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Similarity in mycorrhizal communities associating with two widespread terrestrial orchids decays with distance

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Abstract

Aim: Interactions with mycorrhizal fungi are increasingly recognized as an important factor underlying the distribution and abundance of orchid species. However, the geographical distribution of orchid mycorrhizal fungi (OMF) and how their communities vary over large geographical areas are less well understood. Because climatic and environmental similarity may decrease with geographical distance or because some OMF have limited dispersal capabilities, similarities in orchid mycorrhizal communities can be expected to decrease with increasing distances separating orchid populations. However, up till now empirical evidence is largely lacking.

Location: Eurasia.

Taxa: *Gymnadenia conopsea* (L.) R. Brown and *Epipactis helleborine* (L.) Crantz.

Methods: High-throughput sequencing was used to perform a cross-continental comparison of OMF that associate with two widespread Eurasian terrestrial orchids, *Epipactis helleborine* and *Gymnadenia conopsea*. Both phylogenetic and nonphylogenetic measures of community dissimilarity and their components were calculated and related to geographical distances using Mantel tests.

Results: Our results showed that in both orchid species similarity in mycorrhizal communities decreased significantly with geographical distance. Decomposing the contribution of spatial turnover and nestedness to overall dissimilarity showed that the observed dissimilarity was mainly the result of species replacement between regions, and not of species loss. Similarly, a strong relationship was observed between phylogenetic community dissimilarity and geographical distance. Decomposing PCD values into a nonphylogenetic and phylogenetic component showed that orchid populations located closely next to each other were likely to contain the same operational taxonomic units (OTUs), but that the non-shared taxa came from different phylogenetic clades. Species indicator analyses showed that the majority of OMF OTUs were restricted to particular geographical areas. However, some OTUs occurred in both continents, indicating that some fungi have very wide distributions.

Main conclusions: Overall, these results demonstrate that orchid mycorrhizal communities differ substantially across large geographical areas, but that the distribution of orchids is not necessarily restricted by the distribution of particular OMF. Hence,

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widespread orchid species can be considered mycorrhizal generalists that are flexible in the OMF with which they associate across large geographical areas.

KEYWORDS

Epipactis, fungal community, *Gymnadenia*, mycorrhizal specificity, orchid mycorrhiza, spatial turnover

1 | INTRODUCTION

Mycorrhizal symbioses have been considered as one of the most important symbiotic association in terrestrial ecosystems (van der Heijden et al., 2015). In this mutualism, the soil fungus contributes mineral nutrition and water to the plant that, in turn, contributes photosynthetically fixed carbon back to the fungus, by way of a dual organ made of roots colonized by fungal hyphae, the mycorrhiza (Smith & Read, 2008). Whether a given species is a specialist or generalist largely depends on its ability to associate with a large number of partners and whether its partners have a narrow or a broad geographical range. Many studies have shown that plants are often mycorrhizal generalists (Smith & Read, 2008), in that they can interact with many taxonomically disparate mycorrhizal taxa. Conversely, there are also cases of plants that are mycorrhizal specialists (van der Heijden, Martin, Selosse, & Sanders, 2015), although the precise factors leading to specialist or generalist interactions are not well understood (Shefferson et al., 2019). Interacting with a broad range of partners may increase niche availability and allow survival in a large diversity of environments (Batstone, Carscadden, Afkhami, & Frederickson, 2018).

Since the early discoveries of Noël Bernard (1899; see Selosse, Minasiewicz, & Boullard, 2017), it is widely accepted that orchid species are dependent on mycorrhizal fungi during the early stages of plant development (Dearnaley, Perotto, & Selosse, 2016; Rasmussen & Rasmussen, 2009). Most orchid species maintain associations with mycorrhizal fungi into adulthood as well (Cameron, Leake, & Read, 2006; Rasmussen & Rasmussen, 2009; Waterman et al., 2011). The fungi that form mycorrhizas with green orchids usually are members of the Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae (Dearnaley, Martos, & Selosse, 2012; Jacquemyn, Duffy, & Selosse, 2017; Rasmussen, 1995; Smith & Read, 2008). However, recent research has indicated that many orchid species, including photosynthetic orchids, simultaneously associate with a large diversity of ectomycorrhizal fungi (i.e. fungi usually found as mycorrhizal on tree species) from the Thelephoraceae, Sebacinaceae, Inocybaceae and Tuberaceae (Jacquemyn, Brys, Waud, Busschaert, & Lievens, 2015; Kottke et al., 2010; Waterman et al., 2011; Waud, Busschaert, Lievens, & Jacquemyn, 2016; Yagame, Funabiki, Nagasawa, Fukiharu, & Iwase, 2013; Zhang, Chen, Lv, Gao, & Guo, 2012). Although the ecological function and relevance of these fungi still has to be elucidated (Jacquemyn & Merckx, 2019), the available knowledge suggests that at least in some photosynthetic species their presence has an ecological function (Jacquemyn, Waud, et al., 2017), providing further support for recent claims that fungi may have more complex niches than previously assumed (Selosse, Schneider-maunoury, & Martos, 2018). Therefore, orchids may harbour a large fungal diversity, with broadly distributed orchids having the possibility to sample diverse partners over large geographical ranges.

Although mycorrhizal dependency has been increasingly recognized as an important factor influencing both the distribution and abundance of orchid populations (McCormick & Jacquemyn, 2014; McCormick, Whigham, & Canchani-Viruet, 2018), at present little is known about the geographical distribution of orchid mycorrhizal fungi (OMF; reviewed in Jacquemyn, Duffy, et al., 2017). However, the widespread occurrence of orchids across the globe and in diverse ecosystems (Givnish et al., 2016) suggests that the OMF that are necessary for germination and seedling establishment are also widespread and not necessarily restricted to geographical regions. A major caveat in our current understanding of the biogeographical distribution of OMF is that most of the available data are very fragmentary and that often only a few populations are sampled within a restricted geographical area, making it difficult to draw any general conclusions about the distribution of fungi associated with orchids across larger scales (Jacquemyn, Duffy, et al., 2017).

The few available studies (Davis, Phillips, Wright, Linde, & Dixon, 2015; Duffy, Waud, Schatz, Petanidou, & Jacquemyn, 2019; Girlanda et al., 2006; Irwin, Bougoure, & Dearnaley, 2007; Otero, Flanagan, Herre, Ackerman, & Bayman, 2007; Roy et al., 2009; Selosse, Weiß, Jany, & Tillier, 2002; Taylor, Bruns, & Hodges, 2004; Těšitelová et al., 2015) that have attempted to sample the large-scale distribution of mycorrhizal fungi associating with a particular orchid species have shown that the wide distribution of some orchid species may to some extent be explained by the widespread occurrence of its mycorrhizae. For example, Davis et al. (2015) showed that the Australian orchid *Pheladenia deformis* associates with one or two *Sebacina* sp., but that these fungi have a widespread distribution across the Australian continent. Because many orchid species show more generalist interactions and associate with several different fungi (Girlanda et al., 2006; Jacquemyn, Brys, Lievens, & Wiegand, 2012; Roy et al., 2009; Selosse et al., 2002) and because soil fungal communities can vary strongly in space (Talbot et al., 2014), this possibly leads to turnover in mycorrhizal partners across large geographical areas and a significant decrease in similarity in mycorrhizal communities with increasing distance, that is, distance decay of similarity (Nekola & White, 1999; Soininen, Mcdonald, & Hillebrand, 2007; Talbot et al., 2014). Indeed, recent research has already indicated that OMF diversity decreases with increasing latitude (Duffy et al., 2019), and that the community composition of OMF varies according to habitat (Jacquemyn, Waud, Merckx, et al., 2016).

To improve our knowledge about the geographical distribution of OMF, we performed a cross-continental, Eurasian comparison of the mycorrhizal communities associating with the roots of two widespread terrestrial orchids, *Gymnadenia conopsea* and *Epipactis helleborine*. Given that both species are mycorrhizal generalists that associate with a wide number of mycorrhizal fungi belonging to different fungal families (Jacquemyn, Waud, Lievens, & Brys, 2016; Schweiger, Bidartondo, & Gebauer, 2018; Stark, Babik, & Durka, 2009; Těšitelová et al., 2013; Waud et al., 2016), we hypothesized that the fungal communities associated with *G. conopsea* and *E. helleborine* show large geographical variation, leading to a decay of similarity in mycorrhizal communities with increasing distance. To better understand the causality of the processes underlying variation in OMF diversity, we decomposed the overall dissimilarity into two additive components that account for species replacement and species loss respectively (Baselga, 2010).

2 | MATERIALS AND METHODS

2.1 | Study species

Gymnadenia conopsea (L.) R. Brown is a terrestrial, photosynthetic orchid that is widely distributed across Europe and Asia. Populations have been reported in Anatolia, the Caucasus, the Urals, Siberia and the Far East, including Japan, Korea and China (Meekers, Hutchings, Honnay, & Jacquemyn, 2012). It is one of five species of *Gymnadenia* that occur in China, three of which are endemic. *Gymnadenia conopsea* can be found in a wide range of habitats, including forests, grasslands and waterlogged meadows at altitudes varying between 0 and 4,700 m throughout Europe and temperate and subtropical zones of Asia (Meekers et al., 2012). In China, *G. conopsea* occurs mainly in the provinces Sichuan, Qinghai, Gansu, Tibet, Hebei, Shaanxi and Inner Mongolia. With the overexploitation of *G. conopsea* for traditional medicine as well as over-grazing and habitat destruction, natural populations of *G. conopsea* have declined rapidly in China. Currently, *G. conopsea* has been listed in the grade II section of endangered species in 2000 (Gesang & Gesang, 2010).

Epipactis helleborine (L.) Crantz occurs throughout large parts of Eurasia and North Africa (Delforge, 1995). *Epipactis helleborine* occurs in a broad range of habitat types, including dense forest floors, urban areas, open grasslands with scattered trees and calcareous soils from temperate to boreal zones (Buttler, 1991; Delforge, 1995; Hollingsworth & Dickson, 1997; Salmia, 1986). In North America, *E. helleborine* has become a rapidly spreading species after it was introduced about 150 years ago (Owen, 1879; Soper & Garay, 1954) and it is currently considered as invasive.

Previous studies on the mycorrhizal fungi associating with *G. conopsea* (Schweiger et al., 2018; Stark et al., 2009; Těšitelová et al., 2013; Waud et al., 2016) and *E. helleborine* (Bidartondo, Burghardt, Gebauer, Bruns, & Read, 2004; Jacquemyn, Waud, Lievens, et al., 2016; Ogura-Tsujita & Yukawa, 2008) have shown that both species are mycorrhizal generalists that associate with a wide range of mycorrhizal fungi, including a dominance of ectomycorrhizal fungi in the second species. However, to the best of our knowledge, the mycorrhizal associates of these two orchid species in China remain unknown.

2.2 | Sampling

G. conopsea and *E. helleborine* samples were collected from both Europe and China in July and August 2018 (Figure 1a,b). For *G. conopsea*, four populations (GITA, GITB, GBEA and GPLA) were collected from three countries of Europe (Italy, Belgium and Poland), while six populations (GB, GC, GG, GN, GS and GZ) were sampled in China (Figure 1a, Table 1). For *E. helleborine*, seven populations (EITA, EITB, EBEA, EBEB, EBEC, EBED and EPLA) growing in three countries of Europe (Italy, Belgium and Poland) and seven populations (EJL, ESX, ENJA, ENJB, ENJC, ENX and EBLGZ) growing in three provinces of China (Jilin, Shanxi and Yunnan) were collected respectively (Figure 1b, Table 1). For each population, five individual plants were randomly selected and four root fragments (3–5 cm) from each individual plant were collected. Slight yellowish or opaque roots, a typical feature of OMF infection, were selected, and surface cleaned several times with sterile water to minimize the detection of soil fungi and microscopically checked for mycorrhizal colonization. Roots were stored at −80°C prior to molecular analyses of mycorrhizal associates.

2.3 | Molecular analyses

For DNA extraction, three pieces of colonized roots (2-cm long) were used per plant individual. Genomic DNA was extracted using the E.Z.N.A.® plant DNA Kit (Omega Bio-tek) according to the manufacturer's instructions. To amplify the fungal internal transcribed spacer 2 (ITS2) region of fungi associated with *E. helleborine*, the fungal specific primer pair combination ITS86F (Turenne, Sanche, Hoban, Karlowsky, & Kabani, 1999) and ITS4 (White, Bruns, Lee, & Taylor, 1990) was used, which has been used effectively for the detection of diverse mycobionts in previous studies (Jacquemyn, Waud, Lievens, et al., 2016; Waud et al., 2016). The primer combination of ITS3 (White et al., 1990) and ITS4-OF (Taylor & McCormick, 2008) was used to amplify the ITS2 region of fungi associated with *G. conopsea* according to Waud et al. (2016). PCR reactions were performed in triplicate 50 μl mixture containing 5 μl of 10 × Pyrobest Buffer, 4 μl of 2.5 mM dNTPs, 2 μl of each primer (10 μM), 0.3 μl of Pyrobest DNA Polymerase (TaKaRa) and 30 ng of template DNA. The PCR program was as follows 95°C for 5 min, 30 cycles at 95°C for 30 s, 56°C for 30 s and 72°C for 40 s with a final extension of 72°C for 10 min. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences) according to the manufacturer's instructions and quantified using QuantiFluor™-ST (Promega). The purified amplicon mixture was subjected to high-throughput sequencing by Beijing Allwegene Tech, Ltd using the Illumina Miseq PE300 sequencing platform (Illumina, Inc.) that generated 300 bp long paired-end reads.

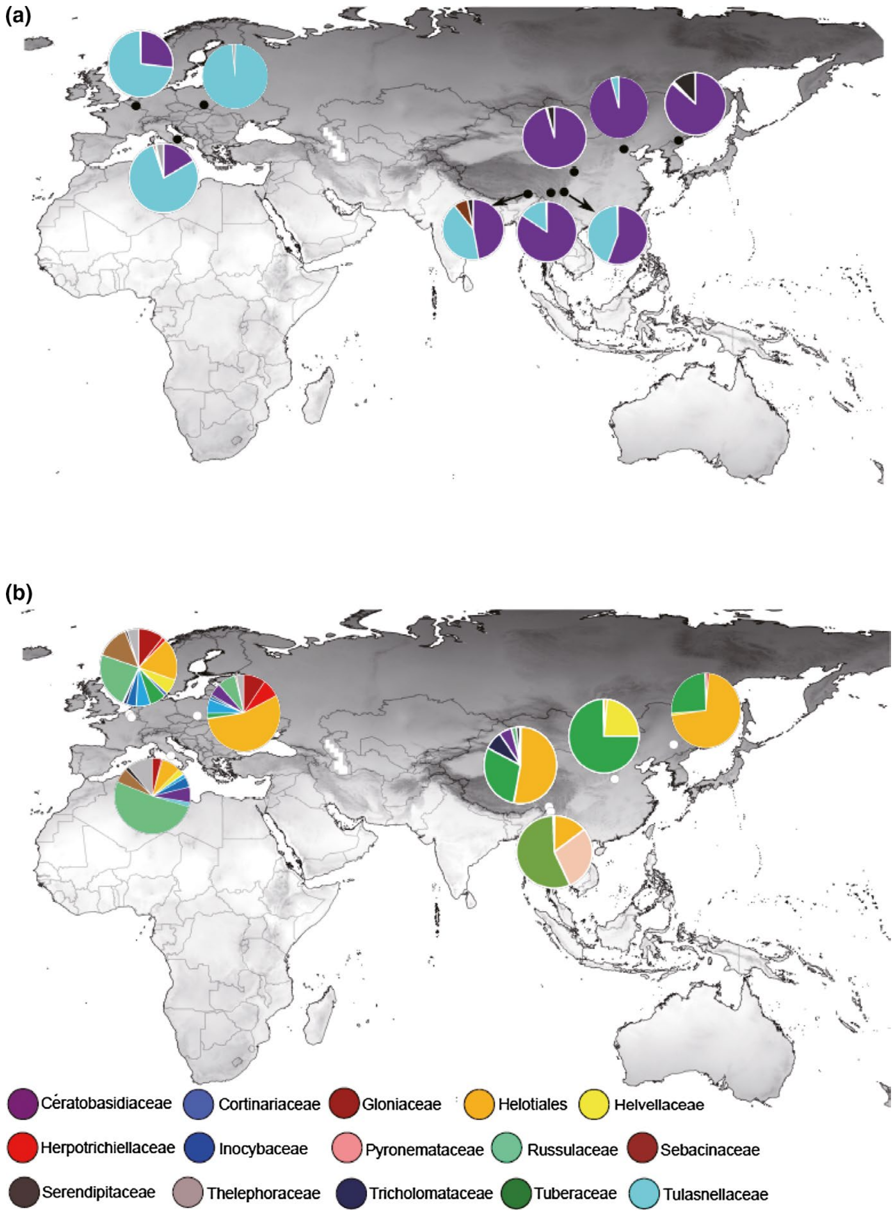

FIGURE 1 Sampling location and distribution of putative mycorrhizal fungi associated with populations of (a) *Gymnadenia conopsea* (closed circles) and (b) *Epipactis helleborine* (open circles), across their Eurasian distributions. Pie charts represent the relative number of reads belonging to each fungal family in each region sampled

2.4 | Data processing and operational taxonomic units delimitation

The extraction of high-quality sequences was firstly performed with the QIIME package (Quantitative Insights Into Microbial Ecology; v1.2.1). Raw sequences were selected based on sequence length, quality, primer and tag, wherein sequence quality was evaluated and enforced according to the following criteria. The raw sequences were selected and the low-quality sequences were removed: (a) raw reads shorter than 110 nucleotides were removed; (b) the 300 bp reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50 bp; (c) exact barcode matching, two nucleotide mismatch in primer matching, reads containing ambiguous characters were removed; (d) only sequences that show an overlap longer than 10 bp were assembled according to their overlap sequence. Reads that could not be assembled were discarded.

The unique sequence set was classified into operational taxonomic units (OTUs) under the threshold of 97% identity using UCLUST (Edgar, 2010). Chimeric sequences were identified and removed using USEARCH (version 10.1). The taxonomy of each representative ITS sequence was analysed by UCLUST against the UNITE database using confidence threshold of 90%. To minimize the risk of retaining sequences that resulted from sequencing errors, global singletons or global doubletons (OTUs represented by only one or two sequence in the entire data set) were removed as it has been shown that this improves the accuracy of diversity estimates (Ihrmark et al., 2012; Waud, Busschaert, Ruyters, Jacquemyn, & Lievens, 2014). Remaining OTUs were assigned

TABLE 1 Sampling sites, the average number of mycorrhizal fungal operational taxonomic units (OTU) and phylogenetic diversity (PD) in each population of *Epipactis helleborine* and *Gymnadenia conopsea* sampled in Europe and China

Population	Region, country	Latitude	Longitude	Number OTUs	PD
Gymnadenia conopsea					
GITA	Passo San Lanciano, Italy	42°10'47''	14°6'39''	15.6	3.03 ± 0.14
GITB	Passo San Leonardo, Italy	42°5'22''	14°1' 55''	14.8	3.15 ± 0.08
GBEA	Bonnerieu, Belgium	50°06'23"	4°43'5"	13.6	2.65 ± 0.46
GPLA	Kalina Lisiniec, Poland	50°21'44"	20°9'37"	22.5	3.63 ± 0.28
GB	Baihua Mountain, Beijing, China	39°49′19″	115°35′35″	17.2	3.20 ± 0.09
GC	Changbai Mountain, Jilin province, China	41°54′31″	128°0′18″	19.6	3.10 ± 0.41
GN	Lanping county, Yunnan province, China	29°24′36″	99°0′15″	23	3.47 ± 0.24
GS	Gongga Mountain, Sichuan Province, China	29°36′4″	102°0′42″	22.2	3.42 ± 0.25
GG	Min county, Gansu province, China	34°24′12″	104°18′12″	19.6	2.82 ± 0.43
GZ	Milin county, Tibet, China	29°7′8″	93°47′14″	19.4	2.76 ± 0.15
			Average:	18.8	3.11 ± 0.09
Epipactis helleborine					
EITA	Acquarotta, Italy	40°52'50"	14°35'52"	62.4	7.34 ± 0.31
EITB	Casafredda, Italy	40°57'20	14°40'54"	88.2	9.70 ± 0.37
EBEA	Bierbeek, Belgium	50°48'50"	4°44'29"	101.6	9.94 ± 0.31
EBEB	Bierbeek, Belgium	50°48'37"	4°44'36"	102.0	9.26 ± 0.08
EBEC	Ave-et-Auffe, Belgium	50°06'04"	5°09'29"	97.6	9.19 ± 0.23
EBED	Belvaux, Belgium	50°06'04	5°10'30"	86.6	8.40 ± 0.35
EPLA	Tunel, Poland	50°27'29''	19°58'41"	86.5	8.48 ± 0.21
EJL	Jiaohe, Jilin province, China	43°48′12″	127°2′42″	25.0	3.41 ± 0.17
ESX	Lingchuan county, Shanxi province, China	35°48′16″	113°24′42″	22.0	3.65 ± 0.25
ENJA	Lanping county, Yunnan province, China	29°24′54″	99°0′15″	17.0	2.74 ± 0.42
ENJB	Lanping county, Yunnan province, China	29°24′35″	99°0′18″	25.4	3.44 ± 0.23
ENJC	Lanping county, Yunnan province, China	29°24′26″	99°0′11″	26.0	2.95 ± 0.27
ENX	Nixi Township, Shangri-la, Yunnan Province, China	28°18′18″	99°24′42″	21.4	3.16 ± 0.28
EBLGZ	Balog Zon, Shangri-la, Yunnan Province, China	28°18′06″	99°24′36″	17.6	2.98 ± 0.44
			Average:	55.2	6.01 ± 0.36

taxonomic identities based on the BLAST (Altschul et al., 1990) results of the OTU representative sequences (selected by UPARSE) using the GenBank nucleotide (nt) and UNITE database (Edgar, 2013).

2.5 | Data analysis

Prior to removal of OTUs known as non-mycorrhizal fungi, MOTHUR (Schloss et al., 2009) was used to generate rarefaction curves for each sample to estimate the overall coverage of the fungal communities studied (Figure S1). The overall diversity and phylogenetic diversity of OMF detected in roots of *E. helleborine* (Figure S2) and *G. conopsea* (Figure S3) were examined respectively. OTUs were manually screened for possible orchid associating mycorrhizal families based on the information of previously detected mycorrhizal fungi from the roots, germinating seeds and protocorms of various *Epipactis* species (Bidartondo et al., 2004; Jacquemyn, Waud, Lievens, et al., 2016; Selosse, Faccio, Scappaticci, & Bonfante, 2004; Těšitelová et al., 2013; Table S3). Mycorrhizal symbionts of *G. conopsea* mainly belong to the fungal families Ceratobasidiaceae, Tulasnellaceae, Thelophoraceae, Serendipitaceae and Sebacinaceae

(Table S1; Stark et al., 2009; Těšitelová et al., 2013; Waud et al., 2016). Here, we restricted our analysis to those fungal families known to associate with the two orchid species. Representative sequences for each mycorrhizal OTU were submitted to GenBank under the accession numbers: MN006041-MN006135 (*G. conopsea*) and MK955493-MK955537, MK956832-MK956905, MK959119-MK959180, MK961130-MK961204, MK962538-MK962579 and MK965742-MK965875 (*E. helleborine*).

To compare the phylogenetic diversity (PD; Faith, 1992) of OMF between Europe and China, we first constructed a ML tree for all the mycorrhizal OTUs identified. The OTU sequences were aligned using Clustal X version 2.0 (Larkin et al., 2007). The best model of evolution was identified using the Akaike Information Criterion implemented in jModelTest 2 (Darriba, Taboada, Doallo, & Posada, 2012). The GTR+G+I and K2+G+I models of evolution were identified as the best-fit models for the *E. helleborine* and *G. conopsea* data sets respectively. The ML phylogeny was constructed with RAxML 7.2.8 (Stamatakis, Hoover, & Rougemont, 2008). The phylogenetic distances between the OTUs from these trees were used to calculate PD of the OTUs associated with each individual orchid plant. All calculations were done using the software package 'picante' (Kembel et al., 2010) in R (R Development Core Team, 2016). Univariate analysis of variance (ANOVA) was used to test whether the number and PD of OTUs per plant associating with differed significantly between European and Asian samples.

To test the hypothesis that similarities in mycorrhizal communities decrease with increasing distance, we first calculated the distances between all sampled populations using Vincenty's inverse solution (Vincenty, 1975), while Sørensen's dissimilarity index (β_{sor}) and phylogenetic community dissimilarity (PCD) were used to assess dissimilarity in mycorrhizal communities between sampled populations. For each pair of populations, Sørensen's pairwise dissimilarity was calculated as $\beta_{\text{sor}} = (b+c)/(2a+b+c)$, with a the number of fungal OTUs common to both populations, b the number of OTUs occurring in the first, but not in the second population and c the number of species occurring in the second but not in the first population. Phylogenetic community dissimilarity was calculated using the formulas outlined in Ives and Helmus (2010). PCD values <1 indicate that mycorrhizal communities are more similar than randomly selected communities, whereas PCD values >1 denote mycorrhizal communities that are more dissimilar than random communities sampled from the total species pool (Ives & Helmus, 2010). To test for a significant relationship between geographical distances and community dissimilarities, Mantel tests (1967) were performed using a total of 9,999 random permutations.

To get better insights into the precise factors determining dissimilarities, we further decomposed the Sørensen dissimilarity measures into components that assess the contribution of spatial turnover β_{sim} =min (b,c)/(a+min (b,c)) and nestedness (Baselga, 2010):

$$\beta_{\text{nes}} = \frac{\max(b,c) - \min(b,c)}{2a + \min(b,c) + \max(b,c)} \times \frac{a}{a + \min(b,c)}.$$

Mantel tests were used again to assess the relationship between geographical distances and similarities derived from spatial turnover and nestedness. For both species, we also calculated the overall multiple-site dissimilarities (β_{sor}) and its individual components (β_{sim} and β_{nes}). Furthermore, PCD values were partitioned into a nonphylogenetic component that reflects shared species between communities (PCDc) and a phylogenetic component that reflects the evolutionary relationships among non-shared species (PCDp; Ives & Helmus, 2010). We then used Mantel tests to see whether PCDc and PCDp values were significantly related to the geographical distance separating communities. All calculations were performed using the R packages betapart (Baselga et al., 2018) and picante (Kembel et al., 2010).

Finally, Species Indicator Analyses were performed to examine whether certain OTUs were characteristic for a given geographical region. We used the multipatt function in the R package indicspecies (De Cáceres, Legendre, & Moretti, 2010) to define indicator OTUs of both orchid species to a particular geographical region (Europe vs. Asia).

3 | RESULTS

3.1 | Fungal OTUs

In total, Illumina Miseq PE300 sequencing generated 1,198,133 (1,943 OTUs) and 848,653 (1,176 OTUs) fungal sequences for *E. helleborine* and *G. conopsea* respectively. After analysis, 98.8% of the total number of sequences in *E. helleborine* (1,183,757 sequences, 1,858 OTUs) and 95.5% of the total number of sequences in *G. conopsea* (810,902 sequences, 1,052 OTUs) could be assigned to Ascomycota and Basidiomycota. Rarefaction curves showed that the number of OTUs was relatively close to saturation for each individual plant (Figure S1).

3.1.1 | *Gymnadenia conopsea*

The most abundant fungi detected in *G. conopsea* belonged to the fungal families of Ceratobasidiaceae (42 OTUs), Tulasnellaceae (9 OTUs) and Serendipitaceae (9 OTUs), as well as to the ectomycorrhizal taxa Thelephoraceae (19 OTUs), Inocybaceae (16 OTUs), Sebacinaceae (16 OTUs), Russulaceae (14 OTUs) and Tuberaceae (6 OTUs). In addition, a large number of other fungal taxa were also detected, including Cantharellaceae (6 OTUs), Tricholomataceae (17 OTUs), Tremellaceae (7 OTUs), Nectriaceae (24 OTUs) and Entolomataceae (17 OTUs; Figure S4d). Members of Ceratobasidiaceae were most abundant (190,338 sequences, 25.95%), followed by Tulasnellaceae (115,167 sequences, 13.58%), Entolomataceae (84,384 sequences, 9.95%), Inocybaceae (76,731 sequences, 9.05%) and Nectriaceae (66,383 sequences, 7.83%). Members of the ectomycorrhizal Sebacinaceae, Thelephoraceae, Inocybaceae, Russulaceae and Tuberaceae represented <1% relative abundance (Figure S4a).

3.1.2 | *Epipactis helleborine*

The most abundant fungi detected in *E. helleborine* belonged to Helotiales (134 OTUs), Ceratobasidiaceae (22 OTUs) and Serendipitaceae (18 OTUs), as well as ectomycorrhizal taxa Thelephoraceae (62 OTUs), Inocybaceae (29 OTUs), Sebacinaceae (25 OTUs), Russulaceae (32 OTUs), Cortinariaceae (30 OTUs), Helvellaceae (14 OTUs), Tuberaceae (13 OTUs) and Hymenogastraceae (2 OTUs). In addition, a number of other fungal taxa previously shown to colonize *Epipactis* spp. were detected, including Tricholomataceae (16 OTUs), Gloniaceae (12 OTUs), Herpotrichiellaceae (9 OTUs), Pyronemataceae (5 OTUs) and Psathyrellaceae (3 OTUs; Figure S5d). Members of Tulasnellaceae were represented by six OTUs, five of which were detected in European samples, and only one from an individual plant collected from China. In terms of relative abundances of sequences, members of Tuberaceae were most abundant (216,586 sequences, 28.86%), followed by Helotiales (176,777 sequences, 23.56%), Russulaceae (101,541 sequences, 13.53%), Pyronemataceae (44,388 sequences, 5.92%), Helvellaceae (41,300 sequences, 5.50%) and Sebacinaceae (38,618 sequences, 5.15%; Figure S5a).

3.2 | Mycorrhizal fungal communities

3.2.1 | *Gymnadenia conopsea*

G. conopsea mainly associated with members of the Ceratobasidiaceae and Tulasnellaceae and ectomycorrhizal fungi from the Sebacinaceae and Thelephoraceae. These fungal associates represented in total 95 OTUs (314,182 sequences, 37.02% of the total sequences; Table S1), of which 71 (80,672 sequences) and 50 (233,510 sequences) were detected in Europe and China respectively (Table S2). Thirty-nine OTUs (47.6% of all OTUs) were shared between Europe and China. In terms of relative abundance, members of the Tulasnellaceae were the most abundant fungal associates in Europe, while members of the Ceratobasidiaceae were the most abundant fungal associates of *G. conopsea* in China (Figure S4b,c,e). About half of the rhizoctonia fungal OTUs were detected in samples from both Europe and China (Figure 1a). In contrast, none of the fungal OTUs of Sebacinaceae was shared between Europe and China. The average number of mycorrhizal OTUs detected on the roots of individuals of *G. conopsea* in Europe (16.6, range: 9–27) was significantly smaller than that found on roots of plants in China (20.2, range: 7–29; $F = 4.047$, $p = .012$; Table 1). However, PD values did not significantly ($F = 2.706$, $p > .05$) differ between plants from Europe (3.117 ± 0.207) and China (3.128 ± 0.121; Table 1).

3.2.2 | *Epipactis helleborine*

Four hundred and thirty-two OTUs (765,472 sequences, 63.89% of the total sequences) previous described as fungal associates from the genus *Epipactis* were detected in this research (Table S3). Three hundred and twenty-six OTUs (359,589 sequences) and 169 OTUs (405,883 sequences) were detected in Europe and China, respectively, and 64 OTUs (196,428 sequences; 14.8% of all OTUs) were shared (Table S4). When excluding the Helotiales (134 OTUs, 176,777 sequences), of the remaining 298 OTUs (588,695 sequences), 234 OTUs (299,690 sequences) and 83 OTUs (289,005 sequences) were detected in Europe and China, respectively, and 20 OTUs (36,968 sequences; 6.7% of all OTUs) occurred in both regions. They belonged to nine different fungal families (Herpotrichiellaceae, 5 OTUs; Gloniaceae, 5 OTUs; Ceratobasidiaceae, 3 OTUs; Serendipitaceae, 2 OTUs; Thelephoraceae 2 OTUs; Psathyrellaceae, Tricholomataceae, Tulasnellaceae and Pyronemataceae, 1 OTU respectively).

The relative abundance of the fungal families differed between Europe and China (Figure 1b). Members of Russulaceae were most abundant in Europe, while members of the Tuberaceae were most abundant in China. Members of Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae were present in relatively low abundance in both Europe and China (Figure S5b,c,e). The average number of OTUs detected on the roots of individuals of *E. helleborine* in Europe (89.3, range: 43–112) was significantly higher than those on the roots of plants from China (22.1, range: 8–39; $F = 3.984$, $p < .001$; Table 1). The difference is still significant when Helotiales is excluded ($F = 3.926$, $p < .001$; Europe 61.9, range: 36–78; China 9.0, range 3–15). Similarly, PD values of the mycorrhizal communities associating with *E. helleborine* in Europe (8.903 ± 0.337) were significantly ($F = 3.980$, $p < .001$) higher than those of the communities associating with *Epipactis* in China (3.189 ± 0.122, Table 1).

3.3 | Comparison of mycorrhizal communities

The estimated overall multiple-site dissimilarity was higher for *E. helleborine* ($\beta_{sor} = .86$) than for *G. conopsea* ($\beta_{sor} = .76$). In both species most of the multiple-site dissimilarity was the result of spatial turnover ($\beta_{sim} = .76$ and 0.74, respectively) and to a much smaller extent of nestedness ($\beta_{nes} = .09$ and .02, respectively), indicating that the observed dissimilarity patterns are the result of taxon replacement and not by taxon loss. In both orchid species, dissimilarity in mycorrhizal communities increased significantly with increasing geographical distance ($r_M = 0.84$, $p < .0001$ and $r_M = 0.65$, $p = .02$ for *E. helleborine* and *G. conopsea*, respectively; Figures 2 and 3). When the contributions of spatial turnover and nestedness were quantified separately, spatial turnover contributed most to the observed patterns of mycorrhizal dissimilarity with nestedness showing little variation with increasing distance.

PCD values were generally low (<1) for populations located close to each other, but increased (>1) when populations were further apart (Figures 2 and 3). For *E. helleborine*, the relationship between PCD values and geographical distances was highly significant ($r_M = 0.82$, $p < .0001$). Furthermore, the compositional component of PCD (PCDc) was also strongly and significantly correlated with geographical distance ($r_M = 0.79$, $p < .0001$), while PCDp was less tightly correlated with geographical distance ($r_M = 0.55$, $p = .0008$; Figure 2). In *G. conopsea*, both PCD and PCDc were significantly correlated with geographical distance (PCD: $r_M = 0.62$, $p = .0135$; PCDc: $r_M = 0.59$, $p = .0195$; Figure 3), but there was no significant relationship between PCDp and geographical distance ($r_M = -0.25$, $p > .05$; Figure 3).

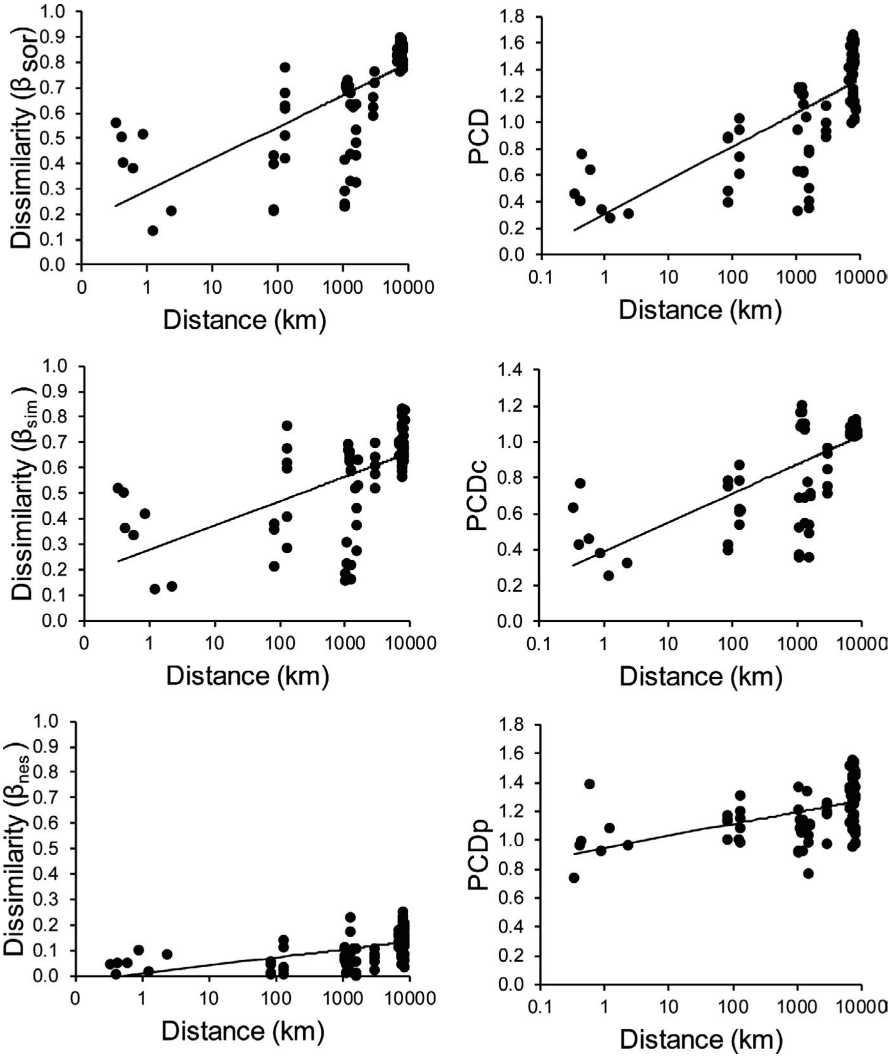

FIGURE 2 Relationships between geographical distances and mycorrhizal dissimilarity (β_{sor}, β_{sim} and β_{nes}) and phylogenetic dissimilarity (PCD, PCDc and PCDp) of populations of *Epipactis helleborine* sampled across Eurasia

Species indicator analyses showed that a large number of fungal OTUs was significantly associated with one of the two geographical regions. In total, 208 and 23 mycorrhizal OTUs were significantly associated with *E. helleborine* growing in Europe and China respectively (Table S5). In *G. conopsea*, 28 OTUs were significantly associated with Europe, while 21 OTUs almost exclusively occurred in China (Table S6).

4 | DISCUSSION

The results of this study show that there is a general increase in dissimilarity with increasing geographical distance in mycorrhizal communities associating with two widespread orchids, *Epipactis helleborine* and *Gymnadenia conopsea*. This pattern is consistent with previous work that has documented a decay in similarity for a wide range of organisms (Nekola & White, 1999; Soininen et al., 2007) and confirms earlier findings that patterns repeatedly observed for macroorganisms may also occur in microorganisms such as fungi (Talbot et al., 2014). In addition, our results showed that most of the observed dissimilarity was the result of spatial turnover and not of nestedness, indicating that both investigated orchid species are mycorrhizal generalists that show large geographical variation in their mycorrhizal communities.

4.1 | Mycorrhizal fungi associating with two widespread orchid species

A recent review on the biogeography of orchid mycorrhizas has suggested that the fungal families that associate with orchids occur in a wide variety of habitats and that some of these fungal species have a very wide distribution (Jacquemyn, Duffy, et al., 2017). Our results are in line with these observations. In our study, *Gymnadenia conopsea* mainly associated with fungi of the Ceratobasidiaceae and Tulasnellaceae, while members of Sebacinales were present at low abundance. In addition, ectomycorrhizal taxa of the Thelephoraceae, Russulaceae, Inocybaceae and Cortinariaceae were also detected. When comparing the OMF community composition of *G. conopsea* between European and Asian populations, around a half of the dominant fungi found in Europe were also found in China, indicating that these fungi have a very broad geographical distribution. Six of nine Tulasnellaceae OTUs, 21 of 42 Ceratobasidiaceae and six of

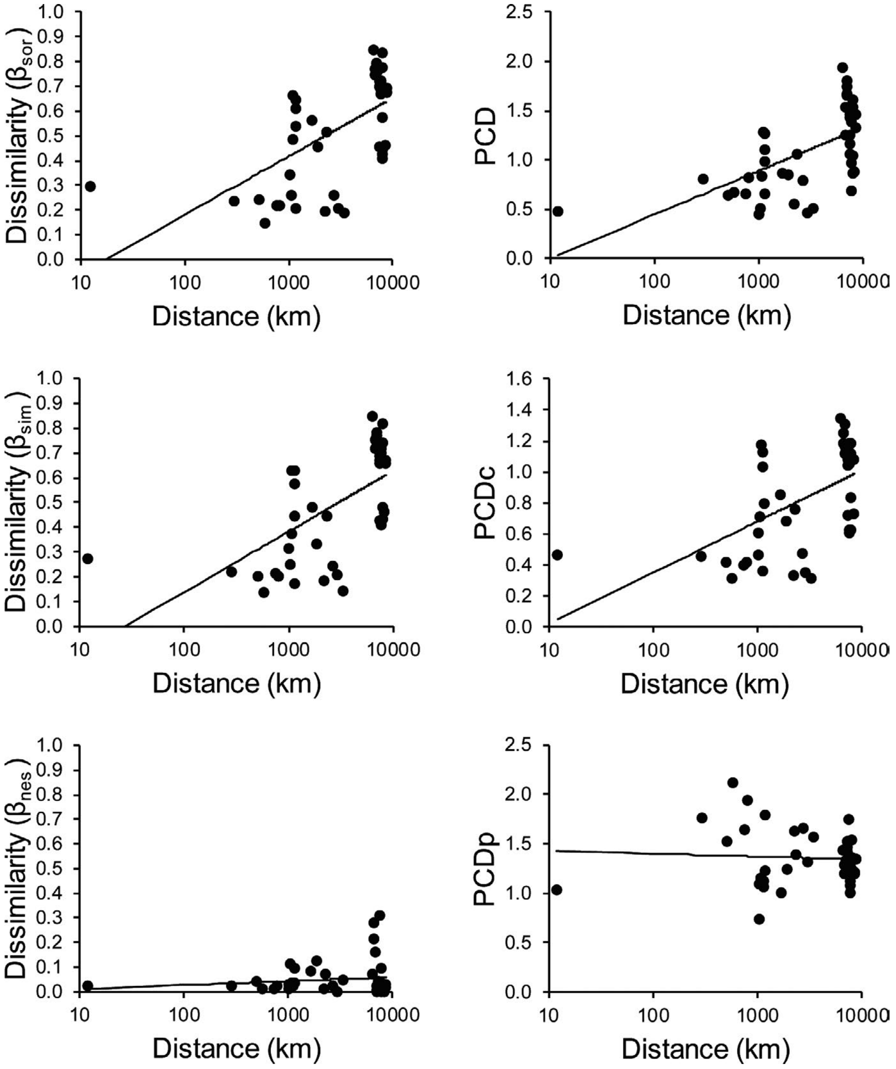

FIGURE 3 Relationships between geographical distances and mycorrhizal dissimilarity (β_{sor}, β_{sim} and β_{nes}) and phylogenetic dissimilarity (PCD, PCDc and PCDp) of populations of *Gymnadenia conopsea* sampled across Eurasia

nine Serendipitaceae OTUs were shared between Europe and China. By allowing seed germination the widespread distribution of these fungal taxa may therefore contribute to the widespread distribution of *G. conopsea*. In contrast, for members of the ectomycorrhizal Thelephoraceae, less than a quarter of the OTUs was found in both Europe and Asia, none of the Sebacinaceae members was found in both areas, indicating that the presence of these fungal members tends to vary across sites and therefore may be of lesser importance than rhizoctonia fungi. This pattern of interaction specificity strongly resembles a pattern that was recently coined 'apparent generalism', in which an orchid species specializes on one or few host species that contribute unique resources, but also associates with other host species that contribute functionally redundant resources (Shefferson et al., 2019).

In contrast, the mycorrhizal communities found in the roots of *E. helleborine* showed a high regional specificity and most of the fungal associates differed between European and Asian populations. Plants of *E. helleborine* mainly associated with members of Helotiales, fungi of Ceratobasidiaceae and Serendipitaceae, and ectomycorrhizal fungi of the Thelephoraceae, Inocybaceae, Sebacinaceae and Russulaceae. In addition, a large number of other ectomycorrhizal taxa known as fungal associates of *E. helleborine* and other *Epipactis* species were detected, mainly including Tuberaceae, Cortinariaceae, Tricholomataceae and Helvellaceae. Only around 15% (64 of 432 putative mycorrhizal OTUs) of the mycorrhizal OTUs were shared between both regions. The dominant fungal associates also differed between European and Asian populations. For example, members of Russulaceae were the most dominant fungal associates in Europe, whereas members of Tuberaceae were most abundant in Asian populations, and not one OTU belonging to these two families was shared between the two regions. Similar patterns were observed for OTUs belonging to Cortinariaceae, Hymenogastraceae, Inocybaceae, Tulasnellaceae, Sebacinaceae and Helvellaceae. This pattern of interaction specificity was recently coined 'true generalism', in which an orchid species associates with multiple hosts that overlap functionally, and that are geographically interchangeable based on opportunity for encounter, leading to frequent host switching (Shefferson et al., 2019). The observed low selectivity towards mycorrhizal fungi and strong spatial turnover in fungal communities may also explain why this species occurs in a wide variety of habitats and seemingly easily colonizes new habitat and even can become invasive (Owen, 1879; Soper & Garay, 1954).

The two investigated species display different nutritional modes: *E. helleborine* is a partially heterotrophic (mixotrophic) orchid species (Bidartondo et al., 2004; Schiebold, Bidartondo, Karasch, Gravendeel, & Gebauer, 2017) that obtains a part of its carbon from its own photosynthesis and the other part from its mycorrhizal fungi. In this species, the fungus may provide 20%–100% of its carbon to the plant, depending on the time in the growth season (Gonneau et al., 2014). In contrast, *G. conopsea* is often considered autotrophic, although a limited amount of carbon may be derived from the fungus (Schweiger et al., 2018). To what extent this biological difference in resource acquisition explains why *E. helleborine* is more opportunistic in fungal associations than *G. conopsea* remains unclear at this point and warrants further investigation.

4.2 | Patterns of dissimilarity

Variation in the species composition of ecological communities can be the result of spatial species turnover and nestedness (Baselga, 2010; Harrison, Ross, & Lawton, 1992). Nestedness refers to the non-random loss of species and leads to progressive dissimilarity between the most species-rich communities and communities that contain only a few species anymore. Spatial turnover, on the other hand, refers to the repeated replacement of one species by another. Both processes can contribute to changes in community composition across large geographical areas and therefore it is important to assess the contribution of both processes to identify the potential causes determining variation in biotic communities (Baselga, 2010).

Our results showed that for both orchid species spatial turnover was the most important factor contributing to the observed variation in mycorrhizal communities. For both species, the relationships between PCD values and geographical distances were also significant, indicating that populations located close to each other have mycorrhizal communities that are more similar than randomly selected communities, whereas populations separated by large distances have mycorrhizal communities that are more dissimilar than random communities. Furthermore, the compositional component of PCD (PCDc) was strongly and significantly correlated with geographical distance, while PCDp was not significantly or only weakly correlated with geographical distance. These results indicate that orchid populations located closely next to each other are likely to contain the same species (PCDc), but that the non-shared taxa come from different phylogenetic clades (PCDp).

The occurrence and geographical variation in local abundance of fungal strains can be influenced by local habitat conditions (Pandey, Sharma, Taylor, & Yadon, 2013) and OMF community composition may therefore be the result of complex interactions between different factors, including extrinsic factors (habitat type, geographical site, soil characteristics, etc.) and intrinsic factors (genetic differentiation, phylogeny of host plants; Chen et al., 2019; McCormick & Jacquemyn, 2014; Swarts & Dixon, 2009; Xing, Ma, Men, Chen, & Guo, 2017). Previous research has, for example, shown that variation in local environmental factors such as soil moisture content, pH, nutrient conditions (especially soil carbon, nitrogen and phosphorus) can generate pronounced differences in mycorrhizal communities (Bunch, Cowden, Wurzburger, & Shefferson, 2013; Jacquemyn et al., 2015). For example, populations of the terrestrial orchid *Neottia ovata* occurring in forest and meadow habitats showed significantly different OM fungal communities (Oja, Kohout, Tedersoo, Kull, & Kõljalg, 2015). Mycorrhizal communities even vary between populations mainly due to differences in soil moisture content and pH (Jacquemyn et al., 2015). In the case of *E. helleborine*, differences in tree species composition and associated ectomycorrhizal communities may explain the observed differences between regions and the role of spatial turnover. More research is needed why some fungal families display very large geographical distributions, whereas others seem to be more restricted.

5 | CONCLUSION

This cross-continental comparison of the mycorrhizal communities associated with two widespread terrestrial orchid *E. helleborine* and *G. conopsea* shows how similarities in fungal communities change with distance. The fungal community composition of the two orchid species differed significantly between Europe and China, leading to significant turnover in mycorrhizal communities and significant decay of similarity across large geographical distances. Nonetheless, some OTUs were found in both continents, suggesting that these fungi have very wide distributions that are not restricted by soil or local climate conditions. Strong turnover in fungal communities and significant decay of similarity with distance indicate that these orchids are generalists in their OMF communities across large geographical areas. More research is needed to understand the relative contribution of mycorrhizal taxa on the fitness of orchid that associate with multiple taxa, and whether orchid populations associated with particular OMF communities in one region are able to readily utilize OMF in another region.

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DATA AVAILABILITY STATEMENT

We confirm that, should the manuscript be accepted, the data supporting the results will be archived in Dryad and Figshare and the data DOI will be included at the end of the article. Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.6bg5473 (Xing, Gao, Zhao, Waud, Duffy, Selosse, Jakalski, Liu, Jacquemyn & Guo, 2019).

All mycorrhizal community data for Gymnadenia conopsea and Epipactis helleborine are available from DRYAD https://doi.org/10.5061/dryad.6bg5473 (Xing, Gao, Zhao, Waud, Duffy, Selosse, Jakalski, Liu, Jacquemyn & Guo, 2019).

Title: Data from: Similarity in mycorrhizal communities associating with two widespread terrestrial orchids decays with distance

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BIOSKETCH

Xiaoke Xing is an ecologist working mainly on the interactions between plants and their fungal endophytes, with strong interests in the role of mycorrhizal interactions on the distribution and conservation of orchid plants.

Author contributions: X.X. and H.J. designed the research. X.X., H.J. and M.W. sampled the root material. Y.G., Z.Z., M.J. and N.L. performed laboratory work. H.J. and M.W. performed the bioinformatic analysis. X.K. and H.J. analysed the data, plotted the figures and wrote the draft manuscript. M.A.S. and H.J. edited the manuscript. X.X., K.J.D., H.J. and G.S. contributed to the final version of the manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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