Factors influencing leaf- and root-associated communities of bacteria and fungi across 33 plant orders in a grassland

Hirokazu Toju[1,2*], Hiroko Kurokawa[3] and Tanaka Kenta[4]

[1]Center for Ecological Research, Kyoto University, Otsu, Shiga 520-2133, Japan

[2]Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

[3]Forestry and Forest Products Research Institute, Matsunosato 1, Tsukuba, Ibaraki 305-8687, Japan

[4]Sugadaira Research Station, Mountain Science Center, University of Tsukuba, Ueda, Nagano 386-2204, Japan

***Correspondence:** Hirokazu Toju (toju.hirokazu.4c@kyoto-u.ac.jp).

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Running head: Leaf and root microbiomes across 33 plant orders

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Abstract.

In terrestrial ecosystems, plants interact with diverse taxonomic groups of bacteria and fungi in the phyllosphere and rhizosphere. Although recent studies based on high-throughput DNA sequencing have drastically increased our understanding of plant-associated microbiomes, we still have limited knowledge of how plant species in a species-rich community differ in their leaf and root microbiome compositions. In a cool-temperate semi-natural grassland in Japan, we compared leaf- and root-associated microbiomes across 138 plant species belonging to 33 plant orders. Based on the whole-microbiome inventory data, we analyzed how sampling season as well as the taxonomy, nativeness (native or alien), lifeform (herbaceous or woody), and mycorrhizal type of host plants could contribute to variation in microbiome compositions among co-occurring plant species. The data also allowed us to explore prokaryote and fungal lineages showing preferences for specific host characteristics. The list of microbial taxa showing significant host preferences involved those potentially having some impacts on survival, growth, or environmental resistance of host plants. Overall, this study provides a platform for understanding how plant and microbial communities are linked with each other at the ecosystem level.

Keywords: biodiversity, endophytes, host specificity, mycorrhizal fungi, plant-associated microbiomes: plant-soil feedbacks

INTRODUCTION

Plants interact with various taxonomic groups of microbes both in the phyllosphere and rhizosphere (van der Heijden et al., 1998;Berendsen et al., 2012;Bai et al., 2015;Peay et al., 2016). Diverse bacteria and yeasts, for example, are present on leaf surfaces, involved in underappreciated metabolic pathways (Mercier and Lindow, 2000;Delmotte et al., 2009;Hacquard et al., 2015). In addition to those epiphytes, a number of bacteria and filamentous fungi are known to inhabit leaf tissue, potentially playing pivotal roles in resistance of host plants against biotic and abiotic environmental stresses (Schardl and Phillips, 1997;Arnold et al., 2003). In root systems, mycorrhizal fungi provide plants with soil phosphorus and/or nitrogen, fueling hosts' growth (Parniske, 2008;Smith and Read, 2008;Tedersoo et al., 2010). Likewise, some endophytic fungal taxa have known to convert organic forms of nitrogen into inorganic forms, contributing to physiological conditions of host plants (Newsham, 2011). Moreover, endophytic bacteria and fungi associated with roots can increase disease resistance of host plants, possibly by stimulating host immune systems (Ramamoorthy et al., 2001;Pieterse et al., 2014;Hacquard et al., 2017) or by suppressing soil pathogens with antibiotic chemicals (Compant et al., 2005;Gao et al., 2010). Thus, understanding of the compositions of plant microbiomes is a prerequisite for understanding the physiology and ecology of plants in terrestrial ecosystems (van der Heijden et al., 2008;Schlaeppi and Bulgarelli, 2015;Toju et al., 2018a).

While exploration of plant microbiomes has been accelerated since the emergence of high-throughput DNA sequencing (Öpik et al., 2009;Lundberg et al., 2012;Bai et al., 2015), we still have limited knowledge of how diverse plant species co-occurring in a grassland or forest ecosystem can differ in their microbiome compositions (Toju et al., 2016a). Moreover, most plant microbiome studies target only bacteria or fungi [but see (Agler et al., 2016)) in either above- or below-ground systems [but see (Bai et al., 2015;Wagner et al., 2016)], precluding comprehensive understanding of microbiome compositions. Given that bacteria and fungi can interact with each other within hosts (Frey-Klett et al., 2007;Hoffman and Arnold, 2010) and that above- and below-ground ecological processes can be interlinked (Bever et al., 2010;Mangan et al., 2010;Van der Putten et al., 2013), the targets of plant

73 microbiome studies need to be expanded towards a better understanding of the processes that
74 organize plant and microbial communities in the wild. Studies comparing microbiome
75 compositions across tens (or more) of plant species co-occurring in natural ecosystems (Toju
76 et al., 2014;Toju et al., 2018b), in particular, will allow us to examine what kinds of host
77 properties can contribute to the organization of leaf- and root-associated microbial
78 communities.

79 In this study, we sampled leaves and roots of 138 plant species representing 112 genera,
80 55 families, and 33 orders in a cool-temperate grassland in Japan, thereby performing a high-
81 throughput sequencing analysis of both prokaryote and fungal communities associated with
82 plants. The sample set of diverse plant species allowed us to examine what host properties can
83 contribute to variation in leaf and root microbiome compositions in an ecosystem.
84 Furthermore, we statistically tested how each prokaryote or fungal genus showed preferences
85 for seasons as well as preference for nativeness (native or alien), lifeform (herbaceous or
86 woody), and mycorrhizal type (ectomycorrhizal, arbuscular mycorrhizal, non-mycorrhizal, or
87 variable mycorrhizal) of host plants. Overall, this study, for the first time, shows how more
88 than 100 plant species in a single ecosystem can differ in their leaf and root microbiome
89 compositions depending on their characteristics. The statistical results on plant–microbe
90 associations shed light on underappreciated diversity of host–symbiont associations in
91 grasslands, providing fundamental information for conserving and restoring terrestrial
92 ecosystems.

93

94 # MATERIALS AND METHODS

95 ## Sampling

96 Fieldwork was conducted in Sugadaira Research Station, Mountain Science Center,
97 University of Tsukuba, Sugadaira, Ueda, Nagano Prefecture, Japan (36.524 ºN; 138.349 ºE;
98 1340 m asl). In Sugadaira Research Station, 6 ha of a semi-natural grassland has been
99 maintained by mowing plants in autumn and thereby preventing the community succession to
100 a forest. Thus, woody plant species that occurred in the grassland are shrubs or saplings of tall

trees colonized from surrounding forests. In total, 200 plant species have been reported from the grassland, including some alien species.

In the grassland, both native and alien plant species were sampled to reveal the compositions of prokaryote and fungal communities associated with leaves and roots through summer and autumn (July 19-20, August 16-18, and September 7-8) in 2017. We targeted only non-reproductive plant individuals that had neither flower buds, flowers, nor fruits so that plant physiology and chemistry would not be affected by reproduction. We tried to sample as many plant species as possible within the sampling days in each month. Note that root systems of multiple plant species were tangled with each other at the study site due to the dominance of perennial plants. Therefore, we sampled 1–8 liters of soil including root systems for each target plant individuals and quickly washed the root system in a nearby laboratory to carefully trace root tips directly connected to above-ground tissue of the target plant. A 1-cm^2 disc of a mature leaf and a 2-cm segment of a terminal root were collected from each plant sample and preserved at -20 ºC until DNA extraction. After the sampling, remaining plant organs of rare plant species were replaced at the original sampling positions. In total, 289 plant individuals representing 138 plant species (112 genera, 55 families, 33 orders) were collected (Supplementary Data 1). The permission of sampling was issued by Sugadaira Research Station, Mountain Science Center, University of Tsukuba.

DNA Extraction, PCR, and Sequencing

Each leaf or root sample was surface-sterilized by immersing it in ×1/100 NaClO (Nacalai Tesque) for 1 min and it was subsequently washed in ethanol twice. DNA extraction was extracted with a cetyltrimethylammonium bromide (CTAB) method after pulverizing the roots with 4 mm zirconium balls at 25 Hz for 3 min using a TissueLyser II (Qiagen) (Toju et al., 2013).

For each of the leaf and root samples, the 16S rRNA V4 region of the prokaryotes and the internal transcribed spacer 1 (ITS1) region of fungi were amplified. The PCR of the 16S rRNA region was performed with the forward primer 515f (Caporaso et al., 2011) fused with

3–6-mer Ns for improved Illumina sequencing quality (Lundberg et al., 2013) and the forward Illumina sequencing primer (5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG- [3–6-mer Ns] – [515f] -3') and the reverse primer 806rB (Apprill et al., 2015) fused with 3–6-mer Ns and the reverse sequencing primer (5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G [3–6-mer Ns] - [806rB] -3') (0.2 μM each). To prevent the amplification of mitochondrial and chloroplast 16S rRNA sequences of host plants, specific peptide nucleic acids [mPNA and pPNA; Lundberg et al. (2013)] (0.7 μM each) were added to the reaction mix of KOD FX Neo (Toyobo). The temperature profile of the PCR was 94 ºC for 2 min, followed by 35 cycles at 98 ºC (denaturation) for 10 s, 78 ºC (annealing of PNA) for 10 s, 60 ºC (annealing of primers) for 30 s, and 68 ºC (extension) for 50 s, and a final extension at 68 ºC for 5 min. To prevent generation of chimeric sequences, the ramp rate through the thermal cycles was set to 1 ºC/sec (Stevens et al., 2013). Illumina sequencing adaptors were then added to respective samples in the supplemental PCR using the forward fusion primers consisting of the P5 Illumina adaptor, 8-mer indexes for sample identification (Hamady et al., 2008) and a partial sequence of the sequencing primer (5'- AAT GAT ACG GCG ACC ACC GAG ATC TAC AC - [8-mer index] - TCG TCG GCA GCG TC -3') and the reverse fusion primers consisting of the P7 adaptor, 8-mer indexes, and a partial sequence of the sequencing primer (5'- CAA GCA GAA GAC GGC ATA CGA GAT - [8-mer index] - GTC TCG TGG GCT CGG -3'). KOD FX Neo was used with a temperature profile of 94 ºC for 2 min, followed by 8 cycles at 98 ºC for 10 s, 55 ºC for 30 s, and 68 ºC for 50 s (ramp rate = 1 ºC/s), and a final extension at 68 ºC for 5 min. The PCR amplicons of the samples were then pooled after a purification/equalization process with the AMPureXP Kit (Beckman Coulter). Primer dimers, which were shorter than 200 bp, were removed from the pooled library by supplemental purification with AMpureXP: the ratio of AMPureXP reagent to the pooled library was set to 0.6 (v/v) in this process.

The PCR of fungal ITS1 region was performed with the forward primer ITS1F_KYO1 (Toju et al., 2012) fused with 3–6-mer Ns for improved Illumina sequencing quality (Lundberg et al., 2013) and the forward Illumina sequencing primer (5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG- [3–6-mer Ns] – [ITS1F_KYO1] -3') and the

158 reverse primer ITS2_KYO2 (Toju et al., 2012) fused with 3–6-mer Ns and the reverse
159 sequencing primer (5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G [3–6-
160 mer Ns] - [ITS2_KYO2] -3'). The buffer and polymerase system of KOD FX Neo was used
161 with a temperature profile of 94 ºC for 2 min, followed by 35 cycles at 98 ºC for 10 s, 58 ºC
162 for 30 s, and 68 ºC for 50 s, and a final extension at 68 ºC for 5 min. Illumina sequencing
163 adaptors and 8-mer index sequences were then added in the second PCR as described above.
164 The amplicons were purified and pooled as described above.

165 The sequencing libraries of the prokaryote 16S and fungal ITS regions were processed in
166 an Illumina MiSeq sequencer (run center: KYOTO-HE; 15% PhiX spike-in). Because the
167 quality of forward sequences is generally higher than that of reverse sequences in Illumina
168 sequencing, we optimized the MiSeq run setting in order to use only forward sequences.
169 Specifically, the run length was set 271 forward (R1) and 31 reverse (R4) cycles to enhance
170 forward sequencing data: the reverse sequences were used only for discriminating between
171 16S and ITS1 sequences based on the sequences of primer positions.

172

173 **Bioinformatics**

174 The raw sequencing data were converted into FASTQ files using the program bcl2fastq 1.8.4
175 distributed by Illumina. The output FASTQ files were demultiplexed with the program
176 Claident v0.2. 2018.05.29 (Tanabe and Toju, 2013;Tanabe, 2018), by which sequencing reads
177 whose 8-mer index positions included nucleotides with low (< 30) quality scores were
178 removed. Only forward sequences were used in the following analyses after removing low-
179 quality 3'-ends using Claident. Noisy reads (Tanabe, 2018) were subsequently discarded and
180 then denoised dataset consisting of 2,973,811 16S and 2,774,197 ITS1 reads were obtained.
181 The sequencing data were deposited to DNA Data Bank of Japan (DDBJ) (DRA007062).

182 For each dataset of 16S and ITS1 regions, filtered reads were clustered with a cut-off
183 sequencing similarity of 97% using the program VSEARCH (Rognes et al., 2014) as
184 implemented in Claident. The operational taxonomic units (OTUs) representing less than 10
185 sequencing reads were subsequently discarded (Supplementary Data 2). The molecular

identification of the remaining OTUs was performed based on the combination of the query-centric auto-*k*-nearest neighbor (QCauto) method (Tanabe and Toju, 2013) and the lowest common ancestor (LCA) algorithm (Huson et al., 2007) as implemented in Claident (Supplementary Data 2). Note that taxonomic identification results based on the combination of the QCauto search and the LCA taxonomic assignment are comparable to, or sometimes more accurate than, those with alternative approaches (Tanabe and Toju, 2013;Toju et al., 2016a;Toju et al., 2016b).

For each combination of target region (16S or ITS1) and sample type (root or soil), we obtained a sample × OTU matrix, in which a cell entry depicted the number of sequencing reads of an OTU in a sample (Supplementary Data 3). The cell entries whose read counts represented less than 0.1% of the total read count of each sample were removed to minimize effects of PCR/sequencing errors (Peay et al., 2015). The filtered matrix was then rarefied to 500 reads per sample using the "rrarefy" function of the vegan 2.5-2 package (Oksanen et al., 2012) of R 3.5.1 (R-Core-Team, 2018). Samples with less than 500 reads were discarded in this process: the numbers of OTUs in the rarefied sample × OTU matrices were 1,470, 5,638, 1,537, and 3,367 for leaf prokaryote, root prokaryote, leaf fungal, and root fungal datasets, respectively (Supplementary Data 4). For each dataset, we also obtained order- and genus-level matrices, which represented order- and genus-level taxonomic compositions of microbes (prokaryotes or fungi), respectively (Supplementary Data 5).

Prokaryote and Fungal Diversity

Relationships between the number of sequencing reads and that of detected OTUs were examined for respective data matrices (leaf prokaryote, root prokaryote, leaf fungal, and root fungal datasets) with the "rarecurve" function of the R vegan package. Likewise, relationships between the number of samples and that of prokaryote/fungal orders or genera were examined with the vegan "specaccum" function. The order-level taxonomic compositions of leaf prokaryotes, root prokaryotes, leaf fungi, and root fungi were visualized in bar graphs for respective plant orders.

214

215 **Factors Contributing to Microbiome Compositions**

216 For each dataset (leaf prokaryote, root prokaryote, leaf fungal, or root fungal dataset), factors
217 contributing to microbial community compositions were examined with the permutational
218 analysis of variance [PERMANOVA; Anderson (2001)] using the vegan "adonis" function
219 (10,000 permutations). Sampling month (July, August, or September) and four variables
220 representing host plant properties were included as explanatory variables. Specifically, order-
221 level plant taxonomy, plant nativeness (native or alien), plant lifeform (herbaceous or woody),
222 and plant mycorrhizal type [ectomycorrhizal (EcM), arbuscular mycorrhizal (AM), non-
223 mycorrhizal (NM), or variable mycorrhizal (NM-AM)] (Brundrett, 2009) were included as
224 variables representing host properties. In each model, a matrix representing order- or genus-
225 level taxonomic compositions of prokaryotes/fungi was used as the input response matrix.
226 The "Bray-Curtis" metric of β-diversity was used in the PERMANOVA analyses.

227

228 **Randomization Analyses of Preferences**

229 To explore prokaryote/fungal genera that preferentially occurred on plant samples with
230 specific properties, a series of randomization tests were performed. In each genus-level matrix
231 (leaf prokaryote, root prokaryote, leaf fungal, or root fungal genus-level matrix), sample
232 information was shuffled among plant samples (100,000 permutations) and then preference of
233 a prokaryote/fungal genus (i) for a sample property (j) was evaluated as follows:

234 $$Preference\ (i, j) = [N_{\text{observed}}\ (i, j) - \text{Mean}\ (N_{\text{ranodomized}}\ (i, j))] \,/\, \text{SD}\ (N_{\text{ranodomized}}\ (i, j)),$$

235 where $N_{\text{observed}}\ (i, j)$ denoted the mean number of the sequencing reads of genus i among
236 property j samples in the original data, and the Mean ($N_{\text{ranodomized}}\ (i, j)$) and SD ($N_{\text{ranodomized}}\ (i,$
237 $j)$) were the mean and standard deviation of the number of sequencing reads for the focal
238 genus–sample property combination across randomized matrices. Based on the index,
239 preferences for sampling month (July, August, or September), plant nativeness (native or
240 alien), plant lifeform (herbaceous or woody), and plant mycorrhizal type were examined,

241 respectively. Because most plant orders included a few plant species in our datasets, the
242 preference analysis was not applied to plant taxonomy. Regarding this standardized
243 preference index, values larger than three generally represent strong preferences [false
244 discovery rate (FDR) < 0.05; Toju et al. (2016a)]: hence, we listed genera whose preference
245 values exceeded three.

246

247 # RESULTS

248 ## Prokaryote and Fungal Diversity

249 After a series of quality filtering and rarefaction procedures, 41.1 (SD = 22.1), 143.4 (SD =
250 37.9), 54.5 (SD = 18.8), and 46.0 (SD = 22.5) OTUs per sample, on average, were detected,
251 from the leaf prokaryote, root prokaryote, leaf fungal, and root fungal datasets, respectively
252 (Supplementary Fig. 1). The numbers of prokaryote orders and genera were higher in root
253 samples than in leaf samples, while those of fungal orders and genera showed opposite
254 patterns (Fig. 2).

255 The leaf prokaryote communities of the examined plants were dominated by the order
256 Rhizobiales, while diverse bacterial taxa constituted the root prokaryote communities (Fig.
257 3A-B). In the leaf fungal communities, the order Capnodiales were the most abundant, while
258 root fungal community compositions varied considerably among host plant orders (Fig. 3C-
259 D).

260

261 ## Factors Contributing to Microbiome Compositions

262 In the PERMANOVA, sampling month had significant effects on the leaf prokaryote, root
263 prokaryote, and leaf fungal community compositions but not on the root fungal community
264 structure (Table 1; Supplementary Table 1). Meanwhile, order-level host taxonomy influenced
265 the root prokaryote, leaf fungal, and the root fungal community compositions but not the leaf
266 prokaryote community structure (Table 1). The nativeness of host plants (native or alien) had

267 significant impacts on the root prokaryote and the root fungal (genus-level) community
268 compositions (Table 1). The analysis also showed that host plant lifeform (herbaceous or
269 woody) had significant effects on the leaf fungal community structure (Table 1).

270

271 **Randomization Analyses of Preferences**

272 In the randomization analyses, the relative abundances of four bacterial and eight fungal
273 genera changed through the sampling months (Table 2). For example, the fungal genera
274 *Leucosporidium*, *Taphrina*, and *Dioszegia* in the leaf fungal community appeared
275 preferentially in July, while the bacterial genera *Amnibacterium*, *Spirosoma*, and
276 *Hymenobacter* preferentially occurred in September (Table 2). Regarding the nativeness of
277 hosts, 14 bacterial and 23 fungal genera showed preferences for alien plant species (Table 3).
278 The list of bacterial genera with preferences for alien plant species included *Deinococcus*,
279 *Dermacoccus*, *Rubrobacter*, *Brevundimonas*, *Paraburkholderia*, and *Virgisporangium*, while
280 that of fungal genera showing preferences for alien plants involved *Phoma*, *Hymenoscyphus*,
281 *Sakaguchia*, *Didymella*, *Curvularia*, *Cylindrocarpon*, and *Meliniomyces* (Table 3). In contrast,
282 two bacterial genera, *Actinoallomurus* and *Singulisphaera* showed preferences for native
283 plant species (Table 3). The randomization analyses also indicated that two bacterial (*Massilia*
284 and *Steroidobacter*) and four fungal (*Veronaea*, *Lophiostoma*, *Agrocybe*, and *Leptodontidium*)
285 genera occurred preferentially on woody plant species (Supplementary Table 2). Although
286 mycorrhizal type of host plants did not have significant effects in the community-level
287 statistical analysis (Table 1), a number of bacterial and fungal genera showed preferences for
288 host mycorrhizal type (Table 4; Supplementary Table 3). For example, the bacterial genera
289 *Ferrimicrobium*, *Kineococcus*, *Sandarakinorhabdus*, and *Microthrix* showed preferences for
290 non-mycorrhizal plant species, while *Flavisolibacter*, *Neochlamydia*, and *Phenylobacterium*
291 showed preferences for ectomycorrhizal plants (Table 4). Fungi in the genera *Colletotrichum*,
292 *Entorrhiza*, *Mycoarthris*, and *Sugiyamaella*, for instance, occurred preferentially on non-
293 mycorrhizal plants, while not only ectomycorrhizal fungal genera (*Laccaria* and *Tomentella*)
294 but also potentially endophytic fungal genera such as *Phialocephala* and *Oidiodendron*

295 appeared preferentially on ectomycorrhizal plant species (Table 4; Supplementary Table 3).
296 Bacteria and fungi with preferences for arbuscular mycorrhizal plants were not detected in the
297 present randomization analyses presumably due to the dominance of arbuscular mycorrhizal
298 plants within the datasets (see Discussion).

299

300 **DISCUSSION**

301 Based on a high-throughput sequencing dataset, we herein compared leaf and root
302 microbiome compositions across co-occurring plant species in a temperate grassland. By
303 targeting one of the most plant-species-rich ecosystems in the cool-temperate climate, we
304 compared leaf- and root-associated microbial communities across 33 plant orders (Fig. 3) and
305 then performed a series of statistical analyses on factors that may influence community
306 compositions of plant-associated microbes (Tables 1–4). Hereafter, we discuss potential
307 contributions of the factors examined, focusing on preferences of each microbial taxon for
308 host characteristics.

309 An interesting finding of this study is that, while the compositions of leaf prokaryote, root
310 prokaryote, and leaf fungal communities changed through the sampling months, root fungal
311 community compositions did not significantly shift during the period (Tables 1–2). This
312 pattern possibly represents difference in basic environmental features between above- and
313 below-ground systems and/or difference in phenological patterns between prokaryote and
314 fungal communities. For example, above-ground biotic/abiotic environments may be more
315 dynamics than below-ground environments, resulting in rapid turnover of microbial
316 communities. Moreover, above-ground parts of plants are more likely to be accessed by wind-
317 dispersed spores and inocula than below-ground parts: hence, above-ground microbiome
318 processes may be susceptible to continual immigration. In addition to potential contrasting
319 features of above- vs. below-ground systems, difference in basic ecology between bacteria
320 and fungi may have contributed to the varied phenological patterns. While mycorrhizal and
321 endophytic fungi usually persist on/around host root systems in the form of hyphal networks
322 (Lian et al., 2006;Smith and Read, 2008), bacterial communities may consist mainly of

323 opportunistic symbionts [*sensu* (Hardoim et al., 2008)], which undergo rapid population
324 growth under favorable environmental conditions and are subsequently replaced by others.
325 Year-round comparative studies on leaf and root microbiomes are awaited for gaining more
326 comprehensive understandings of microbiome dynamics.

327 Among the microbial communities examined, both root-associated prokaryote and fungal
328 communities significantly varied between native and alien plant species (Table 1). The
329 randomization analysis then allowed us to screen for bacterial and fungal genera showing
330 preferences for native or alien plants (Table 3). Among the bacterial genera showing
331 preferences for alien plants, *Paraburkholderia* has been known to include species with
332 nitrogen-fixing abilities (Dall'Agnol et al., 2016), potentially influencing host nutritional
333 conditions. In addition, the analysis showed that various genera in the phylum Actinobacteria
334 (*Rubrobacter*, *Dermacoccus*, *Actinoallomurus*, and *Virgisporangium*) showed preferences for
335 native or alien plant species. Given that many actinomycete bacteria produce chemicals
336 suppressing other microbes (Qin et al., 2011;Bérdy, 2012), their ecological roles in
337 ecosystems are of particular interest. For fungi, although the absence of OTUs displaying
338 preferences for native plants requires careful interpretation (see below), the randomization
339 analysis showed that various fungal taxa could have preferences for alien plant species (Table
340 3). Among them, *Curvularia*, *Didymella*, and *Cylindrocarpon* include well-characterized plant
341 pathogenic species (Alaniz et al., 2007;Akinbode, 2010;Keinath, 2011). In contrast, fungi in
342 the genus *Meliniomyces* have been described as mycorrhizal or endophytic fungi (Hambleton
343 and Sigler, 2005;Ohtaka and Narisawa, 2008;Vohník et al., 2013), possibly contributing to the
344 survival and growth of host plants. Overall, these results suggest that various taxonomic
345 groups of bacteria and fungi are associated with native or alien plant species, potentially
346 affecting invasiveness of alien plants both positively and negatively.

347 At the whole community level, mycorrhizal types of host plants did not have significant
348 effects on plant microbiome compositions, while effects of plant lifeform (herbaceous or
349 woody) were significant in one of the communities examined (i.e., leaf fungal community)
350 (Table 1; Supplementary Table 3). However, the randomization analysis for respective
351 microbial taxa highlighted diverse bacterial and fungal genera showing statistically significant

preferences for host mycorrhizal types (Table 4). Among the bacteria showing preferences for non-mycorrhizal plants, *Ferrimicrobium* includes species adapted to low pH conditions (Johnson et al., 2009), while the genus *Kineococcus* is known to involve species tolerant to salt stress (Bian et al., 2012). Within the fungal community, ectomycorrhizal fungi in the genera *Laccaria* and *Tomentella* showed preferences for ectomycorrhizal plant species as expected based on previous studies on mycorrhizal symbioses (Smith and Read, 2008;Tedersoo et al., 2010). We also found that possibly endophytic fungi in the genus *Phialocephala* (Fernando and Currah, 1996;Grünig et al., 2008) showed preferences for ectomycorrhizal plants. Among the fungal genera showing preferences for non-mycorrhizal plant roots, *Colletotrichum* is of particular interest. Although many *Colletotrichum* species had been known as plant pathogens (Hammerschmidt et al., 1982;O'Connell et al., 2012), recent studies have demonstrated that some species in the genus could work as mutualistic symbionts by providing soil phosphorus to non-mycorrhizal plants such as *Arabidopsis thaliana* (Hiruma et al., 2016;Hiruma et al., 2018). Thus, the list of microbes preferentially associated with non-mycorrhizal plants (Table 4; Supplementary Table 3) sheds light on potential diversity of bacteria and fungi that may partly fill niches of mycorrhizal fungi in non-mycorrhizal plant species.

Although the data collected in this study provide fundamental information of microbial diversity in a grassland ecosystem, the statistical results should be interpreted with caution. First, the small number of samples per plant species may have affected the comparison of microbiome compositions among plant taxa (Fig. 3). The identification of plant roots is time-consuming especially in species-rich grasslands consisting mainly of perennial plants with tangled root systems, limiting the throughput of sampling. Therefore, for more comprehensive profiling of plant microbiomes, we may need to increase the throughput of plant species identification based on molecular taxonomic assignment (i.e., DNA barcoding) of host plants (Hollingsworth et al., 2009;Toju et al., 2013). Second, the presence of unidentified bacteria and fungi in the dataset may have biased the statistical analyses. Although databases of microbes have been continually updated, there remain many bacterial and fungal lineages whose taxonomy has not yet been fixed. In particular, below-ground microbiomes are known

to involve a number of poorly investigated taxa, whose physiological and ecological functions remain to be uncovered (Buée et al., 2009;Fierer, 2017). Thus, with more reference microbial databases, we will be able to examine whether the patterns found in the present analysis hold after assigning unidentified OTUs to right categories. Third, there seems limitation of the randomization method used in this study. In the analysis of host plant nativeness, significant preferences for native plant species were detected only for a few microbial genera (Table 3). Likewise, in the analysis on plant mycorrhizal types, there was no microbial genus showing preferences for arbuscular mycorrhizal plants (Table 4). Given that native and arbuscular mycorrhizal plant species were dominant in the grassland [87.9 % (254/289) and 87.5 % (253/289) of samples, respectively], this kind of randomization analyses may tend to provide conservative results for major categories, while yielding sensitive results for categories with smaller number of samples. Although randomization methods require fewer statistical assumptions than model-based methods [e.g., (Sato et al., 2015)], they may be more suitable for data matrices with equal number of replicate samples across target categories.

We herein revealed how diverse bacterial and fungal taxa were associated with leaves and roots of the 138 plant species co-occurring in a cool-temperate grassland, focusing on potential contributions of host plant characteristics on microbiome compositions. Although recent ecological studies have highlighted possible feedbacks between plant and microbial community dynamics (Bever et al., 2010;Mangan et al., 2010;Van der Putten et al., 2013), we still have limited knowledge of the processes by which species-rich plant communities are maintained by phyllosphere and rhizosphere microbiomes. Accumulating comprehensive inventory data of microbiomes associated with whole plant communities is a prerequisite for advancing our understanding of ecosystem-scale processes. Case studies in various types of terrestrial ecosystems in diverse climatic regions will allow us to elucidate how plant species with different mycorrhizal types often coexist in natural ecosystems (Booth, 2004;Kadowaki et al., in press) or why some ecosystems are resistant against alien plants, while others are heavily disturbed by invasive species (Mitchell and Power, 2003;Reinhart and Callaway, 2006).

AUTHOR CONTRIBUTIONS

HT, HK, and TK designed the work. HT, HK, and TK performed fieldwork. HT conducted molecular experiment and analyzed the data. HT, HK, and TK wrote the manuscript.

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SUPPLEEMENTARY MATERIAL

The Supplementary Material for this article can be found online at XXXXX.

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642

643 **Conflict of Interest Statement:** The authors declare that the research was conducted in the
644 absence of any commercial or financial relationships that could be constructed as conflict of
645 interest.
646

647 **TABLE 1** | Factors contributing to variation in genus-level community compositions of
648 bacteria and fungi.

649

Target	Plant tissue	Taxonomic level	Variable	df	*F*.model	R^2	*P*
Prokaryotes	Leaf	Genus	Month	1	3.62	0.019	**0.0070**
			Order	31	1.37	0.218	0.0140
			Native/alien	1	1.34	0.007	0.2159
			Woody/herbaceous	1	0.93	0.005	0.4221
			Mycorrhizal type	3	1.58	0.024	0.0821
	Root	Genus	Month	1	3.35	0.011	**0.0005**
			Order	32	1.99	0.217	**0.0001**
			Native/alien	1	2.63	0.009	**0.0041**
			Woody/herbaceous	1	1.32	0.005	0.1824
			Mycorrhizal type	3	0.92	0.009	0.5768
Fungi	Leaf	Genus	Month	1	13.35	0.056	**0.0001**
			Order	30	1.63	0.205	**0.0001**
			Native/alien	1	0.95	0.004	0.4333
			Woody/herbaceous	1	5.62	0.024	**0.0001**
			Mycorrhizal type	3	1.34	0.017	0.1338
	Root	Genus	Month	1	1.39	0.005	0.1683
			Order	32	1.30	0.158	**0.0075**
			Native/alien	1	3.25	0.012	**0.0015**
			Woody/herbaceous	1	1.37	0.005	0.1826
			Mycorrhizal type	3	1.23	0.014	0.2030

650

651 A PERMANOVA was conducted for each target community (prokaryotes or fungi). The
652 explanatory variables included in the models were sampling month and four host plant
653 properties [order-level taxonomy, nativeness, lifeform (woody or herbaceous), and
654 mycorrhizal type]. *P* values significant after a Bonferroni correction are shown in bold for
655 each model ($\alpha = 0.05$). See Supplementary Table 1 for results on community compositions at
656 the order level.
657

658 **TABLE 2** | Prokaryote and fungal genera showing preferences for months.

659

Target	Plant tissue	Preferred month	Phylum	Genus	Preference score	FDR
Prokaryotes	Leaf	July	Proteobacteria	*Bradyrhizobium*	3.48	0.00948
		September	Actinobacteria	*Amnibacterium*	3.60	0.00159
		September	Bacteroidetes	*Spirosoma*	3.48	0.00211
		September	Bacteroidetes	*Hymenobacter*	3.08	0.00948
Fungi	Leaf	July	Basidiomycota	*Leucosporidium*	4.32	0.00026
		July	Ascomycota	*Taphrina*	3.93	0.00064
		July	Basidiomycota	*Dioszegia*	3.11	0.01246
		August	Ascomycota	*Bryochiton*	3.28	0.00058
		September	Ascomycota	*Nigrospora*	3.58	0.00052
		September	Ascomycota	*Paraphaeosphaeria*	3.57	0.00007
	Root	July	Basidiomycota	*Agrocybe*	3.50	0.00384
		September	Ascomycota	*Paraphaeosphaeria*	3.01	0.00686

660

661 Prokaryote and fungal genera are listed with their preference scores for specific months. The
662 *P* values obtained in the randomization analysis were converted to false discovery rates
663 (FDRs). Genera whose preference scores exceeded three are shown.
664

665 **TABLE 3** | Prokaryote and fungal genera showing preferences for native/alien plant species.

666

Target	Plant tissue	Preferred host	Phylum	Genus	Preference score	FDR
Prokaryotes	Leaf	Alien	Deinococcus.Thermus	*Deinococcus*	3.02	0.03634
		Alien	Firmicutes	*Staphylococcus*	3.24	0.01210
		Alien	Actinobacteria	*Dermacoccus*	3.60	0.02424
		Alien	Actinobacteria	*Rubrobacter*	3.70	0.00000
		Alien	Nitrospirae	*Nitrospira*	3.70	0.00000
		Alien	Bacteroidetes	*Fibrella*	3.74	0.00000
		Alien	Bacteroidetes	*Mucilaginibacter*	4.16	0.00551
		Alien	Proteobacteria	*Rhizomicrobium*	4.17	0.00075
		Alien	Proteobacteria	*Brevundimonas*	4.33	0.00558
		Alien	Proteobacteria	*Paraburkholderia*	4.88	0.00000
	Root	Native	Actinobacteria	*Actinoallomurus*	3.63	0.00000
		Native	Planctomycetes	*Singulisphaera*	3.50	0.00013
		Alien	Proteobacteria	*Rhodomicrobium*	3.00	0.00000
		Alien	Chloroflexi	*Bellilinea*	3.01	0.00000
		Alien	Tenericutes	*Mycoplasma*	3.01	0.00000
		Alien	Actinobacteria	*Virgisporangium*	3.23	0.01696
Fungi	Leaf	Alien	Basidiomycota	*Filobasidium*	3.01	0.04714
		Alien	Basidiomycota	*Cryptococcus*	3.04	0.07470
		Alien	Basidiomycota	*Simocybe*	3.04	0.01482
		Alien	Ascomycota	*Phoma*	3.16	0.04405
		Alien	Ascomycota	*Preussia*	3.24	0.01482
		Alien	Ascomycota	*Neoceratosperma*	3.24	0.01489
		Alien	Ascomycota	*Hymenoscyphus*	3.31	0.07334
		Alien	Basidiomycota	*Sakaguchia*	3.50	0.00000
		Alien	Basidiomycota	*Sampaiozyma*	3.50	0.00000
		Alien	Ascomycota	*Didymella*	3.52	0.00000
		Alien	Ascomycota	*Dichotomopilus*	3.52	0.00000
		Alien	Ascomycota	*Leohumicola*	3.56	0.00000
		Alien	Ascomycota	*Pleurotheciella*	3.56	0.00000

	Alien	Basidiomycota	*Rhodosporidiobolus*	3.73	0.00349
	Alien	Basidiomycota	*Peniophora*	3.94	0.00431
Root	Alien	Ascomycota	*Candida*	3.00	0.00000
	Alien	Ascomycota	*Curvularia*	3.00	0.00000
	Alien	Ascomycota	*Mycoleptodiscus*	3.00	0.00000
	Alien	Ascomycota	*Cylindrocarpon*	3.01	0.00000
	Alien	Ascomycota	*Microdochium*	3.01	0.00000
	Alien	Chytridiomycota	*Nowakowskiella*	3.02	0.00000
	Alien	Ascomycota	*Drechslera*	3.57	0.01482
	Alien	Ascomycota	*Meliniomyces*	4.00	0.00255

Prokaryote and fungal genera are listed with their preference scores for native/alien plant species. The *P* values obtained in the randomization analysis was converted to false discovery rates (FDRs). Genera whose preference scores exceeded three are shown.

673 **TABLE 4 |** Prokaryote and fungal genera showing preferences for mycorrhizal types of host
674 plant species.

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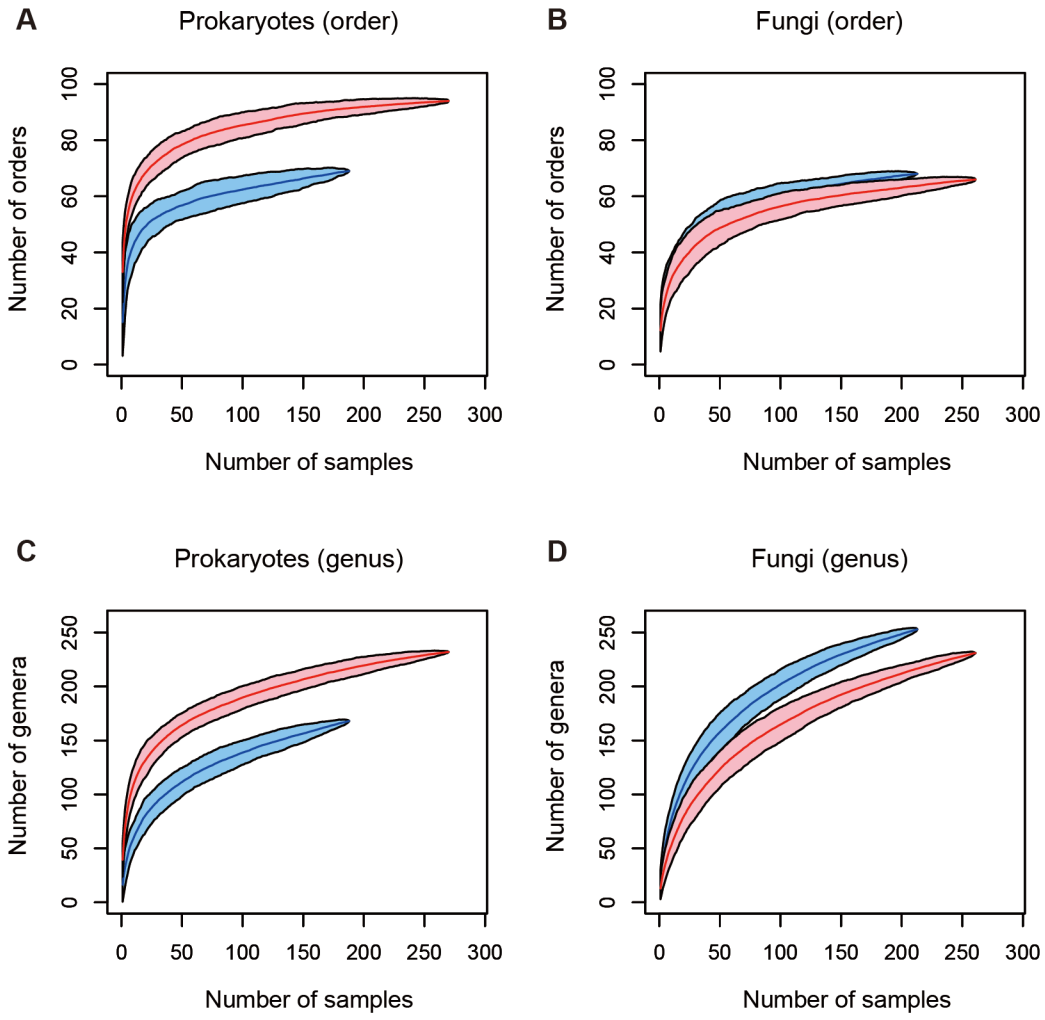
Target	Plant tissue	Preferred host	Phymum	Genus	Preference score	FDR
Prokaryotes	Leaf	NM	Actinobacteria	*Cellulomonas*	4.63	0.00000
		NM	Actinobacteria	*Pseudonocardia*	4.53	0.00343
		EcM	Chlamydiae	*Parachlamydia*	5.40	0.00000
		EcM	Bacteroidetes	*Fibrella*	5.39	0.00000
		EcM	Crenarchaeota	*Fervidicoccus*	5.21	0.00576
		EcM	Proteobacteria	*Steroidobacter*	5.04	0.04904
		EcM	Firmicutes	*Staphylococcus*	4.74	0.00628
	Root	NM-AM	Proteobacteria	*Janthinobacterium*	4.70	0.00000
		NM	Actinobacteria	*Ferrimicrobium*	5.60	0.00364
		NM	Proteobacteria	*Arenimonas*	5.51	0.00048
		NM	Proteobacteria	*Sandarakinorhabdus*	4.81	0.00000
		NM	Actinobacteria	*Kineococcus*	4.77	0.00000
		NM	Actinobacteria	*Microthrix*	4.66	0.00000
		EcM	Chlamydiae	*Neochlamydia*	5.68	0.00000
		EcM	Bacteroidetes	*Flavisolibacter*	4.94	0.00562
		EcM	Proteobacteria	*Phenylobacterium*	4.75	0.04771
Fungi	Leaf	NM-AM	Ascomycota	*Microscypha*	5.74	0.00261
		NM-AM	Ascomycota	*Spegazzinia*	4.95	0.00000
		NM-AM	Ascomycota	*Zygophiala*	4.61	0.03060
		NM	Ascomycota	*Debaryomyces*	5.71	0.00000
		NM	Ascomycota	*Exophiala*	5.65	0.00053
		NM	Ascomycota	*Striaticonidium*	4.53	0.00000
		EcM	Ascomycota	*Apodus*	4.67	0.00000
		EcM	Ascomycota	*Nemania*	4.66	0.00000
		EcM	Basidiomycota	*Pholiota*	4.66	0.00000
		EcM	Basidiomycota	*Hyphodontia*	4.66	0.00000
		EcM	Ascomycota	*Drechslera*	4.64	0.00000
		EcM	Ascomycota	*Pleurotheciella*	4.62	0.00000
		EcM	Ascomycota	*Nigrospora*	4.57	0.02153

Root	NM-AM	Ascomycota	*Stagonospora*	6.06	0.00000
	NM-AM	Ascomycota	*Dendryphion*	5.54	0.00000
	NM-AM	Ascomycota	*Minutisphaera*	5.52	0.00930
	NM-AM	Basidiomycota	*Asterostroma*	5.11	0.02706
	NM-AM	Basidiomycota	*Amphinema*	5.11	0.00570
	NM-AM	Ascomycota	*Nigrospora*	4.91	0.04418
	NM-AM	Ascomycota	*Metapochonia*	4.75	0.01422
	NM	Ascomycota	*Mycoarthris*	5.97	0.00018
	NM	Entorrhizomycota	*Entorrhiza*	4.97	0.00000
	NM	Ascomycota	*Myrothecium*	4.95	0.00000
	NM	Ascomycota	*Infundichalara*	4.92	0.00000
	NM	Ascomycota	*Pleurotheciella*	4.92	0.00000
	NM	Basidiomycota	*Curvibasidium*	4.92	0.00000
	NM	Ascomycota	*Colletotrichum*	4.87	0.03815
	NM	Ascomycota	*Sugiyamaella*	4.81	0.00000
	EcM	Ascomycota	*Leptodontidium*	9.48	0.00000
	EcM	Basidiomycota	*Laccaria*	5.47	0.00000
	EcM	Ascomycota	*Phialocephala*	5.46	0.00398
	EcM	Ascomycota	*Curvularia*	5.46	0.00000
	EcM	Ascomycota	*Mycoleptodiscus*	5.46	0.00000
	EcM	Ascomycota	*Paecilomyces*	5.37	0.01422
	EcM	Ascomycota	*Robillarda*	5.15	0.00476
	EcM	Basidiomycota	*Tomentella*	5.04	0.01568

Prokaryote and fungal genera are listed with their preference scores for mycorrhizal types of plant species. The *P* values obtained in the randomization analysis was converted to false discovery rates (FDRs). The genera whose preference scores exceeded 4.5 are shown. See Supplementary Table 3 for the list of genera whose preference scores were higher than three. EcM, ectomycorrhizal; AM, arbuscular mycorrhizal; NM, non-mycorrhizal; NM-AM, variable mycorrhizal (i.e., non-mycorrhizal or arbuscular mycorrhizal).

FIGURE 1 | Grassland of Sugadaira Research Station.



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690 **FIGURE 2 |** Relationship between the number of leaf/root samples and that of
691 prokaryote/fungal taxa observed. **(A)** Number of prokaryote orders. In total, sequencing data
692 were successfully obtained from 188 leaf and 270 root samples. Blue and red curves represent
693 leaf and root samples, respectively. **(B)** Number of fungal orders. In total, sequencing data
694 were successfully obtained from 213 leaf and 261 root samples. **(C)** Number of prokaryote
695 genera (188 leaf and 270 root samples). **(D)** Number of fungal genera (213 leaf and 261 root
696 samples).

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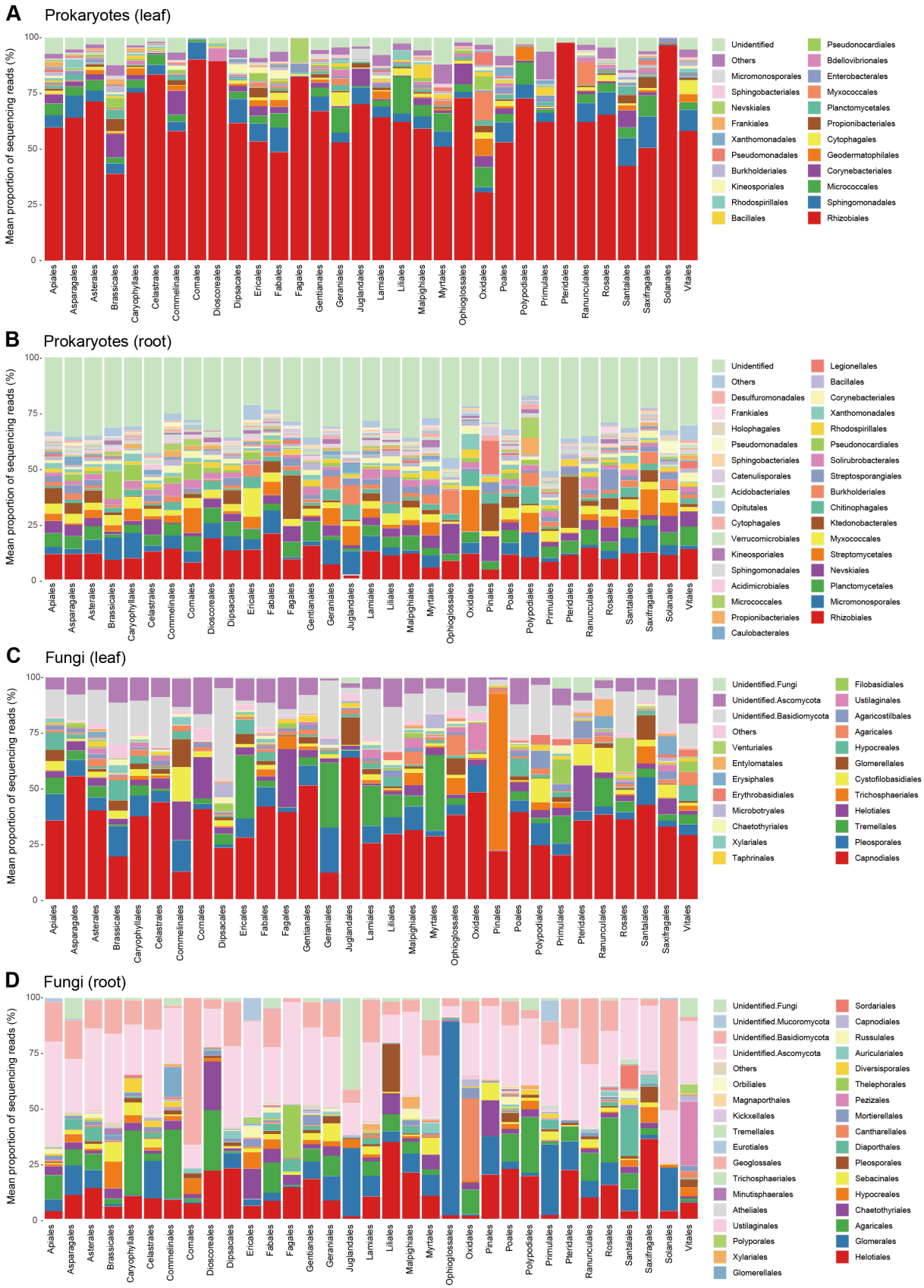

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700 **FIGURE 3 |** Prokaryote and fungal community compositions. **(A)** Order-level compositions
701 of prokaryotes in leaf samples. Mean proportions of sequencing reads are shown for each
702 plant order. In total, sequencing data were successfully obtained from 188 leaf samples. **(B)**
703 Order-level compositions of prokaryotes in root samples (270 root samples). **(C)** Order-level
704 compositions of fungi in leaf samples (213 leaf samples). Mean proportions of sequencing
705 reads are shown for each plant order. **(D)** Order-level compositions of fungi in root samples
706 (261 root samples).