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Specificity and localised distribution of mycorrhizal fungi in the soil may contribute to co-existence of orchid species

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ABSTRACT

Co-occurring orchid species tend to occupy different areas and associate with different mycorrhizal fungi, suggesting that orchid mycorrhizal (OrM) fungi may be unevenly distributed within the soil and, therefore, impact the aboveground spatial distribution of orchids. To test this hypothesis, we investigated spatial variations in the community of potential OrM associates within the roots of three co-habitating orchid species (*Anacamptis morio*, *Gymnadenia conopsea*, and *Orchis mascula*) and the surrounding soil in an orchid-rich calcareous grassland in Southern Belgium using 454 amplicon pyrosequencing. Putative OrM fungi were broadly distributed in the soil, although variations in community composition were strongly related to the proximal host plant. The diversity and frequency of sequences corresponding to OrM fungi in the soil declined with increasing distance from orchid plants, suggesting that the clustered distribution of orchid species may to some extent be explained by the localised distribution of species-specific mycorrhizal associates.

1. Introduction

Fine-scale surveys of the spatial distribution of plant species have shown that many species are not randomly distributed, but instead show highly clustered distribution patterns (Wiegand et al., 2009). Spatial clustering can be the result of many processes that act either independently or synergistically (Punchi-Manage et al., 2014). Limited seed dispersal or abrupt changes in local growth conditions, for example, will restrict seed germination and seedling establishment to small areas around maternal plants (Jacquemyn et al., 2010a), often leading to the significant spatial clustering of many plant species. When multiple species coexist, these processes may interact to generate complex patterns of distribution, varying between complete overlap, partial overlap to spatial segregation (Wiegand et al., 2012).

In species that critically rely on symbioses for completion of their life cycle, spatial distribution patterns and coexistence may be strongly contingent on the spatial distribution of the symbiont. This may be particularly true for orchids as their seeds need to associate with appropriate mycorrhizal fungi to accomplish germination and subsequent growth to a seedling (Smith and Read, 2008; Rasmussen and Rasmussen, 2009). As such, mycorrhizal fungi are an indispensable part of the life cycle of orchids and likely contribute to the spatial distribution of orchids (McCormick and Jacquemyn, 2014). However, what defines suitable microsites for different orchid species will likely depend upon a combination of how specific orchid fungus requirements are and the distribution of biotic and abiotic conditions (Diez, 2007; McCormick et al., 2012; McCormick and Jacquemyn, 2014). It has been hypothesized that an orchid which requires a specific mycorrhizal fungus at any life stage would be more likely to be spatially restricted than one which can associate with many different fungi (McCormick et al., 2006; Jacquemyn et al., 2012a; Riofrío et al., 2013). Moreover, since the fungi that are involved in orchid mycorrhizal (OrM) associations are assumed to be free-living fungi that grow well without the orchid, they are thought to be distributed independently of the orchids they associate with (Dearnaley et al., 2012; McCormick and Jacquemyn, 2014).

Previous works on the spatial distribution of single and multiple orchid species have highlighted some consistent patterns that suggest the importance of mycorrhizal fungi in generating spatial

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distribution patterns of orchids. For example, comparison of the spatial distribution of seedlings and adults has shown that they are not distributed independently, but often show strong spatial correlations (Jacquemyn et al., 2007, 2009). Moreover, seed germination experiments have shown that germination often declines with increasing distance from adult plants, suggesting that the abundance of suitable mycorrhizal fungi decreases as well (McKendrick et al., 2000, 2002; Batty et al., 2001; Diez, 2007; Jacquemyn et al., 2012a). Comparison of the spatial distribution of multiple orchids at a small scale has further shown that species tend to be spatially segregated (Jacquemyn et al., 2012a,b; 2014) and associate with distinct sets of mycorrhizal fungi (Waterman et al., 2011; Jacquemyn et al., 2012a,b; 2014; van der Heijden et al., 2015), again pointing to the overarching impact of fungal distribution patterns on spatial distribution and coexistence of orchid species. However, there are virtually no studies that have directly investigated the spatial distribution of OrM fungi in the soil (but see Masuhara and Katsuya (1994) and McCormick et al. (2009)).

To fill this gap, we investigated the spatial distribution of the mycorrhizal fungi associating with three terrestrial orchid species (*Anacamptis morio*, *Gymnadenia conopsea* and *Orchis mascula*) co-occurring at a single site. Previous research on the aboveground spatial distribution of adult plants and belowground seed germination showed that the three species were spatially segregated from each other, with all three showing significant spatial clustering and spatial aggregation varying by species, and seed germination being largely restricted to sites where the species occurred (Jacquemyn et al. 2012a). Moreover, consequent analysis of the mycorrhizal fungi of these co-occurring orchid species showed distinct differences in patterns of fungal association, with different sets of associating fungi for each investigated orchid species. These results suggest that: (1) mycorrhizal fungi associating with the roots of co-occurring orchids differ largely between species; (2) the fungi themselves are patchily distributed in the soil and (3) decline with increasing distance from adult plants. To test these predictions, we used 454 amplicon pyrosequencing, previously shown to be well-suited for the detection and identification of OrM fungi (Waud et al., 2014), to identify in much greater detail the mycorrhizal fungi associating with the roots of the three species and to investigate the spatial distribution of these fungi in the soil.

2. Materials & methods

2.1. Study system

This study was conducted in a calcareous grassland site located in the Namur province of Southern Belgium. The site contains a broad diversity of plant species, including birch (*Betula pendula*), oak (*Quercus robur*), multiple grass and forb species, and the three orchid species studied herein (*Anacamptis morio*, *Gymnadenia conopsea* and *O. mascula*), which have been previously studied at this site by Jacquemyn et al. (2012a). Within a 70 m × 40 m plot, these orchid species were shown to be spatially clustered and are found growing in shallow soil (~5 cm deep) which consists primarily of decaying plant material (see Jacquemyn et al. (2012a) for more details). Previous research on the fungal associates of these three orchid species has shown their predilection for OrM associations with basidiomycetes belonging to the families Ceratobasidiaceae and Tulasnellaceae, and to a lesser extent Sebacinaceae and Thelophoraceae (Stark et al. 2009; Jacquemyn et al. 2010b, 2011, 2012b; Bailarote et al. 2012; Těšitelová et al., 2013; Ercole et al. 2015). However, there are marked differences in fungal association patterns between the three species, with *G. conopsea* being generalistic and including typical orchid mycorrhizas of the Tulasnellaceae and Ceratobasidiaceae (Stark et al., 2009; Těšitelová et al., 2013). *O. mascula* tends to be specialised towards a single Tulasnellaceae-related fungal symbiont (Jacquemyn et al., 2010b, 2011; 2012a), whereas *A. morio* associates with a large number of mycorrhizal fungi, although the number of associates was often restricted to one or a few mycorrhizal partners within populations (Bailarote et al., 2012; Ercole et al., 2015).

2.2. Sampling

Samples were collected in June 2013 during a period when *O. mascula* and *A. morio* had stopped flowering, but were still visible and *G. conopsea* started flowering. For each of the three studied orchid species, root samples were obtained from six individual plants. Plants were selected randomly, with careful attention to the proximity of other orchid individuals, selecting plants without visible neighbouring orchids within a 1 m radius (Supporting Information, Fig. S1A). Additionally, around each sampled orchid individual, soil samples were collected in each cardinal direction (North, South, East, West) at three different radial distances (5, 15, 50 cm), for a total of twelve radial soil samples per orchid individual. These distances were selected to represent soil within close proximity (5 cm), approaching the perimeter (15 cm), and distant from the observed orchid roots (50 cm), respectively. Soil samples were taken to a depth of 5 cm (approx. 5 g fresh weight) using a unique, sterile sampling tube at each sampling point to avoid cross-contamination. Samples were limited to this depth due to the underlying rocky substratum. The radial samples from around orchid plants were pooled at each distance, resulting in three composite soil samples (5, 15, and 50 cm) for each sampled orchid individual. Additionally, background soil samples were obtained from 41 transect points distributed in 5 m × 10 m intervals across the sampling site (Fig. S1B). Altogether, this resulted in 113 samples (18 root samples, 54 radial soil, 41 transect samples) for further analysis. All root and soil samples were refrigerated at 4 °C until further sub-sampling and processing, which generally occurred within 24 h.

2.3. Molecular analyses

At least three complete roots from each sampled orchid individual were surface sterilized (30 s 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 portion of these roots were sectioned into 5–10 mm fragments and mixed. DNA extractions were performed on two separate 0.5 g mycorrhizal root fragment subsamples from each plant 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 which may contain fungal spores and/or sclerotia were retained. DNA extractions were performed on two separate 0.25 g soil sub-samples per soil sample using the PowerSoil DNA Isolation Kit as described by the manufacturer (Mo Bio Laboratories Inc., Carlsbad, CA, USA). Each pair of DNA extracts was then pooled and stored at −80 °C.

Subsequently, amplicon libraries were created using two complementary PCR primer combinations targeting the fungal internal transcribed spacer (ITS) 2 region, ITS86F/ITS4 (White et al., 1990; Turenne et al., 1999) and ITS3/ITS4OF (White et al., 1990; Taylor and McCormick, 2008). Previous research has shown that these primer pairs were highly complementary and outperformed other primer pairs to characterize OrM communities in both a positive control “mock community” and a similar sampling system (Waud et al., 2014). This “mock community” of 37 reference isolates was