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Summary

- Because orchids are dependent on mycorrhizal fungi for germination and establishment of seedlings, differences in the mycorrhizal communities associating with orchids can be expected to mediate the abundance, spatial distribution and coexistence of terrestrial orchids in natural communities.
- We assessed the small-scale spatial distribution of seven orchid species co-occurring in 25 × 25 m plots in two Mediterranean grasslands. In order to characterize the mycorrhizal community associating with each orchid species, 454 pyrosequencing was used. The extent of spatial clustering was assessed using techniques of spatial point pattern analysis.
- The community of mycorrhizal fungi consisted mainly of members of the Tulasnellaceae, Thelephoraceae and Ceratobasidiaceae, although sporadically members of the Sebacinaceae, Russulaceae and Cortinariaceae were observed. Pronounced differences in mycorrhizal communities were observed between species, whereas strong clustering and significant segregation characterized the spatial distribution of orchid species. However, spatial segregation was not significantly related to phylogenetic dissimilarity of fungal communities.
- Our results indicate that co-occurring orchid species have distinctive mycorrhizal communities and show strong spatial segregation, suggesting that mycorrhizal fungi are important factors driving niche partitioning in terrestrial orchids and may therefore contribute to orchid coexistence.

Introduction

One of the major goals in ecology is to search for the key mechanisms that determine the abundance, spatial distribution and coexistence of species in natural ecosystems (Brown *et al.*, 1995; Tilman & Kareiva, 1997; Hubbell, 2001). Although classical theoretical ecology predicts that two species competing for the same resources cannot stably coexist (Gause, 1934; Tilman, 1982), there are numerous examples of natural systems where the number of competing species exceeds the number of limiting resources (Hutchinson, 1961). Spatially explicit models, on the other hand, have shown that localized dispersal and spatially local interactions can lead to stable coexistence of species because of strong interspecific spatial segregation (Pacala & Levin, 1997). However, in natural environments, the precise factors leading to spatial segregation are not easy to discern and similar patterns of spatial segregation could, for example, also emerge from small-scale habitat heterogeneity (Lundholm, 2009; Brandt *et al.*, 2013). In this case, co-occurring species segregate and coexist, not only because of finite dispersal and local interactions, but also because each species has a competitive advantage in a different habitat type (Pacala & Levin, 1997).

Because orchids are critically dependent on mycorrhizal fungi for completion of their life cycle (Smith & Read, 2008; Rasmussen & Rasmussen, 2009; Dearnaley *et al.*, 2012), coexistence of orchid species can be expected to be mediated by interactions with mycorrhizal fungi. As the dust-like seeds lack the necessary food reserves, associations with mycorrhizal fungi are required to stimulate growth after germination and seedling establishment (Rasmussen & Rasmussen, 2009). Results from previous research have shown that germination and seedling establishment generally decrease with increasing distance from adult plants (Diez, 2007; Jacquemyn *et al.*, 2007, 2012a,b; McCormick *et al.*, 2012), indicating that the presence of adults plants provides a good indication of locations with good environmental conditions and available fungi. Most adult plants also maintain associations with mycorrhizal fungi, possibly contributing to the nutritional requirements of the orchids (Smith & Read, 2008; Girlanda *et al.*, 2011).

Divergent mycorrhizal associations between co-occurring orchid species can therefore be expected to lead to small-scale habitat heterogeneity and reduced competition for resources (van der Heijden *et al.*, 2003; Vandenkoornhuyse *et al.*, 2003), whereas distance-dependent seed germination may contribute to

the highly spatially clustered distribution patterns that are commonly observed in orchids (Chung *et al.*, 2004; Jacquemyn *et al.*, 2007). However, it remains unclear to what extent differences in mycorrhizal communities contribute to spatial segregation and coexistence of orchid species (but see Waterman *et al.*, 2011; Jacquemyn *et al.*, 2012a,b; Těšitelová *et al.*, 2013). One way to gain better insights into the role of mycorrhizal fungi in affecting the spatial distribution of orchids is to combine detailed spatial point pattern analyses with phylogenetic analyses of the mycorrhizal fungi associating with the orchids (Jacquemyn *et al.*, 2012a,b). Because divergent mycorrhizal associations and patchy distributions of orchid mycorrhizal fungi are likely to generate strong spatial clustering of orchids, analyses of spatial point patterns allow the assessment of the spatial association of pairs of species occurring at a given study site (Wiegand *et al.*, 2007). Combined with detailed analyses of the mycorrhizal fungi associating with the orchids, this allows the question of whether spatial clustering or segregation of orchid species is related to differences in mycorrhizal association patterns to be answered.

Although our knowledge of mycorrhizal associations in orchids has increased tremendously during the last few years (Shefferson *et al.*, 2007, 2010; Jacquemyn *et al.*, 2010, 2011, 2012c; Martos *et al.*, 2012), surprisingly little is known about mycorrhizal associations in orchids that co-occur at a given site (but see Waterman *et al.*, 2011; Jacquemyn *et al.*, 2012a,b; Těšitelová *et al.*, 2013). Recent research has shown that orchids commonly associate with several fungi simultaneously (Roy *et al.*, 2009; Lievens *et al.*, 2010; Jacquemyn *et al.*, 2012c) and can form complex networks of interactions (Jacquemyn *et al.*, 2010, 2011; Martos *et al.*, 2012). These findings imply that, to accurately describe mycorrhizal associations in orchids, fungal communities should be assessed rather than the presence of individual fungal species, and that more advanced detection techniques are required than the commonly used Sanger sequencing of cloned PCR products. Although DNA arrays (Lievens *et al.*, 2010) have been successfully used to characterize the mycorrhizal community of orchids (Jacquemyn *et al.*, 2011), the major disadvantage of this technique is that detection is limited to a specific panel of previously selected target fungi. Novel high-throughput sequencing methods, such as 454 pyrosequencing (Margulies *et al.*, 2005), outperform earlier approaches in terms of efficiency and sequencing depth, resulting in detailed characterization of microbial communities and their members. Therefore, these new techniques are likely to offer new insights into fungal community ecology (Lindahl *et al.*, 2013).

In this study, we characterized spatial distribution patterns and mycorrhizal communities associating with nine orchid species belonging to five important genera that are frequently found growing together in Mediterranean grasslands (*Anacamptis*, *Neotinea*, *Orchis*, *Ophrys* and *Serapias*; Delforge, 2005). Two sites were selected in which each plant was meticulously mapped in 25 × 25 m plots. In order to characterize the mycorrhizal communities associating with each orchid species, we used 454 pyrosequencing. For each plot, the extent of spatial aggregation was determined using an index of local dominance of each focal species and related to the phylogenetic breadth of fungi associating with the focal orchid species. Next, species–species associations were studied in detail using methods outlined in Wiegand *et al.* (2007) and contrasted to differences in mycorrhizal communities associating with each species. In particular, we addressed the following questions:

- How does the mycorrhizal community differ between coexisting orchid species within and between sites?
- Does mycorrhizal host breadth affect the extent of spatial clustering of orchid species?
- Do species with similar mycorrhizal communities occupy similar locations?
- To what extent is spatial segregation of orchids related to phylogenetic dissimilarity of the fungal communities associating with the orchids?

Materials and Methods

Study sites and species

The study was conducted in the Gargano National Park in southern Italy. The Gargano National Park covers *c.* 121 118 ha and is renowned for its high orchid diversity, including several representatives of the orchid genera *Anacamptis*, *Dactylorhiza*, *Epipactis*, *Neotinea*, *Ophrys*, *Orchis*, and *Serapias* (Rossini & Quitadamo, 2003). Within the region, two sites were selected that harbored a large diversity of orchids. The sites consisted of Mediterranean grasslands that contained a large number of grass and herb species. The first site (hereafter called 'Foresta Umbra'; 41°44′62″N; 15°56′76″E) was located near a pine plantation and contained *c.* 12 different orchid species. The second site ('Monte Sant'Angelo'; 41°42′85″N; 15°55′54″E) was located outside the village Monte Sant'Angelo and consisted of a series of Mediterranean grasslands that lay scattered throughout the landscape and were separated by stone walls. At least 16 different orchid species were found growing at this site. Both sites are regularly grazed by sheep, but at the time of sampling no apparent signs of grazing were observed.

In spring 2012, within each site, a plot of 25 × 25 m was established. Plots were established in a homogenous part of the site, without apparent topographical discontinuities or major changes in plant community composition. In each site, seven coexisting orchid species were found, belonging to five different genera (*Anacamptis*, *Neotinea*, *Orchis*, *Ophrys* and *Serapias*), yielding a total of nine study species (Supporting Information, Table S1). For each species, all flowering individuals were mapped within the plot. High-precision GPS using differential correction (Trimble Navigation Ltd, Sunnyvale, CA, USA) was employed to determine precisely the position of each individual of the studied species. In this way, 935 and 964 individuals were mapped in the Foresta Umbra and Monte Sant'Angelo sites, respectively (Fig. S1).

Assessment of mycorrhizal communities using 454 pyrosequencing

In spring 2012, roots of four randomly selected plants per orchid species were collected within each study plot to determine

patterns of mycorrhizal associations, yielding a total of 56 study samples. Sampling was done in such a way that the area covered by a species was maximized, minimizing the effect of spatial autocorrelation on mycorrhizal communities. All individuals per species were assigned unique MID (Multiplex Identifier) barcode sequences and treated separately during the initial stages of data analyses. Roots 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, DNA was extracted from 0.5 g mycorrhizal root fragments using the UltraClean Plant DNA Isolation Kit as described by the manufacturer (Mo Bio Laboratories Inc., Solana Beach, CA, USA). This represented *c.* 1 cm of the root tip from at least four roots. Subsequently, an amplicon library was created using barcode-tagged primers for the internal transcribed spacer 2 (ITS-2) targeting fungal specific primer pair ITS86F (5′-GTGAATCATCGAATCTTTGAA-3′; Turenne *et al.*, 1999) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′; White *et al.*, 1990). Whereas ITS86F was originally developed to detect medically important fungal pathogens (Turenne *et al.*, 1999), recently this primer pair has shown power and usefulness in characterizing diverse orchid mycorrhizal communities using 454 pyrosequencing (M. Waud *et al.*, unpublished). Both the forward and reverse primers were synthesized with appropriate 5′ Roche 454 pyrosequencing adapter sequences and the forward primer included a sample-specific 10 bp barcode (Carlsen *et al.*, 2012), facilitating differentiation of the obtained sequences (GC FLX Technical Notes, Roche Applied Science, Mannheim, Germany). Fusion primers were designed according to the scheme provided in Table S2.

Polymerase chain reaction amplification was performed in duplicate in a 25 μl reaction volume containing 0.15 mM of each dNTP, 0.5 μM of each primer, 1 U Titanium *Taq* DNA polymerase, 1X Titanium *Taq* PCR buffer (Clontech Laboratories, Palo Alto, CA, USA), and 5 ng genomic DNA (as measured by a nanodrop instrument; NanoDrop Technologies Inc., Wilmington DE, USA). PCR conditions were as follows: initial denaturation (2 min at 94°C); 35 cycles (45 s at 94°C, 45 s at 59°C, 45 s at 72°C); final extension (10 min at 72°C); hold (10°C). Agarose gel electrophoresis of the generated amplicons illustrated that the majority of products were between 350 to 550 bp in length, a range suitable for analysis using the Roche Genome Sequencer FLX (GS FLX) instrument and GS FLX Titanium chemistry (Roche GS FLX Technical Notes; Youssef *et al.*, 2012). Amplicons were cut from the gel and purified using the Qiagen PCR purification kit (Qiagen). Purified dsDNA amplicons were quantified using the Qubit fluorometer (Invitrogen) and pooled in equimolar quantities. The quality of the resulting amplicon library was assessed using an Agilent Bioanalyzer 2100 and high sensitivity DNA chip (Agilent Technologies, Waldbronn, Germany). Pyrosequencing was performed on the amplicon library using the Roche GS FLX instrument and Titanium chemistry according to the manufacturer's instructions (Roche Applied Science).

Sequences obtained from the GS FLX instrument were assigned to the appropriate PCR reaction based on both barcode and primer sequences using cutadapt 1.0 (Martin, 2011), allowing zero discrepancies in order to ensure high-quality sequences as well as accurate distinction of the samples. Further sequence processing and clustering were performed using MOTHUR, version 1.28 (Schloss *et al.*, 2009). Sequences were trimmed based on a minimum Phred score of 20 (i.e. 1% error rate) over a 50 bp moving window. Minimum and maximum sequence lengths were set to 200 and 400 nucleotides, respectively. Sequences with ambiguous base calls and homopolymers longer than eight nucleotides were rejected and chimeric sequences detected by the Uchime chimera detection program (Edgar *et al.*, 2011) were also removed. For further analysis, sequence data obtained for both replicates were combined for each sample.

Operational taxonomic units (OTUs) were calculated by comparing pairwise sequence alignment identities in CD-HIT-EST 4.5.4 (Li & Godzik, 2006), using the 'accurate but slow' setting. Sequences for which the number of identical base pairs in a pairwise alignment exceeded 95% for the shorter of the two sequences aligned were clustered into the same OTU. A cutoff value of 5% sequence dissimilarity was chosen, as ITS sequences in Tulasnellaceae, in particular, which are regarded as one of the main orchid mycorrhizal fungi (Dearnaley *et al.*, 2012), are known to evolve rapidly (Suarez *et al.*, 2006; Taylor & McCormick, 2008) and, consequently, higher similarity cutoffs may overestimate fungal diversity. OTUs representing only one sequence in the whole dataset (global singletons) were removed from further analysis (Ihrmark *et al.*, 2012). Remaining OTUs were assigned taxonomic identities to the highest taxonomic rank possible/family level based on BLAST (Altschul *et al.*, 1990) results of representative sequences (as indicated by CD-HIT-EST) using GenBank (Benson *et al.*, 2008), including uncultured/environmental entries (a full list of retrieved fungi is provided in Table S3). Subsequently, OTUs were manually screened for possible orchid-associating mycorrhizal families based on the data provided in Table 12.1 in Dearnaley *et al.* (2012). Only OTUs corresponding to known orchid-associating mycorrhizal families were retained for further analysis. For each sampled species, rarefaction curves were calculated using MOTHUR (Schloss *et al.*, 2009).

Data analysis

Phylogenetic analyses Sequences of the 61 retained OTUs (see the Results section) were aligned with MAFFT 7.017 (Katoh *et al.*, 2002) implemented in Geneious Pro 6.1.4 (Biomatters, Auckland, New Zealand). Poorly aligned regions were trimmed from the alignment using the 'automed1' heuristic method implemented in trimAl version 1.2 (Capella-Gutierrez *et al.*, 2009). This resulted in a dataset of 329 characters. A Bayesian relaxed molecular clock analysis was performed with BEAST 1.7.5 (Drummond *et al.*, 2012) using the GTR + G substitution model as selected with jModeltest 2.3.1 (Darriba *et al.*, 2012) under the Akaike information criterion. All Sebacinaceae OTUs were assigned to the outgroup. The uncorrelated lognormal clock model was selected, and a prior with a normal distribution of 1 ± 0.001 was assigned to the root node of the tree. The

distribution of all other priors was set to uniform, except for the uncorrelated relaxed clock standard deviation, to which an exponential prior was assigned. The Bayesian Markov chain Monte Carlo analysis was run for 3×10^7 generations, sampling every 2000th generation. The effective sampling sizes of all parameters were found to exceed 200, suggesting that they are good representations of the posterior distributions. A maximum clade credibility tree was calculated on the last 10 000 sampled trees (Fig. S2). The phylogenetic distances between the OTUs from this tree were used to calculate the phylogenetic diversity (PD; Faith, 1992) and mean pairwise distance (MPD; Webb *et al.*, 2002) of the OTUs associated with each orchid species per site. The OTU abundances were taken into account to calculate the MPD values. The phylogenetic community dissimilarity (PCD; Ives & Helmus, 2010) between the orchid OTU communities was also calculated. PD, MPD and PCD values were obtained using the R package 'Picante' (Kembel *et al.*, 2010). PCD values were determined using all 61 OTUs, and also using only OTUs with a total abundance of > 10% (retaining 25 OTUs).

Based on presence–absence data of fungal virtual taxa in each of the sampled individuals, differences in the fungal community composition associating with the different orchid species were investigated. First, a species-label reallocation scheme using the multiple response permutation procedures (Biondini *et al.*, 1988) test was implemented to test the hypothesis that overall fungal composition differed among species. Analyses were conducted for each site separately. In the case of significant differences, pairwise comparisons were performed to see whether fungal composition differed between particular species. Secondly, to visualize differences in mycorrhizal community structure between species and sites, nonmetric multidimensional scaling ordination techniques were applied using the Bray–Curtis coefficient as distance measure. All analyses were performed using the program PC-ORD version 6 (McCune & Mefford, 2011).

Local dominance index vs mycorrhizal host breadth To answer the question of whether mycorrhizal host breadth affects the extent of spatial clustering of orchid species, we determined, for each species pattern, an index of local dominance. The underlying rationale is that orchid species that have a very narrow range of mycorrhizal hosts should be locally dominant (because of largely unique mycorrhizal hosts), whereas orchid species that have a wide range of mycorrhizal hosts should be spatially more extended and overlap with more other orchid species. To quantify local dominance $L_f(r)$ of a given focal species *f*, we estimated the mean proportion of conspecific neighbors within neighborhoods of radius *r* of the individuals of the focal species (Wiegand *et al.*, 2012). For larger neighborhoods *r*, the local dominance index is basically driven by species abundances and we found that $L_f(r) \approx n_f/n$, where n_f is the number of individuals of the focal species and *n* the total number of individuals in the plot. As we were uncertain as to the best neighborhood radius *r*, we estimated the local dominance $L_f(r)$ for various distances of *r* up to 10 m. Finally, indices of local dominance were related to measures of phylogenetic diversity (PD and MPD) using Spearman rank correlations.

Indices of pairwise spatial segregation vs similarity in associated mycorrhizal communities or phylogenetic community dissimilarity In this analysis, we quantified potential relationships between the degree of spatial overlap of pairs of orchid species and the degree of similarity of their associated mycorrhizal communities. If mycorrhizal communities were the primary driver of the species pattern, we would expect that species with more similar mycorrhizal communities would show a larger overlap than species with more dissimilar mycorrhizal communities. Additionally, we related the degree of spatial overlap between orchid species to their phylogenetic dissimilarity. We expected local coexistence of phylogenetically more dissimilar species, whereas phylogenetically more similar species should be spatially segregated.

To derive an index quantifying the degree of spatial overlap of pairs of orchid species, we used a method presented in Wiegand *et al.* (2012). The goal of this analysis was to quantify the spatial association between individuals of orchid species 1 and 2, that is, how the individuals of orchid species 2 were distributed within local neighborhoods of orchids of a focal species 1. To this end we used two commonly used summary statistics: the bivariate *K*-function, $K_{12}(r)$ (Lotwick & Silverman, 1982), and the nearest neighbor distribution function, $D_{12}(r)$ (Illian *et al.*, 2008). $K_{12}(r)$ can be defined as the mean number of species 2 orchids within neighborhoods with radius *r* around the species 1 orchids, divided by the mean density, λ_2, of species 2 orchids in the plot. $D_{12}(r)$ yields the proportion of species 1 orchids that have at least one orchid 2 neighbor within distance *r*. Note that $\lambda_2 K_{12}(r)$ tests if the mean number of species 2 orchids within neighborhoods around species 1 orchids is below or above the expectation for independence, and $D_{12}(r)$ tests how this mean is distributed (i.e. whether all species 1 orchids have more or less the same number of species 2 neighbors or whether some species 1 orchids have many species 2 neighbors and others have only a few; Wiegand *et al.*, 2012).

To find out if the association of a pair of orchids differed from one that could arise by pure chance, we used a null model, where the spatial pattern of the first species was unchanged but the individuals of the second species were randomized following a homogeneous Poisson process that basically redistributes the orchids of species 2 to random locations within the study area (Wiegand *et al.*, 2012). Note that this null model purposely does not consider the observed clustering of the orchid species, because our objective was to quantify the overlap or segregation of different species but not if they were spatially independent.

To quantify the type of spatial overlap or segregation of pairs of species at a given neighborhood radius *r*, we transformed the initial summary statistics $K_{12}(r)$ and $D_{12}(r)$:

$$\hat{P}(r) = \hat{D}_{12}(r) - D_{12}^{\text{null}}(r) = \hat{D}_{12}(r) - (1 - e^{-\lambda_2 \pi r^2}) \qquad \text{Eqn 1}$$

$$\hat{M}(r) = \log_e(\hat{K}_{12}(r) - K_{12}^{\text{null}}(r)) = \log_e(\hat{K}_{12}(r)) - \log_e(\pi r^2)$$

This was done by subtracting the values of the respective null expectations (i.e. ${D_{12}}^{\text{null}}(r)$ and ${K_{12}}^{\text{null}}(r)$) to yield $P(r) = 0$ and

$M(r) = 0$ under the null model, and that positive or negative departures in $K_{12}(r)$ from independence the null expectation were weighted in the same way (Wiegand *et al.*, 2007).

In the case of segregation, we have fewer species 2 orchids in the neighborhood of species 1 orchids than expected under independence (i.e. $M(r) < 0$) and the nearest orchid 2 neighbor is further away from species 1 orchids than expected (i.e. $P(r) < 0$). In the case of mixing, the opposite is true (i.e. $M(r) > 0$ and $P(r) > 0$). In cases where the number of species 2 orchids that are located in the neighborhood of species 1 orchids is larger than expected ($M(r) > 0$) but the nearest orchid 2 neighbor is further away from species 1 orchids than expected ($P(r) < 0$), we have partial overlap at neighborhood r (Wiegand *et al.*, 2007). An additional type IV association ($M(r) < 0$ and $P(r) > 0$) may only occur if orchids of species 2 are highly clustered and orchids of species 1 overlap the cluster of species 2. To obtain an overview of the spatial association structure of the orchid communities at different neighborhoods r, we counted for each value of r the number of pairs that showed no difference from the null model or one of the four association types.

Finally, the extent of spatial segregation between pairs of species was related to differences in mycorrhizal communities associating with each orchid (i.e. PCD) species using Mantel tests (Mantel, 1967). To derive, for each species pair and a given neighborhood radius r, an index of spatial overlap that has negative values for segregation and positive values for mixing, we used the first principal component of the two-dimensional index ($P(r)$, $M(r)$).

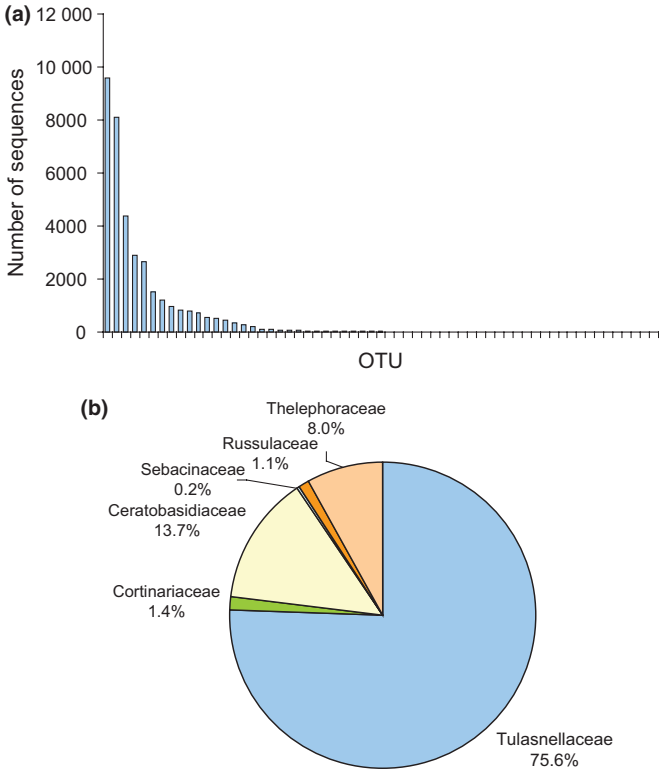

Fig. 1 (a) Sequence frequency distribution has a long tail, with 47 (77%) operational taxonomic units (OTUs) comprising < 1% of all the sequences. (b) Frequency distribution of sequences of OTUs belonging to different fungal families.

Results

Mycorrhizal associations

GS FLX sequencing of the amplicon library yielded a total of 60 790 sequences that passed quality filtering and could be assigned to the different samples. Using a 5% dissimilarity cutoff, a total of 600 OTUs were discovered, a large proportion of which were recovered only once (singletons, 180 OTUs, 30%) or twice (doubletons, 74 OTUs, 12%). BLAST analysis of the representative sequences of each OTU indicated the presence of both mycorrhizal and nonmycorrhizal sequences (with hits to Ascomycetes and other generalist soil fungi (data not shown)). Sixty-one OTUs could be assigned to putative mycorrhizal fungi according to Table 12.1 in Dearnaley *et al.* (2012). These fungi comprised 37 069 sequence reads (61% of all sequences, 20–95% of sequences of each sample; Table S4). The frequency distribution of sequences per OTU was highly skewed (Fig. 1a) with only a few fungi being very abundant and the remaining fungi occurring in low frequencies. Of these, 21 OTUs (28 027 sequences) were assigned to members of the Tulasnellaceae (Fig. 1b). Twenty OTUs (5070 sequences) and five OTUs (2978 sequences) belonged to members of the Ceratobasidiaceae and Thelephoraceae, respectively (Fig. 1b). OTUs related to other fungal families known to associate with orchids (Cortinariaceae, three OTUs, 506 sequences), Sebacinaceae (10 OTUs, 89 sequences) and Russulaceae (two OTUs, 399 sequences) were only sporadically observed (Figs 1b, S2, S3). Representative sequences for each of the 61 mycorrhizal OTUs identified were submitted to GenBank under the accession numbers KF267160–KF267220 (Table S4).

Although the two sites contained almost the same number of OTUs (44 and 43 OTUs at the Forest Umbra and Monte Sant'Angelo sites, respectively; Fig. 2), they differed in mycorrhizal community composition (Figs 2, 3). More specifically, of the 61 detected OTUs, 26 were present in both sites, whereas 18 and 17 OTUs were unique to the Forest Umbra and Monte Sant'Angelo sites, respectively (Fig. 2). The Foresta Umbra site also displayed a higher phylogenetic diversity than the Monte Sant'Angelo site, containing OTUs related to members of Tulasnellaceae, Ceratobasidiaceae, Cortinariaceae, Sebacincaceae, Russulaceae and Thelephoraceae (Fig. 2). OTUs related to members of the Cortinariaceae and Russulaceae were lacking from the Monte Sant'Angelo site, whereas members of Thelephoraceae and Sebacinaceae were very rare. Nonetheless, in both sites, members of the Tulasnellaceae were the most dominant fungal partners. The most common OTUs were OTU29, OTU6, OTU4, OTU73 and OTU23, which were retrieved from 12, 11, and nine species, respectively. With the exception of OTU73, which belonged to the Ceratobasidiaceae, all these OTUs were related to members of the Tulasnellaceae. Most OTUs related to the Sebacinaceae, Russulaceae and Thelephoraceae were associated with one single orchid species and only a few of them were found in the roots of several individuals, indicating that most of

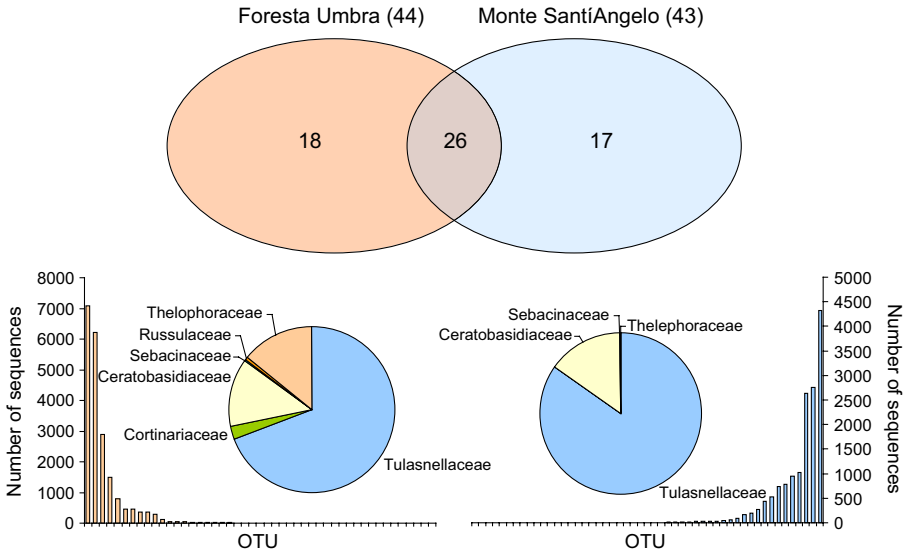

Fig. 2 Comparison of mycorrhizal communities associating with seven orchid species growing at two different sites (Foresta Unmbra and Monte Sant'Angelo) in southern Italy. Numbers in brackets and Venn diagrams represent the number of mycorrhizal operational taxonomic units (OTUs).

these fungi associated only sporadically with the investigated orchid species.

The number of fungal OTUs with which an orchid species was associated varied between five (*Ophrys tenthredinifera*) and 18 (*Anacamptis papilionacea*) at the Foresta Umbra site, and between eight (*O. tenthredinifera*) and 21 (*Neotinea lactea*) at the Monte Sant'Angelo site. For most species, rarefaction curves assessing the overall orchid mycorrhizal OTU richness per plant species approached saturation (Fig. S3). However, in some species (e.g. *N. lactea*, *Anacamptis morio*, *A. papilionacea*) rarefaction curves had not reached saturation, suggesting that in these species our sampling only detected a part of the total fungal diversity. PD values were high, varying between 2.28 and 7.69 (mean = 5.00) at the Foresta Umbra site and between 2.98 and 6.13 (mean = 4.91) for the Monte Sant'Angelo site (Table S1). However, the number of associations and PD values were similar for species that were analyzed at both sites, indicating that the number of associations is relatively constant within species. When focusing on species composition of mycorrhizal fungi, clear differences in association patterns were observed between orchid species both within and between sites (Figs 3, S4). The average within-group distance ($\delta_{obs} = 0.441$ and 0.307 for the Foresta Umbra and Monte Sant'Angelo sites, respectively) was significantly smaller ($P < 0.0001$) than the value based on random reallocation of groups ($\delta_{exp} = 0.716$ and 0.573), yielding chance-corrected within-group agreements $A = 0.383$ and 0.465, respectively. Orchid species from the same genus tended to associate with more similar mycorrhizal communities than species from different genera (Fig. 3). Nonetheless, in some species (e.g. *Ophrys sphegodes* and *A. papilionacea*) pronounced differences in mycorrhizal communities between sites were observed.

As a result of the large diversity of fungi retrieved for each species, PCD values were in general high, varying between 0.533 and 1.411 (mean = 1.087) for the Foresta Umbra site and between 0.563 and 1.044 (mean = 0.802) for the Monte Sant'Angelo site (Table S5), indicating that each orchid species associated with a distinct set of mycorrhizal fungi. Interestingly,

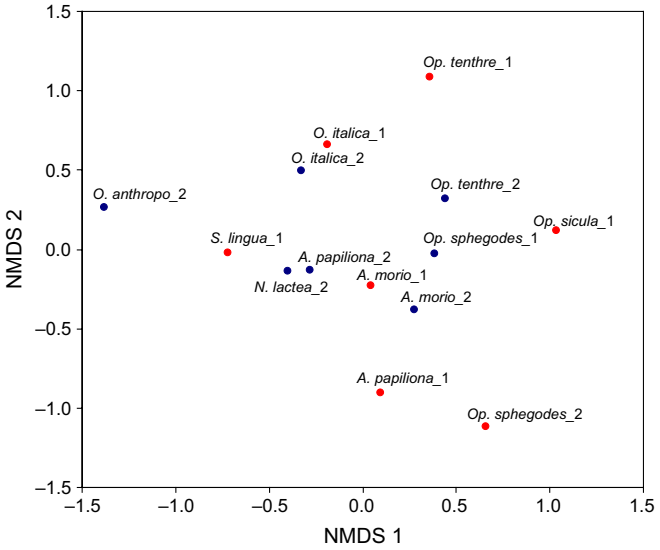

Fig. 3 A nonmetric multidimensional scaling (NMDS) plot of mycorrhizal fungi detected in nine different orchid species sampled at two sites in southern Italy: Foresta Umbra, red; Monte Sant'Angelo, black. See Table 1 for full species names.

PCD values between the same species occurring at different sites were also high, suggesting that mycorrhizal communities associating with orchid species are largely context-dependent (Table S5).

Spatial dominance

At both study sites, all species were locally highly dominant (Table 1). At the Foresta Umbra site, the local dominance at 1 m neighborhoods ranged from 0.63 to 0.87 and that at the 2 m neighborhood ranged from 0.49 to 0.81 (Fig. 4a). At the Monte Sant'Angelo site, the local dominance at 1 m neighborhoods varied between 0.69 and 0.95 and that at the 2 m neighborhood varied between 0.55 and 0.83 (Fig. 4b). This high local dominance is mainly caused by the strong clustering of individual species and little overlap among species (Fig. S1). For small

Table 1 Local dominance indices $L_f(r)$ for the Foresta Umbra site and the Monte Sant'Angelo site and for different neighborhoods (r = 1, 2, 3, 4, 5, 7, and 9 m)

	Neighborhood size						
Species	L_f (1)	L_f (2)	L_f (3)	L_f (4)	L_f (5)	L_f (7)	L_f (9)
Forest Umbra							
Anacamptis morio	0.73	0.66	0.54	0.48	0.42	0.33	0.30
Anacamptis papilionacea	0.87	0.82	0.72	0.66	0.60	0.50	0.42
Ophrys sicula	0.70	0.57	0.40	0.30	0.24	0.16	0.14
Ophrys sphegodes	0.65	0.49	0.37	0.29	0.23	0.16	0.12
Ophrys tenthredinifera	0.63	0.49	0.31	0.24	0.18	0.16	0.14
Orchis italica	0.78	0.65	0.50	0.44	0.39	0.33	0.28
Serapias lingua	0.82	0.71	0.63	0.62	0.60	0.52	0.48
Monte Sant'Angelo							
Anacamptis morio	0.91	0.68	0.55	0.53	0.50	0.39	0.32
Anacamptis papilionacea	0.90	0.77	0.69	0.64	0.61	0.52	0.50
Neotinea lactea	0.69	0.57	0.46	0.38	0.33	0.21	0.16
Ophrys sphegodes	0.95	0.83	0.72	0.64	0.55	0.35	0.27
Ophrys tenthredinifera	0.78	0.57	0.44	0.33	0.28	0.22	0.18
Orchis anthropophora	0.88	0.55	0.38	0.31	0.25	0.15	0.11
Orchis italica	0.79	0.55	0.44	0.38	0.34	0.27	0.20

neighborhood sizes (r = 1 and 2 m), there was no significant (P > 0.05) relationship between local dominance and PD or MPD. However, for larger neighborhood sizes (r > 3 m), local dominance was significantly (P < 0.05) related to both the number and phylogenetic diversity of the mycorrhizal fungi associating with orchids.

Spatial segregation and mycorrhizal communities

The orchid community at the Foresta Umbra site was dominated at neighborhoods of 1 m by segregation, with some pairs showing partial overlap and very few mixing (Fig. 5a). At smaller neighborhoods, the associations were often not significant, but at larger neighborhoods the proportion of significant segregation and partial overlap increased (Fig. 5c). The orchid community at the Monte Sant'Angelo site showed similar association patterns to that at the Foresta Umbra site, but their degree of segregation was generally larger (Fig. 5b,d). At the Foresta Umbra site, we found consistent and positive correlation between the phylogenetic dissimilarity in mycorrhizal communities and the segregation index that measures spatial association (Fig. 6a). Correlations were strongest at the 1 and 1.5 m neighborhoods (with correlation coefficients of *c.* 0.53), but weaker at the 0.5 and 2 m neighborhoods (results not shown). Thus species with similar mycorrhizal communities did occupy, contrary to our initial expectations, different locations. At the Monte Sant'Angelo site, however, we found no correlation between the similarity in mycorrhizal communities and the segregation index that measures spatial association (Fig. 6b). Thus, at this site, species with similar mycorrhizal communities did not necessarily occupy similar locations. At both sites there was no significant correlation between the phylogenetic distance between orchids and the segregation index (Fig. 6c,d), indicating that phylogenetically related orchid species do not occupy similar locations.

Discussion

Mycorrhizal associations

Variation in the mycorrhizal communities associating with co-occurring orchid species may have important ecological consequences and affect the abundance, spatial distribution and, ultimately, the coexistence of orchid species. However, little is presently known about the variation in mycorrhizal communities between co-occurring orchid species, both within and between sites (but see Waterman *et al.* (2011), Jacquemyn *et al.* (2012a,b) and Těšitelová *et al.* (2013)). Here we have shown that Mediterranean grasslands harbor a large diversity of mycorrhizal fungi. Studying two sites in southern Italy, a total of 61 putatively

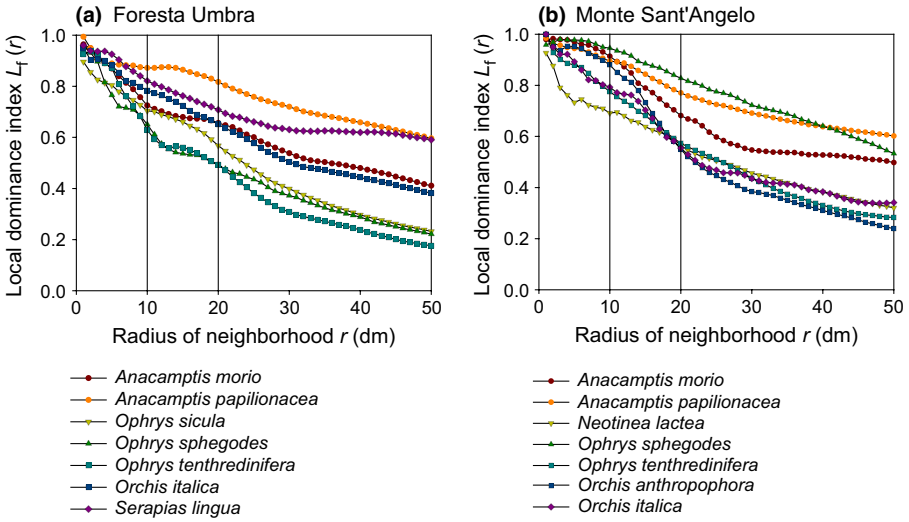

Fig. 4 Dependence of the local dominance index, $L_f(r)$, on the neighborhood radius, r. The local dominance, $L_f(r)$, of a given focal species f is the proportion of conspecific individuals within neighborhoods of radius r around the individuals of the focal species f. Results are shown for Foresta Umbra (a) and Monte Sant'Angelo (b) in southern Italy.

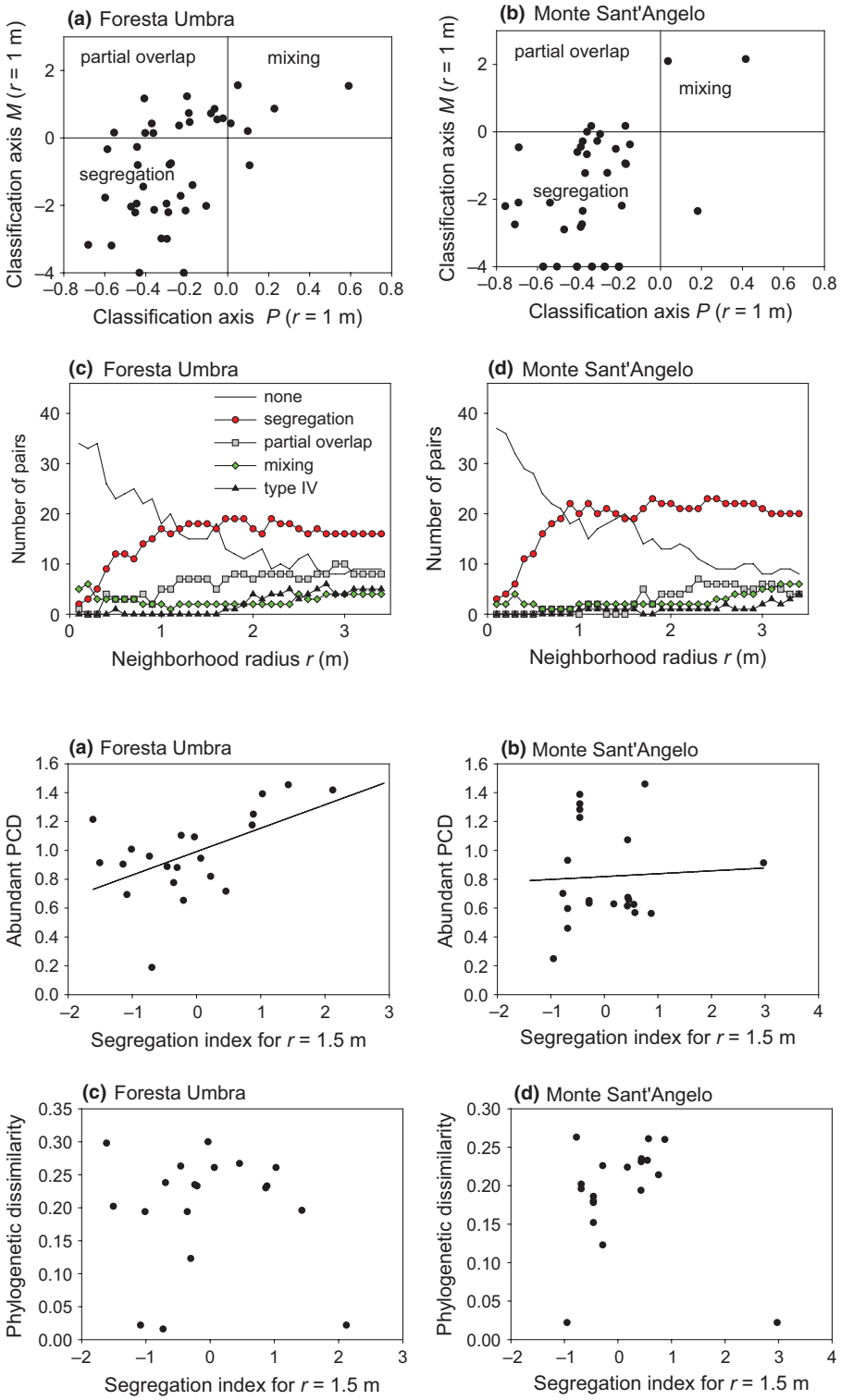

Fig. 5 Assessment of pairwise associations between orchid species for the two study sites in southern Italy (Foresta Umbra and Monte Sant'Angelo). (a, b) The location of the 42 possible species pairs within the two-dimensional scheme defined by the axis $P(r)$ and $M(r)$ at the $r = 1$ m neighborhood. (c, d). Change in the number of association types with neighborhood radius, r.

Fig. 6 Correlation between the index of spatial segregation of pairs of orchid species and the similarity in their mycorrhizal communities and their phylogenetic distance calculated for both study sites in southern Italy (Foresta Umbra and Monte Sant'Angelo). (a, b) Dependence of the phylogenetic community dissimilarity (PCD) index that characterizes the similarity in the mycorrhizal communities of a species pair on the index of spatial segregation of the corresponding pairs of orchid species. A large negative value for the segregation index indicates strong spatial segregation at 1 m neighborhoods, and a large positive value indicates strong spatial overlap. (c, d) Dependence of the phylogenetic distance of a species pair on their index of spatial segregation.

mycorrhizal OTUs were retrieved from 56 individuals of seven different orchid species co-occurring in 25×25 m plots. Although the retrieved OTUs were regarded as mycorrhizal (Dearnaley *et al.*, 2012), it is likely that not all fungi have a clear functional meaning towards the orchid and that they may represent accidental encounters. This is corroborated by the fact that, for some OTUs, only very few sequences were retrieved. Nonetheless, their presence indicates that the diversity of mycorrhizal fungi within Mediterranean grasslands can be high, reaching > 40 different fungal strains within an area of 625 m². This is also confirmed by the fact that, in some of the orchid species studied, rarefaction curves had not yet reached a plateau (Fig. S2). Total diversity is therefore likely to increase with increasing sampling intensity. Clearly, more detailed analyses are needed to

elucidate the total diversity of mycorrhizal fungi within these systems.

The studied orchid species associated predominantly with members of the Tulasnellaceae, and to a lesser extent with members of the Ceratobasidiaceae and Thelephoraceae. These results are largely in accordance with previous results reported for a wide number of related species. For example, species of the genus *Orchis* associated predominantly with Tulasnellaceae and Ceratobasidiaceae (Jacquemyn *et al.*, 2010, 2011; Girlanda *et al.*, 2011). Girlanda *et al.* (2011) also found that members of the Tulasnellaceae and Ceratobasidiaceae were the dominant fungi associating with *Anacamptis laxiflora*, *Ophrys fuciflora* and *Serapias vomeracea*. Bailarote *et al.* (2012) investigated mycorrhizal associations in five populations of *A. morio*, and also found that members of the Tulasnellaceae were the dominant fungi associating with this species, although occasionally associations with members of the Ceratobasidiaceae were found as well. Finally, in *Gymnadenia conopsea*, members of the Tulasnellaceae and Ceratobasidiaceae were also found to be the dominant mycorrhizal fungi (Těšitelová *et al.*, 2013).

Our results further confirm previous findings that Mediterranean orchids commonly associate with several OTUs (Jacquemyn *et al.*, 2010, 2011; Lievens *et al.*, 2010; Girlanda *et al.*, 2011), and in some species (e.g. *N. lactea*, *A. morio*, *A. papilionacea*) more than 15 different OTUs were observed. These results thus indicate that multiple associations and low specificity are common in terrestrial Mediterranean orchids. Nonetheless, marked differences in mycorrhizal community composition were found between species and within species between sites. These results thus suggest that the communities associating with orchid species are, to some extent, context-dependent and may vary from one site to another. This result was previously observed in *A. morio*, which also showed different mycorrhizal communities between populations (Bailarote *et al.*, 2012). Moreover, the total community of mycorrhizal fungi also differed substantially between the two sites, as only 26 out of the 61 identified fungal strains were shared between the two sites.

Spatial patterning

We found consistent and strong spatial structuring of orchid populations located at both study sites. Most species pairs showed either no interaction or significant spatial segregation, whereas only a few species pairs showed partially overlapping or mixed distributions. Moreover, all species showed high local dominance, which was primarily caused by the high spatial clustering of individual plants and strong segregation of individuals from different species. Pronounced spatial clustering appears to be common in terrestrial orchids. Analysis of the spatial distribution of both adults and seedlings of the terrestrial orchids *Orchis purpurea* and *Orchis mascula* has shown that in most populations individuals are not randomly distributed, but commonly occur in high-density clusters (Jacquemyn *et al.*, 2007, 2009). Moreover, comparison of the spatial distribution of both adult plants and seedlings has shown that their distribution often overlaps, indicating that seedlings most often germinate in the immediate neighborhood of mature plants (Jacquemyn *et al.*, 2007, 2009). Seed germination experiments have further shown that the probability of seed germination significantly decreased with increasing distance from the nearest neighboring adult plant (Diez, 2007; Jacquemyn *et al.*, 2012a,b; McCormick *et al.*, 2012). Studies investigating the abundance of fungi in the soil have also indicated that mycorrhizal abundance significantly declines with increasing distance from mature plants (McCormick *et al.*, 2012). These results thus indicate that the nonrandom distribution of orchids results, to some extent, from the presence of suitable mycorrhizal fungi.

However, spatial segregation was not significantly and negatively related to mycorrhizal dissimilarity as expected. If mycorrhizal communities were the main driver of spatial segregation, we would expect that species with similar communities would have a higher probability of co-occurring than species with highly divergent communities. This was clearly not the case. At the Monte Sant'Angelo site there was no relationship between the PCD index and spatial segregation, and at the Foresta Umbra site we even found a positive relationship, implying that species with similar mycorrhizal communities did occupy different locations. An explanation for this could be that competition among orchid species that were too similar overpowered the positive association effect of closely shared mycorrhizal communities. Another explanation is that mycorrhizal divergence was high overall (Table S5), leaving very few possibilities for spatial overlap. Alternatively, it might be the case that the mycorrhizal communities observed in adult plants are different from those associating with germinating seeds and protocorms. Bidartondo & Read (2008), for example, showed that the fungi observed in seedlings were often a subset of those observed in germinating seeds and adult plants. Because fungal community dissimilarity was high overall, the occurrence of fungal specificity bottlenecks during orchid germination and development are likely to occur. However, more research is needed to elucidate the role of fungal bottlenecks in determining the spatial distribution of adult plants in the field.

Coexistence of orchid species

When two species compete for the same resource, theoretical models predict that they cannot stably coexist (i.e. the exclusion principle; Gause, 1934; Tilman, 1982). However, when small-scale habitat heterogeneity is present, species are able to stably coexist in natural environments. Because orchids are critically dependent on mycorrhizal fungi for completion of their life cycle, niche differentiation can be achieved via segregation of mycorrhizal fungi and can therefore be considered a likely factor contributing to the coexistence of orchids. This is because not all fungi are likely to promote seed germination, which will lead to spatial segregation. Moreover, different preferences for mycorrhizal fungi between plant species can also promote coexistence by reducing competition for nutrients (van der Heijden *et al.*, 2003; Vandenkoornhuyse *et al.*, 2003).

In this study, we have shown that different orchid species that co-occurred at a given site associated with distinct mycorrhizal

communities. Similar results have been reported by Waterman *et al.* (2011), who showed that coexisting orchid species of the subtribe Coryciinae tended to associate with different mycorrhizal fungi. Similarly, coexistence of different cytotypes of the terrestrial orchid *G. conopsea* was explained by differences in mycorrhizal communities associating with diploid and tetraploid plants (Těšitelová *et al.*, 2013). However, the possibility that the observed spatial structure results from limited dispersal cannot be ruled out completely, as many orchid species tend to show localized dispersal (Jacquemyn *et al.*, 2007; Jersáková & Malinová, 2007). Spatially explicit models have shown that species can stably coexist based solely on finite dispersal and localized competition. However, this possibility is rather unlikely, as many studies investigating seed germination in relation to the distance of the nearest congener have shown that the probability of seed germination significantly decreases with increasing distance to the nearest plant (McKendrick *et al.*, 2000, 2002; Batty *et al.*, 2001; Leake *et al.*, 2004; Diez, 2007; McCormick *et al.*, 2012). Further evidence showing that seed germination and seedling establishment, and not seed dispersal, are the prime factors determining the spatial distribution of co-occurring orchid species was provided by Jacquemyn *et al.* (2012a). Using seed germination experiments in a site where three orchid species co-occurred, it was clearly shown that spatial variation in seed germination of *A. morio*, *G. conopsea* and *O. mascula* was the limiting factor determining the above-ground spatial distribution of the orchids. Similarly, in three species of the genus *Orchis*, germination of pure and hybrid seeds was strongly related to the above-ground spatial distribution of adult plants, suggesting that the presence of specific mycorrhizal fungi contributed to the spatial distribution and coexistence of pure and hybrid plants (Jacquemyn *et al.*, 2012b).

Conclusions

We have shown that coexisting orchid species have distinctive mycorrhizal communities and display strong spatial segregation. These results may indicate that niche partitioning represents an important mechanism contributing to orchid coexistence in species-rich environments. However, it remains unclear which physiological mechanisms determine mycorrhizal specificity in Mediterranean orchids and how they mediate spatial structuring. Seed germination experiments combined with molecular identification of the associating fungi in a spatial context and *in vitro* seed germination experiments are needed to unambiguously determine the role of mycorrhizal fungi in affecting orchid coexistence.

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Supporting Information

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

Fig. S1 Spatial distribution of seven orchid species in two 25 × 25 m plots sampled in two Mediterranean grasslands in southern Italy (Foresta Umbra and Monte Sant'Angelo).

Fig. S2 Maximum clade credibility tree obtained with Bayesian phylogenetic analysis. Bayesian posterior probabilities of ≥ 0.95 are shown at the nodes.

Fig. S3 Rarefaction curves generated for seven orchid species co-occurring in two 25 × 25 m plots sampled in two Mediterranean grasslands (Foresta Umbra and Monte Sant'Angelo) in southern Italy.

Fig. S4 Nonmetric multidimensional scaling plots of mycorrhizal fungi detected in seven different orchid species sampled at Foresta Umbra and Monte Sant'Angelo.

Table S1 List of sampled species at each site, the number of individual plants present within each 25 × 25 m sampling plot, the number of associated mycorrhizal fungi (operational taxonomic units (OTUs)), phylogenetic diversity (PD) and mean phylogenetic distance (MPD) as determined by assessing the mycorrhizal community in four individuals per orchid species

Table S2 Pyrosequencing concatemer sequences and schema

Table S3 Overview of all operational taxonomic units (OTUs) encountered in this study

Table S4 List of operational taxonomic units (OTUs) corresponding to orchid-associating mycorrhizal families discovered in this study

Table S5 Phylogenetic community dissimilarity (PCD) of orchid mycorrhizal communities associating with different orchid species growing at two sites (Foresta Umbra and Monte Sant'Angelo)

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