, clade C, and subclade super B; subclade super B is represented by the shaded box. Numbers represent the bootstrap value that supports the branch indicated, using neighbor joining and maximum parsimony, respectively. A minus in place of a maximum parsimony value represents branches with no support in the corresponding analysis. CR = Costa Rica (sites: P = Puerto Viejo; S = La Selva); CU = Cuba; PA = Panama; PE = Peru; PR = Puerto Rico (sites: C = Cambalache; D = Dorado; SC = San Cristobal; T = Tortuguero); TT = Trinidad and Tobago. Sequences in boldface type indicate those fungi used in the germination experiment. An asterisk indicates fungi isolated from *Ionopsis satyrioides*.

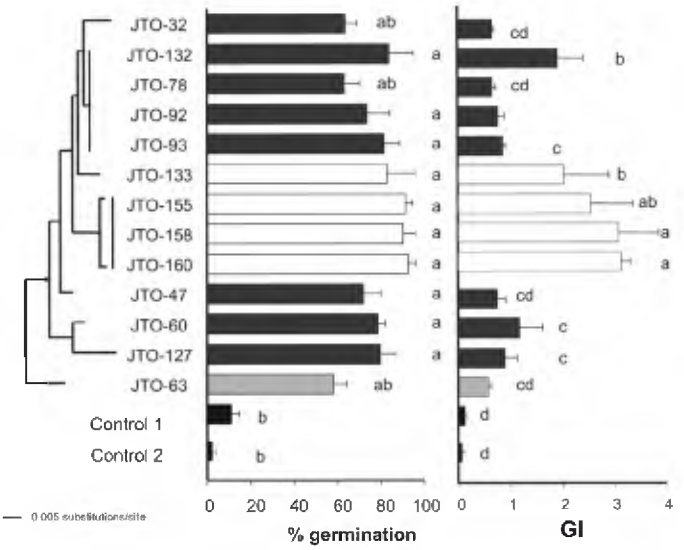

Fig. 2. Associations among percentage of seed germination, seedling growth index (GI) (mean + SD), and fungal phylogeny. Fungal isolates are labeled with their locality of origin (C = Cambalache; D = Dorado; SC = San Cristobal; T = Tortuguero). Bars with the same letter were not significantly different in Tukey multiple comparison tests. Solid bars represent fungi in clade B, excluding super B; open bars represent super B; gray bar represents clade C. Control 1 refers to seeds placed on agar with fungicide. Control 2 refers to seeds on agar without fungicide, but with endophytic fungi present in the seed walls and placenta. The phylogram is a neighbor-joining tree based on the fungi shown.

not seen on plates inoculated with the experimental fungi because the experimental fungi grew much faster. We thus set up two sets of controls to examine the effect of the isolated *Rhizoctonia*-like fungi on seed germination, both with and without the addition of a fungicide (benomyl, 35 mg/L). Levels of germination were low but equivalent in the two sets of control plates lacking *Ceratobasidium* (Fig. 2). Subsequent fungal growth from small clumps of placental tissue on these control plates was assumed to be of endophytic fungi.

Seed germination and growth data were collected by inspecting each plate at 40x magnification at 5, 8, and 11 wk after sowing. On each plate, 30 or more randomly selected seeds were evaluated. The percentage of seed germination was estimated by dividing the number of seeds that had germinated (with germination stages between 1, where the embryo has swelled, and 6, where roots have developed) by the total number of seeds viewed. A standardized growth index (GI) was calculated, modified from Spoerl (1948). The GI varies from 0 (no seeds have germinated) to 6 (all seeds are at seedling stage) (Otero et al., 2004, 2005). Statistical analyses were performed in the program Statistica (StatSoft, Tulsa, Oklahoma, USA). Differences in seed germination and GI were assessed using one-way analysis of variance (ANOVA). The GI data were standardized by dividing each value by six. Because the germination data were percentage values and the standardized GI did not have a normal distribution, both data sets were normalized with an arcsine square-root transformation (Zar, 1999).

RESULTS

All fungi isolated from *I. utricularioides* were allied to *Ceratobasidium* (anamorph *Ceratorhiza*) (Fig. 1). With the exception of one isolate (JTO203 from Panama), all samples formed a well-supported major clade that included the clades A, B, and C first identified in Puerto Rico (Otero et al., 2002). GenBank accessions from *Ceratobasidium* anastomosis groups S and Q (González et al., 2001) and a mycorrhizal isolate from the orchid *Oncidium* sp. in Singapore (*Rhizoctonia* Oss2–2; Ma et al., 2003) also fell in this major clade.

The majority of *I. utricularioides* fungal isolates (29/37 = 78%) were contained within clade B of *Ceratobasidium*. Two isolates from Cuba (JTO485, JTO486) and one from Puerto Rico (JTO063) fell in clade C, and a single isolate (JTO217 from Trinidad) was associated with clade A (Otero et al., 2002). Two other isolates (JTO232 from Cuba and JTO484 from Peru) were grouped together in a previously unidentified clade. No phylogeographic structure was apparent among the fungal isolates, and fungi from clade B were isolated from Cuba, Costa Rica, and Panama, in addition to Puerto Rico. In fact, samples from Puerto Rico, Cuba, and Costa Rica yielded identical ITS sequences (e.g., JTO132, JTO231, JTO487: see Fig. 1).

Population genetic differentiation was investigated in the orchid *I. utricularioides* through DNA sequence variation at the ITS locus. Two genotypes were identified: one was shared by all plants sampled in Costa Rica, while the other was common to plants sampled in both Puerto Rico and Trinidad. These genotypes differed by three transitional base-pair substitutions at positions 126 and 175 of the ITS-1 and

position 203 of ITS-2 (see GenBank accessions DQ088152, Costa Rican genotype; DQ088150, Antillean genotype).

All the *Ceratobasidium* isolates from *I. utricularioides* tested in this experiment promoted germination in vitro, showing that they are potential mycorrhizal fungi (in the sense of Masuhara and Katsuya, 1994). All isolates significantly enhanced germination relative to the controls ($F_{13,28} = 15.41$, $P < 0.0001$), with no significant differences among isolates (Fig. 2).

In contrast to germination, the fungal treatments differed significantly in the seedling growth index (GI) ($F_{13,28} = 23.09$; $P < 0.0001$; Fig. 2). When controls (which had much lower GI than all fungi) were removed from the analysis, differences were still highly significant. The fungi that induced the highest GI fell within a subclade of clade B, here defined as subclade super B (Fig. 1). Isolates in this subclade induced significantly higher GI than other isolates in clade B (Fisher's exact test, two tails, $P = 0.0047$). These fungi were all isolated from a single site in Puerto Rico (Dorado) but were phylogenetically related to fungi from two sites in Costa Rica (Fig. 1). A single isolate outside the super B clade (JTO132) induced similar growth to isolates within super B. This isolate was also derived from a plant from Dorado. All other fungi tested were less effective at promoting seedling growth, with isolates from the remainder of clade B being equivalent to an isolate from clade C (JTO-63).

DISCUSSION

The distributions and diversity of orchid species may largely depend on the specificity of their orchid mycorrhizal (OM) interactions and on the distribution of the OM fungi. Understanding these obligate associations is of crucial importance for orchid ecology and conservation. The study presented here extends our investigations into the specificity of the mycorrhizal associations of the little-studied tropical, epiphytic orchid species.

Our prior studies of OM specificity across a number of photosynthetic, epiphytic neotropical orchid species showed that *I. utricularioides* is significantly more specific in its mycorrhizal associations than related, sympatric species, including *Tolumnia variegata* (Sw.) Braem (Otero et al., 2002, 2004). All but one of the fungal isolates from *I. utricularioides* populations on the island of Puerto Rico fell within a single clade of *Ceratobasidium* (anamorph *Ceratorhiza*) (the clade B discussed here), while sympatric populations of *T. variegata* were associated with fungi from four related clades, including clades A, B, and C (Otero et al., 2004). Our present continental-wide sampling of mycorrhizae of *I. utricularioides* revealed that this orchid shows remarkable fidelity to a single major clade of *Ceratobasidium* fungi (including the clades A, B, and C) across this range. Of 37 fungal isolates from plants collected in Central America, Peru, and the Caribbean islands, only one did not fall in this clade. While broader geographic samplings revealed that the association between *I. utricularioides* and clade B is not as strict as seen in Puerto Rico (Otero et al., 2004), the majority (78%) of the isolates still fell within this clade.

Additionally, a previous study (Otero et al., 2004) showed that seed germination and growth of *I. utricularioides* was more enhanced by fungi isolated from adult plants of this same species than by fungi isolated from adult *T. variegata*. In contrast, seeds from *T. variegata* germinated equally well with fungi isolated from either orchid species. The present study of the germination and seedling growth of *I. utricularioides* with a phylogenetically broad representation of fungal isolates from adult plants of this species reveals that, while all isolates were equally effective at promoting germination, a specific subclade of fungi enhanced seedling growth significantly more than fungi from other subclades. These accumulated data now show a considerable degree of specialization of *I. utricularioides* in its mycorrhizal associations, with the consequence of enhanced germination and early growth with the specific fungus, or, alternatively, a reduced capacity to exploit a broader range of fungal clades; both outcomes may have occurred.

Identification of OM fungi with the pure culture technique has been criticized because only fungi that grow on the specific medium may be identified; nonculturable fungi will not appear (Rasmussen, 2002). In contrast to temperate, terrestrial orchids, which often produce abundant pelotons, tropical, epiphytic orchids often present few intact pelotons amenable to direct PCR identification. The use of pure culture as a method for OM identification in this study was supported by the fact that the few fungi identified through direct PCR of pelotons fell within the same *Ceratobasidium* clade as those identified through pure culture (Table 1, Fig. 1). Furthermore, germination and seedling growth experiments confirmed the mycorrhizal nature of these fungal isolates. The striking observation of the same fungal clade isolated across such a wide geographical distribution of *I. utricularioides* suggests that this clade is indeed an important mycorrhizal partner for this orchid species.

Our broad geographical sampling of OM across the Caribbean region revealed a widespread distribution of the *Ceratobasidium* mycorrhizal clades. Also contained within the major clade were isolates from the *Ceratobasidium* anastomosis groups S and Q, from Florida and Japan, respectively (Gonzalez et al., 2001), and a mycorrhizal fungus isolated from a species of the orchid *Oncidium* in Singapore (GenBank AJ318421). The geographic range of the narrow phylogenetic group (clade B), from Central America to the eastern Caribbean, was also striking, with identical ITS sequences obtained from samples from Puerto Rico, Cuba, and Costa Rica. Little is known about population structure, reproductive mode, and dispersal in *Ceratobasidum*, even in its better-studied relative *Rhizoctonia solani* (= *Thanatephorus*) (Cubeta and Vilgalys, 1997). We sampled orchid roots only, and it is unlikely that this fungus is restricted to orchid roots. However, the extent of its habitat is unknown.

Our functional experiments provide insights into the fitness consequences for the orchid of the mycorrhizal associations. We found that the fungi isolated from adult *I. utricularioides* significantly enhanced orchid seed germination and seedling growth. The large quantity of small seeds produced in a single orchid fruit make it likely that that the life history stage of seedling establishment is a bottleneck in the life cycle of the orchids (Ackerman et al., 1996), and thus seedling development is probably one of the best criteria for measuring functional outcomes (Rasmussen, 2002). The measure of seedling growth may also indicate fitness effects of these OM associations later in the orchid life history; that these fungi were all isolated from adult plants attests to their role in adult plants. Fungal succession in OM associations across orchid life cycles has been described in some studies in terrestrial orchids. In *Spiranthes lacera*, both *Ceratorhiza goodyerae-repentis* (teleomorph: *Ceratobasidium cornigerum*) and *Epulorhiza repens* (teleomorph: *Tulasnella calospora*) were isolated from adult plants, but only *Ceratorhiza goodyerae-repentis* was

isolated from protocorms (Zelmer and Currah, 1997). Similarly, in *Vanilla* sp., *Ceratobasidium, Tulasnella*, and *Thanatephorus* were isolated from adult roots, but only *Ceratobasidium* promoted seed germination in vitro (Porras-Alfaro and Bayman, 2007). These studies suggest that *Ceratobasidium* and their anamorphs are important at the seed germination stage for diverse orchids. In a comparative study of three terrestrial, photosynthetic orchids, McCormick et al. (2004) found that the protocorms of *Tipularia discolor* were associated with a different, more restricted range of fungi than the adult stages. In contrast, in two other species, *Goodyera pubescens* and *Liparis lilifolia*, protocorms and adult plants were associated with the same fungi. While we have no evidence of OM fungal succession in *I. utricularioides*, protocorms and juvenile stages from plants in the wild should be examined to confirm this possibility.

Few studies have examined OM associations across a broad geographic area. The strong association found in this study between *I. utricularioides* and a single clade of *Ceratobasidium* fungi broadly distributed across the orchid's natural range contrasts to the geographical differentiation in OM associations seen across the range of an achlorophyllous, terrestrial orchid (Taylor et al., 2004). The high specificity in *I. utricularioides* is not a consequence of ecological specificity (Perkins and McGee, 1995), i.e., it is not due to a limited number of fungal partners available. Studies of the mycorrhizal fungi of nine sympatric orchid species revealed a broader diversity of fungi available in sites where *I. utricularioides* was collected (Otero et al., 2002), and this orchid species occasionally does associate with these alternative fungi. Clearly, experiments investigating the physiological basis for the enhanced growth with clade super B, in addition to in situ experiments of orchid mycorrhizal function across the geographic range of the species, are the next logical steps to investigate further this highly specific mycorrhizal association.

Although *I. utricularioides* had a strong preference for *Ceratobasidium* clade B across the sampled distribution, the fungus did not have an equivalent specificity. In a previous study, four other species of epiphytic orchids were also associated with fungi in clade B (Otero et al., 2002). These observed fungal associations suggest that this *Ceratobasidium* clade may be an important mycorrhizal associate of a variety of tropical epiphytic orchids. Further sampling of other orchid species sympatric with *I. utricularioides* across its extensive range may reveal that this *Ceratobasidium* clade is associated with many other tropical epiphytic orchids. Interestingly, green house tests with neotropical *Vanilla* species showed that fungal isolates from *I. utricularioides* pertaining to clade super B were significantly more effective at enhancing growth than the fungi isolated from the *Vanilla* (Porras-Alfaro and Bayman, 2007).

Conclusions—The causes and effects of orchid mycorrhizal specificity are still not well understood. In particular, data on epiphytic and tropical orchids, which constitute the greater part of the family, are sorely lacking. Our present findings suggest that the success of *I. utricularioides*, in terms of its broad geographic range and large population sizes, is correlated with its mycorrhizal association to an effective and widely distributed group of fungi. This fungal clade is also likely to be important in mycorrhizal associations of other tropical orchids.

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