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Summary

• The specificity of orchids for their fungi can vary substantially, from highly specialist interactions to more generalist interactions, but little is known about the evolutionary history of the mycorrhizal specificity of orchids.

• Here, we used a network analysis approach to investigate orchid mycorrhizal associations in 16 species of the genus *Orchis* sampled across 11 different regions in Europe. We first examined in detail the structure of the network of associations and then tested for a phylogenetic signal in mycorrhizal specificity and identified the fungi with which the orchids associated.

• We found 20 different fungal lineages that associated with species of the genus *Orchis*, most of them being related to members of the Tulasnellaceae (84.33% of all identified associations) and a smaller proportion being related to members of the Ceratobasidiaceae (9.97%). Species associations formed a nested network that is built on asymmetric links among species. Evolution of mycorrhizal specificity in *Orchis* closely resembles a Brownian motion process, and the interaction between *Orchis* and Tulasnellaceae fungi is significantly influenced by the phylogenetic relationships between the *Orchis* species.

• Our results provide evidence of the presence of phylogenetic conservatism in mycorrhizal specificity in orchids and demonstrate that evolutionary processes may be an important factor in generating patterns of mycorrhizal associations.

Introduction

Within ecological communities, species interact with each other to form complex and often highly structured networks (Bascompte *et al.*, 2003; Olesen *et al.*, 2007; Thébault & Fontaine, 2010). The structure of these networks can vary from gradient, compartmented to highly nested patterns of interactions or even a combination of two patterns (Lewinsohn *et al.*, 2006). The basic description of the architecture of such networks can reveal important insights into the ecological, evolutionary and coevolutionary processes that shape these networks (e.g. Lewinsohn *et al.*, 2006; Rezende *et al.*, 2007; Bascompte, 2010). Moreover, incorporation of phylogenetic relationships allows assessment of the extent to which evolutionary processes are an important factor in determining network structure (Rezende *et al.*, 2007). Although most network analyses to date that have incorporated phylogenetic relationships have been concerned with the community structure of plant–pollinator interactions or plant–frugivore interactions, the same techniques can be used to study broad plant–fungus interactions in a phylogenetic context (Vacher *et al.*, 2008; Jacquemyn *et al.*, 2010).

Most orchid species are dependent on mycorrhizal fungi for completion of their life cycle, at least during the early stages of their development (Smith & Read, 2008; Rasmussen & Rasmussen, 2009). In analogy with plant–animal interactions, the network of orchid–fungus interactions may have either a small number of links among species, indicating an assemblage of ecological specialists, or numerous links, indicating ecological generalists. In the case of orchid species, the architecture of orchid mycorrhizal networks will thus depend on the nature and specificity of the interaction between orchids and their fungi. Previous studies have shown that mycorrhizal specificity may vary considerably between species, ranging from very narrow specificity in nonphotosynthetic and some photosynthetic orchids (e.g. Taylor *et al.*, 2003; Barrett *et al.*, 2010) to broad interactions in other photosynthetic orchids (Shefferson *et al.*, 2007, 2010; Jacquemyn *et al.*, 2010). However, the evolutionary history of species interactions in the Orchidaceae remains largely unexplored. The few studies that have investigated evolutionary changes in mycorrhizal specificity in orchid species involved either nonphotosynthetic orchid species (Taylor *et al.*, 2003; Barrett *et al.*, 2010) or photosynthetic species with relatively narrow specificity (e.g. *Chiloglottis*) (Roche *et al.*, 2010). Little is known about evolutionary trajectories of mycorrhizal specificity in orchid species that have broad specificity, that is, in orchid species that associate with a large number of mycorrhizal fungi (but see Shefferson *et al.*, 2007, 2010).

To gain better insights into the evolutionary history of mycorrhizal specificity in orchid species displaying broad interactions, we investigated mycorrhizal associations in 16 species of the genus *Orchis*. Earlier research on five species of this genus has shown that different species associate with a different number of fungal partners (Jacquemyn *et al.*, 2010), but because of the limited number of species studied it was not possible to unequivocally show whether differences in mycorrhizal specificity were determined by environmental or phylogenetic constraints. Here, we extended a previously developed internal transcribed spacer (ITS)-based DNA array (Jacquemyn *et al.*, 2010) to obtain a comprehensive overview of orchid mycorrhizal associations in the genus. First, clone libraries were constructed and sequenced from a wider range of *Orchis* species as well as from some related species (*Anacamptis morio* and *Gymnadenia coposea*). Based on the obtained sequences the previously developed DNA array was extended, enabling the detection of 23 mycorrhizal fungi. Secondly, based on an analysis of 222 plant individuals collected from 62 orchid populations across Europe, the architecture of the orchid mycorrhizal network was described in detail, testing for both nestedness and modularity (Lewinsohn *et al.*, 2006; Fortuna *et al.*, 2010). Finally, phylogenetic network analyses were used to test the hypothesis that mycorrhizal specificity in the genus *Orchis* and the identity of fungal lineages with which orchid species interact were phylogenetically conserved (Ives & Godfray, 2006; Rezende *et al.*, 2007).

Materials and Methods

Study species

In this study we focused on the genus *Orchis*, which comprises 21 species and several subspecies and varieties that are widely distributed across most of Europe and Asia Minor (Kretzschmar *et al.*, 2007). All species and subspecies belonging to this genus are tuberous, terrestrial perennials that grow in a wide variety of environments, ranging from dry calcareous grasslands through wet meadows to forests. In this study, 16 species of the genus were sampled (Table 1), representing the phylogeny of the genus as fully as possible without having to resort to species that are extremely rare (e.g. *Orchis patens*, *Orchis laeta* and *Orchis adenocheila*). The investigated species differ substantially in distribution area, with some species having a very wide distribution area (e.g. *Orchis mascula* and *Orchis militaris*), whereas others have a very restricted distribution area and some are endemics (e.g. *Orchis brancifortii*, *Orchis galilaea* and *Orchis troodi*).

Sampling

Sampling took place in April–May of 2008, 2009 and 2010. A total of 62 sites distributed across seven countries and 11 different regions in Europe and Israel were sampled (Table 1). At each site, root samples were collected, yielding a total of 222 sampled individuals from one to five populations of the 16 selected species. In most populations, five samples were taken, but in some cases, it was not possible to sample more than one individual. The average sampling size was 3.6 individuals per population.

Molecular assessment of mycorrhizal fungi

All roots were surface-sterilized and microscopically checked for mycorrhizal colonization. After combining multiple mycorrhizally colonized root pieces from the same plant, DNA was extracted from 0.5 g of root material using the UltraClean Plant DNA Isolation Kit as described by the manufacturer (Mo Bio Laboratories Inc., Solana Beach, CA, USA), and then 10 times diluted.

The fungal, and in particular the mycorrhizal, community colonizing the roots was assessed as described previously (Jacquemyn *et al.*, 2010; Lievens *et al.*, 2010). Briefly, in addition to the clone library analysis performed in Jacquemyn *et al.* (2010), ITS-based clone libraries were generated for 20 additional samples from the different studied *Orchis* species as well as from some related species,

Table 1 List of surveyed *Orchis* species, regions and sites sampled, sampling years, and number of populations and individuals sampled at each site

Species	Country	Region	Sampling year	No. pops sampled	No. plants sampled
O. anatolica	Israel	Mount Carmel	2008	1	3
	Cyprus	Akamas Peninsula	2010	1	4
O. anthropophora	Belgium	Southern Belgium	2008	1	3
		Eastern Belgium	2008	2	9
	France	Vercors	2009	1	3
	Italy	Monte Gargano	2009	2	3
O. brancifortii	Italy	Sicily	2008	1	6
O. galilaea	Israel	Mount Carmel	2008	1	3
O. italica	Italy	Sicily	2008	1	4
		Monte Gargano	2009	3	13
	Portugal	Beira Litoral	2009	2	8
O. mascula	Belgium	Eastern Belgium	2008	1	4
		Southern Belgium	2008	3	12
	France	Vercors	2009	2	8
O. militaris	Belgium	Eastern Belgium	2008	1	4
	The Netherlands	South Limburg	2008	1	4
	France	Lorraine	2009	2	7
		Vercors	2009	1	3
O. olbiensis	Portugal	Algarve	2009	1	5
O. pallens	France	Vercors	2009	3	11
O. pauciflora	Italy	Monte Gargano	2009	5	15
O. provincialis	France	Vercors	2009	5	14
O. punctulata	Cyprus	Lemessos	2010	2	7
O. purpurea	Belgium	Eastern Belgium	2008	3	11
	France	Vercors	2009	3	11
O. quadripunctata	Italy	Monte Gargano	2009	5	21
O. simia	Belgium	Southern Belgium	2008	3	11
	France	Vercors	2009	3	9
O. troodi	Cyprus	Lemessos	2010	1	3
		Akamas Peninsula	2010	1	3
			Total	62	222

encompassing *Anacamptis morio* (formerly known as *Orchis morio*) and *Gymnadenia conopsea*. As a result, a total of 50 clone libraries were analysed (representing 22.5% of the sampled individuals). Clone libraries were constructed following PCR amplification with the broad-spectrum basidiomycete primers ITS1-OF and ITS4-OF (Taylor & McCormick, 2008). In a preliminary phase of this study, the effectiveness of several other primer pairs, including ITS1/ITS4-OF, ITS1-OF/ITS4 and ITS1-OF/ITS4-OF, was evaluated to characterize the mycorrhizal community on *Orchis* species. ITS1-OF and ITS4-OF turned out to be the most efficient primer pair as these primers gave the most consistent amplification. Compared with other published primers, this primer pair has the advantage that it does not exclude *Tulasnella* species, and thus should give an accurate view of orchid associations within the Basidiomycota, representing the vast majority of the mycorrhizae found on orchid species (Rasmussen, 1995; Taylor & McCormick, 2008). Additional tests using the other primers did not reveal fungi other than those detected using ITS1-OF and ITS4-OF (partly published in Lievens *et al.*, 2010).

Ninety-six clones were randomly picked from each constructed library and sequenced using the M13 forward primer. DNA sequences from the complete data set were aligned using the MEGA4 software package (Tamura *et al.*, 2007; http://www.megasoftware.net) followed by manual editing. Conserved sequence motifs were identified in the regions flanking each sequence and the sequences were cut to these motifs. Subsequently, sequences were grouped into operational taxonomic units (OTUs), defined by 97% sequence identity. Although it is possible that the use of ITS sequence similarity cut-offs may overestimate or underestimate fungal diversity, this methodology is currently widely used in mycorrhizal research to estimate the richness of specific lineages in a community (McCormick *et al.*, 2009; Jacquemyn *et al.*, 2010; Lievens *et al.*, 2010; Waterman *et al.*, 2011). Because the number of fungal OTUs associating with the different *Orchis* species is unknown, asymptotic species richness and estimators for the sampling effort required to reach the asymptotic richness estimator were determined using methods outlined in Chao *et al.* (2009) with a cut-off value of 97% similarity.

In order to identify the different OTUs, representative sequences for each OTU were queried against GenBank using BLAST. Based on the newly obtained sequences our previously developed DNA array, which enabled the simultaneous detection of 11 OTUs (Jacquemyn *et al.*, 2010), was extended with 12 additional OTUs. For each of these OTUs, four detector oligonucleotides (Supporting Information Table S1) were designed as described previously (Lievens *et al.*, 2003, 2006). Oligonucleotides were designed in such a way that they perfectly matched all sequences of a given OTU, but differed from sequences outside the OTU. In order to enhance the accuracy of identification, oligonucleotide sequences were derived from multiple regions in the ITS sequence. In addition to the OTU-specific oligonucleotides, a nonspecific oligonucleotide (Uni1) and a digoxigenin-labeled control oligonucleotide (Dig1) (Lievens *et al.*, 2003, 2006; Table S1) were used as a control for hybridization and detection, respectively. DNA arrays were produced as described previously (Lievens *et al.*, 2003, 2006), and all oligonucleotides were printed in duplicate. For DNA array analysis, the basidiomycetous ITS regions from all plant individuals listed in Table 1 were PCR-amplified using the primers ITS1-OF and ITS4-OF and simultaneously labeled with alkaline-labile digoxigenin (0.15 mM digoxigenin-11-dUTP mix; Roche Diagnostics GmbH, Mannheim, Germany). DNA samples were amplified according to the following PCR conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of 45 s at 94°C, 45 s at 58°C and 45 s at 72°C, with a final elongation step of 10 min at 72°C. The generated amplicons were subsequently hybridized to the DNA array. Hybridization, washing, detection and analysis of the arrays were performed as previously described (Lievens *et al.*, 2003, 2006). All hybridizations were performed twice to check for consistency of results.

Data analysis

To describe the properties of the network of associations between orchids and their mycorrhizal fungi, we calculated two community-level structural properties that are widely applied in the study of network architecture: nestedness and modularity (Lewinsohn *et al.*, 2006; Fortuna *et al.*, 2010; Thébault & Fontaine, 2010). Two different measures of nestedness were used. Following Bascompte *et al.* (2003), we first calculated $N = (100 - T)/100$, where T is the matrix temperature, a measure of matrix disorder that varies between 0° (perfectly nested) and 100° (perfectly non-nested). Nestedness values close to 1 thus indicate a high degree of nestedness. However, because T may be dependent on the size and shape of the species interaction matrix, we also calculated a recently developed nestedness measure NODF (nestedness metric based on overlap and decreasing fill) that corrects for these flaws (Almeida-Neto *et al.*, 2008). The significance of nestedness was tested using two different null models implemented in ANINHADO (Guimarães & Guimarães, 2006). In the first null model, each cell in the interaction matrix has the same probability of being occupied. This null model is very general and does not take into account that the number of connections per species may vary substantially. A more conservative null model would therefore be a model in which the probability of drawing an interaction is proportional to the level of specialization (Bascompte *et al.*, 2003). In this null model, the probability of each cell being occupied is the average of the probabilities of occupancy of its row and column (Almeida-Neto *et al.*, 2008). All nestedness analyses were performed using ANINHADO 3.0 (Guimarães & Guimarães, 2006).

To estimate the level of modularity and the number of modules, we used the simulated annealing algorithm developed by Guimerà & Amaral (2005), which identifies modules whose nodes have the majority of their links inside their own module. The algorithm provides an index of modularity M:

$$M = \sum_{s=1}^{N_M} \left[\frac{l_s}{L} - \left(\frac{d_s}{2L} \right)^2 \right], \qquad \text{Eqn 1}$$

(N_M, the number of modules; L, the number of links in the network; l_s, the number of links between nodes in module s; d_s, the sum of the number of links of the nodes in module s (Newman & Girvan, 2004).) This measure of modularity has been used before to describe the properties of bipartite networks (e.g. Olesen *et al.*, 2007; Fortuna *et al.*, 2010; Thébault & Fontaine, 2010). To determine the significance of the observed modularity index, 999 random matrices were constructed and the observed modularity index was compared with indices from random matrices.

To test for a phylogenetic signal in the plant–fungus associations, that is, to test whether the number of associations of each plant/fungus taxon is conserved by the phylogenetic relationships between the plant/fungus taxa, we constructed hypotheses of the phylogenetic relationships for both the plants and the mycorrhizal fungi. Each phylogeny was used to measure the K statistic (Blomberg *et al.*, 2003) for the number of associations per taxon. Because measurements of K are directly based on evolutionary rates (branch lengths) estimated by phylogenetic inference, we calculated K both on maximum likelihood (ML) trees, where branch lengths are estimated without a molecular clock assumption and represent genetic distance, and Bayesian relaxed clock (BRC) trees, where branch lengths are estimated under a relaxed molecular clock assumption and represent time. For the plant phylogeny, an aligned ITS data set of 26 *Orchis* species and *Traunsteinera globosa* as outgroup was obtained from Bateman *et al.* (2003). As the ITS sequences were too variable to enable construction of a phylogenetic tree spanning all the fungi found in this study, for the fungi, the

analysis was limited to the Tulasnellaceae OTUs. Alignment of the Tulasnellaceae sequences was performed using the Geneious alignment tool implemented in GENEIOUS PRO 5.0.4 (Drummond *et al.*, 2009). The TrN + G and TIM1 + G models of evolution were identified as the best-fit model for the *Orchis* and Tulasnellaceae data sets, respectively, using Akaike Information Criterion (AIC) implemented in JMODELTEST 0.1 (Posada, 2008). As these models are not available in the phylogenetic analysis software we used, we selected the GTR + G model of evolution for all phylogenetic analyses. For both data sets, an ML phylogeny was constructed with RAXML 7.0.4 (Stamatakis, 2006). Clade support was estimated with RAXML by non-parametric bootstrap analysis on 1000 pseudo-replicate data sets. In addition to the ML trees, we constructed ultrametric trees with a BRC analysis using BEAST 1.5.4 (Drummond & Rambaut, 2007). The uncorrelated lognormal clock model (Drummond *et al.*, 2006) was selected and a *pro forma* calibration point was enforced: the root height was fixed at 1.0. Posterior distributions of parameters were approximated using two independent Markov chain Monte Carlo analyses of 2.0×10^7 generations followed by a discarded burn-in of 2.0×10^6 generations (10%). Convergence of the chains was checked by evaluating the Effective Sample Size (ESS) values of each parameter with TRACER 1.5 (http://tree.bio.ed.ac.uk/software/tracer/).

The *K* statistic was used to measure the phylogenetic signal of the number of fungal associates per *Orchis* species on the *Orchis* ML and BRC trees; the number of Tulasnellaceae associates per *Orchis* species on the *Orchis* ML and BRC trees; and the number *Orchis* species associated with each Tulasnellaceae OTU on the Tulasnellaceae ML and BRC trees. *K* is the ratio of the mean squared error of the trait data divided by the mean squared error of the data calculated from the phylogeny variance–covariance matrix, and this observed ratio is standardized by the ratio expected from Brownian evolution (Blomberg *et al.*, 2003). *K* values near 0 indicate a lack of a phylogenetic signal, and values *c.* 1 typify phylogenetic conservatism. The Anderson–Darling test indicated that our data significantly differed from a normal distribution, and therefore we log-transformed the data before calculating the *K* statistic. Transformations had little effect on the conclusions of this paper. The *K* statistic on both trees was calculated with the 'phylosignal' function of the R package PICANTE (Kembel *et al.*, 2010). The *Orchis* trees were pruned to include only the species present in our mutualistic network ($n = 16$). The statistical significance of *K* was calculated based on variance of phylogenetically independent contrasts relative to tip shuffling randomization (5000 replicates).

Next to the number of mycorrhizal associations, we also tested whether phylogenetic relatedness of *Orchis* species correlates with ecological similarity. The phylogenetic distance between the *Orchis* species was calculated using the 'distance' option in Geneious based on the highest likelihood tree from the ML analysis. Following Rezende *et al.* (2007), the ecological similarity (*S*) of any two *Orchis* species was defined as the number of fungal OTUs with which they both interact divided by the total number of fungal OTUs with which they interact. Ecological distances were calculated as $1 - S$. A simple Mantel test implemented in ZT 1.1 (Bonnet & Van de Peer, 2002) was used to compare phylogenetic distance matrices with matrices of ecological distances between *Orchis* species. Because differences in the number of associated mycorrhizal taxa affect *S* estimates, we also performed partial Mantel tests controlling for the number of associated fungal taxa (the pairwise distance in number of associated taxa was calculated as the absolute difference in number of associated taxa between two *Orchis* species). This partial Mantel test can discern whether phylogeny strictly affects the identity of fungal OTUs with which *Orchis* species interact, independently of the total number of fungal associates of each *Orchis* species (Rezende *et al.*, 2007). Finally, the strength of the phylogenetic signal of the two phylogenies on the *Orchis*–Tulasnellaceae interactions was evaluated using a linear model approach that fits the phylogenetic variance–covariance matrix to the plant–fungi interaction matrix (Ives & Godray, 2006). Using this method, we calculated the independent phylogenetic signals of the *Orchis* (d_O) and Tulasnellaceae (d_T) phylogenies on the interaction matrix (association present/absent) and the strength of the signal of both phylogenies combined (MSE_d). MSE_d was compared with MSE values for a model that assumes no phylogenetic structure (MSE_{star}) and a Brownian evolution model (MSE_b). The model minimizing the mean squared error was considered the best fit. Calculations were performed with the 'pblm' function in PICANTE and were carried out on the ML and BRC *Orchis*–Tulasnellaceae phylogeny sets. Statistical significance of the *d* values was estimated by calculating 95% bootstrap confidence intervals on 100 replicates.

Results

Sequencing of basidiomycetous ITS clone libraries revealed a total of 23 fungal OTUs, the majority of which were related to members of the Tullasnellaceae (12 OTUs) and Ceratobasidiaceae (3 OTUs) (Table 2). OTUs were defined based on a sequence similarity of at least 97%. In comparison with our previous work focusing on *Orchis anthropophora*, *O. mascula*, *O. militaris*, *Orchis purpurea* and *Orchis simia* (OTU1–OTU11) (Jacquemyn *et al.*, 2010; Lievens *et al.*, 2010), 12 additional OTUs were found using a 97% sequence similarity cut-off (OTU12–OTU23). Based on the incidences of the different fungal OTUs and given a 97% similarity cut-off, asymptotic OTU richness was estimated at 27 OTUs. However, an additional 113 clone libraries would be required to reach this estimate.

Table 2 List of fungal operational taxonomic units (OTUs)[a] identified using cloning techniques

			Phylogenetic relationship[c]				
OTU	Representative sequence[b]	Sequence length (bp)	Family	Closest match in GenBank (accession number)	Sequence identity (%)	*S*-value	*E*-value
OTU 1	GQ907249	660	Tulasnellaceae	Uncultured Tulasnellaceae isolate CT96 (GQ241740)	96	1029	0.0
OTU 2	GQ907254	656	Tulasnellaceae	*Epulorhiza* sp. Ep/Sst/07 (EU418851)	98	1135	0.0
OTU 3	GQ907263	656	Tulasnellaceae	Uncultured Tulasnellaceae isolate 968 (DQ925661)	98	1175	0.0
OTU 4	GQ907260	684	Tulasnellaceae	Uncultured Tulasnellaceae isolate CF329 (GQ241761)	97	1196	0.0
OTU 5	GQ907269	687	Tulasnellaceae	*Epulorhiza* sp. RO 02 (AB369933)	98	1273	0.0
OTU 6	GQ907265	699	Tulasnellaceae	Uncultured Tulasnellaceae isolate 451 (EU195344)	97	1092	0.0
OTU 7	GQ907250	677	Tulasnellaceae	Uncultured Tulasnellaceae isolate A1.14 (EU583697)	98	1167	0.0
OTU 8	GQ907283	667	Thelephoraceae	Uncultured ectomycorrhiza (*Tomentella*) isolate UBCOCS640F (EF218835)	93	1015	0.0
OTU 9	GQ907284	702	Cortinariaceae	*Hebeloma senescens* (AY312987)	99	1258	0.0
OTU 10	GU066934	701	Tulasnellaceae	Uncultured Tulasnellaceae isolate CT100 (GQ241745)	96	1206	0.0
OTU 11	GU066936	658	Ceratobasidiaceae	Uncultured Ceratobasidiacaea isolate 7837.2.OR (EU668239)	99	1211	0.0
OTU 12	HQ330992	683	Tulasnellaceae	Uncultured Tulasnellaceae isolate S4.4 (EU583714)	99	1231	0.0
OTU 13	HQ330994	617	Sebacinaceae	Uncultured Sebacinaceae isolate 2008BNE1 (HQ204720)	99	1167	0.0
OTU 14	HQ330996	691	Russulaceae	*Russula ilicis* 563IC52 (AY061682)	99	1277	0.0
OTU 15	HQ330998	678	Tulasnellaceae	Uncultured Tulasnellaceae isolate 209 (DQ925573)	98	1158	0.0
OTU 16	HQ331000	668	Thelephoraceae	Uncultured Thelephoraceae isolate BYD5 (AY748882)	95	1065	0.0
OTU 17	HQ331002	659	Ceratobasidiaceae	*Ceratobasidium* sp. AG-I isolate Ibs1 (DQ102442)	95	1027	0.0
OTU 18	HQ331004	663	Tulasnellaceae	Uncultured Tulasnellaceae isolate 4065 (AY634130)	91	879	0.0
OTU 19	HQ331006	627	Sebacinaceae	Uncultured Sebacinaceae isolate 6.7750.3.R (EU668224)	98	1131	0.0
OTU 20	HQ331008	700	Tulasnellaceae	Uncultured Tulasnellaceae isolate 006_E4 (EF433953)	94	1098	0.0
OTU 21	HQ3311010	594	Atheliaceae	Uncultured ectomycorrhiza (*Amphinema*) isolate 3118AA (FJ210728)	95	858	0.0
OTU 22	HQ331012	669	Unknown	Uncultured fungus genomic DNA sequence (FN397418)	99	1275	0.0
OTU 23	HQ331014	729	Ceratobasidiaceae	*Ceratobasidium* sp. L9Rh-col6 (HM117643)	97	1281	0.0

[a]Fungi were grouped into OTUs defined by 97% internal transcribed spacer (ITS) sequence similarity.
[b]GenBank accession number.
[c]Based on BLAST analysis (February 2011; matches with sequences from our own data were excluded from the analysis).

Although it is generally assumed that the fungal intraspecific variability of the ITS region is relatively low and varies between 0 and 3% (Ciardo *et al.*, 2006), recent findings caution against such a simplified view of species delimitation, as some species can be found with an ITS intraspecific similarity of > 3% (Nilsson *et al.*, 2008). This may be particularly true for taxa having nuclear ribosomal regions with a high rate of evolution such as Tulasnellaceae (Taylor & McCormick, 2008). When using an ITS sequence similarity cut-off of 95% (see e.g. Waterman *et al.*, 2011), the fungi could be grouped into 21 OTUs. If a highly conservative cut-off value of 90% was applied to the Tulasnellaceae

data set, the number of Tulasnellaceae OTUs diminished from 12 to nine, essentially through the grouping of the sequences OTU1, OTU2 and OTU3, and OTU4 and OTU5 into single OTUs (Table S2). In addition, when the more conserved 5.8S rDNA locus was considered (excluding the ITS I and ITS II regions), the number of Tulasnellaceae OTUs was approximately divided by two (Table S2). However, as this region is very short (*c.* 155 bp), the use of 5.8S rDNA sequence similarity cut-offs to define OTUs probably underestimates fungal diversity. Representative sequences for each OTU were deposited in GenBank (accession numbers GQ907249–GQ907285; GU066934–GU066936; HQ330992–HQ331015).

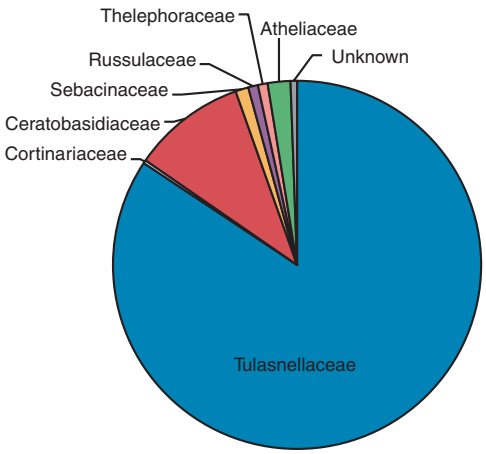

Fig. 1 Frequency distribution of fungal families detected in 16 species of the genus *Orchis*.

Of the 23 identified OTUs, all except OTU15 and OTU20 (both representing tulasnelloid fungi) were found in the sampled *Orchis* individuals. As OTU15 and OTU20 were originally derived from *Gymnadenia conopsea* clone libraries, it was not expected that these would be found in the sampled *Orchis* individuals. The basidiomycetous fungal community associating with *Orchis* species consisted predominantly of fungal OTUs related to Tulasnellaceae (84.33%) and to a lesser extent fungal OTUs related to Ceratobasidiaceae (9.97%) (Fig. 1). OTUs related to other fungal families known to associate with orchids (Athelia ceae, Russulaceae, Sebacinaceae and Thelephoraceae) were only sporadically observed (Fig. 1).

The species degree, that is, the number of fungal OTUs an orchid species associates with, varied between one and nine OTUs (Fig. 2). *Orchis anthropophora*, *Orchis italica*, *O. simia* and *O. militaris* associated with at least eight different OTUs, whereas *Orchis anatolica*, *O. mascula*, *Orchis olbiensis* and *O. troodi* associated with only one or two fungal OTUs. Using two different null models and two different measures of nestedness, nestedness analysis showed that the architecture of the fungal network was significantly

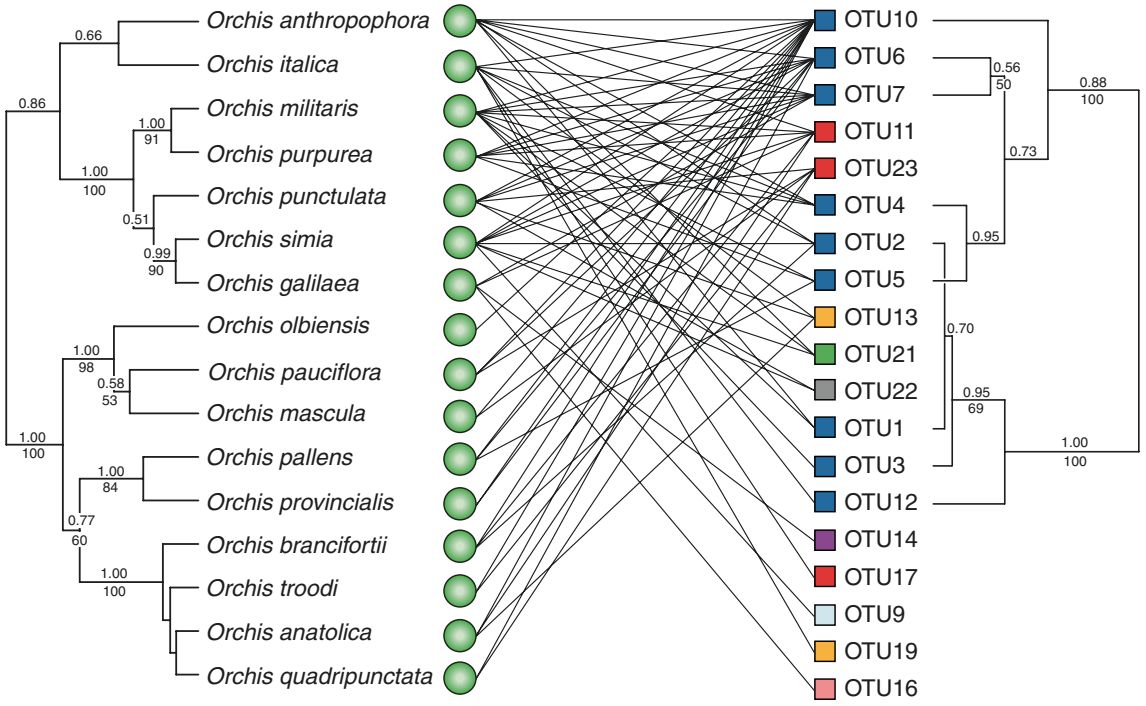

Fig. 2 Network of *Orchis*–mycorrhizal fungi interactions. Lines represent pairwise interactions. Fungal operational taxonomic units (OTUs) are ranked according to the number of interactions. Maximum clade credibility trees from Bayesian relaxed clock analyses are shown for the *Orchis* species and the Tullasnelaceae OTUs. Branch support values above the branches show posterior probabilities. Support values below branches are maximum likelihood nonparametric bootstrap percentages.

nested (N = 0.81, P < 0.001; NODF = 43.88, P < 0.001), indicating that orchid species that associate with a small number of fungal OTUs were always associating with fungal OTUs that associate with orchid species that have a large number of associations. The index of modularity, however, was low (M = 0.3490) and not significantly (P > 0.05) different from that of random networks (95% confidence interval (CI) 0.3316; 0.3865). Four modules were identified, which had, on average, 11.5 links within modules and 12.0 links to other modules.

Phylogenetic analysis of *Orchis* ITS sequences using ML and BRC methods revealed identical well-supported phylogenies, showing no significant differences from earlier hypotheses (Bateman *et al.*, 2003) (Fig. 2). On both ML and BRC phylogenies we measured a significant phylogenetic signal in species degree using Blomberg's K statistic and a randomization test when all fungal OTUs were included (P < 0.01) (Table 3). When the OTUs were restricted to the Tulasnellaceae found on the *Orchis* samples, we only measured a significant K value on the ML *Orchis* tree (P < 0.01) (Table 3). Values of the K statistic, which provides a relative index of the amount of phylogenetic signal relative to that of a trait evolving under Brownian motion, were slightly larger than 1 for both the ML tree and the BRC tree when all OTUs were considered, and for the ML tree when only Tulasnellaceae were considered (Table 3). This suggests that the number of fungal associates of closely related orchid species is more similar than expected under Brownian motion evolution. However, we measured K statistic values < 1 when we tested for a phylogenetic signal in the number of orchid species associated with each Tulasnellaceae OTU using phylogenetic ML and BRC hypotheses for the observed Tulasnellaceae OTUs.

When the similarity of the fungal OTUs with which each *Orchis* species interacted was examined, the simple Mantel test showed that the phylogenetic and ecological distance matrices of *Orchis* species were positively and significantly correlated (Z = 0.3323, P < 0.01). This means that phylogenetically related *Orchis* species tend to interact with a similar set of fungal OTUs. To determine whether this result is influenced by the phylogenetic signal in the number of associated fungal OTUs, we performed a partial Mantel test, controlling for differences in the number of interactions per *Orchis* species. This resulted in a weaker, but still significant positive correlation (Z = 0.1966, P < 0.05) between phylogenetic relatedness and ecological similarity.

Table 3 Results of randomization tests for phylogenetic signal and the K statistic, a quantitative measure of the phylogenetic signal of a trait relative to the expectation given a tree topology and assuming a Brownian motion (BM) evolutionary model

	K	P-value	K	P-value
Phylogenetic tree	All OTUs		Tulasnellaceae only	
ML *Orchis* tree	*1.09543*	**0.00120**	*1.05889*	**0.00152**
BRC *Orchis* tree	*1.12369*	**0.00040**	0.74519	**0.01380**
ML Tulasnellaceae tree			0.38254	0.10658
BRC Tulasnellaceae tree			0.58011	**0.04819**

K < 1 implies a weaker resemblance among relatives than expected under BM, and K > 1 implies stronger resemblance than expected under BM.
Bold values indicate a significant phylogenetic signal and italicized values correspond to traits with K > 1.
BRC, Bayesian relaxed clock; ML, maximum likelihood.

When we incorporated the identity of the interacting taxa in the network, we measured a moderate but significant phylogenetic signal on the *Orchis* phylogeny, both when considering the ML phylogeny (d_O = 0.46855; 95% CI 0.18388–0.67184) and when considering the BRC phylogeny (d_O = 0.50726; 95% CI 0.29479–0.74516). The phylogenetic signal of the Tulasnellaceae phylogenies was close to zero and not significant: for the ML tree, d_T = 0.01160 (95% CI 0–0.12830), and for the BRC tree, d_T = 0.013976 (95% CI 0–0.18047). The overall strength of the phylogenetic signal for the linear model fitted to the actual data (MSE_d = 0.17074 and MSE_d = 0.17095 for the ML and BRC tree sets, respectively) was closer to that found under the assumption of no phylogenetic covariances (MSE_{star} = 0.216335) than for the assumption of maximum phylogenetic signal (MSE_b = 0.36937 and MSE_b = 0.34344 for the ML and BRC tree sets, respectively). These results suggest that only phylogenetic relationships among the *Orchis* species, not the Tulasnellaceae OTUs, impose structure on the interaction matrix, but the overall phylogenetic signal is weak.

Discussion

Species of the genus *Orchis* associated primarily with fungal OTUs related to members of the Tulasnellaceae, and to a lesser extent with OTUs of the Ceratobasidiaceae, confirming previous results reported by Yukawa *et al.* (2009). These fungal families have been recognized as important associates of other temperate orchid genera, such as *Cypripedium* (Tulasnellaceae; Shefferson *et al.*, 2007), *Goodyera* (Ceratobasidiaceae; Shefferson *et al.*, 2010) and *Chiloglottis* (Tulasnellaceae; Roche *et al.*, 2010). In addition, our results are also in accordance with the findings of Schatz *et al.* (2010) and Shefferson *et al.* (2008), who both showed that Tulasnellaceae were the primary associates in *O. anthropophora*, *O. militaris* and *O. simia*. In addition to OTUs associated with Tulasnellaceae and Ceratobasidiaceae, fungal OTUs related to members of, for example, the Sebacinaceae and Atheliaceae were observed, although only very sporadically. Nonetheless, these fungi have been shown to be the dominant fungal associates in species of the terrestrial temperate orchid genus *Caladenia* (Sebacinaceae; Warcup, 1981; Swarts *et al.*, 2010) and in species of the genera *Cephalanthera* and *Corallorhiza*

(Thelephoraceae; Julou *et al.*, 2005; Abadie *et al.*, 2006; Barrett *et al.*, 2010). Members of the Thelephoraceae and Cortinariaceae have also been found in terrestrial orchids, including *Cephalanthera damasonium* (Julou *et al.*, 2005) and *Cephalanthera longifolia* (Abadie *et al.*, 2006). Although the possibility cannot be excluded that not all fungi identified in our study are truly mycorrhizal, the mycorrhizal nature of some of the fungi was confirmed previously by seed germination experiments in the field (Jacquemyn *et al.*, 2011). In addition, it should be noted that the experiments relied on microscopic isolation of mycorrhizal root sections to infer that the obtained OTUs represent mycorrhizal fungi rather than nonmycorrhizal symbionts.

Consistent with our previous study (Jacquemyn *et al.*, 2010), a nested network that was built on asymmetric links among species was found. There were no signs of compartmentalization, suggestive of tight, parallel specialization. These results thus indicate that orchid species that associated with only one or a few fungi relied on the most common fungi, whereas orchid species that associated with a broad range of fungi also associated with fungi that were only sporadically observed. Because obtaining a complete overview of the fungal lineages associating with the studied orchid species is challenging, we still may have underestimated the total diversity of mycorrhizal fungi associating with species of the genus *Orchis*. Members of the Thelephoraceae and Cortinariaceae, for example, represent ectomycorrhizal fungi. This leaves the possibility that other ectomycorrhizal fungi belonging to a phylum other than Basidiomycota may also be present. However, as these fungal lineages were only sporadically observed, the probability of finding other ectomycorrhizal fungi is likely to be small. Moreover, the observed fungal lineages largely correspond with lineages found in other studies that investigated mycorrhizal associations in the genus *Orchis* (Shefferson *et al.*, 2008; Schatz *et al.*, 2010). However, underestimation of total fungal diversity has probably little effect on the architecture of the network, as simulations have shown that, unlike the number of species and links within a network, network structure itself appears to be less sensitive to sampling effort (Nielsen & Bascompte, 2007).

In disagreement with our previous suggestion that mycorrhizal specificity might be environmentally controlled (Jacquemyn *et al.*, 2010), here we showed that mycorrhizal specificity bears a strong phylogenetic imprint. Additionally, when considering the identity of the fungal OTUs with which *Orchis* species interact, we found that closely related *Orchis* species tended to interact with a similar set of fungal OTUs. This suggests that suites of symbiotic hosts evolve to differ more with increasing phylogenetic distance. Phylogenetic constraints on mycorrhizal specificity have been reported in other orchid genera (e.g. in *Cypripedium* (Shefferson *et al.*, 2007) and *Goodyera* (Shefferson *et al.*, 2010)). However, the exact mechanisms leading to the observed nested network structure that is also phylogenetically structured remain unclear. It has been suggested that the choice of fungal hosts and thus mycorrhizal specificity are plant traits on which natural selection may be able to act (Bruns *et al.*, 2002). One possible mechanism to explain the nested network structure in *Orchis* may be that during evolutionary history certain fungal lineages have been abandoned, whereas others have been favored. Possibly, some lineages are more advantageous to orchids than others, and orchids have selected from the potential fungal community the best partner to meet their nutritional demands (Rasmussen & Rasmussen, 2009). Seed germination experiments have shown that in *Orchis* species with broad specificity (*O. anthropophora*, *O. militaris* and *O. purpurea*) protocorms associate with several different fungal lineages (Jacquemyn *et al.*, 2011), which might also suggest that these species have evolved to maximize their nutritional uptake. Similar observations have been reported for *Cypripedium* (Shefferson *et al.*, 2007). In this genus, several species also appeared to be undergoing a broadening of their phylogenetic breadth of mycorrhizal associations.

The alternative hypothesis would be that evolutionary transitions in mycorrhizal specificity are driven by differences in geographical distribution patterns of mycorrhizal fungi (Barrett *et al.*, 2010). In this case, fungal symbionts might have narrow geographic distributions, thus preventing association with the orchids and forcing specificity by the orchid because of the absence of putative symbionts. This would imply that the most common fungal OTUs are at the same time OTUs with a very wide distribution area, whereas OTUs that have been occasionally observed should have very restricted distribution areas. Although at present very little is known about the actual geographic distribution of mycorrhizal fungi in nature, this suggestion appears to be valid for at least some fungal OTUs. OTU10, for example, represents the most common fungal partner, associating with 14 out of the 16 investigated species, and it is also the OTU with the widest distribution area, being found in most sampling sites across Europe. By contrast, fungal OTUs that were only occasionally detected often had limited geographic distribution areas.

We also tested for a phylogenetic signal in the number of orchid species associated with each Tulasnellaceae-related OTU. The observed K statistic values were < 1, suggesting that the species degree for Tulasnellaceae fungi contains less phylogenetic signal than expected from their phylogenetic relationships under a Brownian motion process of evolution. In addition, the Tulasnellaceae phylogeny does not show a significant phylogenetic signal on the interaction with their associated *Orchis* species. Such asymmetric patterns have also been observed in other systems. For example, Bersier & Kehrli (2008) showed that the trophic structure of prey appeared to be more related to phylogeny

than that of predators. Similarly, Vacher *et al.* (2008) showed that compartmentalization of host–parasite interactions reflected major phylogenetic splits, but only in host phylogeny. In the case of orchid mycorrhizal associations, a possible explanation for this asymmetric relationship may be that orchid mycorrhizal fungi are not dependent on orchids for their reproduction and dispersal and can survive as either saprophytes or parasites, and that their distribution is independent of orchids. Therefore, it is unlikely that fungi have evolved substantially in response to the orchids.

To conclude, our results show that orchid mycorrhizal associations in the genus *Orchis* show a significantly nested structure that significantly correlates to the phylogeny of the orchid species, but only weakly with that of the fungi. When the ITS cut-off value for OTU determination was changed from 97 to 95 or even 90%, or a second barcode to resolve species from environmental samples was included, these conclusions were not affected, demonstrating the robustness of our results. From a conservation perspective, nested subset structure and associations with generalist and, probably, widespread fungi suggest that current distribution patterns of orchid species do not reflect mycorrhizal distributions, but are more likely limited by other factors such as habitat fragmentation and destruction, and euthrophication. Seed introduction experiments could be carried out to test this hypothesis, and also shed more light on the factors driving orchid rarity.

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Supporting Information

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

Table S1 List of detector oligonucleotides used in this study

Table S2 Internal transcribed spacer (ITS) (a) and 5.8S rDNA (b) sequence homology percentage between the different Tulasnellaceae operational taxonomic units (OTUs) identified based on an ITS sequence identity of > 97%

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