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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 geographic 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 non-phylogenetic measures of community dissimilarity and their components were calculated and related to geographic 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 geographic 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 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 geographic 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 geographic areas, but that the distribution of orchids is not necessarily restricted by the distribution of particular OMF. Hence, widespread orchid species can be considered mycorrhizal generalists that are flexible in the OMF with which they associate across large geographic 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 et al., 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 et al., 2018).

Since the early discoveries of Noël Bernard (1899; see Selosse et al., 2017), it is widely accepted that orchid species are dependent on mycorrhizal fungi during the early stages of plant development (Rasmussen & Rasmussen 2009; Dearnaley et al., 2016). Most orchid species maintain associations with mycorrhizal fungi into adulthood as well (Cameron et al., 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 (Rasmussen, 1995; Smith &

Read, 2008; Dearnaley et al., 2012; Jacquemyn et al., 2017b). 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 (Waterman et al., 2011, Kottke et al., 2010; Zhang et al., 2012; Yagame et al., 2013; Jacquemyn et al., 2015; Waud et al., 2016). 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 et al., 2017a), providing further support for recent claims that fungi may have more complex niches than previously assumed (Selosse et al., 2018). Therefore, orchids may harbor 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 et al., 2018), at present little is known about the geographic distribution of orchid mycorrhizal fungi (OMF) (reviewed in Jacquemyn et al., 2017b). 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 geographic area, making it difficult to draw any general conclusions about the distribution of fungi associated with orchids across larger scales (Jacquemyn et al., 2017b).

The few available studies (Taylor et al., 2004; Selosse et al., 2002; Girlanda et al., 2006; Irwin et al., 2007; Otero et al., 2007; Roy et al., 2009; Davis et al., 2015; Těšitelová et al., 2015; Duffy et al., 2019) 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 (Selosse et al., 2002; Girlanda et al., 2006; Roy et al., 2009; Jacquemyn et al., 2012) and because soil fungal communities can vary strongly in space (Talbot et al., 2014), this possibly leads to turnover in mycorrhizal partners across large geographic areas and a significant decrease in similarity in mycorrhizal communities with increasing distance, i.e. so-called distance decay of similarity (Nekola & White, 1999; Soininen et al., 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 et al., 2016a).

To improve our knowledge about the geographic distribution of orchid mycorrhizal fungi, 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 et al., 2016b; Stark et al., 2009; Těšitelová et al., 2013; Waud et al., 2016; Schweiger et al., 2018), we hypothesized that the fungal communities associated with *G. conopsea* and *E. helleborine* show large geographic 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 et al., 2012). It is one out 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

147 between 0 and 4700 m throughout Europe and temperate and subtropical zones of Asia
148 (Meekers et al., 2012). In China, *G. conopsea* occurs mainly in the provinces Sichuan,
149 Qinghai, Gansu, Tibet, Hebei, Shaanxi and Inner Mongolia. With the overexploitation
150 of *G. conopsea* for traditional medicine as well as over-grazing and habitat destruction,
151 natural populations of *G. conopsea* have declined rapidly in China. Currently, *G.*
152 *conopsea* has been listed in the grade II section of endangered species in 2000 (Gesang
153 & Gesang, 2010).

154 *Epipactis helleborine* (L.) Crantz occurs throughout large parts of Eurasia and North
155 Africa (Delforge, 1995). *Epipactis helleborine* occurs in a broad range of habitat types,
156 including dense forest floors, urban areas, open grasslands with scattered trees and
157 calcareous soils from temperate to boreal zones (Salmia, 1986; Buttler, 1991; Delforge,
158 1995; Hollingsworth & Dickson, 1997). In North America, *E. helleborine* has become
159 a rapidly spreading species after it was introduced about 150 years ago (Owen, 1879,
160 Soper & Garay, 1954) and it is currently considered as invasive.

161 Previous studies on the mycorrhizal fungi associating with *G. conopsea* (Stark et al.,
162 2009; Těšitelová et al., 2013; Waud et al., 2016; Schweiger et al., 2018) and *E.*
163 *helleborine* (Bidartondo et al., 2004; Ogura-Tsujita & Yukawa, 2008; Jacquemyn et al.,
164 2016b) have shown that both species are mycorrhizal generalists that associate with a
165 wide range of mycorrhizal fungi, including a dominance of ectomycorrhizal fungi in
166 the second species. However, to the best of our knowledge, the mycorrhizal associates
167 of these two orchid species in China remain unknown.

168

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, GPLA) were collected from three countries of Europe (Italy, Belgium and Poland), while six populations (GB, GC, GG, GN, GS, GZ) were sampled in China (Figure 1a, Table 1). For *E. helleborine* samples, seven populations (EITA, EITB, EBEA, EBEB, EBEC, EBED, EPLA) growing in three countries of Europe (Italy, Belgium and Poland) and seven populations (EJL, ESX, ENJA, ENJB, ENJC, ENX, EBLGZ) growing in three provinces of China (Jilin, Shanxi and Yunnan) were collected respectively (Figure 1b, Table 1). For each population, 5 individual plants were randomly selected and 4 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℃ 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, Norcross, GA, U.S.) 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 et al., 1999)

and ITS4 (White et al., 1990) was used, which has been used effectively for the detection of diverse mycobionts in previous studies (Jacquemyn et al., 2016b; 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 50μL mixture containing 5μL of 10 × Pyrobest Buffer, 4μL of 2.5mM dNTPs, 2 μL of each primer (10 μM), 0.3μL 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 30s, 56 °C for 30s, and 72 °C for 40s with a final extension of 72 °C for 10min. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, U.S.).The purified amplicon mixture was subjected to high-throughput sequencing by Beijing Allwegene Tech, Ltd (Beijing, China) using the Illumina Miseq PE300 sequencing platform (Illumina, Inc., CA, USA) that generated 300 bp long paired-end reads.

2.4 Data processing and OTU 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: (i) raw reads shorter than 110 nucleotides were removed, (ii) 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 50bp, (iii) exact barcode matching, 2 nucleotide mismatch in primer matching, reads containing ambiguous characters were removed, (iv) only sequences that 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 analyzed 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 dataset) were removed as it has been shown that this improves the accuracy of diversity estimates (Ihrmark et al., 2012; Waud et al., 2014). Remaining OTUs were assigned 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; Selosse et al., 2004; Těšitelová et al. 2013, Jacquemyn et al., 2016b) (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 et al., 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 et al.,

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 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 $\beta_{sor} = \frac{b+c}{2a+b+c}$, 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 & 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 geographic distances and community dissimilarities, Mantel tests (Mantel, 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 ($\beta_{sim} = \frac{\min(b,c)}{a+\min(b,c)}$) and nestedness ($\beta_{nes} = \frac{\max(b,c)-\min(b,c)}{2a+\min(b,c)+\max(b,c)} \times \frac{a}{a+\min(b,c)}$) (Baselga, 2010). Mantel tests were used again to assess the relationship between geographic 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}). Further, 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 geographic 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 geographic 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 geographic region (Europe vs. Asia).

3 RESULTS

3.1 Fungal OTUs

In total, Illumina Miseq PE300 sequencing generated 1198133 (1943 OTUs) and 848653 (1176 OTUs) fungal sequences for *E. helleborine* and *G. conopsea*, respectively. After analysis, 98.8% of the total number of sequences in *E. helleborine* (1183757 sequences, 1858 OTUs) and 95.5% of the total number of sequences in *G. conopsea* (810902 sequences, 1052 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).

G. 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 (190338 sequences, 25.95 %), followed by Tulasnellaceae (115167 sequences, 13.58%), Entolomataceae (84384 sequences, 9.95%), Inocybaceae (76731 sequences, 9.05%) and Nectriaceae (66383 sequences, 7.83%). Members of the ectomycorrhizal Sebacinaceae, Thelephoraceae, Inocybaceae, Russulaceae and Tuberaceae represented < 1% relative abundance (Figure S4a).

320 ***E. helleborine*** - The most abundant fungi detected in *E. helleborine* belonged to
321 Helotiales (134 OTUs), Ceratobasidiaceae (22 OTUs) and Serendipitaceae (18 OTUs),
322 as well as ectomycorrhizal taxa Thelephoraceae (62 OTUs), Inocybaceae (29 OTUs),
323 Sebacinaceae (25 OTUs), Russulaceae (32 OTUs), Cortinariaceae (30 OTUs),
324 Helvellaceae (14 OTUs), Tuberaceae (13 OTUs) and Hymenogastraceae (2 OTUs). In
325 addition, a number of other fungal taxa previously shown to colonize *Epipactis* spp.
326 were detected, including Tricholomataceae (16 OTUs), Gloniaceae (12 OTUs),
327 Herpotrichiellaceae (9 OTUs), Pyronemataceae (5 OTUs) and Psathyrellaceae (3 OTUs)
328 (Figure S5d). Members of Tulasnellaceae were represented by six OTUs, five of which
329 were detected in European samples, and only one from an individual plant collected
330 from China. In terms of relative abundances of sequences, members of Tuberaceae were
331 most abundant (216586 sequences, 28.86 %), followed by Helotiales (176777
332 sequences, 23.56 %), Russulaceae (101541 sequences, 13.53%), Pyronemataceae
333 (44388 sequences, 5.92%), Helvellaceae (41300 sequences, 5.50%) and Sebacinaceae
334 (38618 sequences, 5.15%) (Figure S5a).
335
336 **3.2 Mycorrhizal fungal communities**
337 ***G. conopsea*** - *G. conopsea* mainly associated with members of the
338 Ceratobasidiaceae and Tulasnellaceae and ectomycorrhizal fungi from the
339 Sebacinaceae and Thelephoraceae. These fungal associates represented in total 95
340 OTUs (314182 sequences, 37.02% of the total sequences) (Table S1), of which 71
341 (80672 sequences) and 50 (233510 sequences) were detected in Europe and China,

342 respectively (Table S2). Thirty-nine OTUs (47.6% of all OTUs) were shared between
343 Europe and China. In terms of relative abundance, Ceratobasidiaceae and
344 Tulasnellaceae were the most abundant in Europe and China. Members of the
345 Tulasnellaceae were the most abundant fungal associates in Europe, while members of
346 the Ceratobasidiaceae were the most abundant fungal associates of *G. conopsea* in
347 China (Figure S4b, c, e). About half of the rhizoctonia fungal OTUs were detected in
348 samples from both Europe and China (Figure 1a). In contrast, none of the fungal OTUs
349 of Sebacinaceae was shared between Europe and China. The average number of
350 mycorrhizal OTUs detected on the roots of individuals of *G. conopsea* in Europe (16.6,
351 range: 9–27) was significantly smaller than that found on roots of plants in China (20.2,
352 range: 7–29; $F = 4.047$, $p = 0.012$) (Table 1). However, PD values did not significantly
353 ($F = 2.706$, $p > 0.05$) differ between plants from Europe (3.117 ± 0.207) and China
354 (3.128 ± 0.121) (Table 1).

355 ***E. helleborine*** - 432 OTUs (765472 sequences, 63.89% of the total sequences)
356 previous described as fungal associates from the genus *Epipactis* were detected in this
357 research (Table S3). 326 OTUs (359589 sequences) and 169 OTUs (405883 sequences)
358 were detected in Europe and China, respectively, and 64 OTUs (196428 sequences;
359 14.8% of all OTUs) were shared (Table S4). When excluding the Helotiales (134 OTUs,
360 176777 sequences), of the remaining 298 OTUs (588695 sequences), 234 OTUs
361 (299690 sequences) and 83 OTUs (289005 sequences) were detected in Europe and
362 China, respectively, and 20 OTUs (36968 sequences; 6.7% of all OTUs) occurred in
363 both regions. They belonged to nine different fungal families (Herpotrichiellaceae, 5

364 OTUs; Gloniaceae, 5 OTUs; Ceratobasidiaceae, 3 OTUs; Serendipitaceae, 2 OTUs;
365 Thelephoraceae 2 OTUs; Psathyrellaceae, Tricholomataceae, Tulasnellaceae, and
366 Pyronemataceae, 1 OTU respectively).

367 The relative abundance of the fungal families differed between Europe and China
368 (Figure 1b). Members of Russulaceae were most abundant in Europe, while members
369 of the Tuberaceae were most abundant in China. Members of Tulasnellaceae,
370 Ceratobasidiaceae and Serendipitaceae were present in relatively low abundance in
371 both Europe and China (Figure S5b, c, e). The average number of OTUs detected on
372 the roots of individuals of *E. helleborine* in Europe (89.3, range: 43–112) was
373 significantly higher than those on the roots of plants from China (22.1, range: 8–39; F
374 = 3.984, $p < 0.001$) (Table 1). The difference is still significant when Helotiales is
375 excluded (F = 3.926, $p < 0.001$; Europe 61.9, range: 36-78; China 9.0, range 3-15).
376 Similarly, PD values of the mycorrhizal communities associating with *E. helleborine*
377 in Europe (8.903 ± 0.337) were significantly (F = 3.980, $p < 0.001$) higher than those
378 of the communities associating with *Epipactis* in China (3.189 ± 0.122, Table 1).
379

380 **3.3 Comparison of mycorrhizal communities**

381 The estimated overall multiple-site dissimilarity was higher for *E. helleborine* (β_{sor} =
382 0.86) than for *G. conopsea* (β_{sor} = 0.76). In both species most of the multiple-site
383 dissimilarity was the result of spatial turnover (β_{sim} = 0.76 and 0.74, respectively) and
384 to a much smaller extent of nestedness (β_{nes} = 0.09 and 0.02, respectively), indicating
385 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 geographic distance ($r_M = 0.84$, $P < 0.0001$ and $r_M = 0.65$, $P = 0.02$ for *E. helleborine* and *G. conopsea*, respectively) (Fig. 2,3). 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 (Fig. 2,3). For *E. helleborine*, the relationship between PCD values and geographic distances was highly significant ($r_M = 0.82$, $P < 0.0001$). Furthermore, the compositional component of PCD (PCDc) was also strongly and significantly correlated with geographic distance ($r_M = 0.79$, $P < 0.0001$), while PCDp was less tightly correlated with geographic distance ($r_M = 0.55$, $P = 0.0008$) (Fig. 2). In *G. conopsea*, both PCD and PCDc were significantly correlated to geographic distance (PCD: $r_M = 0.62$, $P = 0.0135$; PCDc: $r_M = 0.59$, $P = 0.0195$) (Fig. 3), but there was no significant relationship between PCDp and geographic distance ($r_M = -0.25$, $P > 0.05$) (Fig. 3).

Species indicator analyses showed that a large number of fungal OTUs was significantly associated with one of the two geographic 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 geographic 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 macro-organisms 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 geographic 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 et al., 2017b). 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 geographic distribution. Six out of nine Tulasnellaceae OTUs, 21 out of 42 Ceratobasidiaceae, and six out of 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 out 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 et al., 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 to 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 (Harrison et al., 1992; Baselga, 2010). 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 geographic 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 geographic 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 geographic distance, while PCDp was not significantly or only weakly correlated with geographic

496 distance. These results indicate that orchid populations located closely next to each
497 other are likely to contain the same species (PCDc), but that the non-shared taxa come
498 from different phylogenetic clades (PCDp).

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

517

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 geographic 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 geographic 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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Table 1 Sampling sites, average mycorrhizal fungal OTU numbers and phylogenetic diversity (PD) in each population of *Epipactis helleborine* and *Gymnadenia conopsea* growing 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

Figure captions

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.

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

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

Supporting Information

FIGURE S1 Rarefaction curves of Fungal OTU (operational taxonomic unit) richness in individual plant of *Epipactis helleborine* (a) and *Gymnadenia conopsea* (b).

FIGURE S2 Fungal diversity and phylogenetic diversity of the whole fungal communities detected in in each populations of *Epipactis helleborine.*

FIGURE S3 Fungal diversity and phylogenetic diversity of the whole fungal communities detected in each populations of *Gymnadenia conopsea.*

FIGURE S4 Relative abundance of the most abundant fungal families detected in *Gymnadenia conopsea* in total (a), in Europe (b) and in China (c). (d) The number of operational taxonomic units (OTUs) for different fungal families. (e) Relative abundance of fungal families in each *G. conopsea* population.

FIGURE S5 Putative mycorrhizal fungal families detected in *Epipactis helleborine* Relative abundance of each fungal family in the whole mycorrhizal communities in total (a), in Europe (b) and in China (c). (d) The number of operational taxonomic units (OTUs) for different mycorrhizal fungal families. (e) Relative abundance of mycorrhizal families in each *E. helleborine* population.

Fig. 1

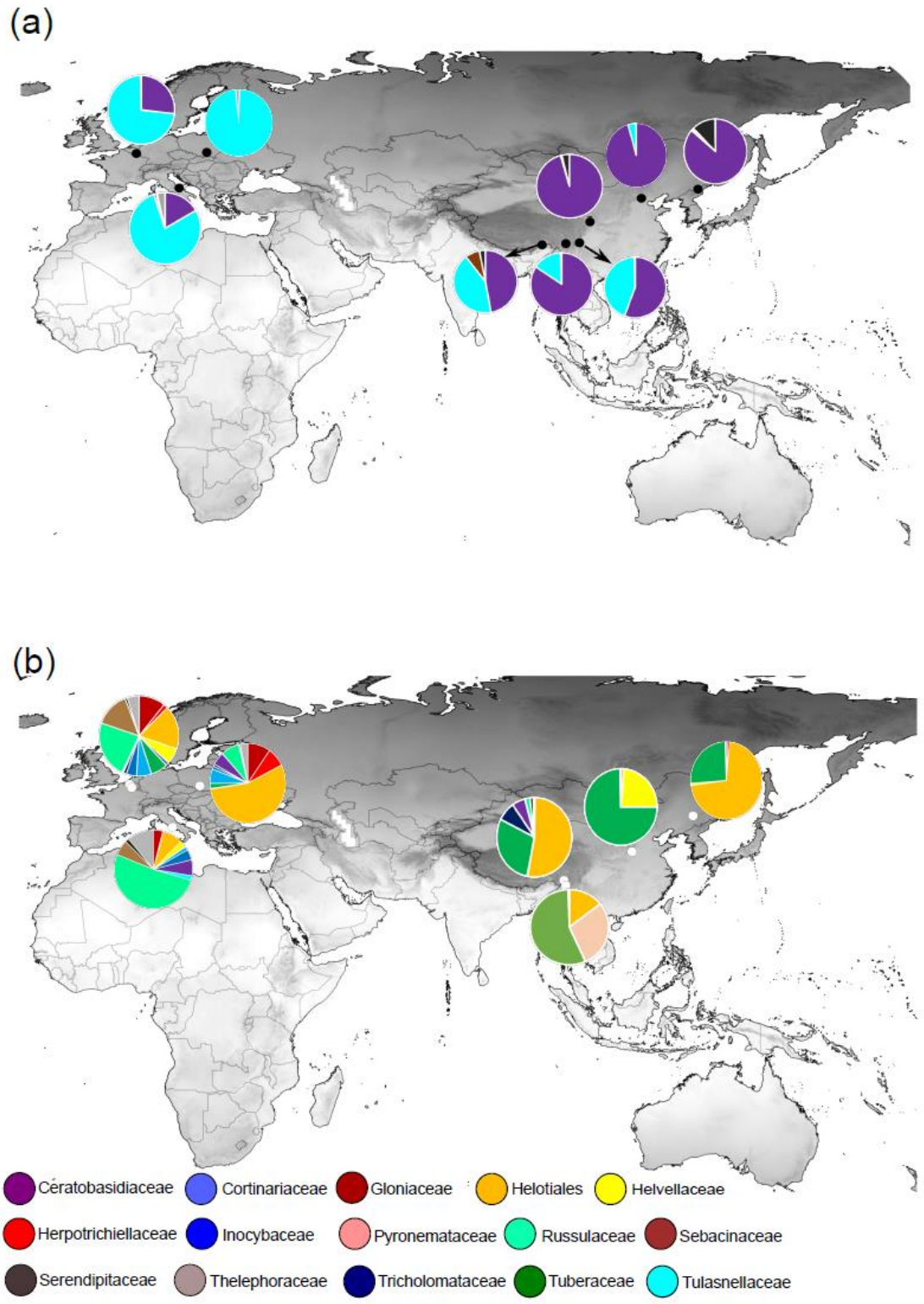

(a)
(b)
Ceratobasidiaceae
Cortinariaceae
Gloniaceae
Helotiales
Helvellaceae
Herpotrichiellaceae
Inocybaceae
Pyronemataceae
Russulaceae
Sebacinaceae
Serendipitaceae
Thelephoraceae
Tricholomataceae
Tuberaceae
Tulasnellaceae

Fig. 2

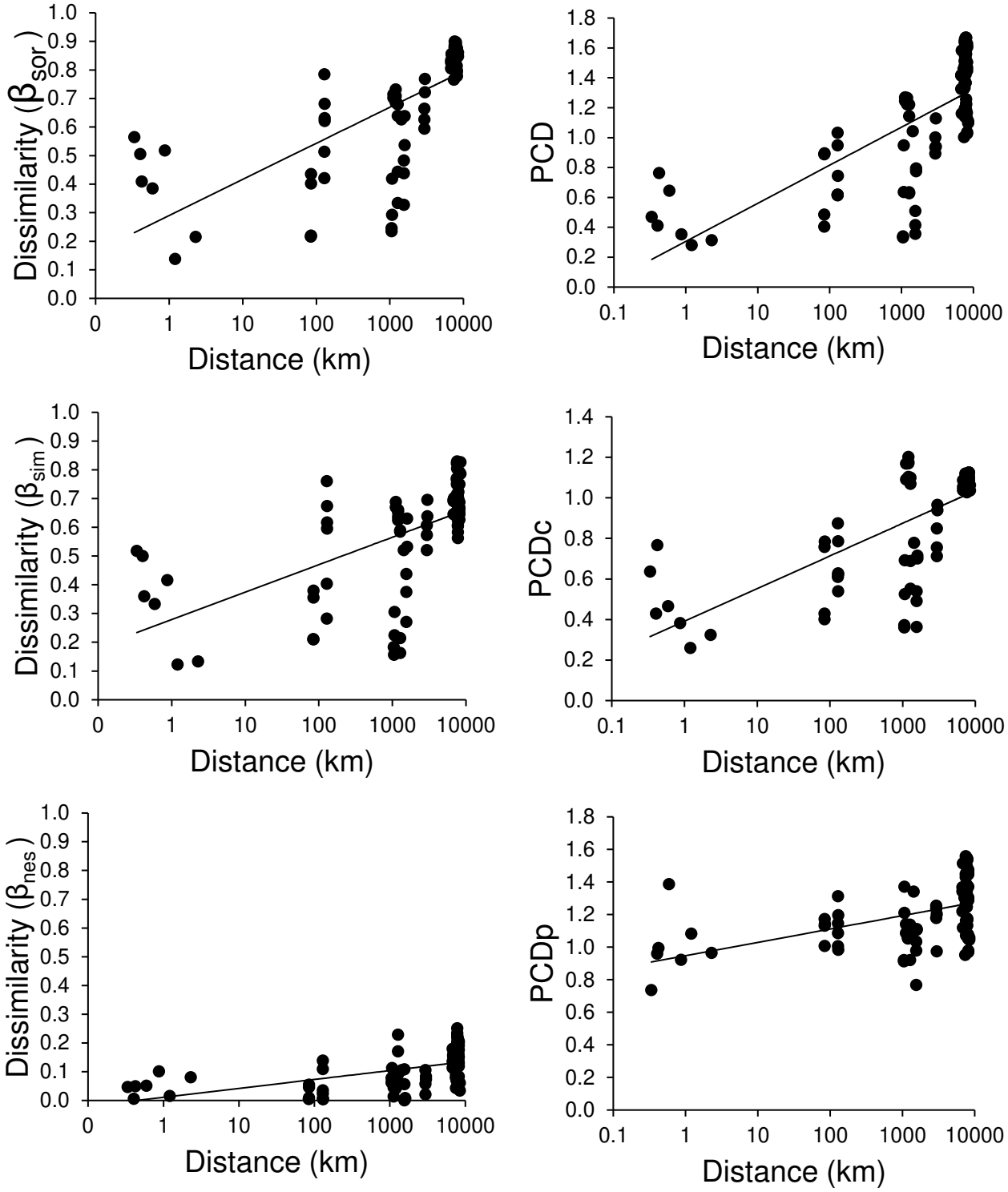
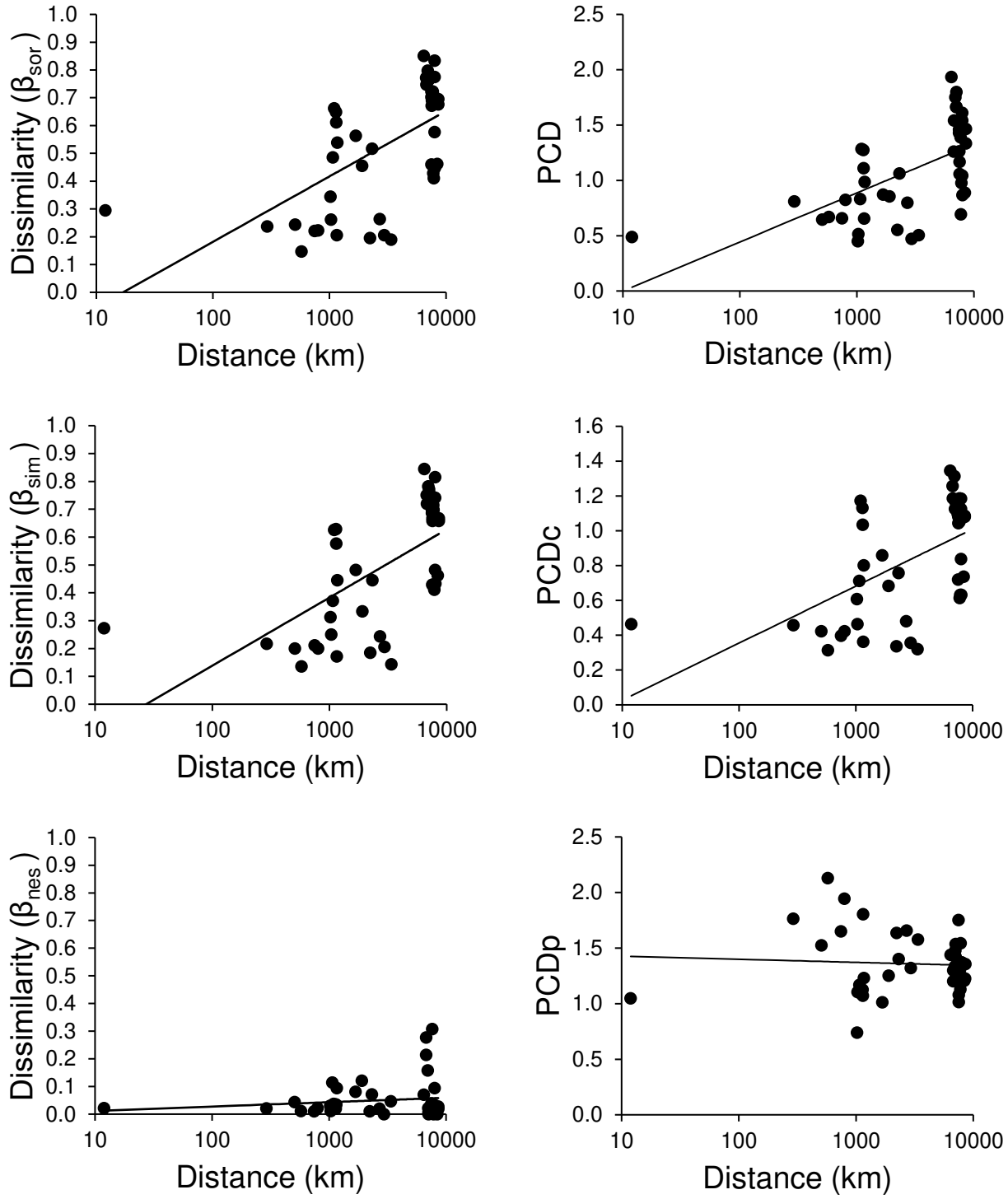

Dissimilarity (βsor)
PCD
Dissimilarity (βsim)
PCDc
Dissimilarity (βnes)
PCDp
Distance (km)
0
1
10
100
1000
10000
0.1

Fig. 3


Dissimilarity (βsor)
PCD
Dissimilarity (βsim)
PCDc
Dissimilarity (βnes)
PCDp
Distance (km)
10
100
1000
10000