1   **Tree species identity and leaf ageing alter the composition of phyllosphere communities**

2   **through changes in leaf traits**

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23   **Abstract**

24   Phyllosphere microorganisms are essential for plant growth and health, notably through their

25   action on nitrogen fixation and pathogens control. However, whether and how the composition of

26   phyllosphere communities vary with plant traits and leaf age remain still unclear. We used

27   high-throughput sequencing to explore the phyllosphere microbial diversity and composition

28   communities in needles of different ages (i.e., originating from different cohorts) for three evergreen

29   coniferous species (*Pinus koraiensis*, *Picea asperata* and *Abies fabri*). We then assessed the

30   relationships between the composition of phyllosphere microorganisms and needle traits. The results

31   showed that needle age explained relatively well the phyllosphere microbiome α diversity, whereas

32   tree species identity explained the phyllosphere microorganisms β diversity. The changes in the

33   composition of phyllosphere microbial communities between newly-formed and perennial needles

34   were greatest in *Pinus koraiensis*. Overall, Cyanobacteria and Gammaproteobacteria were dominant

35   in newly-formed needles. Plant traits such as leaf dry matter content (LDMC), leaf mass per area

36   (LMA) and total phosphorus content (TP) were the main predictors of phyllosphere community.

37   Our results provide new insights into the mechanisms of community assembly among different

38   evergreen tree species and provide a better understanding of the interactions between plant traits and

39   phyllosphere microorganisms during needle ageing.

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41   **Keywords:** community structure, diversity, evergreen coniferous species, needle age, needle traits,

42   phyllosphere microorganisms.

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Introduction

Plants can be considered as holobionts, i.e., the living plants are associated with a variety of microorganisms (Zilber-Rosenberg & Rosenberg, 2008). Although the majority of studies have investigated the role of microbes associated with roots, an increasing number of studies have highlighted that microorganisms colonizing leaves (i.e., the phyllosphere microbiome) can also play a significant role in plant growth and survival (Ruinen, 1965; Vorholt, 2012; Laforest-Lapointe et al., 2017; Leveau, 2019). Phyllosphere microorganisms can interact directly with the plant host by either impacting plant nutrition, for instance through nitrogen fixation (Lindow & Brandl, 2003; Vorholt., 2012; Bashir et al., 2021), or by influencing its adaptability to environmental changes, notably through its impact on water absorption and nutrient use efficiency (Arnold et al., 2003; Beattie, 2011; Laforest-Lapointe et al., 2016). Although there is an increasing recognition that micro-environmental conditions on leaf surface such as ultraviolet radiation, water or nutrient availability may affect microbial community composition or even prevent microbial colonization (Stone & Jackson, 2019; Herrmann et al., 2021; Zhu et al., 2022), whether and how the host plant itself affects the composition and diversity of microbial communities during leaves ageing has rarely been assessed.

Plant species and host plant species genotype are important factors influencing the phyllosphere microbiome (Yang et al., 2001; Kim et al., 2012; Lambais et al., 2006; Wei & Ashman, 2018). It has been shown that the identity of host plant species identity explained 27% of the variation in phyllosphere microbiome among five different tree species (Laforest-Lapointe et al., 2016). Such a variability in the composition of phyllosphere communities can be related to leaf traits related to morphology, chemistry, and physiology (Vacher et al., 2016 Kembel & Mueller, 2014; Kembel et al., 2014). For example, leaf mass per area (LMA) is often considered as one of the main drivers

67 influencing the composition of microbial communities, notably because LMA as part of the leaf

68 economics spectrum is closely related to photosynthetic resource utilization, with high LMA being

69 related to low nutrient exudation and resource utilization efficiency (Wright et al., 2004; Lajoie et al.,

70 2020). Leaf water content can also change the pH at the leaf surface, with further consequences on

71 the diversity and abundance of bacteria (Yadav et al., 2005). For example, low pH may inhibit the

72 growth of microorganisms and reduce microbial diversity, whereas promote the growth of specific

73 bacterial groups such as Acidobacteria (Rodrigues et al., 2013; Chen et al., 2015; Fan et al., 2018).

74 Furthermore, it has been shown that leaves characterized by a thick wax layer impedes moisture

75 seepage whereas low photosynthetic rate may significantly affect the nutrition of certain

76 microorganisms (such as fungal pathogens) in thick leaves (Arnold and Lutzoni, 2007; Würth et al.,

77 2019). Therefore, trait variation can be used to explain changes in the phyllosphere microbiome

78 among different tree species (Whipps et al., 2008; Hunter et al., 2010; Friesen et al., 2011), but

79 whether intraspecific variations in plant functional traits among individuals of the same species may

80 also significantly explain the variability in phyllosphere communities remains poorly known.

81   Intraspecific variation may account for more than 25% of the total variation in leaf functional

82 traits (Siefert et al., 2015). In evergreen plants, needles traits change with the growth of different

83 cohorts of needles that coexist along branches of the same individual (Albert et al., 2010; Kuusk et

84 al., 2018; Liu et al., 2021). The structure, chemistry and function of leaves change with increasing

85 'leaf age', with traits such as LMA, leaf dry matter content (LDMC) and wax layer increasing

86 significantly in 'perennial' leaves compared with 'newly-formed' leaves, whereas the net

87 photosynthetic rate (A) often shows an opposite trend (Field, 1983; Warren, 2006; Niinemets, 2016;

88 Liu et al., 2021). Furthermore, newly-formed leaves often present greater amount of nitrogen (TN)

89　and phosphorus (TP) as they need high amount of nutrients to synthetize proteins that promote cell

90　growth and division, which in turn increase the leaf area for photosynthesis (Radoglou & Teskey,

91　1997; Mediavilla & Escude, 2003; Yuan et al., 2018; Liu et al., 2021). As such, the differentiation in

92　ecological strategies between newly-formed and perennial leaves suggest the existence of an

93　'intraspecific economic spectrum' (Liu et al., 2021), similar to what has been shown among different

94　plant species (Wright et al., 2004; Liu et al., 2021). However, although recent studies have

95　demonstrated that microbial community composition was dependent on both leaf morphological and

96　physiological characteristics (Kembel et al., 2014), it remains unclear whether and how the diversity

97　and structure of phyllosphere communities also vary with leaf age within the same host plant.

98　　　In this study, we investigated how leaf traits influence the diversity and structure of

99　phyllosphere communities among three representative species of evergreen coniferous (*Pinus*

100　*koraiensis*, *Picea asperata* and *Abies fabri*), and how these relationships can vary with increasing

101　leaf age. Therefore, we will use the term 'needles' thereafter and throughout the document, but the

102　concept and ideas are exactly the same than those developed for broadleaved leaves. First, we

103　hypothesized that microbial diversity should increase with needle age because perennial needles

104　should select for specialized microorganisms that are able to survive in a more complex environment

105　(Williams et al., 2013), whereas new needles should stimulate the dominance of a few taxa that are

106　able to compete for high quality resources (H_1) (Würth et al., 2019). Second, we tested the

107　hypothesis that the effect of needle age on the diversity and structure of phyllosphere communities

108　should be stronger for the relatively fast-growing *Pinus koraiensis* species, because the difference

109　between new and perennial needles should be greater compared with slow-growing species as *Picea*

110　*asperata* and *Abies fabri* (H_2) (Kuusk et al., 2018). Finally, we hypothesized that plant traits such as

111 LMA, LDMC and TN, TP should predict the relative proportion of copiotrophic *versus* oligotrophic

112 organisms because these traits should influence nutrient use efficiency and microbial strategies (H$_3$)

113 (Fanin et al., 2014).

114

115 **Materials and methods**

116 **Sample site**

117   The experimental site was located in typical mixed broadleaved-Korean pine forest in

118 Heilongjiang Liangshui National Nature Reserve (47°10'50" N, 128°53'20" E) in northeast China.

119 The altitude is of 300-707 m, the terrain is relatively flat with a slope varying between 10-15°, and a

120 zonal soil considered as dark brown soil. The climate type of this region is temperate continental

121 monsoon climate. The annual average temperature is -0.3 ℃, the annual average precipitation is 676

122 mm, and the precipitation is concentrated from June to August, accounting for more than 60% of the

123 total precipitation of the whole year.

124

125 **Experimental design**

126   Three representative species of evergreen coniferous trees (*Pinus koraiensis*, *Picea asperata*

127 and *Abies fabri*) were selected and sampled from July to August 2021. The diameters at breast

128 height (DBH) range varied from 30 to 50 cm for all the individuals selected (Table S1). Samples

129 were obtained from 7 trees of each tree species. Three branches with 1 to 4 years old branches

130 (considering the polycyclic shoots formed on branches as the basis for distinguishing branch age)

131 were randomly selected for each tree (Li et al., 2006). The needles were divided into newly-formed

132 needles (the 1-year-old branches emerged in 2021 were considered as newly-formed needles) and

133 perennial needles (the 2 - 4 years old needles already existing in the branches). For each needle age,

134 three samples were collected from each individual tree. A total of 126 (2 needle ages × 3 branches ×

135 7 trees × 3 tree species) samples were collected to measure the composition of microbial

136 communities. Additionally, needles were selected at a close distance from the sampling of the

137 phyllosphere microbiome for the measurement of other traits.

138

139 **Needle collection**

140 Needles were collected with sterile gloves and cut off with sterilized branch scissors. The

141 gloves were replaced and branch scissors disinfected. The cut off were put into sterile bags for cold

142 storage (-4 ℃) and quickly brought back to the laboratory. We then weighted 20 g needles on a

143 sterile table and put them into the sterile tube directly after collection. 10 ml of sterile water per

144 gram of sample was then added to extract the phyllosphere microbiome. The samples were subjected

145 to ultrasonic washing for 1 min and vortex vibration for 30 s and this process was repeated twice.

146 The two washing solutions were then mixed and filtered with a 0.22 μm sterile filter membrane. The

147 filtered membranes were snap-frozen in liquid nitrogen and stored at -80 ℃. All samples were

148 shipped on dry ice to Majorbio corporation (Shanghai, China) for DNA extraction and microbial

149 sequencing analysis.

150

151 **Illumina MiSeq Amplicon Sequencing**

152 Total DNA was extracted from the phyllosphere microbiome and examined using 1% agarose

153 gel electrophoresis. PCR use TransGen AP221-02: TransStart Fastpfu DNA Polymerase (PCR: ABI

154 GeneAmp® 9700). PCR products were gel recovered using the axyprepdna gel Recovery Kit

155   (Axygen Corporation), Tris_HCl elution; Detection by 2% agarose electrophoresis. Quantitation of

156   PCR product detection was performed using the QuantiFluor^{-ST} blue fluorescence quantitation

157   system (Promega Corporation). Bacterial and fungal high-throughput sequencing with 16S rRNA

158   and ITS respectively. Bacterial 16S amplification primers were 338F

159   (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT), fungal its

160   primers were ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2R

161   (GCTGCGTTCTTCATCGATGC). All sequencing work was performed at Shanghai Majorbio

162   Bio-pharm Technology Co., Ltd (Shanghai, China) using the MiSeq platform (Illumina, United

163   States).

164

165   **Microbial data extraction**

166   We optimized microbial data by pairing reads which are spliced into a specific sequence. The

167   quality of reads was controlled and filtered (Remove the single sequences without duplicates, and

168   then perform OTU clustering of non-repetitive sequences according to 97% similarity, and remove

169   chimeras in the clustering process to obtain representative sequences of OUT). The samples were

170   then distinguished according to the barcodes and primer sequences at the beginning and end of the

171   sequence. We used the taxonomic databases silva 138/16s_bacteria (Release138

172   http://www.arb-silva.de) and unite 8.0/its_fungi Unite (Release 8.0 http://unite.ut.ee/index.php) to

173   identify bacterial and fungal species. We then used uparse (version 7.0.1090

174   http://drive5.com/uparse/) to cluster OTUs. Sequencing data analyses were performed using the

175   Majorbio Cloud Platform (www.majorbio.com). All the microbial data was deposited in National

176   Center for Biotechnology Information (NCBI) and the number of BioProject is PRJNA979976.

Needle traits determination

Leaf photosynthesis (A) and stomatal conductance (GSW) were measured using a photosynthetic apparatus (Li-COR6800, USA) in clear and cloudless weather in the same conditions with the microbial sampling. LMA was calculated by first measuring leaf volume and then leaf area according to the protocol described in Liu et al. (2019). LDMC was calculated as the ratio between the dry mass of leaves and the fresh mass of leaves. Chlorophyll (Chl) measurement was performed using the acetone method. The total carbon (TC) was measured by combustion method and carbon nitrogen analyzer (multiN/C2100, AnalytikJena, Germany). TN was measured by continuous flow elemental analyzer (AQ400, Seal, Germany) after high-temperature digestion using H_2SO_4-H_2O_2. TP was measured by the Molybdenum antimony anti colorimetry (Allen, 1989) after high-temperature digestion.

Statistical analysis

Variations in microbial species richness (Chao index) and diversity (Shannon index) across needle ages and tree species were analyzed. Venn diagram was then used to analyze the (dis)similarities in OTUs among the different tree species and needle ages. Histograms were used to represent species composition for all the species > 1% at the phylum level. PERMANOVA ($n = 999$ permutations) based on Bray-Curtis distance and principal coordinates analysis (PCoA) were used to assess the effects of tree species and needle ages on the structure of phyllosphere microbial community. One-way ANOVA was used to analyze the trait values that contained leaf morphology (LMA and LDMC) and leaf chemistry traits (concentration of TC, TN, TP and Chl) among different groups, and differences in abundance among the top 10 class-level microbial groups at

199 newly-formed needle and perennial needle of three species. Redundancy analysis (RDA) was used to

200 detect the relationship between needle traits and phyllosphere communities. Heat maps were used to

201 show the relationship between microbial species and needle traits at the top 20 classes of bacteria

202 and fungi.

203

204 **Results**

205 **Diversity and richness of bacteria and fungi of phyllosphere with leaf age among different tree**
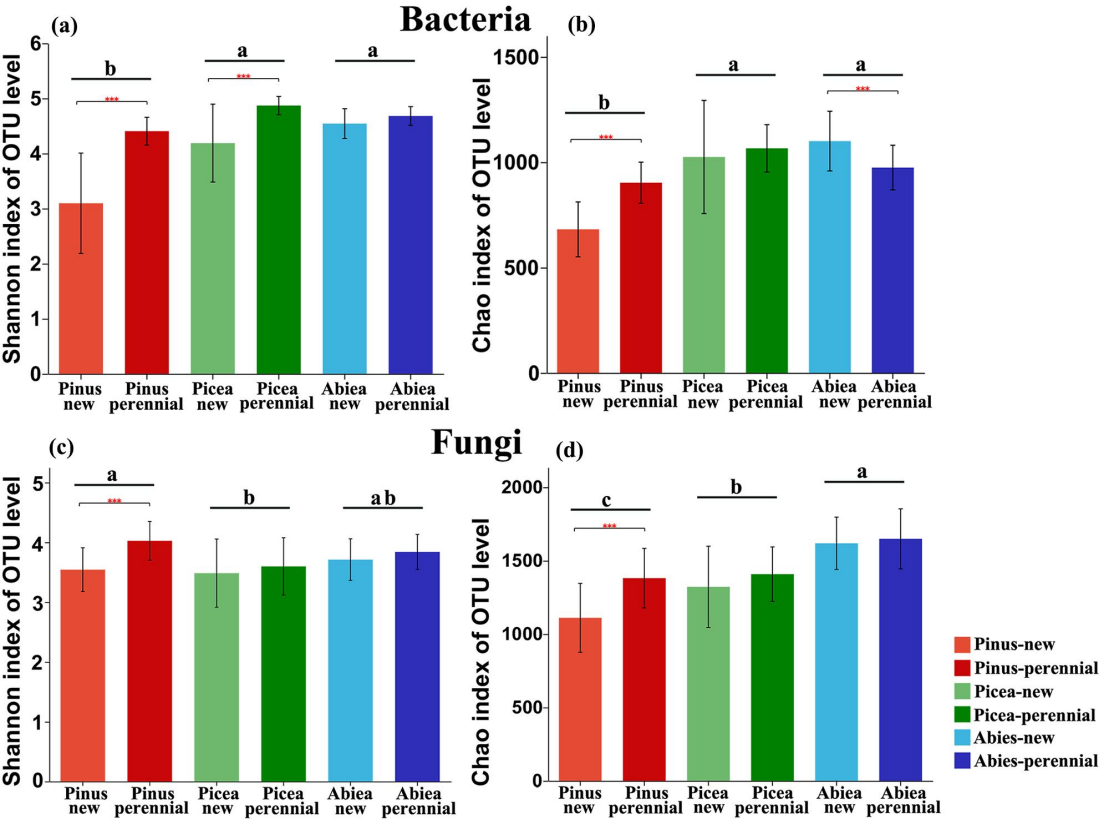
206 **species**

207 The diversity of bacterial and fungal communities varied significantly with needle ages ($P <$

208 0.05) (Table 1). Needle age had a great influence on microbial Shannon index and Chao index, and

209 tree species only affected fungi Chao index. Therefore, needle age had a greater impact on diversity

210 than tree species (Table 1). For bacteria, the Shannon index of *Pinus koraiensis* and *Picea asperata*

211 increased significantly with needle ages ($P < 0.001$) (Fig 1a). The Chao index of *Pinus koraiensis* (P

212 < 0.001) also increased significantly with needle ages (Fig 1b). However, we found a significant

213 decrease in the Chao index for perennial leaves of *Abies fabri* (Fig 1b). The bacterial Shannon

214 index and diversity index of *Pinus koraiensis* were significantly lower than that of *Picea asperata*

215 and *Abies fabri*, and there was no significant difference between *Picea asperata* and *Abies fabri*.

216 Regarding the fungi, the Shannon index and Chao index of *Pinus koraiensis* were significantly

217 affected by needle ages. Among the different species, *Pinus koraiensis* was the species most affected

218 by needle ages ($P < 0.001$) (Fig 1), even though it presented the lowest Chaos index. Interestingly,

219 we found a significant increase in the Chaos index among three coniferous species (Fig 1d), other

220 than that, there is no significant difference between *Picea asperata* and *Abies fabri* in other

221   indicators (Fig 1).

222

223   **Table 1** Effects of tree species and needle age on Shannon index and Chao index of bacteria and
224   fungi

Taxa	Index	Tree species		Needle age		Tree species×needle age	
		F	P	F	P	F	P
bacteria	shannon	0.01	0.908	13.78	<0.001***	0.94	0.338
	chao	0.07	0.789	0.48	0.495	1.29	0.263
fungi	shannon	2.75	0.077	6.34	0.016*	1.59	0.218
	chao	18.47	<0.001***	5.86	0.021*	1.84	0.173

225   Shannon index indicates species diversity and Chao index indicates species richness, * Indicates the
226   significant difference, *P<0.05; **P<0.01; ***P<0.001

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228
229   **Fig 1** Estimates of diversity index (Shannon and Chao) for bacterial (a, b) and fungal (c, d)
230   communities. * Indicates the significant difference between different tree species and leaf age,
231   *P<0.05; **P<0.01; ***P<0.001 (Only the variability between needle ages and tree species are
232   shown). Abbreviations mean *Pinus koraiensis* newly-formed needles (Pinus-new), *Pinus koraiensis*
233   perennial needles (Pinus-perennial), *Picea asperata* newly-formed needles (Picea-new), *Picea*
234   *asperata* perennial needles (Picea-perennial), *Abies fabri* newly-formed needles (Abies-new), *Abies*
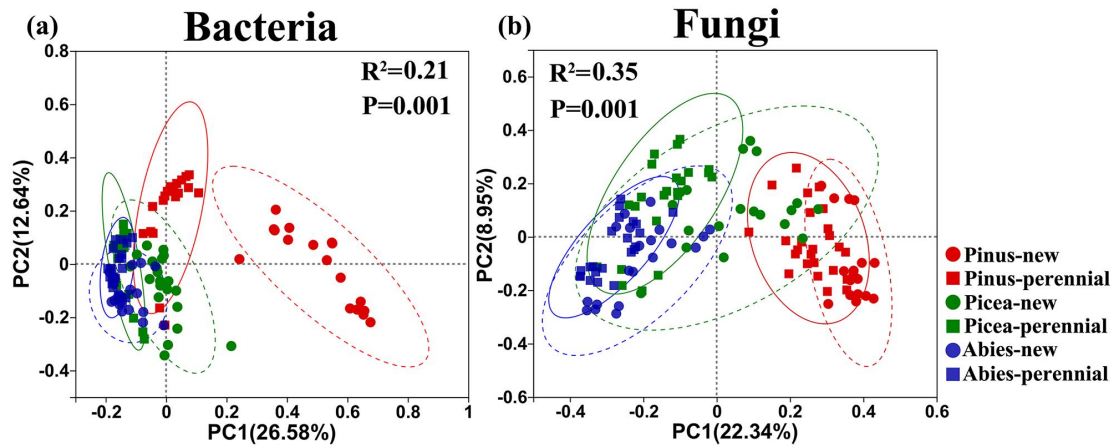235   *fabri* perennial needles (Abies-perennial).

Composition of bacterial and fungal communities

The composition of bacterial and fungal communities was significantly explained by tree species and needle ages as well as by their interactions (Table 2). Tree species was the main factor influencing fungal communities, whereas bacterial communities were mostly influenced by the interaction between tree species and needle ages (Table 2). The first PCoA axis explained 26.58% of the composition in bacterial communities and 22.34% of fungal communities (Fig 2). *Pinus koraiensis* presented a more distinct bacterial community compared with the other species, with a greater differentiation for the newly-formed needles (Fig 2). For the fungal communities, we found a clear gradient among three species, the community structure of *Pinus koraiensis* was significantly different from that of the other two tree species (Fig 2).

Table 2 PERMANOVA analysis of the effects of tree species and needle age on microbial community structure

Taxa	Variable	F	R^2	P
bacteria	needle age	15.623	0.116	0.001
	Tree species	17.712	0.231	0.001
	Tree species*needle age	19.639	0.460	0.001
fungi	needle age	8.338	0.063	0.001
	Tree species	21.304	0.257	0.001
	Tree species*needle age	13.433	0.359	0.001

R^2 value represents the degree of explanation for sample differences; The greater R^2, the higher the degree of interpretation; $P<0.05$ indicating that the reliability of this inspection is high.
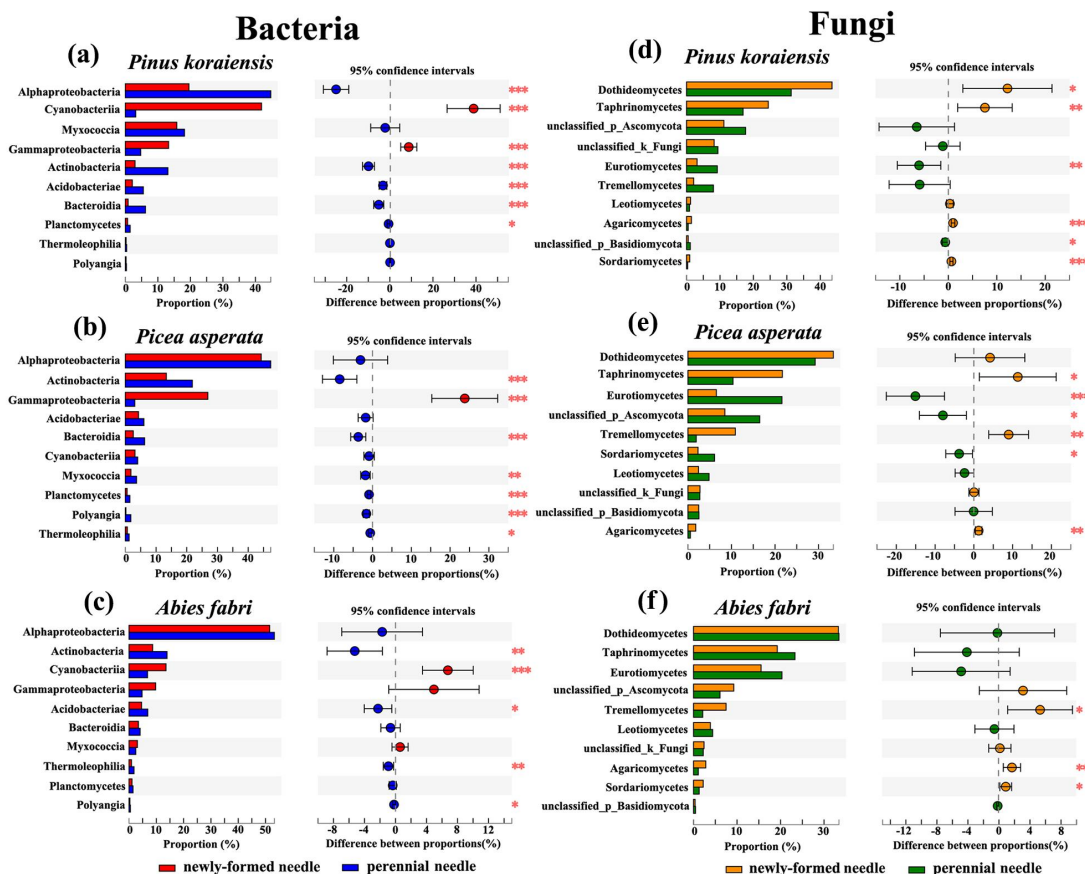
Fig 2 Principal coordinates analysis (PCoA) of bacteria (a) and fungi (b) community across different tree species and needle ages. Ellipses represent 95% confidence intervals. Abbreviations mean *Pinus koraiensis* newly-formed needles (Pinus-new), *Pinus koraiensis* perennial needles (Pinus-perennial), *Picea asperata* newly-formed needles (Picea-new), *Picea asperata* perennial needles (Picea-perennial), *Abies fabri* newly-formed needles (Abies-new), *Abies fabri* perennial needles (Abies-perennial).

There were nearly half of the total number of OTUs of bacteria and fungi shared by the three coniferous species (Fig S1a). For bacteria, the number of specific OTUs was about fourfold higher for newly-formed needles than for perennial needles, but this trend was not confirmed for fungi (Fig S1b). All species shared about 60% or more of OTUs between newly-formed and perennial needles, except for bacteria in *Pinus koraiensis* that shared less than 50% OTUs (Fig S2). The number of unique OTUs can be found in Table S2.

The dominant phylum groups of bacteria were Proteobacteria, Actinobacteria and Cyanobacteria, Myxococcota and Acidobacteria, accounting for about 90% of the total community across the different species (Fig S3). The main phylum division of fungi were Ascomycota (80%) and Basidiomycota (10%) (Fig S3). The variation of Ascomycota (decrease) and Basidiomycota (increase) between newly-formed and perennial needles in *Pinus koraiensis* was opposite to those in *Picea asperata* and *Abies fabri* (Fig S3). From the analysis at the class level, we found that the dominant classes were Alphaproteobacteria, Actinobacteria, Cyanobacteria, Gammaproteobacteria

273 and Myxococcia (Fig S4a). Regarding the fungi, the main classes were Dothideomycetes,

274 Taphrinomycetes, Eurotiomycetes and Tremellomycetes (Fig S4b). Compared to the phyla level, the

275 proportion of Cyanobacteria in *Pinus koraiensis* was very large compared with the other tree species,

276 and particularly on newly-formed needles.

Fig 3 Relative abundance of top classes for bacteria and fungi and results from A one-way ANOVA analysis assessing significant differences among different tree species and needle ages. (a, b and c for bacteria of *Pinus koraiensis*, *Picea asperata* and *Abies fabri*; d, e and f for fungi of *Pinus koraiensis*, *Picea asperata* and *Abies fabri*). * Indicates the significant difference, **P*<0.05; ***P*<0.01; ****P*<0.001.

284 When studying the 10 most abundant classes per treatment, we found that the new leaves

285 exhibited a lower number of dominant classes than perennial needles (Fig 3). The relative abundance

286 of Cyanobacteria was greater in newly-formed needles of *Pinus koraiensis* and *Abies fabri*

287 compared with the old ones (Fig 3a). The relative abundance of Gammaproteobacteria was greater in
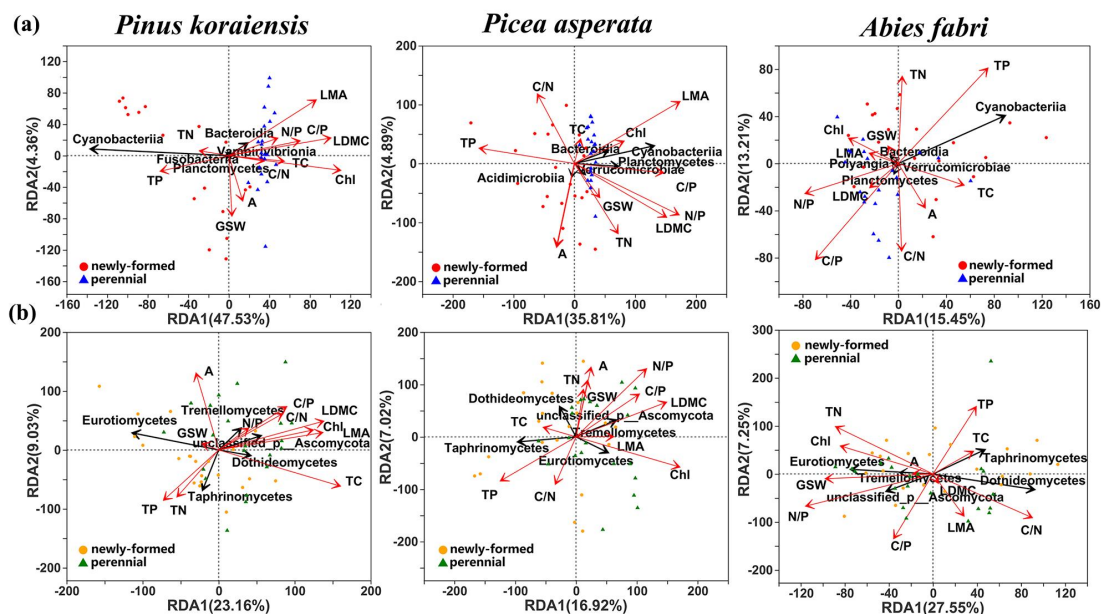
288   newly-formed needles of the three species (Fig 3a, b, and c). In contrast, Alphaproteobacteria and

289   Acidobacteria were dominant in the perennial needles across most species. Regarding the fungi, we

290   found that the classes Dothideomyctes, Taphrinomycetes and Eurotiomycetes were more abundant in

291   newly-formed needles of *Pinus koraiensis* and *Picea asperata* (Fig 3d, e), whereas Tremellomycetes,

292   Agaricomycetes and Sordariomycetes showed a higher abundance in perennial needles of *Abies fabri*

293   (Fig 3f).

294

295   **Relationship between phyllosphere microbiome and needle traits**
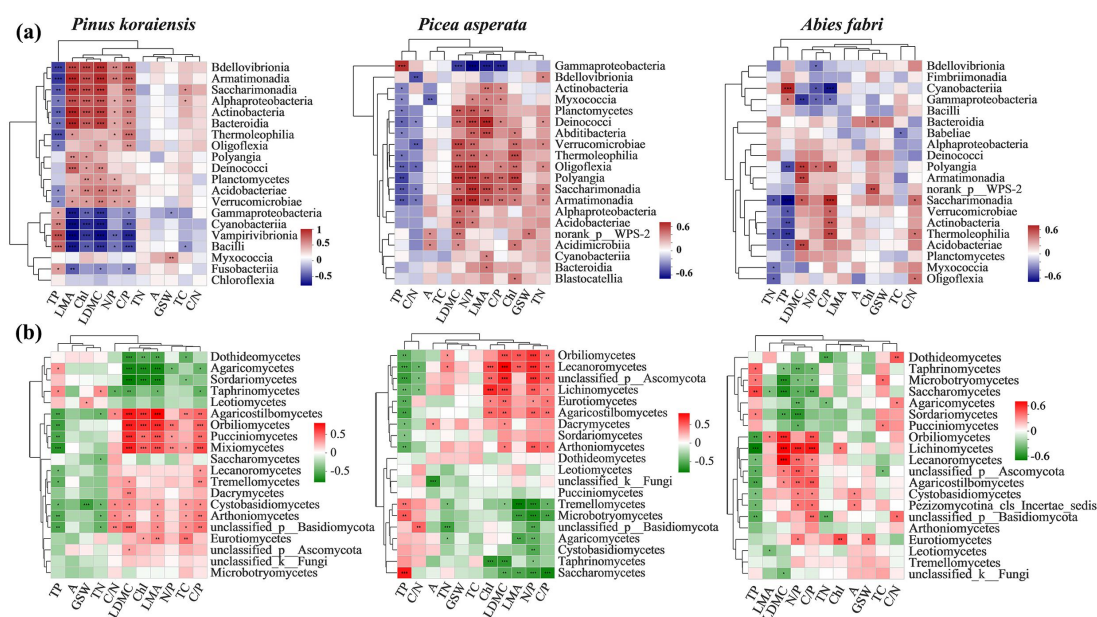
296   Analysis of needle traits revealed that Chl, LMA, LDMC, TP, as well as the ratio of nitrogen

297   and phosphorus (N/P) were significantly different among the different tree species and between two

298   different needle ages (Table S3). The variation in needle traits explained bacterial community

299   structure of *Pinus koraiensis*, *Picea asperata* and *Abies fabri* by 47.53%, 35.81% and 15.45% along

300   the first RDA axis (Fig 4a). RDA Axis 1 also explained 23.16%, 16.92% and 27.55% in the variation

301   of fungal communities for *Pinus koraiensis*, *Picea asperata* and *Abies fabri*, respectively (Fig 4b)

302   and were related to plant traits such as needle nutrient concentrations (N and P contents), LDMC and

303   LMA.

304   Overall, needle traits better explained the composition of phyllosphere bacterial communities in

305   *Pinus koraiensis* at the class level (Fig 4). The same results were obtained at the phylum level (Fig

306   S5). For the three tree species, LMA, LDMC, Chl and TP content and the C/P, N/P ratios were the

307   main needle traits explaining the different classes of bacteria and fungi (Fig 5). Contrary to *Pinus*

308   *koraiensis* and *Picea asperata*, we found that LMA and Chl were related to only a minority of taxa

309   in *Abies fabri* (e.g., bacteria Bacteroidia, norank_p_WPS-2, and Fungi Eurotiomycetes,

Lichinomycetes negatively correlated with Chl, Fungi Orbiliomycetes negatively correlated with

LMA, Saccharymonadia and Leotiomycetes negatively correlated with LMA). Interestingly, the

correlations between TP and bacterial or fungal classes were opposite to most other plant traits (Fig

5). Overall, most of microbial taxa were more abundant on needles presenting high LDMC and LMA

and low TP content (Fig 5). For the bacteria, Saccharymonadia, Actinobacteria, Thermoleophilia,

Verrucomicrobiae, Acidobacteria and Polyangia were positively correlated with LMA, LDMC, but

negatively correlated with TP (Fig 5). In contrast, Cyanobacteria and Gammaproteobacteria were

positively correlated with TP. For the fungi, Agaricostilbomycetes, Eurotiomycetes,

Lecanoromycetes, Lichinomycetes, Orbiliomycetes were positively correlated with LDMC, N/P and

C/P ratios whereas Taphrinomycetes and Saccharomycetes were negatively related to LDMC, N/P

and C/P ratios (Fig 5).

Fig 4 Redundancy analysis (RDA) between the major bacterial (a) and fungal (b) taxa and leaf traits
for each tree species separately. The black arrow indicates species, the red arrow indicates plant traits,
and the length of the arrow represents the degree of influence. The distance between the projection
points and the origin represents the relative influence of plant traits on microbial communities.

Fig 5 Heat map of correlation of the top 20 bacterial (a) and fungal (b) classes and needle traits among different tree species and needle age. The X axis represents the needle traits, and the Y axis represents the name of classes. The right side of the legend shows the range of colors for different R values. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Discussion

Through using needles from three evergreen coniferous tree species, we investigated how cohort of needles of different ages, tree species identity and the variability in plant traits influenced the composition of phyllosphere communities. We found that the diversity of bacterial and fungal communities was greater for perennial than newly-formed needles. On the other hand, the composition of bacterial and fungal communities was mainly driven by tree species identity and its interaction with needle ages, respectively. These changes were related to plant traits, such as morphological traits (i.e., LMA, LDMC) or chemical traits (i.e., N/P, TP) related to leaf economic spectrum, highlighting the importance of understanding the interactions between phyllosphere microorganisms and plant resource acquisition strategies.

17

Diversity of phyllosphere communities across different needle ages

In partial agreement with our first hypothesis, we found an overall lower diversity of phyllosphere microorganisms in the newly-formed needles compared with the perennial needles across different coniferous species, but this effect was particularly apparent for *Pinus koraiensis*. This difference can be due to the fact that the greater effect of light UV on newly-formed leaves may directly alter the composition of phyllosphere communities (Herrmann et al. 2021), while generating a drier environment that favor stress-tolerant phyllosphere microbes (Tim & Sarah, 2006). In contrast, perennial needles are less affected directly by light UV, and the thicker epidermal wax chemicals composed of long-chain hydrocarbons may provide different niches and micro-environments for a variety of phyllosphere microbes including endophytes (Schreiber et al., 2005; Yadav et al., 2005; Beattie et al., 2011; Wang et al., 2018). These results are in line with those of Hermann et al. (2021) showing that microbial diversity increases from the top (light leaves) to the bottom (shaded leaves) of the canopy, probably as a result of harsher environmental conditions and a stronger selective pressure on phyllosphere organisms when they grow on light needles. Furthermore, the newly-formed needles often present higher photosynthetic capacity (Warren, 2006; Albert et al., 2018), which may also promote the absorption of carbon and nitrogen sources and promotes leaf growth (Qi et al., 2021; Takashima et al., 2004). As such, the greater resource availability in newly-formed leaves may have increased the dominance of a few competitive microbial taxa (Kembel et al., 2014). These results are consistent with the results of Williams et al. (2013) that microbial diversity increased as leaves matured, notably because the succession among various microbial taxa with time increases. In line with this idea, our results showed that Proteobacteria, which is often considered as a group mainly composed of copiotrophic microbes (Fierer et al. 2007;

368   Sauvadet et al. 2019), was the dominant group in newly-formed leaves of *Picea asperata* and *Abies*

369   *fabri* (Eva-Maria et al., 2011; Xu et al., 2022). The proliferation of Proteobacteria has been shown to

370   significantly decrease the growth of other microbial groups such as Firmicutes (Chen et al. 2020),

371   which may contribute to explain the observed decreased in microbial diversity compared with

372   perennial leaves (Koskella, 2020). Altogether, these results are in agreement with the microbial

373   succession model of Jackson et al. (2001), who showed that when microorganisms colonized of a

374   new surface, environmental and resource pressures promote competition among microorganisms,

375   whereas microbial diversity increases when new spaces and niches are created over time. However,

376   it is important to note that our results showed an opposite trend for *Abies fabri*, which may be due to

377   the fact that the needles are thicker and bigger compared with the other species, which may provide

378   enough space for the development of various microbial groups, and this even for newly-formed

379   leaves. Altogether, these results suggest that changes in micro-environmental conditions (i.e., light

380   and/or water availability) and physiological characteristics of needles with leaf ageing (i.e.,

381   thickening of the waxy layer and decreasing photosynthetic efficiency) (Niinemets, 2002) can be

382   significant factors affecting the diversity and composition of phyllosphere communities, with

383   microbial diversity increasing in perennial leaves compared with newly-formed leaves.

384

385   **Composition of phyllosphere communities across different coniferous tree species**

386   In line with our second hypothesis, the effect of needle ages on phyllosphere communities was

387   greater for the fast-growing *Pinus koraiensis* compared with slow-growing species *Picea asperata*

388   and *Abies fabri* (Fig 1). In details, Cyanobacteria were particularly abundant in newly-formed

389   needles of *Pinus*, a phylum that is well known for N_2 fixation from the atmosphere (Fay, 1992; Zehr

390   et al., 2008; Fürnkranz et al. 2008; Calabria et al., 2020). The greater N acquisition and

391   photosynthetic capacity has probably contributed to sustain high growth rates in the newly-formed

392   leaves of *Pinus koraiensis*, which may further explain the greater overall plant performance of *Pinus*

393   *koraiensis* compared with the other coniferous species (Fürnkranz et al. 2008). As needles become

394   more and more shaded with tree growth, the decrease in light conditions coupled with changes in

395   needle physiological characteristics have probably decreased the proportion of Cyanobacteria to the

396   benefit of other bacterial taxa that are better adapted to these new environmental conditions (e.g.,

397   Acidobacteria and Actinobacteria) (Jackson & Denney, 2011; Kim et al., 2012; Lambais et al., 2014).

398   These results are in line with those of Rico et al. (2014) showing that diazotrophic diversity

399   increases in dry conditions whereas it decreases when the environmental conditions are wetter. Such

400   a difference in Cyanobacteria between newly-formed and perennial needles was also observed for

401   *Abies fabri*, but the increase in relative abundance was almost tenfold lower compared with *Pinus*

402   *koraiensis*, which was probably not enough to generate significant differences in phyllosphere

403   communities between newly and perennial needles.

404   In addition to Cyanobacteria, we also found that Gammaproteobacteria, which are often

405   considered as copiotrophic organisms (Fierer et al. 2007; Fürnkranz et al. 2008), were greater in

406   newly-formed needles of *Picea asperata* and *Pinus koraiensis*. This result is in line with those of

407   Truchado et al. (2017) showing that Gammaproteobacteria strongly depends on high amount of

408   soluble carbohydrates and nutrients, or alternatively, that this taxa is relatively well adapted to light

409   conditions (Redford & Fierer, 2009; Truchado et al., 2017). In contrast, we found that a multitude of

410   other taxa such as Acidobacteria, Actinobacteria, Myxoccoccia or Bacteroidia were more abundant

411   in perennial leaves for the three coniferous species, but at different proportions according to the tree

species studied. In particular, it has been proposed that Acidobacteria and Myxoccoccia were able to degrade complex polysaccharides such as cellulose and lignin (Ward et al., 2009; Lambais et al., 2014; Kim et al., 2012), which may explain their higher relative contribution in older leaves in *Pinus koraiensis* and *Picea asperata*. Furthermore, a recent study studying leaf position within trees showed that Actinobacteria increased their proportion from the top to the bottom of the canopy (Herrmann et al. 2021), and decrease in abundance as the plant grows (Jia et al., 2020), which confirms that Actinobacteria are relatively intolerant to sunny and dry environmental conditions (Shao et al., 2019).

Regarding the fungi, we also found significant differences with leaf age, with notably a greater relative abundance of Dothideomycetes and Taphrinomycetes in newly-formed leaved of *Pinus koraiensis* and *Picea asperata* (Fig 3). These two groups are often considered as the main taxa of phyllosphere fungi (Delhomme et al., 2015; Qian et al., 2018), probably because they are efficient at competing for nutrients and space in newly-formed needles (Würth et al., 2019). Although many of them are harmless for the plants, some Dothideomycetes can also act as pathogens and attack photosynthetic tissues of plants (Arnold & Lutzoni, 2007). This may be one of the reasons for the lower photosynthetic rate of perennial needles, which also increases competition with other pathogens community such as *Streptococcus glycinica* (Osono et al., 2006; Diaz-Cruz & Cassone, 2022). The decrease in photosynthetic rates and available carbon substrates in perennial leaves may also contribute to explain the changes in composition of fungal communities (Würth et al., 2019). Furthermore, Dothideomycetes are often considered are oligotrophs that can stand harsh environment such as high temperature and high UV of newly needles (Vorholt, 2012; Egidi et al., 2014), which may further contribute to explain why they are more abundant in newly-formed leaves

434 compared with perennial ones. Interestingly, we found that Tremellomycetes and Agaricomycetes

435 were also relatively more abundant in newly-formed needles of *Abies fabri* and *Picea asperata.*

436 Agaricomycetes and Tremellomycetes are well known as saprophytic fungi and yeasts, which can

437 grow on the surface of plants and secrete a large amount of heterologous proteins such as

438 exopolysaccharides to enhance their high tolerance to UV (Huh et al., 2003; Qian et al., 2018; Fanin

439 et al., 2022). Furthermore, Agaricomycetes are often found across a wide range of plant groups due

440 to their capacity to survive in various environment (Qian et al., 2018; Yao et al., 2019; Würth et al.,

441 2019; Ding et al., 2022). Finally, we found that Eurotiomycetes were often more abundant in

442 perennial needles, probably because Tremellomycetes and other fungal taxa limit the growth of

443 Eurotiomycetes in newly-formed leaves, as it has been shown recently in *Picea glauca* (Würth et al.,

444 2019). This demonstrates that the assemblage of phyllosphere communities is achieved through

445 interspecies competition (Würth et al., 2019), and suggests that the reduced nutrients and carbon

446 availability in perennial leaves is no longer suitable for some taxa such as Dothideomycetes and

447 Tremellomycetes.

448

449 **Importance of plant traits for explaining the composition of phyllosphere communities**

450   In line with our third hypothesis, needle traits were important factors explaining the differences

451 in the composition of phyllosphere communities. In particular, we found that the large differences in

452 phenotypic traits such as LMA and LDMC between different cohorts of needles were significantly

453 related to various groups of bacteria and fungi. For instance, it has been shown that LMA and

454 LDMC were indicators of photosynthetic rate and growth rate which predict the survival strategies

455 of trees (Niinemets, 2002). In line with this idea, we found that high LDMC and LMA values in

456   perennial leaves can be used as useful predictors of some microbial taxa such as

457   Gammaproteobacteria, Actinobacteria and Orbiliomycetes during late stages of microbial succession

458   (e.g., notably for the genus Friedmaniella in the Actinobacteria taxa) (Herrmann et al., 2021).

459   Alternatively, because exudation of monosaccharides such as glucose and fructose on the needle

460   surface is often considered as a central factor explaining the composition of phyllosphere

461   communities in newly-formed leaves (Lindow & Brand, 2003), low LDMC and LMA values can be

462   used as useful proxies of copiotrophic taxa during earlier stages of microbial succession (e.g.,

463   notably for the genus Pseudomonas in the Gammaproteobacteria taxa) (Anzai et al., 2000; Das et al.,

464   2017). In addition, it has also been proposed in the literature that nutrient supply was essential to

465   sustain plant growth and succession in the phyllosphere community (Vorholt, 2012; Vacher et al.,

466   2016). In line with this idea, we found that the ratios of N/P and C/P were also significant factors

467   influencing the relative abundance of many bacterial and fungal taxa, with greater abundances of

468   Saccharimonadia and Orbiliomycetes when increasing stoichiometric C:N:P ratios. However,

469   consistent with Yadav et al. (2005), we did not find that total nitrogen was a significant factor

470   affecting the composition of phyllosphere communities in our experiment. This was perhaps because

471   N-fixing bacteria in the phyllosphere (e.g., cyanobacteria) were able to fix N directly from the

472   atmosphere (Freiberg, 1998; Papen et al., 2002; Fürnkranz et al., 2008; Delmotte et al., 2009; Abadi

473   et al., 2021). Alternatively, this suggests that stoichiometric requirements are prevalent over total

474   nutrient contents to predict the composition of phyllosphere communities, at least in the context of

475   our study at the local scale. Finally, we also found that TP affected many microbial taxa such as

476   Saccharimonadia and Orbiliomycetes, but in an opposite direction to LMA and LDMC (Fig 4, Fig 5).

477   This may be because phosphorus-containing compounds are less likely to penetrate the cuticles of

478 perennial needles that often present relatively high LMA and LDMC values (Yadav et al., 2005), or

479 alternatively that fast-growing organisms require more P to maintain high growth rates in

480 newly-formed needles (Mediavilla et al., 2011; Wang et al., 2014; Zhang et al., 2016) Altogether,

481 these findings suggest that needle traits can be used as useful predictors of microbial taxa during leaf

482 ageing.

483

484 **Conclusion**

485 Our study highlights that tree species and needle age are major factors affecting the diversity

486 and composition of phyllosphere communities through changes in plant functional traits and

487 environmental conditions. These result are consistent with our hypotheses and have several

488 implications. First, our results indicate that phyllosphere communities vary within and between tree

489 species, even though phyllosphere communities are thought to be relatively similar within the same

490 tree individual and/or within the same plant functional groups. This suggests the diversity and

491 composition of phyllosphere microbes may strongly vary at the tree or the plot scale in forest, a

492 variability that has rarely been considered in most studies studying plant-microbe interactions in

493 natural ecosystems. Second, they highlight the fact that assessing the succession in microbial

494 communities on leaves as they grow and age (i.e. the shift from newly-formed to perennial leaves)

495 can shed a new light on the function of phyllosphere communities in relation to plant performance.

496 For instance, the role of phyllosphere organisms in sunny leaves has only been recently

497 acknowledged in N-acquisition strategy at the tree scale (Fürnkranz et al., 2008; Vorholt, 2012), and

498 more work will be necessary to quantify the functional role of late successional groups of

499 decomposers during leaf ageing. Finally, our data suggest that changes in environmental conditions

500 can also have functional consequences for tree growth through alterations of phyllosphere

501 communities. For this reason, changes in the balance of N-fixers *versus* endophytes that may result

502 from different positions along branches or due to global changes such as climate warming or N

503 deposition, can have implications for nutrient acquisition and tree productivity in forest ecosystems.

504

511

512 **Author contributions**

513 LW, ZLL and GZJ designed the experiment and collected the samples, LW analyzed data and

514 wrote the first draft of the manuscript in close consultation with ZLL and NF who contributed

515 critically to data interpretation and ideas. CB provided many ideas of the manuscript. All authors

516 contributed to manuscript completion and revision.

517

518 **Data accessibility**

519 All the microbial data was deposited in National Center for Biotechnology Information (NCBI) and

520 the number of BioProject is PRJNA979976.

521

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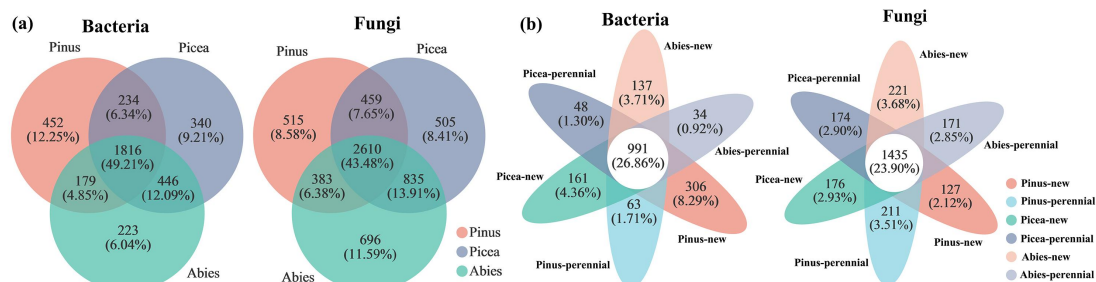
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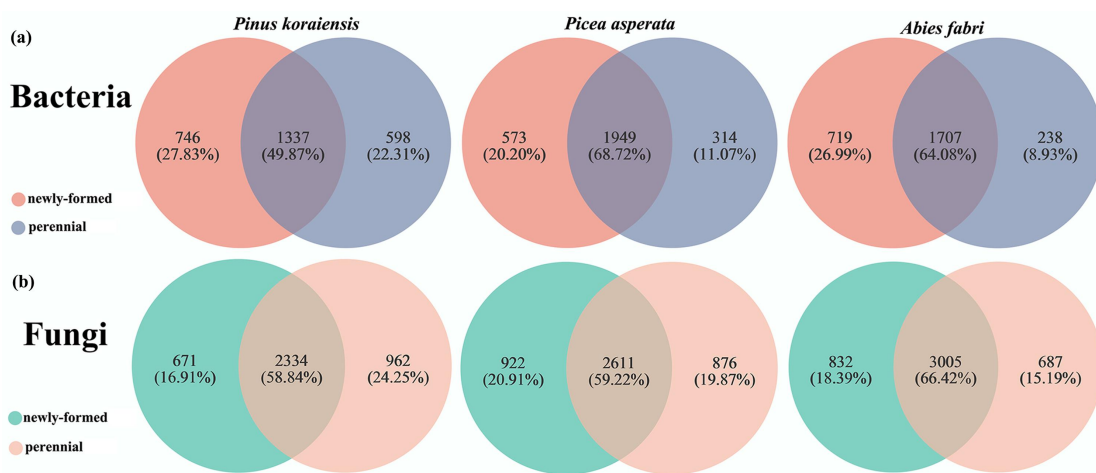
722 **Supplemental Material**

723 **Table S1** Sample trees basic data

724

Identity	Height (m)	DBH (cm)	Height of the first living branch (m)
Pinus koraiensis	18.6	48	5.9
Pinus koraiensis	23.5	47.5	10.4
Pinus koraiensis	25.6	41.1	5.8
Pinus koraiensis	17.5	38.3	3.8
Pinus koraiensis	22.5	44.1	7.5
Pinus koraiensis	18.8	40	10.8
Pinus koraiensis	20.5	38.8	6.5
Picea asperata	27.5	50.2	10.9
Picea asperata	23.8	29.9	12.3
Picea asperata	21.9	36.2	5.5
Picea asperata	24	35	10
Picea asperata	21.7	49.7	9.3
Picea asperata	20.2	47.6	9
Picea asperata	29.3	34.8	15.2
Abies fabri	26	37	6.9
Abies fabri	22.3	42.1	9.5
Abies fabri	17.1	33	6.5
Abies fabri	23.6	40.1	10.3
Abies fabri	19	32.5	8
Abies fabri	21	43.5	5.7
Abies fabri	18.3	31.2	2.6

748

749 DBH (Diameter of a cross-section of a tree trunk 1.3 meters above the ground)

Fig S1 OTU Venn diagram of bacterial and fungal communities among the three different tree species (a) and two needle ages (b). The central numbers of OTUs represents the number of overlaps between the different groups, and the number of OTUs within each circle represents the number of unique OTUs. Abbreviations mean *Pinus koraiensis* newly-formed needles (Pinus-new), *Pinus koraiensis* perennial needles (Pinus-perennial), *Picea asperata* newly-formed needles (Picea-new), *Picea asperata* perennial needles (Picea-perennial), *Abies fabri* newly-formed needles (Abies-new), *Abies fabri* perennial needles (Abies-perennial).
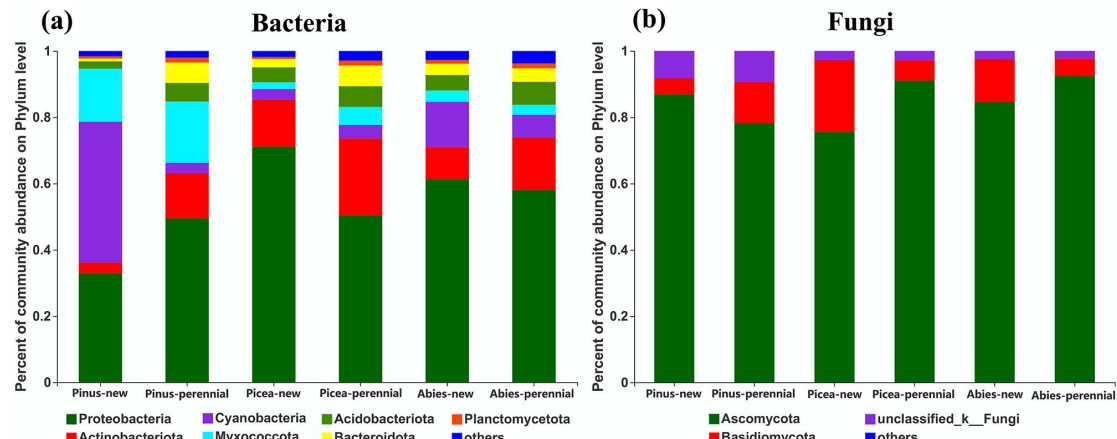

Fig S2 OTU Venn diagram of bacterial (a) and fungal (b) communities of newly-formed needles and perennial needles among the three different tree species. The central numbers of OTUs represents the number of overlaps between the different groups, and the number of OTUs within each circle represents the number of unique OTUs.

Table S2 Orders and classes of specific bacteria and fungi OTU among different tree species and
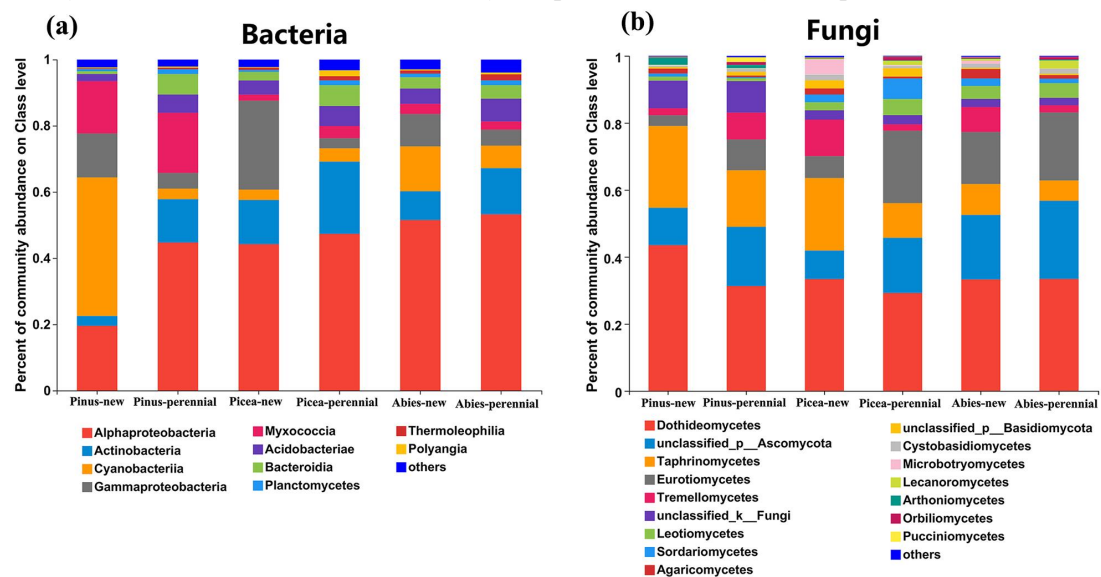
needle ages

	Group	Order	Class
Bacteria	**Pin-new**	o_Thermovenabulales, o_Alteromonadales, o_Leptolyngbyales, o_Beggiatoales, o_Pasteurellales, o_Oxyphotobacteria_Incertae_Sedis, o_Gastranaerophilales, o_Thermotogales, o_norank_c_Limnochordia, o_norank_c_Desulfuromonadia, o_Symbiobacteriales, o_Kryptoniales, o_Desulfotomaculales, o_norank_c_norank_p_Armatimonadota, o_Salinisphaerales, o_Limnochordales, o_Piscirickettsiales, o_0319-7L14, o_RF39, o_Haloplasmatales, o_norank_c_SL56_marine_group, o_Acholeplasmatales, o_Aeromonadales, o_Halanaerobiales, o_Thermales	c_Limnochordia c_Thermovenabulia c_Desulfotomaculia c_norank_p_Armatimonadota c_Kryptonia c_SL56_marine_group c_Desulfuromonadia c_Thermotogae c_Symbiobacteriia c_Halanaerobiia
	Pin-perennial	Alphaproteobacteria_Incertae_Sedis	NS
	Pic-new	o_norank_c_Acidobacteriae o_norank_c_Dojkabacteria o_norank_c_Lineage_IIa, DS-100	c_Lineage_IIa c_Dojkabacteria
	Pic-perennial	o_norank_c_Subgroup_22	c_Subgroup_22
	Abi-new	o_Actinomarinales, Eubacteriales o_norank_c_norank_p_Latescibacterota o_Cloacimonadales o_Clostridia_vadinBB60_group o_Spirochaetales, Syntrophales o_Clostridia_UCG-014, o_norank_c_OLB14	c_norank_p_Latescibacterota c_Cloacimonadia c_OLB14 c_Syntrophia c_Spirochaetia
	Abi-perennial	o_Acidaminococcales	NS
Fungi	**Pin-new**	o_Microbotryaceae, Eocronartiaceae	c_Microbotryales
	Pin-perennial	o_Cystostereaceae o_unclassified_o_Cystofilobasidiales	NS
	Pic-new	o_Tremellales_fam_Incertae_sedis o_Graphiaceae, Basidiobolaceae o_Diaporthaceae o_Rozellomycotina_fam_Incertae_sedis	c_Rozellomycotina_ord_Incertae_sedis c_Basidiobolales
	Pic-perennial	o_Naemateliaceae	NS
	Abi-new	o_Archaeorhizomycetaceae, o_Rhizopodaceae	c_Archaeorhizomycetales
	Abi-perennial	o_Elixiaceae, Pilocarpaceae	c_Umbilicariales

Abbreviations mean *Pinus koraiensis* newly-formed needles (Pinus-new), *Pinus koraiensis*

perennial needles (Pinus-perennial), *Picea asperata* newly-formed needles (Picea-new), *Picea*

asperata perennial needles (Picea-perennial), *Abies fabri* newly-formed needles (Abies-new),

Abies fabri perennial needles (Abies-perennial).

Fig S3 The relative abundance of bacteria (a) and fungi (b) at phylum level for the different tree species at different needle ages. Abbreviations mean *Pinus koraiensis* newly-formed needles (Pinus-new), *Pinus koraiensis* perennial needles (Pinus-perennial), *Picea asperata* newly-formed needles (Picea-new), *Picea asperata* perennial needles (Picea-perennial), *Abies fabri* newly-formed needles (Abies-new), *Abies fabri* perennial needles (Abies-perennial).
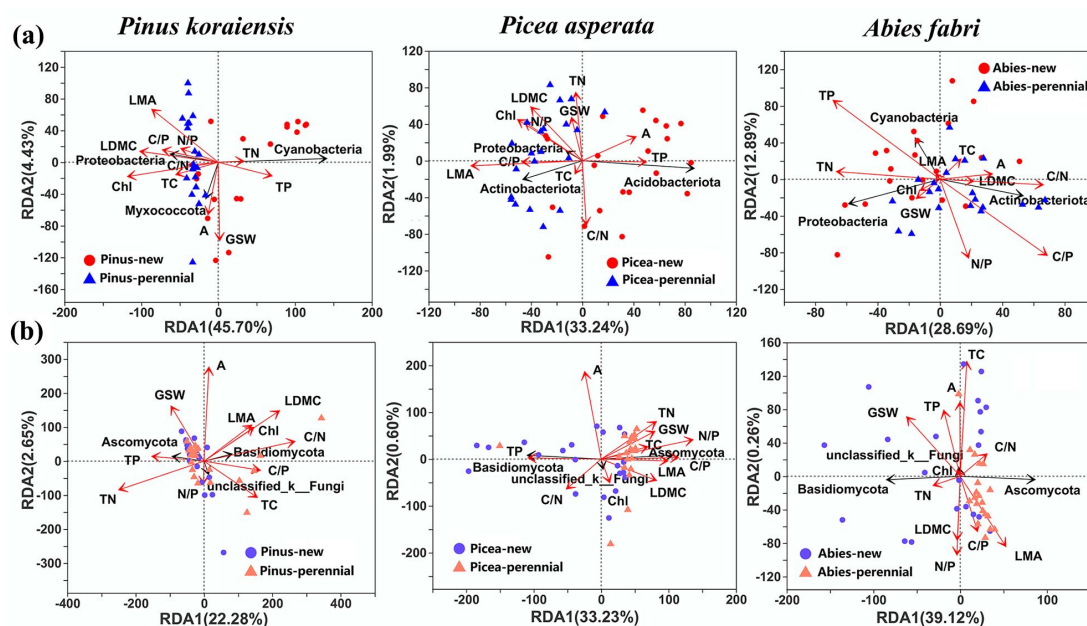

Fig S4 The relative abundance of bacteria (a) and fungi (b) at class level for the different tree species at different needle ages. Abbreviations mean *Pinus koraiensis* newly-formed needles (Pinus-new), *Pinus koraiensis* perennial needles (Pinus-perennial), *Picea asperata* newly-formed needles (Picea-new), *Picea asperata* perennial needles (Picea-perennial), *Abies fabri* newly-formed needles (Abies-new), *Abies fabri* perennial needles (Abies-perennial).

784 **Table S3** Leaf traits among different tree species and needle age.

Taxa	Pinus-new	Pinus-perennial	Picea-new	Picea-perennial	Abies-new	Abies-perennial
Chl (mg/g)	0.94±0.32c	1.60±0.22a	0.76±0.16c	0.94±0.28c	0.94±0.33c	1.16±0.50b
LMA (g/cm²)	0.008±0.001e	0.012±0.004bc	0.013±0.001b	0.016±0.003a	0.010±0.004d	0.010±0.002cd
LDMC (g/g)	0.33±0.03d	0.43±0.03ab	0.37±0.06c	0.44±0.08a	0.40±0.02bc	0.45±0.07a
A (μmol m^{-2} s^{-1})	8.94±2.58ab	9.50±2.32a	7.35±3.74bc	5.90±3.20cd	4.42±2.24d	4.50±2.60d
GSW (mol m^{-2} s^{-1})	0.10±0.03a	0.11±0.03a	0.09±0.04a	0.09±0.04a	0.06±0.02b	0.06±0.03b
TN (mg/g)	14.95±2.11ab	14.41±3.65ab	11.85±2.19c	13.02±2.65bc	15.90±3.79a	14.44±3.94ab
TP (mg/g)	1.83±0.34a	1.31±0.43c	1.54±0.36b	1.13±0.29c	1.82±0.43a	1.18±0.30c
TC (g/kg)	472.6±23.9bc	485.7±11.4ab	461.8±35.1c	473.9±31.6bc	496.7±12.8a	492.7±16.6a
C/N	32.0±4.7c	35.8±9.4abc	40.5±9.1a	38.6±13.0ab	33.3±9.7bc	36.5±9.8abc
C/P	264.9±54.5b	414.4±151.9a	330.7±160.1b	454.3±160.2a	289.9±82.0b	448.5±135.9a
N/P	8.47±1.79b	12.13±5.10a	8.41±4.09b	12.34±5.28a	9.01±2.46b	12.69±3.82a
Needle Length	11.00±1.48b	11.67±1.32c	1.98±0.22a	1.96±0.16a	2.25±0.54a	2.28±0.38a

785 Mean values (± SE) of leaf traits among different tree species and needle age. Significant
786 differences between samples (Duncan test, $P < 0.05$) are denoted with letters (a > b > c).
787 Abbreviations mean Chlorophyll (Chl), Leaf mass per area (LMA), Leaf dry matter content
788 (LDMC), Net photosynthetic rate (A), Stomatal conductance (GSW), Total nitrogen (TN), Total
789 phosphorus (TP), the ratio of nitrogen and phosphorus (N/P).
790

Fig S5 Redundancy analysis (RDA) between the major bacterial (a) and fungal (b) taxa and leaf traits for each tree species separately. The black arrow indicates species, the red arrow indicates plant traits, and the length of the arrow represents the degree of influence. The distance between the projection point and the origin represents the relative influence of plant traits on microbial communities.