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Plant-plant interactions and N fertilization shape soil bacterial and fungal communities

Qingxue Guo [1, †], Lijuan Yan [2, †],

Helena Korpelainen [3], Ülo Niinemets [4], Chunyang Li [1, *]

[1] College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, China

[2] Aquatic Geomicrobiology, Institute of Biodiversity, Friedrich Schiller University Jena, Dornburger Str. 159, D-07743 Jena, Germany

[3] Department of Agricultural Sciences, Viikki Plant Science Centre, P.O. Box 27, FI-00014 University of Helsinki, Finland

[4] Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 1, 51014 Tartu, Estonia

[†] Authors contributed equally to this study

[*] Corresponding author: Chunyang Li, E-mail: licy@hznu.edu.cn

Abstract The impact of conspecific and heterospecific neighboring plants on soil bacterial and fungal communities has never been explored in a forest ecosystem. In the present study, we first investigated soil microbial communities in three plantations: *Larix kaempferi* monoculture, *L. olgensis* monoculture and their mixture. Then, a two-year growth experiment was conducted to investigate the effects of intra- and inter-specific plant interactions of *L. kaempferi* and *L. olgensis* on rhizosphere microbial communities in different nitrogen conditions. The results demonstrated clear differences in the beta-diversity and composition of bacteria and fungi among the three plantations, which implied different effects of plant-plant interactions on soil microbial communities. The results of the pot experiment showed that *L. kaempferi* suffered from greater negative effects from its conspecific neighbor regardless of the N fertilization, whereas the negative effect declined when *L. kaempferi* was grown with *L. olgensis* under N fertilization. Changes in intra- and inter-specific plant interactions significantly impacted the chemical and biological properties of soil under N fertilization, with lower concentrations of NH_4^+, and lower soil microbial biomass (C_{Mic}) and soil carbon nitrogen biomass (N_{Mic}) under intra-specific plant interactions of *L. kaempferi* (KK) compared to inter-specific interactions of *L. kaempferi* and *L. olgensis* (KO). N fertilization increased bacterial and fungal alpha diversities in the rhizosphere soil of KO. For the beta diversity, the PERMANOVA results demonstrated that there was a significant impact of intra- and inter-specific plant interactions on soil microbial communities, with KK significantly differing from intra-specific plant interactions of *L. olgensis* (OO) and KO. The two plant species

and N fertilization showed specific effects on the soil microbial composition, particularly on the fungal community. Both *L. olgensis* and N fertilization increased the abundance of *Ascomycota* but reduced that of *Basidiomycota*, and even shifted the dominance of *Basidiomycota* to *Ascomycota* in KO under N fertilization. Based on our results, we suggest that *L. kaempferi* planted with *L. olgensis* under N fertilization may be an efficient way to promote the productivity of plantations.

Keywords Forest plantations; Plant neighbors; Plant-soil feedback; Fungal communities; Carbon investment

1. Introduction

Many types of forest plantations (mostly monocultures) have been established around the world to provide wood products or restore degraded lands (Paul et al., 2010; Richards et al., 2010). However, the productivity of monocultures has commonly declined due to reasons, such as decreasing nitrogen (N) availability or autotoxicity (O'Hehir and Nambiar, 2010; Chen et al., 2014; Deng et al., 2014). For example, the declined productivity of Chinese fir (*Cunninghamia lanceolata*) monocultures is mainly caused by a novel allelochemical, cyclic dipeptide (Chen et al., 2014), which alters fungal communities in soil by inhibiting the spore germination of *Glomus cunnighamia* and *Gigaspora alboaurantiaca* (Xia et al., 2016). Mixed-forest plantations always have higher productivity than monocultures (Lovelock and Ewel, 2005; Richards et al., 2010). Lovelock and Ewel (2005) have found differences in arbuscular mycorrhizal compositions between different tropical tree mixtures and respective monocultures, and the fungal diversity was correlated with the net primary productivity of plantations. Similarly, Mortimer et al. (2015) have observed that the biomass of soil fungi and bacteria as well as the productivity of tea were higher in a mixture of *Alnus nepalensis* and tea species (*Camellia sinensis* var., assamica) compared with a tea monoculture.

Plants interact intensively and reciprocally with soil microbial communities (Kuzyakov and Xu, 2013). Previous studies have confirmed that plants show specific effects on the structure of their own microbial communities, for example, by secreting organic compounds and through input on litter (reviewed in Bakker et al., 2013a;

89 Cline and Zak, 2015; Kumar et al., 2016; Cheeke et al., 2017). However, the majority
90 of these studies have focused on isolated plants or have overlooked the impacts of
91 plant-plant interactions. Conspecific or heterospecific plant neighbors always cause
92 different effects on processes, such as nitrogen uptake, the secretion of root exudates
93 or growth (McKane et al., 2002; Kozovits et al., 2005; Broz et al., 2010; Kunstler et
94 al., 2015; Guo et al., 2017). If plants suffer from their conspecific or heterospecific
95 neighbors, for example as a result of competition, there may be consequent effects on
96 the microbial communities of soil. As research increasingly demonstrates the
97 connection between plants and microbes, there remains a need to understand how
98 intra- and inter-specific plant-plant interactions impact belowground microbial
99 communities.
100
101 Plant-plant interactions are affected by nitrogen availability (Wilson and Tilman, 1991;
102 Guo et al., 2017; Broadbent et al., 2018) and plant-soil feedback processes (Harrison
103 and Bardgett, 2010; Hendriks et al., 2015). A negative effect means that a particular
104 species changes its biological community or abiotic soil properties in a way that its
105 growth is reduced, while the other species shows less harmful or even beneficial
106 effects (Harrison and Bardgett, 2010). Reduced plant growth can result from changes
107 in the composition of beneficial arbuscular mycorrhizal fungi (Castelli and Casper,
108 2003). It has been discovered that nitrogen fertilization can alleviate plant-soil
109 negative effects and plant-plant interactions (Guo et al., 2017), probably not only by
110 increasing the N level of soil but also by improving microbial communities (Xia et al.,

111 2016). Many studies have reported that nitrogen availability largely impacts bacterial
112 and fungal communities (e.g. Fierer et al., 2012; Dietrich et al., 2017; Carrara et al.,
113 2018; Treseder et al., 2018). Based on a nitrogen fertilization experiment, Yuan et al.
114 (2016) found that *Chloroflexi* and *Bacteroidetes* show positive responses to N
115 fertilization, while *Acidobacteria* and *Verrucomicrobia* have negative responses.
116 Treseder and Allen (2002) discovered that the fungal community of soil tends to shift
117 from *Gigasporaceae* under low N availability to *Glomeraceae* under high N. A few
118 studies have investigated the effects of intra- and inter-specific plant-plant interactions
119 on soil bacterial communities at different N levels in grass lands or agroecosystems
120 (Bakker et al., 2013b, 2014; Pivato et al., 2017). For instance, Pivato et al. (2017)
121 showed that the total bacterial abundance in the rhizosphere of some inter-specific
122 plant-plant mixtures was significantly higher than that of either plant species in a
123 monoculture at a low nitrogen level in an agroecosystem, whereas the difference
124 disappeared at a high nitrogen level. Thus, their results implied that the impacts of
125 plant-plant interactions on soil bacterial communities largely depend on N levels.
126 However, there is less knowledge of the effects of N availability and plant-plant
127 interactions on soil bacterial and fungal communities in forest ecosystems.
128
129 Larch plantations are widely distributed and have a high economic and ecological
130 value across the Northern Hemisphere in areas such as Siberia, north-eastern China
131 and Japan (Agathokleous et al., 2017). The larches *Larix kaempferi* and *L. olgensis*
132 are two important plantation species. *L. kaempferi* grown in isolation has a higher

133 growth rate, biomass production and N accumulation than does *L. olgensis* (Li et al.,
134 2016; Guo et al., 2017). However, our previous results have suggested that the growth
135 of *L. kaempferi* is suppressed when grown in a mixed culture with *L. olgensis* in soil
136 from a *L. kaempferi* plantation (Guo et al., 2017), which may result from the presence
137 of different soil microbial communities. The present study consists of two parts.
138 Firstly, bulk soil samples were collected from three types of plantations (*L. kaempferi*
139 monoculture, *L. olgensis* monoculture and a mixed plantation of these two species).
140 The plantations are characterized by even-aged individuals and there are some
141 herbaceous species in low abundance. This setting enables studies on intra- and
142 inter-specific interactions in natural conditions. Secondly, a two-year pot experiment
143 was established, including isolated plants and combinations of two plants under intra-
144 and inter-specific interactions with and without N fertilization. In the pot experiment,
145 we used soil collected from a mature *L. kaempferi* monoculture to investigate the
146 effects of plant-plant interactions and N fertilization on soil microbial communities.
147 We aimed to answer the following questions: 1) do different plantations contain
148 specific types of soil bacterial and fungal communities? Based on the pot experiment,
149 2) if the intra- or inter-specific plant interactions of the two studied species were
150 different, would the rhizosphere soil bacterial and fungal communities also differ from
151 each other? 3) If N fertilization differently affects intra- and inter-specific plant
152 interactions, how does the interplay of N fertilization and plant interactions affect
153 rhizosphere soil communities?

154 **2. Materials and methods**

In the first part of this study, bulk soil samples were collected from three types of plantations: *L. kaempferi* monoculture plantation, *L. olgensis* monoculture plantation and a mixed-plantation of these two species. In the second part of the study, rhizosphere soil samples were collected from the pot experiment. The pot experiment was conducted outside on an open space at the Qingyuan Experimental Station of Forest Ecology, Institute of Applied Ecology, Chinese Academy of Sciences, Liaoning Province, Northeast China (41º51'N, 124º54'E, 560 m above sea level). The mean annual rainfall is 811 mm and the mean annual air temperature varies between 3.9 ºC and 5.4 ºC. *L. kaempferi* and *L. olgensis* plantations are widely distributed in the study area.

2.1. Plantation selection

The following principles were followed when selecting the three types of plantations: 1) similar age, 2) close location, and 3) similar exposure and slope. Eventually, we found only one pure *L. kaempferi* plantation (about 20 years old, 3.6 ha and 2050 stems per ha), one pure *L. olgensis* plantation (about 20 years old, 4.0 ha and 1750 stems per ha) and one mixed plantation (about 24 years old, 6.0 ha and 600 *L. kaempferi* stems per ha, and 640 *L. olgensis* stems per ha), about 30 km away from the research station. The two monoculture plantations were adjacent, and the *L. olgensis* plantation experienced thinning forest management in 2010 according to the

177 description of a local farmer. The mixed plantation was about 400 m away from the
178 two monoculture plantations but on another hillside.
179
180 In the center of each plantation, ten plots (10 m × 10 m) were established. From each
181 plot, ten bulk soil samples (0-10 cm in depth) were collected from ten subplots (1 m ×
182 1 m) and then mixed to form one composite sample. Finally, ten samples from each
183 plantation were collected. Each bulk soil sample was divided into two equal
184 subsamples: one subsample was used for the analysis of soil properties, while the
185 other subsample was stored at -20 ^{o}C for the subsequent DNA extraction. Five
186 subsamples from each plantation were randomly selected for later DNA extraction.
187
188 *2.2. Pot experiment and sampling*
189
190 For the pot experiment, soil was collected from the *L. kaempferi* plantation near the
191 research station and then homogenized (pH 5.65, C 18.61 g kg^{-1} and total N 1.82 g
192 kg^{-1}) early October, 2013. One-year old seedlings of *L. kaempferi* and *L. olgensis* with
193 approximately the same crown size and height were chosen from a local nursery
194 garden to establish intra- and inter-specific plant interactions. Before planting, all
195 roots were carefully washed with sterile water to remove adhering soil. Two seedlings
196 were planted 10 cm apart from each other into each plastic pot (external diameter and
197 height 56 cm and 33 cm, respectively) late October, 2013. KK refers to two *L.*
198 *kaempferi* seedlings in a pot, KO refers to one *L. kaempferi* and one *L. olgensis* in a

pot, and OO refers to two *L. olgensis* seedlings in a pot. Each seedling combination included thirty-two pots. In May 2014, sixteen pots were randomly selected from each treatment to carry out N fertilization by applying 5.1 g urea (46.3% N). N application was repeated three more times (each time 5.1 g urea), in June 2014, and in May and June 2015. In addition, single *L. kaempferi* seedlings (K) and single *L. olgensis* seedlings (O) were planted into pots in late October, 2013, six pots for each species. Similarly, three pots from both K and O were randomly selected to apply urea as above. The combination of results from the plantations and pot experiment will provide a better understanding of the effects of intra- and inter-specific plant interactions on soil bacterial and fungal communities.

Double-plant pots were harvested twice, late August, 2014 and early September, 2015. The harvested plants were dried at 70ºC for 72h to determine their dry masses. Neighbor effect index (NEI), estimated to measure plant-plant interactions, used the following formulas (Manea and Leishman, 2011):

$NEI_{k/kk} = (Y_{k/kk} - Y_k)/(Y_{k/kk} + Y_k)$, $NEI_{o/oo} = (Y_{o/oo} - Y_o)/(Y_{o/oo} + Y_o)$,

$NEI_{k/ko} = (Y_{k/ko} - Y_k)/(Y_{k/ko} + Y_k)$, $NEI_{o/ko} = (Y_{o/ko} - Y_o)/(Y_{o/ko} + Y_o)$,

where $NEI_{k/kk}$ and $NEI_{o/oo}$ indicate intra-specific plant interactions for *L. kaempferi* and *L. olgensis*, respectively; $NEI_{k/ko}$ and $NEI_{o/ko}$ indicate inter-specific plant interactions for *L. kaempferi* and *L. olgensis*, respectively. Y_k and Y_o are the biomass of *L. kaempferi* and *L. olgensis* grown in isolation, respectively. $Y_{k/kk}$ and $Y_{o/oo}$ are the biomass of *L. kaempferi* and *L. olgensis* with intra-specific plant interactions,

221 respectively. $Y_{k/ko}$ and $Y_{o/ko}$ are the biomass of *L. kaempferi* and *L. olgensis* with
222 inter-specific plant interactions, respectively. Because the biomass of isolated plants is
223 higher than that of plants exposed to plant-plant interactions (Kozovits et al., 2005;
224 Guo et al., 2017), the NEI values are negative. A more negative NEI value indicates
225 that a plant suffers a more negative impact from its neighbor.
226
227 Soils were sampled following the last harvest. Using a soil corer (4 cm in diameter),
228 soil samples were collected from four pots of KK, KO and OO, as well as from
229 corresponding pots with N fertilization. The sampling position was at the center
230 between the two plants in a pot. Then loose soil was removed by careful shaking and
231 the tightly-adhering soil was sampled from root surfaces as rhizosphere soil.
232 Rhizosphere soil samples were collected also from the pots of K and O as well as
233 from corresponding pots with N fertilization. The sampling center was 5 cm away
234 from the basal stem. Each soil sample was divided into two equal subsamples: one
235 subsample was used for the analysis of soil properties, while the other subsample was
236 stored at -20 ºC for the subsequent DNA extraction. Three subsamples from each
237 treatment were chosen for later DNA extraction.
238
239 *2.3. Determination of soil properties*
240
241 Soil pH was determined from soil-water suspensions (1:2.5 v/v). Available
242 phosphorus (AP) was extracted with sodium bicarbonate and then determined using

243 the molybdenum blue method (Xiong et al., 2015) Soil organic matter (SOM) was
244 determined using the potassium dichromate external heating method (Ciavatta et al.,
245 1991). Total N (TN) was determined by the Kjeldahl method (Buchi K370,
246 Switzerland). Soil NH_4^+ and NO_3^- were extracted with 1M KCl and the
247 indophenol-blue colorimetric and double wavelength (220 nm and 275 nm) methods
248 (Nie et al., 2018), respectively. Soil microbial biomass carbon (C_{Mic}), nitrogen (N_{Mic})
249 and phosphorus (P_{Mic}) were determined by the chloroform fumigation extraction
250 method (Yao et al., 2017). For the pot experiment, two-way ANOVAs were applied to
251 check the impact of N application and plant-plant interactions on the soil properties.
252
253 *2.4. DNA extraction and Illumina sequencing*
254
255 Genomic DNA was extracted using the Power Soil kit (MO BIO Laboratories,
256 Carlsbad, CA, USA) following the manufacturer's instructions. The primers used for
257 the amplification of the V4-V5 hypervariable regions of the bacterial 16S rRNA gene
258 were 515F (5′-GTGYCAGCMGCCGCGGTA-3′) and 909R
259 (5′-CCCCGYCAATTCMTTTRAGT-3′) (Yao et al., 2017). The primers used for the
260 amplification of the partial ITS region of fungi were ITS4:
261 5'TCCTCCGCTTATTGATATGC-3' and ITS3_KYO2:
262 5'GATGAAGAACGYAGYRAA-3' (Bokulich and Mills, 2013). The PCR reactions,
263 quality control and purification processes followed the instructions of Yao et al.
264 (2014). A library was constructed and all sequences were generated with the

265 Illumina's MiSeq platform using paired-end reads. All above-mentioned steps were
266 completed at the Environmental Genome Platform of Chengdu Institute of Biology,
267 Chinese Academy of Sciences, China.
268
269 *2.5. Bioinformatics*
270
271 The paired-end reads of the bacterial 16S rRNA gene and fungal ITS region
272 amplicons were processed using the mothur pipeline (V.1.35.1) (Schloss et al., 2009),
273 based on the MiSeq standard operating procedure (Kozich et al., 2013). For quality
274 control, the 16S and ITS sequences that contained ambiguous (N) bases and
275 homopolymers longer than 8 nucleotides were screened out. The remaining sequences
276 were pre-clustered to allow for up to 1 bp difference per 100 bp bases to remove
277 potential sequencing errors before the identification of the chimeric sequences using
278 the UCHIME algorithm. The SILVA full length reference sequences (V.128) were
279 used for the alignments of the bacterial 16S rRNA sequences. For the fungal ITS
280 sequence analysis, due to a lack of reference templates for sequence alignment, we
281 trimmed the raw sequences to the same size (300 bp) after the removal of the forward
282 primer sequence. After removing the chimeric sequences, the unique bacterial 16S
283 rRNA gene sequences were classified using the SILVA reference database (V.128),
284 and fungal ITS sequences were classified using the mothur-formatted UNITE ITS
285 reference database (UNITE v6_sh_99) with the default bootstrapping algorithm
286 (cutoff value 80%). All sequences were assigned to operational taxonomic units

(OTUs) using the OptiClust clustering algorithm at 97% similarity. Bacterial 16S sequences were assigned to OTUs using classify as the split method, whereas fungal ITS sequences were assigned into OTUs using fasta as the split method based on nearest neighbor clustering. Singletons were removed from both bacterial and fungal ITS datasets. For the bacterial OTU dataset, OTUs that were classified as non-bacterial or chloroplast were removed. For the fungal OTU dataset, all non-fungal OTUs were removed. The raw sequencing data were deposited in the NCBI Sequence Read Archive (SRA) database under accession no. SRP125300.

2.6. Alpha diversity estimation

The alpha diversity of bacteria and fungi in each sample was rarefied and estimated at the sampling depths of 2873 and 5558, respectively, using the R package phyloseq (McMurdie and Holmes, 2013). The normalization of the alpha diversity data was visualized using Q-Q plots. The homogeneity of variances was examined using the Bartlett test. The differences of the means between treatments and their interactions were tested using ANOVA, assuming that the alpha diversity data were normally distributed and the variances were equal among treatment groups. The non-parametric Kruskal-Wallis rank sum test of each single treatment factor was used when the data were not normally distributed or the group variances were heterogeneous. Tukey's multiple comparisons of means were used when the differences between groups were significant. The significance of the differences was concluded at the 95% confidence

309 level ($P < 0.05$).

310

311 *2.7. Beta diversity estimation*

312

313 The relative abundance of each OTU was calculated by dividing its read count by the
314 total read count of the corresponding sample, prior to the beta diversity analysis.
315 Principal coordinate analyses (PCoA) were applied to the relative abundance data to
316 visualize the broad pattern of bacterial and fungal communities between treatment
317 groups based on the Bray-Curtis distance, using the R package phyloseq (McMurdie
318 and Holmes, 2013). PERMANOVA was used to assess, whether the treatment groups
319 of Species and N fertilization and their interaction resulted in a different bacterial
320 community composition with the default 999 permutations. Betadisper was used to
321 test, whether the dispersions of observations between the treatment groups were equal
322 with 999 permutations. PERMANOVA and Betadisper were performed based on the
323 Bray-Curtis distance using the R package Vegan (Oksanen et al., 2007). Pairwise
324 PERMANOVA tests were used when the differences were significant between a priori
325 groups following PERMANOVA tests. Constrained analysis of principle coordinates
326 (CAP) was performed to visualize the significant differences in the community
327 composition between the treatment groups based on the Bray-Curtis distance using the
328 R package phyloseq (McMurdie and Holmes, 2013) with 999 permutations. The
329 significance of the differences was concluded at the 95% confidence level ($P < 0.05$).

330

331 *2.8. Biomarker discovery*

332

333 The linear discriminant analysis effect size LEfSe (Segata et al., 2011) was used to
334 determine the differentially abundant OTUs 1) among treatments without interaction
335 (isolated *L. kaempferi* and *L. olgensis*) and with intra-specific and inter-specific
336 interactions, irrespective of the N treatment (Class: Interaction; Subclass: N treatment),
337 and 2) between the N-treated and control pots (Class: N treatment; Subclass:
338 Interaction) in the pot experiment. For the soil sampled from the three plantations,
339 LEfSe was used to explore the OTUs with differential abundance between the
340 different interaction patterns (Class: Interaction).

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351 **3. Results**

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353 *3.1. Neighbor effect index*

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355 *L. kaempferi* suffered stronger negative effects from its conspecific neighbor
356 compared to *L. olgensis*, regardless of N fertilization. However, the negative effect
357 declined when *L. kaempferi* was grown with *L. olgensis* under N fertilization. *L.*
358 *olgensis* suffered less negative effect from *L. kaempferi* when they were grown
359 together (Fig. 1).

360

361 *3.2. Changes in soil properties*

362

363 The mixed plantation soil had higher NH_4^+, C_{Mic} and N_{Mic} contents compared to soil
364 from the two monoculture plantations (Table S1). In the pot experiment, soil pH,
365 SOM and the NO_3- concentration were significantly impacted by N fertilization and
366 plant-plant interactions (Table 1). N fertilization decreased rhizosphere soil pH in
367 each treatment (Table S2). The intra-specific plant interactions of KK showed lower
368 NH_4^+, C_{Mic} and N_{Mic} compared to KO under N fertilization (Table S2).

369

370 *3.3. Taxonomic composition and alpha diversity*

371

372 The bacterial communities of the bulk soil samples from the three plantations and
373 from the pot experiment were dominated by *Protebacteria*, *Acidobacteria* and
374 *Actinobacteria*, whereas the fungal communities were dominated by *Basidiomycota*

375 and *Ascomycota* (Figs. 2 and 3, Supplementary Fig. S1). The abundance of
376 *Basidiomycota* was lower, whereas the abundance of *Ascomycota* became higher in
377 KO and OO relative to KK, especially under N fertilization in the pot experiment (Fig.
378 3).
379
380 In the bulk soil samples from the three plantations, the average Shannon values of the
381 bacterial and fungal communities were 5.98 and 2.52, respectively, and neither intra-
382 nor inter-specific plant interactions had any effects on the bacterial and fungal
383 diversity (P >0.05, Supplementary Fig. S2). In the rhizosphere soil of single-plant
384 pots, N treatment showed no significant effect on bacterial and fungal diversity
385 (P >0.05, Figs. 3a and 4a). However, N treatment increased bacterial diversity (P <
386 0.001, Fig. 4b) and also fungal diversity in the rhizosphere soil of KO (P < 0.05, Fig.
387 5d). In addition, plant-plant interactions had a significant impact on bacterial diversity
388 (P < 0.001, Fig. 4b). For instance, the rhizosphere soil of KK exhibited a lower
389 bacterial diversity compared to KO (P < 0.001) and OO (P < 0.001).
390
391 *3.4. Beta diversity*
392
393 The PCoA showed that the plantation soils had distinct compositions of both bacterial
394 and fungal communities (Supplementary Fig. S3), unlike the rhizosphere soils from
395 the pot experiment (Supplementary Fig. S4). Intra- and inter-specific interactions had
396 a significant effect on bacterial and fungal community structures in soil from the three

plantations (PERMANOVA, $P < 0.01$, Supplementary Fig. S5). In the rhizosphere soil of double-plant pots, plant-plant interactions and N treatment significantly affected both the bacterial and fungal community composition (Supplementary Figs. S6e-h, PERMANOVA, $P < 0.01$). Pairwise PERMANOVA demonstrated that the structure of both bacterial and fungal communities in the rhizosphere soil of KK was different from those of KO and OO (Supplementary Figs. S6e and f, PERMANOVA, $P < 0.05$).

3.5. Biomarker discovery

We used the LEfSe analysis to discover biomarkers (different abundances of bacterial and fungal taxa) in the plantation and pot experiment soil. From phyla to genera, the three plantations showed their specific influence on bacterial and fungal compositions (Supplementary Fig. S7).

In the rhizosphere soil of single plants, the abundance of the bacterial orders like *Ktedonobacterales* and *Sphingomonadales* and the fungal order *Chaetothyriales* and the genus *Ciliophora* decreased, whereas the fungal family *Corynesporascaceae* became more prevalent under N fertilization (Fig. 6a and b). In the rhizosphere soil of double-plant pots, N fertilization significantly increased the abundance of the bacterial orders *Gemmatimonadales*, *Xanthomonadales*, *Propionibacteriales*, *Methylophilales* and *JG37-AG-4*, whereas the abundance of the phylum *Acidobacteria*, including the orders *Solibacterales*, *Blastocatellales* and *Subgroup 6* and the orders *Rhodocyclales*

419 and *Rhodospirillales*, reduced under N fertilization (Fig. 7a). The abundance of
420 *Basidiomycota,* such as class *Tritirachiomycetes* and order *Boletales,* reduced but that
421 of *Ascomycota,* such as the order *Hypocreales,* increased under N fertilization in the
422 double-plant pots (Fig. 7b).
423
424 In the pot experiment, we also identified the bacterial and fungal taxa responding to
425 species and plant interactions, irrespective of N fertilization (Figs. 6c and d, Fig. 8).
426 The orders *Rhodospirillales*, *Rhizobiales*, *Acidimicrobiales* and *Acidobacteriales* were
427 more abundant in the rhizosphere soils of K than O (Fig. 6c). Among fungal taxa,
428 *Ascomycota* preferred the soil of O, whereas *Basidiomycota* and the order
429 *Rhizophydiales* preferred the soil of K (Fig. 6d).
430
431 In the double-plant pots, the bacterial orders *Acidobacteriales* and *Micrococcales*
432 were generally more abundant in the soil of KK, whereas *Actinobacteria* became
433 more prevalent in the soil of KO (Fig. 8a). The taxa from the phyla *Firmicutes*,
434 *Chloroflexi* and *Planctomycetes* were more frequent in the soil of OO (Fig. 8a). The
435 fungal taxa *Basidiomycota* were generally more abundant in the rhizosphere soil of
436 KK, whereas fungi from the other dominant phylum *Ascomycota* were more abundant
437 in the rhizosphere soil of OO (Fig. 8b). In addition, the orders *Microascales* and
438 *Hymenochaetales* became more abundant in the rhizosphere soil of KO (Fig. 8b).
439 **4. Discussion**
440

441 Many plant attributes, such as physiological processes, root exudates and leaf N
442 content largely depend on the identity of a plant's neighbors and on the soil nitrogen
443 content (Broz et al., 2010; Pierik et al., 2013; Chen et al., 2017). It follows that soil
444 microbial communities can be differently impacted depending on, whether plants
445 grow in the absence of neighbors or in the presence of conspecific or heterospecific
446 neighbors in different environments.
447
448 *4.1. Different plantations have specific bacterial and fungal communities*
449
450 Different plantations have specific effects on soil chemical properties (Nazaries et al.,
451 2015; Suz et al., 2017). For example, a pine plantation was found to decrease soil pH
452 and increase the soil nitrate (NO_3^-) content, whereas a birch plantation declined the
453 total carbon and NO_3^- contents (Nazaries et al., 2015). Our results also showed that
454 the chemical traits of soil were different among the three studied plantations,
455 particularly in the KK plantation. Differences in the chemical properties of soil largely
456 depend on plantation characteristics, such as litter production and decomposition
457 (Hättenschwiler et al., 2005; Helfrich et al., 2015), nutrient mineralization (Richards
458 et al., 2010) and root exudates (Chen et al., 2014). Guo et al. (2016) have revealed
459 that inter-specific interactions of *L. kaempferi* and *L. olgensis* decrease leaf C/N
460 compared to intra-specific plant interactions without N fertilization, which may lead
461 to biochemically heterogeneous plant litter in different types of plantations.
462

463 It has been shown that functions, compositions or diversities of soil microbe
464 communities closely correlate with soil properties, such as pH, and soil carbon and
465 nitrogen contents (Bakker et al., 2013b, 2014; Nazaries et al., 2015; Gunina et al.,
466 2017; Suz et al., 2017). Suz et al. (2017) found that there were no significant
467 differences in root colonization by generalist ectomycorrhizal fungi between mixed
468 and pure plantations but, on average, there were more generalist ectomycorrhizal
469 fungi in mixed plantations, where plants connect with their neighbors through
470 common mycorrhizal networks. In this study, the phylum level abundance and the
471 Shannon index of bacterial and fungal communities showed little difference; however,
472 the beta diversity and biomarkers of bacterial and fungal taxa displayed greater
473 dissimilarities, possibly different nutrient translocation or litter decomposition. For
474 example, the abundance of *Basidiomycota* was higher but the abundance of
475 *Ascomycota* was lower in KO and OO plantations compared with KK. The ability to
476 degrade lignin is mainly conserved in *Basidiomycota* (Baldrian, 2006). The
477 accumulation of soil organic matter exerts a positive and significant effect on the
478 fungal beta-diversity (Cline and Zak, 2015), thus implying an important role of soil
479 organic matter in shaping fungal communities and in regulating ecosystem carbon
480 dynamics (Cheeke et al., 2017).
481
482 *4.2. Direct and indirect effects of N fertilization on microbial communities*
483
484 Nitrogen addition directly drives changes in bacterial and fungal communities in

different terrestrial ecosystems by increasing N availability (Cox et al., 2010; Fierer et al., 2012; Yuan et al., 2016; Treseder et al., 2018; Zhu et al., 2018). Our results found a decrease in the abundance of *Acidobacteria* after N fertilization in the pot experiment. An enhanced soil N availability increased the abundance of copiotrophic bacterial taxa, including *Proteobacteria* and *Bacteroidetes*, but lowered the proportion of oligotrophic *Acidobacteria* (Fierer et al., 2012). Similarly, Yuan et al., (2016) have found that *Acidobacteria* show a negative response to N fertilization. For fungal communities, shifts in the fungal composition have been considered to be the main driver of the decomposition response to enhanced N addition (Baldrian, 2006; Cox et al., 2010; Morrison et al., 2016). Cox et al. (2010) have reported that *Russula ochroleuca* and *Thelephora terrestris* respond positively to increasing N, whereas *Pseudotomentella tristis* and *Piloderma* respond negatively to increasing N. In our pot experiment, we discovered a shift from *Basidiomycota* to *Ascomycota* under the combined effects of *L. olgensis* and N fertilization. Changes in bacterial and fungal communities reflect corresponding alterations in functional consequences (Cox et al., 2010; Dietrich et al., 2017).

Nitrogen fertilization affects soil microbial communities also indirectly through changing chemical properties of soil. We found that N fertilization significantly impacted soil pH and soil organic matter, which indirectly control microbial communities (Fierer and Jackson, 2006; Cline and Zak, 2015; Yuan et al., 2016; Carrara et al., 2018). Soil pH is a major driver in shaping soil microbial communities

(Constancias et al., 2015; Ochoa-Hueso et al., 2018). For instance, the relative abundances of *Bacteroidetes*, *Planctomycetes* and *Thaumarchaeota* show a positive correlation with soil pH across an agricultural landscape (Constancias et al., 2015). Fierer and Jackson (2006) have suggested that any significant deviation from extracellular pH should impose stress on single-celled organisms and restrict the survival of taxa exposed to pH beyond their optimum.

4.3. Effects of plant-plant interactions

Mortimer et al. (2015) have reported fewer significant differences in the chemical properties of soil, whereas the ectomycorrhizal biomass, as well as the biomass of Gram-positive, Gram-negative, and actinomycetes bacteria were significantly higher in a mixed plantation than in a monoculture. Our results also demonstrated greater differences in the composition and beta-diversity of soil microbes between monocultures and a mixed-plantation. These results indicated that intra- and inter-specific plant interactions possibly have great impacts on soil bacterial and fungal communities. Our PERMANOVA and LEfse results indicated significant differences between KK and KO as well as KK and OO, with a higher bacterial and fungal diversity in KO compared to KK. At first, we observed a stronger neighbor effect from conspecific species in both species. Available P, total N (under fertilization) and NH_4^+ in the rhizosphere soil of a double-plant *L. kaempferi* (KK) pot were the lowest among all double-plant pots, implying a higher demand for nutrients and a

529 stronger intra-specific competition than previously suggested by Kunstler et al. (2015).
530 Changes in soil nutrients induced by plant-plant competition contribute to alterations
531 in soil microbes (Fierer et al., 2012; Yuan et al., 2016; Treseder et al., 2018; Zhu et al.,
532 2018). Secondly, we observed a weakened neighbor effect caused by *L. kaempferi* on
533 *L. olgensis* in the inter-specific plant interactions. There is a general acceptance that
534 soil microbes are important in determining plant-plant interactions in different
535 environments (Hodge and Fitter, 2013; Keymer and Lankau, 2017). *L. olgensis* has
536 been found to enhance its ability to absorb NO_3^- under inter-specific plant interactions
537 (Guo et al., 2018), which may result from the increase in the fungal phylum
538 *Ascomycota* (Leroy et al., 2017). Finally, we found that after N fertilization, the
539 neighbor effects on both species were less negative in KO, which implied declined
540 inter-specific plant competition compared to KK and OO. In the present study, strong
541 combined effects between plant-plant interactions and N fertilization on the
542 rhizosphere soil were observed, particularly on the fungal communities. Both *L.*
543 *olgensis* and N fertilization increased the abundance of fungal species belonging to
544 *Ascomycota* but reduced abundance of *Basidiomycota* species, and their combined
545 effect even led to a dominance shift from *Basidiomycota* to *Ascomycota* and to a
546 higher fungal diversity. The changes in rhizosphere soil microbes were possibly an
547 important reason to drive changes in plant-plant interactions.
548
549 *4.4. Negative feedback from L. kaempferi plantation conditioned soil*
550

In the pot experiment, the soil was selected from a *L. kaempferi* plantation. Lower bacterial and fungal diversities of KK were probably caused by *L. kaempferi* continuing to prefer its own soil microbial communities, whereas *L. olgensis* selected different microbes and increased bacterial and fungal diversity particularly under N fertilization. Guo et al. (2017) have reported that the *L. kaempferi* conditioned soil showed negative effects on the growth of *L. kaempferi*. The more negative neighbor effect of *L. kaempferi* on its conspecific neighbors also confirmed that. A given plant species alters its biological soil communities and abiotic soil properties that may decrease its own growth rate, resulting in a negative feedback (Harrison and Bardegtt, 2010; Hendriks et al., 2015). The introduction of *L. olgensis* to *L. kaempferi* conditioned soil greatly changed the composition, abundance and diversity of bacterial and fungal communities, particularly under N fertilization. Previously, Van der Putten et al. (2016) have emphasized the role of bacterial and fungal communities in the development of plant-soil feedback under environmental changes through plant species loss or nitrogen enrichment. For example, the negative effects of Chinese fir conditioned soil on Chinese fir is alleviated by an introduced foreign plant species through increasing arbuscular mycorrhizal fungi and improving chemical properties of soil (Xia et al., 2016). The above results suggested that an introduced foreign plant species could improve soil conditions by changing microbial communities to alleviate the negative soil feedback.

5. Conclusions

573

574 Our work demonstrated that intra- and inter-specific plant interactions were
575 differently affected by plant neighbors and N fertilization, and there were distinct
576 changes in soil microbial communities. In turn, the changing soil bacterial and fungal
577 communities probably influence plant-plant interactions. Based on the present study,
578 we suggest that *L. olgensis* and *L. kaempferi* growing together with N fertilization
579 may be an efficient way to promote the productivity of plantations. The introduced
580 foreign plant species and N fertilization could improve the chemical and biological
581 conditions of soil and, consequently, plantation productivity. However, further studies
582 are needed to explore, how soil microbes mediate plant-plant interactions in different
583 ecosystems all around the word. Such knowledge would be crucial for revealing and
584 understanding plant-soil-microbe relationships.

585

586

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591

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824 **Table 1** Effects of plant-plant interactions and N fertilization on soil properties (two-way ANOVA).

		pH	AP	SOM	TN	NH_4^+	NO_3-	C_{Mic}	N_{Mic}	P_{Mic}
N	F	56.674	1.087	10.246	0.173	0.120	0.024	3.358	0.455	1.507
	P	**<0.000**	0.305	**0.003**	0.680	0.732	0.012	0.076	0.505	0.229
Interactions	F	8.297	0.140	26.386	48.904	11.358	7.143	12.470	1.686	0.528
	P	**0.007**	0.711	**<0.000**	**<0.000**	**0.002**	**0.012**	**0.001**	0.203	0.473
N* Interactions	F	0.002	0.301	0.119	1.460	0.003	0.936	1.103	0.153	0.153
	P	0.967	0.587	0.732	0.236	0.960	0.341	0.301	0.699	0.698

825 AP: available phosphorus, SOM: soil organic matter, TN: total nitrogen, C_{Mic}: soil microbial biomass, N_{Mic}: soil carbon nitrogen biomass and

826 P_{Mic}: soil carbon phosphorus biomass.

Table 2 Summaries of PERMANOVA tests with 999 permutations based on Bray-Curtis distances.

Taxonomy		Tree species or interactions		N treatment	
	Experiment	PERMANOVA	Betadisper	PERMANOVA	Betadisper
Bacteria	**Single-plant**	ns	ns	ns	ns
	Double-plant	*	***	**	ns
	KO vs KK	**	ns	ns	ns
	KK vs OO	**	ns	ns	ns
	KO vs OO	ns	ns	ns	ns
	Plantations	***	ns	–	–
	KO vs KK	*	ns	–	–
	KK vs OO	**	ns	–	–
	KO vs OO	ns	ns	–	–
Fungi	**Single-plant**	*	ns	ns	ns
	Double-plant	**	ns	***	ns
	KO vs KK	*	ns	ns	ns
	KK vs OO	**	ns	ns	ns
	KO vs OO	ns	ns	ns	ns
	Plantations	***	ns	–	–
	KO vs KK	**	ns	–	–
	KK vs OO	**	ns	–	–
	KO vs OO	**	ns	–	–

When a plant was grown in isolation, we analyzed the effect of species, and when grown under intra- or inter-specific interactions, we analyzed the effect of plant-plant interactions. KK and OO refer to intra-specific interactions of *Larix kaempferi* and *L. olgensis*, respectively. KO refers to inter-specific interactions of the two species. In the plantations, KK and OO refer to monoculture plantations of *Larix kaempferi* and *L. olgensis*, respectively. KO refers to a mixed plantation of the two species.

843 **Figure legends**

844

845 **Figure 1** Neighbor effect index (NEI) of *L. kaempferi* and *L. olgensis* in different
846 plant-plant interactions. KK and OO refer to *L. kaempferi* and *L. olgensis* in
847 intra-specific plant-plant interactions, respectively. K/KO and O/KO refer to *L.*
848 *kaempferi* and *L. olgensis* in inter-specific plant-plant interactions, respectively. N-
849 and N+ refer to soil without and with N fertilization, respectively. Negative NEI
850 values indicate that a plant suffers negative impacts from its neighbor. Tukey's HSD
851 tests are conducted for multiple comparisons.

852

853 **Figure 2** Taxonomic composition of bacterial communities in the rhizosphere soil at
854 the phylum level under N fertilization. (a) Rhizosphere soil sampled from single-plant
855 pots (without plant-plant interactions); (b) rhizosphere soil sampled from pots with
856 two plants, where KK, KO and OO refer to *L. kaempferi* + *L. kaempferi*, *L. kaempferi*
857 + *L. olgensis* and *L. olgensis* + *L. olgensis*, respectively. N- and N+ refer to pots
858 without and with N fertilization, respectively. For bacteria, only phyla with relative
859 abundance over 1% are shown.

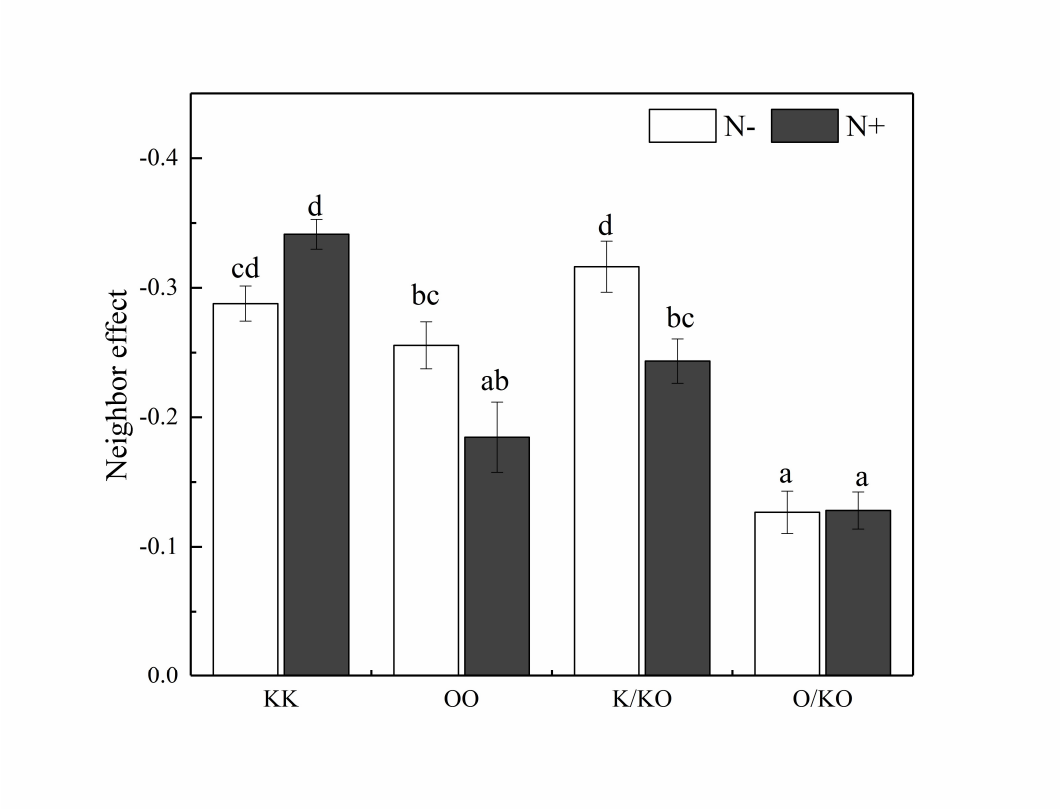
860

861 **Figure 3** Taxonomic composition of fungal communities in the rhizosphere soil at the
862 phylum level under N fertilization. (a) Rhizosphere soil sampled from single-plant
863 pots (without plant-plant interactions); (b) rhizosphere soil sampled from pots with
864 two plants, where KK, KO and OO refer to *L. kaempferi* + *L. kaempferi*, *L. kaempferi*

865 + *L. olgensis* and *L. olgensis* + *L. olgensis*, respectively. N- and N+ refer to pots
866 without and with N fertilization, respectively. For fungi, only phyla with relative
867 abundance over 1% are shown.
868
869 **Figure 4** Effects of different plant-plant interactions on the alpha diversity (Shannon
870 index) of rhizosphere soil bacteria under N fertilization. (a) Rhizosphere soil sampled
871 from single-plant pots (without plant-plant interactions); (b) rhizosphere soil sampled
872 from pots with two plants (intra- or inter-specific interactions). KK, KO and OO refer
873 to *L. kaempferi* + *L. kaempferi*, *L. kaempferi* + *L. olgensis* and *L. olgensis* + *L.*
874 *olgensis*, respectively. N- and N+ refer to pots without and with N fertilization in the
875 pot experiment, respectively. Tukey's HSD tests were conducted for multiple
876 comparisons.
877
878 **Figure 5** Effects of different plant-plant interactions on the alpha diversity (Shannon
879 index) of rhizosphere soil fungi under N fertilization. (a) Rhizosphere soil sampled
880 from single-plant pots (without plant-plant competition); (b) rhizosphere soil sampled
881 from pots with two plants (intra- or inter-specific plant interactions). KK, KO and OO
882 refer to *L. kaempferi* + *L. kaempferi*, *L. kaempferi* + *L. olgensis* and *L. olgensis* + *L.*
883 *olgensis*, respectively. N- and N+ refer to pot experiment without and with N
884 fertilization in the pot experiment, respectively. Tukey's HSD tests were conducted
885 for multiple comparisons.
886

887 **Figure 6** Bacterial (a and c) and fungal (b and d) taxa with different abundance
888 changes in single-plant pots between N-treated (N+) and control (N-) soil, irrespective
889 of plant species (a and b, Class: N treatment; Subclass: Species) and ii) between plant
890 species, irrespective of N treatment (a and b, Class: Species; Subclass: N treatment),
891 as detected by LEfSe analysis. The taxa with the absolute LDA scores over 3 and *P*
892 values less than 0.05 are shown.
893
894 **Figure 7** Bacterial (a) and fungal (b) taxa with different abundance changes in
895 two-plant pots between N-treated (N+) and control (N-) soil, irrespective of
896 plant-plant interactions (Class: N treatment; Subclass: Plant-plant interactions), as
897 detected by LEfSe analysis. The taxa with the absolute LDA scores over 3 and *P*
898 values less than 0.05 are shown.
899
900 **Figure 8** Bacterial (a) and fungal (b) taxa with different abundance changes in
901 two-plant pots between intra- and interspecific plant interactions, irrespective of N
902 treatment (Class: Plant-plant interactions, Subclass: N treatment), as detected by
903 LEfSe analysis. The taxa with the absolute LDA scores over 3 and *P* values less than
904 0.05 are shown. KK, KO and OO refer to plant-plant interactions *L. kaempferi* + *L.*
905 *kaempferi*, *L. kaempferi* + *L. olgensis* and *L. olgensis* + *L. olgensis*, respectively.
906
907
908

909 **Figure 1**



910

911

Figure 2

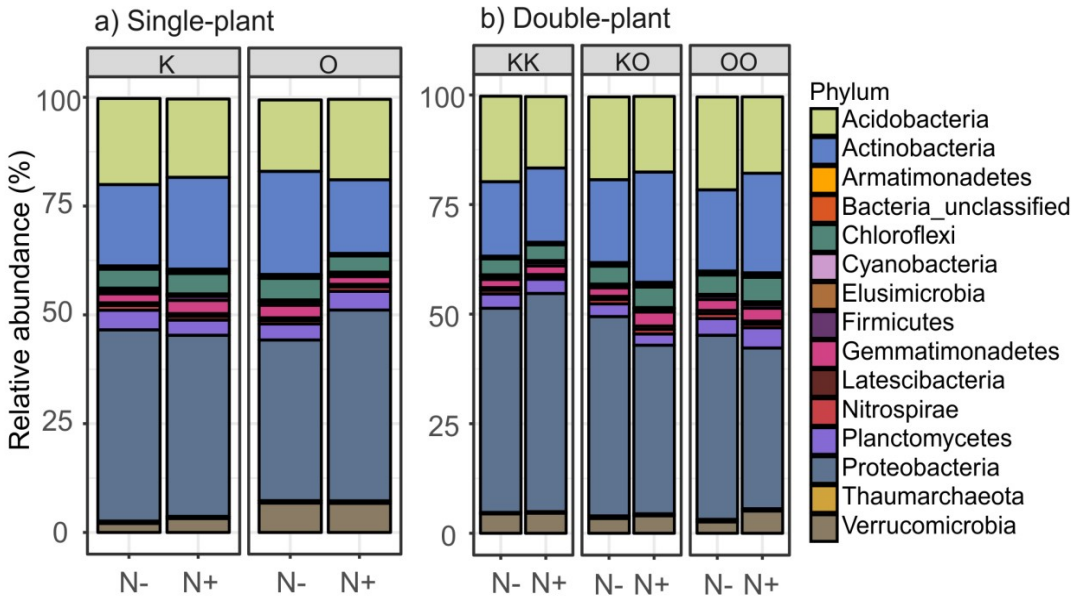

a) Single-plant
b) Double-plant
K
O
KK
KO
OO
Relative abundance (%)
100
75
50
25
0
N-
N+
Phylum
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteria_unclassified
Chloroflexi
Cyanobacteria
Elusimicrobia
Firmicutes
Gemmatimonadetes
Latescibacteria
Nitrospirae
Planctomycetes
Proteobacteria
Thaumarchaeota
Verrucomicrobia

Figure 3

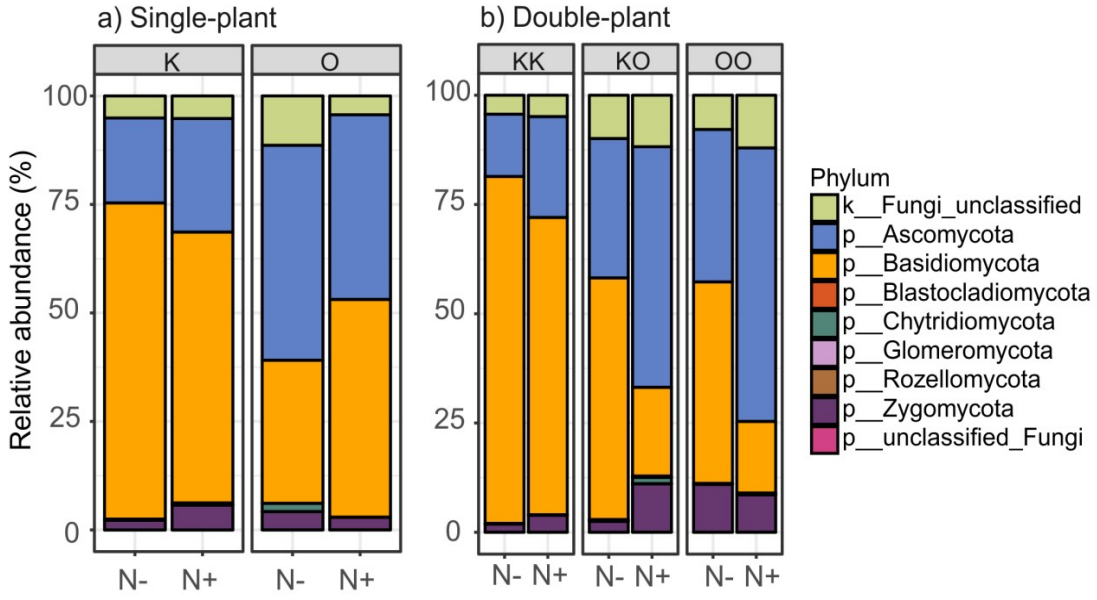
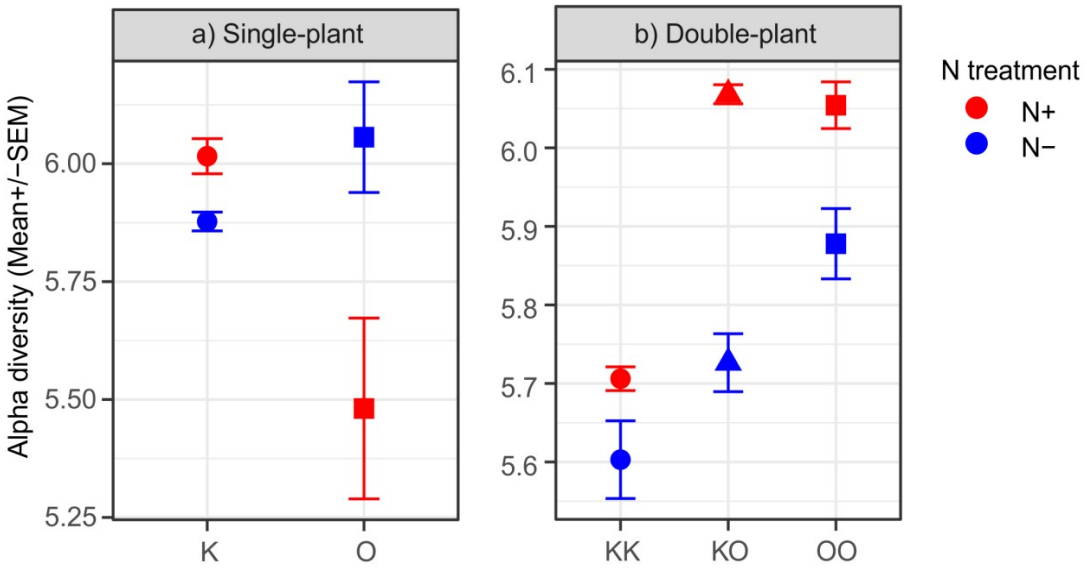
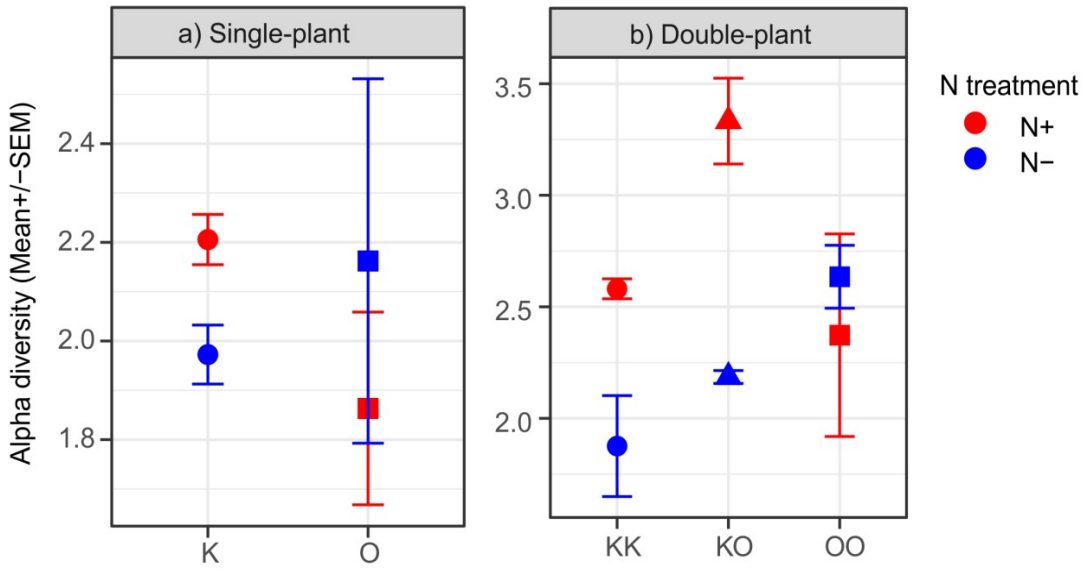

a) Single-plant
b) Double-plant
K
O
KK
KO
OO
Relative abundance (%)
100
75
50
25
0
N-
N+
Phylum
k__Fungi_unclassified
p__Ascomycota
p__Basidiomycota
p__Blastocladiomycota
p__Chytridiomycota
p__Glomeromycota
p__Rozellomycota
p__Zygomycota
p__unclassified_Fungi

Figure 4


a) Single-plant
b) Double-plant
Alpha diversity (Mean+/−SEM)
6.00
5.75
5.50
5.25
6.1
6.0
5.9
5.8
5.7
5.6
K
O
KK
KO
OO
N treatment
N+
N−

960 **Figure 5**



961

962 **Figure 6**

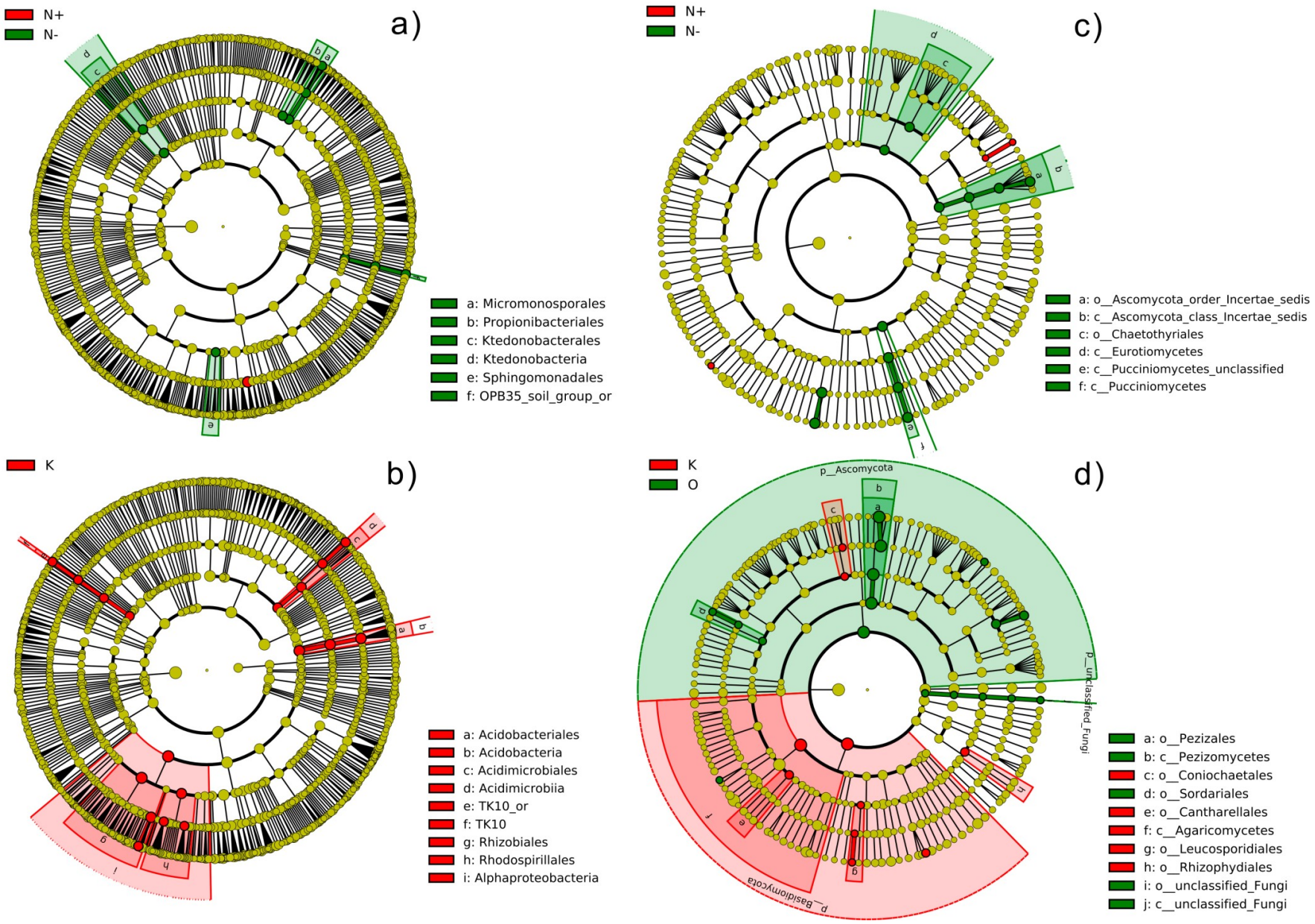
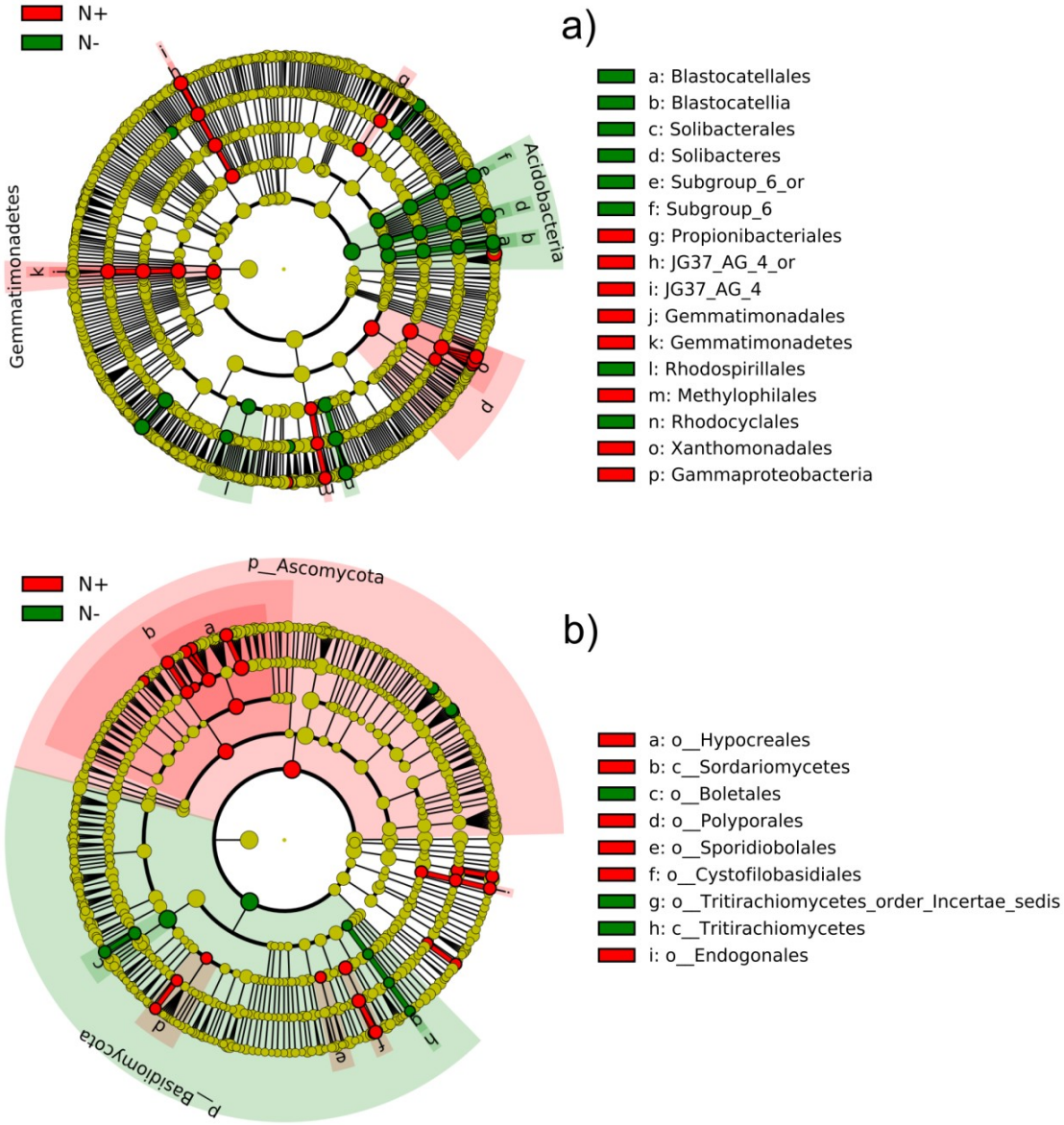
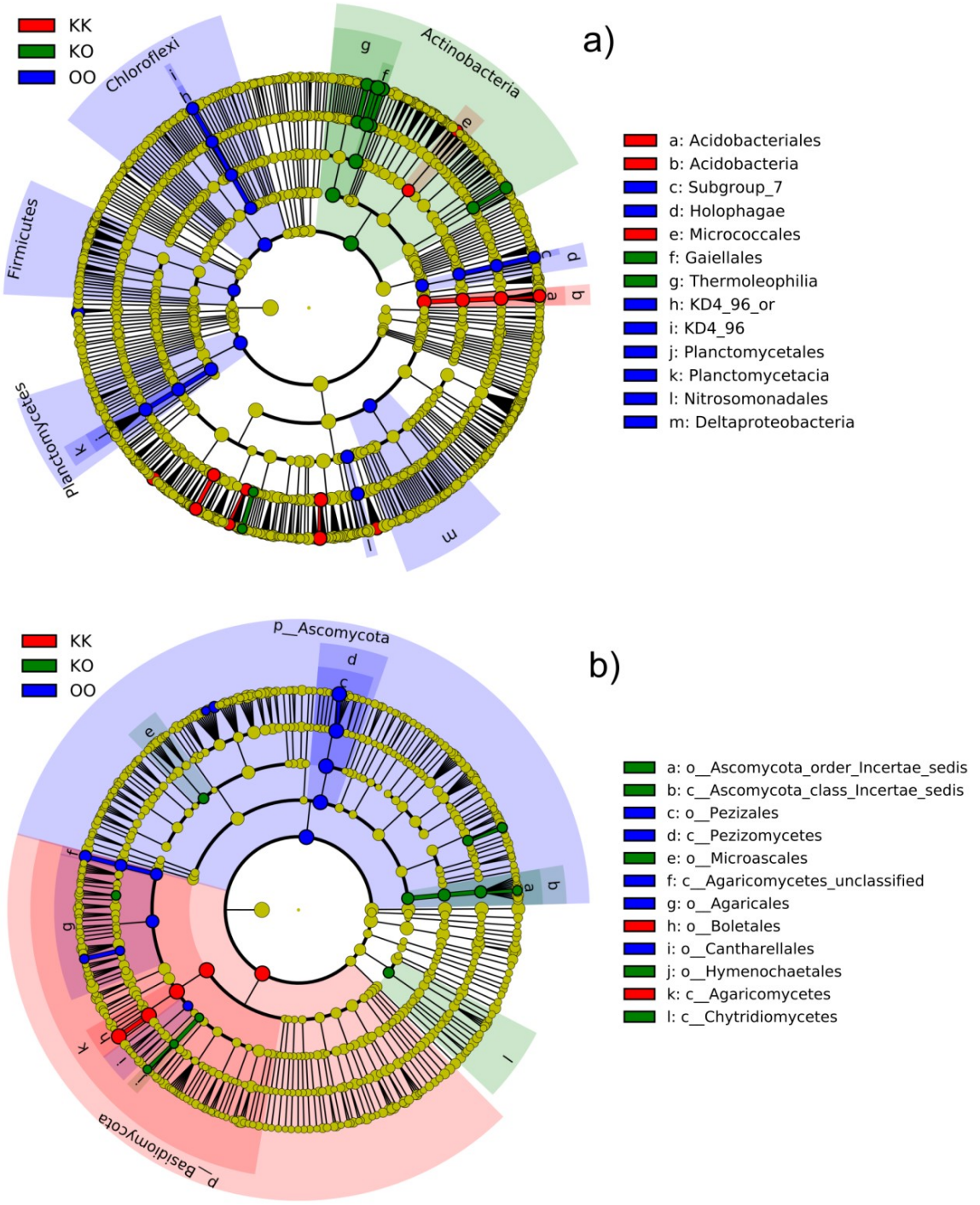

a)
N+
N-
a: Micromonosporales
b: Propionibacteriales
c: Ktedonobacterales
d: Ktedonobacteria
e: Sphingomonadales
f: OPB35_soil_group_or
b)
K
a: Acidobacteriales
b: Acidobacteria
c: Acidimicrobiales
d: Acidimicrobiia
e: TK10_or
f: TK10
g: Rhizobiales
h: Rhodospirillales
i: Alphaproteobacteria
c)
N+
N-
a: o__Ascomycota_order_Incertae_sedis
b: c__Ascomycota_class_Incertae_sedis
c: o__Chaetothyriales
d: c__Eurotiomycetes
e: c__Pucciniomycetes_unclassified
f: c__Pucciniomycetes
d)
K
O
p__Ascomycota
p__Basidiomycota
p__unclassified_Fungi
a: o__Pezizales
b: c__Pezizomycetes
c: o__Coniochaetales
d: o__Sordariales
e: o__Cantharellales
f: c__Agaricomycetes
g: o__Leucosporidiales
h: o__Rhizophydiales
i: o__unclassified_Fungi
j: c__unclassified_Fungi

Figure 7


N+
N-
a)
Acidobacteria
Gemmatimonadetes
a: Blastocatellales
b: Blastocatellia
c: Solibacterales
d: Solibacteres
e: Subgroup_6_or
f: Subgroup_6
g: Propionibacteriales
h: JG37_AG_4_or
i: JG37_AG_4
j: Gemmatimonadales
k: Gemmatimonadetes
l: Rhodospirillales
m: Methylophilales
n: Rhodocyclales
o: Xanthomonadales
p: Gammaproteobacteria
N+
N-
b)
p__Ascomycota
p__Basidiomycota
a: o__Hypocreales
b: c__Sordariomycetes
c: o__Boletales
d: o__Polyporales
e: o__Sporidiobolales
f: o__Cystofilobasidiales
g: o__Tritirachiomycetes_order_Incertae_sedis
h: c__Tritirachiomycetes
i: o__Endogonales

990 **Figure 8**



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