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1 **Mycobiome analysis of asymptomatic and symptomatic Norway spruce trees naturally**
2 **infected by the conifer pathogens *Heterobasidion* spp.**

3 Running title: Mycobiome of Norway spruce

4

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26 **Originality-Significance Statement**

27 This study is the first to address the effect of root and butt rot disease on the composition of fungal
28 communities associated with Norway spruce and the connection between health status of spruce
29 trees and the composition of the resident mycobiota. Presented results showed the significant
30 differences in structure of fungal communities inhabiting wood of symptomatic and asymptomatic
31 spruce trees. Our study provides a new insight into the interaction of fungal plant pathogens with
32 the resident plant microbiota.

33

34 **Summary**

35 Plant microbiome plays an important role in maintaining the host fitness as demonstrated by
36 numerous studies. Despite a significant progress achieved in our understanding of the factors
37 affecting the composition of microbial communities associated with trees, very little is known about
38 the effect of plant pathogens on their structure. We analyzed the mycobiome of different parts of
39 Norway spruce as well as their fungal communities on asymptomatic and symptomatic naturally
40 infected trees. Using *Heterobasidion*-rotted and infected trees as a model, we investigated the
41 primary impact of the disease on fungal communities associated with Norway spruce trees. Our
42 results demonstrate that symptomatic and asymptomatic *Heterobasidion*-infected trees significantly
43 differed in the structure of the fungal communities residing in their wood, but not in other anatomic
44 regions. Each of the investigated tissues (wood, bark, needles and roots) harbored a unique fungal
45 community. Symptomatic trees were more susceptible to co-infection by other wood-degrading
46 fungi.

47

48 **Introduction**

49 All groups of land plants, ranging from mosses to angiosperms, live in close association with a
50 diverse set of microorganisms. Both outer plant surfaces and inner parts of the plant body are

51 colonized by various taxa of bacteria, fungi, archaea, and protists, together comprising plant
52 microbiota (Turner et al., 2013). It is widely accepted that plant microbiota influences host fitness
53 (Vandenkoornhuysen et al., 2015). Certain plant-associated fungi and bacteria contribute to plant
54 growth promotion and resistance against biotic and abiotic stresses (Hardoim et al., 2015).

55 However, dormant pathogens and saprobes equally belong to plant microbiota (Porrás-Alfaro and
56 Bayman, 2011; Hardoim et al., 2015). Thus, the interactions between plants and the associated
57 microorganisms can range from mutualism through commensalism to pathogenicity. The outcomes
58 of specific interactions are influenced by a number of driving forces, including host and microbial
59 genotypes, abiotic factors, and interactions within plant microbiome (Hardoim et al., 2015).

60 The application of metagenomics and metatranscriptomics boosted the studies on plant microbiome
61 function and its role in plant health and stress tolerance (Lebeis, 2015). Nevertheless, the current
62 information on factors driving the composition of plant microbiome and, particularly, forest trees is
63 still very scarce. The available data suggest that microbial communities of the rhizosphere are
64 mainly influenced by soil types, whereas host plant genotype has a limited effect on their
65 composition (Weinert et al., 2011; Bulgarelli et al., 2012; Lundberg et al., 2012). In contrast,
66 microbial communities of phyllosphere and endosphere are predominantly determined by host plant
67 species (Redford et al., 2010; Bulgarelli et al., 2013).

68 Reports on abilities of microbial endophytes to improve host fitness and stress tolerance inspired the
69 idea of using endophytic microorganisms as biocontrol and growth-promoting agents (Backman and
70 Sikora, 2008; Mejía et al., 2008; Blumenstein et al., 2015; Pautasso et al., 2015). However, the
71 research on the impact of microbiome on the plant disease resistance is still in its early stage. The
72 effects of pathogens on microbiome community and *vice versa* observed in a few available studies
73 differed among used experimental models (Hardoim et al., 2015), making it difficult to draw any
74 general conclusions. Nevertheless, some pioneering studies do indicate that there might be a

75 correlation between the structure of microbiome communities and host plant resistance /
76 susceptibility to pathogens (Ardanov et al., 2012; Martin et al., 2013).

77 Butt and root rot disease caused by fungi belonging to *Heterobasidion annosum* species complex
78 has a great economic impact on forest industry in boreal zone (Asiegbu et al., 2005). The pathogen
79 grows necrotrophically in the sapwood of living trees and saprotrophically in dead wood tissues. In
80 Norway spruce, the disease develops slowly resulting in the formation of decay column within the
81 tree trunk, but it rarely causes instant mortality of spruce trees (Asiegbu et al., 2005). Often, decay
82 zone remains limited to heartwood, but occasionally pathogen can reach sapwood. Oliva et al
83 (2013) have observed in field inoculations that heartwood of Norway spruce stumps were more
84 susceptible to *H. parviporum* and *H. annosum* s.s. infection than pine. Current control strategies
85 focus on prevention of fungal infection of tree stumps remaining after harvesting. No absolute
86 protection and elimination of the fungus from already infected trees or stumps are available.
87 Therefore, better understanding of the interactions between *Heterobasidion* fungi, their hosts and
88 other components of host microbiome is needed for the development of novel, more efficient
89 disease management strategies.

90 The aim of the presented study was to investigate the composition of fungal communities associated
91 with different anatomical tissues of Norway spruce trees and to assess the impact of the root and
92 butt rot caused by *Heterobasidion* sp. on the structure of these communities under field conditions.
93 The pathogen establishment is likely to occur in an interaction with a resident microbiota of the
94 infected tree. We hypothesized that there are significant differences in microbial communities of
95 asymptomatic and symptomatic Norway spruce trees. As the extent of the potential effect exerted
96 by *Heterobasidion* infection on spruce fungal communities was difficult to predict, we sampled not
97 only tissues visibly closer to the decay zone (down stem, see Figure 1), but also included in our
98 analysis more distant parts of spruce trees, namely root, bark and needles.

99

100 **Results and discussion**

101 *MiSeq sequencing output*

102 A total of 8 276 762 high quality sequences were generated across root, down stem, upper stem,
103 bark and needle samples in the three sampling sites after sequence denoising and quality filtering.
104 After filtering out unclassified sequences and sequences assigned to plant and animal domains, a
105 core set of 7 673 670 sequences assigned to fungal domain was obtained. Due to technical problem
106 of PCR amplification and sequencing, 16 out of 90 samples had lower number of reads (less than
107 7000) and were excluded from further analysis. The excluded samples also had lower values of
108 Good's coverage index than the remaining ones. The number of sequences in the remaining samples
109 ranged from 194 915 to 22 278 with an average of $103\,390 \pm 44\,630$ (mean \pm SD) sequences.

110

111 **Occurrence of *Heterobasidion* in sampled trees**

112 One of the unexpected findings of our study was that two OTUs assigned to the genus
113 *Heterobasidion* were present not only in diseased trees showing symptoms of wood decay, but also
114 in apparently healthy trees without decay symptoms (Fig. 1). Following this observation, the
115 sampled trees were classified as “symptomatic (with decay)” and “asymptomatic (without decay),
116 respectively.

117 Otu00011 was tentatively classified as *H. annosum*, whereas Otu00048 was assigned to *H.*
118 *parviporum*. Both species occur naturally in Finland. *H. parviporum* predominantly infects Norway
119 spruce trees, whereas *H. annosum* has broader host spectrum and infects both Scots pine and
120 Norway spruce. Several explanations can be proposed for the presence of *Heterobasidion* spp. in
121 asymptomatic trees. First, the entry of pathogen in these trees might have occurred relatively
122 recently, i.e. at the time point of the sample collection the trees were at the initial stages of the
123 disease development, with no detectable symptoms of wood decay. Alternatively, the lack of
124 disease symptoms despite presence of *Heterobasidion* could be related to the genetic resistance

125 background of each individual tree, allowing asymptomatic trees to restrict the fungal growth and
126 invasion. It is known that spruce trees show natural variation in their susceptibility to
127 *Heterobasidion* infection, which could probably explain the absence of disease symptoms despite
128 the identification of the pathogen in the sampled trees. Finally, if two detected OTUs assigned to
129 *Heterobasidion* in fact represented two different species, it is possible that they differed in their
130 virulence. Our data show that Otu00048 was more abundant in asymptomatic trees, whereas
131 Otu00011 had higher abundance in symptomatic trees (Table S1). Taking into account the last
132 observation, we consider the cross-contamination during the sample processing rather unlikely
133 sources of *Heterobasidion*-specific reads in samples from asymptomatic trees. We however cannot
134 rule out completely the possibility that at least some of obtained reads were due to the presence of
135 *Heterobasidion* spores, which landed on the samples surface during harvesting. However, the spore
136 load is expected to be equal for all samples, whereas our data show clear differences in the
137 abundance of two OTUs assigned to the genus *Heterobasidion* among symptomatic and
138 asymptomatic trees.

139

140 ***Richness, diversity and evenness of mycobiome communities of Norway spruce***

141 Quality-filtered fungal sequences were clustered into 4375 OTUs (excluding singletons). The sub-
142 sampled set used to calculate richness, diversity and evenness contained 4315 OTUs. The highest
143 richness of fungal communities in asymptomatic and symptomatic trees were observed in needles
144 and in roots, respectively. The bark had the lowest numbers of OTUs in both symptomatic and
145 asymptomatic trees (Fig. 2A). There were no significant differences in the fungal species richness
146 among asymptomatic and symptomatic trees in any of the sampled tissues. Needles had the highest
147 community diversity in both symptomatic and asymptomatic trees. The lowest community diversity
148 was observed in roots and in down stem in asymptomatic and in symptomatic trees, respectively
149 (Fig. 2B). However, no statistically significant difference in fungal diversity was found in any of the

150 regions among symptomatic and asymptomatic trees. The highest evenness of fungal communities
151 was found in bark of symptomatic trees and in needles of asymptomatic trees, whereas the lowest
152 evenness was observed in roots of both groups (Fig. 2C). Evenness in needles of asymptomatic
153 trees was significantly higher than that of symptomatic trees.

154 The sampled tissues of the spruce trees shared 738 (16.9%) of the total 4375 OTUs. The proportion
155 of the OTUs unique to a certain tissue ranged from 1.6% (69 OTUs; bark) to 13.3% (584 OTUs;
156 roots) (Fig. 2D).

157 The PCoA based on the relative OTUs abundance explained 28.5% of the observed variation and
158 showed distinct clusters for each of the sampled tree tissues (Fig. 3), which were confirmed by
159 PERMANOVA ($p < 0.001$ in all possible pairs). The detailed taxonomic analysis of OTUs detected
160 in specific Norway spruce tissues is presented in Supporting Notes 1 and 2 and in Supporting
161 Figures S1-S6.

162

163 *Impacts of health status on structure of fungal communities of Norway spruce*

164 We hypothesized that there are significant differences in microbial communities of asymptomatic
165 and symptomatic Norway spruce trees. This hypothesis was partially confirmed. There were no
166 significant differences among symptomatic and asymptomatic trees in the structure of fungal
167 communities inhabiting their needles, upper stem bark or roots. At the same time, the
168 PERMANOVA demonstrated that the structures of fungal communities associated with upper stem
169 and down stem of symptomatic and asymptomatic trees were significantly different ($p=0.001$ and
170 $p=0.011$, respectively) (Fig. S7). OTUs that significantly contributed to the shift in fungal
171 community structure among samples from symptomatic and asymptomatic trees are listed in Tables
172 S2 and S3. These results indicate that *Heterobasidion* infection has an effect on fungal communities
173 in the parts of the tree adjacent to the tissues colonized by the pathogen, but no significant effect on
174 more distant parts. However, it was demonstrated recently that *Heterobasidion* spp. infection

175 promotes mycorrhiza development in *Pinus pinea* (Zampieri et al., 2017), indicating that
176 *Heterobasidion* infection might have more profound effects. Several explanations could be
177 proposed for the lack of significant differences in the structure of fungal communities associated
178 with asymptomatic and symptomatic spruce trees in our experiment. First, mycorrhizal fungi
179 constituted only a small fraction in our dataset, and it might be due to our sampling strategy, as we
180 sampled suberized roots and did not collect fine roots. The results might be different if fine roots
181 colonized by ectomycorrhizal fungi were included in the analysis. Second, there might be
182 differences among different tree species in a way they react to *Heterobasidion* infection. Pine trees
183 in the experiment of (Zampieri et al., 2017) showed strong decline in vitality a few months after
184 inoculation. At the same time, sampled spruce trees showed no symptoms of infection except for
185 the heartwood decays, which could be observed only after tree felling. This is in line with the fact
186 that *Heterobasidion* infection in Norway spruce develops slowly and infected trees show little or no
187 symptoms. Thus, it is likely that changes in fungal communities in Norway spruce do not have a
188 systemic effect and are restricted to tissues adjacent to *Heterobasidion* wood decay, at least at the
189 early stages of infection.

190

191 ***OTUs with different relative abundance in asymptomatic and symptomatic trees***

192 The abundance of certain OTUs differed between asymptomatic and symptomatic trees. Some
193 saprotrophic (e.g., *Talaromyces* sp., *Trichoderma atroviridis*, *Penicillium* sp.) and wood-degrading
194 species (e.g., *Inonotus* sp., *S. sanguinolentum*, *A. areolatum*) had higher abundance in symptomatic
195 trees. However, many of them were detected in a limited number of trees. For example, OTU
196 classified as *Inonotus* sp. was abundant only in 2 out of 9 symptomatic trees, and *S. sanguinolentum*
197 and *A. areolatum* were abundant only in a single tree each. These observations might indicate that
198 trees infected by *Heterobasidion* become more susceptible to co-infection with other wood-
199 degrading fungi, but there might be additional factors that determine what particular species will

200 occupy an individual tree and, likely, the stage of the disease and its progression also have their
201 effect.

202 At the same time, the observed difference in abundance of a number of OTUs among asymptomatic
203 and symptomatic trees received statistical support in our analysis. Out of 50 OTUs with the highest
204 abundance, 10 OTUs showed differences of abundance in at least one of the sampled spruce tissues
205 (Tables 1 and S1). Nine of them were more abundant in asymptomatic trees. Only three of those
206 could be assigned to a certain species, namely *Hypogymnia tubulosa*, *Scoliciosporum umbrinum*
207 and *Phialocephala fortinii*. At the same time, Otu00002, classified as *Talaromyces* sp., was more
208 abundant in symptomatic trees. *Talaromyces* is a large genus of saprotrophic fungi frequently
209 isolated from various organic substrates, including plant litter. Some of them are potent producers
210 of secondary metabolites, and *T. flavus* is widely used in the biological control of soil-borne plant
211 pathogen. The corresponding OTU was abundant in all nine sampled symptomatic trees, but the
212 biological significance of this finding merits further investigation.

213 Among OTUs that were more abundant in asymptomatic trees, two belonged to lichens and one – to
214 the root endophyte *P. fortinii*. The higher abundance of *P. fortinii* in asymptomatic trees is
215 noteworthy. Dark septate endophytes of *P. fortinii* species complex are nearly ubiquitously present
216 in root of conifer trees and ericaceous plants (Grunig et al., 2008). Their biological role remains
217 largely unknown, but it was demonstrated that secondary metabolites produced by members of this
218 species complex have an inhibitory effect on plant pathogens (Tellenbach et al., 2013).

219 Additionally, it was shown that a *Phialocephala* isolate protects Norway spruce seedlings from *H.*
220 *parviporum* infection in *in vitro* experiments (Terhonen et al., 2016). In view of these observations,
221 the protective ability of the endophytes of *P. fortinii* species complex against *Heterobasidion*
222 infection in spruce trees deserves further investigation.

223 The higher abundance of two lichen species, *Hypogymnia tubulosa* and *Scoliciosporum umbrinum*,
224 in samples from asymptomatic trees is remarkable, too. Lichens are well-known as producers of

225 biologically active secondary metabolites (Yilmaz et al., 2005). Further work will be required to
226 elucidate whether identified lichen species have any antagonistic properties against root and butt rot
227 pathogens.

228 To the best of our knowledge, only few studies have addressed the effect of plant pathogens on the
229 composition of plant microbiota. This is also the first comprehensive report on mycobiome of
230 different anatomic regions of Norway spruce documented in a single study. The results of our work
231 provide additional evidence that disease progression causes significant changes in the structure of
232 resident microbial communities. However, the situation is likely more complicated, as the effect of
233 the pathogen is rather localized and it might not affect more distant parts of the tree. The observed
234 higher abundance of the dark septate endophyte *P. fortinii* in asymptomatic trees justifies further
235 studies on the role of *Phialocephala* species as natural antagonists to *Heterobasidion* spp. Our data
236 demonstrate the suitability of *Heterobasidion* – spruce pathosystem to address the fundamental
237 question of interactions between plant pathogens and the resident microbiota.

238

239 **Experimental procedures**

240 *Study sites and sample collection*

241 Three Norway spruce (*Picea abies* (L.) Karst.)-dominated forest sites in the municipality of
242 Mäntsälä (Uusimaa region, Southern Finland) were chosen for sampling. The sites are located in
243 privately-owned managed forest and are distributed in three selected plots: (1) plot 1 (60°44'51"N,
244 25°13'17"E), (2) plot 2 (60°45'11"N, 25°13'24"E, and (3) plot 3 (60°45'15" N, 25°15'34" E).

245 All three sampling plots are representative examples of managed spruce forest used for commercial
246 timber production and growing at comparable conditions. Spruce stands at the selected sites were
247 naturally regenerated and of the same age (approximately 55 years at the time of sampling). All
248 three plots are located within an area with a relatively high incidence of *Heterobasidion* infection.

249 The elevation of the sites ranges from 87 to 95 m above sea level. Sample collections were
250 conducted in May 2016.

251 Our sampling was performed simultaneously with the tree harvesting. The samples were collected
252 immediately after tree felling. In this way, we could clearly distinguish asymptomatic and
253 symptomatic trees based on presence or absence of wood decay column at stump height.

254 In each plot, six spruce trees were selected: three trees showing symptoms of *Heterobasidion*-
255 induced wood decay (further referred to as symptomatic trees) (Fig. 1A), and three trees without
256 decay symptoms (further referred to as asymptomatic trees) (Fig. 1B). Diameter of selected trees
257 ranged from 40 to 64 cm. In total, samples of suberized roots, down stem
258 (bark+sapwood+heartwood), upper stem sapwood (referred to as upper stem), upper stem bark
259 (referred to as bark), and needles (Fig. 1) were taken from a total of nine asymptomatic and nine
260 symptomatic trees.

261

262 ***DNA extraction, amplification of ITS2 region and sequencing***

263 Spruce tissues samples were surface-sterilized with 70% ethanol prior to DNA extraction. Total
264 DNA was extracted from grinded spruce tissues following a standard cetyl–trimethyl ammonium
265 bromide (CTAB) method (Chang et al., 1993) with modifications described in (Terhonen et al.,
266 2011). The concentrations and purity of the isolated DNA were measured using NanoDrop ND-
267 1000 spectrophotometer (Thermo Fisher Scientific, USA).

268 PCR amplification of the fungal ITS2 region and sequencing were performed in the facilities of the
269 Institute of Biotechnology (BI, University of Helsinki, Finland). The use of ITS2 region for
270 metabarcoding of fungal communities was advocated in recent reports (Tedersoo et al., 2015;
271 Tedersoo and Lindahl, 2016). Prior to sequencing, a nested PCR was performed. In the first PCR
272 round, extracted DNA samples were used as templates, and the amplification was performed using
273 the primers gITS7 and ITS4 (Ihrmark et al., 2012) containing partial TruSeq adapter sequences at

274 the 5'ends (ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T and GTG ACT
275 GGA GTT CAG ACG TGT GCT CTT CCG ATC T). The combination of primers gITS7 and ITS4
276 is widely used in metabarcoding of fungal communities. They have known limitations, as there are
277 some mismatches between the sequences of the primers and the corresponding sequences of rRNA
278 genes in many Tulasnellaceae, Archaeorhizomycetes and Microsporidia. A newly proposed pair of
279 primers, which should have better performance, was published after the completion of the
280 experimental part of our project (Taylor et al., 2016), and we were not able to assess its suitability.
281 In the second round of PCR, full-length TruSeq P5 and Index containing P7 adapters were used as
282 primers and the products of the first PCR were used as templates. The PCR products were purified
283 and sequenced with Illumina MiSeq platform. Raw sequences were deposited at the European
284 Bioinformatics Institute (EBI) under project accession number PRJEB21787
285 (<http://www.ebi.ac.uk/ena/data/view/PRJEB21787>).

286

287 *Pre-processing and analysis of ITS2 sequences*

288 The raw ITS2 sequences were pre-processed at BI. The read quality was checked with FastQC
289 (Andrews, 2010). Adapter and barcode sequences were removed using Cutadapt (Martin, 2011).
290 The pre-processed data were analyzed using the mothur standard operation pipeline (SOP, v.1.37.6)
291 (Schloss et al., 2011) with the modifications described earlier (Sun et al., 2016). Briefly, pair-end
292 reads were converted to contigs with minimum overlap of 25bp. Sequences containing ambiguous
293 bases (N) and homopolymers longer than eight nucleotides were removed. Processed sequences
294 were pre-clustered with a distance of 2 nt/100 nt using a pseudosingle-linkage algorithm (Huse et
295 al., 2010). Each sequence that passed quality filtering was truncated to a 230-bp length after primer
296 and tag removal. All potential chimeric sequences were identified using the mothur-embedded
297 UCHIME algorithm (Edgar et al., 2011) and removed. Unique sequences were pairwise aligned
298 using the Needleman method (Needleman and Wunsch, 1970). The aligned distance matrices were

299 clustered into operational taxonomic units (OTUs) using the average neighbor algorithm and 97%
300 sequence similarity. All global singletons (OTUs containing only one sequence across all samples)
301 were removed, and the most abundant sequence in each OTU was selected to be the representative
302 sequence. The sequences and OTUs were assigned to taxa using the mothur-formatted UNITE
303 taxonomy reference database (UNITE+INSD, Version 7.2) (Koljalg et al., 2013) with an 80%
304 bootstrap confidence threshold in mothur (Wang et al., 2007).

305 To correct the difference in sample size and ensure comparable estimators across samples, a subset
306 of 22 500 sequences per sample (minimum number of sequences recovered among all samples) was
307 randomly selected to calculate the diversity and to compare the community structure. The following
308 parameters were calculated for all samples: observed and estimated fungal richness (Chao 1),
309 diversity (Inverse Simpson's complement – 1-D), evenness (Simpson's equitability - ED) and
310 Good's coverage (complement of the ratio between local singleton OTUs and the total sequence
311 count).

312 Venn diagrams were constructed from the presence/absence transformed data (without singletons)
313 with venn function from gplots (Warnes et al., 2016). Non-parametric Kruskal-Wallis tests with
314 Hodges-Lehmann estimate were used to identify differences in diversity, species richness and
315 evenness among the sampled tissues in symptomatic and asymptomatic trees. Principal coordinates
316 analysis (PCoA) was used to visualize the fungal community structure with Bray-Curtis similarity
317 using relative abundances of OTUs in PRIMER v.6 (Clarke and Gorley, 2006) with the add-on
318 package of PERMANOVA + (Anderson et al., 2008). Prior to PCoA, the data were square root
319 transformed to meet the analysis criteria. Subsequently, a PERMANOVA test was used to
320 determine the significant difference in community structure between different regions in the tree.

321

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327

328 **Conflict of interest statement**

329 The authors declare no conflict of interest.

330

331 **References**

- 332 Anderson, M., Gorley, R.N., and Clarke, R.K. (2008) *Permanova+ for Primer: Guide to Software*
333 *and Statistical Methods*: Primer-E Limited.
- 334 Andrews, S. (2010) FastQC: a quality control tool for high throughput sequence data. In.
- 335 Ardanov, P., Sessitsch, A., Haggman, H., Kozyrovska, N., and Pirttila, A.M. (2012)
336 *Methylobacterium-Induced Endophyte Community Changes Correspond with Protection of Plants*
337 *against Pathogen Attack. Plos One 7.*
- 338 Asiegbu, F.O., Adomas, A., and Stenlid, J. (2005) Conifer root and butt rot caused by
339 *Heterobasidion annosum* (Fr.) Bref. s.l. *Molecular Plant Pathology 6*: 395-409.
- 340 Backman, P.A., and Sikora, R.A. (2008) Endophytes: An emerging tool for biological control.
341 *Biological Control 46*: 1-3.
- 342 Blumenstein, K., Albrechtsen, B.R., Martin, J.A., Hultberg, M., Sieber, T.N., Helander, M., and
343 Witzell, J. (2015) Nutritional niche overlap potentiates the use of endophytes in biocontrol of a tree
344 disease. *Biocontrol 60*: 655-667.
- 345 Bulgarelli, D., Schlaeppli, K., Spaepen, S., Ver Loren van Themaat, E., and Schulze-Lefert, P.
346 (2013) Structure and functions of the bacterial microbiota of plants. *Annu Rev Plant Biol 64*: 807-
347 838.

- 348 Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F. et
349 al. (2012) Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial
350 microbiota. *Nature* **488**: 91-95.
- 351 Chang, S., Puryear, J., and Cairney, J. (1993) A simple and efficient method for isolating RNA from
352 pine trees. *Plant Molecular Biology Reporter* **11**: 113-116.
- 353 Clarke, K.R., and Gorley, R.N. (2006) *PRIMER v6: User Manual/Tutorial*. Plymouth: PRIMER-E.
- 354 Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., and Knight, R. (2011) UCHIME improves
355 sensitivity and speed of chimera detection. *Bioinformatics* **27**: 2194-2200.
- 356 Grunig, C.R., Queloz, V., Sieber, T.N., and Holdenrieder, O. (2008) Dark septate endophytes (DSE)
357 of the *Phialocephala fortinii* s.l. - *Acephala applanata* species complex in tree roots: classification,
358 population biology, and ecology. *Botany-Botanique* **86**: 1355-1369.
- 359 Hardoim, P.R., van Overbeek, L.S., Berg, G., Pirttila, A.M., Compant, S., Campisano, A. et al.
360 (2015) The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining
361 Functioning of Microbial Endophytes. *Microbiol Mol Biol Rev* **79**: 293-320.
- 362 Huse, S.M., Welch, D.M., Morrison, H.G., and Sogin, M.L. (2010) Ironing out the wrinkles in the
363 rare biosphere through improved OTU clustering. *Environmental Microbiology* **12**: 1889-1898.
- 364 Ihrmark, K., Bodeker, I.T., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J. et al. (2012)
365 New primers to amplify the fungal ITS2 region--evaluation by 454-sequencing of artificial and
366 natural communities. *FEMS Microbiol Ecol* **82**: 666-677.
- 367 Koljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F.S., Bahram, M. et al. (2013)
368 Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology* **22**:
369 5271-5277.
- 370 Lebeis, S.L. (2015) Greater than the sum of their parts: characterizing plant microbiomes at the
371 community-level. *Curr Opin Plant Biol* **24**: 82-86.

- 372 Lundberg, D.S., Lebeis, S.L., Paredes, S.H., Yourstone, S., Gehring, J., Malfatti, S. et al. (2012)
373 Defining the core *Arabidopsis thaliana* root microbiome. *Nature* **488**: 86-90.
- 374 Martin, J.A., Witzell, J., Blumenstein, K., Rozpedowska, E., Helander, M., Sieber, T.N., and Gil, L.
375 (2013) Resistance to Dutch Elm Disease Reduces Presence of Xylem Endophytic Fungi in Elms
376 (*Ulmus* spp.). *Plos One* **8**.
- 377 Martin, M. (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads.
378 *EMBnet journal* **17**: pp. 10-12.
- 379 Mejia, L.C., Rojas, E.I., Maynard, Z., Van Bael, S., Arnold, A.E., Hebbbar, P. et al. (2008)
380 Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. *Biological Control* **46**: 4-14.
- 381 Needleman, S.B., and Wunsch, C.D. (1970) A General Method Applicable to Search for
382 Similarities in Amino Acid Sequence of 2 Proteins. *Journal of Molecular Biology* **48**: 443-+.
- 383 Oliva, J., Bernat, M, Stenlid, J. (2013). Heartwood stump colonisation by *Heterobasidion*
384 *parviporum* and *H. annosum* s.s. in Norway spruce (*Picea abies*) stands. *Forest Ecology and*
385 *Management* **295**: 1- 10.
- 386 Pautasso, M., Schlegel, M., and Holdenrieder, O. (2015) Forest Health in a Changing World.
387 *Microbial Ecology* **69**: 826-842.
- 388 Porrás-Alfaro, A., and Bayman, P. (2011) Hidden fungi, emergent properties: endophytes and
389 microbiomes. *Annu Rev Phytopathol* **49**: 291-315.
- 390 Redford, A.J., Bowers, R.M., Knight, R., Linhart, Y., and Fierer, N. (2010) The ecology of the
391 phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves.
392 *Environ Microbiol* **12**: 2885-2893.
- 393 Schloss, P.D., Gevers, D., and Westcott, S.L. (2011) Reducing the Effects of PCR Amplification
394 and Sequencing Artifacts on 16S rRNA-Based Studies. *Plos One* **6**.

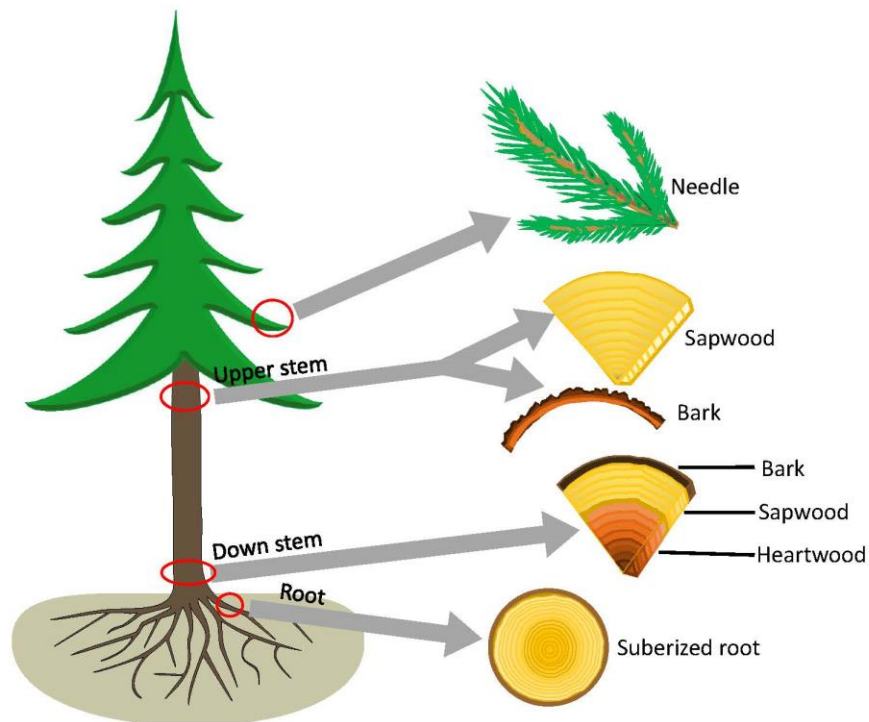
- 395 Sun, H., Santalahti, M., Pumpanen, J., Koster, K., Berninger, F., Raffaello, T. et al. (2016) Bacterial
396 community structure and function shift across a northern boreal forest fire chronosequence.
397 *Scientific Reports* **6**.
- 398 Taylor, D.L., Walters, W.A., Lennon, N.J., Bochicchio, J., Krohn, A., Caporaso, J.G., and
399 Pennanen, T. (2016) Accurate Estimation of Fungal Diversity and Abundance through Improved
400 Lineage-Specific Primers Optimized for Illumina Amplicon Sequencing. *Appl Environ Microbiol*
401 **82**: 7217-7226.
- 402 Tedersoo, L., and Lindahl, B. (2016) Fungal identification biases in microbiome projects. *Environ*
403 *Microbiol Rep.*
- 404 Tedersoo, L., Anslan, S., Bahram, M., Polme, S., Riit, T., Liiv, I. et al. (2015) Shotgun
405 metagenomes and multiple primer pair-barcode combinations of amplicons reveal biases in
406 metabarcoding analyses of fungi. *Mycokeys*: 1-43.
- 407 Tellenbach, C., Sumarah, M.W., Grunig, C.R., and Miller, J.D. (2013) Inhibition of Phytophthora
408 species by secondary metabolites produced by the dark septate endophyte *Phialocephala europaea*.
409 *Fungal Ecology* **6**: 12-18.
- 410 Terhonen, E., Sipari, N., and Asiegbu, F.O. (2016) Inhibition of phytopathogens by fungal root
411 endophytes of Norway spruce. *Biological Control* **99**: 53-63.
- 412 Terhonen, E., Marco, T., Sun, H., Jalkanen, R., Kasanen, R., Vuorinen, M., and Asiegbu, F. (2011)
413 The Effect of Latitude, Season and Needle-Age on the Mycota of Scots Pine (*Pinus sylvestris*) in
414 Finland. *Silva Fennica* **45**: 301-317.
- 415 Turner, T.R., James, E.K., and Poole, P.S. (2013) The plant microbiome. *Genome Biol* **14**: 209.
- 416 Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015) The
417 importance of the microbiome of the plant holobiont. *New Phytol* **206**: 1196-1206.

- 418 Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Naive Bayesian classifier for rapid
419 assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental*
420 *Microbiology* **73**: 5261-5267.
- 421 Warnes, G.R., Bolker, B., Bonebakker, L., Gentleman, R., Liaw, W.H.A., Lumley, T. et al. (2016)
422 Package ‘gplots’. In *Various R Programming Tools for Plotting Data*.
- 423 Weinert, N., Piceno, Y., Ding, G.C., Meincke, R., Heuer, H., Berg, G. et al. (2011) PhyloChip
424 hybridization uncovered an enormous bacterial diversity in the rhizosphere of different potato
425 cultivars: many common and few cultivar-dependent taxa. *FEMS Microbiol Ecol* **75**: 497-506.
- 426 Yilmaz, M., Tay, T., Kivanc, M., Turk, H., and Turk, A.O. (2005) The antimicrobial activity of
427 extracts of the lichen *Hypogymnia tubulosa* and its 3-hydroxyphysodic acid constituent. *Zeitschrift*
428 *Fur Naturforschung C-a Journal of Biosciences* **60**: 35-38.
- 429 Zampieri, E., Giordano, L., Lione, G., Vizzini, A., Sillo, F., Balestrini, R., and Gonthier, P. (2017)
430 A nonnative and a native fungal plant pathogen similarly stimulate ectomycorrhizal development
431 but are perceived differently by a fungal symbiont. *New Phytol* **213**: 1836-1849.

432

433 **Table and Figure legends**

434 **Table 1.** OTUs from the 50 most abundant OTUs in the combined dataset showing significant
435 differences in abundance among asymptomatic and symptomatic trees.



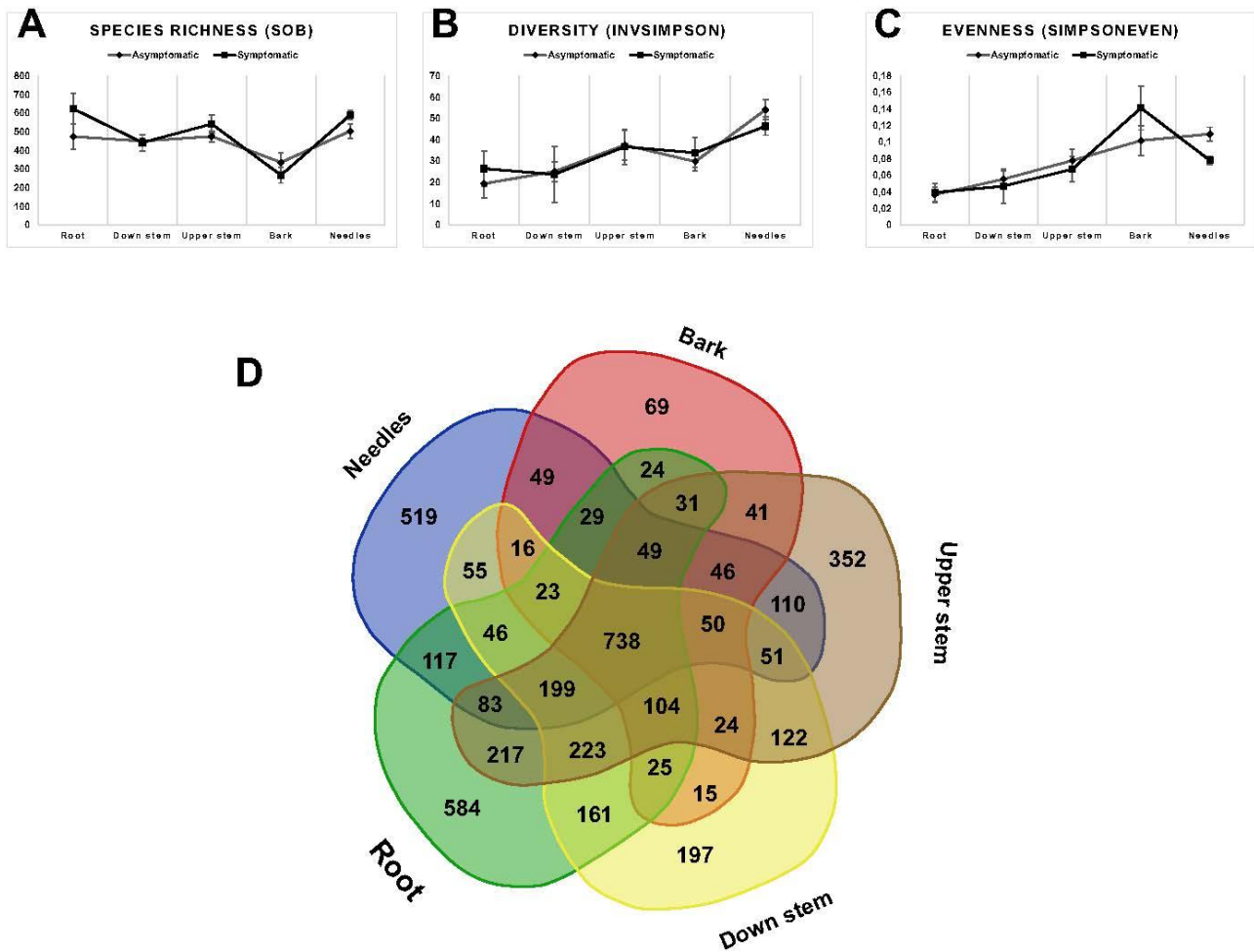
436

437 **Figure 1.** Representative pictures of sampled Norway spruce trees from the sampling site. (A) A
 438 tree classified as symptomatic, with extensive wood decay caused by *Heterobasidion* sp. (B) An
 439 asymptomatic tree without visible symptoms of wood decay. (C) Schematic diagram illustrating the
 440 Norway spruce tissues sampled for the analysis of associated mycobiota.

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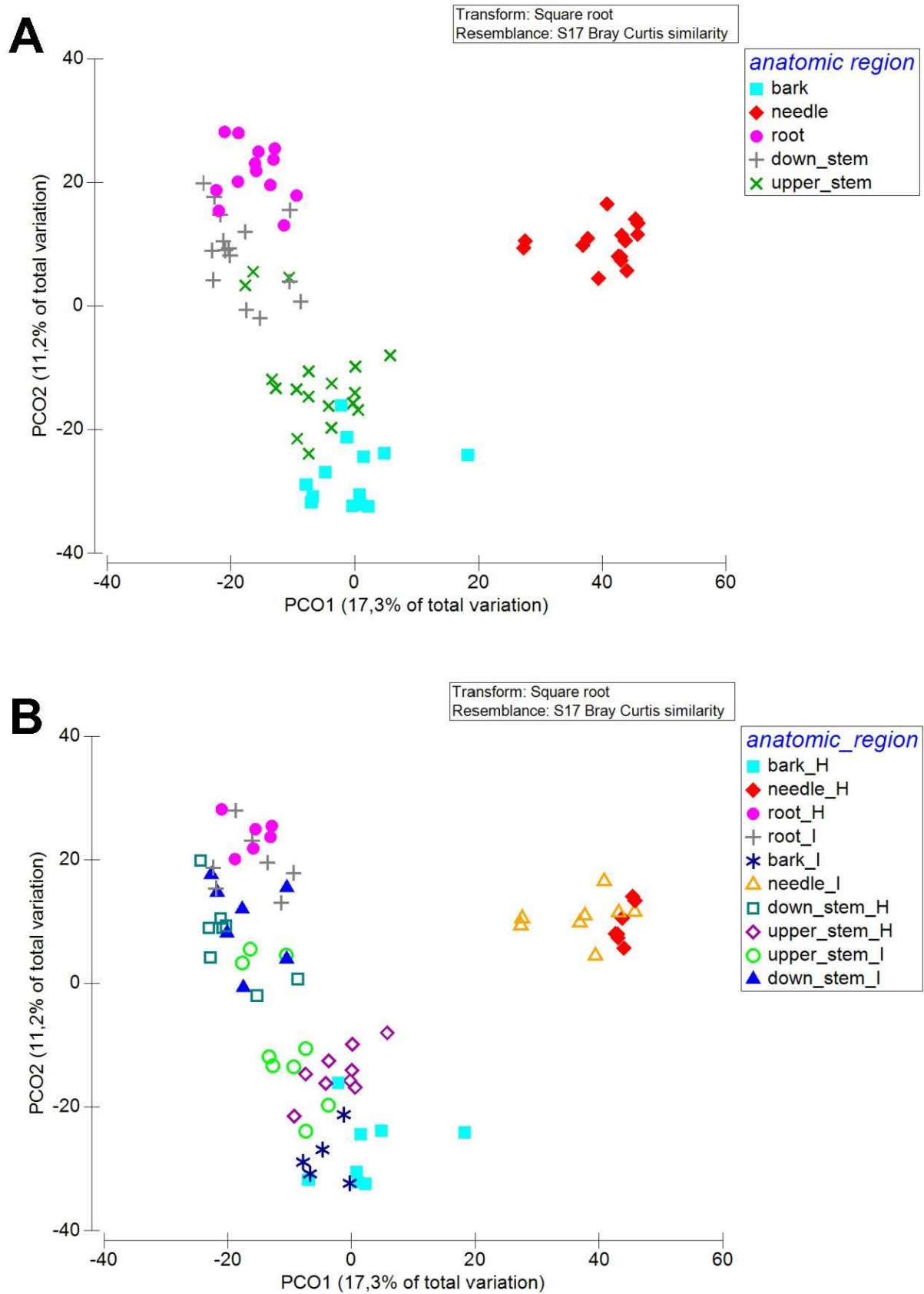
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445 **Figure 2.** (A) Fungal richness (observed OTUs), (B) diversity and (C) evenness indices for ITS2
 446 region libraries from different regions in symptomatic and asymptomatic trees. The mean values
 447 and standard errors are depicted on the graphs. (D) Unique and shared OTUs between different
 448 regions of the tree. The Venn diagram was constructed from the presence/absence transformed
 449 OTUs data (4375 OTUs without singletons).



450

451 **Figure 3.** Principal coordinates analysis (PCoA) based on the relative abundance of fungal OTUs,
 452 showing the differences in fungal community structure in different anatomic regions of the studied

453 Norway spruce trees. (A) Origin of the samples (either from symptomatic or asymptomatic trees) is
454 not indicated. (B) Samples from symptomatic (I) and asymptomatic (H) trees are indicated with
455 different symbols.

456

457 **Supporting Information**

458 **Supporting Note S1.** Mycobiome composition and distribution among Norway spruce tissues.

459

460 **Supporting Note S2.** Dominant OTUs associated with Norway spruce tissues.

461

462 **Supporting Table S1.** Abundance (in %) of the top 50 fungal species in symptomatic and
463 asymptomatic trees

464

465 **Supporting Table S2.** List of OTUs that significantly contributed to the shift in the structure of
466 fungal communities of upper stem among asymptomatic and symptomatic trees.

467

468 **Supporting Table S3.** List of OTUs that significantly contributed to the shift in the structure of
469 fungal communities of down stem among asymptomatic and symptomatic trees.

470 **Supporting Figure S1.** Abundance of fungal phyla (% of the total number of reads) in different
471 tissues of the sampled spruce trees. Ascomycota is the most abundant group in all tissues, but their
472 abundance is the highest in needles, whereas abundance of Basidiomycota increases in woody
473 tissues. Abundance of the remaining groups in all sampled tissues was below 1% of total number of
474 reads. Only phyla with the relative abundance $>0.01\%$ are shown on the diagrams.

475

476 **Supporting Figure S2.** Relative abundance of fungal phyla in different tissues of symptomatic (S)
477 and asymptomatic (A) spruce trees. Phyla with the relative abundance of >0.01% are depicted in
478 each diagram; phyla with the lower abundance are omitted.

479

480 **Supporting Figure S3.** Relative abundance of fungal classes in different tissues of the sampled
481 Norway spruce trees. Only classes with the relative abundance of >0.5% are depicted.

482

483 **Supporting Figure S4.** Relative abundance of fungal orders in various tissues of the sampled
484 Norway spruce trees. Only orders with the relative abundance of >1% are depicted.

485

486 **Supporting Figure S5.** Abundance of the 20 most abundant OTUs (% of total read counts). (A)
487 Abundance in the combined dataset. (B) Abundance in specific tree tissues.

488

489 **Supporting Figure S6.** Ten most abundant OTUs in each of the sampled tissue of asymptomatic
490 (A), (C), (E), (G), (I) and symptomatic (B), (D), (F), (H), (J) Norway spruce trees.

491

492 **Supporting Figure S7.** Principal coordinates analysis (PCoA) based on the relative abundance of
493 fungal OTUs, showing the differences in fungal community structure in upper stem and lower stem
494 of the studied Norway spruce trees.

495

496