Journal of Ecology

British Ecological Society

Journal of Ecology 2016, **104**, 744–754 doi: 10.1111/1365-2745.12556

Germination patterns in three terrestrial orchids relate to abundance of mycorrhizal fungi

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Summary

1. The spatial distribution of plants, which is often generated by patterns of seed recruitment, is an important determinant of population dynamics, especially for orchids with seeds that must be exposed to appropriate mycorrhizal fungi.

2. We compared the distribution and abundance of target mycorrhizal fungi detected in the soil using DNA-based molecular techniques and germination in seed packets of *Goodyera pubescens*, *Liparis liliifolia* and *Tipularia discolor*.

3. We further examined *Tulasnella* spp. associated with *G. pubescens* to determine whether areas with abundant host fungi resulted from multiple genets of the same species or from a single widespread fungal genet.

4. We found that target fungi were more likely to be detected using soil DNA assays than by seed germination. Based on soil DNA, fungi were more widespread than suggested by seed germination, which most often reflected the presence of abundant mycorrhizal fungi in the soil. Fungi were more likely to be abundant close to established orchids. Established plants of *G. pubescens* that were < 50 cm apart associated with a single abundant fungal genet, while those > 50 cm apart associated with multiple fungal genets.

5. *Synthesis*. This study demonstrates the importance of using multiple methods to detect the distribution and abundance of target fungi and suggests that fungal 'hot spots' may be keys to the dynamics of orchid populations.

Key-words: *Goodyera pubescens*, *Liparis liliifolia*, mycorrhizal limitation, mycorrhizas, orchid, Orchidaceae, plant–soil (below-ground) interactions, seed germination, *Tipularia discolor*

Introduction

The spatial distribution of recruitment within plant populations has wide-ranging population effects (Clark, Macklin & Wood 1998; Jacquemyn *et al.* 2007). For example, plant distribution can affect population genetic structure, population density, competition, pollination and the evolution of species (Clark, Macklin & Wood 1998; Otero & Flanagan 2006; Waterman & Bidartondo 2008). Dispersal of seeds into suitable microsites is an important factor determining recruitment and distribution patterns (Jersáková & Malinová 2007; Jacquemyn, Brys & Jongejans 2010). For many plants, a favourable microsite, where seeds germinate and grow to maturity, includes mycorrhizal fungi and other soil symbionts.

The distribution of fungi can influence plant distribution (Swarts *et al.* 2010) and it may be critical for orchids, which have life-history stages that cannot persist unless they are associated with an appropriate mycorrhizal fungus. The distribution of mycorrhizal fungi in the soil has been inferred from the distribution of fruiting structures (Gherbi *et al.* 1999; Bergemann & Miller 2002; Kretzer *et al.* 2005), colonization of roots (Jany, Garbaye & Martin 2002; Hay *et al.* 2015) or both (Gardes & Bruns 1996). The distribution and abundance of hyphae of specific taxa in the soil have only become measureable with the availability of molecular techniques (Masuhara & Katsuya 1994; Buée *et al.* 2009; Douhan *et al.* 2011). It is now also possible to use PCR to detect and quantify fungal hyphae in soils (Hortal, Pera & Parladé 2009; Douhan *et al.* 2011; Bergemann *et al.* 2013). Studies have documented considerable disagreement between fungal distribution and abundance as assessed by fruiting bodies, colonized root tips and extramatrical hyphae in the soil. Some fungi were well represented using each of these assessment methods, while many were detected using a single method. Furthermore, which method estimated abundance to be the greatest differed among taxa (Koide, Xu & Sharda 2005; Landeweert *et al.* 2005; Kjøller 2006). This suggests that these methods are complementary for the detection of fungi in the

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soil and the assessment of fungus distribution can be improved by applying multiple methods.

The potential for mycorrhizal fungi to affect plant distribution is partially governed by the specificity of the association. Approximately 10% of all flowering plants are orchids, all of which form obligate mycorrhizal relationships at one or more stages in their life cycles. Given the importance of orchid–fungus interactions, many efforts have been made to understand the diversity and specificity of orchid mycorrhizal fungi (OMF; Warcup 1971; Taylor & Bruns 1997; McCormick, Whigham & O'Neill 2004; Rasmussen *et al.* 2015). While mycorrhizas occur in all orchid life-history stages, they are obligatory for terrestrial orchid embryos and protocorms, the non-photosynthetic developmental stage between seed germination and production of the first leaf, which are mycoheterotrophic (i.e. they obtain energy, carbon and mineral nutrients from the fungi). Mycoheterotrophic stages may last from months to years (Rasmussen 1995; Gill 1996), and the establishment of a mycorrhizal relationship may be a limiting factor in orchid seedling recruitment (Masuhara & Katsuya 1994; Rasmussen & Whigham 1998; McKendrick *et al.* 2002). Mycorrhizal fungi are necessary for protocorm development, but other factors also contribute to successful seed germination (Těšitelová *et al.* 2012; McCormick & Jacquemyn 2014).

A characteristic of many orchid populations is the sparse distribution of individuals and rarity of populations at local and global scales (Waterman & Bidartondo 2008). These characteristics may be the result of the factors that influence the distribution of specific, required fungi. McCormick *et al.* (2012), for example, found that organic amendments affected the germination of orchid seeds by changing the abundance of mycorrhizal fungi. This suggested that areas with existing orchids might be 'hot spots' of persistently abundant mycorrhizal fungi (Taylor *et al.* 2003; Otero & Flanagan 2006; Waterman & Bidartondo 2008). However, little is known about how 'hot spots' are distributed in soils and whether orchids, through the distribution of 'dust seeds', are regularly able to colonize those 'hot spots' (but see Jacquemyn *et al.* 2007).

The distributions of orchid fungi are often difficult to track because they do not produce conspicuous fruiting bodies (Waterman & Bidartondo 2008; Swarts & Dixon 2009). Even when molecular methods are used, these fungi (especially Tulasnellales) often amplify poorly with general fungal PCR primers (Taylor & McCormick 2008). The most common approach to investigating the distribution and role of orchid fungi in the soil has been the use of seed packets to 'bait' for appropriate fungi (Rasmussen & Whigham 1993), from which fungi within protocorms have been identified via isolation, anastomosis groups and/or molecular techniques (Masuhara & Katsuya 1994; Zettler *et al.* 2011). Packets are used because orchid seeds are too small to be placed in soil then recovered subsequently. However, seed packets can only detect locations where both fungi and environmental conditions are appropriate for seed germination. More recently, Jacquemyn *et al.* (2014) and Oja *et al.* (2014) used 454 sequencing to investigate the relationship between the distributions of fungi and orchids. However, both studies found that OMF were often undetected, even adjacent to orchid roots, where the fungi must occur. For example, Oja *et al.* (2014) found that very few of the fungi that were found in orchid roots were recovered from the adjacent soil and some were not found in any of their soil samples.

At the scale of tens to hundreds of kilometres, it appears that many orchid fungi are widespread and orchid distribution at those scales may be primarily limited by seed dispersal. However, at metre scales, orchid population dynamics may be more driven by the patchy distribution of fungi (McCormick & Jacquemyn 2014). Some OMF have been shown to occur outside of established orchid patches (i.e. >5 m from the nearest conspecific orchid; e.g. McKendrick *et al.* 2002; Těšitelová *et al.* 2012; Oja *et al.* 2014), a situation that can be expected for orchids that depend on fungi that are likely to be free-living saprotrophs, endophytes, or ectomycorrhizal with other plants, with distributions that are independent of orchids. However, other possibilities exist that result in unsuccessful linking of orchids and fungi. Fungi outside of existing orchid populations may represent different, possibly less favourable, species or genotypes of fungi than those within existing populations (as was suggested by McCormick *et al.* 2009). Appropriate fungi outside of orchid patches may be ephemeral, physiologically inappropriate, or in microsites that may not support sufficient fungal biomass to sustain successful seed germination or orchid growth (i.e. microsites may be of variable quality or persistence). Alternatively, the fungi that occur outside orchid populations may be sufficient, but orchid seeds may not be present (i.e. dispersal limitation). Importantly, Jacquemyn *et al.* (2013) found clear evidence of the importance of distribution linkages between orchids and fungi. Orchid species clustered together when they shared fungi, but were spatially segregated when they associated with different fungi. This research strongly suggested that the distribution of individual orchid species and the clustering of species in the community were driven by the distribution of their mycorrhizal fungi. However, they did not examine the distribution of fungi independent of orchids.

Molecular techniques now enable us to examine fungal distribution and abundance in soils (Genney, Anderson & Alexander 2006; Toljander *et al.* 2006; Geml *et al.* 2010; Jacquemyn *et al.* 2014; Oja *et al.* 2014; Taylor *et al.* 2014), and hence to better understand the relationship between OMF and orchid distribution (McCormick *et al.* 2009, 2012). However, the rare and sporadic detection of fungi in soils using these techniques raises the question of how molecular detection of fungi relates to their ability to support orchid germination. Here, we focus on two related issues. First, we address hypotheses related to the distribution of appropriate fungi and seed germination for *Liparis liliifolia*, *Goodyera pubescens* and *Tipularia discolor*, species that, respectively, interact with one or a few closely related fungi or have a specific habitat requirement for seed germination. Secondly, we examined the characteristics of 'hot spots' that support high diversity and/or abundance of compatible fungi. In the first part, we tested

three hypotheses: (i) DNA-based soil analyses would detect appropriate fungi more often than seed germination, (ii) seed germination would reflect the abundance (i.e. density of hyphae) of appropriate fungi, and (iii) appropriate fungi would be more abundant near conspecific plants.

In the second part of the study, we examined the diversity and abundance of *Tulasnella* spp. associated with *G. pubescens*. Previous research has demonstrated that *G. pubescens* plants that survived a drought all did so by switching from one persistent fungal genet, which disappeared during the drought, to a different fungal genet after the drought (McCormick *et al.* 2006). Based on this finding, we hypothesized that 'hot spots', which we define as sites that support multiple individual orchids at all life-history stages, would be sites with a higher density of genets of appropriate fungal taxa, rather than each consisting a single widespread genet.

Materials and methods

STUDY SPECIES AND SITE

Goodyera pubescens R.Br is an evergreen orchid that occurs in mid- and late-successional forests throughout the eastern United States. New leaves are produced primarily in the spring and flowering occurs in mid-summer. Seeds are shed in the fall (Ames 1922) and do not form long-lived seed banks (Whigham *et al.* 2006). Throughout the species range, adults and protocorms associate exclusively with a single clade of *Tulasnella* spp. that appears to be at least predominantly saprotrophic (McCormick, Whigham & O'Neill 2004), although many *Tulasnella* spp. may also function as endophytes (Girlanda *et al.* 2011; Selosse & Martos 2014).

Liparis liliifolia A. Rich ex Lindl. is common in early-successional forests throughout the eastern United States. It produces one or two leaves in early spring and flowers in mid-spring. Seeds are shed in the late fall and are viable for several years, resulting in a long-lived seed bank (Whigham *et al.* 2006). Adults and protocorms associate with a single species of saprotrophic *Tulasnella* (McCormick, Whigham & O'Neill 2004) that is closely related to but genetically distinct from the *Tulasnella* species associated with *G. pubescens*.

Tipularia discolor Nutt. is common in forests of all ages throughout the eastern and southern United States (Whigham & O'Neill 1991). The youngest corm of each plant produces a single leaf that emerges in early autumn and senesces in late spring. Flowering occurs in mid-summer (Whigham & McWethy 1980), and seeds mature and disperse in the late autumn. Seeds of this species also form a long-lived seed bank (Whigham *et al.* 2006). Protocorms of *T. discolor* are almost always found on decomposing wood (Rasmussen & Whigham 1998) and they associate with two clades of fungi of uncertain ecology that may belong to the Auriculariales (McCormick, Whigham & O'Neill 2004). These protocorms often reach 1–3 cm in length before producing a leaf, making it possible to identify protocorms in decomposing wood, often near seedlings. Individuals beyond the protocorm stage associate with a range of saprotrophic and ectomycorrhizal *Tulasnella* spp. as well as other genera (McCormick, Whigham & O'Neill 2004).

The research was conducted in forests at the Smithsonian Environmental Research Center (SERC), which is located in Edgewater, Maryland, USA. Successional and mature forests at SERC are part of the Tulip poplar association (Brush, Lenk & Smith 1980), and the three orchid species occur primarily in mid-successional and mature forests (Rasmussen & Whigham 1998; McCormick, Whigham & O'Neill 2004; Whigham *et al.* 2006). The soils at sites used in this project are described in McCormick *et al.* (2012).

GERMINATION IN SEED PACKETS RELATIVE TO DISTANCE FROM EXISTING PLANTS

In March 2004, we established arrays of seed packets (see Rasmussen & Whigham 1998 for a description of seed packet construction) for each of the three study species to characterize spatial variation in recruitment in relation to environmental conditions and soil fungi. Each seed packet contained 50–300 locally collected seeds, combined from the same 5–10 maternal plants. Initial viability of the seed batches, tested *in vitro* using triphenyltetrazolium chloride, was as follows: *G. pubescens* 95%, *L. liliifolia* 82% and *T. discolor* 42% (reported in Whigham *et al.* 2002, 2006). Seed packets were arrayed in a nested design that originated at a location where appropriate fungi were known to occur, based on the presence of adult orchids for *G. pubescens* and *L. liliifolia* and the presence of naturally occurring protocorms of *T. discolor,* because adults of this species use different fungi than those needed for seed germination. For each orchid, we arranged 28 seed packets in a randomly oriented '+' shaped design centred on an adult orchid (*G. pubescens* and *L. liliifolia*) or protocorms and seedlings (*T. discolor*). We placed six seed packets 5, 10, 50, 100, 200 and 500 cm from the center along each arm of the '+'. Four additional seed packets were placed, one on each diagonal of the '+', 707 cm from the center and 500 cm from each of the four 500-cm seed packets (Fig. 1).

This design was replicated at four isolated plants and in four widely spaced clusters, resulting in eight arrays for *G. pubescens* and *T. discolor*, and six for *L. liliifolia*. Each cluster included 3–5 conspecific orchids within the 5 m × 5 m grid area, while isolated plants were >50 m from another conspecific orchid. Arrays were separated by 100–2500 m. For each array, we measured the distance from each seed packet to the nearest existing adult plant, rather than the distance to the central plant, and used those distances in data analysis (described below). This design provided sufficient power to analyse fungal patchiness and genet size over a range from 5 cm to 10 m with a minimum of 20 sample pairs in each distance class (224 total seed packets for *G. pubescens* and *T. discolor* and 168 seed packets for *L. liliifolia*).

We checked seed packets for germination in the field every 3–6 months by removing and visually examining two seed packets 5 cm from the center of three arrays for each species. Seed packets were returned to the soil after inspection. When protocorms were evident in at least one seed packet in at least two arrays for a species, we collected all packets (including large arrays, see below) for that species. Following this protocol, we collected seed packets of *T. discolor* after 16 months and *G. pubescens* after 19 months. Seed packets of *L. liliifolia* never reached the above criteria, but were collected after 22 months.

In the laboratory, seed packets were rinsed with water to remove excess soil and groups of seed packets were wrapped in damp paper towels, placed in plastic bags, and stored at 4 °C until they could be examined, up to 2 weeks. We counted the number of protocorms in seed packets using a dissecting microscope and measured the largest protocorm in each packet as a measure of fungal ability to support orchid growth beyond germination.

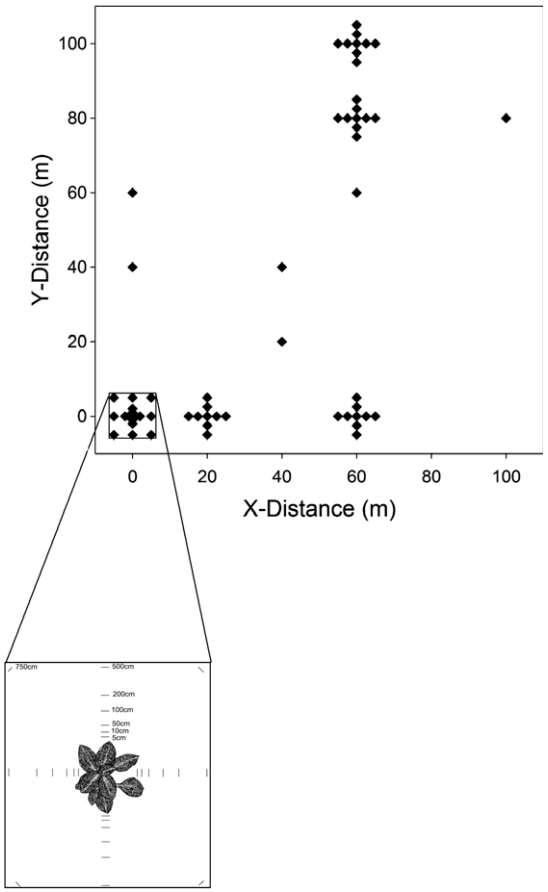

Fig. 1. Diagram showing the layout of seed packets in 100 m × 100 m grids relative to the focal plant and the 500 cm × 500 cm portion of each grid. In the large grid diagram, each diamond indicates the location of a seed packet, while in the close-up seed packet locations are indicated by a dash (–). The close-up shows the focal conspecific plant in the 500 cm × 500 cm portion of each array. Note that seed packet distances from the focal plant in the close-up are not drawn to scale.

In an additional experiment to determine whether fungi were regularly present in locations distant from conspecific orchids, we randomly chose four seed packet arrays (two in orchid clusters and two centred on isolated plants) to act as the anchor point for a larger grid. At each site, for each species, we established a 100 × 100 m grid with 20-m increments to form 36 grid points, with the seed packet array at a random corner (Fig. 1). In each grid, we randomly chose 10 grid points to receive a seed packet, and randomly selected three of those to sample more intensively. These intensively sampled points received eight additional seed packets arrayed in a '+' shape with seed packets 2.5 and 5.0 m from the selected grid point in each of four directions. This resulted in 136 additional seed packets for each species: [10 grid points + (3 intensive sites × 8 packets per site)] × 4 arrays = 136.

DISTRIBUTION OF FUNGI RELATIVE TO GERMINATION

When each seed packet was retrieved, we also collected *c.* 1 g of soil in direct contact with the seed packet mesh. For DNA analysis of the soil, each sample was placed in a −80 °C freezer within 2 h of collection. Frozen samples were lyophilized and ground. For *G. pubescens* and *T. discolor*, we extracted DNA from soil adjacent to each seed packet that had a protocorm as well as all samples ≤1 m from the center of three arrays of each species that had at least one protocorm. To examine small-scale spatial distribution of fungi for *L. liliifolia*, we analysed soils adjacent to seed packets on one randomly selected 'arm' of each of four arrays without protocorms and two 'arms' of the array that had protocorms. We also extracted DNA from the soil associated with 10 randomly selected seed packets without protocorms for each species in each of the large-scale arrays (three arrays for *L. liliifolia*, four each for *G. pubescens* and *T. discolor* for a total of 54, 90 and 88 soil samples, respectively) to determine whether fungi were more widespread than indicated by germination in seed packets. For each soil sample, we determined whether host fungi were present using taxon-specific molecular methods described below and in Appendix S1 in Supporting information. We used a logit regression implemented in Systat 11 for Windows (Systat Software Inc., San Jose, CA, USA) with fungus presence or absence as the independent variable and protocorm presence or absence as the dependent variable to determine whether there was a significant relationship between the presence of fungi and protocorm formation. Species differed considerably in the frequency of occurrence of host fungi in the soil, so each species was analysed separately.

ABUNDANCE OF FUNGI RELATIVE TO GERMINATION

To determine how abundant (i.e. how dense OMF DNA was in a defined volume of soil) host fungi were in each extracted soil sample, we used molecular quantification as described below. We analysed the resulting quantitative data to test for relationships between the presence and total number of protocorms produced and distance from the array center and presence and abundance of appropriate fungi. We used a logit regression with abundance of host fungus as an independent variable and presence or absence of protocorms as a dependent variable. As for analysis of fungal presence, each species was analysed separately.

To determine whether abundance of fungi and seed germination were related to distance from a conspecific orchid, we used an ANOVA for each species, with distance class as a fixed variable, array as a random variable, and abundance of host fungi as a dependent variable. We divided the distance from the array center into distance classes based on approximately equal sampling effort (number of seed packets placed at various distances) because germination was too low to draw meaningful conclusions using distance as a continuous variable.

To validate our approach, we tested whether *L. liliifolia* and *G. pubescens* host fungus abundances as detected by gel band intensity were correlated with those detected by qPCR (see Molecular methods, below) using linear correlations.

DISTRIBUTION OF FUNGAL GENETS

To determine whether patches of orchids (i.e. locations with 5–10 orchids within a 5 m × 5 m area) occurred where there was a single, favourable fungal genet or where multiple genets occurred close together, we examined the distribution of fungal genets in groups of *G. pubescens* plants using ISSR fingerprinting methods described below (McCormick *et al.* 2006).

During the summer of 2002, we mapped the locations (±5 cm) of 60 *G. pubescens* plants in 5 × 5 m grids in three forests. We used *G. pubescens* for this study because fungi are readily culturable and

we had previously found (McCormick *et al.* 2006) that individuals associate with a single fungal genet at a time, thus allowing us to fully sample the fungi associated with each plant with a single root. Pelotons were grown on E-medium agar (Caldwell, Jumpponen & Trappe 2000) with 50 mg L^{-1} Novobiocin, transferred to fresh E-medium agar without Novobiocin and, subsequently, to liquid E-medium for DNA extraction and analysis.

We analysed the relationship between geographic distance and fungal similarity by dividing distances into 10 cm classes and calculating (i) the percentage of samples that were genetically identical and (ii) the genetic dissimilarity of fungi associated with orchids in each distance class based on the number of unshared ISSR bands. This allowed us to distinguish between spatial patterns of genetic relatedness resulting from repeatedly sampling individual genets or from clusters of genetically similar fungi. Comparison between the scale at which genetically identical ISSR patterns were frequently sampled and the size of orchid patches was interpreted to determine whether patches represented the extent of individual genets or clusters of distinct fungi.

MOLECULAR METHODS

Molecular detection of fungi in soil and substrates

The design and testing of fungal species-specific PCR primers are described in Appendix S1. We extracted DNA from freeze-dried, ground 0.4 g subsamples of soil using Fast Spin DNA kits for soil (Qbiogene, Irvine, CA, USA) as per McCormick *et al.* (2013). We amplified DNA of *G. pubescens*-compatible fungi from soil using the microsatellite primers GIS-B159 F/R and SW-2779-59-1 F/R. We amplified DNA from *Tulasnella* spp. specific to *L. liliifolia* using the primer pair ITS-Lip1/ITS4-tul and from fungi specific to *T. discolor* protocorms using Tip14F/Tip14R and Tip2_F1/Tip14R. Amplification reactions of 25 μL were carried out with a final concentration of 0.5 μM each primer, 0.1 μL 10 mg mL^{-1} BSA and 12.5 μL Red Mix Plus PCR Master Mix (PGC Scientifics, Frederick, MD, USA), with an additional 0.65 μL of 25 mM $MgCl_2$ added to each reaction. Amplifications consisted of a 3-min initial denaturation at 94 °C, followed by 25 cycles of 30 s at 94 °C, 30 s at 52–61 °C (depending on the primer, Appendix S1) and 30 s at 72 °C in an MJ Research DNA Engine. Presence of a visible band of appropriate size on a 1% agarose gel was considered indicative of presence of target fungi.

Abundance of fungi in soil

To obtain a semi-quantitative measure of target DNA abundance, we categorized the intensity of each band on an ordinal scale from 0 to 5, with 0 indicating no visible band and 5 an extremely bright band. Duplicate PCRs were carried out, and the products of each subjected to gel electrophoresis twice to verify consistency of band intensity.

To determine whether these semi-quantitative abundance measurements corresponded to more explicitly quantitative measurements of target DNA abundance, DNA from a subset of 20 soils adjacent to *L. liliifolia* and 20 adjacent to *G. pubescens* seed packets was subjected to analysis using real-time quantitative PCR. To quantify fungal abundances, we followed procedures described in McCormick *et al.* (2012). Briefly, 25 μL reactions containing 12.5 μL iQ SYBR Green PCR Super Mix (Bio-Rad Laboratories, Hercules, CA, USA), 10 ng DNA template in 10 μL H_2O, and 1.25 μL (10 mM) of primers ITS-Lip1 and ITS4-tul (*L. liliifolia*) or GIS-B159 F/R (*G. pubescens*) were run on an MJ Research Opticon DNA Engine with Continuous Fluorescence Detection (MJ Research, now Bio-Rad Laboratories) as follows: initial denaturation at 95 °C 5 min followed by 41 cycles of 15 s denaturation at 94 °C, 30 s annealing at 53 °C, and 30 s elongation at 72 °C. Each sample was amplified in triplicate, and quantified using a standard curve. Four serial dilutions of genomic DNA from a pure culture of a *Tulasnella* sp. (isolate # M193; AY373283) or (isolate # M141; AY373264) isolated from *L. liliifolia*, and *G. pubescens*, respectively, were used to construct standard curves (range: 0.001–1 ng target genomic DNA) used in applying the procedures described in McCormick *et al.* (2013). In addition, a melting curve analysis was performed after each analysis to confirm the specificity of the qPCR. Gel band intensity of the *Tulasnella* sp. associating with *L. liliifolia* was strongly correlated with real-time PCR quantification ($r^2 = 0.94$, $P < 0.001$). Gel band intensity of the *Tulasnella* spp. associating with *G. pubescens* was also correlated with qPCR quantification ($r^2 = 0.91$, $P = 0.001$), suggesting that the semi-quantitative measure we employed here was relatively robust. However, primers were not available to amplify a sufficiently small fragment of DNA from *T. discolor* fungi to enable qPCR.

We removed two protocorms from each seed packet where germination occurred and extracted DNA using a modified CTAB extraction process (McCormick, Whigham & O'Neill 2004). We amplified the fungi from each protocorm using ITS5 (White 1990)/ITS4-tul (Taylor & McCormick 2008) for *G. pubescens* and *L. liliifolia* and Tip14F/Tip14R and Tip2_F1/Tip14R for *T. discolor* (Appendix S1). A subset (11 from *G. pubescens*, 2 from *L. liliifolia*, 10 from *T. discolor*; accession No. KU664571–KU664588, *L. liliifolia* sequences matched AY310910) of successfully amplified PCR products was then sequenced bidirectionally to verify the identity of the colonizing fungi. Sequencing reactions were carried out with the original PCR primers, sequenced using Big Dye v. 3.1 chemistry, cleaned with Sephadex and run on an ABI 3700 sequencer (ABI, Inc., Foster City, CA, USA).

ISSRs to distinguish fungal genets

We extracted DNA from fungi grown in liquid E-medium using a TES/CTAB extraction (McCormick, Whigham & O'Neill 2004), amplified using ISSR primer #811 [$(GA)_8C$; obtained from the University of British Columbia Biotechnology Laboratory] and visualized on a 1.5% agarose gel in 1× TBE buffer after staining with ethidium bromide (as per McCormick *et al.* 2006). Bands were visualized on a UV transilluminator, documented using a Fisher Biotech Polaroid photodocumentation system (FB-FDC-34 with FB-PDH-1314 hood), and identity determined visually. Each ISSR banding pattern was coded using presence or absence of each band, and bands were treated as dominant. Multiple DNA isolations of independently cultured but genetically identical fungi have consistently yielded identical banding patterns repeated over several years. To understand how patterns of relatedness contributed to fungal distribution, we calculated the distance separating each pair of fungal isolates and grouped each pairwise comparison into 10 cm distance classes. ISSR data were used to assess the number of different genotypes present in each distance class and the proportion of distinguishable genotypes (PD) was calculated as G/N (Ellstrand & Roose 1987), where G is the number of distinct genotypes and N is the number of individuals sampled. Jaccard's pairwise dissimilarity (J) was used as a measure of genetic distance and geographic distance was calculated from X, Y coordinates on an established grid. We then used Mantel analysis (EcoDist, Goslee & Urban 2007) to determine the relationship between genetic relatedness and physical distance for pairwise

comparisons within each 10 cm distance class. This allowed us to determine the extent to which genetic similarity of fungi associated with nearby plants resulted from resampling of genets, compared to sampling of related fungi.

Results

COMPARING MOLECULAR DETECTION OF FUNGI WITH GERMINATION

Host fungi were nearly always amplified from soils where protocorms were recovered (10 of 11 *G. pubescens*, 2 of 2 *L. liliifolia* and 7 of 8 *T. discolor*). However, appropriate fungi were also amplified frequently from locations where seeds did not germinate (19 of 43 *G. pubescens*, 10 of 20 *L. liliifolia* and 14 of 64 *T. discolor*). This demonstrates that appropriate fungi were broadly but not uniformly distributed at the scales of the arrays that we used (see next section). Regardless of species, appropriate fungi were more likely to be detected in locations where protocorms were produced than in locations with no protocorms (Logit Regression, DF = 1 for each; *G. pubescens* $P < 0.001$, *L. liliifolia* $P = 0.08$, *T. discolor* $P = 0.03$).

DISTRIBUTION OF GERMINATION RELATIVE TO EXISTING PLANTS

Nineteen *G. pubescens* protocorms were found within 11 seed packets in 7 of the 8 arrays (4.9% of seed packets in the 5 m × 5 m section of all arrays). We recovered one *L. liliifolia* protocorm and one seedling from separate seed packets (1.2% of seed packets) in one of the six arrays. The seedling and protocorm were 5 cm and 2 m, respectively, from the array center, but the protocorm was only 12 cm from another *L. liliifolia*. *Tipularia discolor* protocorms ($n = 116$) were retrieved from eight seed packets in four of the eight arrays (3.6% of seed packets). Two seed packets had seedlings that were beginning to produce a leaf and one had also begun to produce a root.

Germination was low at all distances from conspecific plants and across the three species. In only two cases were protocorms found in seed packets that were >50 cm away from a conspecific plant (Fig. 2a), suggesting that conditions appropriate for seed germination were rare where orchids were not present.

ABUNDANCE OF FUNGI RELATIVE TO GERMINATION

For all three species, appropriate fungi were more likely to be abundant in locations where protocorms were produced than in locations with no protocorms (Logit Regression, DF = 1 for each; *G. pubescens* $P < 0.001$, *L. liliifolia* $P = 0.02$, *T. discolor* $P = 0.005$). Most locations with protocorms had abundant (i.e. band intensity of 4–5) host fungus DNA (8 of 10 *G. pubescens*, 2 of 2 *L. liliifolia*, and 5 of 7 *T. discolor* locations). Host fungi detected in locations that did not support germination were only abundant 26% of the time (Fig. 3). We found that the abundance of appropriate

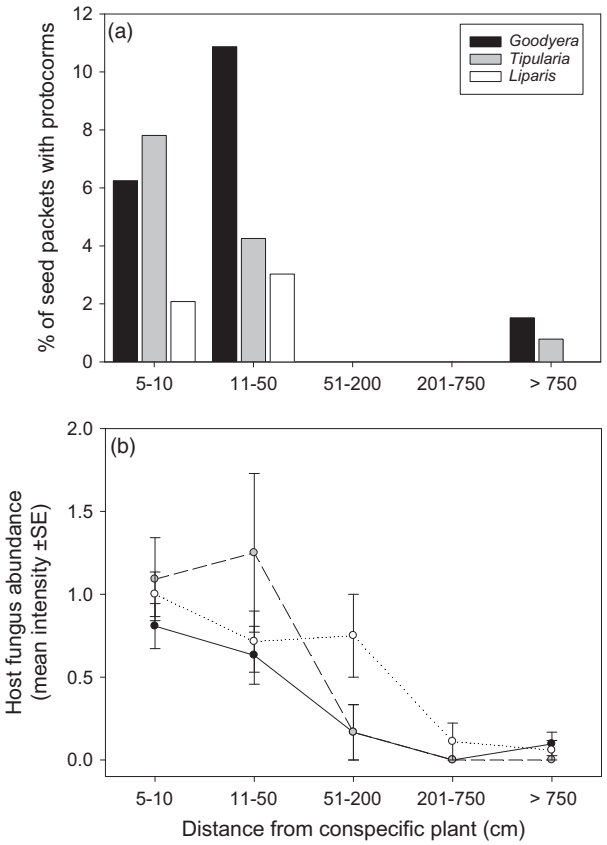

Fig. 2. (a) The percentage of *Goodyera pubescens* (black bars), *Liparis liliifolia* (grey bars) and *Tipularia discolor* (white bars) seed packets within a given distance from array centres producing protocorms in the array experiment. Per cent of seed packets is given to account for variable sampling effort at different distances from the centres of the arrays. (b) The relationship between host fungus abundance, determined semi-quantitatively using PCR amplification of specific taxa from the soil, and distance from a plant of *G. pubescens* (black circles, solid line), *L. liliifolia* (grey circles, dashed line) or *T. discolor* (white circles, dotted line).

fungi decreased as the mean distance (array class) from conspecific plants increased for all three species (ANOVA, *G. pubescens* $P = 0.008$, *L. liliifolia* $P = 0.04$, *T. discolor* $P = 0.04$; Fig. 2b).

DISTRIBUTION OF FUNGAL GENETS

ISSR bands demonstrated that nearly all samples of fungi separated by <40 cm were genetically identical (Fig. 4). Only approximately 40% of fungi sampled 50–70 cm apart were genetically identical, and almost no fungi sampled from more than 70 cm apart were identical. This suggests that patches of plants separated by <40 cm occurred within the boundaries of single fungal genets, while larger orchid patches spanned clusters of genetically distinct fungi.

Discussion

Only a few studies have measured the natural distribution and abundance of orchid mycorrhizal fungi independent of germi-

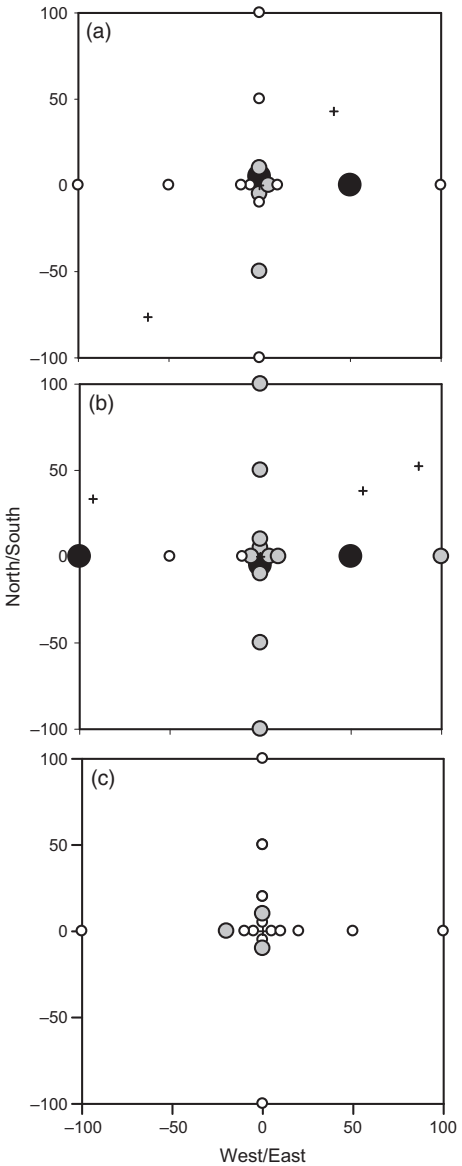

Fig. 3. Three example arrays of *Goodyera pubescens* indicating whether seeds germinated and whether host fungi were present (black = protocorms, host fungi detected, grey = no protocorms, host fungi detected, white = no protocorms, no fungus detected). Abundance of host fungi is reflected by the size of the symbol. A focal plant was located in the center of each array but (a) and (b) show clusters of conspecific plants, with additional plant locations shown by +'s. (c) shows an isolated plant with no conspecific neighbours. Note that locations with germination (black dots) were generally also locations where fungi were abundant (i.e. dots were large) and that locations with OMF (black or grey and larger dots) were clustered near the central, focal plant, except in (a) and (b), where they were also found near other conspecific orchids within the grid.

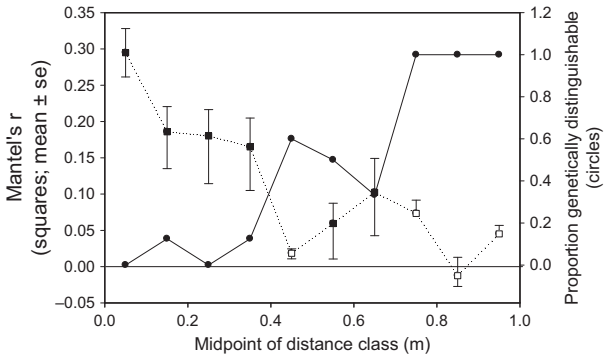

Fig. 4. The genetic similarity of fungi isolated from *Goodyera pubescens* as a function of distance between sampled plants. The per cent of samples in a given distance class that were genetically identical (solid black circles and line, left axis) and the mean genetic similarity (r) between samples in that distance class (solid squares indicate points where genetic similarity is statistically greater than zero; open squares indicate points where genetic similarity is not distinct from zero, dotted line, right axis).

nation in seed packets (Bahram, Peay & Tedersoo 2015). The few that have (McCormick *et al.* 2009; Jacquemyn *et al.* 2014; Oja *et al.* 2014) have only analysed the distribution of OMF using molecular techniques, so it is unclear how molecular detection of fungal distribution relates to their effectiveness in supporting seed germination and protocorm growth. We found that local patterns of germination in all three orchids were related to the spatial distribution and abundance of appropriate mycorrhizal fungi as measured using molecular techniques. Our results suggest that the local distribution and patchiness of some orchids are likely influenced by mycorrhizal fungi. As has been found in other studies using seed packets, we found that appropriate fungi detected as DNA in the soil were more widely distributed than visible adult orchids (Masuhara & Katsuya 1994; Batty *et al.* 2001; McKendrick *et al.* 2002; Bonnardeaux *et al.* 2007; Swarts *et al.* 2010). However, rather than indicating that orchids are not limited by fungi, we suggest that the combination of stochastic dispersal, other environmental requirements and patchy fungal distribution and abundance may together limit orchid recruitment.

FUNGUS DISTRIBUTION RELATIVE TO PLANTS AND GERMINATION

Even though appropriate fungi were both more widely distributed than focal plants and more widespread than indicated by seed germination, germination of all three species was greatest within 50 cm of conspecific plants, which was also where fungi were most abundant in the soil. Previous studies of orchids found similar patterns of higher germination in seed packets near conspecific plants (Perkins & McGee 1995; Diez 2007; Jacquemyn *et al.* 2007; Jersáková & Malinová 2007), suggesting that locations with appropriate environmental conditions and fungi are typically already colonized by orchids. While this does not preclude other factors limiting plant distribution (e.g. soil moisture and nutrients, success of seed production, seed dispersal patterns, seed longevity in the soil or many other possible factors), it suggests that fungal limitation plays an important role.

Although most germination occurred near conspecific plants, two seed packets of *G. pubescens* and one of *T. discolor* that were more than 100 cm from observed conspecific plants produced protocorms. This observation shows that

there are locations lacking adult orchids that have abundant appropriate fungi, but such microsites are uncommon. It is possible that the fungi in those microsites were ephemeral or not in sufficient quantity to support long-term survival of plants and only sites with abundant mature plants consistently support abundant mycorrhizal fungi. It is also possible, based on studies of *Corallorhiza odontorhiza* (McCormick *et al.* 2009), that microsites shift from year to year or that new sites become available over time and allow the population to grow.

ABUNDANCE OF HOST FUNGI COMPARED TO SEED GERMINATION

Germination in seed packets reflected the abundance, rather than solely the distribution, of appropriate fungi (McCormick *et al.* 2012). There were also many locations where abundant appropriate fungi were detected but seeds did not germinate. Much as mushrooms are sometimes poor indicators of fungal extent below-ground (Gardes & Bruns 1996), orchid seed germination only indicated some of the 'hot spots' of host fungus abundance. This finding supports the view that abundant host fungi are necessary but not sufficient for germination. Alternatively, fungi detected in soil adjacent to seed packets may not have reached the seeds within the packet, or may not have been sufficiently abundant. It is also possible that fungal hyphal communities inside and outside of seed packets differed, as has been found for ectomycorrhizal hyphal communities inside and outside of ingrowth cores (Hynes *et al.* 2010). These possibilities provide additional cautionary notes for interpreting seed germination within packets as indicative of fungal distribution.

Locations near mature plants were more likely to have abundant host fungi than distant locations (Fig. 2). However, many microsites near mature plants lacked appropriate fungi and did not support seed germination, reflecting fungal patchiness at very small scales. Small-scale patchiness appears to be typical of fungal communities in soil (Bruns 1995; Taylor *et al.* 2010). The importance of fungal patchiness to orchids was also evidenced by the small percentage of seed packets that supported germination, even very close to conspecific plants (≤14% of seed packets at any distance supported germination; see Fig. 3) and may reflect the patchy distribution of individual fungal genets. Such a local distribution of fungi in a stable population could indicate that suitable sites are essentially saturated with orchids, while a growing population may have many unoccupied suitable sites. Alternatively, it has also been proposed that orchids may increase the local abundance of fungi by protecting them or providing carbon or other necessary nutrients (Bidartondo *et al.* 2000; Selosse & Martos 2014). Only one study has so far provided evidence of orchids contributing carbon to their mycorrhizal fungi (Cameron *et al.* 2008), although others have interpreted orchid carbon isotope composition to suggest it may be more widespread (Hynson, Preiss & Gebauer 2009; Hynson *et al.* 2013). It is also possible, and we suggest more likely, that mature orchids are only found in locations with consistently abundant mycorrhizal fungi. While we cannot distinguish between orchid occupation of most suitable sites and orchid promotion of fungal abundance, the dependence of seed germination on abundance of mycorrhizal fungi that we found in this study holds, regardless of what causes fungi to be abundant.

DISTRIBUTION OF FUNGAL GENETS

The composition and scale of patches of abundant host fungi supporting germination and orchid growth are important. To the extent that patches represent individual fungal genets, then the characteristics and dynamics of the individual host fungus become important to understanding orchid distribution and loss of the genet supporting a patch as a result of environmental changes (e.g. drought or succession) may also result in death of the orchid patch. There are justifiable questions about the repeatability of ISSRs, including poor repeatability, especially from false negatives in bands that are not consistently visible, sensitivity to contaminants that are also amplified and possible non-homology of fragments. However, the primer we used consistently yielded repeatable banding patterns for these fungi from pure cultures, even multiple independent cultures of identical fungi over multiple years. Additionally, inbreeding and local dispersal could produce multiple closely related genets that occur close together, which might not be distinguishable using a single primer. However, we think this is unlikely because with samples of fungi from over 100 *G. pubescens* plants we have never identified fungi that were identical with this primer but which, with additional loci and sequencing, proved to be different genets (M. K. McCormick, unpublished data). While it is certainly still possible that these fungi were closely related, but distinct, genets, the use of additional primers would not have been sufficient to distinguish them.

In our detailed examination of the distribution of fungal genets associated with *G. pubescens,* we found that a single fungal genet was typically associated with plants separated by *c.* <40–70 cm (Fig. 4). The scale at which *G. pubescens* host fungus genets were distributed corresponded closely to the distance from conspecific plants (< 50 cm) at which we saw greater germination in seed packets and more abundant host fungi for all three orchids. This result suggests that small clusters of orchids and local seed germination at distances < 50 cm reflect the spatial extent of host single fungus genets that were genetically or physiologically amenable to supporting seed germination. In contrast, we found that larger patches of *G. pubescens*, with multiple plants scattered over a 5 m × 5 m area, were large enough to include different fungal genets. This finding points to the complex factors that are responsible for the location and abundance of mycorrhizal fungi at submetre scales. There are obvious advantages to the orchids in being located in areas where multiple fungi are potentially available. For example, orchids in larger patches of multiple fungi may be better able to endure stressful conditions by switching to new host genets, compared to orchids reliant on a single fungal genet (McCormick *et al.* 2006). Our results reflect the often-reported relationship between seed

germination and distance from conspecific orchids and suggest that it may be at least partially driven by the distribution of host genets (Jacquemyn *et al.* 2007; Dearnaley, Martos & Selosse 2012). However, fungal genet sizes vary substantially among species and environments and other orchids may exhibit different patterns.

McCormick *et al.* (2012) found that mycorrhizal fungi associated with the three orchids in this study were directly impacted by organic amendments, while seed germination was affected indirectly, as a result of altered fungal abundance. Combined, these two studies provide a multidimensional perspective in which assessment of fungal abundance, genet distribution and microsite conditions were combined with experimental manipulation of environmental conditions and fungus distribution to more fully understand the relationship between substrate quality and fungal diversity and abundance.

These results demonstrate that there are pros and cons of each technique used to assess the distribution of orchid mycorrhizal fungi in the soil. Germination in seed packets may provide the best estimation of the combination of fungus distribution, physiological state and abundance together with appropriate environmental conditions, but it will not detect many other locations where appropriate fungi are growing. Molecular techniques provide more information about the overall distribution and abundance of appropriate fungi in the soil, but due to the miniscule volumes of soil that can be sampled in molecular methods and the high heterogeneity of fungal distribution at fine scales, this method will also fail to identify some locations where appropriate fungi occur. Particular caution may be warranted when general primers are used with next generation sequencing, as recent studies using this technique had very low rates of detection of orchid fungi (Jacquemyn *et al.* 2014; Oja *et al.* 2014). In this study, both germination in seed packets and molecular detection in the soil revealed similar patterns of fungus distribution and abundance relative to established orchids, although neither was sufficient to comprehensively catalog every location in which fungi were present and able to support orchid germination and growth.

In summary, separating the factors that determine successful orchid establishment is a major challenge for conservation and one of the difficulties in achieving this has been limits in knowledge of the distribution of orchid mycorrhizal fungi in the environment. In this study, which represents one of very few cases where orchid fungus distribution in the soil was examined independent of orchids, we found that seed germination and protocorm development reflected both the distribution and abundance of appropriate mycorrhizal fungi. Furthermore, seed germination and DNA-based techniques were complementary ways of detecting mycorrhizal fungi in the soil. Conditions with abundant compatible fungi were often clustered around existing adult plants and, at that small scale, likely reflected the distribution of individual fungal genets. Hence, the distribution of mature orchids likely reflects patterns of host fungus distribution and 'hot spots' of abundance that, in turn, may be related to fine-scale spatial variation in soil conditions or stochastic fungal dispersal. Our results point to a need to explicitly include both seed germination and molecular detection of appropriate mycorrhizal fungi and to examine consistency in seed germination and fungal abundance over time. Such studies will support investigation of the factors that affect the fine-scale abundance of mycorrhizal fungi (McCormick *et al.* 2012). Understanding what factors affect the abundance of specific host fungi will be critical for orchid conservation and may also have implications for the ecology and conservation of other less specific mycorrhizal plants (van der Heijden *et al.* 1998).

Acknowledgements

This work was funded by NSF grant DEB-0316523. Microsatellite development was funded by a grant from the Frank Bertrum Sherry Endowment at the Smithsonian Institution to DFW and MKM. RKB was funded by NSF grant REU-0353759. We also wish to thank John P. O'Neill for field and laboratory help and for maintaining fungal collections and the Smithsonian Laboratory of Analytical Biology for DNA sequencing and microsatellite analysis. The manuscript also benefitted from the constructive suggestions of multiple referees.

Conflict of interests

The authors declare no conflict of interests.

Data accessibility

Seed packet locations, germination and protocorm data, and fungus abundance are available in DRYAD entry doi: 10.5061/dryad.4n5h7 (McCormick *et al.* 2016). Fungal cultures are available from D.F. Whigham and M.K. McCormick upon request.

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Received 29 September 2015; accepted 4 February 2016
Handling Editor: Hans Jacquemyn

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Details on the design and testing the specific PCR primers used to determine the distribution and abundance of specific orchid mycorrhizal fungi in the soil.