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Abstract

● Mycorrhizal fungi are essential for the survival of orchid seedlings under natural conditions. The distribution of these fungi in soil can constrain the establishment and resulting spatial arrangement of orchids at the local scale, but the actual extent of occurrence and spatial patterns of orchid mycorrhizal (OrM) fungi in soil remain largely unknown.

● We addressed the fine-scale spatial distribution of OrM fungi in two orchid-rich Mediterranean grasslands by means of high-throughput sequencing of fungal ITS2 amplicons, obtained from soil samples collected either directly beneath, or at a distance from, adult *Anacamptis morio* and *Ophrys sphegodes* plants.

● Like ectomycorrhizal and arbuscular mycobionts, OrM fungi (tulasnelloid, ceratobasidioid, sebacinoid and pezizoid fungi) exhibited significant horizontal spatial autocorrelation in soil. However, OrM fungal read numbers did not correlate with distance from adult orchid plants, and several of these fungi were extremely sporadic or undetected even in the soil samples containing the orchid roots.

● Orchid mycorrhizal 'rhizoctonias' are commonly regarded as unspecialized saprotrophs. The sporadic occurrence of mycobionts of grassland orchids in host-rich stands questions the view of these mycorrhizal fungi as capable of sustained growth in soil.

Keywords

Fungal communities, Tulasnellaceae, *Tulasnella calospora*, Ceratobasidiaceae, Serendipitaceae, Sebacinales, Pezizaceae, Orchidaceae

INTRODUCTION

Spatial processes play a key role in determining the structure and dynamics of plant communities. In particular, the spatial distribution of soil organisms, such as soil borne mutualists and pathogens of plant roots, likely operates as a driver of spatial patterns of species within plant communities and, ultimately, plant community diversity (Ettema & Wardle, 2002). For instance, spatial heterogeneity in soil mutualists alters the outcome of plant competition (Abbott *et al.*, 2015). Since mycorrhizal fungi play key functions in plant biology (Smith & Read, 2008; van der Heijden *et al.*, 2015), the spatial structure of their communities has attracted considerable interest in the last decade (e.g. Lilleskov *et al.*, 2004; Lekberg *et al.*, 2007; Bahram *et al.*, 2015a). These studies have mainly focused on the communities of ectomycorrhizal (EcM) and arbuscular mycorrhizal (AM) fungi, the dominant symbionts in agricultural and woodland ecosystems. By contrast, spatial patterns of orchid mycorrhizal (OrM) fungi in soil remain largely unknown.

Plant dependency on compatible mycorrhizal fungi is extreme in orchids because germination of the tiny orchid seeds, almost devoid of nutritional reserves, and development of the heterotrophic protocorm require colonization by fungi providing organic carbon (Smith & Read, 2008; Rasmussen & Rasmussen, 2014). Orchids retain their mycorrhizal partnerships at adulthood when, due to their characteristically poorly developed roots, they are thought to be still heavily reliant on mycorrhizal fungi for their mineral nutrition (Waterman & Bidartondo, 2008). In addition, species that develop photosynthetic tissues may still supplement photosynthesis with fungal-derived organic carbon (Selosse & Roy, 2009; Kuga *et al.*, 2014; Stöckel *et al.*, 2014).

Due to their vital role in plant survival, it has been proposed that the spatial distribution of symbiotic fungi could forcefully constrain the establishment and resulting distribution of orchids (McCormick & Jacquemyn, 2014). Many OrM fungi are likely widespread at the scale of tens to hundreds of kilometres, and orchid distribution essentially limited by seed dispersal. By contrast, at the local (metre) scale spatial patterns are presumably driven by other factors (McCormick *et al.*, 2016). Indeed, at this scale many orchid species feature small population sizes and a scattered occurrence. Although seed dispersal limitation across limited distances (Jersáková & Malinová, 2007) has been invoked to account for this highly aggregated distribution, observational studies have provided some evidence that it is not necessarily the primary cause (Jacquemyn *et al.*, 2007, 2009). Rather, such an arrangement suggests recruitment limitation

resulting from patchiness of appropriate (micro)environmental conditions favorable to seed germination and plant development, including the presence of compatible fungi (McCormick & Jacquemyn, 2014). Furthermore, sympatric orchid species often exhibit both strong spatial segregation and association with distinct fungi (Waterman *et al.,* 2011; Jacquemyn *et al.,* 2012, 2014). Taken together, these observations point to a highly patchy distribution of OrM fungi in the environment.

In some cases, orchid seeds can germinate at sites devoid of adults (Těšitelová *et al.,* 2012; McCormick & Jacquemyn, 2014), suggesting that recruitment restrictions may operate at later developmental stages (Selosse, 2014). In other instances, comparisons of the spatial distribution of seedlings and adults in several European (Diez, 2007; Jacquemyn *et al.,* 2007, 2012) and Australian (Batty *et al.,* 2001) orchids revealed that seed germination was restricted to areas where adults occurred, indicating convergent requirements by juveniles and adult plants. These observations suggest that locations where these orchids can reach maturity may be sites with a persistent occurrence of OrM fungi, whereas fungi or environmental conditions in microsites without existing adults may be ephemeral (McCormick & Jacquemyn, 2014; McCormick *et al*., 2016).

The identity of OrM symbionts of adult plants largely depends on the identity and habitat of the orchid host (Dearnaley *et al*., 2012). In particular, photosynthetic orchids in sunny grassland habitats mainly associate, both as seedlings and as adult plants, with fungi in the 'rhizoctonia' complex *sensu lato* (Smith & Read, 2008; Dearnaley *et al.,* 2012; Rasmussen & Rasmussen, 2014), a polyphyletic assemblage encompassing Agaricomycetes belonging to the Serendipitaceae (Sebacinales; Weiß *et al.,* 2016), Ceratobasidiaceae and Tulasnellaceae (Roberts, 1999; Taylor *et al.*, 2002; Weiß *et al.,* 2004). The Tulasnellaceae, in particular, are the most frequently found OrM fungi in both temperate and tropical regions (Dearnaley *et al.,* 2012).

There is a common assumption that most OrM rhizoctonias are unspecialized soil saprotrophs, based on their fast growth *in vitro* (e.g. Smith & Read, 2008; Nurfadilah *et al*., 2013; Bahram, *et al*. 2015a). However, works on Sebacinales and Ceratobasidiaceae in particular, indicate that the phylogenetic diversity of these rhizoctonias parallels a variety of ecological/nutritional strategies, including the ability to establish mycorrhizal or non-mycorrhizal endophytic associations with non-orchid plants (Weiß *et al.,* 2004; Selosse *et al.,* 2002, 2007, 2009; Oberwinkler *et al.,* 2013; Tedersoo & Smith, 2013; Veldre *et al.,* 2013). By contrast, the ecology of the Tulasnellaceae is largely understudied (Selosse, 2014; Selosse & Martos, 2014).

Experimental burial of orchid seed packets confirms that many rhizoctonias can occur in the environment independently of orchid roots (Těšitelová *et al.,* 2012; McCormick & Jacquemyn, 2014). While it is suspected that the main ecological niche of OrM rhizoctonias exists out of orchid hosts (Dearnaley *et al.,* 2012; Selosse & Martos, 2014), the actual extent of their occurrence in the soil habitat, their spatial distribution in the environment, their nutrient demands, and their fine-scale propagation remain largely unknown. OrM fungi are rarely, if ever, retrieved in meta-barcoding studies of soil fungi (e.g. Buée *et al.,* 2009; Orgiazzi *et al.,* 2012; 2013; Schmidt *et al.,* 2013), the main exceptions being investigations targeting EcM communities, due to the reported ability of some OrM fungi to establish EcM symbiosis with tree plants (e.g. Selosse *et al.,* 2002; Tedersoo *et al.* 2008, 2014; McCormick *et al.,* 2009; Tedersoo & Smith, 2013). However, biased fungal community descriptions may derive from the use of primers excluding particular fungal taxa (Bellemain *et al.,* 2010; Ihrmark *et al.,* 2012; Lindahl *et al.,* 2013). The Tulasnellaceae, for instance, exhibit accelerated evolution of the nuclear ribosomal operon, causing most conventional fungal primers to fail in polymerase chain reaction (PCR) amplification of their ITS (Taylor & McCormick, 2008; Waud *et al.,* 2014; Tedersoo *et al.,* 2015). Thus far, however, the occurrence of OrM fungi in soil has not been thoroughly investigated by means of specific primers.

In this study, we addressed the fine-scale spatial distribution of OrM fungi in the soil of two orchid-rich Mediterranean grasslands, by combining high throughput (Illumina MiSeq) sequencing of fungal ITS2 amplicons obtained from soil-extracted DNA using both generalist and taxon-specific primers, and phylogenetic comparison of soil-derived and root-derived sequences. Soil samples were collected underneath and at distance from adult plants of *Anacamptis morio* Bateman, Pridgeon & Chase and *Ophrys sphegodes* Mill., whose mycorrhizal associations had been previously described both in the study area and elsewhere (Illyes *et al.,* 2009; Liebel *et al.,* 2010; Bailarote *et al.,* 2012; Jacquemyn *et al.,* 2014, 2015; Ercole *et al.,* 2014). We specifically hypothesized that: 1) as for other mycorrhizal symbionts, the distribution of OrM fungi in soil is spatially structured, and 2) OrM fungi in soil co-occur, at the fine scale, with their adult orchid hosts.

MATERIALS AND METHODS

Study sites, plant species and sampling

The study area is a Mediterranean grassland in northern Italy. The site is characterized by patches of meadows and pastures (assigned to the association *Festuco-Brometalia*; Braun-Blanquet, 1964), interleaved by woods and shrubs-dominated plots. It is located 460 m asl, in a transition zone between Mediterranean and sub-Atlantic climates. This area features a high richness in orchid species (Girlanda *et al.*, 2006, 2011; Ercole *et al.*, 2014). We focused on *Anacamptis morio* Bateman, Pridgeon & Chase and *Ophrys sphegodes* Mill., two widespread Euro-Mediterranean orchids growing in grassland habitats (Kretzschmar *et al.*, 2007). Both species belong to the Orchideae tribe in the Orchidoideae subfamily of Orchidaceae (Bateman *et al.*, 2003), and have been described as fully photoautotrophic orchids (Liebel *et al.*, 2010; Ercole *et al.*, 2014). They are winter-green perennial tuberous plants in which, after summer dormancy, the underground bulbous tuber produces a basal rosette of leaves and some roots. In these plants, as in most orchidioid species, new roots appear from late summer to autumn, but a few more may form in spring (Rasmussen, 1995). In *A. morio*, seed germination was found to be significantly related to the distance to the nearest congeneric adult (Jacquemyn *et al.*, 2012). Adult plants of both orchid species establish mycorrhizal associations with a diverse fungal spectrum dominated by Tulasnellaceae and Ceratobasidiaceae (Basidiomycota); *A. morio* also frequently associates with fungi in the Pezizaceae (Ascomycota) (Illyes *et al.*, 2009; Liebel *et al.*, 2010; Bailarote *et al.*, 2012; Ercole *et al.*, 2014; Jacquemyn *et al.*, 2014, 2015).

Sampling was carried out in early October 2012 at two sites, located 500 m apart within the study area. At the first site, *A. morio* and *O. sphegodes* co-occurred, although with a limited spatial overlap (Fig. 1). By contrast, *O. sphegodes* did not occur at the second site, where sampling was performed within a dense *A. morio* population (Fig. 1). Plants of either orchid species exhibited positive spatial autocorrelation at either site (Moran's I test, $P<0.0001$). Nearby, adult individuals of other orchid species (mostly *Neotinea tridentata*, *Orchis purpurea* and *Serapias vomeracea*) were also observed at either site.

Soil cores (approx. 10 X 10 cm to 20 cm depth) containing the roots of adult orchid plants were collected at both sites. These plants (six to eight individuals per orchid species per site) were randomly chosen within the respective populations (which consisted of 16-78 individuals). For 10 of these plants (5 per species), root samples were collected (four roots per individual). *A. morio* roots were collected only at site 1. Additional soil samples were collected along 160 cm-long transects, directed away from neighbouring orchids, established around orchid plants located at the margin of each population at either site, and thus running into orchid free vegetation. Around each plant (five individuals per orchid species per site), samples were taken at five distances (0, 20, 40, 80 and 160 cm) from the target plant (Fig. 1). Soil samples were

sieved (2 mm) to remove fine roots and large organic debris, independently stored in ice upon collection and transported to the laboratory. Overall, 80 soil samples and 10 root samples were analyzed.

DNA extraction, PCR amplification and amplicon sequencing

Prior to DNA extraction, soil samples were checked under a stereomicroscope for the absence of orchid seedlings. Following soil homogenization, three 0.5 g subsamples per soil core were taken and total genomic DNA was extracted from the pooled 1.5 g samples. Three independent extractions from each composite soil sample were performed using the FastDNA Kit (MP Biomedicals, LLC, OH, USA) according to the manufacturer's instructions.

Genomic DNA was also extracted from fungal pelotons manually isolated from orchid mycorrhizal roots. Roots were rinsed with tap water and sonicated. Each root (which was approx. 7-8 cm long) was cut into approx. 5-cm-long segments, which were microscopically checked for fungal colonization. Such a microscopic observation revealed the occurrence of many active pelotons in the newly formed roots of both *A. morio* and *O. sphegodes*. Highly colonized root segments were teased with a sterile scalpel in a 6 cm Petri dish containing 5 ml of sterile water, in order to release the pelotons. Pelotons were collected with a micropipette and transferred in PCR tubes. Before PCR amplification, the pool of pelotons obtained from each plant was disrupted by heat shock (10 min at 95°C) in 10 µl 1X PCR buffer (Sigma-Aldrich).

The quality and quantity of DNA samples from soil and roots was assessed by spectrophotometry (ND-1000 Spectrophotometer NanoDropH; Thermo Scientific, Wilmington, Germany). The nuclear ribosomal internal transcribed spacer 2 (ITS2) region was amplified from all DNA extracts by means of a semi-nested PCR approach. In the first PCR, the entire ITS (ITS1-5.8S-ITS2) region was amplified either the generic fungal primer pair ITS1F-ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993) (hereinafter, referred to as the "ITS primer pair"), or the ITS1-OF and ITS4-OF primers, specifically designed for orchid mycorrhizal fungi (Taylor & McCormick, 2008) (hereinafter, the "OF primer pair"). For the second PCR, ITS3mod and ITS4 (White *et al.*, 1990) tagged primers were used to amplify the ITS2 region. ITS3mod is a modified version of ITS3: 5'-CAATCGATGAACAACGYWGC-3'. Each DNA extract was amplified in three replicates.

The first PCR was performed using 0.4U of Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific, Courtaboeuf, France), 1x Phusion HF buffer, 0.5µM of ITS or OF primers, 0.2mM of each dNTPs and 1µl of genomic DNA (20ng), in a final volume of 20µl. For the ITS primer pair, the PCR conditions used were: 5 min at 95°C, 35 cycles of 30 s at 94°C, 45

s at 54°C and 1 min at 72°C, followed by 10 min at 72°C. For OF primers the PCR conditions used were: 2 min at 96°C, 35 cycles of 30 s at 94°C, 40 s at 58°C and 45s at 72°C, followed by 10 min at 72°C. Each PCR product was checked on agarose gel, and diluted at 1/50 to use as template in the nested PCR. The nested PCR was carried out using 1U of Phusion High Fidelity polymerase, 1x HF buffer, 0.5µM of the primers ITS3mod and ITS4 (White *et al.,* 1990) with barcodes, 0.2µM of each dNTPs and 2µl of diluted PCR product, in a total volume of 50µl. PCR conditions were 30 s at 98°C, 30 cycles of 10 s at 98°C, 30 s at 64°C and 20 s at 72°C, followed by 10 min at 72°C. All PCRs were performed using a T3000 thermal cycler (Biometra GmbH, Germany). PCR products were checked on agarose gel, and the three replicates of each sample were pooled and purified using The Wizard® SV Gel and PCR Clean-Up System (Promega, USA) following the manufacturer's instructions. After quantification with Qubit 2.0 (Life Technologies), the purified PCR products were mixed in equimolar amounts to prepare sequencing libraries. The libraries were paired-end sequenced using the Illumina MiSeq technology (2 X 250 bp) by Fasteris (Plan-les-Ouates, Switzerland).

Bioinformatic analyses

Paired-end reads from each library were initially merged using PEAR v0.9.2 (Zhang *et al.,* 2014), with the quality score threshold for trimming the low quality part of a read set at 28 and the minimum length of reads after trimming set at 200 bp.

Assembled reads were then processed using Quantitative Insights into Microbial Ecology (QIIME) v. 1.8 software package (Caporaso *et al.,* 2010). Initial sequence processing and sample assignment were performed with a minimum sequence length cut-off of 200 bp, minimum Phred quality score of 28, calculated over a sliding window of 50 bp, and allowing a maximum mismatch of 3 bp over the forward and reverse primers. Sequences were re-orientated when necessary to 5′ to 3′, and demultiplexed based on the tags and primers. Chimeric sequences were identified and removed performing a *de novo* (abundance based) detection using USEARCH61 (Edgar, 2010), as implemented in the QIIME pipeline. Operational taxonomic units (OTUs) were determined using an open reference-based clustering strategy, with the USEARCH61 method, at 98% similarity; only clusters encompassing at least 10 sequences were retained. The UNITE database version 6 for QIIME was used as a reference for Operational Taxonomic Unit (OTU) picking and taxonomy assignment (Abarenkov *et al.,* 2010; Kõljalg *et al.,* 2013; http://unite.ut.ee, last accessed May 25th, 2015); BLAST algorithm (Altschul *et al.,* 1990) was used as taxonomy assignment method, using 1e^{-5} e-value as threshold. The OTU representative sequences generated in this study (i.e. the most abundant sequence within each OTU) were

submitted to GenBank and recorded under the following string of accession numbers: KX115530-KX116039.

To assess the relatedness with OrM fungi of the OTUs assigned to rhizoctonias and to pezizoid fungi obtained from soil, maximum likelihood (ML) analyses were carried out. Sequences included in the ML analyses comprised best BLAST hits as well as fungal sequences from a variety of terrestrial, including the target species, and epiphytic orchids from different continents and environments, as well as from non-orchid plants, fungal strains and fruitbodies. Due to the phylogenetic distance between the fungi identified (Roberts, 1999), distinct phylogenetic analyses were carried out for Tulasnellaceae, Ceratobasidiaceae, Serendipitaceae and Sebacinaceae (previously referred to as "Sebacinales Clade B" and "Sebacinales Clade A", respectively; Weiß *et al*., 2016). Sequences were aligned using the program Clustal X 2.0 (Larkin *et al.,* 2007) with default conditions for gap opening and gap extension penalty. Alignments were then imported into program MEGA 4.0 (Tamura *et al.*, 2007) for manual adjustment. ML estimation was performed with RAxML v.7.0.4 (Stamatakis, 2006) through 1000 bootstrap replicates (Felsenstein, 1985) using the GTR + GAMMA algorithm to perform a tree inference and search for a good topology. Support values from bootstrapping runs were mapped on the globally best tree using the – f option of RAxML and − x 12345 as a random seed. Nodes receiving a bootstrap support < 70% were not considered as well supported. Alignments and tree topologies are archived in the database TreeBASE (http://www.treebase.org; submission ID 19171). To account for the different intraspecific variation rate in the ITS region for different lineages, the resulting phylogroups (clades supported by ≥70% bootstrap, which included the sequences obtained from soil in this work) were used as taxonomic units in the statistical analyses described below.

Statistical analyses

To allow for comparisons among datasets obtained either from the soil samples collected under the two orchid species at either site, or at different distances from orchid plants, or with the two primer pairs, subsampling at even sequencing depth from each sample (1061 sequences per sample) was performed by means of the *rarefy_even_depth* function in the R package phyloseq (McMurdie & Holmes, 2013)

Chi-square tests were carried out to compare proportions of OTUs and reads obtained with the two primer pairs, assigned to different fungal taxa.

For the taxa which had been retrieved with both primer pairs, data derived from the primer pair yielding the highest read numbers from the highest number of soil samples were used in subsequent analyses.

The effects of orchid species and site on the composition of OrM fungal assemblages in soil samples collected underneath orchid plants were evaluated using permutational multivariate analysis of variance (PERMANOVA, 999 permutations), as implemented in the *adonis* routine of the *vegan* package of R (Oksanen *et al*. 2013, R Development Core Team 2014). The multivariate homogeneity of group dispersions was first assessed by means of the *betadisper* and *permutest* (with 999 permutations) functions in the R package *vegan* (Oksanen *et al.,* 2013). The differences in the composition of OrM fungal communities in orchid roots and soil samples collected beneath were visualized by means of a non-metric multidimensional scaling (NMDS) ordination carried out with the Past3 software (Hammer *et al*., 2001). PERMANOVA was also performed to compare the composition of non-OrM communities in soil. Only taxa occurring in ≥75% of soil samples collected under either orchid species at either site were included in the latter analysis. Indicator species analysis (a classification-based method to measure associations between species and groups of sites; Dufrene & Legendre, 1997) was carried out using the *multipatt* function in the *indicspecies* R package, with 999 permutations (De Cáceres & Legendre, 2009), in order to assess if and which fungi were significantly associated with a particular orchid species/site.

The significance of the relationship between fungal community dissimilarity and geographical distance at either site 1 or site 2 was assessed by use of Mantel tests based on 999 permutations (R software, *ecodist* package; Goslee & Urban, 2007) for each dataset. Bray-Curtis dissimilarity measures were used to generate community distance matrices. Mantel correlograms were also calculated at different distance classes at either site. Significance of Mantel r was adjusted with sequential Bonferroni correction. Mantel tests and correlograms were carried out for the overall OrM and non-OrM fungal assemblages (comprising tulasnelloid, ceratobasidioid, sebacinoid and pezizoid fungi), the single previously mentioned clades, as well as taxonomically-unrelated clades of putatively saprotrophic fungi (Figs S2-S11).

Spatial clustering in read numbers of OrM and non-OrM phylogroups in the soil samples was explored using Moran's I test statistic, as implemented in the R package *ape* (Paradis *et al.,* 2004). For each phylogroup, differences in read numbers among soil samples collected at varying distances along the 160 cm transects were tested for significance by means of Kruskal-Wallis tests conducted using the Past3 software.

RESULTS

Fungal diversity in roots and in soil

After filtering and cleaning, 869,000 and 1,961,000 high-quality sequences were obtained with the OF and ITS primer pairs, respectively. They were clustered in 2959 and 4755 (98% sequence identity) OTUs.

Following subsampling at even sequencing depth for both primer pairs, a diverse array of rhizoctonias (18, 53 and 72 OTUs assigned to tulasnelloid, ceratobasidioid and sebacinoid fungi, respectively) and pezizoid fungi (17 OTUs) was identified in both sites. A higher number of sequences and OTUs assigned to rhizoctonias were obtained with the OF primer pair than with the ITS primer pair ($P=0.011$ and $P<0.001$, respectively, chi-square test; Supporting Information Fig. S1). Other fungi were also differentially amplified, confirming the specificity reported in previous screenings with the same primers (Taylor & McCormick, 2008; Bellemain *et al.*, 2010; Waud *et al.*, 2014; Oja *et al.*, 2015).

Sequences obtained from pelotons isolated from both *O. sphegodes* and *A. morio* roots were predominantly assigned to ceratobasidioid fungi (75.4% and 28.1% of total sequences, respectively). The second most dominant groups were tulasnelloid fungi in *O. sphegodes* (18.7% total sequences) and pezizoid fungi in *A. morio* roots (21.2% total sequences). Sequences assigned to *Hygrocybe spadicea*, *Fusarium oxysporum*, as well as diverse Glomeromycota were also obtained from both orchids, whereas sequences assigned to sebacinoid fungi were not retrieved from roots (Supporting Information Table S1).

Most rhizoctonias identified in soil (94.4%, 88.7%, and 91.7% of tulasnelloid, ceratobasidioid, and Sebacinaceae OTUs, respectively), as well as 35.3% pezizoid OTUs, were phylogenetically closely related to fungi identified in orchid roots at the study sites or elsewhere OrM fungi; Figs S2-S5). None of the tulasnelloid OTUs was closely related to tulasnelloid ECM lineages (Fig. S2). Ceratobasidioid OTUs were distributed in all clades identified by Veldre and co-authors (2013; Fig. S3b).

The soil samples from both sites also hosted common soil fungi (such as *Mortierella* and *Fusarium* spp.), including taxa typical of grassland habitats (such as members of the Clavariaceae and Hygrophoraceae) (Table S2, Figs S6-S11). With a few exceptions, these Ascomycota, Basidiomycota and zygomycetous fungi were unassigned at the species/genus level, but exhibited high sequence identity to environmental sequences from different soils around the world (Table S2).

Influence of orchid species and site factors on the composition of fungal communities in soil

The composition of OrM fungal assemblages in soil differed significantly in the two sites, as assessed by comparing the soil samples collected under *A. morio* at either site (PERMANOVA, Table S3). Such a difference was mainly due to phylogroups *Tul_2* and *Seb_A1*, which were significantly more common in soil samples collected at site 1, and *Cer_18*, which was associated to soil samples collected at site 2 (indicator species analysis, Table S2). Likewise, the assemblage of non-OrM tulasnelloid, ceratobasidioid, sebacinoid and pezizoid fungi also differed at the two sites (PERMANOVA; Table S3), mostly due to *Pez_9* and *Pez_10* (associated to site 1) and *Seb_B4* (associated to site 2; Table S2). Significant differences were also found for non-rhizoctonia and non-pezizoid fungi (PERMANOVA; Table S3), mainly due to a number of Ascomycota (Table S2).

The influence of the orchid species was evaluated for soil samples containing *A. morio* or *O. sphegodes* roots at site 1. No significant difference was found under the two orchid species for either individual taxa of OrM or non-OrM fungi (Indicator species analysis, in Table S2), or their assemblages (PERMANOVA, in Table S3). Similarly, although some taxa exhibited a significant association with a group of soil samples (Table S2), the overall assemblage of non-rhizoctonia and non-pezizoid fungi did not differ significantly between the two groups of soil samples (Table S3).

Most OrM fungi were either absent or infrequent even in the samples collected underneath the orchid plants, occurring in 0-40% of the latter soil samples (Table S2).

Occurrence of OrM fungi in orchid roots and the corresponding soil samples

Sequences obtained from the roots of both orchid species were predominantly assigned to the ceratobasidioid phylogroup *Cer*_2 (Fig. S3a), which accounted for 75.3 and 27.8% of the total number of reads fro*m O. sphegod*es an*d A. mor*io roots, respectively. *Cer*_2 occurred in 60% and 20% of the soil samples containing the respective orchid plants (10 soil samples). *Tul*_2 (the second most dominant fungus in *O. sphegode*s, 13.8% total read number) occurred in 60% of the corresponding soil samples. By contrast, *Pez*_3 (the second most dominant fungus in *A. mori*o, 20.0% total read number), was not found in any of the soil samples containing the corresponding orchid roots. Similarly, *Tulasnella calospora*, which was amplified by the primers we used (Fig. S12), and was retrieved from 40% of the *A. morio* plants analyzed, was not retrieved from any of the soil samples.

The assemblages of the OrM fungi in the soil samples collected under both orchid species were dominated by the ceratobasidioid phylogroup *Cer_11*, which was not amplified from roots. Likewise, *Cer_5* and *Cer_18*, which occurred in all soil samples collected beneath *A. morio*,

were not obtained from roots (Table S1). OrM fungal assemblages, indeed, differed significantly between roots and soil (PERMANOVA; Table S3, see also Fig. 2), mainly due to the significant difference between *A. morio* roots and the corresponding soil samples (PERMANOVA; Table S3). These differences were linked to the indicator ceratobasidioid and pezizoid phylogroups associated with *A. morio* roots and the corresponding soil samples (Table S4), as well as other ceratobasidioid and pezizoid phylogroups which were instead associated with the root samples of both orchids (*Cer_2*, *Pez_3*) or with soil samples, independently of the orchid species (*Cer_11*; Table S4). On the contrary, no significant difference was found between *O. sphegodes* roots and the corresponding soil samples (PERMANOVA; Table S3), which shared phylogroups *Cer_2*, *Tul_2* and *Tul_3* (Tables S1, S2).

Spatial distribution of OrM fungi in soil

Community level analyses

Mantel tests showed significant spatial autocorrelation for the overall OrM fungal assemblage composed by tulasnelloid, ceratobasidioid, sebacinoid and pezizoid fungi (but not for the single groups, when analysed separately) only at site 1 (Table S5). Similarly, significant autocorrelation for the assemblage composed by non-OrM tulasnelloid, ceratobasidioid, sebacinoid and pezizoid fungi (but not for the single taxonomic groups) was found only at site 2 (Table S5).

The Mantel correlograms revealed significant autocorrelation within small distance classes (< 2m on average) for tulasnelloid, ceratobasidioid, sebacinoid and pezizoid fungi (OrM and non-OrM), as well as saprotrophic Psathyrellaceae (Fig. 2a, Table S6). Significant autocorrelation occurred at higher distances for OrM ceratobasidioid and sebacinoid fungi (at 6.25-7.58m and 7.59-8.93m, respectively), non-OrM pezizoid fungi (2.43-3.89m and 2.51-3.42m) and saprotrophic Psathyrellaceae (12.97-14.30m; Fig. 3a, Table S6). The saprotrophic Mycenaceae and Mortierellaceae/Umbelopsidaceae, by contrast, did not exhibit significant distance-decay. Depending on the taxonomic group, significant relationships were found at either or both sites. No difference in the occurrence of significant relationships was found among OrM, non-OrM and saprotrophic taxa (chi-square tests, $P>0.05$; Fig. 3a).

Individual taxon level analyses

When read numbers of each OrM phylogroup were compared in soil samples collected at increasing distances from orchid plants (as a proxy for variation in abundance in soil), no significant difference could be observed for any fungus (P-values of Kruskal-Wallis tests ranging 0.071-1; data not shown).

However, either OrM or non-OrM tulasnelloid, ceratobasidioid, sebacinoid and pezizoid fungi exhibited significant positive spatial autocorrelation (i.e. patchiness), as assessed by means of Moran's tests (the main exception being the three OrM phylogroups, none of which exhibited significant autocorrelation; Table S7). Significant autocorrelation was found at both sites for 11.1% of the (OrM or non-OrM) tulasnelloid, ceratobasidioid, sebacinoid and pezizoid phylogroups exhibiting significant autocorrelation. The same pattern was found for 13.6% of the other (putatively saprotrophic) basidiomycetes tested. Spatial autocorrelation occurred more frequently in some taxonomic groups, e.g. it was particularly rare in the Mortierellaceae/Umbelopsidaceae (Fig. 3b). Autocorrelation occurrence was significantly higher in the OrM than in the non-OrM ceratobasidioid fungi (Fig. 3b).

DISCUSSION

The distribution of OrM fungi in soil is similar to spatial patterns of other mycorrhizal fungi

Although the occurrence of OrM fungi in soil has been taken into account in few studies (McCormick *et al.,* 2009; Bahram *et al.,* 2015a; Oja *et al.,* 2015), the present investigation is one of the first that specifically focuses on spatial patterns of these fungi in soil in relation to the distribution of different orchid species. The composition of the fungal assemblages in soil samples containing orchid roots was not affected by the orchid species, as indicated by the non-significant difference found for the samples taken under *A. morio* and *O. sphegodes*. Soil rhizoctonias were dominated by sebacinoid fungi, followed by ceratobasidioid and tulasnelloid species. In a previous study, Sebacinales was also the most OTU-rich OrM fungal taxon in soil samples collected around roots of *Cypripedium calceolus*, *Neottia ovata* and *Orchis militaris* in two meadow and two forest sites in western Estonia, where a lower richness of Ceratobasidiaceae and Tulasnellaceae was found (Oja *et al.,* 2015). Most sequences derived from our soil samples were phylogenetically closely related to sequences obtained from the roots of the target orchid species (either collected in the study area and in other sites) as well as of different orchid species. The highest proportions of rhizoctonia OTUs unrelated to OrM fungi were assigned to Sebacinales and Ceratobasidiaceae, consistent with the high taxonomic and functional diversity of these taxa (Weiß *et al.,* 2004; Selosse *et al.,* 2002, 2007, 2009; Oberwinkler *et al.,* 2013, 2014; Tedersoo & Smith, 2013; Veldre *et al.,* 2013), and to Pezizaceae, which also exhibit varied ecological strategies, encompassing saprotrophic, mycorrhizal and endophytic fungi (Tedersoo *et al.,* 2013).

As observed for other mycorrhizal fungi, the distribution of both OrM fungal assemblages and individual taxa in soil featured non-random spatial distribution, as indicated, respectively, by significant Mantel and Moran's I tests. Such patterns were reported thus far for EcM, AM fungi or the general soil fungal community at small scales (e.g. Lilleskov *et al.,* 2004; Lekberg *et al*., 2007; Peay & Bruns, 2014; Bahram *et al.,* 2013, 2015a). In particular, we found significant autocorrelation for OrM fungal assemblages at distances up to approx. 10m, which is comparable to the spatial autocorrelation range of AM fungi in temperate ecosystems (Bahram *et al.,* 2015a). Such patterns may depend on random dispersal processes. However, spatial patterns of soil fungi are also known to depend strongly on habitat type (Bahram et al., 2013, 2015b). Although we did not measure environmental variables at the two study sites (which are located in a relatively homogeneous landscape), the differences in the spatial patterns between the two stands are suggestive of a role for environmental variation in shaping the distribution of OrM fungi in the area. Non-OrM fungal assemblages varied significantly at the two sites, suggesting different biotic environments.

At both the community and the individual taxon level, we found evidence of clade-specific differences in spatial patterns of OrM fungi, as already observed for EcM symbionts (e.g. Lilleskov *et al.,* 2004; Bahram *et al.,* 2013). We also found a significantly higher frequency of spatial autocorrelation in OrM than in non-OrM ceratobasidioid fungi. This may reflect either different dispersal patterns, or different trophic strategies, as reported for different EcM or plant pathogenic fungi which exhibited stronger spatial structure in soil, compared to saprotrophic fungi (Bahram et al., 2015b). Studies making use of larger datasets of OrM may clarify these two possibilities.

Widespread OrM fungi may exhibit sporadic occurrence in soil

Orchid-rich areas have been suggested to exhibit persistently high abundances of OrM fungi to provide either sufficient nutrients or a high probability of the fungus encountering seeds (McCormick & Jacquemyn, 2014). This suggestion was mainly based on seed germination, indicating greater occurrence of fungal symbionts close to adult plants (Batty *et al.*, 2001; Diez, 2007; Jacquemyn *et al.,* 2012). In our work, higher read numbers of sequences obtained from *O. sphegodes* and *A. morio* roots did not correlate with shorter distances from adult plants. Moreover, we found that several fungi dominating in orchid roots were extremely sporadic or were not detected at all even in soil samples containing the roots of orchid plants colonized by the same fungi. By contrast, other OrM fungi predominated in the same soil samples. Recent quantitative PCR analyses focusing on dominant OrM fungi in other orchid species showed that

their abundance declined rapidly with distance from the adult host plants (McCormick *et al.*, 2016; Waud *et al.*, 2016). It remains unknown whether this discrepancy is due to different soil conditions or the plant and fungal taxa involved.

Although the possibility of a non-exhaustive coverage of our soil samples cannot be entirely ruled out, our results point to an extremely patchy occurrence of several OrM fungi, heterogeneously distributed in soil even at the scale of the soil cores that were sampled. Another caution concerns the simultaneous examination of roots and surrounding soil. The timing of fungal colonization, development of pelotons and subsequent lysis has not been investigated for these orchids, and the possibility of rapid dynamics of OrM fungi in soil, as opposed to orchid roots, cannot be dismissed. In other terms, OrM fungi which were initially abundant in soil (at the moment of root colonization) could have disappeared from it afterwards. However, the rapid peloton collapse and degeneration observed in the orchid species investigated to date (with lysis sometimes taking less than 24 hours; Smith & Read, 2008) suggest that the presence of active hyphal coils is evidence of recent colonization from the environment. In their study of temporal changes in root and rhizosphere fungal communities of *C. calceolus*, *N. ovata* and *O. militaris* in Estonian meadows and forests, Oja *et al.* (2015) observed a slight but significant turnover of OrM fungal OTUs inside roots. By contrast, the soil OrM fungal community remained fairly stable, with negligible turnover over the vegetation period. This temporal investigation thus highlighted mismatches in the fungi dominating in roots and soil, as we did on a spatial basis. Both observations therefore suggest an active selection, by orchid plants, of compatible fungi from the surrounding environment. A similar concept of orchid preference was formulated by McCormick *et al.* (2009) based on differences between the arrays of OrM fungi (tomentelloid OTUs) recovered from mycorrhizae of *Corallorhiza odontorhiza* and soil at a study site in eastern United States.

The lack of detection of OrM fungi in the soil cores containing orchids roots colonized by the same fungi indicates limited, if any, development of extraradical fungal mycelium. The occurrence of OrM extraradical mycelium is to be verified morphologically under natural conditions. To the best of our knowledge, nothing is currently known about either mycelium- or spore-based, short- and long-distance dispersal mechanisms of OrM fungi in soil. Exploration for new, uncolonized host roots is a crucial function of the extraradical mycorrhizal mycelium. In EcM fungi, several functional groups, so-called “exploration types”, have been defined based on the amount, range, and differentiation of the mycelial structures emanating from the hyphal mantle into the soil (Agerer, 2001, 2007). Such morphological features determine the fungal ability to explore different volumes of soil around colonized root tips (Agerer & Raidl, 2004;

Weigt *et al.*, 2012). In a recent study addressing the relationship between EcM exploration types and root density in a *Pinus muricata* forest, Peay and colleagues (2011) found that long-distance exploration types were more prevalent in areas of low root density, while short-distance types were more common in areas of high root density, supporting the idea that when roots are densely packed, short-range exploration would be an effective strategy and may be more efficient in terms of carbon expenditure than longer distance types. Considering that orchid plants occur in dense patches and their roots are also in close contact with the dense root systems of co-occurring grasses, the extraradical mycelium of OrM fungal species may only explore a limited volume of soil in the close rhizosphere of their host plants.

The most notable example of an OrM fungus amplified from orchid roots but undetected in soil samples is *Tulasnella calospora*, one of the main orchid symbionts at the study sites as well as in other regions (e.g. Roberts, 1999; Girlanda *et al.*, 2011; De Long *et al.*, 2013). Genome sequencing of a *T. calospora* strain isolated from an *A. laxiflora* plant at the study area revealed a robust genetic apparatus for the degradation of crystalline cellulose (Kohler *et al.*, 2015), lending further credit to the assumption, based on earlier observations of *in vitro* growth on complex organic polymers (Smith & Read, 2008), of a strong saprotrophic competence of this fungus. Our findings, however, point to a reduced competitive ability of *T. calospora* in soil under natural conditions. The rare occurrence, if not absence, of this and other common OrM fungi even in the soil beneath their orchid hosts raises the question as to whether orchid roots represent a "refuge" for these fungi, as discussed by Selosse & Martos (2014). Similarly, based on their observations of a declining fungal abundance with increasing distance from the adult host plants, McCormick and colleagues (2016) and Waud *et al.* (2016) have suggested that orchids maintain fungal communities to some extent, so that the distribution of orchid plants determines the distribution of their OrM associates. OrM fungi could use their host plants for survival and persistence in the environment (Selosse, 2014; Oja *et al.*, 2015). Alternatively, OrM fungi could be stimulated to grow and proliferate into roots. Orchids have a much more reduced root system than most EcM and AM plants (Rasmussen, 1995). Therefore, they can supposedly offer a rhizosphere habitat spanning shorter distances than other plants. This situation, as well as the possible limited dispersal ability of OrM fungi, may contribute to their restricted distribution in soil.

In conclusion, we have found evidence of spatial autocorrelation in all main taxonomic groups of OrM fungi in the study areas. An intriguing result is that some widespread root symbionts were found to be quite rare even in host-dense soils. Future investigations should explore alternative niches of common OrM fungi found to be infrequent in soil at the small scale and also address

the functional role of the extraradical OrM mycelium. Soil microsites are likely the key to understand habitat preferences in this group of mycorrhizal fungi.

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AUTHOR CONTRIBUTIONS

All authors planned and designed the research. S.V. and E.E. conducted field work and performed the experiments. S.V., E.E. and S.G. analysed data. S.P. and M.G. wrote the manuscript; all authors contributed to manuscript revision.

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FIGURE LEGENDS

Figure 1. Spatial distribution of adult plants of *Anacamptis morio* (purple circles) and *Ophrys sphegodes* (yellow circles) at both study sites. The position of adult individuals of other orchid

species (white circles) is also reported. The *A. morio* and *O. sphegodes* plants under which soil samples were collected are numbered. Straight lines indicate the 160 cm-long transects along which further soil samples were collected. Sampling along these transects was done at the edge of the population into orchid free vegetation.

Figure 2. Nonmetric multidimensional scaling (NMDS) ordination of OrM fungal assemblages (ITS2 sequences) in orchid roots (circles) and soil samples collected underneath the same plants (triangles). Vectors represent the correlation coefficients between the "orchid" (*Anacamptis morio*, *Ophrys sphegodes*) or "habitat" (roots, soil) variables and the NMDS scores. The length of the vectors are arbitrarily scaled to make a readable biplot, so only their directions and relative lengths have to be considered. *O. sphegodes*, open symbols; *A. morio*, filled black symbols. Stress: 0.1398. R^2= 0.6698 and = 0.1335 for axis 1 and axis 2, respectively.

Figure 3. Frequency of significant spatial autocorrelation (P<0.05) in soil for different orchid mycorrhizal (OrM) and non-orchid mycorrhizal (non-OrM) fungal groups. (**a**) Mantel correlograms (Table S6): percentage of total distance classes for which significant autocorrelation was found. (**b**) Moran's I tests (Table S7): percentage of phylogroups exhibiting significant autocorrelation. White bars, OrM fungi; grey bars, non-OrM fungi; shaded bars, entire clade (OrM and non-OrM fungi); black bars, sums of different clades. Tul., tulasnelloid fungi; Cer., ceratobasidioid fungi; Seb., sebacinoid fungi; Pez., pezizoid fungi; Hygro., *Hygrocybe* spp.; Lepiot., Lepiotaceae; Myce., Mycenaceae; Psathy., Psathyrellaceae, Clav., Clavariaceae; Asco., Ascomycota; Mort., Mortierellaceae and Umbelopsidaceae. Phylogroups/OTUs comprised in each fungal group are listed in the legends of Tables S5-S7. Bars with different letters differ significantly according to chi-square tests (P<0.05, pairwise comparisons, small letters, comparisons between white, gray or shaded bars; capital letters, comparisons between black bars).

SUPPLEMENTARY INFORMATION

Figure S1. Read and OTU numbers for different fungal groups obtained with the OF (blue bars) or the ITS (purple bars) primer pairs. Rhizo., rhizoctonias; Basidio., other Basidiomycota; Asco., Ascomycota; Zygo., zygomycetous fungi; Glomero., Glomeromycota; Chytridio., traditional Chytridiomycota; unident., unidentified fungi.

Figure S2. Maximum likelihood tree obtained from the ITS2 sequence alignment of tulasnelloid fungi. *Multiclavula corynoides* was used as an outgroup taxon. Alignment length: 863 bp. Bootstrap support values above 70% (1000 maximum likelihood replicates) are reported. Sequences obtained from soil samples in this study are indicated in red; sequences obtained from

951 pelotons in *Anacamptis morio* or *Ophrys sphegodes* roots at the time of soil sampling are
952 indicated in blue.
953 **Figure S3a**. Maximum likelihood tree obtained from the ITS2 sequence alignment of
954 ceratobasidioid fungi. The phylogram is midpoint rooted. Alignment length: 499 bp. Bootstrap
955 support values above 70% (1000 maximum likelihood replicates) are reported. Sequences
956 obtained from soil samples in this study are indicated in red; sequences obtained from pelotons
957 in *Anacamptis morio* or *Ophrys sphegodes* roots at the time of soil sampling are indicated in
958 blue.
959 **Figure S3b**. Placement of ceratobasidioid sequences, obtained in this study, within the
960 phylogenetic reconstruction by Veldre *et al.* (2013). The maximum likelihood tree is rooted at
961 the /fusisporus clade. Alignment length: 644 bp. Bootstrap support values above 70% (1000
962 maximum likelihood replicates) are reported. Sequences obtained from soil samples in this study
963 are indicated in red; sequences obtained from pelotons in *Anacamptis morio* or *Ophrys*
964 *sphegodes* roots at the time of soil sampling are indicated in blue. Sequences obtained from
965 orchids at the study sites in previous investigations (Girlanda *et al.* 2011, Ercole *et al.* 2014) are
966 indicated in green.
967 **Figure S4a**. Maximum likelihood tree obtained from the ITS2 sequence alignment of fungi
968 assigned to Sebacinaceae. *Paulisebacina allantoidea* was used as an outgroup taxon. Alignment
969 length: 408 bp. Bootstrap support values above 70% (1000 maximum likelihood replicates) are
970 reported. Sequences obtained from soil samples in this study are indicated in red; sequences
971 obtained from pelotons in *Anacamptis morio* or *Ophrys sphegodes* roots at the time of soil
972 sampling are indicated in blue.
973 **Figure S4b**. Maximum likelihood tree obtained from the ITS2 sequence alignment of fungi
974 assigned to Serendipitaceae. *Paulisebacina allantoidea* was used as an outgroup taxon.
975 Alignment length: 419 bp. Bootstrap support values above 70% (1000 maximum likelihood
976 replicates) are reported. Sequences obtained from soil samples in this study are indicated in red;
977 sequences obtained from pelotons in *Anacamptis morio* or *Ophrys sphegodes* roots at the time of
978 soil sampling are indicated in blue.
979 **Figure S5**. Maximum likelihood tree obtained from the ITS2 sequence alignment of pezizoid
980 fungi. *Ascobolus* spp. were used as outgroup taxa. Alignment length: 426 bp. Bootstrap support
981 values above 70% (1000 maximum likelihood replicates) are reported. Sequences obtained from
982 soil samples in this study are indicated in red; sequences obtained from pelotons in *Anacamptis*
983 *morio* or *Ophrys sphegodes* roots at the time of soil sampling are indicated in blue.

Figure S6. Maximum likelihood tree obtained from the ITS2 sequence alignment of *Hygrocybe* spp. *Hygroaster albellus* was used as outgroup taxon. Alignment length: 493 bp. Bootstrap support values above 70% (1000 maximum likelihood replicates) are reported. Sequences obtained from soil samples in this study are indicated in red.

Figure S7. Maximum likelihood tree obtained from the ITS2 sequence alignment of *Lepiota* spp. *Macrolepiota procera* was used as outgroup taxon. Alignment length: 373 bp. Bootstrap support values above 70% (1000 maximum likelihood replicates) are reported. Sequences obtained from soil samples in this study are indicated in red.

Figure S8. Maximum likelihood trees obtained from the ITS2 sequence alignment of fungi in the tricholomatoid clade. *Mycena* spp. were used as outgroup taxa in Figs S8a,b. *Entoloma prunuloides* and *Xeromphalina campanella* were used as outgroup taxa in Fig. S8c and Fig. S8d, respectively. Alignment lengths: 500 bp, 500 bp, 407 bp, 745 bp. Bootstrap support values above 70% (1000 maximum likelihood replicates) are reported. Sequences obtained from soil samples in this study are indicated in red.

Figure S9. Maximum likelihood trees obtained from the ITS2 sequence alignment of *Lepiota* spp. Phylograms are midpoint rooted. Alignment lengths: 608 bp, 609 bp, 366 bp, 375 bp. Bootstrap support values above 70% (1000 maximum likelihood replicates) are reported. Sequences obtained from soil samples in this study are indicated in red.

Figure S10. Maximum likelihood trees obtained from the ITS2 sequence alignment of fungi in the Mortierellales. Phylograms are midpoint rooted. Alignment lengths: 493 bp, 414 bp, 469 bp, 516 bp, 440 bp, 413 bp, 457 bp, 387 bp. Bootstrap support values above 70% (1000 maximum likelihood replicates) are reported. Sequences obtained from soil samples in this study are indicated in red.

Figure S11. Maximum likelihood trees obtained from the ITS2 sequence alignment of fungi in the Clavariaceae. *Plicaturopsis crispa* was used as outgroup taxon in Fig S11a. *Hyphodontiella multiseptata* and *Clavaria asperulospora* were used as outgroup taxa in Fig. S11b and Fig. S11c, respectively. Alignment lengths: 448 bp, 448 bp, 375 bp. Bootstrap support values above 70% (1000 maximum likelihood replicates) are reported. Sequences obtained from soil samples in this study are indicated in red.

Figure S12. Amplification of *Tulasnella calospora* (strains MUT4182 and MUT4233) DNA by means of the tagged primers used in this study. 1, 3, 5: strain MUT4182; 2, 4, 6: strain MUT4233. 1, 2: 20 ng DNA; 3, 4: 10 ng DNA; 5, 6: 2 ng DNA. C1-C2, negative controls; M, 100 bp marker (Sigma-Aldrich).

Table S1. Fungi amplified from pelotons of all *Ophrys sphegodes* or *Anacamptis morio* plants analyzed. Read number percentages (with respect to the total number of reads from each orchid species), taxonomic assignation and best BLAST hits are reported for each phylogroup/OTU. Other tulasnelloid, ceratobasidioid, sebacinoid and pezizoid fungi retrieved from roots at the time of soil sampling are reported in Figs. S2-S5.

Table S2. Fungal distribution in the soil samples containing *Anacamptis morio* or *Ophrys sphegodes* roots at either site. OrM and non-OrM tulasnelloid, ceratobasidioid, sebacinoid and pezizoid fungi, as well as other fungi (occurring in ≥75% of soil samples collected under either orchid species at either site) are reported. Percentages of soil samples the fungus was amplified from, taxonomic assignation and best BLAST hits are indicated for each phylogroup/OTU. Results of indicator species analysis for both binary (presence/absence) and non-binary (OTU read numbers) data are included (P-value, the statistical significance of the relationship as assessed with 999 random permutations). AM1, soil samples containing *A. morio* roots collected at site 1; AM2, soil samples containing *A. morio* roots collected at site 2; OS1, soil samples containing *O. sphegodes* roots collected at site 1.

Table S3. Results of the permutational multivariate analysis of variance (PERMANOVA) and the test for the multivariate homogeneity of group dispersions (betadisper and permutest) for orchid mycorrhizal (OrM) and non-orchid mycorrhizal (non-OrM) fungal assemblages in soil samples containing orchid roots at either site. For non-OrM fungal assemblages, only taxa occurring in ≥75% of soil samples collected under either orchid species at either site were included in the analyses. AM1, soil samples containing *A. morio* roots collected at site 1; AM2, soil samples containing *A. morio* roots collected at site 2; OS1, soil samples containing *O. sphegodes* roots collected at site 1. NA, not ascertainable.

Table S4. Results of indicator species analysis for the comparison of OrM fungal assemblages in either *Anacamptis morio* (AM) or *Ophrys sphegodes* (OS) roots and soil samples containing the orchid roots. Results for both binary (presence/absence) and non-binary (OTU read numbers) data are included (P-value, the statistical significance of the relationship as assessed with 999 random permutations).

Table S5. Results of Mantel tests [Mantel.cor, Mantel r statistics; P-value, two-tailed p-value (null hypothesis: r=0) adjusted with sequential Bonferroni correction], based on 999 permutations, for different orchid mycorrhizal (OrM) and non-orchid mycorrhizal (non-OrM) fungal groups in soil. P values <0.05 are indicated in red. OS, *Ophrys sphegodes*; AM, *Anacamptis morio*.

Table S6. Results of Mantel correlograms (Mantel.cor, Mantel r statistics; P-value, P-value (null hypothesis: r = 0) adjusted with sequential Bonferroni correction), based on 999 permutations, for different orchid mycorrhizal (OrM) and non-orchid mycorrhizal (non-OrM) fungal groups. For site 1, both results for all soil samples, and samples collected under either orchid species (OS, *Ophrys sphegodes*; AM, *Anacamptis morio*) or along transects starting from either orchid are reported. P values <0.05 are indicated in red. Phylogroups/OTUs within each fungal group are listed in Table S5.

Table S7. P-values of Moran's I tests for spatial autocorrelation of read numbers of each tulasnelloid, ceratobasidioid, sebacinoid and pezizoid OrM and non-OrM phylogroup at either site. Several non-rhizoctonia and non-pezizoid fungi were also tested. For site 1, both results for all soil samples, and samples collected under either orchid species (OS, *Ophrys sphegodes*; AM, *Anacamptis morio*) or along transects starting from either orchid are reported. OrM phylogroups are indicated in blue. P-values <0.05 are indicated in red. n.a., not ascertainable.