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Research

Nonrandom seedling establishment corresponds with distance-dependent decline in mycorrhizal abundance in two terrestrial orchids

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Summary

- In plant species that critically rely on mycorrhizal symbionts for germination and seedling establishment, distance-dependent decline of mycorrhizal fungi in the soil can be hypothesized to lead to significant spatial clustering as a result of nonrandom spatial patterns of seedling establishment. To test this hypothesis, we investigated the abundance and distribution of mycorrhizal fungi in the soil and how they relate to spatial patterns of adults and seedling recruitment in two related orchid species.
- We combined assessments of spatial variation in fungal abundance using quantitative PCR (qPCR) with spatial point pattern analyses based on long-term demographic data and cluster point process models.
- qPCR analyses showed that fungal abundance declined rapidly with distance from the adult host plants. Spatial point pattern analyses showed that successful recruitment in both species was clustered significantly around adult plants and that the decline in the neighborhood density of recruits around adults coincided with the decline of fungal abundance around adult plants.
- Overall, these results indicate that the distribution and abundance of fungal associates in the soil may have a strong impact on the aboveground distribution of its partner.

Introduction

Comprehensive investigations of spatial patterns of seedling recruitment in plants have shown that most recruits do not establish randomly in populations, but often show highly aggregated distribution patterns and are often spatially associated with their parents (Nathan & Muller-Landau, 2000; Wiegand *et al.*, 2007, 2009; but see Getzin *et al.*, 2014). This highly aggregated distribution pattern is generally attributed to various mechanisms, including seed dispersal across limited distances (Jersáková & Malinová, 2007), clumped seed dispersal by animals (Howe, 1989) and/or limitation of suitable habitat patches wherein germination and recruit establishment can occur (Jacquemyn *et al.*, 2010a). Recent advances in spatial point pattern analyses have shown that spatial patterns of seedling recruitment can be fairly complex and may include patterns with multiple critical scales of clustering and isolated recruits (Wiegand *et al.*, 2009). Thus, relatively complex point process models need to be used to describe the clustered pattern of seedling recruitment, which, in turn, can help to unravel the potential drivers of variation in spatial patterns of seedling recruitment (Wiegand & Moloney, 2014).

In plant species that critically rely on mycorrhizal fungi, the spatial distribution and abundance of suitable mycorrhizal fungi may be primary factors influencing spatial patterns of seed germination and seedling recruitment (McCormick & Jacquemyn, 2014). Previous research has shown that germination, subsequent seedling recruitment and establishment in orchids are critically dependent on the presence of orchid mycorrhizal (OrM) fungi (Smith & Read, 2008; Rasmussen & Rasmussen, 2009). The spatial distribution and abundance of OrM fungal associates, however, is generally assumed to be distributed independently from their orchid associates, as they themselves are not dependent on this relationship for survival (Dearnaley *et al.*, 2012; McCormick *et al.*, 2012; McCormick & Jacquemyn, 2014; van der Heijden *et al.*, 2015), but direct proof for this has not yet been given.

Orchid species have been shown to associate with distinct sets of mycorrhizal fungi and often exhibit spatial segregation by species (Lievens *et al.*, 2010; Waterman *et al.*, 2011; Jacquemyn *et al.*, 2012a,b, 2014). It can therefore be expected that the spatial occurrence of these OrM fungi in the soil may be responsible, at least in part, for the clustered structure of recruits and adult

plants in natural orchid populations (Diez, 2007; McCormick *et al.*, 2009). Observational studies have indeed repeatedly shown strong correlations in spatial distribution of orchid adults and recruits, and have provided some evidence that seed dispersal is necessarily not the primary cause (Jacquemyn *et al.*, 2007, 2009b). This suggests that the local availability and abundance of suitable OrM associates declines with increasing distance from parent plants, therefore reducing the likelihood of successful germination with increasing distance from parent plants. This hypothesis has been supported by seed germination experiments, which have shown that germination often declines with increasing distance from adult plants (McKendrick *et al.*, 2000, 2002; Batty *et al.*, 2001; Diez, 2007; Jacquemyn *et al.*, 2012b). However, to our knowledge, the distance-dependent decline in OrM fungal presence with increasing distance from parent plants has not yet been quantified.

In addition, none of the studies investigating the spatial variation in seed germination have also monitored subsequent successful growth to the recruit stage. Given that recruits have been shown to associate with a narrower range of mycorrhizal fungi than the more promiscuous seeds and adults (Bidartondo & Read, 2008) and because it may take several years before an orchid seed develops into a seedling (Rasmussen, 1995), it remains unclear whether habitat patches that are suitable to promote seed germination are also suitable to support successful recruit establishment. In addition to mycorrhizal availability, we expect that a multitude of other factors may influence germination and seedling recruitment in orchids. These factors include, but are not necessarily limited to, physical microsite conditions (light, moisture, temperature) and inter/intraspecific competition (reviewed in Rasmussen *et al.*, 2015). Given these diverse factors, many of which are diffuse or temporal, it therefore remains unclear what truly defines suitable establishment sites and subsequent distribution patterns for different orchid species (McCormick *et al.*, 2012; McCormick & Jacquemyn, 2014).

In this study on two natural populations of the phylogenetically related orchid species *Orchis mascula* and *O. purpurea*, we investigated the abundance and distribution of mycorrhizal fungi in the soil and how they relate to the spatial patterns of adults and seedling recruitment. Previous results have shown that both species are characterized by significant spatial clustering of adult individuals within populations (Jacquemyn *et al.*, 2007, 2009b). We therefore hypothesize that the mycorrhizal fungi associating with the roots of these orchid species rapidly decline in abundance with increasing distance from their orchid host, and that spatial patterns of orchid recruit establishment are related to fungal availability. To test these hypotheses, we first assessed spatial variation in fungal abundance using quantitative PCR (qPCR). More specifically, we used qPCR to determine the abundance of two OrM Tulasnellaceae fungi within roots of their species-specific *Orchis* hosts and the surrounding soil at different distances from adult plants. The output of the molecular analyses was then combined in a second step with spatial point pattern analyses based on long-term demographic observations, wherein the spatial location of adult individuals and seedling recruitment was rigorously recorded. We fitted cluster point process models to the spatial data to describe the spatial pattern of recruitment and to investigate if (and how) the density of recruits declined with distance to adults.

Materials and Methods

Study species

Orchis mascula L. (Early Purple Orchid) and *O. purpurea* Huds. (Lady Orchid) are tuberous perennial orchid species that are widely distributed throughout Europe, western Asia and northern Africa (Kretzschmar *et al.*, 2007). Both species occur primarily in limestone woodlands, although they can also be found in calcareous grasslands. The two species flower in late spring from mid-May until mid-June (Nilsson, 1983; Jacquemyn *et al.*, 2009a). Previous research has shown that the majority of *Orchis* OrM fungal associates are members of the Tulasnellaceae family, with *O. mascula* individuals reportedly forming exclusive associations with one Tulasnellaceae fungus and *O. purpurea* sometimes found in association with more than one Tulasnellaceae taxon simultaneously (Jacquemyn *et al.*, 2010b, 2011, 2012b; Lievens *et al.*, 2010; Girlanda *et al.*, 2011).

Study system and monitoring of seedling establishment

The study was conducted in two previously described natural *Orchis* populations within Belgium, one for each studied species. The study site of *O. mascula* was located in the Calestienne region (southern Belgium; 50°04′N, 4°35′E) and consisted of a species-rich woodland that has been undisturbed for > 30 yr (site 2 in Jacquemyn *et al.*, 2009b). Within this site, a 10 × 10-m² plot was established and all individual plants were accurately mapped and monitored between 2006 and 2013. The study population of *O. purpurea* was situated in a species-rich forest in Voeren (western Belgium; 50°44′N, 5°50′E). Here, the species has increased in abundance over the past 10 yr after the forest was coppiced and cleared of shrubs (see Jacquemyn *et al.*, 2007 for more details). At this site, a 10 × 16-m² plot was established and, within this plot, the spatial distribution and seedling recruitment were intensively monitored between 2003 and 2015. In both plots, all individuals were mapped to the nearest centimeter and their life history stages were determined, adults being defined as plants with at least two large leaves and recruits being defined as small individuals with only one or two small leaves (Jacquemyn *et al.*, 2007, 2009b).

Sampling for assessment of fungal abundance

For each of the two studied orchid species, root samples were obtained from 10 adult plants within both study sites. Plants were selected haphazardly from among orchids without visible neighboring adult orchids within a radius of 1 m. In addition, soil samples were collected in each cardinal direction (north, south, east, west) at five different distances (5, 10, 15, 25, 50 cm) around each sampled orchid individual (Supporting Information Fig. S1). Soil plug samples were taken to a depth of 5 cm (*c.* 5 g

FW) using a sterile sampling tube at each sampling point to avoid cross-contamination. Altogether, this resulted in 420 unique samples (10 *O. mascula* plants, 10 *O. purpurea* plants, 20 soil samples per *Orchis* plant) for DNA extraction and subsequent molecular analysis. All plant and soil samples were initially stored at 4°C and processed (subsampling, DNA extraction) within 96 h of sampling.

At least three complete roots from each sampled orchid individual were surface sterilized (30 s of submergence in 1% sodium hypochlorite, followed by three 30-s rinse steps in sterile distilled water) and microscopically checked for mycorrhizal colonization. Subsequently, the distal 5-cm portions of colonized roots were sectioned into 5–10-mm fragments, which were then mixed to create a homogeneous mycorrhizal root sample for each individual plant and to facilitate tissue breakdown during DNA extraction. From each mycorrhizal root sample, two 0.25-g subsamples were selected and separate DNA extractions were performed using the UltraClean Plant DNA Isolation Kit as described by the manufacturer (Mo Bio Laboratories Inc., Carlsbad, CA, USA). Soil samples were individually homogenized and visible debris (stones, twigs, roots, etc.) was manually removed, although fine particles that may contain fungal propagules were retained. DNA extractions were performed on two separate 0.25-g soil subsamples per soil sample using the PowerSoil DNA Isolation Kit as described by the manufacturer (Mo Bio Laboratories Inc.). Each pair of DNA extracts was then pooled, quantified using a Qubit fluorometer with the high-sensitivity DNA reagent kit (Invitrogen, Carlsbad, CA, USA) and stored at −80°C.

Assessment of fungal abundance

454-amplicon pyrosequencing revealed that the most abundant OrM fungi associating with the investigated orchid species corresponded to two distinct Tulasnellaceae taxa (operational taxonomic units, OTUs) that were detected on (and exclusive to) either *O. purpurea* (OTU1) or *O. mascula* (OTU2) roots (Table S1); they had both been observed previously on their respective hosts (Jacquemyn *et al.*, 2010b; Lievens *et al.*, 2010). For each of these OTUs, a qPCR assay was developed and validated based on the internal transcribed spacer (ITS) 2 region (Table 1). The developed assays were utilized to quantify target Tulasnellaceae OTU DNA from each root and soil DNA sample on an ABI StepOnePlus real-time PCR instrument (Life Technologies, Carlsbad, CA, USA). Reactions were performed in a total volume of 25 µl consisting of 12.5 µl of iQ SYBR Green PCR Super Mix (Bio-Rad Laboratories, Hercules, CA, USA), 1.0 µl DNA template, 9.0 µl H_2O and 1.25 µl (10 mM) of each forward and reverse primer, depending on the target OTU (Table 1). Amplifications were run as follows: initial denaturation for 5 min at 95°C, followed by 40 cycles of 15 s of denaturation at 94°C, 30 s of annealing at 54.5°C (OTU1) or 55.5°C (OTU2), and 30 s of elongation at 72°C. Each sample extract was amplified in duplicate and quantified using a standard curve amplified in triplicate. Standard curves (range, 1.0E + 03–1.0E + 08 mol μl^{-1}) were generated using six 10-fold dilutions of target DNA amplified from *O. mascula* (OTU2) or *O. purpurea* (OTU1) roots. A melting curve analysis was performed after each analysis to confirm product specificity. In addition, amplification accuracy was verified by Sanger sequencing of a number of generated amplicons. In this way, product identity was confirmed for all samples considered to be positive using the qPCR assays (Ct value < 29 and a single melting peak at the expected melting temperature; Table 1; Fig. S2a,c). Additional positive (DNA extract from roots colonized with OTU2, *O. mascula*, or OTU1, *O. purpurea*, fungi) and negative control reactions (no template DNA) were included in triplicate with all analyses to verify target-specific amplification and the absence of contaminants, respectively.

Analysis of plant spatial structure

To analyze the spatial patterns of seedling recruits and adult orchids, we conducted two analyses. In a first step, we characterized the spatial pattern of the recruits in detail and, in a second analysis, we characterized the spatial association pattern of recruits relative to adults. Our working hypothesis was that recruits were clustered around the adults as a result of a distance-dependent decline in seedling establishment caused by a declining abundance in OrM fungi.

To quantify the spatial point pattern of the recruits, we used four summary statistics that are together able to characterize complex clustered point patterns (Wiegand *et al.*, 2013). First, the pair correlation function $g(r)$ describes the expected density of recruits at distance r from the typical recruit, divided by the

Table 1 Primer combinations designed and used for the amplification of target operational taxonomic units (OTUs) from *Orchis mascula* and *O. purpurea* roots and their surrounding soil using quantitative PCR (qPCR) including closest GenBank match (Benson *et al.*, 2008)

Primer combination	Target OTU (source)	GenBank match	Primer name (direction)	Sequence (5′–3′)	Annealing temp. (°C)	Melting temp. (°C)	Product length (bp)
OTU1f_1g/Tul_r1	OTU1 (*O. purpurea*)	GQ907280.1 Tulasnellaceae clone OpuE02_B_2	OTU1f_1g (Forward)	ATGATCATCTCAA ACCTTKCGYTTC	54.5	84.7	315
			Tul_r1 (Reverse)	AGCGGGTARTCC YACCCGAG			
OTU2f_2g/Tul_r3	OTU2 (*O. mascula*)	GU066934.1 Tulasnellaceae clone OmaF04_A_2	OTU2f_2g (Forward)	CCAATGCTCTGA CGAAAGCACTC	55.5	81.8	299
			Tul_r3 (Reverse)	CAGCGGGTARTCC YACCCGAGTTG			

overall density λ of recruits (i.e. number of recruits divided by the area of the observation window W). Second, we also used the K-function, the cumulative version of the pair correlation function, that is, $K(r) = \int_{r'=0}^{r} g(r')2\pi r' dr'$, which was based on the expected number of recruits within distance r from the typical recruit, and its square-root transformation $L(r) = \sqrt{K(r)/\pi} - r$. Finally, important additional information was provided by the probability $D(r)$ that the typical recruit has its nearest neighbor within distance r, and by the probability $H_s(r)$ that a random test point has its nearest recruit within distance r (Illian *et al.*, 2008; Wiegand & Moloney, 2014). $D(r)$ characterizes the internal cluster structures, whereas $H_s(r)$ characterizes the 'holes' in the pattern (Wiegand & Moloney, 2014).

To quantify the relationship of recruits relative to the adult orchids, we used the bivariate counterparts $g_{12}(r)$, $L_{12}(r)$ and $D_{12}(r)$ of $g(r)$, $L(r)$ and $D(r)$, respectively. For example, the bivariate pair correlation function $g_{12}(r)$ describes the mean density of recruits (indicated by subscript 2) at distance r from the typical adult (adults are indicated by subscript 1), divided by the intensity λ_2 of recruits.

Analysis of recruits To describe the spatial pattern of seedling recruitment, we fitted a series of increasingly more complex Thomas point process models to the spatial data (Wiegand & Moloney, 2014, their section 4.1.4). For details, see Methods S1.

The 'simple Thomas process' consists of randomly and independently distributed clusters of points. The cluster centers are characterized by their density ρ, the n points are randomly assigned to a cluster and the points of a cluster are scattered around their cluster center following a two-dimensional normal distribution with standard deviation σ. These three rules generate patterns with one critical scale of clustering defined by the parameter σ (where the cluster radius is *c.* 2σ). Analytical formulae exist for $g(r)$ and $K(r)$ (see Eqn S1), which allows for straightforward fitting of the parameters to the observed $g(r)$ and $K(r)$ (Wiegand *et al.*, 2007, 2009; Wiegand & Moloney, 2014).

However, the cluster centers (i.e. the adults) may themselves show a clustered distribution pattern. To consider this, the 'nested double-cluster Thomas process' (Eqn S2) assumes that the cluster centers (e.g. adults) follow a simple Thomas process where small clusters (of recruits) are scattered around the clustered adults (Wiegand *et al.*, 2007, 2009). The parameters describing the two critical scales of clustering are σ_1 (large scale, adults) and σ_s (small scale, recruits).

Finally, a number of isolated recruit individuals may appear which are not related to a nearby adult. To accommodate such isolated recruits, we modeled an independent superposition of a random pattern with a nested double-cluster Thomas process (Eqn S3; Wiegand *et al.*, 2007, 2009). The new parameter p_C, the proportion p_C of points belonging to the clustered component pattern, does not affect $g(r)$ and $K(r)$, but $D(r)$ and $H_s(r)$, which are therefore used to fit p_C (Wiegand *et al.*, 2009).

Analysis of adult–recruit relationship We hypothesized that seedling recruitment is scattered around the locations of adults. This suggests the use of a 'bivariate parent–offspring Thomas process with clustered parents', where the (clustered) adults (focal pattern 1) form the cluster center of the recruits (pattern 2) (Eqn S4).

We observed that not all adults had recruits in their immediate neighborhood. We therefore modified the bivariate Thomas process and allowed for a proportion p_e of 'empty' clusters (i.e. adults not being the center of a recruit cluster). The bivariate $g_{12}(r)$ and $K_{12}(r)$ are not influenced by the parameter p_e, but the bivariate distribution of nearest-neighbor distances $D_{12}(r)$ is sensitive to changes in p_e and can be used to fit p_e.

The parameters of the double-cluster Thomas processes fitted to the recruits should coincide with the parameters of the bivariate parent–offspring Thomas process with clustered parents. This allowed us to check whether the fitted point process models were consistent.

Results

Fungal abundance and distribution in the soil

qPCR standard curves based on known concentrations of target DNA showed a linear correlation (OTU1, $r^2 = 0.998$; OTU2, $r^2 = 0.997$) between log values of input DNA and qPCR threshold cycles over at least six orders of magnitude (Fig. S2b,d), enabling accurate quantification of our target fungi in terms of target DNA molecules μl^{-1} DNA extract (Table S2). The highest concentrations of target OrM DNA were consistently obtained from orchid root samples, with the average OTU1 concentration obtained from *O. purpurea* roots being 235 149 mol μl^{-1} DNA extract (SE = 14 064 mol μl^{-1}) and the average OTU2 concentration from *O. mascula* roots being 415 292 mol μl^{-1} DNA extract (SE = 90 943 mol μl^{-1}). The concentration of these target OTUs in the soil surrounding their orchid host decreased rapidly with increasing distance from the orchid plant (Fig. 1), with OTU1 decreasing from an average concentration of 28 234 mol μl^{-1} (SE = 5912 mol μl^{-1}) at a distance of 5 cm from *O. purpurea* plants to 345 mol μl^{-1} (SE = 143 mol μl^{-1}) at 50 cm. Likewise, the average OTU2 concentration of 10 201 mol μl^{-1} (SE = 1472 mol μl^{-1}) at 5 cm from *O. mascula* plants decreased to 1456 mol μl^{-1} (SE = 704 mol μl^{-1}) at 50 cm. Nevertheless, there were some intervals wherein the target OTU was not detected at one radial sampling point, but was detected at a more distal location, although the general trend of decline prevailed (Table S2).

Plant spatial structure

Results for *O. purpurea* The recruits of *O. purpurea* showed very strong clustering (Fig. S3b), and 80% of the recruits had their nearest neighbor within 17 cm (Fig. S3e). However, 11% (18) of the recruits had no nearest neighbor within 40 cm (Fig. S3e), indicating the presence of some isolated recruits.

Although the nested double-cluster Thomas process (Eqn S2) fitted $g(r)$ and $K(r)$ well (Fig. S3b,c; see Table S3 for parameter estimates), it did not fit the nearest-neighbor summary statistics

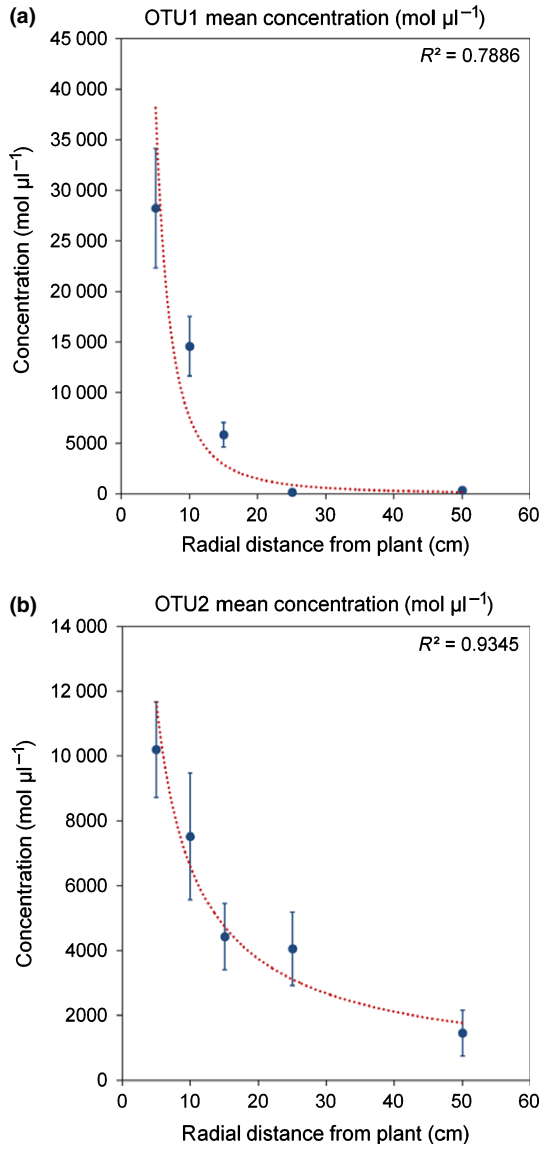

Fig. 1 Quantitative PCR (qPCR) results for (a) OTU1 and (b) OTU2 (Tulasnellaceae identified orchid mycorrhizal (OrM) fungal operational taxonomic units (OTUs) detected in association with *Orchis purpurea* and *Orchis mascula* roots, respectively), including mean concentration of target DNA (mol μl^{-1}) calculated from radial soil sample qPCR results obtained at five distances from *Orchis* plants (5, 10, 15, 25 and 50 cm), where each data point indicates the mean of 80 samples with error bars representing ± standard error (SE).

$D(r)$ and $H_s(r)$ (Fig. S3d,e). The departures in $H_s(r)$ and $D(r)$ indicated that there were more isolated recruits than expected by this point process. Consequently, we found that a Thomas process with superposition of random points (Eqn S3) produced, for 25 isolated points (16% of all recruits), a reasonable agreement in all four summary statistics (Fig. S3b,c,f,g). With this parameter (i.e. $p_C = 0.84$), we obtained an estimate of 46 $p_C^2 = 33$ large clusters (Table S3).

The bivariate pattern of recruits around adult individuals of *O. purpurea* is illustrated in Fig. 2(a). There was a substantial proportion of adults that had no recruits within 40 cm, and there were isolated recruits having no adult within 40 cm (blue circles). In a first step of the bivariate adult–recruit analysis, we fitted a simple Thomas process (Eqn S1) to the pattern of all adults and found a strong small-scale clustering (with a cluster radius of some 5 cm), but otherwise basically a random pattern at larger distances (Fig. S4). As indicated by $D(r)$, only few adults (say 10%) had a nearest neighbor within the cluster diameter (Fig. S4c). This indicates that the clustering is caused by a few adults which are located close to each other (see also Fig. S3).

In a second step of our adult–recruit analysis, we assumed that the recruits were clustered around the clustered adults, using the corresponding bivariate Thomas process (Eqn S4). Because we were interested in the small-scale adult–recruit relationship, we excluded the isolated recruits from the bivariate analysis. The fit with Eqn S4 revealed a parameter $\sigma_r = 10.0$ cm that coincided well with the parameter $\sigma_1 = 11.9$ cm of the large clusters of the recruit pattern (Fig. S3) and a parameter $\rho_r = 0.000046$ (corresponding to 72 cluster centers; note that there are 72 adults). This suggested that the large-scale clustering of the (non-isolated) recruits was caused by their scattering around adults. Interestingly, both adults and recruits showed an additional small-scale clustering cluster radius of *c.* 5 cm (Table S3).

However, the point process of Eqn S4 substantially overestimates the bivariate $D_{12}(r)$ (Fig. 2d), which means that not all adults are surrounded by a recruit cluster. Using a variant of this point process (where a proportion p_e of the adults had no associated recruit cluster), we found the best fit of $D_{12}(r)$ for a value of $p_e = 0.5$ (Fig. 2e). This yielded 36 adults with an associated recruit cluster, which coincided well with the estimate from the univariate analysis of recruits, which yielded 33 large clusters (Table S3). The results of the two point process models were therefore in good agreement, suggesting that they described the data well.

Results for *O. mascula* The recruits of the species *O. mascula* also showed strong clustering (Fig. S5b) and three-quarters of the recruits had their nearest neighbor within 20 cm (Fig. S5c). The nested double-cluster Thomas process (Eqn S2) fitted $g(r)$ and $L(r)$ well (Fig. S5b,c; Table S4). We found, for 10 isolated points (9% of all recruits), a reasonable agreement in the nearest-neighbor summary statistics $D(r)$ and $H_s(r)$ (Fig. S5d,e). Considering the 9% of the recruits belonging to the random component pattern (i.e. $p_C = 0.91$), we obtained an estimate of 96 small clusters (Table S4).

Figure 3(a) shows the bivariate recruit–adult pattern of *O. mascula*. There was a substantial proportion of adults that had no recruits within 20 cm, and there were isolated recruits having no adult within 20 cm (blue circles). Again, we excluded the isolated recruits from the bivariate analysis. In a first step, we fitted a simple Thomas process (Eqn S1) to the pattern of all adults and found a large-scale clustering with parameter $\sigma_a = 24.6$ cm (coinciding well with the critical scale of large-scale clustering of the recruits of $\sigma_1 = 26.7$ cm) (Table S4). However, the adults showed an additional small-scale clustering with parameter $\sigma_s = 5.2$ cm (Fig. S6) distributed in 187 small clusters. In a second step, we assumed that the recruits were clustered around the adults, used

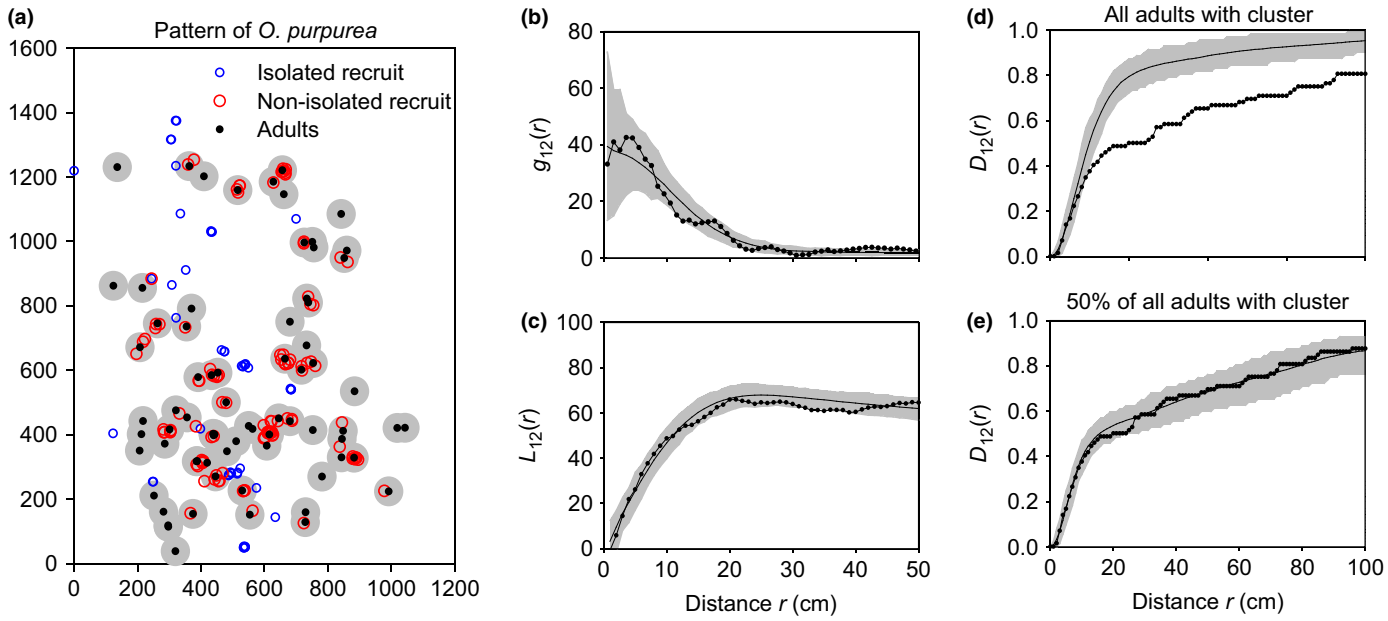

Fig. 2 Analysis of the spatial association of recruits and adults of the species *Orchis purpurea*. (a) Spatial pattern of adults and recruits. Isolated recruits (open blue circles) have no nearest adult neighbor within 40 cm (the 40-cm neighborhood is indicated by the gray shaded circles around the adults). Recruits with a nearby adult are indicated by open red circles. (b–e) Results of the fitted bivariate parent–offspring Thomas process with clustered parents (Supporting Information Eqn S4). Closed circles, observed summary statistics; black solid line, expectation under the cluster process; gray band, simulation envelopes being the fifth largest and highest value of the summary statistic taken from 199 simulations of the cluster model. (d) The bivariate nearest-neighbor distribution function $D_{12}(r)$ for the cluster process in which all adults have a recruit cluster and (e) for the case in which only a proportion $p_A = 0.5$ of all adults have a recruit cluster. Table S3 shows the fitted parameter values.

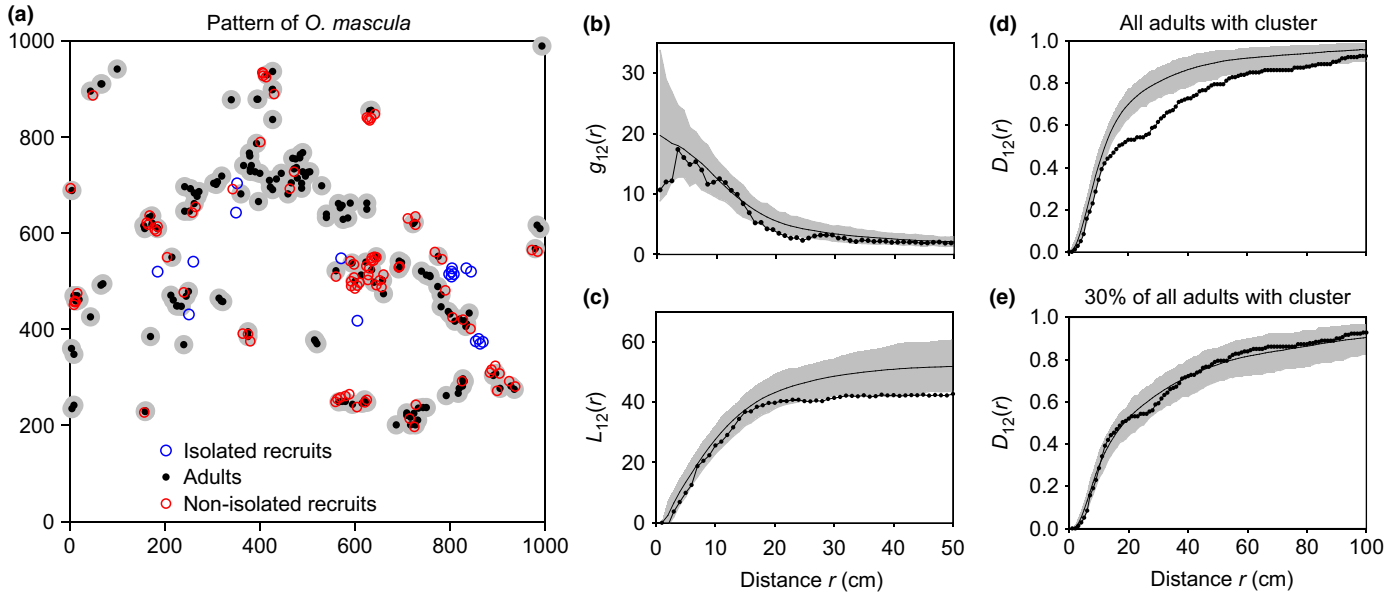

Fig. 3 Analysis of the spatial association of recruits and adults of the species *Orchis mascula*. (a) Spatial pattern of adults and recruits. Isolated recruits (open blue circles) have no nearest adult neighbor within 40 cm (the 40-cm neighborhood is indicated by the gray shaded circles around the adults). Recruits with a nearby adult are indicated by open red circles. (b–e) Results of fitted bivariate parent-offspring Thomas process with clustered parents (Supporting Information Eqn S4). Closed circles, observed summary statistics; black solid line, expectation under the cluster process; gray band, simulation envelopes being the fifth largest and highest value of the summary statistic taken from 199 simulations of the cluster model. (d) The bivariate nearest-neighbor distribution function $D_{12}(r)$ for the cluster process in which all adults have an associated recruit cluster, and (e) for the case in which only a proportion $p_A = 0.7$ of all adults have a recruit cluster. Table S4 shows the fitted parameter values.

the parameters σ_a and ρ_a of the large-scale clustering of adults, and considered that only a proportion p_e of adults had a recruit cluster. Fitting the corresponding bivariate Thomas process (Eqn S4) revealed a parameter $\sigma_r = 8.27$ cm, which coincided well with the parameter $\sigma_l = 5.6$ cm of the large clusters of the recruit pattern (Fig. S5). This point process fitted $g(r)$ and $L(r)$

reasonably well (Fig. 3b,c), and we found that only 30% of all adults have an associated recruit cluster (i.e. $p_e = 0.7$) (Fig. 3d,e). This yielded 70 adults with a recruit cluster, which coincided reasonably well with the 96 small recruit clusters found in the univariate analysis of the recruits (Table S4).

Linking patterns of fungal abundance to patterns of orchid recruitment

Figure 4 links the results of the analysis of the spatial distribution of mycorrhizal fungi around adult orchids with the spatial point pattern analysis that revealed how the recruits were distributed around the adults. For both species, we found a remarkable concordance between the decrease in the concentration of target DNA (mol μl^{-1}) with distance r from the adults and the decrease in the density of recruits with distance from the adults as described by the pair correlation function (Fig. 4). For *O. mascula*, we found that a large-scale component was involved in the decline of the concentration of target DNA, which coincided largely with the large-scale clustering of the adults (Fig. S6) with a parameter σ of *c.* 25 cm. Thus, the decline over the first 15 cm reflected the mycorrhizal fungi corresponding to the small clusters of the focal adult (with a radius of *c.* 16 cm), whereas the 'tail' of the mycorrhizal fungi that extended from 15 cm to somewhat > 50 cm corresponded to the mycorrhizal fungi of other adults of the same large cluster (with a radius of *c.* 50 cm). By contrast, the adults of the species *O. purpurea* showed no second critical scale of larger scale clustering. Therefore, we do not observe such a tail for *O. purpurea*.

Discussion

Successful recruit establishment in orchids is dependent on the complex interaction of multiple abiotic (resources, microsites, etc.) and biotic (competition, predation, etc.) factors (McCormick *et al.*, 2012; McCormick & Jacquemyn, 2014; Rasmussen *et al.*, 2015). Previous seed germination experiments have shown that, beyond spatial clustering near conspecific plants, seed germination is also affected by soil moisture, pH and organic content (Batty *et al.*, 2001; Diez, 2007). However, whether these factors affect orchids directly or whether seed germination is affected indirectly through mycorrhizal fungi has not been examined. In this study, we combined the assessment of OrM fungal abundance using qPCR with detailed spatial point pattern analyses based on long-term demographic data to elucidate the processes determining seedling recruitment in two terrestrial orchids. To assess their distribution and abundance in the soil surrounding their respective orchid host, qPCR methodology was successfully designed and applied for the detection of two distinct Tulasnellaceae OrM associates that have been described previously within the roots of either *O. purpurea* (OTU1) or *O. mascula* (OTU2) (Jacquemyn *et al.*, 2010b; Lievens *et al.*, 2010). Our results showed that successful seedling recruitment exhibited complex spatial patterns with two critical nested spatial scales of clustering, and that the adult plants formed the cluster centers of the recruits. More importantly, we found a good correlation between the decline in recruit density and the decline in OrM fungal abundance with increased distance from the adult plant. These results suggest that the observed distance-dependent decline in fungal abundance is a strong driver in determining recruitment patterns in both orchid species.

Abundance of mycorrhizal fungi declines sharply with increasing distance from orchid roots

Previous molecular studies based on assignment tests have shown that seeds are dispersed across distances that are much larger than the average distances separating adult plants and recruits (Jacquemyn *et al.*, 2007, 2009b), suggesting that limited seed dispersal is an unlikely source of the observed patterns of seedling recruitment in both populations. The observed spatial clustering of recruits in close proximity to adult plants therefore more likely

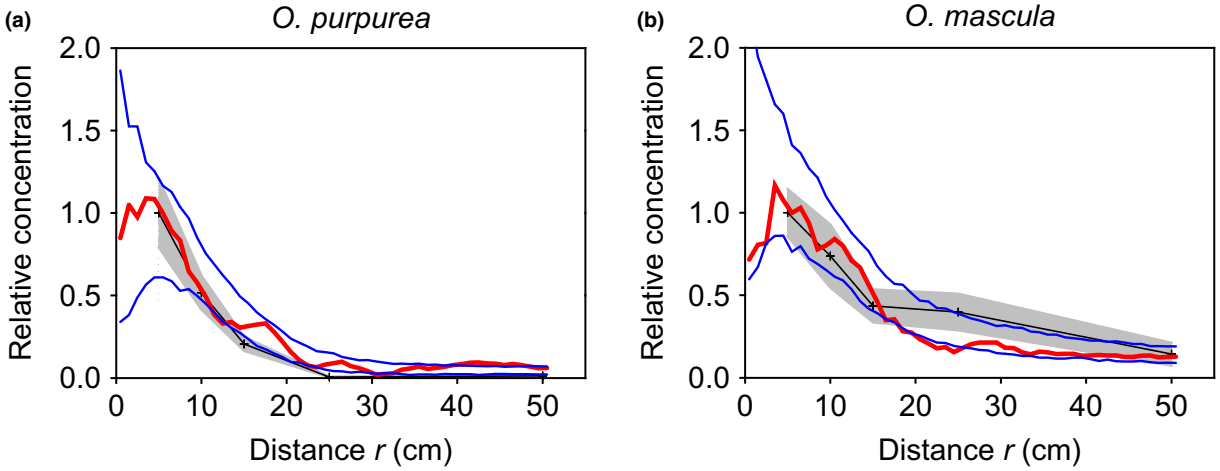

Fig. 4 Combination of the results of the spatial mycorrhizal fungal analysis with those of the spatial point pattern analysis for the species (a) *Orchis purpurea* and (b) *Orchis mascula*. The gray envelope shows the range of the standard errors of the (scaled) mean concentration of target DNA (mol μl^{-1}) at five distances from *Orchis* plants, and the red line shows the (scaled) pair correlation function describing how the density of recruits around adult orchids (i.e. the cluster center) declines with distance r. The blue lines are the simulation envelopes of the fitted cluster process.

reflects the occurrence of localized concentrations of suitable OrM fungi and thus suitable patches for seedling establishment (Rasmussen, 1995; Otero & Flanagan, 2006; Bidartondo & Read, 2008; McCormick *et al.*, 2009; McCormick & Jacquemyn, 2014). These expectations were confirmed by our qPCR analyses, which clearly showed that the abundance of a known OrM fungal associate declined sharply with increasing distance from adult plants in exactly the same way as recruit density declined with distance from adults (Fig. 4). We have subsequently observed similar results in experiments on other orchid species (*Anacamptis morio*, *Gymnadenia conopsea* and *Orchis mascula*), wherein the similarity in fungal communities associating with adult plants and in soil samples taken at various distances from adult plants sharply declined with increasing distance (Waud *et al.*, 2016).

A declining abundance of mycorrhizal fungi with increasing distance from adult plants may suggest that orchids maintain fungal communities to some extent, so that the distribution of orchid plants determines the distribution of their OrM associates. However, our results also showed that both OrM fungal OTUs may be sporadically observed with low abundances at larger distances from adult plants. Interestingly, OTU1 was more sporadically distributed in the soil surrounding *O. purpurea* plants than was OTU2 in the soil surrounding *O. mascula*, despite both qPCRs showing similar sensitivity. In addition, there were rare occurrences of these OTUs being detected at low concentrations (< 1000 mol μl^{-1}) within soil with no neighboring positive sampling points. These variations indicate that different fungal strains may have highly complex distribution patterns of low abundance in the soil outside the close proximity of orchid associates, and may explain the sporadic occurrence of isolated recruits in our study populations.

Orchid distribution and establishment

As mentioned previously, recruits were not randomly distributed within the studied populations of either *Orchis* species, but showed clear signs of spatial clustering around adult plants. This indicates that nonrandom seedling establishment contributes to the significant aggregation patterns that have been repeatedly observed in natural orchid populations (Chung *et al.*, 2005; Jacquemyn *et al.*, 2007, 2009b). In both species, recruits were strongly clustered around central adult plants, although not all adults had (visible) recruits nearby, and not all recruits were clustered with an adult (Figs 2a, 3a). These 'isolated' individuals may result from some flowering adults not setting seeds, the 4-yr time lag between seed production and seedling emergence from the soil, occasional seed dispersal at larger distances, or OrM mycorrhizal availability at locations at which current adult plants are scarce (Jacquemyn *et al.*, 2007, 2009b).

There were some marked differences between the two species in the spatial clustering of individuals. When excluding recruits that were placed far away from adults from the adult–recruit analysis, and fitting a cluster point process to the data in which the adults were the cluster centers and the recruits were tightly clustered around the adults, the recruits of *O. purpurea* were clustered at two critical scales, with the larger scale coinciding well with the clustering around adults. For *O. mascula*, this pattern was less clear because the *O. mascula* adults were themselves clustered with two critical scales (in almost exactly the same way as their recruits). These differences may be explained by differences in seed production and subsequent dispersal. Whereas flowering in *O. purpurea* was quite regular and most flowering plants set seed, flowering in *O. mascula* was much more irregular, and a large proportion of flowering plants failed to set, either as a result of herbivory (most often by snails) or because of insufficient pollination (Jacquemyn *et al.*, 2009a). Moreover, flowering plants of *O. purpurea* are much taller than flowering plants of *O. mascula*, which may increase seed dispersal distances. Low and irregular seed production, combined with smaller seed dispersal distances, may restrict the chance of seeds being dispersed further away from mother plants, leading to the formation of clusters of adult plants in time.

Interestingly, *O. purpurea* has been shown previously to be capable of associating with multiple OrM fungi, whereas *O. mascula* has generally been observed in more specialized association with one Tulasnellaceae OrM fungus (Jacquemyn *et al.*, 2010b, 2011, 2012b; Lievens *et al.*, 2010). These differences in fungal association specificity between *Orchis* species may contribute to the differences observed in spatial clustering, wherein the broader specificity of *O. purpurea* may lend itself to successful recruitment in disparate patches of an alternative OrM fungal associate, thus contributing to the likelihood of isolated recruits of this species. However, in the investigated population of *O. purpurea*, re-analysis of the associated sequences, together with newly generated sequences obtained through next-generation sequencing, indicated that the majority probably belong to the common OTUs studied herein, when clustered at 97% sequence similarity. Overall, these observations therefore indicate that, in both species, a single fungal OTU appears to be responsible for seed germination and subsequent seedling recruitment, although the possibility that fungi other than those detected here have contributed to seedling establishment cannot be ruled out completely. However, previous research has shown that seedlings tend to have the highest specificity towards mycorrhizal fungi (Bidartondo & Read, 2008), whereas seeds and adults tend to have broad specificity. In any case, orchid species that exhibit more complex OrM association patterns would therefore require further investigation to definitively ascertain what constitutes suitable establishment sites and distribution patterns (McCormick *et al.*, 2012; McCormick & Jacquemyn, 2014).

Conclusions

Our results have clearly shown that OrM abundance significantly declines with increasing distance from adult plants and that successful seedling establishment to a large extent follows this pattern of declining OrM associate abundance. Given that more seeds germinate than typically establish as seedlings, this suggests that protocorm survival and subsequent seedling establishment are more dependent on the presence of species-specific OrM fungi, or that higher abundances of these fungi must be attained

before a germinating seed can grow into a seedling (Bidartondo & Read, 2008; Smith & Read, 2008; Rasmussen & Rasmussen, 2009). As a result, the distribution and abundance of the OrM fungal associate in the soil strongly influences the aboveground distribution of its orchid partner in the field. Clearly, the relationship between OrM abundance and orchid distribution through seedling establishment is complex and probably species specific; thus, more research on patterns of seedling establishment in natural orchid populations is needed to unequivocally reveal the impact and fine details of these difficult to observe belowground interactions.

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Author contributions

M.W., B.L. and H.J. planned and designed the research. M.W., R.B. and H.J. conducted the fieldwork. M.W., T.W. and H.J. performed the experiments and analyzed the data. M.W., T.W., R.B., B.L. and H.J. wrote the manuscript.

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Supporting Information

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

Fig. S1 Radial soil sampling plan.

Fig. S2 Quantitative PCR characteristics.

Fig. S3 Spatial analysis of *Orchis purpurea* recruits.

Fig. S4 Spatial analysis of *Orchis purpurea* adults.

Fig. S5 Spatial analysis of *Orchis mascula* recruits.

Fig. S6 Spatial analysis of *Orchis mascula* adults.

Table S1 Summary of fungal operational taxonomic units (OTUs) detected on orchid roots during preliminary screening

Table S2 Mean concentration of target DNA detected during quantitative PCR (qPCR) analysis

Table S3 Summary of statistical results for *Orchis purpurea*

Table S4 Summary of statistical results for *Orchis mascula*

Methods S1 Detailed description of cluster point process models.

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