# Mycorrhizal diversity in photosynthetic terrestrial orchids

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## Summary

- Specific orchid–fungal associations are known for nonphotosynthetic orchids but fungal diversity in photosynthetic orchids is thought to be quite broad. Specific fungal associations will figure prominently in conservation efforts, while diverse associations may require less attention. We combined culture techniques with ITS and mtLSU sequences and phylogenetic analysis to determine the genetic diversity of mycorrhizal fungi associated with an evergreen, a spring-green, and a winter-green orchid and compared this diversity with that published for a nonphotosynthetic orchid.
- Mycorrhizal diversity in two of the three photosynthetic orchids was lower than for the nonphotosynthetic orchid. Mycorrhizal diversity in protocorms of the third species was also equal to, or less than, the fungal diversity associated with the nonphotosynthetic species, but adult fungal diversity was greater.
- We found that photosynthetic orchids do not necessarily have more diverse mycorrhizal associations than nonphotosynthetic orchids. Similarly, evergreen orchids do not necessarily have greater mycorrhizal diversity than seasonally green orchids. Thus, orchid mycorrhizal diversity may not be determined by adult photosynthetic capacity.

**Key words:** *Cephalanthera austinae*, *Goodyera pubescens*, *Liparis lilifolia*, *Tipularia discolor*, Orchidaceae, mycorrhizal diversity.

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## Introduction

The Orchidaceae is one of the largest plant families on Earth, including almost 10% of all flowering plant species (Dressler, 1981). Members of this diverse family grow in a wide range of habitats and have a substantial variety of life history strategies ranging from epiphytic to terrestrial, and from evergreen to nongreen species. The orchid family's unique characteristics and much of its diversity may be attributable to its distinctive relationship with mycorrhizal fungi (Burgeff, 1909; Benzing, 1981; Zettler *et al.*, 2004). The initial developmental stage of all orchids is a nonphotosynthetic protocorm that is myco-heterotrophic (Alexander & Hadley, 1985; Leake, 1994; Rasmussen, 1995; Rasmussen & Whigham, 2002). The role of fungal associates in mature orchids is poorly understood and little is known about what role fungal diversity plays in affecting an orchid's distribution, population size, and genetic diversity.

For orchids that require specific fungi, availability of appropriate symbionts may determine which habitats allow orchid growth and what environmental factors are critical for orchid recruitment. Specificity, as we use it in this study, refers to genetic sequence diversity interpreted in relative phylogenetic terms. Such specificity may be absolute where an orchid species can only associate with a single fungal clade, or it may be ecological, where the fungi needed to support orchid growth differ among environments.

There have been relatively few studies of nonphotosynthetic orchids (Warcup, 1981a; Taylor & Bruns, 1997, 1999a; McKendrick *et al.*, 2000). However, each nonphotosynthetic species studied has associated with only a few closely related fungi in a single genus (Taylor & Bruns, 1997, 1999; McKendrick *et al.*, 2000, 2002; Taylor *et al.*, 2002). In one case, Taylor & Bruns (1999a) found that two species of *Corallorhiza* growing sympatrically each associated with several *Russula* spp., but they never shared fungal species, demonstrating higher than expected specificity in both orchids.

The use of molecular techniques to identify fungal associates has dominated the study of fungi associated with nonphotosynthetic orchids, largely because fungal associates of these plants are generally difficult or impossible to isolate axenically, often belonging to the Russulaceae and Thelephoraceae. Similarly, researchers studying other myco-heterotrophic plants

have also used molecular techniques and found substantial fungal specificity (e.g. Bidartondo & Bruns, 2001; Bidartondo *et al.*, 2002, 2003). As a result, the mycorrhizal specificity of nonphotosynthetic orchids has been attributed to their life-long myco-heterotrophism and the studies of a few species have been generalized to represent most nonphotosynthetic orchids.

Theories about orchid fungal diversity typically divide orchids into groups based on their photosynthetic ability (Taylor *et al.*, 2002; Zettler *et al.*, 2004) and ecology (epiphytic vs terrestrial). All orchids are initially myco-heterotrophic (Leake, 1994) but most eventually produce leaves and become photosynthetic. Most studies of orchid fungal associations have focused on mature terrestrial photosynthetic orchids (Burgeff, 1909; Benzing, 1981; Hadley, 1985; Richardson *et al.*, 1993; Otero *et al.*, 2002). These studies have often identified considerable breadth among the fungi associated with individual species (e.g. Hadley & Pegg, 1989; Bayman *et al.*, 1997; Zelmer & Currah, 1997; Zettler *et al.*, 2004). Some early researchers felt that fungal specificity was lacking in most photosynthetic orchids because their seeds regularly germinated in the laboratory with a wide range of fungi (Knudson, 1922; Curtis, 1939). It was suggested that fungal specificity found in some photosynthetic orchids could be a function of the fungi that are available in the narrow range of habitats where the orchids occurred (ecological specificity; Perkins & McGee, 1995) and might not reflect absolute specificity in fungal associations (Curtis, 1939). This view of fungal specificity in photosynthetic orchids is not limited to early researchers. More recently, Zelmer *et al.* (1996) identified multiple fungal genera in 9 of 14 photosynthetic orchids from which they obtained fungal cultures.

However, some researchers have found specific fungal associations in photosynthetic orchids. Warcup (1971, 1973, 1981b) found at least genus-level specificity in the fungal associates of several photosynthetic Australian orchids. Perkins *et al.* (1995) found that fungal associates of *Microtis parviflora* included only two fungi and Masuhara & Katsuya (1994) found that a single fungus dominated roots of adult *Spiranthes sinensis*.

By contrast to fungi associated with nonphotosynthetic orchids, fungi associated with photosynthetic orchids are often easy to culture. Perhaps for this reason, molecular techniques have rarely been used (Kristiansen *et al.*, 2001; Otero *et al.*, 2002). However, these culturable fungi often remain asexual in culture and lack many features used to identify and differentiate fungi (Warcup, 1981b). Because they lack many informative features, orchid mycorrhizae are often characterized as belonging to one of several form genera: *Epulorhiza*, *Ceratorhiza*, and *Moniliopsis*. Even characterization at this level has been questionable (Warcup, 1981b; Zettler *et al.*, 2004). On the rare occasions where orchid fungi have been induced to produce reproductive structures in laboratory conditions and identified beyond form genus, fungal associates have been found to include at least five teleomorph genera (*Ceratobasidium*, *Oliveonia*, *Sebacina*, *Thanatephorus*, and *Tulasnella*) as well as members of several other genera of Basidiomycete fungi (Currah *et al.*, 1997; Taylor *et al.*, 2003; Zettler *et al.*, 2004). However, identification of fungi beyond form genus and determination of fungal diversity generally requires DNA-based molecular techniques (Kristiansen *et al.*, 2001; Otero *et al.*, 2002).

Fungal specificity in photosynthetic orchids remains contentious (Otero *et al.*, 2002). The source of much of this contention can be traced to differences in culture techniques and difficulties in identifying resulting cultures. Cultural techniques have differed substantially among studies. Some studies have included fungi associated with orchid roots both internally and externally, while others included only fungi that form pelotons within orchid root cells. Depending on how fungi are cultured, functional mycorrhizae may not be reliably distinguished from surface contaminants and pathogens. When a target fungus is not culturable or difficult to culture, the chance of mistaking a contaminant for a mycorrhizal fungus may be high even in peloton-based cultures.

One attempt to distinguish functional, culturable mycorrhizal fungi from opportunistic contaminant soil fungi has been to assume that all appropriate symbionts must support seed germination (e.g. Burgeff, 1909). However, this too is problematic. Depending on the media chosen, germination in the laboratory may be supported by a much greater range of fungi than are found associated with field plants (e.g. Masuhara & Katsuya, 1994; Perkins *et al.*, 1995; Rasmussen, 2002) or germination may be prevented with fungi consistently found associated with field protocorms (McCormick, personal observation). Germination is especially unlikely *in vitro* when peloton-forming fungi are ectomycorrhizal and grow slowly, if at all, in the absence of a photosynthetic host plant (McCormick, unpublished data). Also, fungi associated with photosynthetic adults may not always include fungi found associated with conspecific protocorms (e.g. Clements, 1988; Zelmer *et al.*, 1996; Peterson *et al.*, 1998; Salman *et al.*, 2002). This germination criterion precludes the possibility of fungal succession as the orchid develops beyond complete fungal dependence (Zelmer *et al.*, 1996).

DNA-based techniques have the advantage of allowing direct identification of the dominant fungi contained within an orchid root (generally the peloton-forming fungi) and are not limited by culturability or the presence of contaminants that may outgrow orchid mycorrhizal fungi. Combined with culture-based studies, which allow in-depth studies of isolated fungi, DNA-based identification of fungi associated with photosynthetic and nonphotosynthetic orchids can go a long way towards settling the debate about orchid fungal specificity and its causes. We have used a combination of culture and DNA-based techniques to determine the fungal specificity of three co-occurring photosynthetic orchids at both protocorm and adult stages. We have also conducted a germination study with two of these species to determine whether specificity measured on naturally occurring plants (ecological specificity) reflects absolute fungal specificity at the protocorm stage.

### Study species

*Goodyera pubescens* R.Br is an evergreen orchid that is found primarily in mid and late successional forests throughout the eastern USA. New leaves are produced primarily in the spring and autumn, and flowering occurs in mid-summer. Plants spread clonally through the branching of rhizomes that eventually degrade, leaving independent ramets. Pelotons of mycorrhizal fungi are present year-round in older roots and appear in new roots shortly after they are produced (Rasmussen & Whigham, 2002).

*Liparis lilifolia* A. Rich ex Lindl. is a spring-green orchid, producing one or two large green leaves in early spring. It is a common orchid in early successional forests throughout the eastern USA. Flowers are produced in mid-spring and fruits mature through the summer. After senescing in the autumn, seeds are shed and one or occasionally two corm offshoots are produced, from which the next year's leaves will be produced. After a new shoot is produced the old corm degrades (Whigham & O'Neill, 1991). Pelotons are present year-round at the base of the corm near the points of root attachment. Roots are very thin and do not contain pelotons (Rasmussen & Whigham, 2002).

*Tipularia discolor* Nutt. is a winter-green orchid, producing a single leaf in late autumn. Leaves range in colour from green to purple, often with purple or white spots. It is a common orchid in forests of all ages throughout the eastern and southern USA (Whigham & O'Neill, 1991). Protocorms of *T. discolor* are consistently associated with decomposing wood, often occurring on fallen trees and stumps (Rasmussen & Whigham, 1998). Leaves senesce in early spring and each mature corm produces a single flower stalk in late summer when no leaves are present. New corms form at the base of new leaves in late autumn. Only new corms produce leaves, but several old corms may remain attached to new corms. Plants reproduce clonally by producing two new shoots rather than one (Whigham & O'Neill, 1991).

*Cephalanthera austinae* (A. Gray) Heller is a nonphotosynthetic orchid that produces a white flowering stalk with white flowers during mid summer. It is uncommon and limited to deep coniferous forests of the Pacific Northwest from southern British Columbia through California.

In this paper, we examine fungi associated with three co-occurring green orchids; two seasonally photosynthetic orchids, one spring-green and one winter-green, an evergreen species, and a nonphotosynthetic species. Our goal was to use molecular techniques to evaluate the genetic diversity of fungi associated with adults and protocorms of species that have similar geographic ranges and also grow in similar habitats. We then compared fungal sequence diversity in three photosynthetic species to sequence diversity found in *Cephalanthera austinae* by Taylor & Bruns (1997). Our hypothesis was that if fungal specificity was a reflection of adult photosynthetic capacity then fungal specificity would be lower in the evergreen species than in the seasonally green species. We also expected that the three photosynthetic species would have lower fungal specificity than the nonphotosynthetic species. To augment our molecular approach to identification of appropriate fungal symbionts, we also examine the utility of a germination requirement in two species that are amenable to germination in the laboratory.

## Methods

### Sample collection

We collected roots of *G. pubescens* and *T. discolor* and corms of *L. lilifolia* from throughout their ranges in the eastern and mid-western USA (Table 1). Sampling for all species occurred between January 1997 and November 2001 during the season when plants had leaves and/or reproductive stalks aboveground. We evaluated intra-habitat diversity of peloton-forming fungi by sampling fungi associated with adults and protocorms at the Smithsonian Environmental Research Center (SERC, Edgewater, Maryland, USA). Samples at SERC included multiple populations of each orchid, and naturally occurring protocorms and seedlings of *T. discolor*. We sampled additional protocorms of *G. pubescens*, and *L. lilifolia* from seed packets (Rasmussen & Whigham, 1993) placed in multiple field sites adjacent to, and distant from, existing populations at SERC. For comparison, fungi associated with each species were also collected from adults at other locations within the geographic range of the species.

Within 1 wk of collection, we isolated fungal pelotons following Rasmussen (1995) and plated them individually on modified E-medium (Caldwell *et al.*, 1991) with 50 mg Novobiocin $l^{-1}$. We also preserved aliquots of approx. 20 pelotons each and remaining pieces of infected plant tissue by freezing at −20°C in Eppendorf tubes. These preserved samples were used for DNA analysis of *T. discolor* protocorm fungi, which we were repeatedly unable to culture axenically. For each isolation from *G. pubescens*, and *L. lilifolia*, we arbitrarily chose one growing peloton from each plant to transfer to liquid culture (E-medium without agar or Novobiocin). Only one peloton was sampled from these species because intersimple sequence repeat (ISSR) analysis (Zhou *et al.*, 1999) demonstrated that we never recovered more than one fungal individual from a single plant (McCormick, unpublished data). For *T. discolor*, ISSR analysis demonstrated that multiple fungal individuals were commonly present and could be easily distinguished morphologically (McCormick, unpublished data). Therefore, in *T. discolor* one fungus of each morphotype was transferred to liquid culture. We subsampled fungi from liquid culture and rinsed them in sterile water prior to extracting DNA.

### DNA extraction

We extracted DNA from each cultured fungus or bulked fungal pelotons (approx. 20 pelotons) using a CTAB (cetyltrimethylammonium bromide) extraction modified

**Table 1** Information about fungal isolates included in this study: fungal isolate numbers, the orchid from which they were isolated, developmental stage of the host plant, its sampling location and GenBank accession numbers of ITS or mtLSU sequences reported here

| Isolate | Orchid | Stage | Sampling Location | mtLSU | ITS |
|---|---|---|---|---|---|
| 101 | *Goodyera* | Adult | SERC population TF, MD | AY382794 | AY373266 |
| 109 | *Goodyera* | Adult | SERC population TF, MD | | AY373263 |
| 120 | *Liparis* | Adult | SERC population FP, MD | | AY310910 |
| 128 | *Tipularia* | Adult | SERC population T, MD | AY382806 | AY373300 |
| 140 | *Liparis* | Adult | SERC population CO, MD | | AY373281 |
| 141 | *Goodyera* | Adult | SERC population CO, MD | | AY373264 |
| 144 | *Goodyera* | Protocorm | SERC seed packet TF, MD | | AY373265 |
| 145 | *Goodyera* | Protocorm | SERC seed packetT, MD | | AY373276 |
| 146 | *Goodyera* | Protocorm | SERC seed packet FC, MD | | AY373274 |
| 148 | *Goodyera* | Protocorm | SERC seed packet T, MD | | AY373267 |
| 149 | *Goodyera* | Protocorm | SERC seed packet FC, MD | | AY373273 |
| 166 | *Liparis* | Protocorm | SERC seed packet T, MD | AY382803 | AY373280 |
| 169 | *Tipularia* | Adult | SERC population TF, MD | AY382795 | AY373301 |
| 170 | *Tipularia* | Adult | SERC same plant as 169 | AY382807 | AY373302 |
| 175 | *Tipularia* | Adult | SERC population BC, MD | | AY373304 |
| 179 | *Goodyera* | Adult | SERC population CO, MD | | AY373275 |
| 181 | *Tipularia* | Adult | SERC population CO, MD | | AY373305 |
| 184 | *Tipularia* | Adult | SERC population WT, MD | | AY373306 |
| 185 | *Tipularia* | Adult | SERC population BC, MD | AY382800 | |
| 186 | *Tipularia* | Adult | SERC population CO, MD | AY382808 | AY373307 |
| 188 | *Tipularia* | Adult | SERC population CO, MD | | AY373308 |
| 191 | *Tipularia* | Adult | SERC population FP, MD | | AY373299 |
| 192 | *Tipularia* | Adult | SERC population FP, MD | | AY373309 |
| 193 | *Liparis* | Adult | Kellogg Experimental Forest, MI | AY382802 | AY373283 |
| 196 | *Liparis* | Adult | SERC population CO, MD | | AY373284 |
| 197 | *Liparis* | Adult | SERC population FP, MD | | AY373285 |
| 213-1 | *Tipularia* | Adult | SERC population FP, MD | | AY373310 |
| 213-2 | *Tipularia* | Adult | SERC population FP, MD | | AY373311 |
| 218 | *Tipularia* | Adult | SERC population CO, MD | | AY373312 |
| 219 | *Tipularia* | Adult | SERC population CO, MD | | AY373313 |
| 230 | *Goodyera* | Adult | Barry State Game Area, MI | AY382796 | AY373268 |
| 233 | *Liparis* | Adult | Lee County, IA | | AY373287 |
| 234 | *Liparis* | Adult | Bear Gardens, VA | | AY373286 |
| 238 | *Liparis* | Adult | Johnson County, IA | | AY373288 |
| 239 | *Goodyera* | Adult | Coweeta Hydrologic Laboratory, NC | | AY373269 |
| 241 | *Goodyera* | Adult | Coweeta Hydrologic Laboratory, NC | | AY373270 |
| 243 | *Goodyera* | Adult | White Hall Woods, GA | AY382797 | AY373271 |
| 244 | *Goodyera* | Adult | Coweeta Hydrologic Laboratory, NC | | AY373272 |
| 245 | *Goodyera* | Adult | Coweeta Hydrologic Laboratory, NC | | AY373282 |
| 247 | *Goodyera* | Adult | Coweeta Hydrologic Laboratory, NC | | AY373277 |
| 248 | *Goodyera* | Adult | Coweeta Hydrologic Laboratory, NC | | AY373278 |
| 249 | *Goodyera* | Adult | Coweeta Hydrologic Laboratory, NC | | AY373279 |
| 252 | *Tipularia* | Adult | Smithgall Woods, GA | | AY373315 |
| 253 | *Tipularia* | Adult | White Hall Woods, GA | | AY373314 |
| 254 | *Tipularia* | Adult | White Hall Woods, GA | AY382809 | AY373316 |
| 258 | *Liparis* | Adult | Bear Garden, VA | | AY373289 |
| Tp1 | *Tipularia* | Protocorm | SERC population T1 | AY382816 | AY382817 |
| Tpc1 | *Tipularia* | Protocorm | SERC population T2 | | AY382819 |
| Tpc2 | *Tipularia* | Protocorm | SERC population T2 | | AY382818 |
| TpCO2 | *Tipularia* | Protocorm | SERC population CO2 | AY382810 | AY382824 |
| TpCO22 | *Tipularia* | Protocorm | SERC population CO2 | | AY581893 |
| TpCS2 | *Tipularia* | Protocorm | SERC population CS | | AY382823 |
| TSJB1 | *Tipularia* | Seedling | Jug Bay Wetlands Sanctuary, MD | | AY382820 |

Samples without isolate numbers at the end of the table correspond to fungi from *Tipularia* protocorms from which isolations were not attempted, but to which molecular techniques were applied.

from Piercey-Normore & Depriest (2001). The procedure for extracting DNA from plant tissues containing pelotons was the same as for cultured fungi except that 200 µl $l^{-1}$ 2-Mercaptoethanol was added to the extraction buffer.

### DNA amplification

Nuclear DNA from the first and second internal transcribed spacers and the 5.8 s subunit of the ribosome gene (hereafter referred to collectively as ITS) was amplified from each sample of fungal DNA using the eukaryotic universal primer pair ITS4/ITS5 (White *et al.*, 1990). Amplification reactions of 25 µl were carried out with final concentrations of 0.5 µM for each primer and 50% Taq PCR Master Mix (Promega Corporation, Madison, Wisconsin, USA). Samples from *T. discolor* protocorms also included plant DNA so the ITS was amplified using the fungus-specific primer ITS1F (Gardes & Bruns, 1993) in place of ITS5. Amplifications consisted of 35 cycles in an MJ Research DNA Engine 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 54°C and elongation for 1 min at 72°C. Negative control reactions without template DNA were performed with each set of amplifications.

To determine relationships between distantly related groups of fungi, we also amplified the CML7.5/MLIN3 (Bruns *et al.*, 1998) region of the mitochondrial large subunit from two fungi within each of the groups determined by ITS sequences. For *T. discolor* protocorms, attempts to amplify the mtLSU were unsuccessful so we amplified part of the nrLSU using the primer pair ITS1F/LR21 (Tedersoo *et al.*, 2003). Amplification reactions were carried out as for ITS. Amplification of this region consisted of a 1 min initial denaturation at 95°C prior to thermocycling, followed by a 1 min denaturation at 95°C, a 1 min annealing at 57°C, and 3 min elongation at 72°C for each cycle. The final cycle was followed by 7 min extension at 72°C.

For each sample PCR products from two 25 µl reactions were combined and cleaned using Wizard PCR preps (Promega Corporation, Madison, Wisconsin, USA). Cleaned samples were eluted with 50 µl sterile water and quantified in preparation for conducting sequencing reactions. Sequencing was carried out using automated sequencing methodology of the ABI Prism Terminator Cycle Sequencing Ready Reaction Kit (original dyes with Amplitaq DNA Polymerase, Perkin Elmer, Foster City, California, USA) at half reaction volumes. Products were cleaned in Sephadex G-50 (fine) Centri-Sep spin columns (Princeton Separations, Adelphia, New Jersey, USA). Samples were dried under vacuum and run on an ABI 377 (Applied Biosystems, Foster City, California, USA) autosequencer at the Smithsonian Institution's Laboratory of Molecular Systematics (Suitland, Maryland, USA). Sequencher (v.3.1) was used to combine forward and backward sequences and multiple sequences were manually aligned in Se-Al version 1.0 (Rambaut, 1996). Alignments were visually examined and manually optimized. New ITS and mtLSU sequences from this study have been deposited in GenBank under the accession numbers listed in Table 1.

### Analyses

All analyses were conducted in PAUP* 4.11 (beta test version, Swofford, 2002). Topology was determined by maximum parsimony analysis for the ITS sequences. Robustness of clades was estimated by bootstrap analysis (Felsenstein, 1985). Bootstrap analysis used 100 random addition replicates with TBR branch swapping. Neighbour-joining analysis was also used to generate trees with qualitatively identical results. Because the ITS region was extremely variable to the point where long branch attraction was a serious concern in determining relationships among distant groups, relationships among major clades were also examined using only the less variable 5.8S gene of the nuclear ribosomal repeat and ML5/ML6 region of the mitochondrial large subunit. The ML5/ML6 and 5.8S sequences were then compared with sequences in GenBank through a BLAST search. Each analysis (ITS and mtLSU) included sequences of several known taxa for comparison (Table 2).

### Germination tests

To determine whether fungi isolated from adult orchids were also able to support germination, we tested an arbitrary selection of genetically distinct fungi from each of the major groups of fungi obtained from co-occurring orchids, all of which belonged to the genus *Tulasnella*, for their ability to support germination of *L. lilifolia*, *G. pubescens*, and *T. discolor* seeds *in vitro*. We used only *Tulasnella* spp. because the only non-*Tulasnella* fungi we identified were not culturable axenically. We conducted these tests of germination on lean wood media (2 g $l^{-1}$ ground *Liriodendron tulipifera* wood with 12 g $l^{-1}$ agar), which closely approximates germination in field conditions (i.e. germination occurs at similar rates and only with fungi we find associated with conspecific adults and protocorms in the field; Whigham *et al.*, 2002). We measured percent germination (defined as production of the first rhizoids) and protocorm growth 12 wk after inoculation with the fungi being tested. We have found that little germination occurs after 12 wk and protocorm growth at 12 wk is indicative of subsequent growth.

## Results

### Fungal identity and diversity

The ITS alignment for tulasnelloid fungi (Fig. 1) contained 557 parsimony-informative characters of 987 aligned characters. Based on ITS sequences, fungal associates of *G. pubescens* all belonged to the genus *Tulasnella*. These isolates likely represented

**Table 2** Identified fungi used to construct phylogenetic trees

| Fungal Species | mtLSU | ITS |
|---|---|---|
| *Albatrellus confluens*[9] | | AF506393 |
| *Albatrellus ellisii*[7] | AD001539 | |
| *Albatrellus peckianus*[7] | AD001541 | |
| *Amanita muscaria*[7] | AD001549 | |
| *Auricularia auricula-judae*[10,11] | AF393090 | AF291268 |
| *Auricularia fuscosuccinea*[7] | | AF291270 |
| *Boletus satanas*[7] | AD001566 | |
| *Botryobasidium subcoronatum*[10] | | AF393048 |
| *Cantharellus cinnabarinus*[7] | AD001574 | |
| *Ceratorhiza goodyerae-repentis*[1] | AF345556 | |
| *Coniophora arida*[7] | AD001579 | |
| *Exidia glandulosa*[12] | | AY509555 |
| *Exidiopsis* sp. RJB1182112 | | AY509549 |
| *Gyrodon merulioides*[7] | AD001590 | |
| *Hebeloma crustuliniforme*[7] | AD001592 | |
| *Heterobasidion annosum*[7] | AD001593 | |
| *Heterochaetella brachyspora*[12] | | AY509552 |
| *Hygrophoropsis aurantiaca*[7] | AD001595 | |
| *Hygrophorus sordidus*[7] | AD001597 | |
| *Laccaria laccata*[7] | AD001602 | |
| *Lactarius volemus*[7] | AD001604 | |
| *Leccinum manzanitae*[7] | AD001606 | |
| *Paxillus involutus*[7] | AD001615 | |
| *Phylloporus rhodoxanthus*[7] | AD001618 | |
| *Pisolithus arhizus*[7] | AD001620 | |
| *Pseudohydnum gelatinosum*[13] | | AF384861 |
| *Ramaria araiospora*[7] | AD001624 | |
| *Rhizopogon vinicolor*[7] | AD001631 | |
| *Russula brevipes*[8] | AF156913 | |
| *Sebacina* sp.[7] | AD001635 | |
| *Serpula incrassata*[7] | AD001639 | |
| *Suillus tomentosus*[7] | AD001644 | |
| Thelephoraceae Taylor #3[5] | | U83466 |
| Thelephoraceae Taylor #2[5] | | U83467 |
| Thelephoraceae Taylor #4[5] | | U83468 |
| Thelephoraceae Taylor #10[5] | | U83469 |
| Thelephoraceae Taylor #6[5] | | U83470 |
| Thelephoraceae Taylor #9[5] | | U83471 |
| Thelephoraceae Taylor #5[5] | | U83472 |
| Thelephoraceae Taylor #1[5] | | U83473 |
| Thelephoraceae Taylor #14[5] | | U83474 |
| Thelephoraceae Taylor #11[5] | | U83475 |
| Thelephoraceae Taylor #7[5] | | U83476 |
| Thelephoraceae Taylor #12[5] | | U83477 |
| Thelephoraceae Taylor #13[5] | | U83478 |
| Thelephoraceae Taylor #8[5] | | U83479 |
| *Thelephora terrestris*[6] | AY010258 | |
| *Tomentella atrorenicolor*[3] | | AJ421254 |
| *Tomentella badia*[2] | | AF272937 |
| *Tomentella* cf. *coerulea*[4] | AY010274 | AY010259 |
| *Tomentella fusco-cinera*[2] | | AF272942 |
| *Tomentella lapidum*[2] | | AF272941 |
| *Tomentella galzanii*[3] | | AJ421255 |
| *Tomentella lateritia*[2] | | AF272941 |
| *Tomentella pilosa*[3] | | AJ421252 |
| *Tomentella punicea*[3] | | AF272943 |
| *Tomentella* sp.1 | | AY382821 |
| *Tomentella stuposa*[4] | | AY010277 |
| *Tomentella subclavigera*[6] | AY010260 | |
| *Tomentella subtestacea*[3] | | AJ421256 |

**Table 2** *continued*

| Fungal Species | mtLSU | ITS |
|---|---|---|
| *Tomentella terrestris*[2] | | AF272911 |
| *Tomentella umbrinospora*[2] | | AF272920 |
| *Tomentellopsis zygodesmoides*[14] | | AJ410761 |
| *Trechispora kavinioides*[15] | | AF347086 |
| *Tricholoma manzanitae*[7] | AD001653 | |
| *Tulasnella albida* KC110 | AY382804 | AY373294 |
| *Tulasnella bifrons* BPI724849 | | AY373290 |
| *Tulasnella deliquescens* DAOM47.8 (= *T. calospora*) | AY382801 | AY373291 |
| *Rhizoctonia repens* CBS326.47 (= *T. calospora*) | AY382798 | AY373298 |
| *Tulasnella danica* KC388 | AY382805 | AY373297 |
| *Tulasnella eichleriana* KC725 | AY382799 | |
| *Tulasnella eichleriana* KC852 | | AY373292 |
| *Tulasnella irregularis*[1] | AF345560 | |
| *Tulasnella pruinosa* DAOM17641 | AY382811 | AY373295 |
| *Tulasnella tomaculum* KC429 | AY382812 | AY373296 |
| *Tulasnella violea* DAOM22200 | AY382815 | AY373303 |
| *Tulasnella violea* KC151 | AY382814 | |
| *Tulasnella violea* KC851 | AY382813 | AY373293 |
| *Waitea circinata*[7] | AD001658 | |
| *Xerocomus chrysenteron*[7] | AD001659 | |

Sources for sequences obtained from GenBank are indicated as follows: [1]Kristiansen *et al*. (2001), [2]Kõljalg *et al*. (2000), [3]Kõljalg *et al*. (2001), [4]Lilleskov *et al*. (2002), [5]Taylor & Bruns (1997), [6]Lilleskov *et al*. (1999), [7]Bruns *et al*. (1998), [8]Taylor & Bruns (1999b), [9]Larsson & Larsson (2003), [10]Binder & Hibbett (2002), [11]Weiss & Oberwinkler (2001), [12]Wells *et al*. (2003), [13]Lim & Berbee (2001), [14]Kõljalg *et al*. (2002), [15]Larsson (2001). No superscript indicates sequences generated in this study.

one or two distinct species, one of which may be *T. bifrons* (Fig. 1). Isolates from within SERC encompassed the full range of genetic variation present across the sampled range from Michigan to Georgia. Fungi obtained from protocorms were not distinct from those obtained from adults.

All isolates from *L. lilifolia* belonged to a single clade of *Tulasnella* that was closely related to fungi from *G. pubescens* (Fig. 1). Genetic variation, even within the extremely variable ITS regions, included only two variable bases across the full sequence length, although each isolate constituted a unique genetic individual based on ISSR banding patterns (McCormick, unpublished data). Again, samples from within SERC included the full range of fungal sequence diversity and fungi from protocorms and adults were indistinguishable (Fig. 1).

Fungi isolated from *T. discolor* spanned a dramatically greater range of variation than those from any of the other orchids we examined. On the basis of ITS sequences, fungi isolated from adult plants appeared to include at least four distinctly different groups of tulasnelloid fungi (Fig. 1). ITS sequences from these four groups of fungi were so different that they were very difficult to align unambiguously, as were the sequences of *Tulasnella tomaculum* KC429, *T. violea* KC851, and *T. eichleriana* KC852, reflecting the substantial ITS genetic diversity within the genus *Tulasnella* (Taylor *et al*., 2003). Isolates from *T. discolor* adults included some fungi closely related to associates of *G. pubescens*, some closely related to associates of *L. lilifolia*, and two other groups of distantly related tulasnelloid fungi. Additionally, protocorm fungi were distinctly different from adult fungi (Fig. 2). A BLAST search of ITS sequences obtained from protocorm fungi revealed no close relatives in the GenBank database (all sequences with < 50% sequence identity). However, when ITS sequences were extended into the nrLSU region a BLAST search identified *T. discolor* protocorm fungi as likely belonging to the Auriculariales (> 90% sequence identity). Attempts to amplify the mtLSU region for these fungi were largely unsuccessful. Of 10 samples, only two produced readable sequences and these were closely related to the genus *Tomentella* (> 90% sequence identity). It seems likely that the mtLSU primers used here, possibly ML6, since it was used in all sequencing attempts in order to exclude plant DNA, do not amplify the primary protocorm fungi and only produced clean sequences for the two samples where additional fungi were present. A phylogeny based on ITS sequences separated the protocorm fungi into two clades. Protocorm fungi were unculturable and their persistence in adults is still unknown, but in preliminary investigations we have found at least some persistence.

Overall, the phylogeny based on ITS sequences was better resolved than the mtLSU phylogeny. However, relationships between fungal clades based on mtLSU sequences were consistent with relationships estimated from ITS sequences. Phylogenetic trees generated from the less variable ML5/ML6 region were similar to trees generated from the full MLIN3/CML7.5 region, but they were less well supported and are not

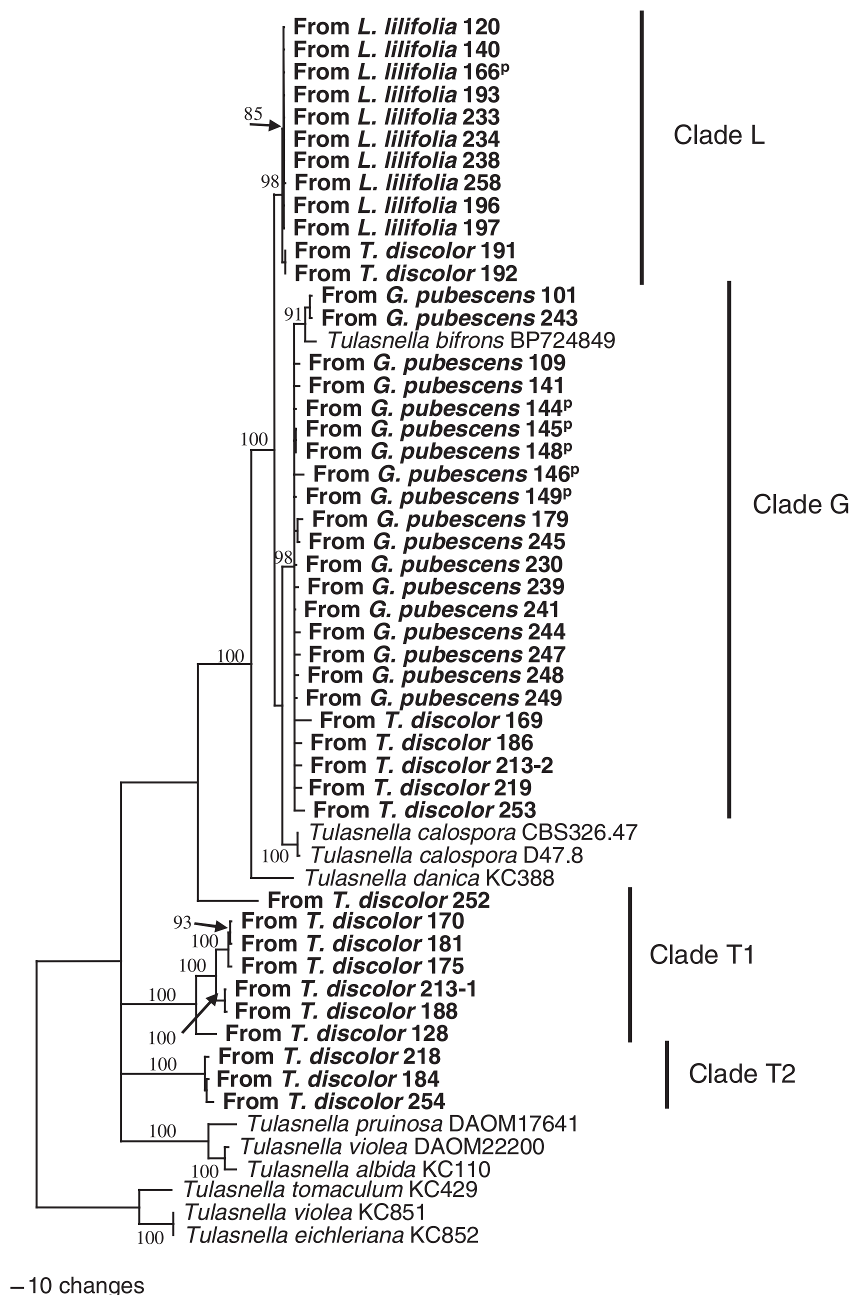


**Fig. 1** Phylogenetic tree constructed from ITS sequences of cultured tulasnelloid fungi isolated from *Goodyera pubescens*, *Liparis lilifolia*, *Tipularia discolor* and identified *Tulasnella* spp. and *Sebacina* spp. p indicates fungi isolated from protocorms. All other fungi were isolated from adult orchids. Bootstrap values > 50 are given above each branch. The tree is rooted with *Tulasnella tomaculum* and *T. eichleriana* as the outgroups based on mtLSU sequences. Clades L, G, T1, and T2 indicate fungi isolated from or indistinguishable from those isolated from *L. lilifolia*, *G. pubescens*, or two groups from adult *T. discolor*, respectively.

presented here. MtLSU sequences from fungi associated with *G. pubescens* and *L. lilifolia* were very similar. MtLSU sequences were most useful in verifying that the four distinct groups of fungi from *T. discolor* all belonged within *Tulasnella* (Fig. 3).

*Goodyera pubescens* and *L. lilifolia* both had a much lower genetic diversity among their fungal associates than was found in *C. austinae*. Sequence variation among *T. discolor* protocorms was similar to the amount of variation among isolates from *C. austinae* (Figs 3 and 4). However, fungi associated with *T. discolor* adults had more diverse sequences than those associated with *G. pubescens*, *L. lilifolia*, or *C. austinae*.

## Germination tests

All fungi isolated from *G. pubescens* adults and protocorms supported seed germination, rhizoid formation, and subsequent biomass accumulation of *G. pubescens* protocorms in the laboratory. Fungi isolated from *T. discolor* adults that were genetically similar to those isolated from *G. pubescens* also supported *G. pubescens* growth (Fig. 5). One *L. lilifolia*-type fungus also supported *G. pubescens* growth and might be compatible. All fungi isolated from *L. lilifolia* adults and protocorms supported growth of *L. lilifolia* protocorms in the laboratory. Of the other fungi tested, only fungi isolated from *T. discolor* adults that were

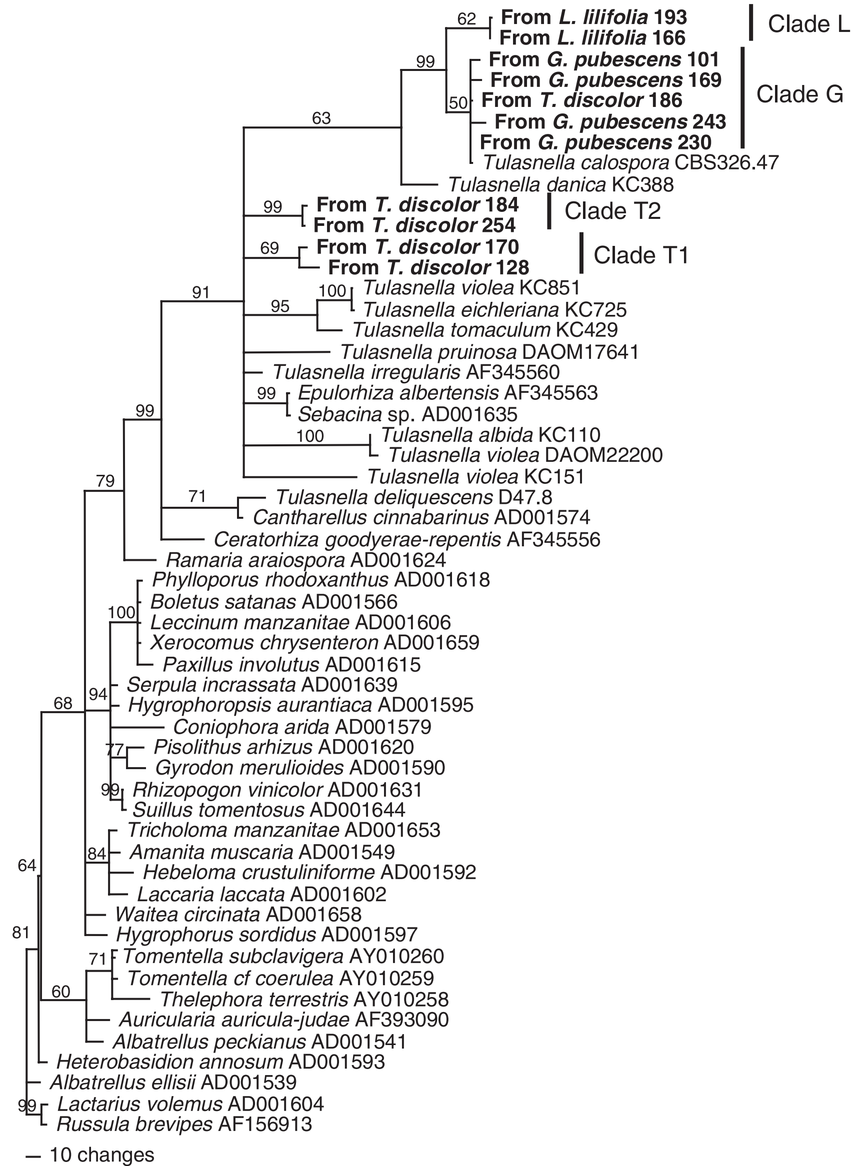


**Fig. 2** Phylogenetic tree constructed from mtLSU sequences of two representative fungi from each of the major tulasnelloid clades identified by ITS sequence data. Bootstrap values > 50 are given above each branch. All sequences are from cultured fungi. The tree includes representative fungi from the major clades identified in Bruns *et al*. (1998) and is rooted with Russulaceae as the outgroup based on Bruns *et al*. (1998).

genetically indistinguishable from *L. lilifolia* isolates supported *L. lilifolia* growth (Fig. 5). No germination was observed for *T. discolor*, probably because protocorm fungi were not axenically culturable and hence were not included in this study. Protocorm fungi from *T. discolor* may require a photosynthetic ectomycorrhizal host or another fungus as a source of carbon.

## Discussion

The ITS sequence diversity of fungi associated with these three photosynthetic terrestrial orchids was lower than or equal to that of fungi associated with the nonphotosynthetic *C. austinae* (at least during the protocorm stage), not greater than as we had predicted. Marked adult fungal specificity was evident in two of the three species we examined. ITS sequence variation among fungal associates was lower in *G. pubescens* and *L. lilifolia* than in the nonphotosynthetic *C. austinae*, and similar to that found within host races of *Corallorhiza maculata* (Taylor *et al.*, 2004). Other nonphotosynthetic orchids also associated with fungi from a single genus but because different regions of DNA were sequenced, the sequence variation found here cannot be quantitatively compared (e.g. *Corallorhiza maculata* (Taylor & Bruns, 1997), *Corallorhiza trifida* (McKendrick *et al.*, 2000), *Corallorhiza maculata* (Taylor *et al.*, 2004)) despite similar or greater geographic distribution and number of plants sampled. Each of these nonphotosynthetic orchids associated with multiple fungi from a single genus, however, *L. lilifolia* associated with a single fungal species and *G. pubescens* likely associated with two species. This extremely low sequence variation among fungi from *L. lilifolia* and *G. pubescens* may indicate greater fungal

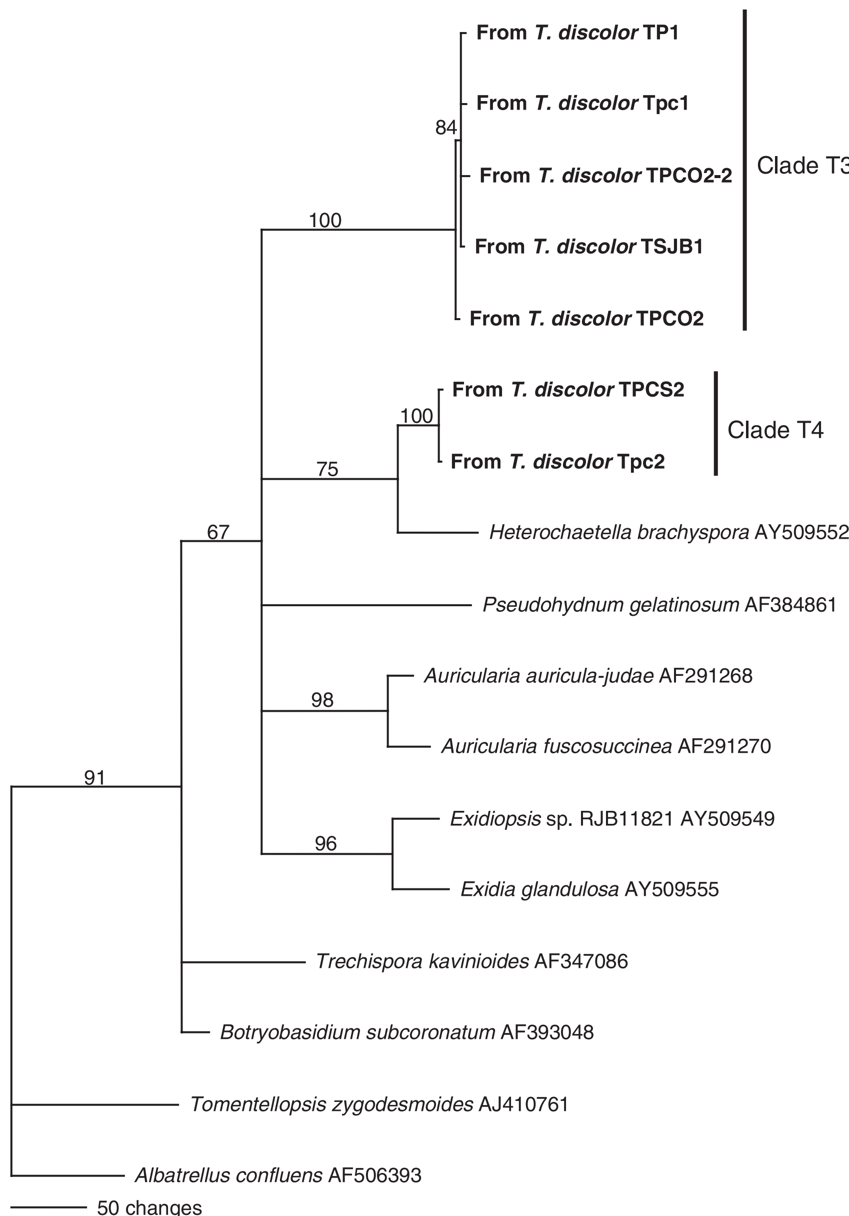


**Fig. 3** Phylogenetic tree constructed from ITS and nrLSU sequences of fungi isolated from *Tipularia discolor* protocorms and published sequences of fungi that were similar to *T. discolor* fungi based on a BLAST search of GenBank. Bootstrap values > 50 are given above each branch. The tree is rooted with *Albatrellus confluens* as an outgroup, based on the mtLSU tree.

specificity relative to the nonphotosynthetic species considered, but such a pattern could also result from differences in accumulation of genetic changes among different groups of fungi.

Fungi from adults and protocorms were not genetically distinguishable in *G. pubescens* and *L. lilifolia* and seed germination on lean wood media was correspondingly only successful with fungi isolated from adults of these species. Only *T. discolor* had a broad range of fungal associates and different adult and protocorm fungi as was predicted for photosynthetic orchids. Sequences among the two clades of *T. discolor* protocorm fungal associates were sufficiently different that they probably include at least two related species, but they included substantially less fungal diversity than was found within even single mature plants. However, a limited number of protocorms were examined for each species and all were from sites at SERC. Future studies will expand protocorm studies to include other sites.

The photosynthetic orchids that we sampled in this study all grow within relatively closed-canopy forests. It is possible that in such a low light environment they have a greater dependence on their fungal associates than species growing in the open, especially when their peak reproductive demands occur during the summer when plants are most heavily shaded (*G. pubescens*) or when they are without leaves (*T. discolor*). However, we have found similar specificity in more limited studies of 15 other photosynthetic terrestrial orchids, most of which grow in the open (McCormick, unpublished data). Indeed, Gebauer & Meyer (2003) used natural abundances of

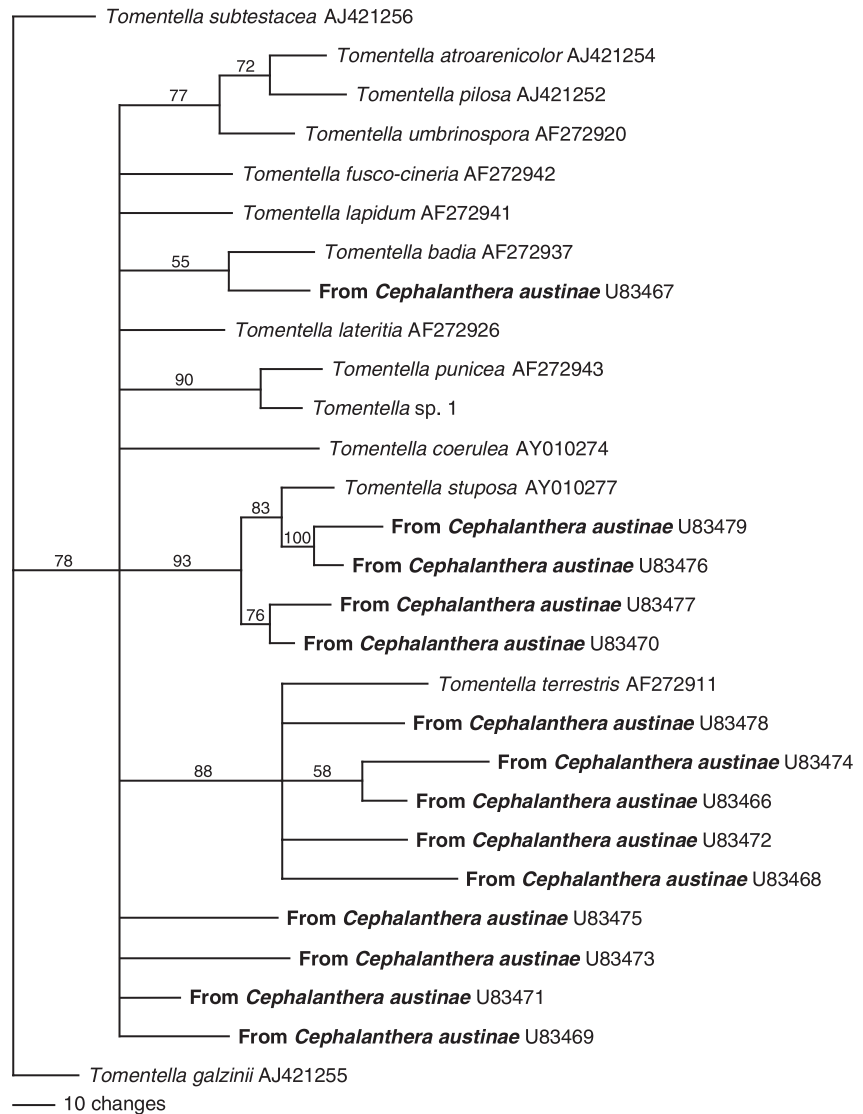


**Fig. 4** Unrooted phylogenetic tree constructed from published ITS sequences of selected thelephoroid fungi and fungi from *Cephalanthera austinae*. Bootstrap values > 50 are given above each branch.

$^{13}C$ and $^{15}N$ to demonstrate that some photosynthetic orchids, both from forests and more open grasslands, depended on fungi as a significant source of carbon and nitrogen.

In each of these orchids, extensive sampling in populations at SERC revealed all major clades of fungal associates. More limited sampling at other sites throughout the species range increased the genotypes within each clade, but did not increase the number of clades. This suggests that orchids within a site are not locally adapted to the fungi present at that location, but are more likely to associate with fungi that are present throughout their range in similar habitats. Similarly, studies of genetic variation within species of orchids have found that the great majority of genetic variation within an orchid species occurred within rather than among populations (e.g. Case, 1993; Smith *et al.*, 2002; McCormick, unpublished data).

Despite the broad range of fungi associated with adult *T. discolor*, fungal diversity in protocorms was much lower. Although *T. discolor* protocorm fungi were assessed in a limited number of samples, ITS sequence diversity was similar to the ITS sequence diversity of the nonphotosynthetic *C. austinae* and that found in the nonphotosynthetic *Corallorhiza odontorhiza* (McCormick, unpublished data), which co-occurs with the photosynthetic orchids sampled here. Each of these photosynthetic orchids had very specific fungal requirements, at least at the nonphotosynthetic protocorm stage. This suggests that the generalization that nonphotosynthetic orchids have specific fungal associations while photosynthetic orchids associate with more diverse fungi, may be unwarranted. The greater fungal specificity of *T. discolor* protocorms indicates the importance of sampling fungi from protocorms in addition to those from adult orchids. Substantial specificity in photosynthetic orchids may be more common than has previously been reported, especially during the protocorm stage. In species where very diverse adult fungal associates have been

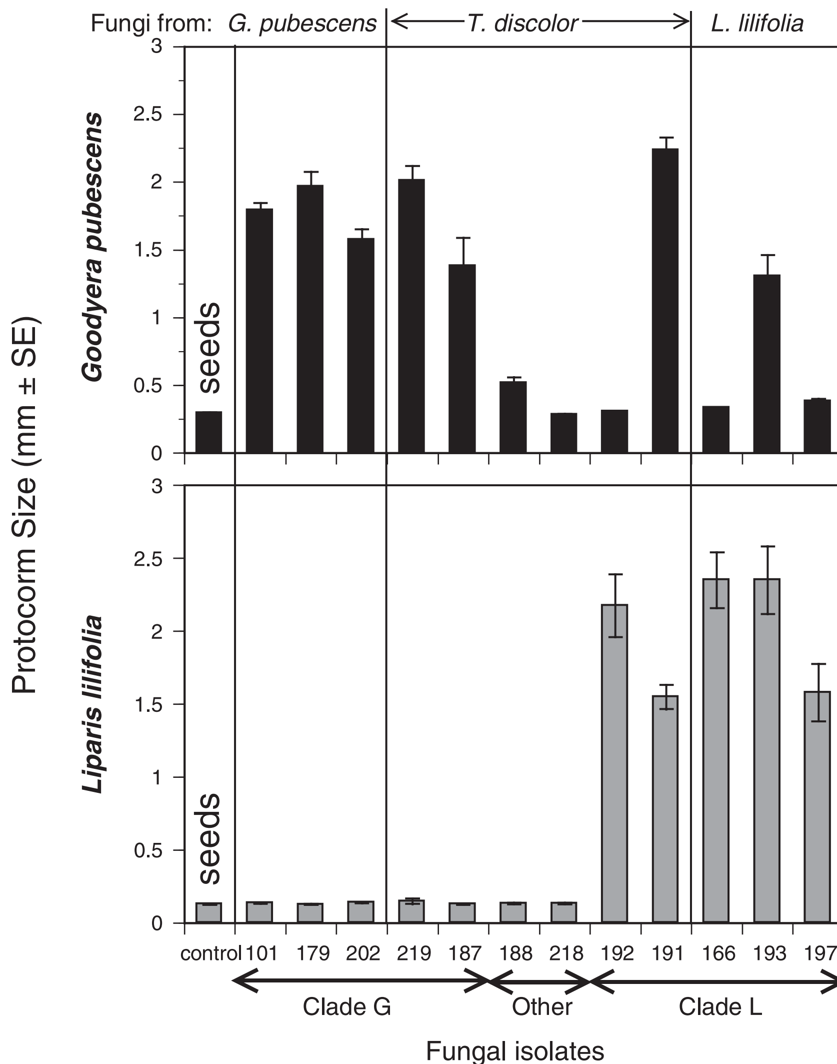


**Fig. 5** Size (mm + 1 SE) of *Goodyera pubescens* (closed bars) and *Liparis lilifolia* (open bars) protocorms after 16 week on lean wood media supported by some Tulasnelloid fungi isolated from *G. pubescens*, *Tipularia discolor*, and *L. lilifolia*. Clade G indicates fungi genetically similar to fungi isolated from *G. pubescens*. Clade L indicates fungi genetically similar to fungi isolated from *L. lilifolia* (see Fig. 1). Other indicates Tulasnelloid fungi belonging to Clades T1 and T2 from *T. discolor* that are distantly related to fungi in Clades G and L (see Fig. 1). Numbers on the *x*-axis refer to isolate numbers in Table 1. The control treatment shows size of seeds without fungi.

found, additional studies focused on the fungal specificity of the protocorm stage may be warranted.

*Tipularia discolor* adults associated with a broad range of culturable tulasnelloid fungi. Because *T. discolor* adults often had multiple fungi within a single root, it was very difficult to obtain single PCR products for direct sequencing without axenic isolation or cloning. As a result, only culturable fungi are represented here. A much greater diversity of fungi is almost certainly present within adult orchids, as is represented by a member of the Russulaceae identified by D.L. Taylor (1997) in a *T. discolor* adult from SERC. It is also likely that fungi associated with protocorms persist in at least some adult orchids. We are currently working to fully assess the range of fungi present in adult *T. discolor*.

All fungi associated with adult *L. lilifolia* and *G. pubescens* were able to support growth of conspecific protocorms on wood media in the laboratory. Ability to support seed germination would be a good criterion for identifying functional orchid mycorrhizal fungi in these species. Molecular techniques and genetic similarity also successfully identified fungi capable of forming functional orchid mycorrhizae. Other fungi associated with orchid roots in the field, which may have been isolated using surface-sterilized root sections, cannot be excluded as important to orchid germination and growth, but apparently did not form pelotons in these orchids.

However, no fungi cultured from pelotons in adult *T. discolor* were able to support conspecific seed germination in the laboratory. This was expected because all fungi identified using molecular methods from protocorms were unculturable. One of two closely related fungi was consistently identified in each protocorm. Thus, easily culturable contaminants and pathogens, presumably present in small quantities, were not a problem for molecular fungal identification. We are currently utilizing similar molecular techniques to identify unculturable peloton-forming fungi in *T. discolor* adults.

Germination of *T. discolor* has been observed in seed packets in the field when wood was transferred from areas with naturally occurring protocorms to areas where germination

otherwise does not occur (Rasmussen & Whigham, 1998). Such germination was not observed when sterile wood was transferred. This suggests that the fungus is more limited in its distribution than are appropriate host trees (if the fungus is ectomycorrhizal) or other external conditions. Field observation suggests that protocorm fungi are limited to wood of a particular decomposition stage. This may be because the fungi are wood degraders, ectomycorrhizal fungi with a strong preference for decomposing wood, as Tedersoo *et al.* (2003) found for Sebacinaceae and resupinate thelephoroid and athelioid fungi, or parasites on fungi belonging to one of these groups.

If the specificity found here is common to many other species of photosynthetic terrestrial orchids, then conservation efforts must include identification and conservation of the specific fungi required for germination and growth of the protocorm stage. Without conservation of required fungi, orchid conservation and reintroductions cannot succeed. In such specific associations, the ecological requirements of appropriate fungi may also have a strong effect on orchid ability to tolerate disturbance and environmental change.

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