LANKESTERIANA 13(1–2): 119—128. 2013.

TULASNELLA IRREGULARIS (BASIDIOMYCOTA: TULASNELLACEAE) FROM ROOTS OF *ENCYCLIA TAMPENSIS* IN SOUTH FLORIDA, AND CONFIRMATION OF ITS MYCORRHIZAL SIGNIFICANCE THROUGH SYMBIOTIC SEED GERMINATION

Lawrence W. Zettler*, Laura L. Corey, Alishia L. Jacks, Luke T. Gruender & Alyssa M. Lopez

Department of Biology, Illinois College, 1101 West College Avenue, Jacksonville, IL 62650, U.S.A.
*Author for correspondence: lwzettle@mail.ic.edu

Abstract. Epiphytic orchids remain understudied with respect to their obligate mycorrhizal relationships – a key component of the integrated conservation model. Existing studies have revealed that these plants, like their terrestrial counterparts, commonly associate with ubiquitous basidiomycetes (*e.g*., Tulasnellaceae); however, few studies have verified their physiological role(s). Two strains of mycorrhizal fungi (UAMH 11541, UAMH 11543) were isolated from roots of an epiphytic orchid in south Florida, *Encyclia tampensis*; one was acquired from a seedling and one from a mature specimen. Seeds of four epiphytic taxa were subsequently inoculated (separately) with both fungal strains *in vitro*: *E. tampensis*, *Epidendrum amphistomum*, *Epidendrum nocturnum,* and *Prosthechea cochleata*. More than one-third of inoculated *E. tampensis* and *E. nocturnum* seeds developed leaves in total darkness after 100 days. No significant differences were detected between the two strains on germination, nor any interaction between fungus and seed source (ANOVA, $\alpha = 0.05$). Using ITS amplification and sequencing, both strains were identified as the teleomorph, *Tulasnella irregularis* (Basidiomycota: Tulasnellaceae), and both were genetically identical with a high (98%) degree of certainty. Thus, symbiotic germination and ITS sequencing results are in agreement that both strains are indeed the same fungus. This paper is meant to shed additional light into epiphytic orchid-fungal interactions and highlights the need to identify, test (through symbiotic germination) and safeguard mycorrhizal fungi necessary for conservation.

Resumen. Las relaciones micorríticas obligadas de las orquídeas epífitas – un componente clave en el modelo integrado de conservación – siguen desconocidas. Los estudios existentes han revelado que estas plantas, así como sus equivalentes terrestres, se asocian normalmente con basidiomicetos ubícuitos (*e.g*. Tulasnellaceae); sin embargo pocos estudios han verificado su papel fisiológico. Dos cepas del hongo micorrítico (UAMH 11541, UAMH 11543) fueron aisladas de las raíces de una especie de orquídea epifítica del sur de Florida, *Encyclia tampensis* – una obtenida de una plántula joven y la otra obtenida de una planta madura. Las semillas de cuatro taxones epífitos fueron inoculadas por separado con los dos hongos aislados *in vitro*: *E. tampensis*, *Epidendrum amphistomum*, *Epidendrum nocturnum* y *Prosthechea cochleata*. Más de un tercio de las semillas de *E. tampensis* y *E. nocturnum* (ambos inoculados) desarrollaron hojas cuando fueron mantenidos en total obscuridad por más de cien días. No se detectaron diferencias significativas entre las dos cepas del hongo con respecto a la germinación y la interacción entre el hongo y las semillas procedentes de diferentes fuentes (ANOVA, $\alpha = 0.05$). Usando amplificación y secuenciación ITS, ambas cepas fueron identificadas como teleomorfos, *Tulasnella irregularis* (Basidiomycota: Tulasnellaceae) y ambas resultaron genéticamente idénticas con un elevado grado (98%) de seguridad. De esta manera, tanto la germinación simbiótica así como los resultados de la secuenciación ITS concuerdan en identificar con certeza ambas cepas como el mismo hongo. Este artículo trata de elucidar las interacciones entre orquídeas epífitas y hongos micorríticos y también subraya la necesidad de identificar, comprobar (a través de germinación simbiótica), y preservar los hongos micorríticos necesarios para fines de conservación.

Key words: Conservation, epiphytic orchids, mycorrhizal relationships, physiology, ITS sequencing, Basidiomycota, *Tulasnella irregularis, Encyclia tampensis*, *Epidendrum*

Orchids occur naturally on all vegetated continents (Dressler 1981), but about three-quarters of all known species (73%) exist as epiphytes within the tropics (Atwood 1986). Of the family's estimated 17,000-35,000 species worldwide (Atwood 1986; Dressler, 1993), orchid diversity is richest in the New World (Cribb *et al*. 2003). In light of accelerated destruction of the world's tropical forests in this century and the last, many orchid species face almost certain extinction unless effective strategies aimed at their long-term conservation are swiftly implemented. For orchids in particular, this will be a daunting task given the high degree to which these plants rely on other biotic agents (*e.g*., pollinators, mycorrhizal fungi) for their reproduction and survival needs. As a result, integrated conservation — blending ecological/genetic studies with *ex situ* and *in situ* research — has emerged as a more complete, inclusive approach to orchid conservation (see Swarts & Dixon 2009), evidenced by studies in Australia (Swarts 2007) and North America (Stewart 2007).

Compared to temperate terrestrial orchids, tropical epiphytes remain understudied with respect to their obligate mycorrhizal relationships (Otero *et al*. 2007), a key component of the integrated model. However, a growing number of studies have emerged in recent years that document the identity of mycorrhizal fungi associated with epiphytic orchids worldwide using DNA and TEM methods (*e.g*., Aggarwal *et al.* 2012; Ma *et al*. 2003; Martos *et al*. 2009; Pereira *et al*. 2003, 2005; Roy *et al.* 2009; Herrera *et al*. 2010; Kottke *et al*. 2010; see review by Dearnaley *et al.* 2013). Like their temperate terrestrial counterparts, epiphytic orchids (so far) appear to associate commonly with ubiquitous basidiomycetes assignable to Ceratobasidiaceae, Tulasnellaceae and Sebacinales. While interesting as this new information may be, most studies have targeted only mature plants rather than seedlings, and most have merely identified these fungi without verifying their physiological role(s). For integrated conservation to be successfully applied to the epiphytes, studies must also isolate, identify and preserve mycorrhizal fungi, including those from early growth stages (protocorms, seedlings), but this may be viewed as problematic given that locating diminutive seedlings on arboreal substrates is not always easy or practical. To facilitate the recovery of protocorm stages, a seed-baiting technique modified for epiphytic orchids (Zettler *et al.* 2011) may hold some promise in capturing fungi that initiate the germination processes *in situ*. Though access to tree limbs may be physically challenging, locating tiny leaf-bearing seedlings on arboreal substrates is relatively easy once practiced with a well-trained eye. Seedlings subsequently recovered may likewise harbor mycorrhizal strains that play a key role in the orchid's growth and development.

In nature, all orchids are thought to have a critical need for mycorrhizal fungi as a carbon source to propel orchid growth stages to completion. For horticultural purposes, the use of fungi to germinate orchid seeds *in vitro* (= symbiotic seed germination) has been largely ignored for the epiphytes because of the ease with which these plants can be grown on asymbiotic (carbon-based) media, unlike temperate (hardy) terrestrials that are notorious for having fastidious germination requirements (Rasmussen 1995). As a propagation tool, symbiotic germination (Clements *et al.* 1986; Dixon 1987) not only appears to have merit for epiphytic orchids (*e.g*., Aggarwal *et al*. 2012; Zettler *et al*. 2007; see Bayman 2012) but can also be used to verify the physiological role(s) of peloton-forming fungi. Although *in vitro* outcomes may or may not reflect what actually occurs *in situ*, the use of symbiotic germination for this purpose does provide some baseline for assessing mycorrhizal fungi for the purposes of conservation when viewed in the proper context.

In this paper, we describe the isolation of two strains of mycorrhizal fungi from roots of an epiphytic orchid in south Florida, *Encyclia tampensis* (Lindl.) Small, spanning two growth stages (leaf-bearing seedling, mature plant). The use of ITS amplification and sequencing was carried out to identify these strains, and *in vitro* symbiotic seed germination was applied to verify their physiological role. Seeds from three other epiphytic taxa (*Epidendrum amphistomum* A.Rich., *E. nocturnum* Jacq., *Prosthechea cochleata* (L.) W.E.Higgins) from the same region (Collier County, Florida, USA) were also inoculated to test for fungal specificity. The goal of this study is to augment long-term conservation of *E. tampensis* and other epiphytic orchids by emphasizing mycorrhizal fungus recovery, use, assessment, and preservation.

FIGURES 1-4. *Encyclia tampensis* from the Florida Panther National Wildlife Refuge. 1. Close-up of an *E. tampensis* flower. Though still common, this species is commercially exploited for its appealing floral display. Scale bar = 1 cm. 2. Young seedlings of *E. tampensis* growing on the host tree, *Quercus virginiana* Mill., beneath a mature orchid. The narrow, pale green strap leaf and pseudobulb on each seedling are indicative of *E. tampensis*. Scale bar = 2 cm. 3. A tiny *E. tampensis* seedling growing in close proximity to larger seedlings seen in Fig. 2. Scale bar = 2 cm. 4. Subsequent removal of the seedling in Fig. 3 showing roots that yielded *Tulasnella irregularis* (UAMH 11543).

Material and methods

Orchid material and study site — Roots and mature seeds of *E. tampensis* (Fig. 1, 2) were collected from the Florida Panther National Wildlife Refuge (FPNWR) located in remote Collier County, Florida (USA) within the Big Cypress Basin eco-region of south Florida. Roots were collected from a small, leaf-bearing seedling (Fig. 3, 4) and a mature (flowering) specimen affixed to the SW-facing bark of *Quercus*

virginiana Mill. (Fagaceae) on 29 June 2011. The seedling was located ca. 0.5 m below the mature donor plant and was one of ca. 20-30 seedlings (Fig. 2) visible in various growth stages along the length of the host tree's bark. Their identification as *E. tampensis* was based on the narrow, pale green, rigid strap leaves, and characteristic ovoid pseudobulbs subtending the leaves on the larger seedlings. Although *E. tampensis* is occasionally found in wetter habitats (cypress domes) in association with other epiphytic taxa in the FPNWR, it is the only leaf-bearing species to occupy the drier upland sites suitable for *Q. virginiana*. Using a small spatula, the donor seedling with two roots (Fig. 4) was gently lifted from the surface of the substrate and placed into a pre-sterilized glass vial. One 10 cm-long root from the mature plant was likewise gently lifted from the substrate using a scalpel and placed into a separate pre-sterilized glass vial. Both sets of roots were then transported to the laboratory and refrigerated (6° C) for one week leading up to fungal isolation.

Seeds from four epiphytic orchid taxa were collected from Collier County, Florida, for use in symbiotic germination experiments: *E. tampensis*, *E. amphistomum*, *E. nocturnum,* and *Prosthechea cochleata*. Although *E. tampensis* is considered a common species, it is commercially exploited for its showy floral display (Fig. 1). The other three taxa are listed as state-endangered (Brown 2005). Three seed sources were collected from *E. tampensis* (S194, S195, S196) and one source for each of the other three taxa: *E. amphistomum* (S197), *E. nocturnum* (S20), and *P. cochleata* (S177) (Table 1). Mature capsules that appeared to be in the act of opening naturally were collected and placed over $CaSO_4$ desiccant (Drierite, W.A. Hammond Co., Xenia, Ohio, USA) in separate vials for transport to the laboratory. Within seven days of collection, capsules were placed over fresh Drierite desiccant at ambient temperature until seeds were thoroughly dry. Seeds were then removed by gently tapping the outer surface of the capsule over aluminum foil, then placed in sealed glass vials and stored at -7º C in darkness until use.

Fungal isolation, initial identification and preservation — Mycorrhizal fungi were isolated from the root cortical region using standard procedures (*e.g*., Currah *et al*. 1987, 1990; Richardson *et al.* 1993; Zettler *et al*. 2003). Roots were surface-sterilized for 1 min. in a solution of 5% absolute ethanol (EtOH), 5% Clorox bleach (5.25% NaOCl; Clorox Co., Oakland, California, USA) and 90% sterile DI water, followed by two 1 min. rinses in sterile DI water. Clumps of macerated cortical cells containing pelotons were immersed in Fungal Isolation Medium (FIM) containing streptomycin sulfate (Clements & Ellyard 1979) and incubated at ambient temperature. After 1-4 days, hyphal tips that emerged from the cortical region and/or pelotons were subcultured to potato dextrose agar (PDA, Difco™, Becton, Dickinson and Co., Sparks, Maryland, USA) using a sterile scalpel and dissection microscope. Orchid mycorrhizal strains were initially distinguished

Table 1. Six seed sources from four epiphytic orchid species utilized in symbiotic germination experiments. All seeds were derived from mature capsules on specimens that grew naturally in Collier Co., FL, and all were obtained from the Florida Panther NWR with the exception of S20.

Seed Source	Orchid	Date collected	Notes
S20	*Epidendrum nocturnum*	1 June 2002	Fakahatchee Strand, ca. 15 km S of FPNWR
S177	*Prosthechea cochleata*	14 March 2009	McBride's Pond (cypress dome)
S194	*Encyclia tampensis*	20 November 2011	Both S194 and S195 from two separate plants ca. 1 km SE of McBrides' Pond
S195	*Encyclia tampensis*	20 November 2011	See above
S196	*Encycila tampensis*	6 January 2012	Dry site ca. 100 m E of McBride's Pond
S197	*Epidendrum amphistomum*	28 June 2011	McBride's Pond (cypress dome)

from common molds using previously published descriptions (Currah *et al.* 1987; 1990; Richardson *et al*. 1993; Zettler *et al.* 2003). Fungi that yielded cultural characteristics on PDA assignable to the anamorphic form-genus *Rhizoctonia* and *Epulorhiza* in particular (Currah *et al.* 1997a; Moore 1987), were stored at Illinois College under refrigeration (4º C) in darkness on PDA slants in screw-cap tubes for eventual use in symbiotic germination experiments. Subcultures of these *Epulorhiza* strains were also deposited in the University of Alberta (Canada) Microfungus Collection and Herbarium (UAMH) for permanent safekeeping and future reference as: UAMH 11541, UAMH 11542, and UAMH 11543.

Molecular identification of fungi — Two of the fungi deposited into UAMH, one from the *E. tampensis* seedling (UAMH 11543) and one from the mature plant (UAMH 11541), were identified further via sequencing of the ITS regions of ribosomal DNA. To facilitate DNA isolation, colonies of pure fungus cultures were grown on liquid media (potato dextrose broth, Difco™) on a shaker at ambient temperature until harvesting, ca. 1 month after inoculation. DNA was isolated from liquid fungal cultures using the Omega EZNA Fungal DNA Mini Kit protocol for fresh/frozen samples (Omega Biotek, Doraville, Georgia, USA). The ITS regions of DNA isolates were amplified using primers ITS1-OF-T and ITS4-OF (Taylor & McCormick 2008). The reactions contained 1x EZNA Taq Buffer, 0.1 mM dNTPs, 25 pmoles ITS1-OF-T, 25 pmoles ITS4-OF, 12.5 units Taq polymerase and 5 µl of DNA sample. The amplification was performed in a programmable thermal cycler (Labnet, Edison, New Jersey, USA) programmed for 45 cycles. Each cycle consisted of 94°C for 30 sec, 52°C for 30 sec and 72°C for 60 sec. Amplification products were visualized by electrophoresis on 2% agarose gels containing 0.1 mg/ml ethidium bromide.

In vitro *symbiotic seed germination* — The protocol for symbiotic seed germination closely followed the procedure outlined by Zettler *et al*. (2007), except seeds were pipetted directly onto the agar surface and not onto filter paper. Briefly, seeds were surface-sterilized using the same bleach/EtOH rinse described above for fungal isolations, and ca. 50-100 seeds were dispensed onto the surface of an oat-based medium (2.5 g rolled oats, 7.0 g agar, 1 L DI water; Dixon 1987) within a 9 cm diam. petri plate using a sterile glass pipette. For each of the six seed sources, 10 replicate plates were prepared and inoculated with the seedling-derived fungus (UAMH 11543), and 10 replicate plates received the fungus isolated from the mature orchid (UAMH 11541). Five replicate plates for each of the six seed sources did not receive fungal inoculation and served as controls. To promote evaporation of the suspension droplet and seed/agar contact, petri plates were wrapped in Parafilm "M" (Pechiney Plastic Packaging, Menasha, Wisconsin, USA) ca. 24 hrs after sowing and inoculation. All plates were then wrapped tightly in aluminum foil to exclude light and incubated at ambient temperature (22º C) for 100 days. Using a dissecting microscope, seed germination and seedling development were assessed on a scale of 0-5 where: Stage 0 = no germination; Stage 1 = swollen embryo/ production of one or more rhizoids; Stage 2 = embryo enlargement leading to rupture of the testa; Stage 3 = appearance of shoot region (protomeristem); Stage 4 = emergence of first leaf; Stage 5 = leaf elongation. To test the hypothesis that both fungal strains (UAMH 11541, UAMH 11543) were the same fungus, data were analyzed using general linear model procedures multivariate analysis of variance ($P < 0.05$) and mean separation at $\alpha = 0.05$ by SPSS 12.0 for Windows subprogram (SPSS, Chicago, Illinois, USA). The experiment was repeated once.

Results and discussion

Fungal identification — Root sections of *E. tampensis* harbored a variety of endophytic fungi evident in petri plates containing FIM, most of which were later identified as common saprophytic molds (*e.g*., *Pestalotia*) on PDA. Of the ubiquitous anamorphic genera known to commonly associate with orchids worldwide, only *Epulorhiza* was evident in the samples. This initial identification was based on the creamy white colony appearance on PDA, coupled with mostly submerged/adnate mycelium with entire margins and relatively slow-growing hyphae (< 0.10 mm/hr) at ambient temperature (Currah *et al.* 1987, 1990; Richardson *et al.* 1993; Zettler *et al.* 2003). Three different strains of *Epulorhiza* were isolated, two of which originated from a different region of the mature plant's root (UAMH 11541, UAHM 11542) and the

third from the seedling root (UAMH 11543). On PDA, all three appeared indistinguishable from one another and closely resembled two other *Epulorhiza* strains isolated previously from orchids in northern Florida: UAMH 9824 from *Spiranthes brevilabris* Lindl. in Levy County (Stewart *et al*. 2003) and UAMH 9203 from *Epidendrum magnoliae* Muhl. (syn. *Epi. conopseum* R.Br.) in Alachua County (Zettler *et al*. 1997). Additional *Epulorhiza* strains have been isolated from Florida orchids including *Habenaria macroceratitis* Willd. in central Florida (Stewart & Kane 2006) and *E. nocturnum* from the Florida Panther NWR (L.W. Zettler, unpubl. data). To what extent these *Epulorhiza* isolates are genetically similar remains unknown, but the use of molecular techniques could be applied to those strains currently in storage at UAMH to resolve this question. In this study, sequencing of the ITS regions of ribosomal DNA revealed that both *Epulorhiza* isolates were assignable to the teleomorph *Tulasnella irregularis* Warcup & Talbot (Basidiomycota, Tulasnellaceae). Not only were these two strains of the same taxon, they both appeared to be genetically identical with a high (98%) degree of certainty. The fact that one strain was isolated from an *E. tampensis* seedling (UAMH 11543) and the other from a mature plant (UAMH 11541) indicates that different growth stages in this orchid are nutritionally tied to this one fungus.

Seed germination and seedling development — Both fungal isolates (UAMH 11541, UAMH 11543) facilitated seed germination and seedling development spanning all four orchid taxa *in vitro* 100 days after sowing and inoculation (Table 2). Percent germination exceeded 50% for all three *E. tampensis* seed sources (S194 = >81%; S195 = >50%; S196 = >84%) as well as seeds from *E. nocturnum* (S20 = >68%), whereas seeds from the other two taxa (*P. cochleata*, *E. amphistomum*) failed to develop beyond Stage 2 (Table 2). More than one-third of *E. tampensis* and *E. nocturnum* seeds inoculated with *T. irregularis* initiated and developed leaves (Stage 5) in total darkness. Two seed sources in particular, S20 (*E. nocturnum*) and S194 (*E. tampensis*), resulted in the highest percentage (>64%) of seeds developing to Stage 5 (Table 2). In contrast, seeds sown on the oat-based medium in the absence of fungi (control) largely failed to germinate (<7%). Thus, the presence of *T. irregularis* had a stimulatory effect on seed germination and development. No significant differences were detected between the two strains on seed germination nor any interaction between fungus and seed source (ANOVA, $\alpha = 0.05$). While there was a main effect of seed, both fungal isolates mirrored one another in their ability to germinate and prompt development across seed sources. Thus, symbiotic germination and ITS sequencing results are in agreement that both strains are indeed the same fungus.

Ecological implications — Although orchid seeds do contain small traces of food reserves (Rasmussen 1995), the long-held assumption is that mycorrhizal fungi are required as a carbon source to propel orchid seedlings to a photosynthetic stage. For epiphytic orchids, this concept has received more scrutiny given that these plants would have more access to sunlight in the host tree's canopy, compared to seedlings of terrestrials that remain buried underground. In this study, young protocorms of *E. tampensis* and *E. nocturnum* are fully capable of exploiting fungi for their growth and developmental needs, evidenced by advanced growth stages in the absence of light. This outcome supports a similar finding by Zettler *et al.* (1999) for seeds of *E. tampensis* inoculated with a fungus (*Epulorhiza* sp., UAMH 9203) from *E. magnoliae* Muhl. and incubated in darkness for 13 weeks. Likewise, seeds of *E. magnoliae* and *E. nocturnum* also developed leaves in darkness following inoculation with *Epulorhiza* (Zettler *et al*. 1998, 2007). To what extent that protocorms of other epiphytic orchids rely on mycotrophy remains to be determined, but the evidence indicates that this nutritional capability is not restricted to terrestrial orchids, at least under a controlled laboratory setting.
The presence of the same strain of *T. irregularis* acquired from a seedling and mature plant alike supports the hypothesis that *E. tampensis* relies on one fungus and also indicates that mycotrophy may continue to play a nutritional role as the orchid matures. This concept also makes sense from the perspective of seedling recruitment and survival. For example, mature orchids that retain mycorrhizal fungi would likely impart a survival advantage to nearby seedlings because the fungus required for germination and seedling development would be more likely to persist on a common substrate. Indeed, Batty *et al.* (2001) and

Table 2. *In vitro* symbiotic seed germination of four native epiphytic orchid species from south Florida (Collier County) using a mycorrhizal fungus (*Tulasnella irregularis*) from *Encyclia tampensis*, 100 days after sowing. Fungal strains UAMH 11543 and UAMH 11541 originated from an *E. tampensis* seedling and mature plant, respectively, that grew naturally on the same host tree (*Quercus virginiana*) within the Florida Panther NWR. No significant differences were detected between the two fungal strains on germination, nor any interaction between fungus and seed source (ANOVA, $\alpha = 0.05$).

	Orchid	Seed Source [1]	*n*[2]	# Seeds	# Stage 0	# Stage 1	# Stage 2	# Stage 3	# Stage 4	# Stage 5*	Mean % Germination
UAMH 11543	*P. cochleata*	S177	10	1,407	1,189	0	218	---	---	---	15.5
	E. amphistomum	S197	9	350	338	0	12	---	---	---	3.4
	E. nocturnum	S20	10	285	79	0	2	0	4	200 (70.2)	72.3
	E. tampensis	S194	10	384	40	0	0	0	5	339 (88.3)	89.6
	E. tampensis	S 195	9	504	231	3	13	6	4	231 (45.8)	54.2
	E. tampensis	S196	9	1,507	232	0	3	70	5	636 (42.2)	84.6
UAMH 11541	*P. cochleata*	S177	10	1,438	1,272	0	166	---	---	---	11.5
	E. amphistomum	S197	9	235	228	0	7	---	---	---	3.0
	E. nocturnum	S20	10	410	128	0	0	0	19	263 (64.1)	68.8
	E. tampensis	S194	10	425	79	0	0	1	14	331 (77.9)	81.4
	E. tampensis	S195	10	597	296	3	6	3	51	238 (39.9)	50.4
	E. tampensis	S196	9	1,194	188	1	3	39	504	459 (38.4)	84.3
Control	*P. cochleata*	S177	4	709	709	---	---	---	---	---	0.0
	E. amphistomum	S197	2	20	20	---	---	---	---	---	0.0
	E. nocturnum	S20	5	199	199	---	---	---	---	---	0.0
	E. tampensis	S194	5	220	219	0	1	---	---	---	0.5
	E. tampensis	S195	4	153	143	6	4	---	---	---	6.5
	E. tampensis	S196	5	909	908	0	1	---	---	---	0.1

[1]All seeds were obtained from ripe capsules on plants within the Florida Panther NWR except S20 (collected from the Fakahatchee Strand State Preserve).

[2]Number of replicate petri plates for a given treatment; unequal subsample sizes resulted after contaminated plates were discarded.

Growth stages: 0=no germination, 1=swollen embryo/production of rhizoids, 2=embryo enlargement/rupture of testa, 3=appearance of shoot, 4=emergence of first leaf, 5=leaf elongation.

* Numbers in parentheses reflect the percentage of total seeds that developed to Stage 5.

Diez (2007) both reported higher survival for seedlings in close proximity to the host plant and attributed this to the presence of mycorrhizal fungi. Similarly, Bare (2012) reported that 20 of 22 orchid individuals produced seedlings on the same host tree or within a 1 m radius, implying a mycorrhizal link. This study is the first to confirm the mycorrhizal link between a mature plant and seedling on the same host tree using molecular identification augmented with *in vitro* symbiotic germination. Additional studies are needed to determine if *T. irregularis* is more widespread in *E. tampensis* and other epiphytic orchids.

The question of whether or not orchids display specificity for certain kinds of mycorrhizal fungi has been the subject of interest in recent years, but it is generally assumed that more common and/or widespread orchids exploit a broad range of fungi (= generalists), whereas rare and/or restricted orchids target specific fungal groups (= specialists) (Swarts & Dixon 2009). Few studies (*e.g.*, Otero and Bayman 2009) have attempted to answer this question for epiphytic orchids using *in vitro* symbiotic germination as a tool. In this study, *E. nocturnum* was shown to be capable of utilizing *T. irregularis* acquired from an orchid that typically grows in areas more prone to desiccation (*e.g.*, on *Q. virginiana* host trees). *Encyclia tampensis*, on the other hand, is more widespread throughout Florida where it frequents the drier landscape, but it is also known to occupy more humid habitats (*e.g.*, host trees in cypress domes) in association with other epiphytic orchid species, including *E. nocturnum*. Thus, it is conceivable that *E. nocturnum* could colonize drier sites as an associate of *E. tampensis* if given the opportunity; yet this is often not the case, at least within the Florida Panther NWR. When leaves of these two orchid species are compared, those of *E. tampensis* appear to be better adapted to desiccation (*e.g.*, more narrow, paler, subtended by pseudobulbs), perhaps indicating that *E. nocturnum* may be more restricted by lack of moisture, not by mycorrhizal fungi. Thus far, at least one strain of *Epulorhiza* has been isolated from *E. nocturnum* from a cypress dome in the Florida Panther NWR (L.W. Zettler, unpubl. data). If ITS sequencing verifies that this strain is also *T. irregularis*, this could help explain why both orchids live as associates in wetter habitats, lending further support for moisture as the primary limiting factor in their distribution.

The future of orchid conservation in south Florida — About half (106) of North America's orchid species are found in Florida, and half of these species are restricted to the Big Cypress Basin eco-region in the southernmost part of the state (Brown 2005). With two exceptions (*E. tampensis*, *E. magnoliae*), all of North America's epiphytic orchids are confined to this region where subfreezing temperatures are infrequent. Many of these epiphytes (*e.g.*, *Dendrophylax lindenii* (Lindl.) Benth. ex Rolfe, *E. amphistomum*) are also found in the West Indies and even farther south. As such, the Big Cypress Basin eco-region could be viewed as the northern outpost for epiphytic orchid research in the Western Hemisphere. During the past decade, a number of studies have been published involving epiphytic and terrestrial orchids in south Florida, and in the Florida Panther NWR in particular (*e.g.*, Dutra *et al*. 2008, 2009).

Much of this work has been made possible through private, state and federal agencies (*e.g.*, Naples Orchid Society, US Fish & Wildlife Service) that have provided funds as well as facilities. At the Florida Panther NWR, a lab equipped with an autoclave and sterile hood have made it possible to study orchid seed germination requirements *in vitro* as well as *in situ*, and a greenhouse located adjacent to the lab has been used for propagation. In nearby urban areas (Naples, Miami), a strong core of orchid hobbyists provide enthusiasm, some of which is sparked by local and national media coverage (*e.g.*, *USA Today*) and vice-versa. The recently formed North American Orchid Conservation Center (NAOCC), based at the Smithsonian Environmental Research Center in Maryland, is expected to play a key role in orchid conservation this decade, and plans are underway for that organization to adopt the orchid-fungal model. Taken together, the orchids in the Big Cypress Basin eco-region are in a favorable position to receive additional, multi-dimensional study aimed at their long-term conservation. The findings presented in this paper are meant to shed additional light into epiphytic orchid-fungal interactions, and highlight the need to identify, test (though symbiotic germination), and safeguard the mycorrhizal fungi necessary for integrated conservation to be successful.

Acknowledgments. We warmly thank Larry W. Richardson (U.S. Fish & Wildlife Service) for ongoing and meaningful research collaborations within the Florida Panther NWR and Elizabeth Rellinger Zettler (Illinois College) for statistical analysis. We also acknowledge Andy Stice and Korrie Edwards (Illinois College) for technical assistance, Scott L. Stewart (Morton College), and Lee Hoffman (Native Orchid Restoration Project) for collecting S20 seed, Lynne Sigler (UAMH) for accessioning our fungal isolates, Haleigh Ray (University of Florida) and John McCormick (East Tennessee State University) for field assistance. For photos, we thank Emily Massey and Haleigh Ray (University of Florida) for Fig. 1, and Fig. 2 and 3, respectively. Funding was provided by Illinois College's Student-Faculty Research Committee, Illinois Orchid Society, Prairie State Orchid Society, and U.S. Fish & Wildlife Service, for which we are grateful.

Literature cited

Aggarwal, S., C. Nirmala, S. Beri, S. Rastogi & A. Adholeya. 2012. *In vitro* symbiotic seed germination and molecular characterization of associated fungi in a commercially important and endangered Indian orchid, *Vanda coerulea* Griff. ex Lindl. Eur. J. Env. Sci. 2: 33-42.

Atwood, J.T. 1986. The size of the Orchidaceae and the systematic distribution of epiphytic orchids. Selbyana 9: 171-186.

Bare, M.C. 2012. Surviving deforestation in Ecuador: orchid taxa that respond favorably to transplantation in a restored neotropical forest. Orchids 81: 432-438.

Batty, A.L., K.W. Dixon, M. Brundrett & K. Sivasithamparam. 2001. Constraints to symbiotic germination of terrestrial orchid seed in a Mediterranean bushland. New Phytol. 152: 511-520.

Bayman, P. 2012. Growing epiphytic orchids from seed: a simple, non-sterile, symbiotic method. Orchids 8: 564-567.

Brown, P.M. 2005. Wild orchids of Florida. University Press of Florida, Gainesville.

Clements, M.A. & R.K. Ellyard. 1979. The symbiotic germination of Australian terrestrial orchids. Amer. Orchid Soc. Bull. 48: 810-816.

Clements, M.A., H. Muir & P.J. Cribb. 1986. A preliminary report on the symbiotic germination of European terrestrial orchids. Kew Bull. 41: 437-445.

Cribb, P.J., S.P. Kell, K.W. Dixon & R.L. Barrett. 2003. Orchid conservation: a global perspective. Pp. 1-24 *in*: K.W. Dixon, S.P. Kell, R.L. Barrett & P.J. Cribb (eds.), *Orchid conservation*. Natural History Publications (Borneo), Kota Kinabalu, Sabah.

Currah R.S., L. Sigler & S. Hambleton. 1987. New records and new taxa of fungi from the mycorrhizae of terrestrial orchids of Alberta. Canad. J. Bot. 65: 2473-2482.

Currah R.S., E.A. Smreciu & S. Hambleton. 1990. Mycorrhizae and mycorrhizal fungi of boreal species of *Platanthera* and *Coeloglossum* (Orchidaceae). Canad. J. Bot. 68: 1171-1181.

Currah, R.S., C.D. Zelmer, S. Hambleton & K.A. Richardson. 1997. Fungi from orchid mycorrhizas. Pp. 117-170 *in*: J. Arditti & A.M. Pridgeon (ed.) Orchid biology: reviews and perspectives, VII. Kluwer. Academic Publishers, UK.

Currah R.S., L.W. Zettler & T.M. McInnis. 1997. *Epulorhiza inquilina* sp. nov. from *Platanthera* (Orchidaceae) and a key to *Epulorhiza* species. Mycotaxon 61: 335-342.

Dearnaley, J.D.W., F. Martos & M.-A. Selosse. 2013. Orchid mycorrhizas: molecular ecology, physiology, evolution, and conservation aspects. Pp. 207-230 *in*: Fungal associations (2nd ed.). The Mycota IX. B. Hock (ed.) Springer-Verlag, Berlin.

Diez, J.M. 2007. Hierarchical patterns of symbiotic orchid germination. J. Ecol. 95: 159-170.

Dixon, K.W. 1987. Raising terrestrial orchids from seed. Pp. 47-100 *in*: W.K. Harris (ed.) Modern orchid growing for pleasure and profit. Orchid Club of S. Australia, Inc., Adelaide, S. Australia.

Dressler, R.L. 1981. The orchids,: natural history and classification. Harvard University Press, Cambridge, Massachusetts.

Dressler, R.L. 1993. Phylogeny and classification of the orchid family. Dioscorides Press, Portland, Oregon.

Dutra, D., T.R. Johnson, P.J. Kauth, S.L. Stewart, M.E. Kane & L.W. Richardson. 2008. Asymbiotic seed germination, *in vitro* seedling development, and greenhouse acclimatization of the threatened terrestrial orchid *Bletia purpurea*. Plant Cell Tiss. Organ Cult. 94: 11-21.

Dutra, D., M.E. Kane, C.R. Adams & L.W. Richardson. 2009. Reproductive biology of *Cyrtopodium punctatum in situ*: implications for conservation of an endangered Florida orchid. Plant Spec. Biol. 24: 92-103.

Herrera, P., J.P. Suárez & I. Kottke. 2010. Orchids keep the ascomycetes outside: a highly diverse group of ascomycetes colonizing the velamen of epiphytic orchids from tropical mountain rainforest in Southern Ecuador. Mycol. 1: 262-268.

Kottke, I., J.P. Suárez, P. Herrera, D. Cruz, R. Bauer, I. Huang & S. Garcia. 2010. Atractiellomycetes belonging to the "rust" lineage (Pucciniomycotina) form mycorrhizae with terrestrial and epiphytic neotropical orchids. Proc. Biol. Sci. 277: 1289-1298.

Ma, M., T. Koon & S.M. Wong. 2003. Identification and molecular phylogeny of *Epulorhiza* isolates from tropical orchids. Mycol. Res. 107: 1041-1049.

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

Moore, R.T. 1987. The genera of *Rhizoctonia*-like fungi: *Ascorhizoctonia*, *Ceratorhiza* gen. nov., *Epulorhiza* gen. nov., *Moniliopsis*, and *Rhizoctonia*. Mycotaxon 29: 91-99.

Otero, J.T. & P. Bayman. 2009. Germinación simbiótica y asimbiotica en semillas de orquideas epifitas. Acta Agronom. 58: 270-276.

Otero, J.T., N.S. Flanagan, E.A. Herre, J.D. Ackerman & P. Bayman. 2007. Widespread mycorrhizal specificity correlates to mycorrhizal function in the neotropical epiphytic orchid *Ionopsis utricullariodes* (Orchidaceae). Amer. J. Bot. 94: 1944-1950.

Pereira, O.L., C.L. Rollemberg, A.C. Borges, K. Matsuoka & M.C.M. Kasuya. 2003. *Epulorhiza epiphytica* sp. nov. isolated from mycorrhizal roots of epiphytic orchids in Brazil. Mycoscience 44: 153-155.

Pereira, O.L., M.C.M. Kasuya, A.C. Borges & E. Fernandes de Araújo. 2005. Morphological and molecular characterization of mycorrhizal fungi isolated from neotropical orchids in Brazil. Canad. J. Bot. 83: 54-65.

Rasmussen, H.N. 1995. Terrestrial orchids: from seed to mycotrophic plant. Cambridge University Press, Cambridge, Massachusetts.

Richardson, K.A., R.S. Currah & S. Hambleton. 1993. Basidiomycetous endophytes from the roots of neotropical epiphytic Orchidaceae. Lindleyana 8: 127-137.

Roy, M., S. Watthana A. Stier, F. Richard, S. Vessabutr & M.-A. Selosse. 2009. Two mycoheterotrophic orchids from Thailand tropical dipterocarpacean forests associate with a broad diversity of ectomycorrhizal fungi. BMC Biol. 7: doi 10.1186/1741-7007-7-51.

Stewart, S.L. 2007. Integrated conservation of Florida Orchidaceae in the genera *Habenaria* and *Spiranthes*: model orchid conservation systems for the Americas. Ph.D. dissertation, University of Florida, Gainesville.

Stewart, S.L. & M.E. Kane. 2006. Symbiotic seed germination of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell Tiss. Organ Cult. 86: 159-167.

Stewart, S.L., L.W. Zettler & J. Minso. 2003. Symbiotic germination and reintroduction of *Spiranthes brevilabris* Lindley, an endangered orchid native to Florida. Selbyana 24: 64-70.

Swarts, N.D. 2007. Integrated conservation of the rare and endangered terrestrial orchid *Caladenia huegelii* H.G. Reichb. Ph.D. dissertation, University of Western Australia, Perth.

Swarts, N.D. & K.W. Dixon. 2009. Terrestrial orchid conservation in the age of extinction. Ann. Bot. 104: 543-556.

Taylor, D.L. & M.K. McCormick. 2008. Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. New Phytol. 177: 1020-1033.

Zettler, L.W., J.C. Burkhead & J.A. Marshall. 1999. Use of a mycorrhizal fungus from *Epidendrum conopseum* to germinate *Encyclia tampensis in vitro*. Lindleyana 14:102-105.

Zettler, L.W., L.L. Corey, L.W. Richardson, A.Y. Ross & L. Moller-Jacobs. 2011. Protocorms of an epiphytic orchid (*Epidendrum amphistomum* A. Richard) recovered *in situ*, and subsequent identification of associated mycorrhizal fungi using molecular markers. Eur. J. Env. Sci. 1: 108-114.

Zettler, L.W., S.B. Poulter, K.I. McDonald & S.L. Stewart. 2007. Conservation-driven propagation of an epiphytic orchid (*Epidendrum nocturnum*) with a mycorrhizal fungus. HortScience 42: 135-139.

Zettler, L.W., J. Sharma & F.N. Rasmussen. 2003. Mycorrhizal diversity. Pp. 185-203 *in*: K.W. Dixon, S.P. Kell, R.L. Barrett & P.J. Cribb (ed.) Orchid conservation. Natural History Publications (Borneo), Kota Kinabalu, Sabah.

Zettler, L.W., T. Wilson Delaney & J.A. Sunley. 1998. Seed propagation of the epiphytic green-fly orchid (*Epidendrum conopseum*) using its endophytic fungus. Selbyana 19: 249-253.