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Seed Germination of *Habenaria repens* (Orchidaceae) *in situ* Beyond its Range, and its Potential for Assisted Migration Imposed by Climate Change

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ABSTRACT All orchids require free-living, mycorrhizal fungi to complete their life cycles in nature and consequently, orchid conservation must take into account both organisms. In light of climate change now underway, orchids and other plants must be capable of migrating to higher latitudes, either on their own or with human intervention (=assisted migration). In this paper, we describe the symbiotic germination of a common terrestrial orchid, *Habenaria repens*, *in situ* using seeds from a southern ecotype (Florida) placed at latitudes at and above the species' current natural range. To recover fungi *in situ*, 500 nylon packets containing 25,000–50,000 seeds were buried at 5 field sites within the Atlantic coastal plain in North Carolina, Maryland, and Virginia. After ca. 5 months, a total of 10 leafless seedlings (protocorms) were recovered from two North Carolina sites, one harboring an extant *H. repens* population, the other an extirpated population. These protocorms yielded mycorrhizal fungi assignable to the anamorphic genus *Epulorhiza*. The physiological significance of each isolate was confirmed after *H. repens* seeds germinated *in vitro* following fungal inoculation (=symbiotic germination). This study demonstrates that seeds of *H. repens* from a southern ecotype (Florida) are indeed capable of germinating at higher latitudes where fungi already persist. Consequently, the mycotrophic demands of orchids like *H. repens* might be met by assisted migration involving seed release alone.

INTRODUCTION In nature, orchids require free-living, mycorrhizal fungi to prompt seed germination and seedling development to circumvent the lack of nutrient reserves (endosperm) in their minute, dust-like seeds (Clements 1988). After release from a capsule, copious numbers of orchid seeds become airborne and may be carried considerable distances on wind currents (Rasmussen 1995) until they eventually become affixed to a substrate (e.g., tree limb, leaf litter) harboring a diverse mycoflora. Hyphae from certain groups of fungi, especially basidiomycetes of *Rhizoctonia sensu lato* (e.g., *Ceratorhiza*, *Epulorhiza*; Zelmer and Currah 1995), physically

contact the orchid seed and infect the embryo forming intracellular coils (pelotons, Peterson et al. 2004). These pelotons are subsequently digested by the embryo in a controlled manner, releasing water, carbohydrates, vitamins, minerals and hormones, and prompting development. During the course of the orchid's life, fungi may be digested off and on into adulthood (Rasmussen 2002), supplementing (or replacing) photosynthesis (Hadley and Pegg 1989, Rasmussen 1995, Zettler 2005). Moreover, different taxonomic groups of fungi may be utilized along the way that are different from those that initially prompted seedling development (=fungal succession, Dijk et al. 1997, Zelmer et al. 1996). This process of "feeding" on fungi (mycotrophy) is apparently unique to the Orchidaceae and

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may even have contributed to the family's evolutionary success (Rasmussen 1995, 2002; Taylor et al. 2003). However, the reliance on mycotrophy renders the Orchidaceae especially vulnerable, compared to other angiosperms, because without fungi orchids could not complete their life cycles *in situ*. In light of climate change now underway, orchid conservation must take into account orchid food (=fungi) as well as the orchid.

Habenaria repens Nuttall, is a semi-aquatic terrestrial orchid native to the West Indies, Central America, Mexico, and coastal plain of the southeastern United States (Williams and Williams 1983, Brown 2002). The species is apparently ephemeral and may utilize a broad range of fungi (=“generalist strategy,” Rasmussen and Rasmussen 2007) based on *in vitro* germination studies (Keel 2007, Stewart and Zettler 2002). Photoperiod experiments conducted in conjunction with *in vitro* symbiotic seed germination (Keel 2007) suggest this species has the potential to migrate farther north, taking advantage of longer day lengths. Thus, the current northern limit of its range (east central North Carolina) may be restricted by cooler temperatures and/or the absence of suitable fungi needed for germination, not by limits imposed by photoperiod. If cooler temperatures primarily limit the northern extent of *H. repens*, and not mycorrhizal fungi, it is conceivable that this terrestrial orchid may be capable of migrating farther north given climate change. Under the worst-case climate change scenario (for this study we chose to focus on climate change driven latitudinal migration and not address other issues such as sea-level rise) projected for the next 50–100 years (5.8°C increase), plants will need to expand their northern range by ca. 580 km (Hansen et al. 2001, Davis 1989). For *H. repens*, the northern limit would extend into southern Pennsylvania (Keel 2007). As a species with lightweight, wind-dispersed seeds, *H. repens* might be capable of extending its northern range naturally or via assisted migration (=intentional introduction by humans beyond its natural range, Keel 2005). The latter is a distinct possibility given the ease by which this species can be artificially cultivated (Stewart and Zettler 2002). This goal would be meaningless, however, if introduced seedlings were unable to

spawn seedlings themselves due to the absence of suitable mycorrhizal fungi in the immediate area (Rasmussen 1995). In this paper, we describe the symbiotic germination of *H. repens in situ* using seeds from Florida placed at latitudes at the northern boundary and beyond the species' natural range (North Carolina, Maryland, Virginia). In addition, we provide a description of the mycorrhizal fungi capable of prompting seedling development in these northern latitudes. These two studies were undertaken to understand what factors need consideration in planning for the assisted migration of orchids in general and *H. repens* in particular.

METHODS

Seed Collection, Storage and In Situ Sowing

Seed collection and storage followed the techniques outlined by Zettler (1997). Seeds were obtained from yellowing, mature capsules just prior to dehiscence from four sources, all originally obtained from Florida (Figure 1). Immediately after collection, capsules were placed over Drierite (CaSO₄) desiccant at ca. 22°C up to 91 days. Seeds were removed from capsules assisted by a sterile scalpel, added to sterile vials, and promptly stored at –7 to –10°C in darkness (L:D 0:24 hrs) until use.

To obtain mycorrhizal fungi *in situ*, seeds served as “baits” (Masuhara and Kutsuya 1994, Rasmussen and Whigham 1993, van der Kinderen 1995, Zelmer et al. 1996, Zettler et al. 2005). Briefly, ca. 50–100 seeds were placed in retrievable nylon mesh packets (95 µm pore size, #FR65-2222M Carolina Biological Supply Co., Burlington, North Carolina) which were then folded and inserted into plastic 35 mm slide mounts. Staples were used to secure the packets within the frame. A total of 500 packets were constructed, and each packet was selected at random for eventual placement *in situ*. A small hole was drilled through one or two corners of each slide mount and the packets were tied with 5 kg test nylon fishing line. Packets were tied in two rows of 5 packets with 15 cm between packets within the rows and 30 cm between the rows. Packets were selected at random for the groups of 10. Each group of 10 packets was placed in a plastic sandwich bag, and 10 bags of packets were selected at random for

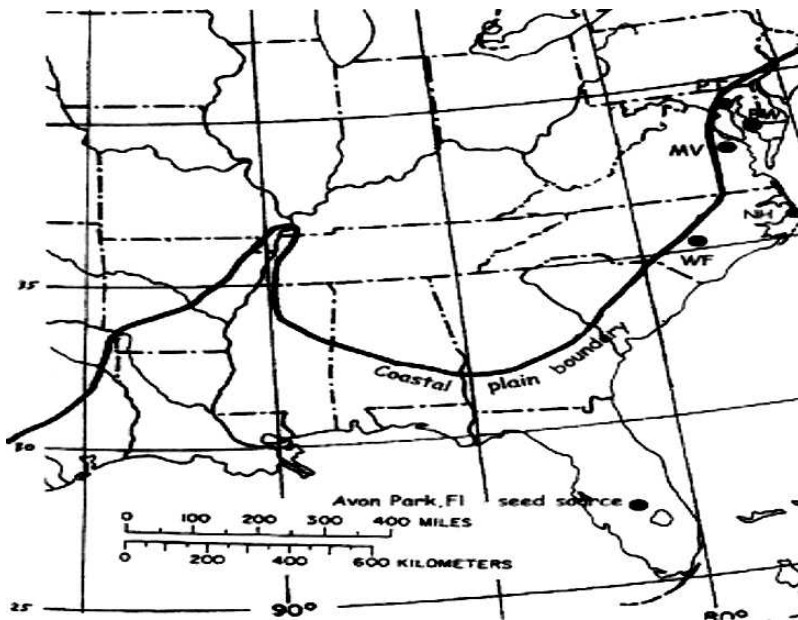


Figure 1. Map of SE United States showing the current range of *Habenaria repens*, the original origin of seeds utilized in this study, and the 5 sites sampled for the presence of mycorrhizal fungi. Only one of the 5 sites (WF Site, Cumberland Co., North Carolina) harbored an existing population of *H. repens*. Dark line is boundary of coastal plain.

each of the research areas. At each field site two rows of packets were sown parallel to the shore line with the first row ca. 2.5 cm from the water's edge (Figure 2). The 5 field sites were located within the Atlantic coastal plain of North Carolina, Maryland, and Virginia (Figure 1 and Table 1). These sites were chosen because they contained ponds with shoreline habitats that appeared suitable for *Habenaria repens* (Figure 3). Two of the sites were in North Carolina, one which harbored an extant *H. repens* population (WF; Figure 1 and Table 1), the other an extirpated population from the 1950s or 1960s (NH; Figure 1 and Table 1). The other three sites (MV, BW, PT; Figure 1 and Table 1) lacked *H. repens* at the time of sowing and prior, based on herbarium records. All seed packets were buried between 11–18 May 2005. Each burial site was selected based on its resemblance to the microhabitat preference of *H. repens* observed in Florida, i.e., generally an open area with sparse vegetation adjacent to standing freshwater. A clean knife was used to make a slit in the soil, and packets were inserted at a 45 degree angle just deep enough so that the top edge of the packet was

completely covered by soil. The knife was wiped clean and surface-disinfected with 70% EtOH between site visits. Packets remained underground for ca. 5 months.

Fungal Isolation and Identification

Packets containing leafless seedlings (protocorms) were retrieved from the five sites during 10–17 October 2005, placed in separate plastic bags and stored briefly at ca. 5°C, then promptly returned to the laboratory within 1 week of retrieval. Fungal isolation followed the procedure outlined by Zettler (1997). Protocorms were carefully removed from packets, rinsed under tap water to remove surface debris, and surface disinfected for 1 min in a solution of 5 ml absolute EtOH, 5 ml 6.0% NaOCl (Clorox® bleach) and 90 ml sterile DI water. After vigorous agitation, the solution was decanted, and the protocorms were rinsed twice in sterile DI water (1 min each rinse). After the second rinse, protocorms were removed using a forceps, added to a drop of DI water placed within a plastic Petri plate, and emasculated with a scalpel—all using sterile techniques under a laminar hood. Molten (50°C) fungal isolation medium

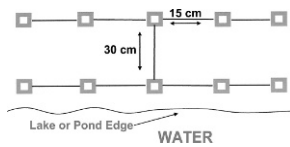


Figure 2. Seed packet configuration showing placement at the packet sites within the six project field sites.

(FIM, Angle et al. 1991) containing an antibiotic (streptomycin sulfate) was added to the emasculated protocorm tissue, gently swirled, allowed to solidify, and incubated overnight at 30°C. Hyphal tips originating from pelotons were clearly visible under a dissection microscope, and were excised using a sterile scalpel. Pure cultures were obtained by removing a single hyphal tip from FIM to potato dextrose agar (PDA; Carolina Biological Supply Co., Burlington, North Carolina). Colony growth on PDA was observed during a 3 week period, and cultural characteristics (hyphal growth rate, width, monilioid cell appearance) were documented using the protocol outlined by Currah et al. (1987, 1990). Corn meal agar (CMA; Carolina Biological Supply Co., Burlington, North Carolina) was used in the assessment of hyphal widths and monilioid cell (asexual propagules that are one of the distinguishing features of the anamorphic genus *Epulorhiza*) length and width, and PDA was used to determine hyphal growth rates, which were carried out during a 37 day period at 22–24°C. Voucher specimens (isolates) that resembled published descriptions of orchid mycorrhizal fungi (Currah et al. 1987, 1990) were deposited into the Herbarium and Microfungus Collection (UAMH), University of Alberta for future reference.

Soil Sample Collection and Analysis

At each retrieval site, after removal of the 10 seed packets at each site, a ca. 100 g soil sample was taken at the approximate center of the 40 × 85 cm site. Indeed, multiple soil samples taken at each site would have been preferable. In related studies, “grab samples” (10 soil samples selected at random along a transect) are often obtained. In this study, however, the length of each of the two transects (rows of seed packets) spanned a short distance (centimeters vs. meters), and the decision was made to obtain one soil sample from the immediate location where seed packets were buried. Samples were later analyzed for pH and characterized by texture and organic matter by N. Burt, United States Forest Service Soil Scientist. Soil texture was characterized using standard United States Department of Agriculture (USDA) Natural Resources Conservation Service protocols documented in the Soil Survey Manual (USDA Handbook No. 18: p. 136–141). A Hellige Soil Reaction pH Tester Kit with a resolution and accuracy of 0.5 units was used for soil pH. Organic soils were identified and classified based on USDA Natural Resources Conservation Service, Keys to Soil Taxonomy (1996, p. 51–53).

Fungal Confirmation (In Vitro Symbiotic Germination)

Pelotons constitute a mass of fungal hyphae, either from one fungus or a mixture of strains. Pelotons are then typically digested (mycotrophy) and this process supplies the orchid with its primary source of carbon. Thus, the fungi that comprise pelotons are assumed to be of some physiological significance to the

Table 1. Site codes of the five sites sampled with seed packets, and descriptions of the site locations

Site Code	Description
MV	Caroline Co., Virginia. Private research station containing marsh, pond and <i>Sphagnum</i> bog. (39°6'N, 76°48'W)
BW	Dorchester Co., Maryland. Blackwater National Wildlife Refuge. Natural and artificial ponds for waterfowl. (38°24'N, 76°6'W)
PT	Prince George's/Anne Arundel Cos. Maryland. Patuxent National Wildlife Refuge. Natural and artificial ponds for research purposes. (38°5'N, 76°6'W)
NH	Dare Co., North Carolina. Nags Head Ecological Preserve (The Nature Conservancy), Outer Banks. Habitat largely shaded by secondary growth, mixed hardwood forest. This site once harbored <i>H. repens</i> in the 1950s or 1960s, based on NCSC Herbarium specimens (no date) and Krings (curator, personal communication) when the site was actively logged and farmed. (35°59'N, 75°40'W)
WF	Cumberland Co., North Carolina. Lakewood River Farm (Williams Farm). Privately owned, containing both artificial and natural ponds. Extant <i>H. repens</i> site. (34°57'N, 78°50'W)



Figure 3. *Habenaria repens* (water-spider orchid) is a terrestrial to aquatic orchid native to Central America, West Indies, Mexico, North Carolina south to Florida and west to southeastern Arkansas and Texas.

orchid during germination and development. To determine if the fungi isolated from pelotons of protocorms might be of physiological significance, each isolate was initially screened by means of symbiotic seed germination. Seeds of *H. repens* were inoculated *in vitro* following the protocols outlined by Dixon (1987) and Stewart and Zettler (2002). Three seed sources were utilized (S119, S121, S122), one of which (S119) was the same utilized *in situ* previously (Table 2). All three sources were obtained from hand-pollinated flowers on plants cultivated in a greenhouse. It is certainly possible that hand pollination may have contributed to some level of artificially high seed vigor, but we assume this affect was negligible. Plants undergoing anthesis *in situ* often display visual signs of pollination (evident by dark-colored stigmatic surfaces followed by rapid fruit set vs. pale-colored stigmatic surfaces and no fruit set), and very often most (sometimes all) available flowers on one plant set fruit. Of the specimens grown under greenhouse conditions

that eventually yielded seed, none of these plants were provided with an additional source of nitrogen or other nutrients, and none were treated with pesticides. The substrate (peat moss) used was pre-sterilized and ground, with no additional nutrients added at the time of packaging, at least to our knowledge. Thus, every attempt was made to mimic natural growing conditions in a greenhouse leading to hand-pollination. The plants used for the pollination were obtained from a previous *in vitro* symbiotic germination study (Stewart and Zettler 2002) that utilized seed from a natural population in Florida (Highlands Co., Avon Park). The seed sources differed in the time the capsules were harvested in 2002: S119 contained mature seed collected 23 September, S 121 contained mature seed collected 16 September, and S122 consisted of mature and immature seed collected 11 September. Epiphytic orchids are readily cultivated on asymbiotic media using immature seed. For terrestrial species, symbiotic germination often utilizes mature seed

Table 2. Codes of the seed lots used in the seed packets, and descriptions of the seed lot sources

Seed Code	Description
SFL1	From natural population in Highlands Co., Florida, containing ca. 100 plants in anthesis. Collected 3 August 2004 by S. L. Stewart.
S119	Seeds derived 10–12 capsules from artificially cross-pollinated plants cultivated at Illinois College, Jacksonville, Illinois, on 23 September 2002 (Stewart and Zettler 2002). Original population located in Avon Park Highlands Co., Florida that yielded seeds on 9–10 October 1999.
S79	Seeds collected from population from Avon Park, Highlands Co., Florida, on 9–10 October 1999.
SNY1	Seeds derived from artificial cross-pollination between a second generation plant cultivated with S119 seed and a plant collected at Avon Park, Highlands Co., Florida, on 23 May 2004. Collected 29 September 2004 by B. G. Keel.

instead. Given that the oat medium in symbiotic germination is low in nutrients compared to standard asymbiotic media (e.g., Stewart and Zettler 2002), it seems unlikely that the germination from the third seed lot would be artificially inflated. Seeds were removed from cold storage (-7°C), surface disinfected as reported previously for protocorms (above), and rinsed twice in DI water (1 min each rinse). Using a sterile inoculation loop, between 50–100 seeds were placed over the surface of a filter paper strip (Whatman No. 4) on the surface of modified oat medium (2.5 g rolled oats, 7.0 g agar, 1 L DI water, Dixon 1987) within a Petri plate. A 0.5 cm^3 block of fungal inoculum was added to one side of the paper strip, and each plate was immediately wrapped in Parafilm "M" (Am. National Can., Greenwich, Connecticut) to retain moisture and minimize contamination. Plates were then wrapped tightly in aluminum foil to exclude light, and incubated at 22°C for up to 10 weeks. Ten replicate plates were prepared per fungus treatment. Seed germination and development were assessed on a scale of 0–5: Stage 0 = no germination; Stage 1 = appearance of rhizoid(s); Stage 2 = rupture of testa by enlarging embryo (germination); Stage 3 = appearance of shoot; Stage 4 = emergence of leaf from shoot region; Stage 5 = leaf elongation. Statistical analysis (ANOVA) was carried out using SPSS software (SPSS, Inc., Chicago, Illinois).

In vitro symbiotic germination was repeated a second time using freshly collected seed inoculated with four of the isolates (UAMH 10751, 10752, 10753, 10754) as well as a control (no fungus). This experiment was of particular interest because all four isolates were previously stored via cryopreservation at the UAMH culture collection (Alberta, Canada),

offering an opportunity to compare germination before and after their deposition there. When the fungi were initially isolated, they closely matched published descriptions of *Epulorhiza* strains typical of those that have been used with success in other studies, including one that involved *H. repens* (Stewart and Zettler 2002). Seeds sown on oat-based media without a fungus typically do not develop beyond Stage 2 (embryo swelling), whereas seeds sown (inoculated) with effective strains often display rapid growth and development (Stage 3–5). The initial *in vitro* seed germination experiment we employed served mostly as a pilot study which we used to initially screen our isolates. Although these strains appeared to be *Epulorhiza* in culture, we wanted to screen them using symbiotic germination as a tool before we used the strains in the 2nd experiment. Seeds for the second experiment were obtained from yellowing capsules on 3 October 2008 from the same cultivated *H. repens* specimen that yielded seed in the previous experiment. Immediately after collection, capsules were placed over Drierite desiccant at 22°C for 25 days. Seeds that were subsequently released were then placed in cool (6°C) storage in darkness for 35 days until sowing. Seed sowing mirrored the previous protocol, except that seeds in the control plates were not inoculated with a fungus. Eight to ten plates for each treatment and 13 control plates were prepared (Table 5). Statistical analysis (ANOVA, Tukey test for multiple comparison of means) was carried out using SPSS software (SPSS, Inc., Chicago, Illinois).

RESULTS AND DISCUSSION

Germination In Situ

Of 500 seed packets containing ca. 25,000–50,000 seeds released *in situ*, all were success-

fully recovered, but only four packets yielded protocorms, five months after sowing. These four packets were retrieved from the two North Carolina sites and contained a total of 10 Stage 3 protocorms, representing <0.05% of the seed total. Seven of the protocorms came from the Cumberland Co. (WF) site, and the remaining three from the Dare Co. (NH) site. Interestingly, the Cumberland Co. site harbored an extant population of *Habenaria repens* at the time of sowing suggesting that the area contained an active mycoflora capable of spawning another generation of *H. repens*. One could probably assume that a site already harboring an orchid population (*H. repens*) may contain traces of the fungal inoculum that prompted initial germination there. However, the whereabouts of such fungi are thought to be sporadic. The recovery of protocorms from the Dare Co. site is noteworthy because it suggests that the area still harbors a mycoflora capable of supporting *H. repens* even though the species has not been seen in the immediate area for 40 or 50 years. As the seed bait technique aptly demonstrates, it may be possible to re-establish *H. repens* in an area like Dare Co. that contains habitat conducive to this orchid's establishment (e.g., pond shorelines and marshy areas devoid of tree canopies).

The lack of protocorms from the other three sites (Maryland, Virginia) was somewhat surprising considering that the habitats appeared similar to those in North Carolina, and were located just beyond (275–380 km) the northern-most (extinct?) population. However, as this study shows, and is supported by research elsewhere (Batty et al. 2001, Masuhara and Katsuya 1994, Zettler et al. 2005), most seed packets do not harbor protocorms when retrieved, implying that orchid fungi have a patchy distribution across the landscape (Scade et al. 2006). Thus, orchid seed germination *in situ* is likely a "hit or miss affair," mostly the latter, necessitating that the Orchidaceae produce copious numbers of seed to ensure a sufficient number of "hits." It is conceivable then that the mycoflora required by *H. repens* may still be present at the Maryland and Virginia sites, and that seed packets were simply not placed in an area containing an active mycoflora.

Given that seed packets were retrieved after only five months in soil, it is also possible that seeds in additional packets, including those in and beyond the North Carolina sites, would have yielded protocorms given more time (e.g., >1 year). Nevertheless, our recovery of Stage 3 protocorms in such a short time period mirrors the developmental time observed *in vitro* (Stewart and Zettler 2002), and supports the concept that *H. repens* employs a rapid germination strategy aimed at exploiting wetland habitats that are prone to desiccation.

Fungal Identification

All 10 protocorms recovered from the four packets sown in North Carolina yielded six fungal strains assignable to the anamorphic genus *Epulorhiza* (Moore 1987), based on morphological characteristics in pure culture (Table 3) that closely matched published descriptions in North America (Currah et al. 1987, 1990; Stewart et al. 2003). This genus is one of the most commonly isolated mycorrhizal associates of orchids worldwide (Currah and Zelmer 1992, Zettler et al. 2003) and is apparently ubiquitous. To date, six species of *Epulorhiza* have been described (Currah et al. 1997, Pereira et al. 2003), the most common being *E. repens* (Bernard) Moore. Two strains of this species originating from unrelated taxa in Florida (*Spiranthes brevilabris* Lindl. and *Epidendrum conopseum* (= *E. magnoliae* Muhl.), were used effectively to cultivate *H. repens in vitro* (Stewart and Zettler 2002). Our recovery of *Epulorhiza* from *H. repens in situ*, therefore, is not surprising. Little is still known about the whereabouts, the ecological role(s) and the diversity of orchid fungi (Rasmussen 1995, Zettler et al. 2003), as well as the biotic and abiotic factors that influence fungal distribution. Analysis of the soil from the North Carolina sites (Table 4) revealed a uniformly low pH range (4.2–4.3), but variable organic matter content. Similarly, *Epulorhiza* strains have been frequently isolated from other taxa inhabiting acidic bogs (Currah et al. 1997, Zettler 2008). In Midwestern prairies, which generally have higher pH ranges (6.0–7.3, Bowles et al. 2005), the genus *Ceratorhiza*—not *Epulorhiza*—is the primary terrestrial orchid associate (Sharma et al. 2003, Zettler et al. 2001, Zettler et al. 2005). According to Arditti (1992), terrestrial orchid

Table 3. Descriptions of the six strains of mycorrhizal fungi (*Epulorhiza* sp.) recovered from *Habenaria repens* protocorms *in situ*

UAMH Strain and Origin	Hyphal Growth Rate (PDA; mm/hr)	Hyphal Width (μm , CMA)	Monilioid Cell Length \times Width (μm , CMA)	Colony Appearance (PDA)
10749/WF	0.05	4–6	15–22 \times 14.21	Cream-colored, entire margin, dense surface and submerged mycelium, little aerial
10750/WF	0.05	5–8	11–20 \times 11–19	Cream-colored, irregular margin, dense surface and aerial mycelium
10751/WF	0.11	8–9	12–20 \times 11–20	Cream-colored, finger-like irregular margin, dense surface, partly submerged but mostly aerial mycelium
10752/WF	0.10	5–8	11–20 \times 11–20	Cream-colored, irregular margin, dense surface and submerged mycelium, little aerial
10753/NH	0.10	6–9	11–20 \times 11–17	Cream-colored, finger-like irregular margin, dense surface and submerged mycelium, little aerial
10754/NH	0.11	8–9	14–20 \times 13–20	Cream-colored, finger-like irregular margin dense surface and submerged mycelium, little aerial

seed germination is optimal at a lower pH range (4.2–5.0). It is conceivable that, at least some North American terrestrial orchids, including *H. repens*, frequent acidic environments because (at least in part) these plants exploit the *Epulorhiza* strains that are adapted to such conditions. However, *Epulorhiza* may also have the ability to colonize slightly alkaline soils. This is evidenced by Stewart et al. (2003) who adjusted their oat medium to a pH of 8.0 for their symbiotic germination of *Spiranthes brevilabris* to parallel the presumed pH value of the calcareous habitat in which they found *S. brevilabris*.

Fungal Confirmation

In the initial screening experiment, seeds from the three sources (S119, S121, S122) inoculated with the six *Epulorhiza* isolates germinated and yielded leaf-bearing (Stage 4) seedlings, 42 days after sowing. After 70 days, seed germination percentages exceeded 25% for two of the seed sources (S121, S122) inoculated with four of the isolates

(UAMH 10751, 10752, 10753, 10754). Seeds inoculated with these four isolates also developed to the advanced (Stage 5) seedling stage. One strain in particular (UAMH 10754) appeared to be especially effective at inducing a large percentage of seeds to develop to the highest growth stage (43% for S121, 63% for S122). Considering that seeds from the two sources developed to Stage 5 in a short period of time (70 days) on a medium low in nutrients (modified oats medium) suggests that these four isolates may have played a stimulatory role *in situ*. This possibility was confirmed by the second experiment when nearly all seeds incubated in the absence of a fungus (control) failed to germinate, whereas seeds inoculated with the four fungal strains developed to Stage 5 after 54 days (Table 5). In the initial experiment, the two Dare Co. strains (UAMH 10753, 10754) prompted the highest seed germination percentages, particularly UAMH 10754 (73.3%). In the second experiment, however, fewer seeds germinated using this strain (13.6%) compared to the

Table 4. Analysis of soil from the two North Carolina sites where *Epulorhiza* strains were recovered

Site ¹	pH	Texture	Organic Matter Content
Cumberland Co. (WF-7)	4.2	heavy sandy loam	very low
Cumberland Co. (WF-10)	4.3	sandy loam	low
Dare Co. (NH-3)	4.3	organic/hemic (moderately decomposed organic matter)	high

¹Numbers listed after population code (e.g., WF) denote one of 10 microsites where seed packets were buried.

Table 5. *In vitro* symbiotic germination of *Habenaria repens* using the four *Epulorhiza* isolates recovered from *H. repens* protocorms *in situ*. Values denoted by the same letter are not significantly different ($P > 0.05$). Data in this table is from the second germination experiment

Fungus	<i>n</i>	# Seeds	# Seeds in Stage						% Germination (±SE)
			0	1	2	3	4	5	
10751	8	1,127	917	0	10	29	52	119	20.0 (2.38) a
10752	8	1,348	1,066	0	16	70	76	120	22.4 (4.58) a
10753	10	1,684	1,268	0	225	117	59	15	25.8 (3.53) a
10754	9	1,887	1,640	1	39	54	60	93	13.6 (1.38) a
Control	13	2,328	2,327	1	0	0	0	0	0.03 (0.03) b

n = denotes the number of replicate Petri plates per treatment. Unequal subsample sizes resulted when contaminated plates were discarded.

other three isolates (Table 5). Germination percentages were also lower overall using these four isolates compared to those observed in the initial experiment. Although these differences could be attributed to a number of factors that do not involve the fungus (e.g., differences in seed storage), the act of cryopreservation should not be ruled out as one possible explanation for the drop in seed germination percentages. Subsequent studies should be attempted to determine if safeguarding mycorrhizal fungi via cryopreservation remains a reliable, preferred option for orchid conservation. Until then, we advocate that fungi recovered from protocorms of other species *in situ* be screened by *in vitro* symbiotic germination, and deposited into culture collections to augment conservation.

SUMMARY Given the rapid rate of climate change now underway, many plant species may not be capable of migration to higher latitudes (and altitudes) fast enough to keep pace with the predicted rise in temperatures this century (Neilson et al. 2005). Consequently, high risk species would probably benefit from assisted migration. The minute size and dust-like nature of the orchid seed suggests that these plants might be capable of migrating naturally. However, the Orchidaceae's extreme dependence on mycorrhizal fungi (mycotrophy) suggests otherwise if suitable fungi are not already present at the new site(s). It remains unclear how orchid fungi are dispersed naturally over long-distances, but it is conceivable that monilioid cells and basidiospores, produced by the anamorphic and teleomorphic stages, respectively, serve this role. Under laboratory conditions, orchid fungi rarely produce a perfect

(teleomorphic) stage (Currah 1987, Warcup and Talbot 1980). Even if copious numbers of spores are released naturally, the probability that a viable orchid seed will make physical contact with hyphae from a suitable fungus originating from distant areas seems unlikely.

Our study, using a common fungal "generalist" (*H. repens*) as a model, demonstrates that orchid seeds from a southern ecotype (Florida) are indeed capable of germinating at more northern latitudes, separated by 850 km. Consequently, orchid "generalists" like *H. repens* may be candidates for assisted migration through seed release alone, if fungi are found to be present at intended introduction sites. Other species, with more fastidious germination requirements, may benefit from the assisted migration of both orchids and fungi. The latter might be accomplished in at least three ways: 1) introducing fungal mycelium to soil (Hollick 2004) in suitable areas prior to seed sowing, 2) releasing seeds encapsulated with inoculum (Tan et al. 1998, Wood et al. 2000), and 3) releasing fungus-infected orchid seedlings that were acquired via *in vitro* symbiotic germination (Stewart et al. 2003). In the latter, orchid seedlings would serve as a source of fungal inoculum which might make it possible for both biotic agents to persist in new areas. Given the complexity of the orchid life cycle with its obligatory reliance on other organisms (e.g., specific pollinators, fungi), long-term conservation will be better served using an integrated approach (Stewart 2007, Swarts 2007, Stewart and Kane 2007, Swarts et al. 2007, Swarts and Dixon 2009). This study offers hope that the mycotrophic demands for the assisted migration of orchids might be met involving seed release alone. Due to

concerns that introducing mycorrhizal fungi might be ecologically detrimental (Schwartz et al. 2006), we urge future workers to utilize the seed baiting technique in sites where assisted migration might be practiced before releasing any new mycorrhizal strains from distant areas.

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LITERATURE CITED

- Angle, J.S., S.P. McGrath, and R.L. Chaney. 1991. New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution. *Appl. Environ. Microbiol.* 57:3674–3676.
- Arditti, J. 1992. *Fundamentals of orchid biology*. John Wiley and Sons, New York, New York.
- Batty, A.L., K.W. Dixon, M. Brundrett, and K. Sivasithamparam. 2001. Constraints to symbiotic germination of terrestrial orchid seed in a mediterranean bushland. *New Phytologist* 152:511–520.
- Bowles, M.L., L.W. Zettler, T. Bell, and P. Kelsey. 2005. Relationships between soil characteristics, distribution and restoration potential of the Federal threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nutt.) Lindl. *Amer. Midl. Naturalist* 154:273–285.
- Brown, P.M. 2002. *Wild orchids of Florida*. University of Florida Press, Gainesville, Florida.
- Clements, M.A. 1988. Orchid mycorrhizal associations. *Lindleyana* 3:73–86.
- Currah, R.S. 1987. *Thanetophorus pennatus* sp. nov. isolated from the mycorrhizal roots of *Calypso bulbosa* (Orchidaceae) from Alberta. *Can. J. Bot.* 65:1957–1960.
- Currah, R.S. and C.D. Zelmer. 1992. A key and notes for the genera of fungi mycorrhizal with orchids, and a new species in the genus *Epulorhiza*. *Rep. Tottori Mycolog. Inst.* 30:43–59.
- Currah, R.S., E.A. Smreciu, and S. Hambleton. 1990. Mycorrhizae and mycorrhizal fungi of boreal species of *Platanthera* and *Coe-loglossum* (Orchidaceae). *Can. J. Bot.* 68: 1171–1181.
- Currah, R.S., L. Sigler, and S. Hambleton. 1987. New records and new taxa of fungi from the mycorrhizae of terrestrial orchids in Alberta. *Can. J. Bot.* 65:2473–2482.
- Currah, R.S., L.W. Zettler, and T.M. McInnis. 1997. *Epulorhiza inquilina* sp. nov. from *Platanthera* (Orchidaceae), and a key to *Epulorhiza* species. *Mycotaxon* 61:335–342.
- Davis, M.B. 1989. Lags in vegetation response to greenhouse warming. *Climate Change* 15:75–82.
- Dijk, E., J.H. Willems, and J. van Anandel. 1997. Nutrient responses as a key factor to the ecology of orchid species. *Acta Bot. Neerlandica* 46:339–363.
- Dixon, K.W. 1987. Raising terrestrial orchids from seed. p. 47–100. *In*: Harris, W.K. (ed.). *Modern orchid growing for pleasure and profit*. Orchid Club of South Australia, Inc., Adelaide, South Australia, Australia.
- Hadley, G. and G.F. Pegg. 1989. Host-fungus relationships in orchid mycorrhizal systems. p. 57–71. *In*: Pritchard, H.W. (ed.). *Modern methods in orchid conservation*. Cambridge University Press, Cambridge, United Kingdom.
- Hansen, A.J., R.P. Neilson, V.H. Dale, C.H. Flather, L.R. Iverson, D.J. Currie, S. Shafer,

- R. Cook, and P.J. Bartlein. 2001. Global change in forests: responses of species, communities, and biomes. *BioScience* 51: 765–779.
- Hollick, P.S. 2004. Mycorrhizal specificity in endemic Western Australian terrestrial orchids (tribe Diurideae): implications for conservation. Ph.D. Dissertation, Murdoch University, Perth, Western Australia, Australia.
- Keel, B.G. 2005. Assisted migration. p. 36. *In*: Allaby, M. (ed.). *Dictionary of Ecology*. Oxford University Press, Oxford, United Kingdom.
- Keel, B.G. 2007. Assisted migration as a conservation strategy for rapid climate change: Investigating extended photoperiod and mycobiont distributions for *Habenaria repens* Nuttall (Orchidaceae) as a case study. Ph.D. dissertation, Antioch University New England, Keene, New Hampshire.
- Masuhara, G. and K. Katsuya. 1994. *In situ* and *in vitro* specificity between *Rhizoctonia* spp. and *Spiranthes sinensis* (Persoon) Ames. var. *amoena* (M. Bieberstein) Hara (Orchidaceae). *New Phytologist* 127:711–718.
- Moore, R.T. 1987. The genera of *Rhizoctonia*-like fungi: *Ascorhizoctonia*, *Ceratorhiza* gen. nov., *Epulorhiza* gen. nov., *Moniliopsis* and *Rhizoctonia*. *Mycotaxon* 29:91–99.
- Neilson, R.P., L.F. Pitelka, A.M. Solomon, R. Nathan, G.F. Midgley, J.M.V. Fragoso, H. Lischke, and K. Thompson. 2005. Forecasting regional to global plant migration in response to climate change. *BioScience* 55:749–759.
- Pereira, O.L., C.L. Rollemberg, A.C. Borges, K. Matsuoka, and M.C.M. Kasuya. 2003. *Epulorhiza epiphytica* sp. nov. isolated from mycorrhizal roots of epiphytic orchids in Brazil. *Mycoscience* 44:153–155.
- Peterson, R.L., H.B. Massicott, and L.H. Melville. 2004. *Mycorrhizas: anatomy and cell biology*. NRC Research Press, Ottawa, Ontario.
- Rasmussen, H.N. 1995. *Terrestrial orchids: from seed to mycotrophic plant*. Cambridge University Press, Cambridge, United Kingdom.
- Rasmussen, H.N. 2002. Recent developments in the study of orchid mycorrhiza. *Plant and Soil* 244:149–163.
- Rasmussen, H.N. and D.F. Whignam. 1993. Seed ecology of dust seeds *in situ*: a new study technique and its application in terrestrial orchids. *Amer. J. Bot.* 80:1374–1378.
- Rasmussen, H.N. and F.N. Rasmussen. 2007. Trophic relationships in orchid mycorrhiza diversity and implications for conservation. *Lankesteriana* 7:334–341.
- Scade, A., M.C. Brundrett, A.L. Batty, K.W. Dixon, and K. Sivasithamparam. 2006. Survival of transplanted terrestrial orchid seedlings in urban bushland habitats with high or low weed cover. *Australian J. Bot.* 54:383–389.
- Schwartz, M.W., J.D. Hoekesma, C.A. Gehring, N.C. Johnson, J.N. Kironomos, L.K. Abbott, and A. Pringle. 2006. The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. *Ecology Letters* 9:501–515.
- Sharma, J., L.W. Zettler, and J.W. Van Sambeek. 2003. A survey of mycobionts of Federally threatened *Platanthera praeclara* (Orchidaceae). *Symbiosis* 34:145–155.
- 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, Florida.
- Stewart, S.L. and L.W. Zettler. 2002. Symbiotic germination of three semi-aquatic rein orchids (*Habenaria repens*, *H. quinqueseta*, *H. macroceratitis*) from Florida. *Aquat. Bot.* 72:25–35.
- Stewart, S.L. and M.E. Kane. 2007. Symbiotic seed germination and evidence for *in vitro* mycobiont specificity in *Spiranthes brevibrabis* (Orchidaceae) and its implications for species-level conservation. *In Vitro Cell. Dev. Biol.-Plant* 43:178–186.
- Stewart, S.L., L.W. Zettler, J. Minso, and P.M. Brown. 2003. Symbiotic germination and reintroduction of *Spiranthes brevibrabis* Lindley, and 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, Crawley, Western Australia, Australia.

- Swarts, N.D. and K.W. Dixon. 2009. Terrestrial orchid conservation in the age of extinction. *Annals of Botany* 104:543–556.
- Swarts, N.D., A.L. Batty, S. Hopper, and K.W. Dixon. 2007. Does integrated conservation of terrestrial orchids work? *Lankesteriana* 7:219–222.
- Tan, T.K., W.S. Loon, E. Khor, and C.S. Loh. 1998. Infection of *Spathoglottis plicata* (Orchidaceae) seeds by mycorrhizal fungus. *Plant Cell Rep.* 18:14–19.
- Taylor, D.L., T.D. Bruns, T.M. Szaro, and S.A. Hodges. 2003. Divergence in mycorrhizal specialization within *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid. *Amer. J. Bot.* 90:1168–1179.
- van der Kinderen, G. 1995. A method for the study of field-germinated seeds of terrestrial orchids. *Lindleyana* 10:68.
- Warcup, J.H. and P.H.B. Talbot. 1980. Perfect states of *Rhizoctonias* associated with orchids. III. *New Phytologist* 86:267–272.
- Williams, J.G. and A.E. Williams. 1983. *Field guide to orchids of North America*. Universe Books, New York, New York.
- Wood, C.B., H.W. Pritchard, and A.P. Miller. 2000. Simultaneous preservation of orchid seed and its fungal symbiont using encapsulation-dehydration is dependent on moisture content and storage temperature. *CryoLetters* 21:125–136.
- Zelmer, C.D. and R.S. Currah. 1995. *Ceratohiza pernacatena* and *Epulorhiza calendulina* spp. nov.; mycorrhizal fungi of terrestrial orchids. *Can. J. Bot.* 73:1981–1985.
- Zelmer, C.D., L. Cuthbertson, and R.S. Currah. 1996. Fungi associated with terrestrial orchid mycorrhizas, seeds and protocorms. *Mycoscience* 37:439–448.
- Zettler, L.W. 1997. Terrestrial orchid conservation by symbiotic seed germination: techniques and perspectives. *Selbyana* 18: 188–194.
- Zettler, L.W. 2005. Nature's fungal connoisseurs. *Orchids* 74:292–297.
- Zettler, L.W. 2008. An overview of the orchid-fungal association, and its application to orchid conservation in the Americas. p. 315–318. *In: Proceedings of the 19th World Orchid Conference, Miami, Florida.*
- Zettler, L.W., J. Sharma, and F.N. Rasmussen. 2003. Mycorrhizal diversity. p. 185–203. *In: Dixon, K., P. Cribb, S. Kell, and R. Barrett (eds.). Orchid conservation*. Natural History Publications, Kota Kinabalu, Sabah, Malaysia.
- Zettler, L.W., K.A. Piskin, S.L. Stewart, J.J. Hartsock, M.L. Bowles, and T.J. Bell. 2005. Protocorm mycobionts of a Federally threatened orchid, *Platanthera leucophaea*, and a technique to promote leaf formation in seedlings. *Stud. Mycology* 53:163–171.
- Zettler, L.W., S.L. Stewart, M.L. Bowles, and K.A. Jacobs. 2001. Mycorrhizal fungi and cold-assisted symbiotic germination of the federally threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nuttall) Lindley. *Amer. Midl. Naturalist* 145:168–175.