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High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*

RICHARD P. SHEFFERSON,*¶ MICHAEL WEIß,† TIIU KULL‡ and D. LEE TAYLOR§
*Department of Integrative Biology, University of California, 3060 Valley Life Sciences Building, #3140, Berkeley, California 94720 USA, †Botanisches Institut, Universität Tübingen, Auf der Morgenstelle 1, D-72076 Tübingen, Germany, ‡Institute of Zoology and Botany, Estonian Agricultural University, Riia 181, Tartu 51014 Estonia, §Institute of Arctic Biology, University of Alaska, 311 Irving I Building, 902 N. Koyukuk Drive, Fairbanks, Alaska 99775–7000 USA

Abstract

Lady's slipper orchids (*Cypripedium* spp.) are rare terrestrial plants that grow throughout the temperate Northern Hemisphere. Like all orchids, they require mycorrhizal fungi for germination and seedling nutrition. The nutritional relationships of adult *Cypripedium* mycorrhizae are unclear; however, *Cypripedium* distribution may be limited by mycorrhizal specificity, whether this specificity occurs only during the seedling stage or carries on into adulthood. We attempted to identify the primary mycorrhizal symbionts for 100 *Cypripedium* plants, and successfully did so with two *Cypripedium calceolus*, 10 *Cypripedium californicum*, six *Cypripedium candidum*, 16 *Cypripedium fasciculatum*, two *Cypripedium guttatum*, 12 *Cypripedium montanum*, and 11 *Cypripedium parviflorum* plants from a total of 44 populations in Europe and North America, yielding fungal nuclear large subunit and mitochondrial large subunit sequence and RFLP (restriction fragment length polymorphism) data for 59 plants. Because orchid mycorrhizal fungi are typically observed without fruiting structures, we assessed fungal identity through direct PCR (polymerase chain reaction) amplification of fungal genes from mycorrhizally colonized root tissue. Phylogenetic analysis revealed that the great majority of *Cypripedium* mycorrhizal fungi are members of narrow clades within the fungal family Tulasnellaceae. Rarely occurring root endophytes include members of the Sebacinaceae, Ceratobasidiaceae, and the ascomycetous genus, *Phialophora*. *C. californicum* was the only orchid species with apparently low specificity, as it associated with tulasnelloid, ceratobasidioid, and sebacinoid fungi in roughly equal proportion. Our results add support to the growing literature showing that high specificity is not limited to nonphotosynthetic plants, but also occurs in photosynthetic ones.

Keywords: Ceratobasidiaceae, myco-heterotrophy, *Rhizoctonia*, Sebacinaceae, specificity, Tulasnellaceae

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Introduction

Symbiotic relationships are important to the evolution and conservation of many, perhaps all, organisms. For plants, symbioses are particularly important for sexual reproduction, as when animals act as pollinators or seed dispersers in return for nectar or a portion of the seed crop (Jordano 1993; Pellmyr *et al*. 1996; Kawakita & Kato 2004).

Correspondence: Richard P. Shefferson, ¶Microbial Ecology Laboratory, Forestry and Forest Products Research Institute, Tsukuba 305-8687, Japan, Fax: (+81) 29 859 0644;
E-mail: dormancy@gmail.com

At least as important to plants are microbial symbioses resulting in significant nutritional advantage. Primary among these is the mycorrhiza, a plant-fungus symbiosis from which most plants gain the majority of their nutrients, including those limiting their growth (Smith & Read 1997). Mycorrhizal fungi are thought to have evolved at the same time as land plants, allowing the latter to colonize and radiate on land (Wilkinson 2001; Brundrett 2002). Most plants respond mutually to such gains by allowing the fungus a share of photosynthetically-fixed carbon, which limits fungal growth (Smith & Read 1997). However, some plants, including the entire orchid family, Orchidaceae, have evolved a different kind of mycorrhizal

relationship in which carbon is not supplied to the fungus (Taylor *et al*. 2002). Orchid mycorrhizae have been studied for nearly a century now, and yet the notoriously cryptic fungi involved in these symbioses have limited even our understanding of their taxonomic identities and evolutionary relationships (Rasmussen 1995). Of all orchids that have been studied, few have been the object of mycorrhizal inquiry for as long as the primary genus of temperate lady's slipper orchids, *Cypripedium*.

Mycorrhizal infection is a prerequisite for the germination and growth of all orchid seeds in the wild (Bernard 1904). For *Cypripedium*, life begins as a 'dust seed', a seed so small as to be almost microscopic (Curtis 1943; Rasmussen 1995; Kull 2002), with recruitment estimated at less than 0.06% in *Cypripedium calceolus* (Kull 2002). Upon infection by a compatible mycorrhizal fungus, the seed germinates and develops into a seedling that consumes fungal sugars, a condition known as 'myco-heterotrophy' (Beau 1920; Smith 1967; Alexander & Hadley 1985; Leake 1994; Rasmussen 1995). The myco-heterotrophic orchid seedling may grow for several years prior to developing into a 'green' seedling (Rasmussen 1995; Kull 1999). As adults, *Cypripedium* species undergo 'adult dormancy', periods of one or more years during which no sprouts are produced and no photosynthesis takes place (Lesica & Steele 1994; Shefferson *et al*. 2003), suggesting that these plants may retain myco-heterotrophy into adulthood (Gill 1989).

Efforts to understand mycorrhizal associations in this group generally focus on attempts at seed germination and propagation with various species of the fungal genus *Rhizoctonia*. However, since MacDougal's (1899) classic description of *Cypripedium* mycorrhizal morphology, studies of this interaction have either failed to yield germination of *Cypripedium* seeds or have led to inconsistent results (Curtis 1939; Zelmer *et al*. 1996; Ramsay & Stewart 1998). This may be the result of confusion surrounding this fungal genus. *Rhizoctonia* is often noted for its associations with most other orchids (Rasmussen 1995; Case *et al*. 1998; Ramsay *et al*. 1998). It is polyphyletic, including fungi from across the fungal division Basidiomycota, particularly from the families Tulasnellaceae, Sebacinaceae, and Ceratobasidiaceae (Andersen 1996; Muller *et al*. 1998). However, because *Rhizoctonia* is an artificial genus based on asexual stages, the identities of and phylogenetic relationships among *Rhizoctonia* spp. have only begun to be evaluated now with the dawn of molecular phylogenetics (Andersen 1996; Muller *et al*. 1998; Taylor 2000; Ma *et al*. 2003).

If *Cypripedium* species specialize on particular mycorrhizal fungi, then these fungi may limit their distribution. Soil microbial diversity plays important roles in determining plant abundance (Klironomos 2002), and seed germination in some orchids only occurs near adults (Batty *et al*. 2001). Some have suggested that *Cypripedium* spp. must be mycorrhizal generalists because of inoculation trials showing germination of their seeds by fungi isolated from distantly related orchids (Curtis 1939; Hadley 1970; but see Tomita & Konno 1998). However, orchid seeds form mycorrhizae with a greater breadth of fungi *in vitro* than in the wild (Masuhara & Katsuya 1994). By current standards, in which specificity is defined not by the number of fungal species that a plant can associate with but by the phylogenetic breadth of symbionts (Molina *et al*. 1992; Taylor *et al*. 2002; McCormick *et al*. 2004), the mycorrhizal specificity of *Cypripedium* has never been rigorously assessed.

We assessed the identity and breadth of mycorrhizal fungi that associate with seven *Cypripedium* species from North America and Europe. We directly PCR (polymerase chain reaction)-amplified nuclear and mitochondrial genes previously established as useful for this purpose directly from mycorrhizal tissue. Mycorrhizal identity and specificity were assessed by sequencing these genes and performing phylogenetic analyses. We further examined whether these associations differed with *Cypripedium* species and with geography.

Materials and methods

Sample collection

Seven *Cypripedium* species were chosen for this study based on their accessibility and geographical distribution. From least to most widely distributed, according to distribution maps provided in Cribb (1997), they are: *Cypripedium californicum* A. Gray, *Cypripedium fasciculatum* Kellogg ex S. Watson, *Cypripedium candidum* Mühl ex Willd., *Cypripedium montanum* Douglas ex Lindl., *Cypripedium guttatum* Sw., *Cypripedium parviflorum* Salisb., and *Cypripedium calceolus* L. While taxonomic controversy surrounds the unusually high levels of genetic variation in *C. calceolus* (Case 1994; Brzosko *et al*. 2002), and uncertainty over the taxonomic status of *C. parviflorum* continues to this day (Cribb 1997), all current phylogenetic hypotheses suggest that the seven species used in this study are at the very least distinct from one another (Cox *et al*. 1997; Cribb 1997). Although the entire genus is listed on CITES (Convention on International Trade of Endangered Species) Appendix 2 (World Conservation Union 1963), the distributions vary, and the first four species are exceptionally restricted geographically. *C. californicum* has the narrowest range, occurring only in northern California and southern Oregon (Cribb 1997). *C. fasciculatum* occurs over the same range as *C. californicum*, but also includes parts of Washington, British Columbia, and the central Rocky Mountains. *C. candidum* occurs only in the northern central United States and southernmost Ontario. *C. montanum* occurs only in the Pacific Northwest of the United States

Table 1 List of surveyed *Cypripedium* species, regions and locales sampled, years harvested, and numbers of populations and individuals sampled at each locale. Numbers in parentheses under the '# Pops sampled' and '# Plants sampled' columns indicate the number of populations and plants, respectively, yielding PCR product with fungal nucLSU or mtLSU primers. All plants exhibited at least a small amount of peloton-containing root tissue

Cypripedium species	Country or US State	Region (county/park/forest)	Year sampled	# Pops sampled	# Plants sampled
calceolus	Estonia	Baltic coast	2003	5 (2)	11 (2)
californicum	California	Sonoma County	2002	6 (3)	9 (4)
		Mendocino National Forest	2002	1 (1)	3 (3)
		Del Norte National Forest	2002	3 (1)	7 (3)
candidum	Illinois	Lake County	2001	1 (1)	2 (2)
	Kentucky	Hardin County	2001	2 (2)	5 (4)
fasciculatum	California	Klamath National Forest	2002	2 (2)	3 (3)
		Mendocino National Forest	2002	2 (1)	3 (1)
		Plumas National Forest	2002	2 (2)	8 (6)
		Six Rivers National Forest	2002	2 (2)	8 (6)
guttatum	Alaska	Fairbanks	2003	2 (2)	5 (2)
montanum	California	Klamath National Forest	2000–2002	9 (8)	23 (12)
parviflorum	Illinois	Lake County	2001	1 (1)	7 (5)
	Kentucky	Hardin County	2001	4 (4)	4 (4)
		Powell County	2001	2 (2)	2 (2)
Total				44 (34)	100 (59)

and Canada, extending from northern California to the Alaskan Panhandle. At the other extreme, *C. calceolus*, the sole European species, occurs across the widest territory, from Great Britain to Japan, and from Spain to Scandinavia. *C. parviflorum* is the most widespread North American species, occurring throughout Canada, the eastern and central United States, and in pockets in the American Rocky Mountains and Pacific Coast. *C. guttatum* has an unusual distribution including a small pocket in eastern Alaska, but extending throughout central Siberia, northeastern and south-central China, Japan, and North Korea.

Sampling was conducted between May and October every year from 2000 to 2003, with a total of 100 plants sampled across four US states and in Estonia (Table 1). As many plants were sampled from as many populations as regional managers and landowners allowed, although most populations were too small to offer more than a limited number of sampled plants. When possible, we chose plants representing a range of life stages, from small, vegetative sprouts to large, multistemmed and flowering individuals. Between two and six roots representing a range of root ages were taken per plant. Two or three whole plants, including adults and photosynthetic seedlings, were also collected from each study species. All roots were surface-sterilized using 10% to 20% bleach solution (Taylor & Bruns 1997). Using a compound microscope, we sampled *Cypripedium* roots in 0.5–1.0 cm intervals looking for the presence of pelotons, mycorrhizal hyphal coils growing within orchid root cortical cells (Smith & Read 1997), resulting in five to 20 mycorrhizal root samples per plant.

Molecular methodology

Characterization of *Cypripedium* mycorrhizae involved: (i) extraction of fungal and plant DNA from mycorrhizal plant tissue, (ii) amplification of fungal genomic regions useful in determining fungal identity, (iii) DNA sequencing, and (iv) phylogenetic analysis for identification of mycorrhizal fungi and assessment of specificity.

Fungal and plant DNAs were extracted from mycorrhizal root samples using the QIAGEN DNeasy Plant Mini DNA kit (QIAGEN). Some sections containing either no morphological evidence of mycorrhizal infection, or morphology suggesting parasitic or other fungal infections, were also taken to provide controls.

To assess candidate groups of mycorrhizal fungi, we first attempted to PCR amplify DNA extracts using primers from the internal transcribed spacer, hereafter ITS: ITS1F–ITS4 (White *et al*. 1990; Gardes & Bruns 1993), ITS1–ITS4B (Gardes & Bruns 1993), and ITS1F–ITS4-Tul (Taylor 1997). Only the ITS1F–ITS4 combination yielded PCR product, so we used the fungal-specific primers ITS1F and cNL2F, the latter allowing us to amplify an *c.* 300 bp portion of the highly conserved 28S nuclear large subunit ribosomal gene, hereafter nucLSU (Taylor *et al*. 2003). Concurrently, we amplified the mitochondrial large subunit ribosomal gene (hereafter, mtLSU) of all samples with the primer pairs ML5–ML6 and MLIN3–ML6 (Bruns *et al*. 1998). Although both mitochondrial primer pairs amplified DNA, the primer combination MLIN3–ML6 produced sequences strikingly similar to plant chloroplast DNA, and was not used further. PCR involved 35 cycles with an

annealing temperature of 55 °C, using an MJ PTC-200 Thermocycler (MJ Research). Representative samples were chosen for each plant via RFLP (restriction fragment length polymorphism) analysis of ITS-nucLSU PCR product using the restriction enzymes *Hin*fI and *Mbo*I (Gardes & Bruns 1996).

In some cases, RFLP analysis revealed the presence of multiple fungi in a root section. These PCR products were cloned using Stratagene XL-1 Blue Supercompetent cells (Stratagene) and the pDrive cloning vector (QIAGEN), and blue-white screened on LB medium infused with X-gal, ampicillin, and kanamycin. Plasmid DNAs were purified with the QIAprep Spin Miniprep Kit (QIAGEN), after which the cloned PCR product was re-amplified with primers ITS1F and cNL2F and screened with RFLP analysis to assess the diversity of ITS-nucLSU PCR products per plate. Clones representative of the major RFLP-types found in each sample were chosen for sequencing, as were some duplicates and all ITS-nucLSU and mtLSU products from samples not requiring PCR cloning.

Sequencing began with purification of PCR samples with the MinElute PCR Purification Kit (QIAGEN). We cycle sequenced each PCR sample with BigDye version 3.1 chemistry (Applied Biosystems) modified with BetterBuffer (The Gel Company). Cycle sequenced samples were electrophoresed with an ABI 377–96 Genetic Analyser (Applied Biosystems). The resulting sequences were edited in SEQUENCHER (Gene Codes) and analysed with BLAST (Altschul *et al*. 1997) against the NCBI sequence database (National Center for Biotechnology Information, GenBank: http://www.ncbi.nlm.nih.gov) to detect similar sequences of known phylogenetic placement.

Phylogenetic analysis

We began by grouping sequences by family association as determined in BLAST, and conducting phylogenetic analyses with representatives of each endophyte family. Initial phylogenetic analysis involved adding nucLSU (ITS4 to cNL2F) sequences into an alignment representing the major plant-associated groups of Basidiomycetes and Ascomycetes. Next, a similar analysis was conducted focusing on Basidiomycetes. The poor fit of the major fungal clade in the Basidiomycete nucLSU tree led us to take representative samples from this clade for further amplification and sequencing of an *c.* 600 bp region of the nucLSU using the primer set ITS1F–TW14 (White *et al.* 1990), followed by phylogenetic analysis as before. Further analyses involved adding sequences to alignments representing narrower phylogenetic breadth. After nucLSU analysis, mtLSU sequences were added to an alignment of mycorrhizal fungal species (Bruns *et al*. 1998).

Sequences were aligned using CLUSTALX (Thompson *et al*. 1997). Phylogenetic analysis involved maximum likelihood searches in METAPIGA 1.0.2b for Windows XP (Lemmon & Milinkovitch 2002) using the HKY85 (Hasegawa-Kishino-Yano) nucleotide substitution model (Hasegawa *et al*. 1985), with rate heterogeneity and invariant sites allowed, transition : transversion rates estimated from the data, and eight populations and eight trees per population with random starting trees in the metapopulation search algorithm. Branch support was estimated via 250 maximum likelihood replicates in METAPIGA with four populations and four trees per population, yielding 1000 trees per run, and all other parameters as before (Lemmon & Milinkovitch 2002). These support values are considered estimates of the probabilities that the respective clades are monophyletic given the sequence data (Lemmon & Milinkovitch 2002). Rarely encountered fungi with strong BLAST support were not phylogenetically analysed, although they are presented with BLAST results in this study. Sequences generated in this study have been deposited in GenBank under accession nos. AY578184–AY578251, AY578268–AY578284, AY585831, AY674054-AY674056 and AY682107.

Inference of mycorrhizal interaction from direct DNA amplification is complicated by the high likelihood of encountering nonmycorrhizal fungi in root tissue found in nature. For example, peloton-containing root tissue may also include other endophytes. Absolute proof of the interaction is perhaps impossible in this system because experimental tests of mycorrhizal colonization are precluded by the 10–16 years required for the plant to grow to maturity (Shefferson *et al*. 2001), by the rarity of the plants (Cribb 1997), because cultivation is notoriously difficult and often unsuccessful (Cribb 1997), and because fungal isolations from members of this genus generally fail (Ramsay *et al.* 1998). We assumed that fungal groups were more likely to be mycorrhizal when they included uncloned sequences, because, if they contain pelotons, the resulting sequences are most likely to have come from the peloton-forming fungi. We also assumed that large clades of sample sequences represent fungi more likely to be mycorrhizal than single sequences spread widely across the Kingdom Fungi. Amplification of multiple fungi would result in greater difficulty in parsing out which of the groups are mycorrhizal, after all. Lastly, a greater potential for mycorrhizal status was suggested with fungal groups known to form such associations with other orchids.

Results

In contrast to previous research suggesting that evidence of mycorrhizal infection can only be found in protocorms and young plants (Vinogradova & Andronova 2002), we found partially digested, brownish pelotons in all *Cypripedium* plants in the study (Fig. 1). Roots, rather than rhizomes, were mycorrhizal in adults, although rhizomes were sometimes colonized in seedlings. Mycorrhizal colonization was observed at sparse, irregular intervals along

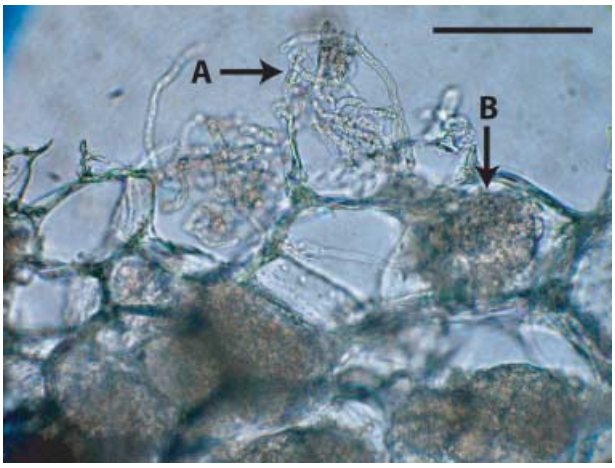

Fig. 1 Cross-section of mycorrhizal hyphal coils (i.e. pelotons) observed in *Cypripedium guttatum* root cortical cells. Arrows indicate mycorrhizal fungal hyphae, separated from the intact peloton (A), and still within an intact peloton (B). Scale bar represents 100 μm.

the length of the root system, with young roots usually devoid of mycorrhizae, old roots generally decaying, and intermediate aged roots displaying the most colonization. No completely undigested pelotons were observed, although *Cypripedium guttatum* pelotons appeared most intact. Isolations were not attempted, as a result of a long history of unsuccessful attempts by other researchers (Rasmussen 1995). Furthermore, root sampling conducted throughout the growing season across all years of study failed to reveal the annual time of mycorrhizal establishment. PCR amplification of mycorrhizal tissue revealed that although ITS, nucLSU, and mtLSU could be amplified for all six North American species, only the mtLSU could be amplified for *Cypripedium calceolus*, and PCR product could be obtained for a total of 59 out of 100 sampled plants (Table 1). Amplification failures could have resulted from a combination of spotty colonization, digested pelotons, and small root samples, which could not be avoided because of the threatened status of this genus. Lack of nucLSU amplification in *C. calceolus* may have also resulted from inappropriate primer sets. Negative controls in PCRs, utilizing ultra-filtered water further sterilized with ultraviolet light, surface-sterilized nonmycorrhizal root tissue without noticeable fungal endophytes, surface-sterilized stem tissue, and leaf tissue rinsed with ultra-filtered water, consistently yielded no PCR product. When checked on 1.5% agarose gels, PCR tests of root tissue appearing infected by nonmycorrhizal fungi yielded multiple bands and smears inconsistent with mycorrhizal PCR product.

Mycorrhizal symbionts and other endophytes

Analysis of nucLSU and mtLSU sequences congruently suggested that mycorrhizal tissue was dominated by fungi from the family Tulasnellaceae (clades snLT1 and 2, Fig. 2; clade mT1, Fig. 3), a result confirmed in an analysis involving twice-longer sequences generated using primers ITS1F–TW14 and reference sequences from a broad phylogenetic scope of Hymenomycetes (not shown). When all samples were accounted for, this clade accounted for the vast majority of mycorrhizal infections in *Cypripedium* root tissue, with 82.7% of basidiomycetous nucLSU PCR product. The only fungal group found in all *Cypripedium* species, it was also found in 39 of the 59 total plants (66.1%) that yielded fungal nucLSU and/or mtLSU PCR product, more than any other endophyte (Table 2).

Two tulasnelloid clades were identified. Of these, clade snLT1, sister to all other taxa of Tulasnellaceae in our

Table 2 Fungal root endophytes in *Cypripedium*. Fungal groups represent closest-related taxa from nucLSU and mtLSU phylogenies of Basidiomycota and Ascomycota, and from NCBI BLAST results. Each row lists the corresponding numbers of plants associated with each fungal group. Numbers of plants yielding multiple fungi, and hence requiring cloning of PCR products, are listed in parentheses. Total numbers of plants of each species yielding PCR product are listed in the final row, and total numbers of *Cypripedium* plants yielding each fungal group are listed in the final column. Column sums may be greater than totals listed in the final row, because some plants contained multiple fungi and are listed multiple times per column

Fungal group	*calceolus*	*californicum*	*candidum*	*fasciculatum*	*guttatum*	*montanum*	*parviflorum*	Total Plants
Tulasnellaceae	2 (0)	3 (1)	2 (1)	14 (7)	0 (2)	10 (7)	6 (4)	39
Sebacinaceae	—	2 (2)	—	1 (1)	—	—	1 (1)	4
Ceratobasidiaceae	—	3 (0)	—	—	—	—	—	3
Thelephoraceae	—	—	1 (1)	—	—	1 (1)	—	2
Russula	—	—	—	—	—	—	1 (1)	1
Agaricales	—	—	—	—	—	—	1 (1)	1
Phialophora	—	3 (3)	4 (4)	3 (3)	—	4 (4)	4 (4)	18
Glomus	—	1 (1)	—	—	—	—	1 (1)	2
Total Plants	2	10	6	16	2	12	10	

Fig. 2 Fungal nuclear large subunit phylogeny of the Tulasnellaceae, showing the overwhelming majority of *Cypripedium* mycorrhizal fungi which fall within this family. Of two clades, the majority of fungi belong to clade snLT1 (short for 'short nuclear large tulasnelloid 1'), which includes fungi from all *Cypripedium* species sampled except *Cypripedium californicum*, *Cypripedium candidum* and *Cypripedium calceolus*, and is sister to the remaining Tulasnellaceae. Clade snLT2, smaller than snLT1, falls firmly within the Tulasnellaceae and includes fungi from *Cypripedium californicum* and *C. candidum*. Phylogeny constructed using a 295 bp alignment of the 28S nucLSU rDNA region, and rooted with *Auricularia auricula-judae*. The best tree resulting from heuristic maximum likelihood analysis is presented, with support values derived using maximum likelihood replicates (only values ≥ 50% shown); for details, see text. Sequences generated from fungal symbionts in this study are noted by a one-letter code designating the source *Cypripedium* species: F—*Cypripedium fasciculatum*, G—*Cypripedium guttatum*, L—*Cypripedium californicum*, M—*Cypripedium montanum*, N—*Cypripedium candidum*, and P—*Cypripedium parviflorum*. This is followed by parentheses containing the plant number (given as 'pXXX'), clone number (given as 'cXX', if applicable; if clone number is missing, then no cloning was performed), and an identifier for the geographical location of the plant. Geographic codes are as follows: DCa—Del Norte National Forest, California, USA; FAk—Fairbanks, Alaska, USA; HKy—Hardin Co., Kentucky, USA; KCa—Klamath National Forest, California, USA; LII—Lake County, Illinois, USA; PCa—Plumas National Forest, California, USA; PKy—Powell Co., Kentucky, USA; SCa—Sonoma Co., California, USA; and 6 Ca—Six Rivers National Forest, California, USA. Sources for reference sequences used in this figure: (a)—Bidartondo *et al.* (2003), (b)—Taylor *et al.* (2003), (c)—Kottke *et al.* (2003), (d)—Langer, unpublished, (e)—Hibbett *et al.* (2000), and (f)—Weiß & Oberwinkler (2001).

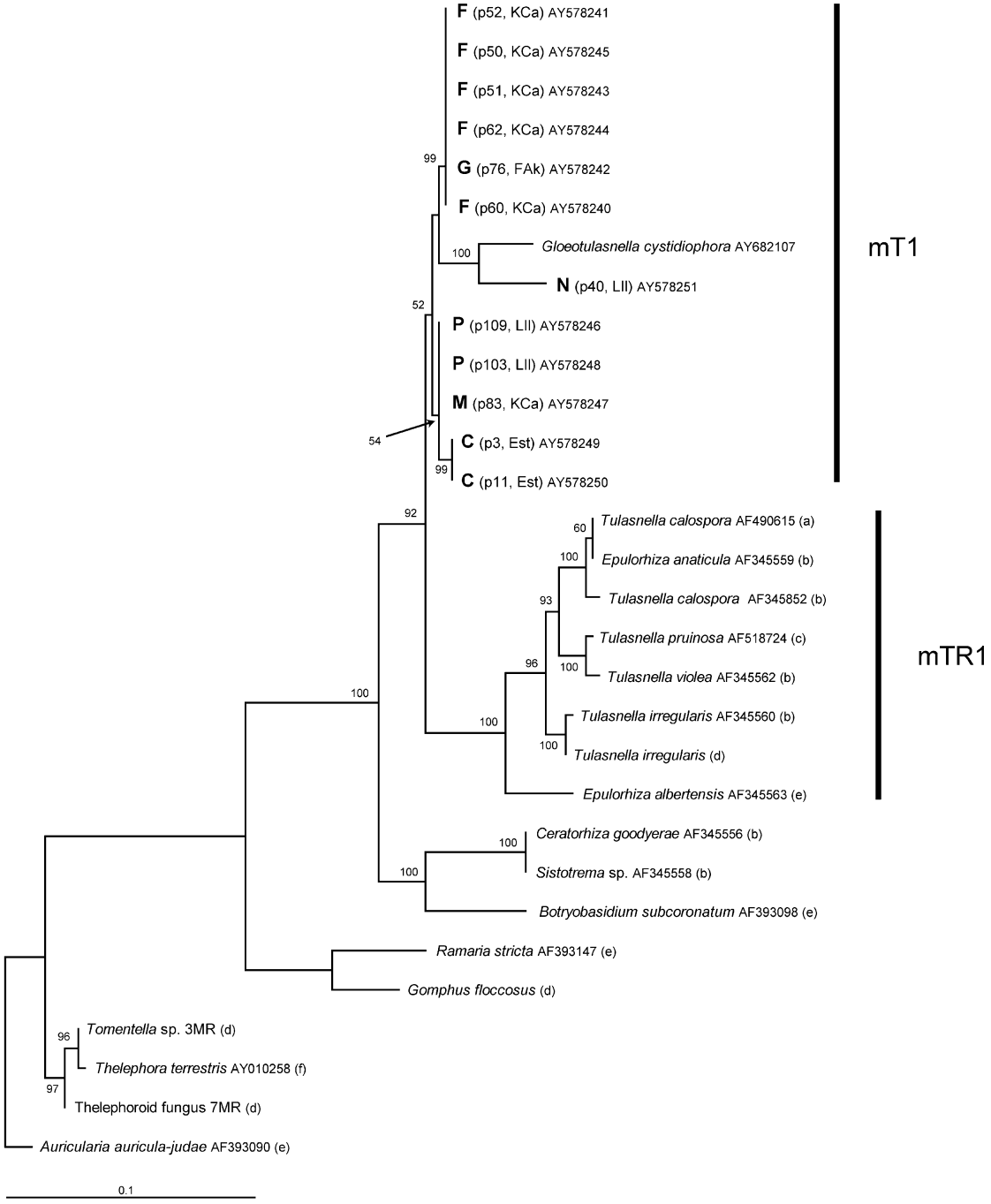

Fig. 3 Fungal mitochondrial large subunit phylogeny supporting the hypothesis that the main fungi mycorrhizal with *Cypripedium* are primarily within the family Tulasnellaceae. Clade mTR1 (short for 'mtLSU tulasnelloid reference 1') and *Gloeotulasnella cystidiophora* include all tulasnelloid mtLSU sequences on GenBank as of the writing of this manuscript. This clade is basal to clade mT1, which includes all mtLSU samples in this study. Phylogeny constructed using a 282 bp alignment of the mtLSU, rooted with *Auricularia auricula-judae*. The best tree resulting from heuristic maximum likelihood analysis is presented, with support values derived using maximum likelihood replicates (only values ≥ 50% shown); for details, see text. Sequences generated from fungal symbionts in this study are noted by a one-letter code designating the source *Cypripedium* species: C—*Cypripedium calceolus*, F—*Cypripedium fasciculatum*, G—*Cypripedium guttatum*, L—*Cypripedium californicum*, M—*Cypripedium montanum*, N—*Cypripedium candidum*, and P—*Cypripedium parviflorum*. This is followed by parentheses containing the plant number (given as 'pXXX'), clone number (given as 'cXX', if applicable; if clone number is missing, then no cloning was performed), and an identifier for the geographical location of the plant. Geographic codes are as follows: Est—Estonia; FAk—Fairbanks, Alaska, USA; HKy—Hardin Co., Kentucky, USA; KCa—Klamath National Forest, California, USA; LII—Lake County, Illinois, USA; SCa—Sonoma Co., California, USA; and 6 Ca—Six Rivers National Forest, California, USA. Sources for reference sequences used in this figure are as follows: (a)—Kristiansen *et al*. (2004), (b)—Kristiansen *et al*. (2001), (c)—Hibbett & Binder (2002), (d)—Bruns *et al*. (1998), (e)—Binder & Hibbett (2002), and (f)—Lilleskov, Fahey, Horton, and Lovett, unpublished.

Table 3 Nuclear large subunit BLAST search results for fungi occurring rarely in *Cypripedium* mycorrhizal tissue. Only potentially mycorrhizal fungi are listed. The nearest taxon listed is as described in the GenBank accession. Overlap length corresponds to the number of base pairs in the sample sequence that fit a pairwise alignment with the nearest taxon accession in GenBank. The last column gives the number of matching base pairs divided by the length of the overlap with the nearest taxon

Orchid host species	Sample accession	Sequence length	Nearest taxon	Accession of nearest taxon	Overlap length	% match
Cypripedium californicum	AY578184	249	Uncultured *Glomus* sp.	AY138144	244	98%
C. californicum	AY578185	249	*Glomus clarum*	AJ510242	245	94%
C. californicum	AY578228	252	*Ceratobasidium* sp.	AY293171	253	99%
C. californicum	AY578229	251	*Ceratobasidium* sp.	AY293171	252	98%
C. californicum	AY578233	252	*Sebacina vermifera*	AF291366	248	97%
C. californicum	AY578234	252	*Sebacina vermifera*	AF291366	247	98%
C. californicum	AY674054	251	*Ceratobasidium* sp.	AY293171	252	99%
C. candidum	AY578230	254	Thelephoraceous ectomycorrhiza	AF430277	250	98%
C. fasciculatum	AY578232	252	Sebacinaceous endomycorrhiza	AF440652	252	99%
C. montanum	AY578231	254	Thelephoraceous ectomycorrhiza	AF430277	255	98%
C. parviflorum	AY578186	250	*Glomus mosseae*	AF396788	256	86%
C. parviflorum	AY578235	251	Sebacinaceous mycorrhiza	AF298948	252	91%
C. parviflorum	AY674055	256	*Russula sardonia*	AF325318	256	99%
C. parviflorum	AY674056	257	*Pluteus ephebeus*	AF261574	257	92%
Goodyera oblongifolia	AY578227	254	*Thanatephorus cucumeris*	AF354119	254	99%

analysis, included 63.5% of all Basidiomycete nucLSU sequences (Fig. 2). Clade snLT2 was smaller but was less phylogenetically distinct from reference Tulasnellaceae taxa. This clade included the only tulasnelloid sequences from *Cypripedium candidum* and *Cypripedium californicum*, as well as some from *Cypripedium montanum* and *Cypripedium parviflorum* (Fig. 2). Although the nuclear ribosomal loci in this family evolve at a faster rate than those of other Basidiomycetes (Taylor 1997), distances among tulasnelloid mycorrhizal samples within these two clades were small, suggesting high specificity to these clades rather than to other tulasnelloid groups.

Apart from these basidiomycetous fungi, sequences of ascomycetous endophytes corresponding to the polyphyletic genus, *Phialophora*, were commonly recovered. These were found in representatives of all *Cypripedium* species studied except *C. guttatum* and *C. calceolus*, and were associated with 18 of 59 plants (30.5%; Fig. 4; Table 2). All of these fungi were most closely related to parasitic and/or saprotrophic groups of *Phialophora* (Fig. 4), especially *Phialophora gregata*, the causal disease agent of soybean brown stem rot (Malvick *et al*. 2003), the root- and stem-rotting fungus *Phialophora malorum* (Sugar & Spotts 1992; Blok *et al.* 1995), and the obscure rot-fungus *Phialophora melinii* (Shigo 1974). A small group was associated with *Chalara* sp. and *Xenochalara juniperi* (Fig. 4), both relatively obscure species (Coetsee *et al*. 2000). In no cases did these Ascomycetes appear closely related to the ectendomycorrhizal species *Phialophora finlandia* (Harney *et al*. 1997; Vrålstad *et al*. 2002). Because of this evidence, because all *Phialophora* sequences were clones from a small subset of *Cypripedium* plants associating with tulasnelloid mycorrhizal fungi, and because this association was minor in the well-sampled species *Cypripedium fasciculatum* and *Cypripedium montanum* (Table 2), we suggest that these fungi are most likely either parasites of the orchids or of their basidiomycetous mycorrhizal fungi, or rotters of dead or dying root tissue.

Other fungi were also occasionally found (Table 3). Fungi from family Ceratobasidiaceae associated with *C. californicum* plants from the Del Norte National Forest, but no other plants (Table 3). Although ceratobasidioid associations were observed in *Goodyera oblongifolia* plants sympatric with one *C. fasciculatum* population at the Plumas National Forest, California (Table 3), the latter associated only with tulasnelloid fungi. *C. californicum* was the only well-sampled species to exhibit mycorrhizal patterns counter to the general patterns we observed, with most uncloned nucLSU sequences belonging to the Ceratobasidiaceae, and approximately equal numbers of cloned sequences belonging to the Tulasnellaceae and Sebacinaceae (Tables 2 and 3). Other endophytes, belonging to Thelephorales, Russulales, Agaricales, and Glomeromycota, were found in one or two plants each, and only in samples co-occurring with other fungi (i.e. requiring PCR cloning; Table 2).

Discussion

Identity of the mycorrhizal fungi

The primary mycorrhizal symbionts of *Cypripedium* are within the family, Tulasnellaceae. This obscure family includes many known orchid mycorrhizal fungi (Warcup

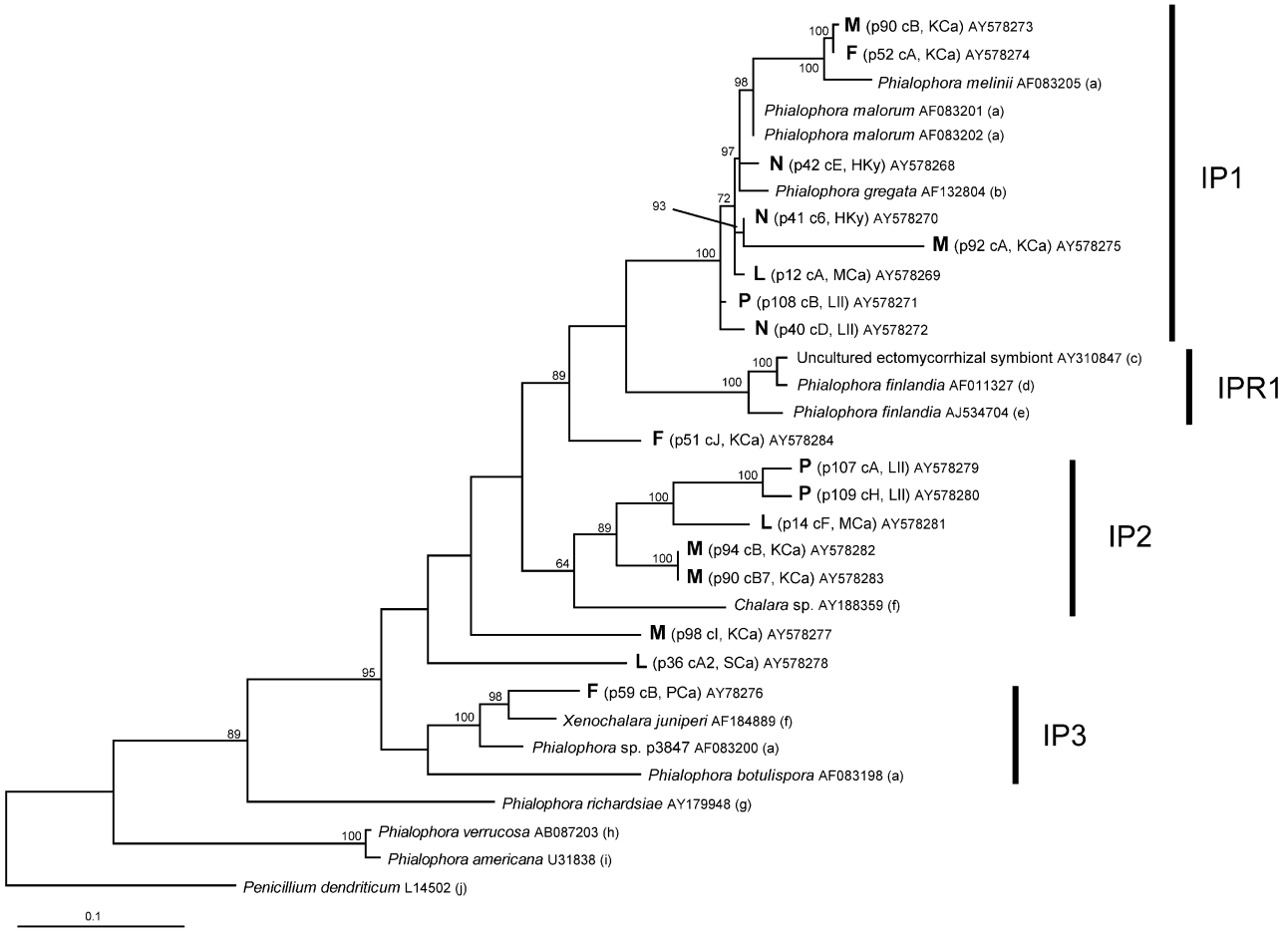

Fig. 4 Fungal internal transcribed spacer phylogeny suggesting that ascomycetous *Cypripedium* root endophytes are likely saprotrophic or parasitic members of the polyphyletic genus *Phialophora*. Clade IP1 (short for 'ITS phialophoroid 1') includes *Phialophora gregata*, *Phialophora melinii*, and *Phialophora malorum*, all rot fungi. Clades IP2 and IP3 include *Xenochalara juniperi* and *Chalara* sp., which are not well-described fungi. Clade IPR1 (short for 'ITS phialophoroid reference 1') includes the only mycorrhizal species, *Phialophora finlandia*, which appears relatively unrelated to any *Cypripedium* root endophyte. Tree constructed using a 454 bp alignment of the internal transcribed spacer (ITS), and rooted with *Penicillium dendriticum* (LaBuglio *et al*. 1993). The best tree resulting from heuristic maximum likelihood analysis is presented, with support values derived using maximum likelihood replicates (only values ≥ 50% shown); for details, see text. Sequences generated from fungal symbionts in this study are noted by a one-letter code designating the source *Cypripedium* species: F—*Cypripedium fasciculatum*, L—*Cypripedium californicum*, M—*Cypripedium montanum*, N—*Cypripedium candidum*, and P—*Cypripedium parviflorum*. This is followed by parentheses containing the plant number (given as 'pXXX'), clone number (given as 'cXX', if applicable; if clone number is missing, then no cloning was performed), and an identifier for the geographical location of the plant. Geographic codes are as follows: HKy—Hardin Co., Kentucky, USA; KCa—Klamath National Forest, California, USA; LII—Lake County, Illinois, USA; MCa—Mendocino National Forest, California, USA; PCa—Plumas National Forest, California, USA; and SCa—Sonoma Co., California, USA. Sources for reference sequences used in this figure are as follows: (a)—McKenry, Rogers, and Wang, unpublished (b)—Harrington *et al*. (2000), (c)—Kennedy *et al*. (2003), (d)—Saenz & Taylor (1999), (e)—Tedersoo *et al*. (2003), (f)—Golonka, unpublished, (g)—Coetsee *et al*. (2000), (h)—Mostert *et al*. (2003), (i)—Abliz *et al*. (2003), (j)—Yan *et al*. (1995), and (k)—LaBuglio *et al*. (1993).

& Talbot 1967; Warcup & Talbot 1971; Rasmussen 1995), and even some liverwort symbionts (Bidartondo *et al*. 2003; Kottke *et al*. 2003). This group has been noted as a primary mycorrhizal symbiont of *Neuwiedia veratrifolia* (Kristiansen *et al*. 2004), a member of the Apostasioideae, an orchid subfamily immediately basal to the Cypripedioideae and sister to the remaining Orchidaceae, suggesting that family Tulasnellaceae may be the ancestral family of mycorrhizal fungi in the Orchidaceae. However, *Cypripedium*-mycorrhizal tulasnelloids are not similar enough to sequences from other Tulasnellaceae in GenBank to infer a genus-level classification at this time. *Rhizoctonia sclerotica*, previously isolated from *Cypripedium reginae*, and *Rhizoctonia subtilis*, previously isolated from both *Cypripedium candidum* and

Cypripedium parviflorum (Curtis 1939), may be members of this group, although no molecular data on these cultures exist (Andersen 1996; Shan *et al*. 2002).

Some other orchid-associating fungi were also observed. Fungi in family Sebacinaceae have been isolated from a broad range of orchid species, including *Caladenia* spp., *Elythranthera* spp., *Eriochilus* spp., *Glossodia major*, *Microtis unifolia*, and *Platanthera orbiculata* (Warcup & Talbot 1967; Warcup 1971; Warcup 1981; Currah *et al.* 1990). Sebacinoids are the mycorrhizal symbionts of the achlorophyllous orchids *Neottia nidus-avis* and *Hexalectris spicata* (McKendrick *et al*. 2002; Selosse *et al*. 2002b; Taylor *et al*. 2003), which utilize this fungal group for carbon nutrition. They are also the rhizoctonias isolated from the Australian green orchids *Cyrtostylis reniformis* and *Microtis uniflora*, having been assigned to the broad species complex of *Sebacina vermifera* by induction of their perfect stages (Warcup 1981; Warcup 1988; Weiß *et al*. 2004). Fungi in family Ceratobasidiaceae, which associate with *Cypripedium californicum* in Del Norte National Forest of northern California, have been previously isolated from the Australian orchid *Acianthus reniformis* (Warcup & Talbot 1967). Related fungi have been found in a wide variety of orchid species around the world (Warcup 1981; Otero *et al*. 2002). Members of the Russulaceae family have been observed in *Corallorhiza maculata* and *Corallorhiza mertensiana*, and the Thelephoraceae have been observed in *Cephalanthera austinae* (Taylor & Bruns 1997; Taylor & Bruns 1999). Lastly, *Phialophora* endophytes may be related to *Phialocephala victorinii*, previously observed as a root endophyte in some populations of *C. parviflorum* (Vujanovic *et al*. 2000), although the phylogenetic relationships among *Phialophora* and *Phialocephala* species and their ecological functions are not clear (Jacobs *et al*. 2003).

Tulasnelloid fungi may serve to transfer carbon to the mature *Cypripedium* plant. Ectomycorrhizal members of the Tulasnellaceae have been found to transfer carbon from birch seedlings to the nonphotosynthetic liverwort *Cryptothallus mirabilis* (Bidartondo *et al*. 2003). Even some sebacinoids are ectomycorrhizal (Warcup 1988; Glen *et al*. 2002; Selosse *et al*. 2002a; Urban *et al*. 2003; Weiß *et al*. 2004), suggesting that even *C. californicum* may always associate with fungi capable of contributing carbon. ^{13}C and ^{15}N isotope ratios assessed in green orchids in comparison to their achlorophyllous counterparts suggest that some green orchids obtain some of their carbon from ectomycorrhizal sources (Gebauer & Meyer 2003; Bidartondo *et al*. 2004).

Specificity and distribution

Cypripedium mycorrhizal specificity appears generally high. We suggest, however, that habitat characteristics may be as important limiters of *Cypripedium* distribution as mycorrhizal fungi. *C. californicum*, for example, is not only the least mycorrhizal-specific orchid in this study, but the only one limited to serpentine sites with standing or running water (Coleman 1995; Cribb 1997). Associations with multiple families of fungi suggest that *C. californicum* species may switch among mycorrhizal fungal families relatively easily.

Cypripedium montanum and *Cypripedium fasciculatum*, well-sampled species that associated only with the Tulasnellaceae, may indeed be limited by the distribution of their fungal partners. *C. candidum* associated with only a subset of the fungi associating with sympatric *C. parviflorum*, and was rarer in these sites (the former associating with clade snLT2, and the latter with both clades snLT1 and 2; Fig. 2). Even common and widespread temperate, terrestrial orchids, such as *Goodyera repens*, are thought to be fungus-limited (Rasmussen & Whigham 1994). Many *Tulasnella* species are considered rare enough to warrant inclusion on European Red Lists (Arnolds & de Vries 1993), lending support to the suggestion that mycorrhizae are likely to be important factors affecting *Cypripedium* distribution, although this fungal genus has been largely ignored and may be more common than thought. However, unlike other *Cypripedium* species, *C. calceolus*, *C. guttatum* and *C. montanum* were only sampled in one region each. Furthermore, the former two species are represented by symbionts from only two plants each (Table 1). Thus, the high specificity that we report for these taxa may be artefactual.

C. californicum's lack of specificity appears to be anomalous. Given that *C. californicum* is generally limited to wet, serpentine sites, the toxicity of serpentine sites and the ability of some mycorrhizal fungi to reduce plant uptake of toxic elements (Joner *et al*. 2000; Meharg & Cairney 2000) may result in different mycorrhizal needs for the plant, perhaps even resulting in a need for a greater breadth of fungi. Such interactions between stratum and specificity have been explored in epiphytic orchids by Otero *et al*. (2004), but have not been well-explored in terrestrial orchids.

Patterns of association and specificity closely resemble previously reported results for other orchids, in that specificity is often, although not always, high. In a study of three varieties of the nonphotosynthetic orchid *Hexalectris spicata*, the primary association was with the Sebacinaceae, although a ceratobasidioid fungus was also found associated with one variety (Taylor *et al*. 2003). *Dactylorhiza majalis* associates primarily with the Tulasnellaceae, but also forms occasional associations with members of the genus *Laccaria*, a rather unrelated group (Kristiansen *et al*. 2001). McCormick *et al*. (2004) found a range in specificity among unrelated photosynthetic orchids, each with a different ecology, and inferred a lack of correspondence to any ecological characteristics. Otero *et al*. (2002) and Otero *et al*. (2004), in examining tropical, epiphytic orchids, found some, like *Tolumnia variegata*, to be generalists, while others, including *Ionopsis utricularioides*, a close relative of

Tolumnia variegata, exhibited evidence of high specificity. Our results also concur with those of other green orchids, including *Caladenia*, *Dendrobium*, and *Pterostylis* (Warcup 1981), and *Liparis* (McCormick *et al*. 2000).

Conclusion

Mycorrhizal dynamics in photosynthetic orchids have long been controversial. *In vitro* and *in situ* approaches to mycorrhizal specificity often lead to conflicting results (Masuhara & Katsuya 1994), and isolations are inherently biased by choice of and response to growth medium (Allen *et al*. 2003; McCormick *et al*. 2004). The growing consensus on mycorrhizal specificity in mature, green orchids seems to be that they may run the gamut of specialization. Some widespread green orchids are less specific and associate with a wider array of fungal species than nonphotosynthetic orchids, but others are more specific (McCormick *et al*. 2000). The genus, *Cypripedium* appears to be predominantly specialist, although *Cypripedium californicum* appears to associate with three fungal families. However, our inference was limited by the difficult life histories of these plants, which precludes laboratory experimentation with mature individuals.

Because mycorrhizal specificity does not correspond with geographical distribution (i.e. no gradient in specificity correlates with geographical extent across species), we must ask what this gradient in specificity means ecologically. The possible influence of habitat on the distribution of *C. californicum* suggests that nutrient conditions and perhaps the presence of toxic elements may play a large role. Furthermore, some green orchids exhibit nitrogen and carbon stable-isotope ratios suggesting that fungal carbon may provide them with energy throughout their lives (Bidartondo *et al*. 2004), which proposes that carbon requirements, or other nutrient requirements related to mycorrhizal fungi, may govern mycorrhizal associations in *Cypripedium*. In particular, although *Cypripedium* plants are photosynthetic and do not appear to need external carbon sources, do times of adult dormancy translate to times of mycoheterotrophy (Lesica & Steele 1994; Rasmussen 1995; Shefferson *et al*. 2001; Kull 2002; Shefferson *et al*. 2003)? Further research should elaborate on these possible connections between mycorrhizal association, habitat conditions, life history stages, and demographic trends.

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This research was conducted as part of the Ph.D. dissertation of Richard Shefferson, and is part of a collaborative effort to determine the causal agents of and factors affecting adult dormancy in geophytes. Michael Weiß focuses on the systematics and phylogenetics of basal hymenomycetes. Tiiu Kull studies the population ecology of rare plants, particularly terrestrial orchids. D. Lee Taylor studies microbial-plant symbioses, both mycorrhizal and rhizobial.
