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# Limitations on orchid recruitment: not a simple picture

MELISSA K. McCORMICK,* D. LEE TAYLOR,† KATARINA JUHASZOVA,* ROBERT K. BURNETT JR,*[1] DENNIS F. WHIGHAM* and JOHN P. O'NEILL*

*Smithsonian Environmental Research Center, PO Box 28, Edgewater, MD 21037, USA, †Institute of Arctic Biology, University of Alaska, Fairbanks, Fairbanks, AK, USA

## Abstract

**Mycorrhizal fungi have substantial potential to influence plant distribution, especially in specialized orchids and mycoheterotrophic plants. However, little is known about environmental factors that influence the distribution of mycorrhizal fungi. Previous studies using seed packets have been unable to distinguish whether germination patterns resulted from the distribution of appropriate edaphic conditions or the distribution of host fungi, as these cannot be separated using seed packets alone. We used a combination of organic amendments, seed packets and molecular assessment of soil fungi required by three terrestrial orchid species to separate direct and indirect effects of fungi and environmental conditions on both seed germination and subsequent protocorm development. We found that locations with abundant mycorrhizal fungi were most likely to support seed germination and greater growth for all three orchids. Organic amendments affected germination primarily by affecting the abundance of appropriate mycorrhizal fungi. However, fungi associated with the three orchid species were affected differently by the organic amendments and by forest successional stage. The results of this study help contextualize the importance of fungal distribution and abundance to the population dynamics of plants with specific mycorrhizal requirements. Such phenomena may also be important for plants with more general mycorrhizal associations.**

*Keywords*: fungal distribution, *Goodyera pubescens*, *Liparis liliifolia*, mycorrhizae, Orchid, Orchidaceae, *Tipularia discolor*, *Tulasnella*

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## Introduction

Identifying the factors that affect plant distribution and abundance is a central goal of plant ecology. The main factors on which most research has been focused are nutrient, water, and light availability and competition, which affects access to these factors. However, researchers are increasingly recognizing that microbial communities, especially symbiotic (e.g. Bever *et al.* 2010 and references therein) and pathogenic microbes (e.g. Mills & Bever 1998; Packer & Clay 2000; Schnitzer *et al.* 2011), mediate plant responses to both abiotic conditions and competition and thus influence plant distribution. Central among the microbes likely to influence plant distribution are mycorrhizal fungi, which are required by the vast majority of land plants (Brundrett 2009).

Mycorrhizal fungal communities in terrestrial ecosystems are diverse and play major roles in connecting above- and below-ground nutrient cycles and establishing and maintaining ecosystem structure and function (e.g. Saikkonen *et al.* 1998; van der Heijden *et al.* 2008; Horton & Bruns 1998; Claridge 2002; Helgason *et al.* 2002). Mycorrhizal fungi have been found to influence the distribution and diversity of plant communities (e.g. Allen *et al.* 1995; Grime 1997; Hartnett & Wilson 2002; Toljander *et al.* 2006), and specific strains of arbuscular mycorrhizal (AM) fungi differentially affect plant physiology (van der Heijden *et al.* 1998), clonal architecture (Streitwolf-Engel *et al.* 1997) and growth (Kiers *et al.* 2000; Lovelock & Miller 2002). The benefit conferred by mycorrhizal associations to both plants and fungi is known to depend on soil conditions, especially fertility,

Correspondence: Melissa K. McCormick,
Fax: +1 443 482 2380; E-mail: mccormickm@si.edu
[1]Present address: Department of Plant Science and Landscape Architecture, University of Maryland, College Park, College Park, MD, USA.

but very little is known about the factors that influence the distribution of mycorrhizal fungi, either as communities or individually.

Orchid mycorrhizae are an ideal system in which to examine the potential for soil resources to influence plant distribution indirectly, that is, through impacts on mycorrhizal fungi. As in many plants, recruitment from seed is critical in orchid population dynamics and probably plays a substantial role in determining where orchids occur (Masuhara & Katsuya 1994; Rasmussen & Whigham 1998b; Bidartondo & Read 2008; Jacquemyn *et al.* 2009). Unlike most plants, all orchids are obligate mycoheterotrophs and require resources obtained from fungi to grow until they develop into photosynthetic seedlings. This mycoheterotrophic stage may last from several months to the entire life cycle (Rasmussen 1995; Rasmussen & Whigham 1998b) and often requires very specific mycorrhizal fungi (reviewed in Taylor *et al.* 2002; McCormick *et al.* 2004).

The extreme fungal specificity of many orchids (e.g. Taylor & Bruns 1997; Taylor *et al.* 2002; McCormick *et al.* 2004; Otero *et al.* 2004) simplifies the assessment of the distribution of compatible fungal partners in the soil. Furthermore, the obligate fungal dependence of early orchid life history stages suggests that orchids are likely to be strongly affected by the distribution of particular fungi. Three lines of evidence suggest that the ability of appropriate fungi to support orchid germination and development (hereafter, 'host fungi') may depend on edaphic conditions that, in turn, affect the ability of host fungi to support orchids. First, two studies by McCormick *et al.* (2009, unpublished data) showed that the distribution of a temperate, mycoheterotrophic orchid and three green orchids coincided closely with locations at which the required mycorrhizal fungi were both present and abundant. Second, multiple studies using seed packets have shown that fungi are often more widespread than existing orchid populations (e.g. Masuhara & Katsuya 1994; McKendrick *et al.* 2000; Bonnardeaux *et al.* 2007), although this distribution may be transitory (Wright *et al.* 2009). Third, Diez (2007) found that seed germination, which requires appropriate fungi, was affected by edaphic conditions (soil moisture, % organic matter and pH).

Because many orchid species are rare, numerous studies have attempted to determine what conditions limit their distribution, and several of these have focused on fungal distribution (e.g. Perkins & McGee 1995; McKendrick *et al.* 2000; Batty *et al.* 2001; Brundrett *et al.* 2003; Otero *et al.* 2007), but nearly all of these studies have exclusively used seed packets (but see Perkins & McGee 1995; McCormick *et al.* 2009). While seed packets can indicate when both fungi and environmental conditions are appropriate, they cannot separate the two factors (as per Nantel & Neumann 1992). Unlike AM and ECM fungi, orchid fungi are not thought to be obligately associated with orchids, so the distribution of orchid mycorrhizal fungi is likely independent of the distribution of orchids. This means that the presence of orchids or their germinating seeds cannot be used to indicate all the locations where host fungi occur or to identify the factors governing fungal distribution.

Limited fungal distribution, driven by edaphic factors, has been suggested to explain the results of several recent studies that demonstrated decreased orchid seed germination with increasing distance from adult plants (e.g. Batty *et al.* 2001; Diez 2007; Jacquemyn *et al.* 2009). However, while fungal limitation has been suggested, it has never been demonstrated with certainty, and we still know little about factors driving the distribution of most fungi at scales relevant to plant populations (e.g. Read & Perez-Moreno 2003, Toljander *et al.* 2006).

Here, we used a combination of seed packets, organic amendments and fungal inocula to separately examine the effects of edaphic conditions and availability of host fungi on the distribution of three photosynthetic terrestrial orchids (*Goodyera pubescens*, *Liparis liliifolia* and *Tipularia discolor*). We asked three questions. (i) Does forest successional stage or organic amendment affect seed germination or protocorm growth? (ii) Do these factors affect the presence or abundance of host fungi? (iii) Are effects of these environmental factors on host fungi, germination and protocorm growth independent or do environmental effects on host fungi mediate effects on orchids? We combined molecular detection of host fungi in the soil with seed packet and resource manipulations to determine the extent to which forest successional stage and soil organic conditions affected seed germination and growth directly, as opposed to indirectly via effects on host fungi. We predicted that forest successional stage and organic amendments would primarily affect the distribution and abundance of host fungi, which would, in turn, limit orchid seed germination and protocorm growth.

## Materials and methods

### *Species*

*Goodyera pubescens* R.Br (Orchidoideae; Cranichideae; Goodyerinae) is an evergreen orchid that is found primarily in mid- and late successional forests throughout the eastern United States. Both adults and protocorms associate exclusively with a single clade of saprotrophic *Tulasnella* spp. (McCormick *et al.* 2004). *Liparis liliifolia* A. Rich ex Lindl. (Epidendroideae; higher Epidendroideae; Malaxideae) is a spring-green orchid and is common in early successional forests throughout the

eastern United States. Both adults and protocorms associate with a single species of saprotrophic *Tulasnella* that is closely related to fungi associated with *G. pubescens* (McCormick *et al.* 2004). *Tipularia discolor* Nutt. (Epidendroideae; higher Epidendroideae; Calypsoae) is a winter-green orchid and is common in forests of all stages throughout the eastern and southern United States (Whigham & O'Neill 1991). Protocorms of *T. discolor* are consistently associated with decomposing wood (Rasmussen & Whigham 1998a) and associate with two closely related clades of fungi in the Auriculariodes, although adults have broader associations. Additional details on the ecologies of these species at the Smithsonian Environmental Research Center (SERC) may be found in Whigham & O'Neill (1991), McCormick *et al.* (2004) and Whigham *et al.* (2006).

### *Study site*

SERC is located in Edgewater, Maryland, USA. Successional and mature forest sites at SERC are part of the Tulip poplar association (Brush *et al.* 1980; Parker *et al.* 1989; Brown & Parker 1994). The canopy in mature forests is dominated by *Liriodendron tulipifera* L., *Fagus grandifolia* Ehrh., *Quercus* spp. and *Carya* spp. Successional forest study sites are dominated by *L. tulipifera, Liquidambar styraciflua, Acer rubrum* and *F. grandifolia.*

The soils at all the study sites are classified as associations of the Collington sandy loam (fine-loamy mixed, active, mesic Typic Hapludult) and the Monmouth fine sandy loam (fine, mixed, active, mesic Typic Hapludult) (Soil Survey Staff http://soils.usda.gov/technical/classification/osd/index.html). Differences in successional stage, agriculture and logging have produced only minor differences in mineralogy and soil chemistry within the SERC forest (Pierce 1974; Correll 1974; Szlavecz *et al.* 2011).

### *Wood amendment experiment*

In autumn 1997, a long-term amendment study was initiated to determine the extent to which decomposing wood of different stages affected germination and growth of five orchid species. Here, we discuss only the results for *G. pubescens, L. liliifolia* and *T. discolor.* This experiment used seed packets to monitor the effects of organic amendments on germination in natural forest environments and is described in detail in Whigham *et al.* (2002, 2006). Briefly, in each of three forests, we removed the leaf and humus layers from one 2 × 2 m area in which we excavated nine 18 × 35.5 × 10 cm deep subplots, which we lined with fine mesh fibreglass screen. We constructed 270 seed packets of each species, containing approximately 50–300 locally collected seeds of the study species. Initial seed viability for each species was previously tested and was as follows: *G. pubescens* 95%, *L. liliifolia* 82% and *T. discolor* 42% (Whigham *et al.* 2002, 2006). Packets were placed vertically into linear plastic trays designed for slide projectors. Each tray received five seed packets of each species. The trays were buried in eight different types of substrate amendments at each of three forest sites (Whigham *et al.* 2002).

The substrate amendments included wood of two tree species (*L. tulipifera* and *Quercus alba*), each in three stages of decomposition (fresh, 5–15 years decomposition and >15 years decomposition), and local humus along with a local soil control. Naturally decomposing wood was collected from one of the forest sites, dried for 1 week at 60 °C, processed through a commercial wood chipper and further ground in a Wiley Mill prior to experimental use. No further treatments were applied to this wood prior to use. Each excavated subplot received amendment in the bottom, two trays of seed packets (10 packets of each species), and more amendment packed around the tray. Six of the nine subplots in each forest were filled with ground wood from the decomposition series described above (two tree species × three decomposition stages), one was filled with homogenized, sieved (1 cm) local humus, and two with homogenized, sieved (1 cm) local soil.

For *G. pubescens*, we visually counted the number of protocorms in seed packets after 1 year as a surrogate for seed germination. This technique was used because *G. pubescens* embryos will swell and break the testa (i.e. germinate) asymbiotically, but they will not develop beyond this point without mycorrhizal colonization. Thus, we defined germination for this species as symbiotic germination that produced protocorms as described by Whigham *et al.* (2002). Very few seeds of *T. discolor* and *L. liliifolia* germinated in the first year; we therefore counted protocorms after 4 years.

### *Litter and fungus amendment experiment*

In May 2004, we began a second amendment study in which we incorporated molecular techniques to assess the distribution of host fungi independent of seed germination. We established study plots at six sites in SERC, three in mature forests (120–150 years post-abandonment) and three in earlier successional forests (50–70 years post-abandonment). Plots were located so that they did not include trees, recent decomposing wood or any of the studied orchids. Each plot was divided into 36 subplots. Four seed packets (as per Rasmussen & Whigham 1998b) of the three species were placed vertically in the soil, with the top 1 cm of the slide mount extended above the soil surface, and

arranged in a square around the centre of each subplot. Each subplot then received a 25-cm-diameter ring to contain amendments (Fig. 1). Each ring was constructed of plastic baseboard material (Armstrong 4-inch-wide plastic wallbase) cut so that each strip was 5 cm wide. Strips were 86 cm long and included 4–6 small vents to allow water drainage. The strips were then wrapped in a circle, and the overlapping ends were stapled together to form a 25-cm-diameter ring that was staked to the soil surface.

The three amendment treatments were 1000 $cm^3$ of chipped *L. tulipifera* wood, 1000 $cm^3$ hand-crushed *L. tulipifera* leaves or no amendment. We chose *L. tulipifera* wood and leaves because it is the dominant species in both the mature and successional forests at SERC and is known to support growth of orchid fungi and protocorms (Rasmussen & Whigham 1998a; Whigham *et al.* 2002). These amendments were reapplied in the spring of each year and were crossed with two fungal treatments: inoculated with fungi appropriate to the orchid or uninoculated. All treatment combinations were replicated twice in each plot. The complete factorial design thus included three orchid species × three amendments × two fungal treatments × six sites × two replicates = 216 subplots and 864 seed packets in total.

Fungi were inoculated as a small pinch (~2 g wet weight) of damp wood with fungal inoculum that was lightly mixed into the soil surface and amendments in the centre of the seed packets in each subplot. An equal amount of damp sterile wood was added to uninoculated subplots.

Fungi for *G. pubescens* and *L. liliifolia* were grown in the laboratory from axenic cultures that originated as pelotons from adults of these two orchids. Each culture was grown from a plug of E-medium agar in 20 mL liquid E-medium (per 1 L distilled $H_2O$: 10 g glucose, 0.25 g ammonium tartrate, 0.4 g $KH_2PO_4$, 0.5 g $MgSO_4$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.05 g yeast extract, 0.005 g ferric citrate, 0.005 g $MnSO_4 \cdot 4H_2O$ and 0.004 g $ZnSO_4 \cdot 7H_2O$; Caldwell *et al.* 2000) for approximately 3 weeks. Chipped *L. tulipifera* wood was moistened and then placed into GA-7 culture vessels (Magenta Corp., Chicago, IL, USA) and autoclaved for 30 min to sterilize. After cooling, hyphae were mixed into the wood and allowed to grow for 4–6 weeks until the wood was well colonized. Colonization was tested by removing four random pieces of wood from each container and allowing fungi to grow out on solid E-medium. Fungi growing out were observed to be sure that they were the desired fungi and were uncontaminated by bacteria or other fungi. A subset was also genotyped using ISSR techniques (see McCormick *et al.* 2006) to verify visual identification.

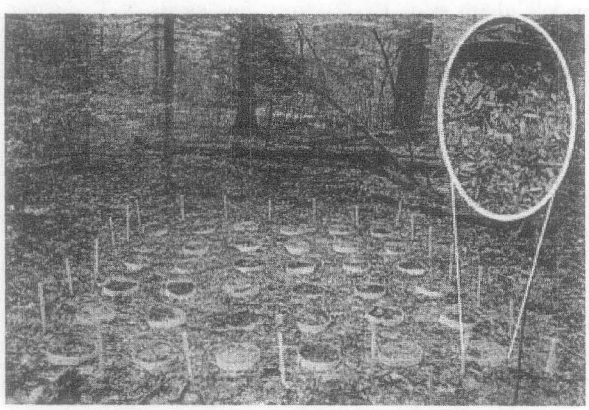

Fig. 1 Field layout of amended subplots within a 3 × 3 m array of 36 subplots. Inset shows a close-up of one subplot with two seedlings of *Goodyera pubescens* emerging from seed packets in a wood-amended subplot. The edges of two slide mounts containing seed packets are visible as white and grey lines near the bottom of the inset.

Fungi associated with *T. discolor* protocorms have not been culturable, but are consistently associated with decomposing wood. For each inoculation, we collected wood surrounding naturally occurring protocorms at three locations 1 day before adding it to amendment plots. Wood from the three locations was mixed in a plastic bag prior to addition and was added the same way as the cultured *Tulasnella* spp. We extracted DNA from three subsamples of mixed wood inoculum and successfully amplified both taxa of *T. discolor* host fungi using the techniques described in *Molecular analyses*, ensuring that the wood contained the desired fungi. Fungi were inoculated in spring of each year following the first rain after amendment additions.

Seed packets of *G. pubescens* were collected after 16 months. Seed packets of *L. liliifolia* and *T. discolor* were collected after 24 months. Collected packets were stored at 4 °C until they could be examined. We counted all protocorms using a dissecting microscope and measured the largest protocorm in each packet.

During June 2005, four soil samples ~2.5 cm diameter and 2 cm deep were removed from each 25-cm-diameter subplot. These four samples were equally spaced halfway between the seed packets and ring. After collection, each soil sample was stored in a −80 °C freezer before lyophilization (Labconoco 195 Freeze dry system, Kansas City, MO, USA). Each sample was then ground using a sterile mortar and pestle, and 0.075 g of each of the four samples from each subplot was composited to provide a single soil sample. We conducted preliminary tests to determine that pooling of samples within subplots provided a technique that allowed us to detect heterogeneously distributed host fungi without destroying the plot for future sampling (M. K. McCormick, unpublished data).

### *Molecular analyses*

DNA was extracted from each 0.4 g composited soil subsample using Fast Spin DNA kits for soil (Qbiogene, Irvine, CA, USA). We amplified fungal DNA from the soil DNA using PCR primers specific to the target group(s) of fungi (Table 1). For *G. pubescens* and *L. liliifolia*, we first amplified *Tulasnella* spp. from 20 ng total DNA using ITS5 (White *et al.* 1990)/ITS4-tul (Taylor & McCormick 2008) and examined the number of *Tulasnella* 'taxa' in each soil sample, considering each amplicon size in automated ribosomal intergenic spacer analysis (ARISA) to represent a taxon. These reactions were also used to indicate the abundance of Tulasnelloid fungi. One microlitre of each sample was then run on an ABI 3100 sequencer (ABI, Inc., Foster City, CA, USA) and analysed using GeneMapper version 4.0 (Applied Biosystems, Inc., Foster City, CA, USA), and amplicon sizes were grouped using a TRFLP peak sorting function for Excel (Rees *et al.* 2004, http://www.wsc.monash.edu.au/~cwalsh/treeflap.xls). To verify that appropriate *G. pubescens* host fungi were present within the larger group of core *Tulasnella* amplified by ITS5/ITS-4tul, we also subjected each *G. pubescens* soil sample that had some peaks detected in the ARISA to PCR amplification using two pairs of microsatellite primers (B159 and SW-2779-59-1) specific to the clade of fungi associated with *G. pubescens*. The presence and abundance of *G. pubescens* host fungi were calculated using these microsatellite fragments rather than the abundance of Tulasnelloid fungi as a group. Each *L. liliifolia* soil sample was subjected to amplification with ITS-Lip1/ITS4-tul, and each *T. discolor* soil sample was subjected to amplification with TipC1F/-TipR and TipC2F/TipR to cover the two related clades of *Tipularia* protocorm fungi (McCormick *et al.* unpublished data; Table 1). Amplification reactions of 25 μL were carried out with a final concentration of 0.5 μM each primer, 0.1 μL BSA and 50% Red Mix Plus PCR Master Mix (PGC Scientifics, Frederick, MD, USA). An additional 0.65 μL of 25 mM $MgCl_2$ was added to each reaction. Amplifications consisted of 25 cycles in an MJ Research DNA Engine and employed a 3-min initial denaturation at 94 °C before and elongation for 10 min at 72 °C after thermocycling. Each cycle consisted of a 30-s denaturation at 94 °C, followed by an annealing step of 30 s at 52–61 °C (depending on the primer, Table 1) and elongation for 30 s at 72 °C.

We tested for the presence of inoculated fungi, which were pure cultures except for *T. discolor* fungi, as opposed to naturally occurring fungi, in two ways. First, we sequenced the ITS region of fungi found in *G. pubescens* protocorms from seed packets and compared them to the added fungus. Second, we sequenced target fungi amplified from the soil around seed packets. The primers Lip1/ITS 4-Tul produced single bands and were sequenced directly. Tulasnelloid PCR products amplified by ITS 5/ITS-4tul from the soil around 12 *G. pubescens* seed packets contained DNA from multiple organisms and required cloning to sequence. Cloning was performed using TopoTA 4.0 vector with chemically competent cells (Invitrogen, Carlsbad, CA, USA) per manufacturer's instructions. We then chose 20 colonies with appropriate-size inserts from each sample to amplify using PCR with the vector primers M13f/M13r. Each single-amplicon sample or clone was sequenced using Big Dye version 3.1 chemistry and run on an ABI 3100 sequencer (ABI, Inc.).

### *Statistical analyses*

*Germination*. Statistical analyses of symbiotic seed germination in the wood amendment experiment were conducted using Systat 11 for Windows (Systat Software Inc., San Jose, CA, USA). Logit regression was used to test whether wood of different decomposition

**Table 1** PCR primers used in the present study

| Name | Sequence | *T* | Target taxa |
|---|---|---|---|
| ITS-Lip1† | CGTCTCCCTGTGTTACCTCTTT | 54 | *Liparis liliifolia* host fungi |
| ITS4-Tul‡ | CCGCCAGATTCACACATTGA | 54 | *Tulasnella* spp. |
| ITS5§ | GGAAGTAAAAGTCGTAACAAGG | 54 | Fungi |
| GIS-B159_F† | TTGACTTTCGACAATATCAGAG | 51 | *Goodyera pubescens* host fungi |
| GIS-B159_R† | AGGGCTGTGAGAGAGTTATC | 53 | *G. pubescens* host fungi |
| SW-2779-59-1F† | CTGTTGCACATCGACCTCAG | 56 | *G. pubescens* host fungi |
| SW-2779-59-1R† | AGCYAACTCTGTACCCGCT | 56 | *G. pubescens* host fungi |
| TipC1F† | TGCGAATGTGTCCCTCACAC | 58 | Clade 1 fungi hosting *Tipularia discolor* protocorms |
| TipC2F† | CGTGTTCATCATCCTCACACCT | 58 | Clade 2 fungi hosting *T. discolor* protocorms |
| TipR† | TGCATTTCGAGACGAGCCG | 58 | Fungi hosting *T. discolor* protocorms |

*T* indicates the annealing temperature (°C) at which superscripts indicate primer references †McCormick *et al.* unpublished data; ‡Taylor & McCormick 2008; §White *et al.* 1990.

stages was more or less likely to result in germination. The numbers of protocorms produced in wood from different species and decomposition stages were compared using ANOVA on log-transformed data.

In the litter and fungus amendment experiment, we analysed the likelihood of symbiotic seed germination (i.e. whether any seeds in a packet germinated) in different amendments using logit regression, as above. The number of protocorms (i.e. extent of symbiotic germination) produced in different stage forests, with different amendments and with the presence or absence of host fungi, was compared using ANOVA on log-transformed data. The three orchids had different patterns of seed germination, as indicated by significant species × factor interactions in the full ANOVA analysis, so we present analyses of each species separately for clarity.

To determine whether differences in seed germination among treatments resulted from direct effects of treatments on germination or from effects on fungi, which then affected germination, we conducted a stepwise regression analysis (backward, $\alpha$ to include or remove = 0.15) with fungus presence, amendment treatment and forest successional stage as factors predicting number of protocorms produced (=symbiotic seed germination; log transformed).

A separate set of analyses were conducted using abundance of host fungi, rather than simply presence, because the two perspectives provide qualitatively different interpretations (i.e. presence of fungi might be sufficient to stimulate germination but might not support protocorm growth). To obtain a measure of fungal abundance, we used total fluorescent intensity (peak height) summed across the different microsatellite fragments from 10 nmol of DNA subjected to 25 cycles. Interpreting end-stage fluorescent intensity of PCR product quantitatively has some justifiable criticisms, especially in mixed DNA samples, but has been found to generally preserve relative DNA abundances in similar soil samples (Polz & Cavanaugh 1998). Our analyses were conducted across similar soils, and preliminary tests we conducted (e.g. spiking mixed DNA samples with known quantities of target DNA) suggested that interpretation was robust across these samples (M.K. McCormick, unpublished data). Additionally, these semi-quantitative measures were compared to quantitative PCR abundance measurements and found to be strongly correlated ($r^2 = 0.94$, $P < 0.001$; McCormick *et al.* unpublished data).

*Protocorm growth.* We analysed size of the largest protocorm separately from symbiotic germination (number of protocorms) because conditions sufficient for seed germination might not be the same as conditions supporting robust protocorm growth. Analyses identical to those for germination were conducted with the same variables but with protocorm size (log transformed) instead of number of protocorms as the dependent variable. Bonferroni corrections to *P*-values were used to account for multiple tests.

*Host fungus abundance.* We analysed the direct effects of forest stage and amendment on the distribution and abundance of host fungi, independent of orchids, with the same methods (logit regression and ANOVA) as were used to examine germination and protocorm growth. We analysed differences in *Tulasnella* and host fungus abundance, measured as cumulative peak heights from the microsatellite analysis, using ANOVA. To determine whether inoculated fungi became established and supported seed germination, we compared fungal DNA sequences obtained from soil clones and protocorms to those of the added fungi.

## Results

### *Seed germination*

In the wood amendment experiment, we obtained 208 *Goodyera pubescens* protocorms in 37 seed packets, 91 protocorms of *Liparis liliifolia* in three seed packets and 11 protocorms of *Tipularia discolor* in three seed packets. All protocorms were produced in wood-amended plots. The three *T. discolor* seed packets with protocorms were in three different plots at two sites. These seed packets were in both species of wood and at both fresh and >15 years decomposition stages. All three *L. liliifolia* seed packets with protocorms were from a single plot amended with fresh *Quercus* wood. Only *G. pubescens* produced protocorms in a sufficient number of seed packets to examine effects of wood species and decomposition stage on seed germination.

The number of *G. pubescens* protocorms differed significantly with decomposition stage ($P < 0.001$; Fig. 2), with fresh and <15-year-old wood having more protocorms than >15-year-old wood. Wood species was not significant ($P = 0.641$), and the decomposition stage × wood species interaction was almost significant ($P = 0.082$). The likelihood of germination occurring (i.e. the number of seed packets with some germination) varied little among decomposition stages or wood species.

In the litter and fungus amendment experiment, we obtained 120 *G. pubescens* protocorms from 36 seed packets in 18 subplots in four sites. All but one of the protocorms were in mature forest sites (Fig. 4a, b). The likelihood of symbiotic germination in seed packets was significantly influenced by forest successional stage ($P = 0.005$). Protocorm presence was also predicted by

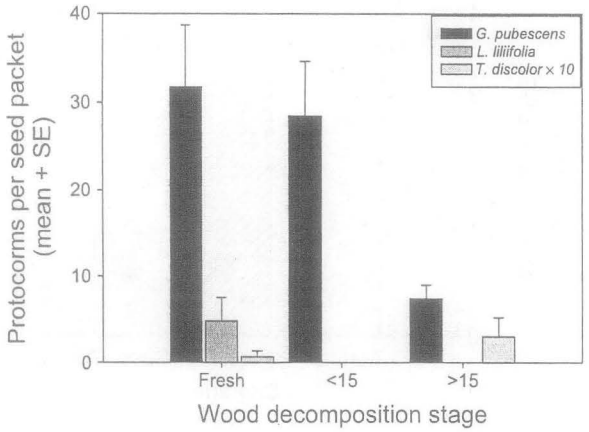


Fig. 2 The mean number (±SE) of protocorms produced per seed packet ($N$ = 30 seed packets/stage) of *Goodyera pubescens* (black bars), *Liparis liliifolia* (dark grey bars) and *Tipularia discolor* (light grey bars; values shown ×10 to increase visibility) in decomposing wood amendments as a function of wood decomposition stage (fresh, <15 years, or >15 years).

host fungi in the soil, regardless of whether host fungi were assessed by the presence or abundance in the analysis ($P \leq 0.002$; Table 2). Interactions between main effects could not be analysed because there were many cases where no protocorms were produced in an interaction category.

We obtained 21 *L. liliifolia* protocorms from 14 seed packets in eight subplots in four sites (Fig. 3a, b). Germination was significantly related to fungus ($P = 0.009$) when fungal abundance, rather than just presence, was considered and was nearly significantly related to amendment when the presence of host fungi was considered ($P = 0.068$). All but one protocorm developed in subplots where host fungi were added. The one subplot that produced a protocorm without added fungi had been disturbed by small mammal digging that unearthed two of the four seed packets.

Nine *T. discolor* protocorms, all of which were very small (<0.5 mm), were obtained from five seed packets in five subplots in four sites (Fig. 3a, b). The number of seed packets with protocorms was too small for meaningful statistical analyses, but no clear patterns were apparent. Two seed packets with protocorms developed in subplots amended with leaves, two with no amendment and one with wood. Three of the five seed packets were in subplots with fungus added (Fig. 3a, b). Neither fungal abundance nor amendment was a significant predictor of the occurrence of *T. discolor* protocorms.

Stepwise regression analysis of factors affecting germination consistently removed amendment treatment, but retained forest successional stage, when host fungus abundance was included as a variable.

### *Protocorm growth*

While forest successional stage and fungal abundance affected the likelihood of germination in *G. pubescens*, the size of the largest protocorms was significantly predicted by fungal abundance and amendment treatment (all $P \leq 0.002$). In *L. liliifolia*, the size of protocorms in seed packets was significantly predicted only by abundance of host fungi ($P \leq 0.010$; Table 2). For *T. discolor*, stepwise regression gave identical results for protocorm size as for germination, namely that forest successional stage and host fungus abundance were retained as predictor variables.

### *Host fungus abundance*

The presence of natural, uninoculated *Tulasnella* spp. in soils differed significantly depending on forest successional stage (logit Regression; $P < 0.001$, Fig. 4a) and amendment (logit Regression; $P = 0.013$; Fig. 4b), as did *Tulasnella* abundance, measured as cumulative peak heights from microsatellite fragments (forest stage,

**Table 2** Significant factors in logit analyses relating the probability of seed packets producing protocorms (protocorm presence) and ANOVA relating the size of protocorms (Protocorm size) produced by *Goodyera pubescens*, and *Liparis liliifolia* to the presence and abundance (band fluorescent intensity) of host fungi in the soil adjacent to seed packets in the amendment and fungus addition study. Analyses were conducted separately for each species in the interest of clarity because of significant species × main factor interactions. For each species and for both analyses (logit and ANOVA), the full model, reflected in the $r^2$ or $\rho^2$ values, included amendment, forest successional stage and fungi detected as main effects. Only $P$-values for significant and marginally significant effects are given. $P$-values for all other factors were >0.20

| | Protocorm presence | Protocorm size |
|---|---|---|
| Fungus presence | | |
| *G. pubescens* | Successional Stage: $P = 0.005$, $\rho^2 = 0.283$ | Amendment: $P < 0.001$, Fungus: $P = 0.001$, $r^2 = 0.813$ |
| *L. liliifolia* | Amendment: $P = 0.069$, $r^2 = 0.119$ | Fungus: $P = 0.030$, $r^2 = 0.106$ |
| Fungus abundance | | |
| *G. pubescens* | Successional Stage: $P = 0.004$, $\rho^2 = 0.300$ | Amendment: $P = 0.002$, Fungus: $P = 0.002$, $r^2 = 0.783$ |
| *L. liliifolia* | Fungus: $P = 0.012$, $\rho^2 = 0.263$ | Fungus: $P < 0.001$, $r^2 = 0.211$ |

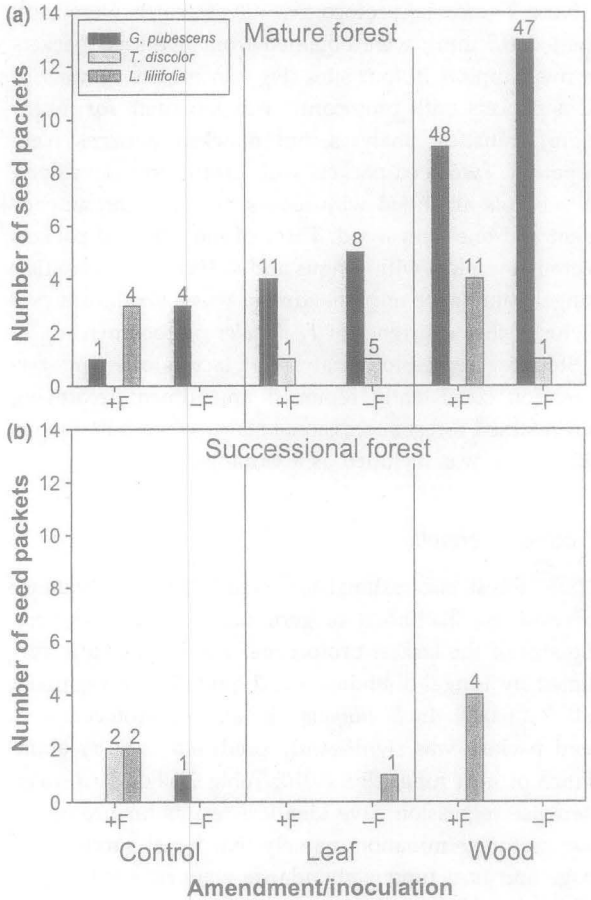


Fig. 3 Germination in *Goodyera pubescens* (black bars), *Liparis liliifolia* (dark grey bars) and *Tipularia discolor* (light grey bars) seed packets placed in amendment study plots in (a) mature and (b) successional forests. *X*-axis is separated into three sections based on organic amendments (control/no amendment, leaf, wood). Each section is further divided by fungal inoculation treatment (+F = inoculation with host fungi, –F = no inoculation). The height of each bar indicates the number of seed packets with some protocorms. The number above each bar indicates the total number of protocorms found in the seed packets from that treatment.

$P < 0.001$; amendment, $P = 0.002$; Fig. 4c). This was mostly a result of many fewer subplots in successional forest sites that had any *Tulasnella* taxa compared to mature forest sites. When only subplots with at least some *Tulasnella* taxa were considered, the successional and mature forest sites were very similar in richness ($P = 0.374$, Fig. 4a). *Tulasnella* spp. needed by *G. pubescens* and *L. liliifolia* and host fungi needed by *T. discolor* were all present more often in mature than in successional forests ($P < 0.001$, $P = 0.048$, $P = 0.043$, respectively). Host fungi needed by *G. pubescens* and *T. discolor*, but not *L. liliifolia*, were also more abundant in mature than in successional forests ($P < 0.001$, $P = 0.040$ and $P = 0.264$, respectively).

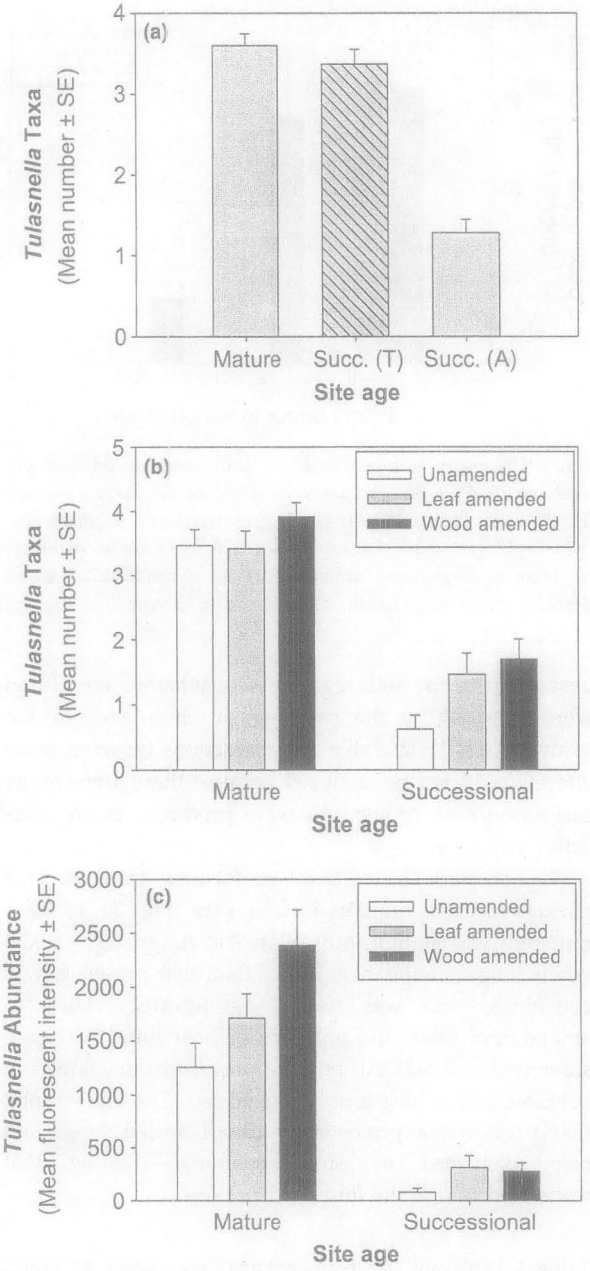


Fig. 4 The *Tulasnella* taxa amplified from amendment study plots as a function of forest successional stage and amendment. (a) Number of Tulasnelloid fungi in all plots in mature and successional [Succ. (A)] forest subplots and in only successional subplots with at least some Tulasnelloid fungi [Succ. (T) with cross-hatch]. (b) The number of *Tulasnella* taxa amplified in wood-amended compared to leaf litter-amended and control plots. (c) The abundance (mean fluorescent intensity of amplified microsatellite fragments) of *Goodyera pubescens* host taxa that were amplified in mature successional forest subplots amended with wood, leaves or unamended.

Inoculation of fungi resulted in somewhat more common (mean detection 0.33 ± 0.08 vs. 0.16 ± 0.06; $P = 0.101$) and significantly more abundant (mean

fluorescent intensity 0.64 ± 0.16 vs. 0.28 ± 0.12; $P = 0.051$) host fungi across the three species. For *G. pubescens*, host fungi were widespread, and most DNA sequences did not match the added fungus (Genbank, AY373267). Inoculated fungi were recovered from protocorms in three of 36 (8.3%) *G. pubescens* seed packets with protocorms. Other *Tulasnella* taxa that did not match inoculated fungi but belonged to the clade of *G. pubescens* host fungi were recovered from protocorms in other seed packets, and taxa belonging to this and other *Tulasnella* clades were recovered from soil clones (Genbank accessions JQ247545-JQ247568). Each seed packet had only a single host fungus but soils had 1–5 taxa, corresponding to the number of taxa detected using ARISA.

For *L. liliifolia*, host fungi were detected significantly more often in subplots to which fungi had been added and sequences of these fungi matched the added fungus (Genbank, AY373283). Host fungi were detected in soils from *T. discolor* subplots to which fungi had been added as well as some without fungi added (Genbank accessions JQ247533-JQ247544). Because we added a mixture of fungi rather than a pure culture, we cannot be certain whether the detected fungi were those we added or those naturally occurring at each site, but fungi were significantly more likely to be present in subplots to which fungi were added than those without added fungi ($P = 0.032$).

## Discussion

Germination of all three orchids appeared to be primarily limited by factors that influenced host fungus abundance, which was a significant predictor of presence, number of protocorms and protocorm size across all three species. If orchid distribution is often limited by occurrence of mycorrhizal hosts, then understanding the factors governing both fungal distribution and abundance may be critical to conservation of many threatened and endangered orchid species (e.g. Swarts *et al.* 2010; Wright *et al.* 2009; Phillips *et al.* 2011). The type of amendment added had a significant effect on symbiotic seed germination only if host fungus abundance was not included in the analysis, suggesting that the effect of amendment on germination was mediated by effects on host fungi, rather than direct effects on orchid seeds. Forest successional stage appeared to have both a direct effect on orchid seed germination and an indirect effect, mediated via effects on the abundance of host fungi.

### *Germination*

In the wood amendment experiment, in which no fungi were added, protocorms were recovered from seed packets only in wood-amended plots. In contrast, Whigham *et al.* (2002) reported that *Goodyera pubescens* germination was higher in humus and soil than in wood. Whigham *et al.*, however, included seeds that had germinated asymbiotically and did not develop into symbiotic protocorms. In the present study, we only counted seeds as having germinated when they had developed into symbiotic protocorms and that only occurred in wood-amended plots. Despite the apparent importance of wood to all three orchids, there were differences in the stage of wood decomposition in which the protocorms occurred. Protocorms of *G. pubescens* were more abundant on fresh and <15-year-old decomposing wood, while *Liparis liliifolia* protocorms were only found on fresh wood and *Tipularia discolor* protocorms were most abundant on old (>15 years decomposition) wood. These results suggest that wood amendments might promote growth of fungi needed to support many orchids, but it is important to determine the characteristics of the wood that the host fungi would grow in.

In the litter and fungus amendment experiment, germination of *G. pubescens* was affected by both the availability of decomposing wood and forest successional stage. Protocorms were almost exclusively produced in mature forests and were substantially larger and more abundant in wood-amended subplots, regardless of whether host fungi had been added. In contrast, the germination of *L. liliifolia* was similar across amendment treatments and sites, suggesting that organic amendments were relatively unimportant for this species. However, *L. liliifolia* germination occurred almost exclusively in plots to which host fungi had been added, suggesting that it was strongly limited by the distribution of host fungi. Correspondingly, *G. pubescens* is found primarily in mature forests, although it occurs abundantly in a few areas in successional forests, while *L. liliifolia* is found primarily in successional forests and only occasionally in mature forests.

Very few *T. discolor* protocorms developed in amendment subplots, suggesting that none of our amendments effectively mimicked needed conditions. Previous studies have demonstrated that *T. discolor* is consistently associated with decomposing wood (Rasmussen & Whigham 1998a). Considering that *T. discolor* germinated primarily on old decomposing wood in our wood amendment experiment, it may be that the wood we added in this experiment had not yet decomposed sufficiently to provide appropriate conditions. However, it is also possible that fungi detected in soil adjacent to seed packets may not have reached seeds within, as has been found for ectomycorrhizal hyphal communities inside and outside of in-growth cores (e.g. Hynes *et al.* 2010), or seed packet design may have inhibited germination.

One effect of our wood amendments was to increase soil moisture and C/N ratios (Timothy Filley, personal communication). In our initial wood amendment experiment, wood increased symbiotic germination of all three orchid species, but two of the study species first germinated after 4 years, immediately following a year of relatively high precipitation. This suggests that the effect of wood on soil moisture content may have been important, especially as McCormick *et al.* (2006) found that *G. pubescens* host fungi appear to be sensitive to drought. Similarly, Diez (2007) found that seed germination of *G. pubescens* increased with higher soil moisture and organic matter content.

### *Host fungus abundance*

Forest successional stage had a major effect on both the distribution and abundance of mycorrhizal fungi, especially for *G. pubescens*. While *Tulasnella* spp. were diverse and widespread in mature forests, they were far less diverse and abundant in successional forests. Host fungi from *G. pubescens* were rarely found in the soil in successional forests, even when they had been added, suggesting that environmental differences limited both their distribution and abundance. Abundance of fungi associated with *L. liliifolia* was primarily affected by whether they had been added to subplots, regardless of successional stage. Fungi associated with *T. discolor* were somewhat more abundant in mature than successional forests. While it is possible that host fungi are abundant in limited environments that were less often sampled in younger successional forests, our results suggest that fungal associates of the three orchids we studied may be affected by forest successional stage and that the role of fungal distribution in response to successional stage may warrant further investigation in other orchids.

We found that the abundance of host fungi associated with the three study orchids was also affected by organic amendments. The fungi needed by *G. pubescens* and core *Tulasnella* spp. as a group were more abundant on decomposing wood. By 'core *Tulasnella*', we refer to those species in the clade containing *Tulasnella violacea* (=*T. lilacina*), the type species of the genus, and most cultured *Tulasnella* species. Host fungi needed by *L. liliifolia* and *T. discolor* were not significantly affected by amendment in this study, but tended to be more abundant in control and wood-amended, compared to leaf-amended, subplots.

Researchers have repeatedly hypothesized that appropriate mycorrhizal fungi could limit orchid distribution but have found that host fungi are not limited to locations supporting orchid populations (e.g. Masuhara & Katsuya 1994; Bonnardeaux *et al.* 2007; Swarts *et al.* 2010; Phillips *et al.* 2011). Most of these examples come from terrestrial orchids. The distribution of host fungi associated with epiphytic orchids is less well studied, perhaps because seed packet techniques are less successful on branches than in the ground, but many epiphytic orchids have similarly specific fungal requirements and may also be affected by the distribution and abundance of their mycorrhizal fungi (Otero *et al.* 2004, 2007). Of the three orchids we studied, only *L. liliifolia* was limited almost entirely by the availability of host fungi. Seeds of *L. liliifolia* only germinated in subplots to which fungi had been added, regardless of the amendment treatment applied or forest successional stage. Successional stage and amendment treatments affected germination of the other two study species primarily by affecting host fungus distribution and abundance, which then explained effects on seed germination. This suggests that fungal presence was not sufficient to support germination and protocorm growth, which also required that host fungi be abundant.

If orchid germination requires abundant host fungi, then changing or ephemeral conditions might cause temporary increases in host fungus abundance (e.g. Wright *et al.* 2009) that might allow fungi to support germination but might not be sufficiently consistent to support growth to maturity. There is evidence for such inter-annual changes of *G. pubescens'* host fungi in response to drought (McCormick *et al.* 2006), and McCormick *et al.* (2009) suggested that the dynamics of a population of *Corallorhiza odontorhiza* might be driven by the distribution of particularly drought-resistant fungi. Understanding the roles of successional stage and edaphic conditions, which in turn affect the distribution and abundance of host fungi, may provide guidance for improving orchid conservation and restoration success by identifying conditions favouring persistent growth of host fungi (e.g. Gale *et al.* 2010).

While the distribution of ectomycorrhizal fungi as a group has repeatedly been shown to limit the distribution of ectomycorrhizal plants (e.g. Nara 2006; Collier & Bidartondo 2009), the importance of distribution of particular host fungi for affecting arbuscular and ectomycorrhizal associations, which often involve many more diverse fungi that are often obligately associated with host plants, will be more difficult to demonstrate than for orchid mycorrhizae. The increasing recognition that, as a result of differential host plant benefit and altered competitive dynamics, particular fungi may be needed to allow some plants to persist in some communities (e.g. Bever *et al.* 2010) combined with the broader findings that many mycorrhizal fungi may be more sensitive to edaphic conditions than plant hosts (e.g., Nantel & Neumann 1992) suggests that effects of soil factors on fungi may be important in governing distribution of

many plants. However, this effect on generalist plants will likely be more subtle than for plants that are obligately dependent on a narrow phylogenetic range of fungi.

The perception that arbuscular and ectomycorrhizal associations are quite general has led researchers to ignore the distribution of particular mycorrhizal fungi as a factor potentially affecting plant distribution. However, recent studies have shown that success of a plant species in a community can be increased in the presence of particularly beneficial mycorrhizal fungi or decreased when only other fungi are available (e.g. van der Heijden *et al.* 2008; Bever *et al.* 2010 and references therein). In this study, we found that recruitment of three species of terrestrial orchids was affected by the distribution and abundance of mycorrhizal fungi. This demonstrates that mycorrhizal distribution and abundance can be critical in determining the distribution of plants with specific mycorrhizal requirements and suggests that it may also be important for plants with more general mycorrhizal associations.

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M.K.M., D.F.W. (senior authors) and J.P.O. (senior technician) have conducted orchid-mycorrhizal research for many years. They have collaborated in several projects related to expanding our understanding of the mechanisms and consequences of plant-fungal, especially orchid-fungal, interactions. D.L.T. (senior author) is interested in the community structure of soil fungi and the evolutionary dynamics of plant-microbe interactions. K.J. and R.K.B. were involved in the project as student interns.

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## Data accessibility

DNA sequences from *G. pubescens* protocorms: Genbank accessions JQ247533-JQ247568.

DNA sample descriptions and individual Genbank accession no. (Appendix S1, Supporting information).

Wood amendment experiment: treatment, site, seed packets with germinating seeds and protocorms: DRYAD entry doi: 10.5061/dryad.c770013c.

Litter and fungus amendment experiment: treatment, site, seed packets with germinating seeds and protocorms, protocorm size: DRYAD entry doi: 10.5061/dryad.c770013c.

Abundance and size of microsatellite fragments detected in soils associated with *G. pubescens* seed packets (Appendix S2, Supporting information).

## Supporting information

Additional supporting information may be found in the online version of this article.

**Appendix S1** DNA sample descriptions and individual Genbank accession numbers. Each sample is described by the Genbank identifier and accession number, followed by a description with source (soil or protocorm), site number and a letter-number combination that describes the subplot location within the study plot, whether the subplot was inoculated with host fungi, whether the subplot was amended with wood or leaf litter, and the age (young or old) of the forest where the plot was located.

**Appendix S2** Abundance and size of microsatellite fragments detected in soils associated with *G. pubescens* seed packets. Each sample is identified by a site number (1-6), a letter-number combination that describes the subplot location within the study plot, the amendment treatment applied to that subplot, and whether it was inoculated with host fungi. The microsatellite fragments (size in base pairs) detected in each sample are separated by primer (B159 or SW-2779-59-1) and the height of each ARISA peak (fluorescent intensity) produced by 20ng DNA.

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