1 Subsoil arbuscular mycorrhizal fungal communities in arable soil differ from
2 those in topsoil

3 Moisés A. Sosa-Hernández^{1,2}, Julien Roy^{1,2}, Stefan Hempel^{1,2}, Timo Kautz³, Ulrich
 4 Köpke³, Marie Uksa^{4,5}, Michael Schlöter^{5,6}, Tancredi Caruso⁷, Matthias C. Rillig^{1,2*}.

5 ¹ *Institute of Biology, Plant Ecology, Freie Universität Berlin, Berlin, Germany*

6 ² *Berlin-Brandenburg Institute of Advanced Biodiversity Research, Berlin, Germany*

7 ³ *Institute of Organic Agriculture, University of Bonn, Bonn, Germany*

8 ⁴ *Institute of Soil Science and Land Evaluation, Soil Biology, University of Hohenheim,*
 9 *Stuttgart, Germany*

10 ⁵ *Research Unit for Comparative Microbiome Analysis, Helmholtz Zentrum München,*
 11 *Neuherberg, Germany*

12 ⁶ *Chair for Soil Science, Technische Universität München, Freising, Germany*

13 ⁷ *School of Biological Sciences and Institute for Global Food Security, Queen's*
 14 *University of Belfast, Belfast, Northern Ireland (UK)*

15

16

17 * Correspondence to Moisés A. Sosa Hernández, Freie Universität Berlin, Plant Ecology,
 18 Altensteinstr. 6, D-14195 Berlin, Germany; Email: moisessh@zedat.fu-berlin.de Tel.
 19 +49 (0)30 838-53143

20

21 **Abstract**

22 **Arbuscular mycorrhizal fungi are recognized as important drivers of plant health**
 23 **and productivity in agriculture but very often existing knowledge is limited to the**
 24 **topsoil. With growing interest in the role of subsoil in sustainable agriculture, we**
 25 **used high-throughput Illumina sequencing on a set of samples encompassing**
 26 **drilosphere, rhizosphere and bulk soil, in both top- and subsoil. Our results**
 27 **show subsoil AMF communities harbor unique Operational Taxonomic Units**
 28 **(OTUs) and that both soil depths differ in community structure both at the OTU**
 29 **and family level. Our results emphasize the distinctness of subsoil AMF**
 30 **communities and the potential role of subsoil as a biodiversity reservoir.**

31 **Keywords:** subsoil; drilosphere; arbuscular mycorrhiza; agriculture; Illumina MiSeq;
 32 soil depth

33

34 Arbuscular mycorrhizal fungi (AMF) belong to the monophyletic subphylum
 35 Glomeromycotina (Spatafora et al., 2016) and form a symbiotic relationship with the
 36 roots of most land plants including the majority of agricultural crops (Smith and Read,
 37 2008). This symbiosis is of great relevance for sustainable agriculture due to its ability
 38 to increase productivity (Lekberg and Koide, 2005), nutrient uptake (Smith and Smith,
 39 2011), soil aggregation (Leifheit et al., 2014), and plant protection (Veresoglou and
 40 Rillig, 2012). In arable fields, the subsoil (i.e. the soil beneath the plough layer)
 41 contains more than two thirds of the soil nutrient pool (Kautz et al., 2013). Thus,
 42 understanding how AMF communities vary with depth and what factors drive their
 43 community assembly is a prerequisite for developing appropriate subsoil management
 44 strategies.

45 Previous studies have shown that AMF spore abundance and diversity in agricultural
46 fields decrease with soil depth (Muleta et al., 2008; Oehl et al., 2005; Yang et al.,
47 2010), however under some circumstances subsoil spore diversity may be greater than
48 in the topsoil (Oehl et al., 2005). Moreover, some species' spores seem to be
49 associated with particular soil layers, suggesting a vertical variation in community
50 composition (Muleta et al., 2008; Oehl et al., 2005; Yang et al., 2010). These spore-
51 based results have recently been supported by small ribosomal subunit (SSU) cloning-
52 sequencing approaches, finding differences in community composition between AMF
53 communities at different depths (Moll et al., 2016). Nonetheless, we are just starting to
54 unearth subsoil AMF diversity, and the community assembly processes remain
55 unknown.

56 To shed light on subsoil AMF communities, we used Illumina MiSeq sequencing to
57 analyze a set of soil samples encompassing drilosphere (soil directly influenced by
58 earthworms), rhizosphere (soil directly influenced by roots) and bulk soil (without roots
59 or earthworm burrows), in both top- (10-30 cm, i. e. above the ploughing layer) and
60 subsoil (60-75 cm). Sampling the different compartments was intended to add greater
61 resolution to our results. The first 10 cm were not sampled due to difficulties in
62 differentiating the mentioned compartments at this depth. Samples were collected in
63 May 2011 in a field planted with *Cichorium intybus* L. (see Uksa et al. (2014) for
64 details).

65 We performed amplicon-based AMF specific metabarcoding using the AMF specific
66 primer sets described in Krüger et al. (2009) and the primers LR3 and LR2rev
67 (Hofstetter et al., 2002). The final product of amplification is a 350-440 bp region in the
68 LSU including the variable D1-D2 region. Samples were paired-end sequenced on an
69 Illumina MiSeq platform. Bioinformatics details are given in the supplementary material.
70 Briefly, following quality filtering and chimera removal, sequences were *de novo*

71 clustered at a 97% similarity level into operational taxonomic units (OTUs).
72 Representative sequences of these OTUs have been deposited at ENA under
73 accession numbers LT855246-LT855309.

74 Taxonomic assignment of the OTUs was carried out using BLAST+ (Camacho et al.,
75 2009) against Glomeromycotina (i.e. AMF) reference sequences published in Krüger et
76 al. (2009) and against the EMBL nucleotide database (Kanz et al., 2005). Following a
77 similar approach as in Martínez-García et al. (2015) for SSU sequences, we
78 considered matches with $\geq 97\%$ similarity a species level match, $\geq 90\%$ a genus level
79 match, $\geq 80\%$ a family level match and $\geq 70\%$ a subphylum level match. A species level
80 match refers to how confidently we assign a name to our OTU based on known
81 sequences, and does not imply that these OTUs are to be considered equivalent to
82 those species. In total, 64 OTUs were confidently assigned to the subphylum
83 Glomeromycotina. Of these, we were able to assign 11 OTUs at the species level, 34
84 at the genus level, 16 at the family level and 3 at the subphylum level.

85 The resulting OTU table was analyzed with R version 3.3.1 (R Core Team, 2016).
86 Relative abundance data were obtained by rarefaction of all the samples to the lowest
87 number of reads in a sample (34377 reads), by random subsampling without
88 replacement. Details on the analyses are given in the supplementary material.

89 Our results present high-throughput molecular evidence that the subsoil AMF
90 community is not simply a subset of the topsoil community, but harbors unique OTUs
91 and that the two soil depths differ in structure both at the family level (**Fig. 1**) and at the
92 OTU level (**Fig. 2**). We detected 64 Glomeromycotina OTUs belonging to 7 families
93 (Ambisporaceae, Archaeosporaceae, Claroideoglomeraceae, Diversisporaceae,
94 Gigasporaceae, Glomeraceae and Paraglomeraceae, **Table S1**). OTU accumulation
95 curves for both top- and subsoil reached a plateau, indicating that we captured the
96 majority of the diversity (**Fig. S2**). We observed a highly significant community shift

when comparing the top- and the subsoil (PERMANOVA, $F_{1,16} = 8.67$, $P < 0.001$, **Fig. 3**).

Most remarkably, Claroideoglomeraceae and Diversisporaceae exhibit inversely proportional relative abundances across the studied soil profiles (**Fig. 1**). In topsoil OTUs assigned to Diversisporaceae represented 41.8% of the reads whereas in subsoil they represented 7.3% (GLM, $F_{1,15} = 50.83$ $P < 0.001$). Conversely, OTUs assigned to Claroideoglomeraceae represented 15.0% of the reads in topsoil but 59.9% in subsoil (GLM, $F_{1,15} = 17.87$ $P < 0.001$). The greater relative abundance of the family Claroideoglomeraceae in subsoil is not correlated with its nominal diversity but with a modest increase in relative richness (**Fig. S3a,b**). This finding may point to some species in the Claroideoglomeraceae family being subsoil specialists and particularly dominant in this compartment. This hypothesis is supported by previous results where *Claroideoglomus etunicatum* spores were more commonly found in deeper soil layers (Oehl et al., 2005; Yang et al., 2010). Conversely, both nominal and relative diversity in the Diversisporaceae decreased in subsoil showing no evidence for specialization in subsoil within this family. The family Glomeraceae however, is detected at a mostly constant relative abundance across topsoil (34.1%) and subsoil (28.4%), but decreases in abundance from rhizosphere (49.0%) to bulk soil and drilosphere considered together (23.6%; GLM, $F_{1,15} = 6.79$ $P = 0.02$) (**Fig. 1**). Members of the Glomeraceae are known to preferentially allocate biomass inside the root while producing limited biomass in the soil (Powell et al., 2009). Our observations broadly support the idea that, due to producing a limited extraradical mycelium, Glomeraceae species are expected to preferentially colonize the direct surroundings of the root and to rapidly decrease in abundance outside the rhizosphere. We hypothesize that species with a preferentially intraradical lifestyle are less responsive to abiotic factors outside the root and therefore can readily colonize different soil horizons. In turn, those intraradical lifestyles would be mostly affected by host characteristics.

In our subsoil samples OTU richness (27.4 ± 5.9) was significantly lower than in topsoil (41.6 ± 6.0 ; GLM, $F_{1,15}=23.83$ $P<0.001$). Nonetheless we detected a total of 49 OTUs in subsoil, with two OTUs (OTU_40 subphylum Glomeromycotina and OTU_68 genus *Glomus*) exclusively found in the subsoil, also before normalization (data not shown). Agricultural soils are subjected to a set of disturbances including fertilization or plant removal during harvest; topsoils are additionally subjected to high disturbance in form of tillage, negatively influencing AMF diversity (Kabir, 2005). Applying the C-S-R (competitor, stress tolerator, ruderal) framework to AMF (Chagnon *et al.*, 2013), we would expect topsoil to be dominated by more ruderal species (i.e. elevated growth rates, rapid and abundant spore production, etc.) and subsoil by stress tolerators (i.e. low growth rates, long lived mycelium, etc.). We believe this may be one of the major factors explaining the observed differences in the communities across depth.

In our study no compartment effect was detected for the communities (PERMANOVA, $F_{2,16}= 0.68$, $P=0.66$), besides the mentioned change in relative abundance of the family Glomeraceae (see above). However, previous studies conducted with the same samples show that bacterial communities exhibit a clear compartmentation in the subsoil (Uksa *et al.*, 2015). This difference might be related to the linear, hyphal growth habit of fungi and their unique ability to integrate over larger soil volumes.

We were able to show that subsoil communities are clearly different and not only a subset of topsoil communities, and found contrasting patterns of abundance for different families. Whether this shift in community composition also means a shift in function remains unknown; but the clear difference in dominant families with depth suggests turnover also in functional traits (Powell *et al.* 2009). Our results emphasize the need to account for subsoil when designing agricultural management strategies and highlight the potential role of deeper soil layers as a biodiversity reservoir.

149

150 **Acknowledgements**

151 MR acknowledges funding through the Federal Ministry of Education and Research
 152 (BMBF) initiative 'BonaRes - Soil as a sustainable resource for the bioeconomy' for the
 153 project Soil³. TC was supported by the project SENSE (Structure and Ecological Niche
 154 in the Soil Environment; EC FP7 - 631399 - SENSE). Illumina sequencing was carried
 155 out at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv, Berlin,
 156 Germany). All authors thank Doreen Fischer and Marta Fogt for their involvement in the
 157 sampling and sample preparation processes, respectively. MS-H thanks Margarita
 158 Moreno Bayón for constant support.

159

160

161 **References**

- 162 Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K.,
 163 Madden, T.L., 2009. BLAST+: architecture and applications. BMC Bioinformatics
 164 10, 421. doi:10.1186/1471-2105-10-421
- 165 Chagnon, P.L., Bradley, R.L., Maherali, H., Klironomos, J.N. 2013. A trait-based
 166 framework to understand life history of mycorrhizal fungi. Trends in Plant Science
 167 18: 484–491.
- 168 Hofstetter, V., Clémenton, H., Vilgalys, R., Moncalvo, J.-M., 2002. Phylogenetic
 169 analyses of the Lyophylleae (Agaricales, Basidiomycota) based on nuclear and
 170 mitochondrial rDNA sequences. Mycological Research 106, 1043–1059.
 171 doi:10.1017/S095375620200641X

172 Kabir, Z. 2005. Tillage or no-tillage: impacts on mycorrhizae. Canadian Journal of Plant
173 Science 85: 23–29.

174 Kanz, C., Aldebert, P., Althorpe, N., Baker, W., Baldwin, A., Bates, K., Browne, P., van
175 den Broek, A., Castro, M., Cochrane, G., Duggan, K., Eberhardt, R., Faruque, N.,
176 Gamble, J., Diez, F.G., Harte, N., Kulikova, T., Lin, Q., Lombard, V., Lopez, R.,
177 Mancuso, R., McHale, M., Nardone, F., Silventoinen, V., Sobhany, S., Stoeher, P.,
178 Tuli, M.A., Tzouvara, K., Vaughan, R., Wu, D., Zhu, W., Apweiler, R., 2005. The
179 EMBL Nucleotide Sequence Database. Nucleic Acids Research 33, D29-33.
180 doi:10.1093/nar/gki098

181 Kautz, T., Amelung, W., Ewert, F., Gaiser, T., Horn, R., Jahn, R., Javaux, M., Kemna,
182 A., Kuzyakov, Y., Munch, J.C., P??tzold, S., Peth, S., Scherer, H.W., Schlöter, M.,
183 Schneider, H., Vanderborght, J., Vetterlein, D., Walter, A., Wiesenberg, G.L.B.,
184 Köpke, U., 2013. Nutrient acquisition from arable subsoils in temperate climates: A
185 review. Soil Biology and Biochemistry 57, 1003–1022.
186 doi:10.1016/j.soilbio.2012.09.014

187 Krüger, M., Stockinger, H., Krüger, C., Schüssler, A., 2009. DNA-based species level
188 detection of *Glomeromycota*: one PCR primer set for all arbuscular mycorrhizal
189 fungi. New Phytologist 183, 212–23. doi:10.1111/j.1469-8137.2009.02835.x

190 Leifheit, E.F., Veresoglou, S.D., Lehmann, A., Morris, E.K., Rillig, M.C., 2014. Multiple
191 factors influence the role of arbuscular mycorrhizal fungi in soil aggregation-a
192 meta-analysis. Plant and Soil 374, 523–537. doi:10.1007/s11104-013-1899-2

193 Lekberg, Y., Koide, R.T., 2005. Is plant performance limited by abundance of
194 arbuscular mycorrhizal fungi? A meta-analysis of studies published between 1988
195 and 2003. New Phytologist 168, 189–204. doi:10.1111/j.1469-8137.2005.01490.x

196 Martínez-García, L.B., Richardson, S.J., Tylianakis, J.M., Peltzer, D.A., Dickie, I.A.,

197 2015. Host identity is a dominant driver of mycorrhizal fungal community
198 composition during ecosystem development. *New Phytologist* 205, 1565–1576.
199 doi:10.1111/nph.13226

200 Moll, J., Hoppe, B., König, S., Wubet, T., Buscot, F., Krüger, D., 2016. Spatial
201 Distribution of Fungal Communities in an Arable Soil. *PLoS ONE* 11, 1–17.
202 doi:10.1371/journal.pone.0148130

203 Muleta, D., Assefa, F., Nemomissa, S., Granhall, U., 2008. Distribution of arbuscular
204 mycorrhizal fungi spores in soils of smallholder agroforestry and monocultural
205 coffee systems in southwestern Ethiopia. *Biology and Fertility of Soils* 44, 653–
206 659. doi:10.1007/s00374-007-0261-3

207 Oehl, F., Sieverding, E., Ineichen, K., Ris, E.A., Boller, T., Wiemken, A., 2005.
208 Community structure of arbuscular mycorrhizal fungi at different soil depths in
209 extensively and intensively managed agroecosystems. *New Phytologist* 165, 273–
210 283. doi:10.1111/j.1469-8137.2004.01235.x

211 Powell, J.R., Parrent, J.L., Hart, M.M., Klironomos, J.N., Rillig, M.C., Maherali, H.,
212 2009. Phylogenetic trait conservatism and the evolution of functional trade-offs in
213 arbuscular mycorrhizal fungi. *Proceedings of the Royal Society B: Biological*
214 *Sciences* 276, 4237–45. doi:10.1098/rspb.2009.1015

215 R Core Team, 2016. R: A language and environment for statistical computing. R
216 Foundation for Statistical Computing, Vienna, Austria.

217 Smith S. E., Read D., 2008. *Mycorrhizal Symbiosis*, 3rd Edn. San Diego, CA:
218 Academic Press.

219 Smith, S.E., Smith, F.A., 2011. Roles of Arbuscular Mycorrhizas in Plant Nutrition and
220 Growth: New Paradigms from Cellular to Ecosystem Scales. *Annual Review of*

221 Plant Biology 62, 227–250. doi:10.1146/annurev-arplant-042110-103846

222 Spatafora, J.W., Chang, Y., Benny, G.L., Lazarus, K., Smith, M.E., Berbee, M.L.,
 223 Bonito, G., Corradi, N., Grigoriev, I., Gryganskyi, A., James, T.Y., O'Donnell, K.,
 224 Roberson, R.W., Taylor, T.N., Uehling, J., Vilgalys, R., White, M.M., Stajich, J.E.,
 225 2016. A phylum-level phylogenetic classification of zygomycete fungi based on
 226 genome-scale data. *Mycologia* 108, 1028–1046. doi:10.3852/16-042

227 Uksa, M., Fischer, D., Welzl, G., Kautz, T., Köpke, U., Schlöter, M., 2014. Community
 228 structure of prokaryotes and their functional potential in subsoils is more affected
 229 by spatial heterogeneity than by temporal variations. *Soil Biology and*
 230 *Biochemistry* 75, 197–201. doi:10.1016/j.soilbio.2014.04.018

231 Uksa, M., Schlöter, M., Endesfelder, D., Kublik, S., Engel, M., Kautz, T., Köpke, U.,
 232 Fischer, D., 2015. Prokaryotes in subsoil-evidence for a strong spatial separation
 233 of different phyla by analysing co-occurrence networks. *Frontiers in Microbiology*
 234 6, 1–13. doi:10.3389/fmicb.2015.01269

235 Veresoglou, S.D., Rillig, M.C., 2012. Suppression of fungal and nematode plant
 236 pathogens through arbuscular mycorrhizal fungi. *Biology Letters* 8, 214–217.
 237 doi:10.1098/rsbl.2011.0874

238 Yang, F.Y., Li, G.Z., Zhang, D.E., Christie, P., Li, X.L., Gai, J.P., 2010. Geographical
 239 and plant genotype effects on the formation of arbuscular mycorrhiza in *Avena*
 240 *sativa* and *Avena nuda* at different soil depths. *Biology and Fertility of Soils* 46,
 241 435–443. doi:10.1007/s00374-010-0450-3

242

243

244 Figures

245 Fig. 1 Relative abundance of reads per family in different soil compartments.

246 Proportion of reads assigned to each family for depth (panel A), compartment (B) and
 247 depth and compartment (C). Families are coded by color. The category “Others”
 248 comprises the families Ambisporaceae, Archaeosporaceae, Gigasporaceae and
 249 Paraglomeraceae, as well as OTUs assigned only at the subphylum level. Topsoil =
 250 10-30 cm, subsoil = 60-75 cm. Extended results are presented in the supplementary
 251 materia

252

253 Fig. 2 Relative abundance of the 10 most abundant OTUs for each soil

254 **compartment.** Relative abundance of the OTU is represented by the area of the
 255 square (see scale below figure panel). The OTU list corresponds to the 10 most
 256 abundant OTUs for each environment. Topsoil = 10-30 cm, subsoil = 60-75 cm.
 257 Taxonomic assignment of each OTU is given (also see Table

258 S1).

259

260

261 **Fig. 3 Community ordination of AMF in different soil compartments.** Non-metric
262 multidimensional scaling (NMDS) of Bray-Curtis pairwise community dissimilarities.
263 The OTU table was normalized to the minimum amount of reads per sample. Ellipses
264 represent one standard deviation around the centroid of each soil depth. Lines link
265 each sample to the centroid of the group. Topsoil = 10-30 cm, subsoil = 60-75 cm.
266 Depth is coded by color and compartment by symbol shape.

267

268