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ORCHID–FUNGUS FIDELITY: A MARRIAGE MEANT TO LAST?

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Abstract. The characteristics of plant–mycorrhizae associations are known to vary in both time and space, but the ecological consequences of variation in the dynamics of plant–fungus interactions are poorly understood. For example, do plants associate with single fungi or multiple fungi simultaneously, and do the associations persist through a plant's lifetime or do plants support a succession of different fungi? We investigated these and other questions related to plant–fungus interactions in *Goodyera pubescens*, an evergreen terrestrial orchid of the eastern United States, that interacts with closely related fungi in the genus *Tulasnella*.

Unlike the mycorrhizal associations of other plants, orchid–mycorrhizal associations only benefit the orchid, based on current evidence. Many terrestrial orchids have been found to associate with specific groups of fungi. This characteristic could potentially limit orchids to relatively narrow ranges of environmental conditions and may be a contributing factor in the decline of many orchids in the face of changing environmental conditions.

We found that *G. pubescens* protocorms (developing embryos prior to leaf production) and adults associated with only one fungal individual at a time. The orchid–fungus association persists for years, but during a drought period that was associated with the death of many plants, surviving plants were able to switch to new fungal individuals. These results suggest that *G. pubescens* interacts with the same fungal partner during periods of modest environmental variation but is able to switch to a different fungal partner. We hypothesize that the ability to switch fungi allows *G. pubescens* to survive more extreme environmental perturbations. However, laboratory experiments suggest that switching fungi has potential costs, as it increases the risk of mortality, especially for smaller individuals. Our findings indicate that it is unlikely that switching fungi is a common way to improve tolerance of less severe environmental fluctuations and disturbances. These findings may have important implications for plant responses to severe climatic events or to more gradual environmental changes such as global warming.

Key words: *environmental change;* Goodyera; *mycorrhiza; orchid; Orchidaceae;* Tulasnella.

Introduction

The great majority of terrestrial plants depend on mycorrhizal fungi for at least some of their nutrients, and all woodland herbs that have been examined thus far have been shown to be mycorrhizal (Whigham 2004). Ecologically, mycorrhizal relationships in woodland herbs enable the plants to obtain nutrients in nutrient-limited environments (Boerner 1986, 1990). Many mycorrhizal relationships are considered obligate for both plant and fungal partners (Smith and Read 1997, Bruns et al. 2002), and there has been an implicit assumption that little specificity exists in ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) relationships (Bruns et al. 2002, Sanders 2002). However, with the application of molecular technologies to mycorrhizal ecology, it is becoming increasingly apparent that at least some mycorrhizal relationships may be quite specific from the perspective of either the plant or fungal host (e.g., Bruns et al. 2002, Sanders 2002).

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Difficulty applying molecular techniques to AM fungi (Sanders 2002, Pawlowska and Taylor 2004) and the long life span of most ECM hosts (Helm et al. 1996, Bruns et al. 2002) has made it difficult to assess the diversity and temporal continuity of most mycorrhizal partnerships. Helm et al. (1996) and Boerner et al. (1996) found substantial changes in the mycorrhizal communities associated with plants distributed across successional gradients, but these changes were associated with plant species and communities, not with individual plants. Changes in the mycorrhizal communities associated with long-lived species such as perennial herbs, shrubs, and trees suggests that individual plants must be able to switch among fungi to tolerate environmental changes and changes in resources such as those that occur during succession. The ability to switch fungi would have a dramatic impact on species that are not able to utilize multiple fungi. This may be a particular problem among orchids, which often form mycorrhizal relationships with a limited range of fungi (e.g., McCormick et al. 2004), thus limiting their tolerance of disturbance and, to the extent that suitable fungi

differ in their ability to grow under different conditions, also environmental change.

A particularly intimate symbiotic association with mycorrhizal fungi is a defining characteristic of the orchid family (Leake 1994). All orchids begin life as minute, dust-like seeds with little stored nutrients. All orchids also pass through a non-photosynthetic protocorm stage during which they are entirely dependent on their mycorrhizal fungi for all nutrition (Rasmussen 2002). Orchids are colonized by mycorrhizal fungi at or around the time of germination. Throughout their lives, orchids restrict the growth of their mycorrhizal fungi to specific cells through the production of fungicidal phytoalexins (Beyrle et al. 1995). Nutrients are obtained by digesting fungal pelotons (coils of fungal hyphae), and there appears to be little opportunity for the two-way exchange of nutrients that is characteristic of most plant–mycorrhizal interactions (Alexander and Hadley 1985). The lack of two-way nutrient exchange has yet to be conclusively demonstrated but nutrients obtained through a two-way exchange are likely to be of substantially smaller magnitude than those obtained from digestion. Protocorms are clearly dependent on their fungi, but the relationship between adult orchids and their fungi is less clear. Most orchids become photosynthetic as adults and thus are no longer entirely dependent on their mycorrhizal fungi for carbon. However, roots of adult terrestrial orchids are consistently heavily colonized by pelotons of mycorrhizal fungi (e.g., Zelmer et al. 1996, Rasmussen and Whigham 2002), representing a potentially substantial source of carbon and other nutrients. Gebauer and Meyer (2003) have used stable isotopes to demonstrate that many adult orchids continue to obtain a substantial amount of carbon and nitrogen from their fungi.

While many non-photosynthetic (and thus completely myco-heterotrophic) orchids and some green orchids, especially those growing in deep shade, associate with presumably long-lived ectomycorrhizal fungi that are likely to have access to long-term carbon sources (i.e., trees; McKendrick et al. 2000, Taylor et al. 2002, Selosse et al. 2004, Julou et al. 2005), most photosynthetic terrestrial orchids studied to date have associated with saprotrophic fungi. If, as suggested, intermittent inputs of accessible organic material lead to short life spans for saprotrophic fungi, then photosynthetic orchids may be compelled to form associations with new fungi upon the death of their fungal host (Rasmussen 2002). However, many photosynthetic orchids associate with the same group of fungi as juveniles and adults and some species form associations with very specific fungal taxa, sometimes only a single species (McCormick et al. 2004). It is unknown whether these specific associations are continuous with a single fungal individual or whether they are the result of repeated colonizations by multiple fungal individuals (Rasmussen and Whigham 2002, Zettler et al. 2003).

The way an orchid incorporates multiple fungi into its life may also be important. A single orchid may associate with multiple fungi at once, analogous to polygamy, or with one fungus at a time, switching from one to another, analogous to serial monogamy. A polygamous orchid may experience shifts in abundance of one fungal partner or another but will likely never be without fungi. A serially monogamous orchid may at times find itself without a fungal partner and so may need to be able to survive independent of its fungus. If the fungal partner is more susceptible to environmental change than the plant partner, these times without fungi may coincide with times of severe environmental stress. It seems likely that the polygamous strategy would be favored unless interactions between multiple fungal partners in some way decrease benefits to the orchid.

Also, the benefits or adverse effects of associating with multiple fungi may change with orchid life stage. The duration of the protocorm stage is poorly described in temperate terrestrial orchids (Rasmussen 1995), but may last as little as six months (Rasmussen and Whigham 1993) or possibly as long as several years, while the adult stage may be much longer-lived. A short-lived protocorm may be able to rely on a single fungus, while a longer-lived adult may require multiple fungi. The small size of a protocorm may also affect its interaction with fungi. If there are any negative interactions between multiple fungi, they may be especially intense in the tight confines of a protocorm, whereas multiple fungi may be distributed among multiple roots or ramets of an adult and so not come in contact with one another.

We examined the ability of a photosynthetic terrestrial orchid to associate with multiple fungi simultaneously and to switch among fungi. We asked the following questions. (1) Are protocorms able to associate with multiple fungi? (2) Do adult orchids have multiple fungi within single roots, among roots, or among ramets? (3) Can protocorms switch fungi? (4) Do individual plant–fungus associations persist over time?

We examined these questions in *Goodyera pubescens* R.Br., a common, evergreen terrestrial orchid found in mid/late-succession forests throughout the eastern U.S. and into southeastern Canada (see Plate 1). *Goodyera pubescens* spreads clonally through branching rhizomes that eventually decompose, leaving independent ramets. We have previously found that *G. pubescens* has a very specific fungal association, and protocorms and adults both associate with a small group of closely related *Tulasnella* spp. (McCormick et al. 2004). Preliminary isotopic studies indicate that adult *G. pubescens* are strongly photosynthetic but fungi also contribute substantially to adult orchid carbon and nitrogen (M. K. McCormick and T. R. Filley, *unpublished data*). Field studies of adult plants were conducted at the Smithsonian Environmental Research Center (SERC) in Edgewater, Maryland, USA, and at the Coweeta Hydrologic Laboratory in Otto, North Carolina, USA.

PLATE 1. Rosette of adult *Goodyera pubescens* at the Smithsonian Environmental Research Center. Photo credit: M. McCormick.

MATERIALS AND METHODS

Association with multiple fungi

Protocorms.—To determine whether *G. pubescens* protocorms could associate with multiple fungi, we established two fungi on opposite sides of Petri dishes with lean wood/agar media (either 2 g/L ground *Liriodendron tulipifera* or *Quercus alba* wood and 12 g/L agar). When the two fungi had grown to the point at which their hyphae began to overlap (~4 d), we placed ~20 surface-sterilized *G. pubescens* seeds (shaken for 2 h in a saturated solution of calcium hypochlorite with 0.05% Tween80 [Fisher Scientific International, Hampton, Massachusetts, USA]; Whigham et al. 2002) in the region of overlapping hyphae. We conducted this experiment with six randomly chosen combinations of five fungal isolates on each of the media with five replicate plates per fungal combination per medium. All five fungi (fungal isolate numbers 101, 109, 149, 159, 179) had been isolated from *G. pubescens* protocorms or adults in SERC forests and support protocorm growth on wood media in the laboratory (Whigham et al. 2002; M. K. McCormick, *personal observation*). Although species concepts are difficult in the fungi, especially in the genus *Tulasnella*, these isolates likely represent different species based on ITS DNA sequence variation (~2% sequence variation; GenBank accession numbers AY373266, AY373263, AY373273, AY373275; isolate 159 is genetically identical to isolate 145, AY373276; McCormick et al. 2004).

Twelve weeks after seeds were placed on the Petri dishes, we scored percentage of germination and measured the length of all resulting protocorms. We randomly chose two protocorms from each Petri dish (500 protocorms total) and extracted and plated 10 pelotons from each on E+N plates (E-medium as per Caldwell et al. [2000] plus 50 μg/L novobiocin). Of these 500 protocorms, we randomly selected seven per fungal combination (35 protocorms total) to identify the genotype of all pelotons that grew out using inter-simple sequence repeats (ISSRs). We analyzed a single peloton using ISSRs from an additional 60 of the remaining 465 protocorms.

In order to obtain genotypes of fungi from each peloton that grew, we transferred a small plug of agar with fungal hyphae from each Petri dish to a sterile flask with ~25 mL liquid E-medium. After ~7 d of growth in liquid, we removed all fungal tissue from the liquid, rinsed it in sterile distilled water, and removed the agar plug. We then divided the fungal hyphae in half and extracted DNA from one half, preserving the remaining half by freezing at −20°C in a sterile 1.5-mL Eppendorf tube.

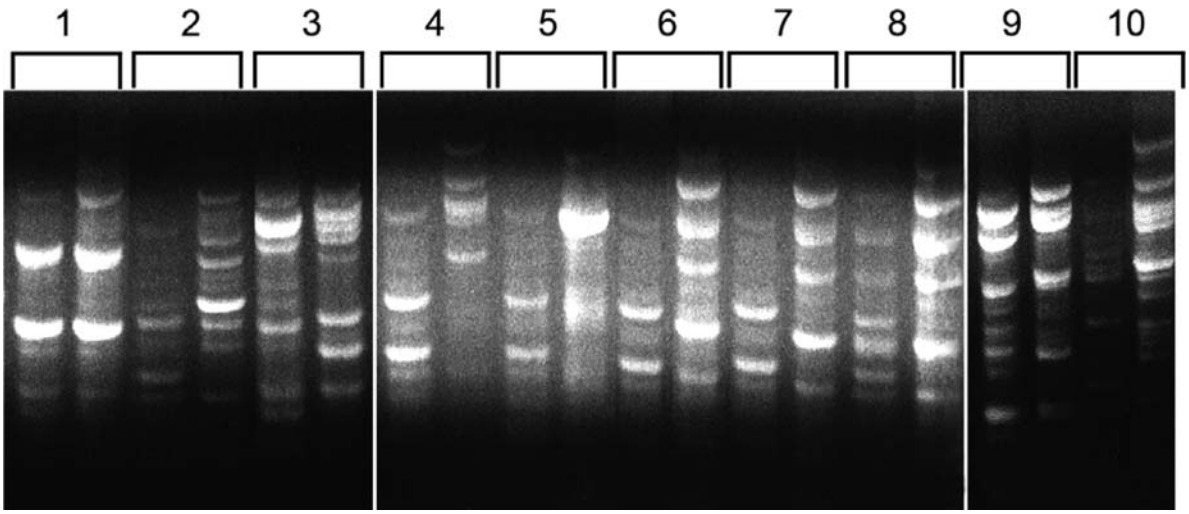

FIG. 1. Inter-simple sequence repeat (ISSR) gels from 2003 showing change in fungi isolated from single plants over time. Each pair of fungi from a single plant is indicated by a numbered bracket. Only plant number 1 retained its initial fungus. Samples were separated by two (numbers 2, 4, 5, 6, 7, 8, 10) or four (numbers 1, 3, 9) years.

DNA was extracted using a Tris–ethylenediaminetetraacetate (EDTA)–sodium dodecyl sulfate/hexadecyl trimethylammonium bromide (TES/CTAB) extraction (McCormick et al. 2004) and amplified using ISSR primer number 811 ($(GA)_8C$; obtained from the University of British Columbia Biotechnology Laboratory, Vancouver, British Columbia, Canada). Amplification reactions of 25 μL were carried out with a final concentration of 0.5 μmol/L primer 811 and 50% Taq polymerase chain reaction (PCR) Master Mix (Promega, Madison, Wisconsin, USA). Amplifications consisted of 35 cycles in an MJ Research DNA Engine (MJ Research, Waltham, Massachusetts, USA) and employed a 1-min initial denaturation at 96°C before thermocycling. Each cycle consisted of a 1-min denaturation at 94°C, followed by an annealing step of 1 min at 44°C and elongation for 1 min at 72°C. Reactions with no template DNA were performed on each amplification to ensure no contaminants were present. Inter-simple sequence repeat (ISSR) banding patterns were characterized on a 1.5% agarose gel in 1× Tris–boric acid–EDTA (TBE) buffer. The ISSR bands were stained by a 20-min immersion in 2 mg/L ethidium bromide in distilled water, followed by a 20-min destaining in distilled water. Bands were visualized on a UV transilluminator, documented using a Fisher Biotech Polaroid photodocumentation system (FB-FDC-34 with FB-PDH-1314 hood; Polaroid, Norton, Massachusetts, USA), and identity determined visually.

Adults.—To determine whether adult *G. pubescens* associated with multiple fungi, we tested for the presence of multiple fungi within individual roots, among roots within a ramet, and among ramets within a genet. For the individual root test, we compared fungi grown from multiple pelotons from a single plant root from 40 plants sampled throughout the geographic range (see the Appendix). For the among-root test, we sampled two roots from each of 10 adult plants in three SERC populations in 2001–2003. In each of these cases, the two roots were sampled from a single ramet. In spring 2002 we sampled two roots from each of four more plants growing in two populations at the Coweeta Hydrologic Laboratory. For the among-ramets test, we sampled pairs of ramets from 13 plant pairs at SERC in 2002–2003, sampling one root from each of two ramets that retained an intact or recently intact physical connection. We used ISSR banding patterns to distinguish individual fungi (e.g., Anderson et al. 1998, Zhou et al. 1999).

Switching fungi

Protocorms.—To determine whether *G. pubescens* protocorms could switch fungi, we established five fungi on lean *Liriodendron* wood media and allowed *G. pubescens* seeds to germinate and grow for eight weeks. We then randomly selected 32 protocorms to receive each treatment. We transferred four protocorms to each of eight replicate plates per treatment. Individual protocorms were identified by different colored ink dots on the underside of each plate, and the length of each was measured at the time of transfer. We established eight treatments: Fungal isolate 101→109, 101→149, 109→179, 149→159, and the reverse of each. We also established two control plates for each fungal isolate, in which protocorms were transferred to a new plate with the same fungus as their germination fungus (e.g., 101→101). These control plates allowed us to separate growth effects resulting from transferring protocorms to new plates from effects resulting from different fungi. The growth of switched and control protocorms were compared using an ANOVA with fixed treatment (i.e.,

TABLE 1. The number of protocorms infected by dominant and subordinate fungi when germinated with two fungi on a plate (association with multiple fungi) and the number of protocorms analyzed after transfer to a second fungus (fungal switching).

Fungal isolates in treatment†	Association with multiple fungi (no. protocorms infected)		Fungal switching (no. protocorms infected)	
	Dominant	Subordinate	D→S	S→D
101, 109	16	3	7 (6)	3 (2)
101, 149	14	3	6 (6)	3 (3)
109, 149	15	3	na	na
159, 109	5	1	na	na
179, 109	19	0	7 (7)	1 (1)
159, 149	16	0	1 (1)	1 (1)

Notes: Dominant fungal isolates were identified in each treatment when *Goodyera pubescens* seeds were germinated in the presence of two fungi. For association with multiple fungi, the number of protocorms infected by the dominant and subordinate fungal isolates, analyzed by inter-simple sequence repeat (ISSR) banding patterns, are given for each two-fungus combination. For fungal switching, we report the number of protocorms that were switched from a dominant to a subordinate fungus (D→S) or a subordinate to a dominant (S→D) fungus for which infecting fungi were identified using ISSR banding patterns. The number in parentheses gives the number of the analyzed protocorms that were identified as "growing" at the time of analysis. The abbreviation "na" indicates fungal combinations for which the ability to switch fungi was not analyzed.

†Dominant fungal isolate, followed by subdominant fungal isolate.

switched vs. control) and random plate main effects and size at the time of transfer as a covariate.

Treatments were chosen to include a "dominant" and "subordinate" fungus in each combination. We defined the dominant fungus as the one that infected the majority of protocorms and also produced higher protocorm growth rates in the laboratory experiment with multiple fungi (see *Association with multiple fungi: Protocorms*, above). After 12 wk of growth on new plates, we remeasured the length of all protocorms and randomly selected 8–10 protocorms from each treatment for which we would use ISSRs to identify all fungi growing from pelotons (maximum of six pelotons per protocorm). This allowed us to determine whether individual protocorms could switch fungi and whether multiple fungi were present. We compared the growth of fungi started with a dominant vs. subordinate fungus using an ANOVA with fixed dominance and random plate main effects and size at transfer as a covariate.

Adults.—To determine whether individual plant–fungus associations persisted and hence whether there was evidence of adult plants switching among fungi, we resampled plants in three populations at SERC. Four plants were sampled in 2002, one year after they had previously been sampled. Ten additional plants were resampled in 2003. For seven of these plants, 2003 was two years after initial sampling, and for three plants 2003 was four years after they were initially sampled. Based on results from the examination of association with multiple fungi, only one peloton from each root was grown out on E-medium and transferred to liquid medium, and DNA was extracted and amplified using methods outlined above and ISSR primer 811. It was previously determined that fungi sampled from *G. pubescens* in these populations were sufficiently variable in banding patterns with primer 811, so only this primer was needed to distinguish even closely related individuals (see Fig. 1).

RESULTS

Association with multiple fungi

Protocorms.—When grown on a plate with overlapping hyphae of two fungi, each *G. pubescens* protocorm had pelotons composed of a single fungus. In four of the six fungal combinations each fungal isolate infected some protocorms. However, even when each fungus infected some protocorms, the isolates were not equally successful in infecting protocorms (Table 1). The fungus that infected most protocorms in each pairing was designated the "dominant" fungus. In each case, the dominant fungus in terms of infection success was also the fungus that was better able to support protocorm growth when protocorms were grown with that fungus alone (fungus 101, 2.94 ± 0.21 mm; fungus 109, 2.49 ± 0.12 mm; fungus 149, 1.48 ± 0.18 mm; fungus 159, 2.75 ± 0.19 mm; fungus 179, 3.10 ± 0.09 mm; mean ± SE). Size of protocorms grown with two fungi in this experiment was comparable to size grown with the dominant fungus alone (1.30 ± 0.05 mm with one fungus, 1.33 ± 0.03 mm with two fungi, $P > 0.7$, F ratio = 0.110), but germination was significantly higher with two fungi than with one (76.1 ± 4.2% with one fungus, 96.2 ± 0.5% with two fungi, $P = 0.002$, F ratio = 10.825). No seeds germinated in the no-fungus control. Ungerminated seeds were 0.34 ± 0.05 mm. The two growth media were indistinguishable with respect to protocorm growth and infection success of the two fungi on a plate (all $P > 0.5$), so they were combined in all analyses.

Adults.—In the 35 plants in which we attempted to identify the fungi that formed multiple pelotons, all of the pelotons grown from a single root had identical ISSR banding patterns (data not shown). Similarly, in each of the 10 adult *G. pubescens* from SERC and four from Coweeta, fungi that came from different roots on the same plant had identical ISSR banding patterns.

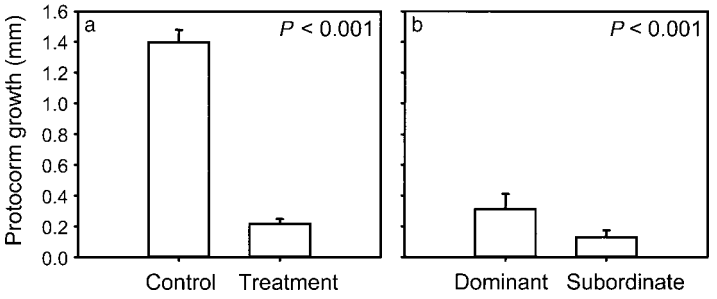

FIG. 2. (a) Protocorm growth (means + SE) 12 weeks after transfer to new plates of different (treatment) or the same (control) fungi with which they initially germinated and (b) treatment protocorms grown initially with dominant and subordinate fungi. *P* values given on each graph indicate significance of the difference between the two *x*-coordinate groups after transfer, using size at transfer as a covariate in an ANOVA: (a) treatment vs. control; (b) dominant vs. subordinate.

Fungi sampled from different ramets of a genet (13 genets) also had identical ISSR banding patterns.

Switching fungi

Protocorms.—When protocorms grown with one fungus were transferred to a Petri dish with a different fungus they grew significantly less than if they were transferred to a Petri dish with their initial fungus ($P < 0.001$; Fig. 2a). Protocorms grown with a dominant initial fungus grew significantly better after switching to a subordinate fungus than the reverse ($P < 0.001$; Fig. 2b). Fewer than 30% of protocorms that were transferred to plates with different fungi continued to grow (Fig. 3). Those protocorms that did not grow all died by ~16–20 wk after transfer. We had more success growing fungi from pelotons sampled from protocorms >1 mm long. As a result, we were only able to identify the fungi infecting two protocorms that had not grown. Also, some fungal combinations had very few protocorms that continued growing (see Table 1) and so were poorly represented in our ISSR identifications. The two non-growing protocorms both contained only pelotons with ISSR banding patterns matching the fungus with which they initially germinated. All protocorms that continued growing contained only pelotons with banding patterns that matched the second fungus (i.e., the fungus on the plate to which they were transferred).

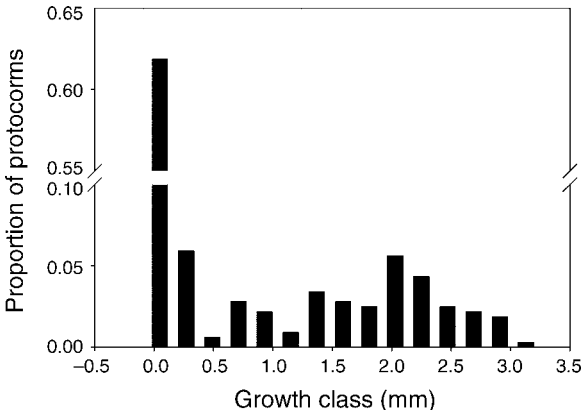

FIG. 3. Frequency histogram showing growth of protocorms that were switched to plates of fungi that differed from their initial fungi (i.e., treatment protocorms, $n = 257$). Protocorms growing only 0 or 0.5 mm after transfer were designated non-growers. The remaining protocorms were designated growers. Note the large axis break between 0.10 and 0.55 necessary to show the distribution of protocorms among the less-represented size classes.

Adults.—All four plants we resampled in 2002 had fungi with ISSR banding patterns that matched those of the fungi we sampled one year prior (data not shown). However, nine of 10 plants resampled in 2003 had switched fungi (Fig. 1). The one plant that had not switched fungi had retained the same fungus for four years. In one case (numbers 6 and 7 in Fig. 1), two nearby plants had identical fungi when initially sampled in 2001, and both had switched to the same new fungus in 2003.

DISCUSSION

Association with multiple fungi

We found that protocorms of *G. pubescens* always contained only one fungal individual when seeds were germinated in the presence of two fungi. The presence of multiple fungi increased germination ($P < 0.001$) but had no effect on growth ($P > 0.7$). When forced to switch to a new fungus, protocorms still had only one living fungus, and even those that successfully formed an association with a new fungus endured a pause in their growth that may have resulted from the time between when the first fungus was fully digested and when the new fungus had formed extensive pelotons.

In the germination experiment, one fungus in each fungal pair was dominant in terms of establishing mycorrhizal relationships with the majority of germinating seeds (Table 1). In each case, the dominant fungus was also the one that supported larger protocorms after 12 wk. It is not entirely clear what could cause a fungus to be dominant over another in supporting germination and also growth. Dominance may result from a fungus infecting seeds faster so that protocorms infected with the faster fungus began to grow earlier, although in the course of conducting many experiments with this species

we have never observed differences among fungi in time of germination even at the earliest measurement times. Dominance could also result from a fungus being better able to obtain nutrients from the media. While not a definitive test of nutrient acquisition ability, we have found that in each pair of fungi used in this study the dominant fungus grew faster on medium with cellulose as the sole carbon source than the non-dominant fungus did (12–64% greater growth, paired t test, $P = 0.008$). The fungi used in this study did not differ in their ability to support protocorm growth among the two wood media used, but in other studies we have found that there was an interaction between fungal isolate and growth medium in fungal ability to support *G. pubescens* growth such that some fungi best supported growth on one medium while others better supported growth on a different medium. Perhaps a better nourished fungus provides more nutrients to germinating seeds and growing protocorms. Regardless of the mechanism, protocorms that germinated with a dominant fungus were more frequently able to take up a second fungus than those that germinated with a subordinate fungus (Fig. 2b). This suggests that protocorms that were more vigorous were better able to endure a period without a fungus (or with minimal fungus available) before a new fungus became established and spread throughout the available cells. This may lend support to the hypothesis that photosynthetic protocorms and seedlings may also be better able to switch fungi. We are currently testing this theory.

Adult *G. pubescens* plants in the field also never had more than one fungus regardless of whether single roots, multiple roots, or multiple ramets were sampled. This finding that plants never associated with multiple fungi is in contrast to the majority of AM (e.g., Lovelock and Miller 2002), ECM (e.g., Bruns 1995), and at least some ericoid (Allen et al. 2003) fungal associations, which clearly include multiple fungi at one time. Even within the Orchidaceae, photosynthetic adults of *Tipularia discolor* (McCormick et al. 2004), *Platanthera* spp. (Zelmer et al. 1996, Sharma et al. 2003), multiple orchids of shaded forests (Bidartondo et al. 2004), and two non-photosynthetic orchids (Selosse et al. 2002, Julou et al. 2005) associated with more than one fungus at a time. Of these studies, association of protocorms with multiple fungal individuals has only been addressed in *T. discolor*. Although adult *T. discolor* associated with multiple fungal individuals, protocorms had much more specific fungal requirements and associated with single fungi (McCormick et al. 2004).

This fidelity (inability to associate with multiple fungi) may be widespread among orchids. *Liparis liliifolia* protocorms and adults also appear unable to associate with multiple fungi (M. K. McCormick, *unpublished data*), and Shefferson et al. (2005), examining only adult plants, found only a single plant that associated with multiple fungi, so this phenomenon likely applies more generally than just to *G. pubescens*. Other specialized plant–fungus associations, such as in the Monotropaceae and other completely or partially myco-heterotrophic plants, may also have limited ability to associate with multiple fungi at a time, but this has not yet been investigated.

Association with multiple fungi simultaneously, especially fungi that may differ in their ability to access nutrients under different conditions, may allow some orchids to "hedge their bets" such that some of their fungi will be successful in any of a wide range of environmental conditions. This bet-hedging strategy may be especially important in completely myco-heterotrophic species. However, it seems unlikely that this strategy is utilized by *G. pubescens*.

Switching fungi

Protocorms were able to switch from one fungal isolate to another in the laboratory. Because the second fungi were already established on the plates to which protocorms were transferred, there was little chance for the initial fungus to grow out of the protocorms and become established. As a result, transferred protocorms, which were entirely non-photosynthetic, were forced to either accept a new fungus or be cut off from all nutrients. Transfer to a plate with a different fungus was associated with $>70\%$ mortality. Transfer to a new plate containing the initial protocorm fungus had $<1\%$ mortality, so the high mortality was not simply a function of the transfer process.

It is unclear what mechanism prevented simultaneous association with multiple fungi. It may be that the first fungus that colonizes a germinating seed physically or chemically prevents colonization by another fungus. These same fungi isolated from *G. pubescens* have also been found in nearby *Tipularia discolor* adults in which they co-occur with other species of fungi, suggesting the fungi do not exclude all other fungi but they may exclude closely related fungi, such as those utilized by *G. pubescens*. It is also possible that a developing protocorm possesses some mechanism to prevent colonization by multiple fungi. Perhaps colonization by only a single fungus would be advantageous because protocorms may get "caught in the crossfire" of antagonistic interactions between multiple colonizing fungi. Although we cannot be sure what mechanism prevented association with multiple fungi at once or whether it was driven by the fungus or the plant, the substantial increase in mortality associated with switching fungi suggests that fungal switching likely is not as common and is less successful in the protocorm stage compared to the adult stage. As in the experiment to examine association with multiple fungi, protocorms never had pelotons of more than one fungus, so it appeared that the initial fungus was first digested and cleared out of protocorm cells before a second fungus was taken up.

Adult plants were also able to switch fungi. In the field, adult plant fungal switching occurred during 2002, the driest growing season on record in Maryland (Wolman 2004), perhaps when the initial fungi used by the plants were killed by the dry conditions. The extreme

drought that occurred when many adult plants switched fungi also resulted in the death of 38% of 37 marked adult plants. In the previous three years (spring 1999–2002), annual mortality of marked adult plants was <5%. Although adult mortality increased in the field during the drought, mortality was lower than that found for protocorms in the laboratory, perhaps because the photosynthetic adult plants were better able to endure time without a fungus than an entirely myco-heterotrophic protocorm. We are currently testing this possibility.

Conclusions

We found that protocorms and adults of the orchid *G. pubescens* never associated with multiple fungi simultaneously but both protocorms and adults were able to switch fungi. This might be expected, since a plant that associated with only a single fungus would be very vulnerable to environmental perturbations that adversely affected its fungus. However, fungal switching seemed only to occur under rather extreme conditions, during which the initial fungus likely died, and it was also associated with substantial mortality. Thus, it seems that switching fungi in *G. pubescens* is likely to be a last resort in the face of extreme conditions rather than a response to minor environmental fluctuations. Other orchids that similarly associate with single fungi (e.g., *Cypripedium* spp., Shefferson et al. 2005; *Liparis liliifolia*, M. K. McCormick, D. F. Whigham, and J. P. O'Neill, *unpublished data*) may have a similar ability to accept new fungi in extreme conditions, but this remains to be seen.

The ability to switch fungi, regardless of whether plants associate with multiple fungi, may prove critical for plant tolerance of environmental changes, especially changes that are associated with changes in the fungal community. Some invasive earthworms, for example, cause significant changes in the microbial community of forests, especially a decrease in the abundance of fungi compared to bacteria (Bohlen et al. 2004). Changes of this type might be quite detrimental to plants that form mycorrhizal associations and are not able to shift from one fungus to another. Over evolutionary time most plant lineages have experienced climate changes (e.g., glacial and interglacial intervals) that likely required them to switch mycorrhizal fungi. However, the possibility of changes within a single plant lifetime has never been evaluated. The ease or difficulty with which different plant species change fungal associates may affect the way that plant communities respond to short-term extreme environmental conditions such as a drought, longer-term changes such as succession, or changes brought about by global warming.

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APPENDIX

A list of all fungi used in laboratory experiments and those field plants sampled multiple times (*Ecological Archives* E-087-052-A1).