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1 **Fine-scale spatial distribution of orchid mycorrhizal fungi in the soil of host-rich**
2 **grasslands**

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23 **Abstract**

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

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

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

37 • Orchid mycorrhizal ‘rhizoctonias’ are commonly regarded as unspecialized saprotrophs. The
38 sporadic occurrence of mycobionts of grassland orchids in host-rich stands questions the view of
39 these mycorrhizal fungi as capable of sustained growth in soil.

40

41 **Keywords**

42 Fungal communities, Tulasnellaceae, *Tulasnella calospora*, Ceratobasidiaceae, Serendipitaceae,
43 Sebaciniales, Pezizaceae, Orchidaceae

44

45 INTRODUCTION

46

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

59 Plant dependency on compatible mycorrhizal fungi is extreme in orchids because
60 germination of the tiny orchid seeds, almost devoid of nutritional reserves, and development of
61 the heterotrophic protocorm require colonization by fungi providing organic carbon (Smith &
62 Read, 2008; Rasmussen & Rasmussen, 2014). Orchids retain their mycorrhizal partnerships at
63 adulthood when, due to their characteristically poorly developed roots, they are thought to be
64 still heavily reliant on mycorrhizal fungi for their mineral nutrition (Waterman & Bidartondo,
65 2008). In addition, species that develop photosynthetic tissues may still supplement
66 photosynthesis with fungal-derived organic carbon (Selosse & Roy, 2009; Kuga *et al.*, 2014;
67 Stöckel *et al.*, 2014).

68 Due to their vital role in plant survival, it has been proposed that the spatial distribution of
69 symbiotic fungi could forcefully constrain the establishment and resulting distribution of orchids
70 (McCormick & Jacquemyn, 2014). Many OrM fungi are likely widespread at the scale of tens to
71 hundreds of kilometres, and orchid distribution essentially limited by seed dispersal. By contrast,
72 at the local (metre) scale spatial patterns are presumably driven by other factors (McCormick *et al.*,
73 2016). Indeed, at this scale many orchid species feature small population sizes and a
74 scattered occurrence. Although seed dispersal limitation across limited distances (Jersáková &
75 Malinová, 2007) has been invoked to account for this highly aggregated distribution,
76 observational studies have provided some evidence that it is not necessarily the primary cause
77 (Jacquemyn *et al.*, 2007, 2009). Rather, such an arrangement suggests recruitment limitation

78 resulting from patchiness of appropriate (micro)environmental conditions favorable to seed
79 germination and plant development, including the presence of compatible fungi (McCormick &
80 Jacquemyn, 2014). Furthermore, sympatric orchid species often exhibit both strong spatial
81 segregation and association with distinct fungi (Waterman *et al.*, 2011; Jacquemyn *et al.*, 2012,
82 2014). Taken together, these observations point to a highly patchy distribution of OrM fungi in
83 the environment.

84 In some cases, orchid seeds can germinate at sites devoid of adults (Těšitelová *et al.*, 2012;
85 McCormick & Jacquemyn, 2014), suggesting that recruitment restrictions may operate at later
86 developmental stages (Selosse, 2014). In other instances, comparisons of the spatial distribution
87 of seedlings and adults in several European (Diez, 2007; Jacquemyn *et al.*, 2007, 2012) and
88 Australian (Batty *et al.*, 2001) orchids revealed that seed germination was restricted to areas
89 where adults occurred, indicating convergent requirements by juveniles and adult plants. These
90 observations suggest that locations where these orchids can reach maturity may be sites with a
91 persistent occurrence of OrM fungi, whereas fungi or environmental conditions in microsites
92 without existing adults may be ephemeral (McCormick & Jacquemyn, 2014; McCormick *et al.*,
93 2016).

94 The identity of OrM symbionts of adult plants largely depends on the identity and habitat of
95 the orchid host (Dearnaley *et al.*, 2012). In particular, photosynthetic orchids in sunny grassland
96 habitats mainly associate, both as seedlings and as adult plants, with fungi in the ‘rhizoctonia’
97 complex *sensu lato* (Smith & Read, 2008; Dearnaley *et al.*, 2012; Rasmussen & Rasmussen,
98 2014), a polyphyletic assemblage encompassing Agaricomycetes belonging to the
99 Serendipitaceae (Sebacinales; Weiß *et al.*, 2016), Ceratobasidiaceae and Tulasnellaceae
100 (Roberts, 1999; Taylor *et al.*, 2002; Weiß *et al.*, 2004). The Tulasnellaceae, in particular, are the
101 most frequently found OrM fungi in both temperate and tropical regions (Dearnaley *et al.*, 2012).

102 There is a common assumption that most OrM rhizoctonias are unspecialized soil
103 saprotrophs, based on their fast growth *in vitro* (e.g. Smith & Read, 2008; Nurfadilah *et al.*,
104 2013; Bahram, *et al.* 2015a). However, works on Sebacinales and Ceratobasidiaceae in
105 particular, indicate that the phylogenetic diversity of these rhizoctonias parallels a variety of
106 ecological/nutritional strategies, including the ability to establish mycorrhizal or non-
107 mycorrhizal endophytic associations with non-orchid plants (Weiß *et al.*, 2004; Selosse *et al.*,
108 2002, 2007, 2009; Oberwinkler *et al.*, 2013; Tedersoo & Smith, 2013; Veldre *et al.*, 2013). By
109 contrast, the ecology of the Tulasnellaceae is largely understudied (Selosse, 2014; Selosse &
110 Martos, 2014).

111 Experimental burial of orchid seed packets confirms that many rhizoctonias can occur in the
112 environment independently of orchid roots (Těšitelová *et al.*, 2012; McCormick & Jacquemyn,
113 2014). While it is suspected that the main ecological niche of OrM rhizoctonias exists out of
114 orchid hosts (Dearnaley *et al.*, 2012; Selosse & Martos, 2014), the actual extent of their
115 occurrence in the soil habitat, their spatial distribution in the environment, their nutrient
116 demands, and their fine-scale propagation remain largely unknown. OrM fungi are rarely, if ever,
117 retrieved in meta-barcoding studies of soil fungi (e.g. Buée *et al.*, 2009; Orgiazzi *et al.*, 2012;
118 2013; Schmidt *et al.*, 2013), the main exceptions being investigations targeting EcM
119 communities, due to the reported ability of some OrM fungi to establish EcM symbiosis with
120 tree plants (e.g. Selosse *et al.*, 2002; Tedersoo *et al.* 2008, 2014; McCormick *et al.*, 2009;
121 Tedersoo & Smith, 2013). However, biased fungal community descriptions may derive from the
122 use of primers excluding particular fungal taxa (Bellemain *et al.*, 2010; Ihrmark *et al.*, 2012;
123 Lindahl *et al.*, 2013). The Tulasnellaceae, for instance, exhibit accelerated evolution of the
124 nuclear ribosomal operon, causing most conventional fungal primers to fail in polymerase chain
125 reaction (PCR) amplification of their ITS (Taylor & McCormick, 2008; Waud *et al.*, 2014;
126 Tedersoo *et al.*, 2015). Thus far, however, the occurrence of OrM fungi in soil has not been
127 thoroughly investigated by means of specific primers.

128
129 In this study, we addressed the fine-scale spatial distribution of OrM fungi in the soil of two
130 orchid-rich Mediterranean grasslands, by combining high throughput (Illumina MiSeq)
131 sequencing of fungal ITS2 amplicons obtained from soil-extracted DNA using both generalist
132 and taxon-specific primers, and phylogenetic comparison of soil-derived and root-derived
133 sequences. Soil samples were collected underneath and at distance from adult plants of
134 *Anacamptis morio* Bateman, Pridgeon & Chase and *Ophrys sphegodes* Mill., whose mycorrhizal
135 associations had been previously described both in the study area and elsewhere (Illyes *et al.*,
136 2009; Liebel *et al.*, 2010; Bailarote *et al.*, 2012; Jacquemyn *et al.*, 2014, 2015; Ercole *et al.*,
137 2014). We specifically hypothesized that: 1) as for other mycorrhizal symbionts, the distribution
138 of OrM fungi in soil is spatially structured, and 2) OrM fungi in soil co-occur, at the fine scale,
139 with their adult orchid hosts.

140

141

142 **MATERIALS AND METHODS**

143 *Study sites, plant species and sampling*

144 The study area is a Mediterranean grassland in northern Italy. The site is characterized by
145 patches of meadows and pastures (assigned to the association *Festuco-Brometalia*; Braun-
146 Blanquet, 1964), interleaved by woods and shrubs-dominated plots. It is located 460 m asl, in a
147 transition zone between Mediterranean and sub-Atlantic climates. This area features a high
148 richness in orchid species (Girlanda *et al.*, 2006, 2011; Ercole *et al.*, 2014). We focused on
149 *Anacamptis morio* Bateman, Pridgeon & Chase and *Ophrys sphegodes* Mill., two widespread
150 Euro-Mediterranean orchids growing in grassland habitats (Kretzschmar *et al.*, 2007). Both
151 species belong to the Orchideae tribe in the Orchidoideae subfamily of Orchidaceae (Bateman *et*
152 *al.*, 2003), and have been described as fully photoautotrophic orchids (Liebel *et al.*, 2010; Ercole
153 *et al.*, 2014). They are winter-green perennial tuberous plants in which, after summer dormancy,
154 the underground bulbous tuber produces a basal rosette of leaves and some roots. In these plants,
155 as in most orchidoid species, new roots appear from late summer to autumn, but a few more
156 may form in spring (Rasmussen, 1995). In *A. morio*, seed germination was found to be
157 significantly related to the distance to the nearest congeneric adult (Jacquemyn *et al.*, 2012).
158 Adult plants of both orchid species establish mycorrhizal associations with a diverse fungal
159 spectrum dominated by Tulasnellaceae and Ceratobasidiaceae (Basidiomycota); *A. morio* also
160 frequently associates with fungi in the Pezizaceae (Ascomycota) (Illyes *et al.*, 2009; Liebel *et al.*,
161 2010; Bailarote *et al.*, 2012; Ercole *et al.*, 2014; Jacquemyn *et al.*, 2014, 2015).

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

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

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

181
182 *DNA extraction, PCR amplification and amplicon sequencing*
183 Prior to DNA extraction, soil samples were checked under a stereomicroscope for the absence of
184 orchid seedlings. Following soil homogenization, three 0.5 g subsamples per soil core were taken
185 and total genomic DNA was extracted from the pooled 1.5 g samples. Three independent
186 extractions from each composite soil sample were performed using the FastDNA Kit (MP
187 Biomedicals, LLC, OH, USA) according to the manufacturer's instructions.

188 Genomic DNA was also extracted from fungal pelotons manually isolated from orchid
189 mycorrhizal roots. Roots were rinsed with tap water and sonicated. Each root (which was approx.
190 7-8 cm long) was cut into approx. 5-cm-long segments, which were microscopically checked for
191 fungal colonization. Such a microscopic observation revealed the occurrence of many active
192 pelotons in the newly formed roots of both *A. morio* and *O. sphegodes*. Highly colonized root
193 segments were teased with a sterile scalpel in a 6 cm Petri dish containing 5 ml of sterile water,
194 in order to release the pelotons. Pelotons were collected with a micropipette and transferred in
195 PCR tubes. Before PCR amplification, the pool of pelotons obtained from each plant was
196 disrupted by heat shock (10 min at 95°C) in 10 µl 1X PCR buffer (Sigma-Aldrich).

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

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

212 s at 54°C and 1 min at 72°C, followed by 10 min at 72°C. For OF primers the PCR conditions
213 used were: 2 min at 96°C, 35 cycles of 30 s at 94°C, 40 s at 58°C and 45s at 72°C, followed by
214 10 min at 72°C. Each PCR product was checked on agarose gel, and diluted at 1/50 to use as
215 template in the nested PCR. The nested PCR was carried out using 1U of Phusion High Fidelity
216 polymerase, 1x HF buffer, 0.5µM of the primers ITS3mod and ITS4 (White *et al.*, 1990) with
217 barcodes, 0.2µM of each dNTPs and 2µl of diluted PCR product, in a total volume of 50µl. PCR
218 conditions were 30 s at 98°C, 30 cycles of 10 s at 98°C, 30 s at 64°C and 20 s at 72°C, followed
219 by 10 min at 72°C. All PCRs were performed using a T3000 thermal cycler (Biometra GmbH,
220 Germany). PCR products were checked on agarose gel, and the three replicates of each sample
221 were pooled and purified using The Wizard® SV Gel and PCR Clean-Up System (Promega,
222 USA) following the manufacturer's instructions. After quantification with Qubit 2.0 (Life
223 Technologies), the purified PCR products were mixed in equimolar amounts to prepare
224 sequencing libraries. The libraries were paired-end sequenced using the Illumina MiSeq
225 technology (2 X 250 bp) by Fasteris (Plan-les-Ouates, Switzerland).

226

227 *Bioinformatic analyses*

228 Paired-end reads from each library were initially merged using PEAR v0.9.2 (Zhang *et al.*,
229 2014), with the quality score threshold for trimming the low quality part of a read set at 28 and
230 the minimum length of reads after trimming set at 200 bp.

231 Assembled reads were then processed using Quantitative Insights into Microbial Ecology
232 (QIIME) v. 1.8 software package (Caporaso *et al.*, 2010). Initial sequence processing and sample
233 assignment were performed with a minimum sequence length cut-off of 200 bp, minimum Phred
234 quality score of 28, calculated over a sliding window of 50 bp, and allowing a maximum
235 mismatch of 3 bp over the forward and reverse primers. Sequences were re-orientated when
236 necessary to 5' to 3', and demultiplexed based on the tags and primers. Chimeric sequences were
237 identified and removed performing a *de novo* (abundance based) detection using USEARCH61
238 (Edgar, 2010), as implemented in the QIIME pipeline. Operational taxonomic units (OTUs) were
239 determined using an open reference-based clustering strategy, with the USEARCH61 method, at
240 98% similarity; only clusters encompassing at least 10 sequences were retained. The UNITE
241 database version 6 for QIIME was used as a reference for Operational Taxonomic Unit (OTU)
242 picking and taxonomy assignment (Abarenkov *et al.*, 2010; Kõljalg *et al.*, 2013;
243 <http://unite.ut.ee>, last accessed May 25th, 2015); BLAST algorithm (Altschul *et al.*, 1990) was
244 used as taxonomy assignment method, using $1e^{-5}$ e-value as threshold. The OTU representative
245 sequences generated in this study (i.e. the most abundant sequence within each OTU) were

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

248 To assess the relatedness with OrM fungi of the OTUs assigned to rhizoctonias and to
249 pezizoid fungi obtained from soil, maximum likelihood (ML) analyses were carried out.
250 Sequences included in the ML analyses comprised best BLAST hits as well as fungal sequences
251 from a variety of terrestrial, including the target species, and epiphytic orchids from different
252 continents and environments, as well as from non-orchid plants, fungal strains and fruitbodies.
253 Due to the phylogenetic distance between the fungi identified (Roberts, 1999), distinct
254 phylogenetic analyses were carried out for Tulasnellaceae, Ceratobasidiaceae, Serendipitaceae
255 and Sebacinaceae (previously referred to as “Sebacinales Clade B” and “Sebacinales Clade A”,
256 respectively; Weiß *et al.*, 2016). Sequences were aligned using the program Clustal X 2.0
257 (Larkin *et al.*, 2007) with default conditions for gap opening and gap extension penalty.
258 Alignments were then imported into program MEGA 4.0 (Tamura *et al.*, 2007) for manual
259 adjustment. ML estimation was performed with RAxML v.7.0.4 (Stamatakis, 2006) through
260 1000 bootstrap replicates (Felsenstein, 1985) using the GTR + GAMMA algorithm to perform a
261 tree inference and search for a good topology. Support values from bootstrapping runs were
262 mapped on the globally best tree using the *-f* option of RAxML and *-x* 12345 as a random
263 seed. Nodes receiving a bootstrap support < 70% were not considered as well supported.
264 Alignments and tree topologies are archived in the database TreeBASE
265 (<http://www.treebase.org>; submission ID 19171). To account for the different intraspecific
266 variation rate in the ITS region for different lineages, the resulting phylogroups (clades
267 supported by $\geq 70\%$ bootstrap, which included the sequences obtained from soil in this work)
268 were used as taxonomic units in the statistical analyses described below.

269

270 *Statistical analyses*

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

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

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

281 The effects of orchid species and site on the composition of OrM fungal assemblages in soil
282 samples collected underneath orchid plants were evaluated using permutational multivariate
283 analysis of variance (PERMANOVA, 999 permutations), as implemented in the *adonis* routine
284 of the *vegan* package of R (Oksanen *et al.* 2013, R Development Core Team 2014). The
285 multivariate homogeneity of group dispersions was first assessed by means of the *betadisper* and
286 *permutest* (with 999 permutations) functions in the R package *vegan* (Oksanen *et al.*, 2013). The
287 differences in the composition of OrM fungal communities in orchid roots and soil samples
288 collected beneath were visualized by means of a non-metric multidimensional scaling (NMDS)
289 ordination carried out with the Past3 software (Hammer *et al.*, 2001). PERMANOVA was also
290 performed to compare the composition of non-OrM communities in soil. Only taxa occurring in
291 $\geq 75\%$ of soil samples collected under either orchid species at either site were included in the
292 latter analysis. Indicator species analysis (a classification-based method to measure associations
293 between species and groups of sites; Dufrene & Legendre, 1997) was carried out using the
294 *multipatt* function in the *indicspecies* R package, with 999 permutations (De Cáceres &
295 Legendre, 2009), in order to assess if and which fungi were significantly associated with a
296 particular orchid species/site.

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

306 Spatial clustering in read numbers of OrM and non-OrM phylogroups in the soil samples was
307 explored using Moran's I test statistic, as implemented in the R package *ape* (Paradis *et al.*,
308 2004). For each phylogroup, differences in read numbers among soil samples collected at
309 varying distances along the 160 cm transects were tested for significance by means of Kruskal-
310 Wallis tests conducted using the Past3 software.

311

312 **RESULTS**

313 **Fungal diversity in roots and in soil**

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

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

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

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

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

344
345 **Influence of orchid species and site factors on the composition of fungal communities in soil**

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

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

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

365

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

367 Sequences obtained from the roots of both orchid species were predominantly assigned to the
368 ceratobasidioid phylogroup *Cer_2* (Fig. S3a), which accounted for 75.3 and 27.8% of the total
369 number of reads from *O. sphegodes* and *A. morio* roots, respectively. *Cer_2* occurred in 60% and
370 20% of the soil samples containing the respective orchid plants (10 soil samples). *Tul_2* (the
371 second most dominant fungus in *O. sphegodes*, 13.8% total read number) occurred in 60% of the
372 corresponding soil samples. By contrast, *Pez_3* (the second most dominant fungus in *A. morio*,
373 20.0% total read number), was not found in any of the soil samples containing the corresponding
374 orchid roots. Similarly, *Tulasnella calospora*, which was amplified by the primers we used (Fig.
375 S12), and was retrieved from 40% of the *A. morio* plants analyzed, was not retrieved from any of
376 the soil samples.

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

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

390

391 **Spatial distribution of OrM fungi in soil**

392 *Community level analyses*

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

398 The Mantel correlograms revealed significant autocorrelation within small distance classes
399 (< 2m on average) for tulasnelloid, ceratobasidioid, sebacinoid and pezizoid fungi (OrM and
400 non-OrM), as well as saprotrophic Psathyrellaceae (Fig. 2a, Table S6). Significant
401 autocorrelation occurred at higher distances for OrM ceratobasidioid and sebacinoid fungi (at
402 6.25-7.58m and 7.59-8.93m, respectively), non-OrM pezizoid fungi (2.43-3.89m and 2.51-
403 3.42m) and saprotrophic Psathyrellaceae (12.97-14.30m; Fig. 3a, Table S6). The saprotrophic
404 Mycenaceae and Mortierellaceae/Umbelopsidaceae, by contrast, did not exhibit significant
405 distance-decay. Depending on the taxonomic group, significant relationships were found at either
406 or both sites. No difference in the occurrence of significant relationships was found among
407 OrM, non-OrM and saprotrophic taxa (chi-square tests, $P > 0.05$; Fig. 3a).

408

409 *Individual taxon level analyses*

410 When read numbers of each OrM phylogroup were compared in soil samples collected at
411 increasing distances from orchid plants (as a proxy for variation in abundance in soil), no
412 significant difference could be observed for any fungus (P-values of Kruskal-Wallis tests ranging
413 0.071-1; data not shown).

414 However, either OrM or non-OrM tulasnelloid, ceratobasidioid, sebacinoid and pezizoid
415 fungi exhibited significant positive spatial autocorrelation (i.e. patchiness), as assessed by means
416 of Moran's tests (the main exception being the three OrM phylogroups, none of which exhibited
417 significant autocorrelation; Table S7). Significant autocorrelation was found at both sites for
418 11.1% of the (OrM or non-OrM) tulasnelloid, ceratobasidioid, sebacinoid and pezizoid
419 phylogroups exhibiting significant autocorrelation. The same pattern was found for 13.6% of the
420 other (putatively saprotrophic) basidiomycetes tested. Spatial autocorrelation occurred more
421 frequently in some taxonomic groups, e.g. it was particularly rare in the
422 Mortierellaceae/Umbelopsidaceae (Fig. 3b). Autocorrelation occurrence was significantly higher
423 in the OrM than in the non-OrM ceratobasidioid fungi (Fig. 3b).

424

425

426 **DISCUSSION**

427 **The distribution of OrM fungi in soil is similar to spatial patterns of other mycorrhizal** 428 **fungi**

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

448 As observed for other mycorrhizal fungi, the distribution of both OrM fungal
449 assemblages and individual taxa in soil featured non-random spatial distribution, as indicated,
450 respectively, by significant Mantel and Moran's I tests. Such patterns were reported thus far for
451 EcM, AM fungi or the general soil fungal community at small scales (e.g. Lilleskov *et al.*, 2004;
452 Lekberg *et al.*, 2007; Peay & Bruns, 2014; Bahram *et al.*, 2013, 2015a). In particular, we found
453 significant autocorrelation for OrM fungal assemblages at distances up to approx. 10m, which is
454 comparable to the spatial autocorrelation range of AM fungi in temperate ecosystems (Bahram *et al.*,
455 2015a). Such patterns may depend on random dispersal processes. However, spatial patterns
456 of soil fungi are also known to depend strongly on habitat type (Bahram *et al.*, 2013, 2015b).
457 Although we did not measure environmental variables at the two study sites (which are located
458 in a relatively homogeneous landscape), the differences in the spatial patterns between the two
459 stands are suggestive of a role for environmental variation in shaping the distribution of OrM
460 fungi in the area. Non-OrM fungal assemblages varied significantly at the two sites, suggesting
461 different biotic environments.

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

470

471 **Widespread OrM fungi may exhibit sporadic occurrence in soil**

472 Orchid-rich areas have been suggested to exhibit persistently high abundances of OrM fungi to
473 provide either sufficient nutrients or a high probability of the fungus encountering seeds
474 (McCormick & Jacquemyn, 2014). This suggestion was mainly based on seed germination,
475 indicating greater occurrence of fungal symbionts close to adult plants (Batty *et al.*, 2001; Diez,
476 2007; Jacquemyn *et al.*, 2012). In our work, higher read numbers of sequences obtained from *O.*
477 *sphogodes* and *A. morio* roots did not correlate with shorter distances from adult plants.
478 Moreover, we found that several fungi dominating in orchid roots were extremely sporadic or
479 were not detected at all even in soil samples containing the roots of orchid plants colonized by
480 the same fungi. By contrast, other OrM fungi predominated in the same soil samples. Recent
481 quantitative PCR analyses focusing on dominant OrM fungi in other orchid species showed that

482 their abundance declined rapidly with distance from the adult host plants (McCormick *et al.*,
483 2016; Waud *et al.*, 2016). It remains unknown whether this discrepancy is due to different soil
484 conditions or the plant and fungal taxa involved.

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

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

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

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

545
546 In conclusion, we have found evidence of spatial autocorrelation in all main taxonomic groups of
547 OrM fungi in the study areas. An intriguing result is that some widespread root symbionts were
548 found to be quite rare even in host-dense soils. Future investigations should explore alternative
549 niches of common OrM fungi found to be infrequent in soil at the small scale and also address

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

552

553

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560

561

562 **AUTHOR CONTRIBUTIONS**

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

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914 **FIGURE LEGENDS**
915 **Figure 1.** Spatial distribution of adult plants of *Anacamptis morio* (purple circles) and *Ophrys*
916 *sphogodes* (yellow circles) at both study sites. The position of adult individuals of other orchid

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

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

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

941

942 SUPPLEMENTARY INFORMATION

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

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

951 pelotons in *Anacamptis morio* or *Ophrys sphegodes* roots at the time of soil sampling are
952 indicated in blue.

953 **Figure S3a.** Maximum likelihood tree obtained from the ITS2 sequence alignment of
954 ceratobasidioid fungi. The phylogram is midpoint rooted. Alignment length: 499 bp. Bootstrap
955 support values above 70% (1000 maximum likelihood replicates) are reported. Sequences
956 obtained from soil samples in this study are indicated in red; sequences obtained from pelotons
957 in *Anacamptis morio* or *Ophrys sphegodes* roots at the time of soil sampling are indicated in
958 blue.

959 **Figure S3b.** Placement of ceratobasidioid sequences, obtained in this study, within the
960 phylogenetic reconstruction by Veldre *et al.* (2013). The maximum likelihood tree is rooted at
961 the /fusisporus clade. Alignment length: 644 bp. Bootstrap support values above 70% (1000
962 maximum likelihood replicates) are reported. Sequences obtained from soil samples in this study
963 are indicated in red; sequences obtained from pelotons in *Anacamptis morio* or *Ophrys*
964 *sphogodes* roots at the time of soil sampling are indicated in blue. Sequences obtained from
965 orchids at the study sites in previous investigations (Girlanda *et al.* 2011, Ercole *et al.* 2014) are
966 indicated in green.

967 **Figure S4a.** Maximum likelihood tree obtained from the ITS2 sequence alignment of fungi
968 assigned to Sebacinaceae. *Paulisebacina allantoidea* was used as an outgroup taxon. Alignment
969 length: 408 bp. Bootstrap support values above 70% (1000 maximum likelihood replicates) are
970 reported. Sequences obtained from soil samples in this study are indicated in red; sequences
971 obtained from pelotons in *Anacamptis morio* or *Ophrys sphegodes* roots at the time of soil
972 sampling are indicated in blue.

973 **Figure S4b.** Maximum likelihood tree obtained from the ITS2 sequence alignment of fungi
974 assigned to Serendipitaceae. *Paulisebacina allantoidea* was used as an outgroup taxon.
975 Alignment length: 419 bp. Bootstrap support values above 70% (1000 maximum likelihood
976 replicates) are reported. Sequences obtained from soil samples in this study are indicated in red;
977 sequences obtained from pelotons in *Anacamptis morio* or *Ophrys sphegodes* roots at the time of
978 soil sampling are indicated in blue.

979 **Figure S5.** Maximum likelihood tree obtained from the ITS2 sequence alignment of pezizoid
980 fungi. *Ascobolus* spp. were used as outgroup taxa. Alignment length: 426 bp. Bootstrap support
981 values above 70% (1000 maximum likelihood replicates) are reported. Sequences obtained from
982 soil samples in this study are indicated in red; sequences obtained from pelotons in *Anacamptis*
983 *morio* or *Ophrys sphegodes* roots at the time of soil sampling are indicated in blue.

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

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

992 **Figure S8.** Maximum likelihood trees obtained from the ITS2 sequence alignment of fungi in the
993 tricholomatoid clade. *Mycena* spp. were used as outgroup taxa in Figs S8a,b. *Entoloma*
994 *prunuloides* and *Xeromphalina campanella* were used as outgroup taxa in Fig. S8c and Fig. S8d,
995 respectively. Alignment lengths: 500 bp, 500 bp, 407 bp, 745 bp. Bootstrap support values above
996 70% (1000 maximum likelihood replicates) are reported. Sequences obtained from soil samples
997 in this study are indicated in red.

998 **Figure S9.** Maximum likelihood trees obtained from the ITS2 sequence alignment of *Lepiota*
999 spp. Phylograms are midpoint rooted. Alignment lengths: 608 bp, 609 bp, 366 bp, 375 bp.
1000 Bootstrap support values above 70% (1000 maximum likelihood replicates) are reported.
1001 Sequences obtained from soil samples in this study are indicated in red.

1002 **Figure S10.** Maximum likelihood trees obtained from the ITS2 sequence alignment of fungi in
1003 the Mortierellales. Phylograms are midpoint rooted. Alignment lengths: 493 bp, 414 bp, 469 bp,
1004 516 bp, 440 bp, 413 bp, 457 bp, 387 bp. Bootstrap support values above 70% (1000 maximum
1005 likelihood replicates) are reported. Sequences obtained from soil samples in this study are
1006 indicated in red.

1007 **Figure S11.** Maximum likelihood trees obtained from the ITS2 sequence alignment of fungi in
1008 the Clavariaceae. *Plicaturopsis crispa* was used as outgroup taxon in Fig S11a. *Hyphodontiella*
1009 *multiseptata* and *Clavaria asperulospora* were used as outgroup taxa in Fig. S11b and Fig. S11c,
1010 respectively. Alignment lengths: 448 bp, 448 bp, 375 bp. Bootstrap support values above 70%
1011 (1000 maximum likelihood replicates) are reported. Sequences obtained from soil samples in this
1012 study are indicated in red.

1013 **Figure S12.** Amplification of *Tulasnella calospora* (strains MUT4182 and MUT4233) DNA by
1014 means of the tagged primers used in this study. 1, 3, 5: strain MUT4182; 2, 4, 6: strain
1015 MUT4233. 1, 2: 20 ng DNA; 3, 4: 10 ng DNA; 5, 6: 2 ng DNA. C1-C2, negative controls; M,
1016 100 bp marker (Sigma-Aldrich).

1017 **Table S1.** Fungi amplified from pelotons of all *Ophrys sphegodes* or *Anacamptis morio* plants
1018 analyzed. Read number percentages (with respect to the total number of reads from each orchid
1019 species), taxonomic assignment and best BLAST hits are reported for each phylogroup/OTU.
1020 Other tulasnellid, ceratobasidioid, sebacinoid and pezizoid fungi retrieved from roots at the
1021 time of soil sampling are reported in Figs. S2-S5.

1022 **Table S2.** Fungal distribution in the soil samples containing *Anacamptis morio* or *Ophrys*
1023 *sphogodes* roots at either site. OrM and non-OrM tulasnellid, ceratobasidioid, sebacinoid and
1024 pezizoid fungi, as well as other fungi (occurring in $\geq 75\%$ of soil samples collected under either
1025 orchid species at either site) are reported. Percentages of soil samples the fungus was amplified
1026 from, taxonomic assignment and best BLAST hits are indicated for each phylogroup/OTU.
1027 Results of indicator species analysis for both binary (presence/absence) and non-binary (OTU
1028 read numbers) data are included (P-value, the statistical significance of the relationship as
1029 assessed with 999 random permutations). AM1, soil samples containing *A. morio* roots collected
1030 at site 1; AM2, soil samples containing *A. morio* roots collected at site 2; OS1, soil samples
1031 containing *O. sphogodes* roots collected at site 1.

1032 **Table S3.** Results of the permutational multivariate analysis of variance (PERMANOVA) and
1033 the test for the multivariate homogeneity of group dispersions (betadisper and permutest) for
1034 orchid mycorrhizal (OrM) and non-orchid mycorrhizal (non-OrM) fungal assemblages in soil
1035 samples containing orchid roots at either site. For non-OrM fungal assemblages, only taxa
1036 occurring in $\geq 75\%$ of soil samples collected under either orchid species at either site were
1037 included in the analyses. AM1, soil samples containing *A. morio* roots collected at site 1; AM2,
1038 soil samples containing *A. morio* roots collected at site 2; OS1, soil samples containing *O.*
1039 *sphogodes* roots collected at site 1. NA, not ascertainable.

1040 **Table S4.** Results of indicator species analysis for the comparison of OrM fungal assemblages in
1041 either *Anacamptis morio* (AM) or *Ophrys sphogodes* (OS) roots and soil samples containing the
1042 orchid roots. Results for both binary (presence/absence) and non-binary (OTU read numbers)
1043 data are included (P-value, the statistical significance of the relationship as assessed with 999
1044 random permutations).

1045 **Table S5.** Results of Mantel tests [Mantel.cor, Mantel r statistics; P-value, two-tailed p-value
1046 (null hypothesis: $r=0$) adjusted with sequential Bonferroni correction], based on 999
1047 permutations, for different orchid mycorrhizal (OrM) and non-orchid mycorrhizal (non-OrM)
1048 fungal groups in soil. P values < 0.05 are indicated in red. OS, *Ophrys sphogodes*; AM,
1049 *Anacamptis morio*.

1050 **Table S6.** Results of Mantel correlograms (Mantel.cor, Mantel r statistics; P-value, P-value (null
1051 hypothesis: $r = 0$) adjusted with sequential Bonferroni correction), based on 999 permutations,
1052 for different orchid mycorrhizal (OrM) and non-orchid mycorrhizal (non-OrM) fungal groups.
1053 For site 1, both results for all soil samples, and samples collected under either orchid species
1054 (OS, *Ophrys sphegodes*; AM, *Anacamptis morio*) or along transects starting from either orchid
1055 are reported. P values <0.05 are indicated in red. Phylogroups/OTUs within each fungal group
1056 are listed in Table S5.

1057 **Table S7.** P-values of Moran's I tests for spatial autocorrelation of read numbers of each
1058 tulasnelloid, ceratobasidioid, sebacinoid and pezizoid OrM and non-OrM phylogroup at either
1059 site. Several non-rhizoctonia and non-peizoid fungi were also tested. For site 1, both results for
1060 all soil samples, and samples collected under either orchid species (OS, *Ophrys sphegodes*; AM,
1061 *Anacamptis morio*) or along transects starting from either orchid are reported. OrM phylogroups
1062 are indicated in blue. P-values <0.05 are indicated in red. n.a., not ascertainable.