1 **Determining species diversity of microfungal communities in forest**

2 **tree roots by pure-culture isolation and DNA sequencing**

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Abstract

16

17 Pure-culture isolation from roots was compared with transformation of total DNA from

18 roots followed by sequence analysis of ITS 1/2 rDNA of representative clones as

19 methods for determining the abundance and composition of microbiota in roots of *Betula*

20 *pendula, Fagus sylvatica, Larix decidua, Prunus serotina* and *Quercus petraea*. The

21 results from the two methods differed greatly, with no overlap between the taxa

22 identified. Pure-culture isolation revealed greater species diversity (47 taxa), the most

23 frequent fungi being *Ascomycota*, including *Penicillium* spp., *Phialocephala fortinii*,

24 *Pochonia bulbillosa, Sesquicillium candelabrum* and *Trichoderma* spp. Transformation

25 of total DNA and sequencing revealed less diversity (22 taxa), the most frequent taxa

26 being *Basidiomycota*, including *Coprinus fissolanatus* and *Mycena* spp*.,* and

27 *Ascomycota*, including *Podospora–Schizothecium* spp., *Helgardia anguioides* and

28 *Microdochium* sp. Communities characterized by either method showed slightly greater

29 fungal diversity and less species dominance on *F. sylvatica* than on roots of other trees,

30 whilst DNA sequencing showed least diversity and greatest species dominance on *Q.*

31 *petraea*.

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1. Introduction

Fungi and fungus-like organisms inhabiting roots play a key role in forest ecosystems and constitute an essential part of forest biodiversity. Together with the soil and rhizosphere organisms they represent a significant biological potential. They form associations with plants that are beneficial, pathogenic or neutral. They have a significant impact on plant health, growth and productivity (Peace, 1962; Wilcox, 1983; Mańka, 1998; Sinclair et al., 1987).

Most studies of fungi inhabiting trees roots have focussed on mycorrhizal species (Clapp et al., 1995; Gloud, 2000; Dahlberg, 2001; Horton and Bruns, 2001; Taylor, 2002; Allen et al., 2003; Brundrett, 2004; Bruns and Shefferson, 2004; Koide and Mosse, 2004; Simard and Durall, 2004; Menkis et al., 2005). Only a few studies have investigated other than mycorrhizal diversity. Kwaśna (1996 a, b; 1997 a, b; 2001, 2002, 2003, 2004) investigated microfungal communities on/in tree roots in relation to their involvement in resistance to root pathogens and changes in their activity resulting from different forest management practices. Microfungal communities in roots have also been studied in relation to decline or death of *Fraxinus* and *Quercus* trees in Europe (Jung et al., 1996; Przybył, 2002; Halmschlager and Kowalski, 2004), and to decay of conifer seedlings (Hamelin et al., 1996; Kernaghan et al., 2003; Menkis et al., 2006).

Information on density, diversity and functioning of fungal communities in tree roots still remains limited. Greater understanding of the processes of fungal establishment, survival, and relationships within communities, particularly in the early stages of forest successions, is required and has implications for future forest practices (Horton et al., 1999).

Effective detection and classification are important prerequisites for studies on the ecology and behaviour of microorganisms. Morphological species recognition (MSR) (Taylor et al., 2000), used in classical taxonomic classification, based on the

morphology of sexual or asexual reproductive structures, has several shortcomings. It often does not consider adequately the within–taxon variability and is useless in cases of morphological similarity, indistinctness, sterility of fungi and the presence of non-culturable organisms (Wetzel et al., 1996; Harney et al.,1997; Sieber, 2002). Therefore misclassification is possible. Despite that, the classical approach has led to recognition of the ecology and activity of some important tree-root fungi, including pathogens (Holdenrieder and Sieber, 1992; Guillaumin et al., 1993; Erwin and Ribiero, 1996; Woodward et al., 1998; Vettraino et al., 2002). Phylogenetic species recognition (PSR), with the concept of genealogical concordance, now offers additional tools for species definition and recognition (Taylor et al., 2000). PCR–based molecular methods used increasingly in studies on the diversity of root microbiota can detect latent pathogens, slow-growing endophytes and non–culturable species (Donaldson et al., 1995; Hamelin et al., 1996; Hantula et al., 2002; Kernaghan et al., 2003; Menkis et al., 2006).

Identification based on the 18S rRNA, useful in bacteria, is problematic in fungi and mostly limited to genus or family level. This is because of the lack of variation in conserved regions within 18S rRNA, resulting from the relatively short period of fungal evolution compared with that of bacteria (Hugenholz and Pace, 1996). This makes the identification of closely related members in most fungal genera impossible. Therefore reference sequences for fungal 18S rRNA in databases are limited, with interest in other sequences, such as the ITS rRNA region, increasing.

The ITS 1/2 rRNA (internal transcribed spacer) is the non-coding rRNA region located between the 18S rRNA and 28S rRNA genes and incorporating the 5.8S rRNA gene. Its advantage is a fast rate of evolution, resulting in greater sequence variation between closely related species. Fungal ITS sequences generally provide good taxonomic resolution and so are useful in studies on relationships between fungi at lower taxonomic levels (Gardes and Bruns, 1993: Cullings and Vogler, 1998; Redecker et al., 1999; Lord et al., 2002; Anderson et al., 2003 a; Roose–Amsaleg et al., 2004).

Cloning and sequencing of ITS 1/2 rDNA PCR products have shown that the alternative classical method provides only a selective view of fungal diversity in soil (Borneman and Hartin, 2000; Viaud et al., 2000; Anderson and Cairney, 2004; Hunt et al., 2004). Cloning and sequencing 18S rDNA and ITS 1/2 rDNA from fungal communities in roots of grasses showed high diversity in the mycobiota and resulted in the discovery of novel fungal lineages at higher taxonomic levels (Vandenkoornhuyse ey al., 2002; Neubert et al., 2006). All known fungal phyla were represented and a large

99 proportion of fungi detected could not be related to any known rDNA gene sequence.
100 This raises questions about fungal diversity on/in roots of other plants, about the
101 ecological implications of the occurrence and roles of novel taxa in specific ecological
102 niches.

103   This prompted a study on fungal communities from tree roots in which a classical
104 approach (isolation, culturing and identification of fungi by morphology) and a molecular
105 approach (isolation of total DNA from roots and amplification, cloning and sequencing of
106 ITS 1/2 rDNA) were both applied to limit the possibility of overlooking components of
107 fungal biodiversity. The objective was to demonstrate and compare biodiversities of the
108 mycobiota of tree roots using the two different methods. This was intended to increase
109 our understanding of (i) relationships between plants and the mycobiota, (ii) the type of
110 association involved, whether an active interaction resulting from nutritional preferences
111 or passive and accidental contact, and (iii) spatial heterogeneity of mycobiota. We
112 hypothesized that the two methods would identify two different community structures
113 from each root system but with many species overlapping. Sampling different tree
114 species of the same age and from one location was intended to eliminate the effects of
115 environmental differences and ensure similarity of habitat. Implications for the ecological
116 significance of the composition and diversity of the communities identified by each
117 method might then be obtained.
118

119 ## 2. Materials and methods
120

121 *2.1. Sampling site*
122

123 A root complex was collected from the B horizon (20–30 cm deep) from each of five 6–
124 year–old trees of five species: *Betula pendula* Roth., *Fagus sylvatica* L., *Larix decidua*
125 Mill., *Prunus serotina* Ehrh. and *Quercus petraea* Liebl. The trees were grown within a
126 4700–ha conifer–hardwood complex, after *Pinus sylvestris* L., in the Zielonka Forest
127 District, western Poland (17° 10' E, 52° 50' N). The different species were evenly
128 distributed among each other. The distance between sampled plants was 5–6 m. The
129 soil profile was a podzol with very dark mineral horizon rich in organic matter
130 (cryptopodzol). The thickness of the humus horizon was (8.5–) 10 (–12.5) cm, with a pH
131 value in water of 4.1 and a C/N ratio of about 30. The mineral soil was a sandy loam

132 with particle fractions: 3% gravel 20 mm, 7% gravel 6 mm, 28% sand, 33% fine sand,

133 21% silt, 8% clay. Humus stains reached a depth of 40 cm and decalcification was

134 observed below 200 cm. The sampled horizon had a weak structure and was coarsely

135 grained with sands. The physical and chemical properties of the soil were similar under

136 all trees sampled.

137   The ground–cover vegetation was mainly *Deschampsia flexuosa* (L) Trin.,

138 *Brachypodium* and *Calamagrostis epigeios* (L.) Roth.

139

140 *2. 2. Root samples*

141

142 Roots were shaken to remove loosely adhering soil, washed three times for 10 min in

143 sterile distilled water and dried on blotting paper. Fifty pieces of thin, suberized roots, 1

144 cm long and 2–5 mm in diameter, were cut off from five root complexes of each tree

145 species (10 pieces from each complex). Twenty-five pieces were used for DNA

146 extraction and another 25 for fungal isolation in culture.

147

148 *2. 3. DNA extraction, amplification and sequencing*

149 Root pieces were freeze–dried and ground in liquid nitrogen. The total DNA was

150 extracted using the method of Ward et al. (2005), followed by additional purification with

151 SureClean (Bioline Ltd, London, Cat. No. BIO37042). PCR amplification of the ITS 1/2

152 rDNA was done with DNA diluted (10^{-2}) in deionized water. Primers used were: ITS 4 (5'

153 TCC TCC GCT TAT TGA TAT GC) and ITS 5 (5' GGA AGT AAA AGT CGT AAC AAG

154 G) (White et al., 1990). Each 25 µl PCR mixture consisted of 0.2 µM of each primer,

155 0.25 U of *Taq* polymerase (MBI Fermentas, St. Leon–Rot, Germany), buffer (10 mM

156 Tris–HCl pH 8.8, 50 mM KCl, 0.08% Nonidet P–40, 0.1 mg ml^{-1} BSA, 1.5 mM MgCl$_2$),

157 0.2 mM deoxyribonucleoside triphosphates (dNTPs) and 2 µl (200 ng) diluted total DNA.

158 Cycling conditions were: an initial denaturation at 94°C for 10 min, followed by 30 cycles

159 of 94°C for 30 s, 42°C for 1 min and 72°C for 2 min, and a final extension of 72°C for 10

160 min. The PCR products were checked by electrophoresis of 5 µl of product in a 1%

161 agarose gel containing ethidium bromide (0.5 µg ml^{-1}) to stain the DNA. PCR products

162 were purified using the MinElute PCR purification kit (Qiagen, Crawley, UK) and cloned

163 into pGEM–T Easy (Promega Corporation Madison, WI, USA) following the

manufacturer's instructions. The rDNA amplicons from each tree species were used to generate individual libraries. Inserts (100 for each library) were primarily selected in blue/white screening on X–gal medium. For each insert a small amount of culture from a transfected cell was added to 300 µl 10 mM Tris pH 8, boiled for 10 min and centrifuged. Each insert was next amplified by PCR with primers ewfitsrev 1 (5' CTC CGC TTA TTG ATA TGC TTA AAC) and ITS 5 using the protocol described above. The amplified ITS 1/2 rDNA region included 600–650 bp. Representative clones were selected in two separate RFLP analyses of ITS 1/2 rDNA with *Cfo*I and *Hae*III restriction enzymes. Digestion was carried out at 37°C for 20 h. The products were electrophoresed in gels (2% NuSieve agarose, Cambrex, BioScience, Wokingham, UK + 1% standard agarose) in 1 x TBE stained with 0.5 µg ml^{-1} ethidium bromide.

Restriction digestion patterns were compared for each library. DNA band sizes were determined by comparison with bands of a *ΦX*174 DNA *Hae*III digest and the Low DNA Mass Ladder (Invitrogen Ltd, Paisley, UK) using GeneTools gel analysis Software, (Syngene). An operational taxonomic unit (OTU) was defined as a group of clones with identical restriction digestion pattern. All members of a single OTU in an individual library were considered to belong to the same taxon. Plasmid DNA was prepared from representative clones from each OTU with QIAprep spin miniprep kit (Qiagen, Crawley, UK).

Plasmids were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (AB Applied Biosystems, Foster City, CA 94404, USA) using primer ITS 1 (5' TCC GTA GGT GAA CCT GCG G) and M 13 fwd (5' GCC AGG GTT TTC CCA GTC ACG A). The purified products were run at the DNA Sequencing Facility, Oxford University, UK (http://polaris.bioch.ox.ac.uk/dnaseq/index.cfm). Each sequence was identified to the lowest taxonomic rank common to all of the top BLAST hits using the taxonomy of Kirk et al. (2001).

Chimeric sequences, which comprised at least two partial sequences resulting in cross-over artefacts, were excluded on the basis of variability introduced by them within a set of reference sequences. The reference sequences used were the closest relatives to the chimeric sequence. They were obtained from GenBank database with BLAST. A comparison of reference sequences provided variability within references. This was compared with the variability between chimeric and reference sequences. Comparisons were performed on fragments of the full-length sequences. Multiple alignments were

197   performed by CLUSTALW (Thompson et al., 1994) followed by manual inspection of its
198   results. The process of alignment and chimera evaluation was automated by a program
199   Ccode   (Chimera   and   cross–over   detection   and   evaluation)
200   (http://www.irnase.csic.es/users/jmgrau/index.html).

201   DNA sequences were assembled using the STADEN package (Medical Research
202   Council, Laboratory of Molecular Biology, Cambridge, UK). Representative sequences
203   for each OTU were queried against GenBank database using BLAST. Sequences with
204   most similarity were downloaded from GenBank (Table 1) and used for alignment using
205   the GCG programs PILEUP (gap creation penalty = 4, gap extension penalty = 0.2) and
206   CLUSTALX (Thompson et al., 1997). The alignments were edited manually using
207   GENEDOC (Nicholas and Nicholas, 1997). Ambiguously aligned regions were excluded
208   from the alignment. Phylogenetic analyses were carried out using programs in PHYLIP
209   version 3.6 (Felsenstein, 2004). Genetic distances between pairs of fungi were
210   calculated with the program DNADIST using the Kimura two–parameter method.
211   Phylogenetic trees were constructed using the distance method NEIGHBOR using the
212   original data set and 1000 bootstrapped data sets generated by the program
213   SEQBOOT. Trees were displayed using TREEVIEW (Page, 1996).

214   Sequences were deposited in the Rothamsted Research database under the
215   accession numbers of their OTUs (Table 1).

216

217   *2.4. Isolation and identification by morphology of fungi in pure culture*

218

219   Root fragments (25 from each tree species) were cut into 0.5 cm pieces and placed on
220   synthetic nutrient agar (SNA: Nirenberg, 1976; 1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4·7$
221   H_2O, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 20 g agar, 1 L distilled water). SNA is a
222   low-nutrient agar that stimulates sporulation of a vast range of fungi and fungus-like
223   organisms and guarantees a high level of effectiveness in their detection (Bateman and
224   Kwaśna, 1999; Kwaśna 2001, 2002, 2003). Plates were incubated at 20°C for 5 days in
225   darkness, at 20°C for a further 7 days under continuous black (near-ultra violet) light,
226   and at $20–25^{o}$C for a further 30 days in natural day/night conditions. Fungi growing from
227   the root pieces were identified on the basis of their morphology on 2% potato dextrose
228   agar (PDA), Czapek solution agar (CzA), Czapek yeast autolysate agar (CYA), 2% malt

229 extract agar (MEA), 1% carrot decoct agar (CDA) and SNA using microscopy (Domsch
230 et al., 1980; Pitt, 1980; de Hoog et al., 2000 a, b).

231

232 *2. 5. Statistical analyses*

233

234 Species richness and structure of fungal communities were determined for each tree
235 species. Isolates of the same species or clones with the same OTU were grouped, and
236 the frequency of each species or OTU was determined. A number of diversity indices
237 (Magurran, 1988) were calculated for each community or library. These indices included
238 three different species richness indicators: (i) the total number of species in the
239 community or OTUs per library, (ii) Shannon's diversity index (H'), a general diversity
240 index that considers both species richness and evenness, and (iii) Margalef's index
241 (D_{Mg}), which shows richness from the ratio between number of species or OTUs and
242 their ln function (Magurran, 1988; Hill et al., 2003). Three different indices were also
243 calculated for evenness and dominance: (i) Shannon's evenness index (E), which is the
244 ratio of Shannon's diversity index to the maximum possible value with the observed
245 number of species or OTUs, (ii) Simpson's index (D), which gives the probability that
246 two isolates or clones chosen at random will be from the same species or OTU, and (iii)
247 Berger-Parker's index (d), which is the relative abundance of the most abundant species
248 or OTU (Magurran, 1988; Hill et al., 2003). The similarity between fungal communities
249 on roots of any two tree species was determined by calculating the qualitative
250 Sorensen's similarity index (C_N) from the number of co-occurring species or OTUs
251 (Magurran, 1988; McCaig et al., 1999).

252

253 **3. Results**

254

255 *3. 1. Fungal identification by DNA cloning and sequencing*

256

257 The total DNA was extracted from roots of *B. pendula, F. sylvatica, L. decidua, P.*
258 *serotina* and *Q. petraea*. The ITS 1/2 rDNA was amplified from the total DNA of each
259 tree species and cloned into pGEM–T Easy. One hundred clones from each library were
260 analysed by RFLP, separately with two restriction enzymes: first with *Hae*III, then with
261 *Cfo*I.

262　　　In five libraries (one for each tree species) there were 55 unique restriction
263　digestion patterns obtained with *Cfo*I. The number of clones with unique restriction
264　digestion pattern in one library ranged from 1 to 84 (Table 1). The number of unique
265　restriction patterns with *Cfo*I designated to one species ranged from 1 to 5. In total, 47
266　clones representing 31 unique and the most frequent restriction digestion patterns with
267　*Cfo*I were sequenced. Between six (*L. decidua*) and 19 (*F. sylvatica*) clones per library
268　were sequenced.

269　　　The entire lengths of the 47 sequences were used as queries in BLAST searches
270　to find related sequences (Table 1). A threshold of 97% similarity was used as an
271　approximation to differentiate closely similar sequences at the species level. This is
272　analogous to the practice used to distinguish bacterial species by their 16S rRNA
273　sequences. It has also been reported that the level of intraspecific variation in the
274　*Basidiomycota* commonly ranges from 0 to 3% (Zervakis et al., 2004).

275　　　Thirty sequences (64%) represented fungi with at least 97% similarity to database
276　entries from other, unrelated studies. Seventeen sequences were novel, with novelty
277　defined by the difference criterion of at least 3%. Twenty-two distinct taxa were detected
278　by cloning and sequencing (Table 2).

279　　　Phylogenetic analysis was performed in order to insert the sequences into a
280　molecular taxonomic framework provided by the closest matches from the databases.
281　The analysis was restricted to 200 bp and included the 5.8S DNA and short lengths of
282　ITS1 and ITS2. The phyla *Mycota*, including *Ascomycota* and *Basidiomycota* (larger
283　clade)*, Protozoa* and *Oomycota* (smaller clade) were correctly separated (Fig. 1).
284　Sequences within the same class or order grouped together. Most of the OTUs (79%)
285　grouped closely with their BLAST database matches. Seven OTUs linked to
286　*Podospora–Schizothecium* complex, and six OTUs to *Helgardia anguioides*
287　(*Ascomycota*)*.* Four and nine OTUs linked to *Mycena* spp. and *C. fissolanatus*
288　(*Basidiomycota*). *Protozoa* with *Polymyxa graminis* and *Oomycota* with three different
289　*Pythium* species formed two separate sub-clades with very strong bootstrap support.
290　The *Ascomycota* and *Basidiomycota* comprised several novel sequences based on the
291　3% difference criterion. They include HK 413, HK 411, HK 403, HK 406, HK 388, HK
292　374, EW 817, HK 405 and HK 378. They were located with no context anticipated from
293　the BLAST search, or, if located, had low similarity matrix values to the closest matches.
294　A distinct branch for HK 405 suggests novelty at a lower taxonomic level. OTUs of

295 *Basidiomycota* were located mostly on shorter branches, relatively close to their
296 database matches.

297

298 *3. 2. Morphological identification*

299

300 A total of 522 isolates was detected on SNA from the thin, suberized roots of *B.*
301 *pendula, F. sylvatica, L. decidua, P. serotina* and *Q. petraea.* An average number of
302 isolates from a root ranged from 2.8 in *Q. petraea* to 5.4 in *F. sylvatica*. The number of
303 isolates from a single tree ranged from 69 in *Q. petraea* to 133 in *F. sylvatica*. Forty-nine
304 distinct fungal taxa were recorded by morphotyping and morphological identification.
305 The number of fungal taxa detected ranged from 12 in *L. decidua* to 32 in *F. sylvatica*.
306 Most fungal isolates (86%) were identified at least to genus level. The most frequently
307 isolated fungi were members of *Ascomycota* (*Penicillium* spp., *Phialocephala fortinii*,
308 *Pochonia bulbillosa*, *Sesquicillium candelabrum* and *Trichoderma* spp.).
309   Fungal communities from different plant species had component fungi in common.
310 Twenty-four fungal species (51%) were recorded from a single tree species, six fungal
311 species from two tree species, and 11, four and two fungal species from three, four and
312 five tree species, respectively (Table 3).

313

314 *3. 3. Fungal community structure*

315

316 Twice as many taxa (47) were identified by pure-culture isolation as by sequencing ITS
317 1/2 rDNA from representative clones (22) (Tables 2, 3). The total number of taxa in the
318 combined data set was 69, of which most (85%) were identified at least to genus level.
319 There was no overlap between taxa identified by the two methods.
320   The relatively small number of fungal taxa identified by the molecular method and
321 the infrequent occurrence of most of them resulted in relatively small diversity indices
322 based on species richness (D_{Mg}) and the proportional abundance of species (H') (Table
323 4). Species richness was less on roots of *Q. petraea* than on roots of other species. The
324 dominance of one taxon in these communities resulted in small values for Shannon's
325 evenness index (E) and high values for dominance indices (D and d). Most unevenness
326 occurred in the community from *Q. petraea*, which was dominated by *Coprinus* and had
327 the least diversity (Fig. 2). There was most similarity in community structure between *B.*

328 *pendula* and *L. decidua* and least similarity between *F. sylvatica* and *Q. petraea* (Table
329 5).

330   A different community structure was identified by morphology, resulting in different
331 diversity and evenness indices (Table 4). Species richness was smaller on roots of *L.*
332 *decidua* than on roots of other species. No dominance of one taxon resulted in high
333 values for Shannon's evenness index (*E*) and low values for dominance indices (*D* and
334 *d*). There was most unevenness in communities from *Q. petraea* and *L. decidua*, which
335 were dominated by *Penicillium* spp. There was most similarity between communities
336 from *L. decidua* and *P. serotina* and least similarity between those from *L. decidua* and
337 *Q. petraea* (Table 5). *Fagus sylvatica* tended to have the most diverse and most even
338 communities according to both methods.

339

4. Discussion

341

342 Microbiota from roots of five species of 6-year-old forest trees were characterized by (i)
343 cloning of ITS 1/2 rDNA PCR products isolated from root-associated organisms and
344 sequencing of representative clones, and (ii) pure-culture isolation from individual root
345 pieces, which involved morphotyping and identification on the basis of morphology.
346 Similar numbers of clones and isolates were characterized by each method (approx.
347 500 per method).

348   The two methods detected 69 fungal species that were mostly root saprotrophs.
349 The number of taxa detected by the molecular method was 22 and by pure-culture
350 isolation was 47. The detection of a greater number of fungal species by culturing and
351 morphotyping than by the PCR-based molecular method is consistent with other studies
352 (Burke et al., 2005; Allmer et al., 2006; Menkis et al., 2006).

353   The total number of species recorded seems to relate to the age of the trees.
354 Greater species richness has been found in mature trees: 90-year-old *Pseudotsuga*
355 *menziesii* (Mirb.) Franco and *Tsuga heterophylla* (Raf.) Sarg., and 350-400-year-old
356 *Abies* yielded 200 and 80 morphotypes, respectively (Luoma et al., 1997; Bidartondo et
357 al., 2000). Younger trees usually yield fewer than half the numbers of fungi that occur in
358 mature trees (Baxter et al., 1999).

359   There were large differences in the abundance and composition of microbiota
360   determined by molecular method and the pure-culture isolation, and there were no
361   species common to both communities. In other studies, only two species of fungi from
362   soil were common to 67 taxa culturable on agar and 51 taxa detected by PCR–RFLP of
363   ITS 1/2 rDNA (Viaud et al., 2000). Six years later, 10.7% of taxa from decayed conifer
364   seedling roots were common for communities identified using classical and molecular
365   approaches (Menkis et al., 2006). This suggests that as more sequence information
366   becomes available in the public databases the species overlap between communities
367   found by classical and molecular approach is being increased. Our results do not
368   support this trend, however. The absence of overlapping species may indicate
369   inefficiency in both techniques. In pure-culture isolation, this may result from the
370   specificity of microbiota colonizing roots of trees. It is often dominated by endophytes
371   and ectomycorrhizal species that are difficult to culture, have specific, often unknown,
372   nutritional and environmental preferences and are often suppressed by more vigorous
373   and aggressive taxa. Inefficiency of PCR amplification may result from incompatibility of
374   primers unable to amplify DNA with complicated structure or heterogeneous nature. The
375   problem posed by heterogeneous groups of fungi has been countered by using
376   degenerate primers (Jacobsen et al., 2005) or including degenerate nucleotides in the
377   primer sequences (Landgraf, 2006).

378   Cloning and sequencing representative clones from root-associated organisms
379   resulted in the detection of members of *Ascomycota, Basidiomycota*, *Oomycota,*
380   *Protozoa*, but no *Zygomycota* were found.

381   Most diversity and differentiation was found in the *Ascomycota. Podospora*
382   *glutinans, H. anguioides* and *Microdochium* sp. from, respectively, the orders
383   *Sordariales, Helotiales* and *Xylariales* were the most common. *Cladophialophora* sp.,
384   *Lewia infectoria*, *Ophiosphaerella* sp. and *Tetracladium furcatum* occurred rarely and
385   only in roots of one tree species. *Podospora glutinans* was detected in roots of *B.*
386   *pendula, F. sylvatica* and *L. decidua*, suggesting that it occurs commonly, at least in the
387   area of study. *Schizothecium miniglutinans* was also detected in roots of *F. sylvatica*.
388   The *Podospora–Schizothecium* complex includes coprophilous fungi reported so far
389   only from opossum and rabbit dung in New Zealand and Australia (CBS 113105) (Bell
390   and Mahoney, 1997) and bearberry (*Arctostaphylos uva-ursi* (*Ericaceae*) in Switzerland
391   (CBS 134.83). The current record is the first for these fungi in trees tissúes. *Helgardia*
392   *anguioides* and *Microdochium* sp. were detected in roots of *B. pendula, F. sylvatica, L.*

393 *decidua* and *Q. petraea*. These fungi were so far known only from cereals and grasses.
394 Their occurrence in tree roots may have resulted from their growth in the proximity of
395 roots of grasses in the ground-cover vegetation. Such a phenomenon is not unknown.
396 Kennedy et al. (2003) showed that formation of mycorrhizal networks between the
397 canopy trees and understorey plants in an evergreen forest is possible and even
398 common. According to Johnson et al. (2003) and Hawkes et al. (2006) invading plants
399 may strongly influence the diversity of endophytes and mycorrhizas in roots of native
400 plants. Competition between fungal species invading from bordering regions and the
401 indigenous fungal population may alter the overall structure of a fungal community and
402 influence the nutrient cycling processes. The other, less frequently occurring
403 *Ascomycota* have also never been recorded as specialist colonists of forest tree tissues.
404 *Cladophialophora* sp., although apparently predominant in tropical and subtropical
405 regions (de Hoog et al., 2000 b), can also occur in colder habitats. Clones matching
406 *Cladophialophora* sp. have been found in the receding forefront of Lyman glacier, North
407 Cascade Mountains, USA (Jumpponen, 2003). *Lewia* and *Ophiosphaerella* sp. are
408 grass pathogens and *T. furcatum* is an aquatic fungus.

409 The most frequently occurring fungi were *Basidiomycota*. Between one and three
410 species of *Basidiomycota* from *Agaricales* and *Ceratobasidiales* were recorded in roots
411 of a single tree. *Coprinus fissolanatus* was the most common, particularly in *Q. decidua*
412 (92% of OTUs) and *B. pendula* (61% of OTUs). The occurrence of *C. fissolanatus* within
413 sterile fungal morphotypes detected in *Pinus tabulaeformis* Carr., studied using rDNA
414 sequencing, has been suggested (Wang et al., 2005). *Coprinus disseminatus* (Pers.)
415 Gray and *Coprinus* sp. were reported from roots of *Pinus sylvestris* and *Picea abies* (L.)
416 H. Karst seedlings in Lithuania (Menkis et al., 2006). Before being detected in forest tree
417 roots *Coprinus* species were known to be free-living saprotrophs, particularly common in
418 beech forest stands in central and eastern Europe (Burel, 2004; Fukiharu et al., 2005;
419 Quere et al., 2006). *Mycena* and *Arrhenia obscurata* were detected less frequently. Two
420 species of *Mycena* were also reported from roots of *P. sylvestris* seedlings in Lithuania
421 (Menkis et al., 2006). So far, the genus was known to be abundant in forest soil,
422 particularly under mixed hardwood in the temperate region of the USA (O'Brien et al.,
423 2005).

424 *Oomycota* was represented by three different species of *Pythium.* One of them
425 colonized roots of *B. pendula* and the other two were in roots of *F. sylvaticus. Pythium*
426 spp. are important root pathogens, causing damping-off and decay. The efficiency of

427   their detection without *Pythium*–specific primers is significant. Menkis et al. (2005)
428   suggested that detection of *Pythium* with fungal consensus rDNA primers is difficult.
429   Only the molecular method detected *Protozoa*, represented by *P. graminis* from
430   *Plasmodiophorales*. It could not be detected earlier using morphological procedures
431   because it is non-culturable *in vitro* by known methods. Generally, it is recognized as an
432   obligately biotrophic, non-pathogenic colonizer of plant roots (Simpson and Roger,
433   2004: Adl et al., 2005). So far it was considered as almost entirely restricted to the
434   *Poaceae,* with a reservoir in *w*ild grasses. It is of considerable agronomic importance
435   because of its ability to acquire and transmit a range of plant viruses pathogenic on
436   cereal crops (Kanyuka et al., 2003). This is the first record of its occurrence in tree roots
437   and a forest habitat. The extent to which it colonizes trees and the degree of host
438   specialization are largely unknown. In this study it was recorded on *F. sylvatica* and *Q.*
439   *petreaea* roots.

440   Cloning and sequencing detected neither *Penicillium* nor *Trichoderma* spp.
441   (A*scomycota, Eurotiales, Hypocreales*), which are common fungi in tree roots (Kwaśna
442   1996 a, b; 1997 a, b; 2001, 2002, 2003, 2004). Neither genus was recorded by
443   oligonucleotide fingerprinting of rRNA (Valinsky et al., 2002), direct isolation of SSU and
444   ITS rRNA by PCR and high-throughput sequencing of cloned fragments (O'Brien et al.,
445   2005) or direct sequencing of ITS rDNA (Menkis et al., 2006). Anderson et al. (2003 b)
446   was able to detect *Eupenicillium* and *Penicillium* species in ITS analysed by DGGE
447   (denaturing gradient gel electrophoresis). Methods in which the 18S rDNA region was
448   analysed with fungal-automated rRNA intergenic spacer analysis (F–ARISA), and
449   cloning and sequencing, were more effective for their detection (Jumpponen, 2003;
450   Hansgate et al., 2005). It seems, so far, that only the arbuscular mycorrhizal (AM) fungi
451   have the sufficient variation in the 18S rRNA gene for discrimination between species
452   and below species level, because of the early radiation of *Glomeromycota*
453   (Vandenkoornhuyse and Leyval, 1998).

454   Pure-culture isolation allowed detection of at least 40 species of *Ascomycota* (mostly
455   *Fusarium* spp., *Penicillium* spp.*, Phialocephala fortinii*, *Trichoderma* spp. from
456   *Eurotiales, Helotiales* and *Hypocreales*), three species of *Zygomycota* (*Mortierella* spp.)
457   but no *Basidiomycota*. The majority of *Ascomycota* detected are ubiquitous saprotrophs
458   and root endophytes. They are successful competitors culturable on artificial media and
459   easy to detect by isolation and morphotyping (Grünig et al., 2006). Identification of
460   some, e.g. *P. fortinii*, was made possible by the use of SNA, which stimulates

sporulation and helps identification by morphology. In earlier studies, *P. fortinii* was categorized as a dark septate endophyte (DSE) (Jumpponen, 2001). It is usually detected on roots of younger trees (Chlebicki, 2004; Menkis et al., 2005, 2006) and it is thought that under some conditions *P. fortinii* is capable of forming mutualistic associations functionally similar to mycorrhiza (Chlebicki, 2004; Jumponnen et al., 1998).

Neither of the methods detected ectomycorrhizal species. There are no reports on a mycorrhizal habit of *Coprinus, Mycena* or *A. obscurata.* Mycorrhizas were absent probably because thin (2–5 mm diam.) suberized roots were used and they were from a young (6-year-old) stand. Suberized roots are usually non–mycorrhizal (Smith and Read, 1997). Disturbance of soil while planting the trees 5 years earlier may have led to insufficient time for mycorrhizal establishment. A complex community of mycorrhizal fungi usually develops over many years (Deacon and Fleming, 1992). Mycorrhizas may develop earlier in fast-growing trees such as *Populus* and *Salix* (Heijden et al., 1999; Baum and Makeschin, 2000; Khasa et al., 2002). Greater frequency of mycorrhizal fungi is often not associated with greater diversity; only one mycorrhizal species, *Rhizopogon rubescens* (Tul. & C. Tul.) Tul. & C. Tul. was found in 55% of root tips of *Pinus pinea* L. seedlings (El–Karkouri et al., 2002).

Among the microbiota detected, 21% of OTUs did not have matches in the current EMBL/GenBank database at the species threshold of 97% similarity. This is likely to be a consequence of poor taxon coverage in the database, even though the ITS rDNA is the best-represented locus for the *Mycota* and the most commonly used marker in studies on fungi. Novel sequences were found more often within *Ascomycota* (nine OTUs) than *Basidiomycota* (two OTUs).

Most fungal species were recorded too infrequently to draw general conclusions about their distribution. Identification by cloning and sequencing indicated, however, considerable species dominance (Fig. 2). In the situation of low community diversity in *Q. petraea*, some specificity for the dominant *C. fissolanatus* was indicated. Similar dominance by a single taxon was recorded in the community from roots of *Q. ilex* in old-growth Mediterranean forest (Richard et al., 2005) and in roots of healthy reed (Neubert et al., 2006). By contrast, identification by morphology suggested a relatively even distribution of species. The different tree species had similar evenness indices, providing no information on host-specificity among the fungi. There was no apparent effect of tree species on the structure of microbial communities. There was no apparent

specialization for resources, host defence reaction or competition by other fungi, or other factors that differentiated the communities. The most common and most frequent fungal species (49% of the total number of isolates) were usually shared by two to five tree species. A similar pattern of colonization was reported for mycorrhizal fungi in a mixed *Pinus contorta* Dougl. and *Picea engelmannii* Parry ex Engelm. forest in the USA (Cullings et al., 2000). Communities identified by morphology showed greater diversity among and within tree species.

This study showed that each method provides only a selective view of diversity of the biota in a natural environment. The two methods differed more in their indication of diversity (taxon detected) than density (number of individual taxon per site). The molecular methods effectively detected fungi of the *Basidiomycota,* which usually form important mycorrhizal associations, and *Oomycota*, which are often pathogenic on young roots. Pure-culture isolation effectively detected saprotrophs and endophytes, particularly within the *Ascomycota*. The results emphasize that only a combination of morphological and molecular techniques provides an effective approach for assessment of diversity and density of root biota. Advantages and limitations of molecular and morphological methods for determining the abundance and composition of biota in plant and soil have also been discussed elsewhere (Menkis et al., 2005, 2006; O'Brien et al., 2005; Tiquia, 2005; Allmer et al., 2006; Girlanda et al., 2006).

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Descriptions

Fig. 1 – Phylogenetic tree obtained by NEIGHBOR analysis of the ITS 1/2 rDNA showing relationships between organisms from roots of deciduous trees. Bootstrap values based on 1000 replicates are indicated above the branches. Sequences from the study are labelled **HK**.

Fig. 2 – Diversity in communities of microbiota from tree roots

Table 1 – Database typing of ITS sequences

Table 2 – Frequency of microbiotal taxa estimated by the molecular method

Table 3 – Frequency of fungi estimated by the pure–culture isolation method

Table 4 – Diversity indices for microbiota communities from roots of five tree species calculated from cloned OTUs and isolates produced by pure–culture isolation

Table 5 – Similarity coefficients based on pairwise comparisons of community structures of microbiota on roots of five tree species

Table 1 – Fungal sequences from GenBank showing most similarity to OTUs from roots of *B. pendula*, *F. sylvatica*, *L. decidua*, *P. serotina* and *Q. petraea.*

Taxon	Order	Accession No.	Depositor	No. of clones in OTU	*Cfo*I RFLP pattern	Host species	OTU
Arrhenia obscurata (D.A. Reid) Redhead, Lutzoni, Moncalvo & Vilgalys	*Agaricales*	U66448	Lutzoni, F. Aug 1997[a]	4	350	*F. sylvatica*	HK 378
Ascomycete sp.		AY787739	Lygis, V., Vasiliauskas, R., Larsson, K.-H. Nov 2004[b]	6	200,180,120,100	*F. sylvatica*	HK 374
Cladophialophora sp.	*Chaetothyriales*	AY781217	Vasiliauskas, R., Larsson, E., Larsson, K.-H., Stenlid, J. Dec 2004[b]	1	300,220	*Q. petraea*	HK 399
Coprinus fissolanatus Kemp	*Agaricales*	AF345812	Park, DS., Shin, HS., Moncalvo, JM. Feb 2001[c]	1	600	*Q. petraea*	EW 816
				2	600,150,100	*Q. petraea*	HK 366
				60	400,300	*B. pendula*	HK 367
				17	400,300	*F. sylvatica*	HK 373, HK 387
				45	400,300	*L. decidua*	HK 433
				84	400,300	*Q. petraea*	HK 365
				2	400,250,150,140	*Q. petraea*	HK 402
				3	320,300,100	*Q. petraea*	HK 404
Helgardia anguioides (Nirenberg) Crous & W. Gams	*Helotiales*	AY266144	Stewart, EL., Liu, Z., Crous, P., Szabo, L.J. Mar 2003[d]	4	410,280	*B. pendula*	HK 407
				2	380	*F. sylvatica*	HK 385, HK 386
				6	280,100,80	*F. sylvatica*	HK 376
				2	280,100,80	*L. decidua*	HK 391, HK 435
Lewia infectoria (Fuckel) M.E. Barr & E.G. Simmons	*Pleosporales*	Y17066	McKay, GJ. Apr 1998[e]	2	280,180,140,100	*B. pendula*	HK 408
Microdochium sp.	*Xylariales*	AJ279481	Wirsel, SGR. Dec 1999[f]	5	300,180	*B. pendula*	HK 406
				6	300,180	*F. sylvatica*	HK 379
				1	280,180	*B. pendula*	HK 411
				2	280,180	*Q. petraea*	HK 403
Mycena epipterygia (Scop.) Gray	*Agaricales*	AY805613	Menkis, A., Allmer, J., Vasiliauskas, R., Lygis, V., Stenlid, J., Finlay, R. Nov 2004[b]	5	380,350	*B. pendula*	HK 405
				8	380,350	*F. sylvatica*	HK 377
				5	380,350	*L. decidua*	HK 434
Mycena aff. *murina* Murrill	*Agaricales*	AF335444	Berbee, ML., Inderbitzin, P., Zhang, G. Jan 2001[g]	1	320,200,180,100	*L. decidua*	HK 437
				1	320,200,180,100	*Q. petraea*	HK 401

Species	Order	Accession	Reference	n	Bands	Host	Code
Ophiosphaerella sp.	*Pleosporales*	AJ246157	Carter, JP. Jan 1999[h]	2	350,180,150	*F. sylvatica*	HK 380
Podospora glutinans (Cain) Cain	*Sordariales*	AY615207	Krug, JC., Schulz, MJ., Jeng, RS. Apr 2004[i]	5	230,120,80	*B. pendula*	EW 818
				17	230,120,80	*F. sylvatica*	HK 375, HK 382
				4	230,120,80	*L. decidua*	HK 436
				4	230,220,120,100,80	*F. sylvatica*	HK 392
Polymyxa graminis Ledingham	*Plasmodiophorales*	Y12826	Ward, E., Adams, MJ. Apr 1997[j]	1	380,250	*Q. petraea*	HK 400
				4	350,80,70	*F. sylvatica*	HK 381
Pythium arrhenomanes Drechsler	*Pythiales*	AF330180	Heelan, LA., Croft, BJ., Dietzgen, RG., Maclean, DJ. Dec 2000[k]	2	600,280,75	*F. sylvatica*	HK 383
Pythium insidiosum De Cock, L. Mend., A.A. Padhye, Ajello & Kaufman	*Pythiales*	AY151179	Schurko, AM., Mendoza, L., Levesque, CA., Desaulniers, NL., de Cock, AW., Klassen, GR. Sep 2002[l]	6	700,180,120	*B. pendula*	EW 819
Pythium sylvaticum W.A. Campb. & F.F. Hendrix	*Pythiales*	AY598645	Levesque, CA., de Cock, AWAM. Apr 2004[m]	2	400,110,90,70	*F. sylvatica*	HK 384
Schizothecium miniglutinans (J.H. Mirza & Cain) N. Lundq.	*Sordariales*	AY515362	Debuchy, R., Bell, AE., Mahoney, DP. Dec 2003[n]	1	400,240,80	*F. sylvatica*	HK 389
Tetracladium furcatum Descals	*Incertae sedis*	AF411026	Nikolcheva, LG., Baerlocher, FJ. Aug 2001[o]	2	380,300	*B. pendula*	HK 409
Thanatephorus cucumeris (A.B. Frank) Donk	*Ceratobasidiales*	AY154300	Kuramae, EE., Buzeto, AL., Ciampi, MB., Souza, NL. Sep 2002[p]	1	390	*B. pendula*	HK 410
Tricladium splendens Ingold	*Helotiales*	AY204635	Baschien, C., Marvanova, L., Manz, W., Szewzyk, U. Dec 2002[r]	15	190,120	*F. sylvatica*	HK 388
				1	190,120	*Q. petraea*	EW 817
Mycorrhizal ascomycete of *Rhododendron*		AB089660	Usuki, F., Abe, J., Kakishima, M. Aug 2002[s]	1	200,170,120	*F. sylvatica*	HK 390
Uncultured fungus		AF504837	Anderson, IC., Campbell, CD., Prosser,JI. Apr 2002[t]	3	600	*B. pendula*	HK 412
Uncultured Zygomycete		AY969893	O'brien, HE., Parrent, JL., Jackson, JA., Moncalvo, JM., Vilgalys, R. Mar 2005[u]	1	700,400,300	*B. pendula*	HK 413

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Table 2 – Frequency of microbiotal taxa estimated by the molecular method.

	Taxa	Betula pendula	Fagus sylvatica	Larix decidua	Prunus serotina	Quercus petraea
		% of OTUs				
1.	*Arrhenia obscurata*	1.0	4.0	3.0	3.0	
2.	Ascomycete sp.		6.0		8.0	
3.	*Cladophialophora* sp.					1.0
4.	*Coprinus fissolanatus*	61.0	19.0	45.0	36.0	92.0
5.	*Helgardia anguioides*	4.0	9.0	10.0	2.0	
6.	*Lewia infectoria*	2.0				
7.	*Microdochium* sp.	6.0	6.0	17.0	3.0	2.0
8.	*Mycena epipterygia*	5.0	8.0	5.0	14.0	
9.	*Mycena* aff. *murina*	1.0		3.0	6.0	1.0
10.	*Ophiosphaerella* sp.		2.0		1.0	
11.	*Podospora glutinans*	5.0	21.0	9.0	8.0	
12.	*Polymyxa graminis*		4.0		3.0	1.0
13.	*Pythium arrhenomanes*	1.0	2.0		8.0	
14.	*Pythium insidiosum*	6.0		4.0	3.0	2.0
15.	*Pythium sylvaticum*		2.0			
16.	*Schizothecium miniglutinans*	1.0	1.0			
17.	*Tetracladium furcatum*	2.0				
18.	*Thanatephorus cucumeris*	1.0			2.0	
19.	*Tricladium splendens*		15.0	2.0	3.0	1.0
20.	Zygomycete sp.	1.0				
21.	Mycorrhizal ascomycete of *Rhododendron*		1.0	2.0		
22.	Uncultured fungus	3.0				
	Number of clones studied	100	100	100	100	100
	Number of taxa detected	15	14	10	14	7
	Number of taxa per root	0.6	0.6	0.4	0.6	0.3

Table 3 – Frequency of fungi estimated by the pure–culture isolation method.

Taxa	Betula pendula	Fagus sylvatica	Larix decidua	Prunus serotina	Quercus petraea
	% of the total number of isolates				
1. *Arthrinium phaeospermum* (Corda) M.B. Ellis				2.9	
2. *Aspergillus fumigatus* Fresenius		0.8			
3. *Cladosporium cladosporioides* (Fresenius) G.A. de Vries					1.5
4. *Coniothyrium fuckelii* Saccardo			7.1		
5. *Cylindrocarpon didymum* (Harting) Wollenweber		1.5			
6. *Fusarium avenaceum* (Fries : Fries) Saccardo		1.5			1.5
7. *Fusarium oxysporum* Schlechtendal : Fries		1.5	1.0	1.9	
8. *Fusarium redolens* Wollenweber				0.9	
9. *Fusarium* sp.	1.7				
10. *Geotrichum candidum* Link ex Leman		1.5			
11. *Gliocladium roseum* Bainier				3.9	
12. *Humicola grisea* Traaen				1.9	
13. *Mortierella elongata* Linnem.		1.5			
14. *Mortierella gamsii* Milko		3.0			
15. *Mortierella humilis* Linnem. ex W. Gams		1.5			2.9
16. *Paecilomyces farinosus* (Holmskjold : Fries) A.H.S. Brown & G. Smith		3.8			
17. *Papulaspora* sp.				0.9	
18. *Penicillium adametzii* Zaleski	6.9	1.5	10.1	13.5	20.0
19. *Penicillium canescens* Sopp		1.5	1.0		
20. *Penicillium cyclopium* Westling	3.4	3.0		1.9	
21. *Penicillium daleae* Zaleski	18.8	12.0	3.1	13.5	21.7
22. *Penicillium herquei* Bainier & Sartory				1.9	
23. *Penicillium janczewskii* Zaleski	23.9	9.1		2.9	18.8
24. *Penicillium restrictum* J.C. Gilman & E.V. Abbott				1.9	
25. *Penicillium simplicissimum* (Oudem.) Thom	10.3	0.8	28.4	17.3	
26. *Penicillium steckii* Zaleski	12.0			1.9	4.3
27. *Penicillium vinaceum* Gilman et Abbott		9.1	17.2	14.4	
28. *Penicillium* spp.		0.8			
29. *Phialocephala fortinii* C.J.K. Wang & H.E. Wilcox		3.0	8.0		4.3

30.	*Phialophora botulispora* Cole & W.B. Kendr.		0.8			
31.	*Phialophora richardsiae* (Nannf.) Conant		1.5			1.5
32.	*Phoma* cf. *lingam* (Tode) Desm.					2.9
33.	*Pochonia bulbillosa* (W. Gams & Malla) Zare & W. Gams	5.1	6.0	8.0		1.5
34.	*Sesquicillium candelabrum* (Bonorden) W. Gams		3.0	2.0	1.9	5.8
35.	*Sporothrix schenckii* Hectoen et Perkins		4.5		1.9	
36.	*Tolypocladium inflatum* W. Gams		4.5			2.9
37.	*Trichoderma aureoviride* Rifai				4.9	
38.	*Trichoderma harzianum* Rifai		0.8			
39.	*Trichoderma koningii* Oudemans		5.3	11.0	9.7	
40.	*Trichoderma viride* Pers. ex Gray		5.3	3.1		1.5
41.	*Trichoderma* sp.	9.4				
42.	*Varicosporium elodeae* W. Kegel					1.5
43.	*Verticillium* sp.		2.2			
44.	non–sporulating, black-mycelium	3.4	2.2			1.5
45.	non–sporulating, brown-mycelium	1.7	2.2			1.5
46.	non–sporulating, grey-mycelium	1.7	2.2			2.9
47.	non–sporulating, white-mycelium	1.7	2.2			1.5
	Number of isolates	117	133	99	104	69
	Number of taxa detected	13	32	12	19	19
	Number of taxa per root	0.5	1.3	0.5	0.8	0.8
	Number of isolates per inoculum	2.4	2.7	2.0	2.2	1.4
	Number of isolates per root	4.8	5.4	4.0	4.4	2.8

Table 4 – Diversity indices for microbiotal communities from roots of *B. pendula*, *F. sylvatica*, *L. decidua*, *P. serotina* and *Q. petraea* calculated from cloned OTUs and isolates produced by pure–culture isolation.

| Tree species | No. of OTUs/ isolates | Species richness indices | | Evenness or dominance indices | | |
		Margalef's index (D_{Mg})	Shannon's index (H)	Shannon's evenness index (E)	Simpson's index (D)	Berger-Parker's index (d)
Community identified by molecular method						
Betula pendula	100	3.04	1.61	0.59	0.38	0.61
Fagus sylvatica	100	2.83	2.27	0.86	0.12	0.21
Larix decidua	100	1.96	1.75	0.76	0.25	0.45
Prunus serotina	100	2.83	2.15	0.81	0.17	0.36
Quercus petraea	100	1.30	0.37	0.19	0.85	0.92
Community identified by morphology						
Betula pendula	117	2.52	2.21	0.86	0.13	0.24
Fagus sylvatica	133	6.34	3.17	0.92	0.05	0.12
Larix decidua	99	2.40	2.11	0.85	0.14	0.28
Prunus serotina	104	3.88	2.52	0.86	0.10	0.17
Quercus petraea	69	4.25	2.32	0.79	0.23	0.22

Table 5 – Similarity coefficients based on pairwise comparisons of community structures of microbiota on roots of *B. pendula*, *F. sylvatica*, *L. decidua*, *P. serotina* and *Q. petraea*.

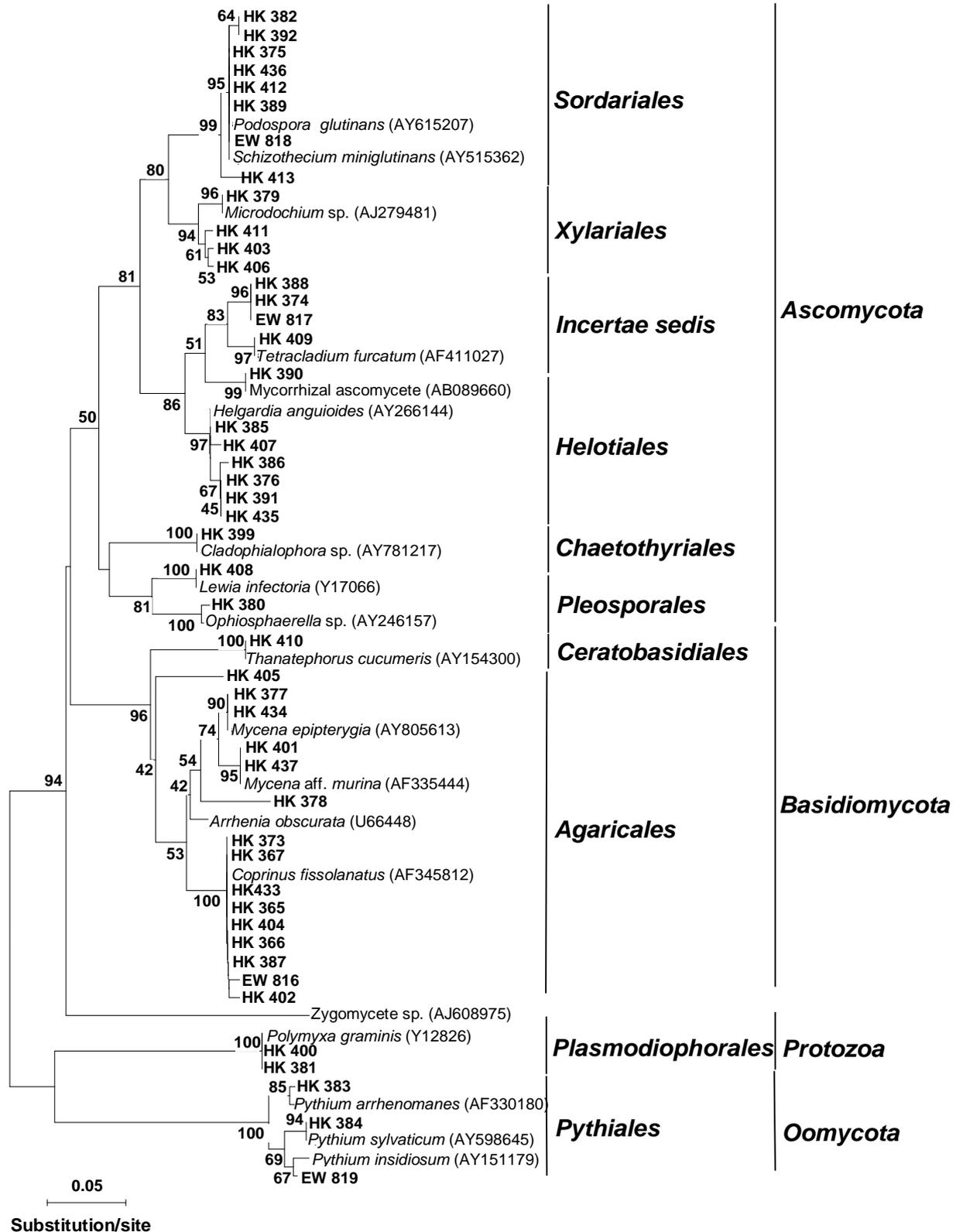| Tree species | Sorensen's similarity index (C_N) | | | |
	B. pendula	*F. sylvatica*	*L. decidua*	*P. serotina*
Community identified by molecular method				
F. sylvatica	0.42			
L. decidua	0.71	0.54		
P. serotina	0.58	0.52	0.65	
Q. petraea	0.66	0.23	0.51	0.43
Community identified by morphology				
F. sylvatica	0.40			
L. decidua	0.27	0.38		
P. serotina	0.37	0.39	0.58	
Q. petraea	0.48	0.49	0.24	0.40

Fig. 1 – Phylogenetic tree obtained by NEIGHBOR analysis of the ITS 1/2 rDNA showing relationships between organisms from roots of *B. pendula*, *F. sylvatica*, *L. decidua*, *P. serotina* and *Q. petraea*. Bootstrap values based on 1000 replicates are indicated above the branches. Sequences from the study are labelled **HK** or **EW**.
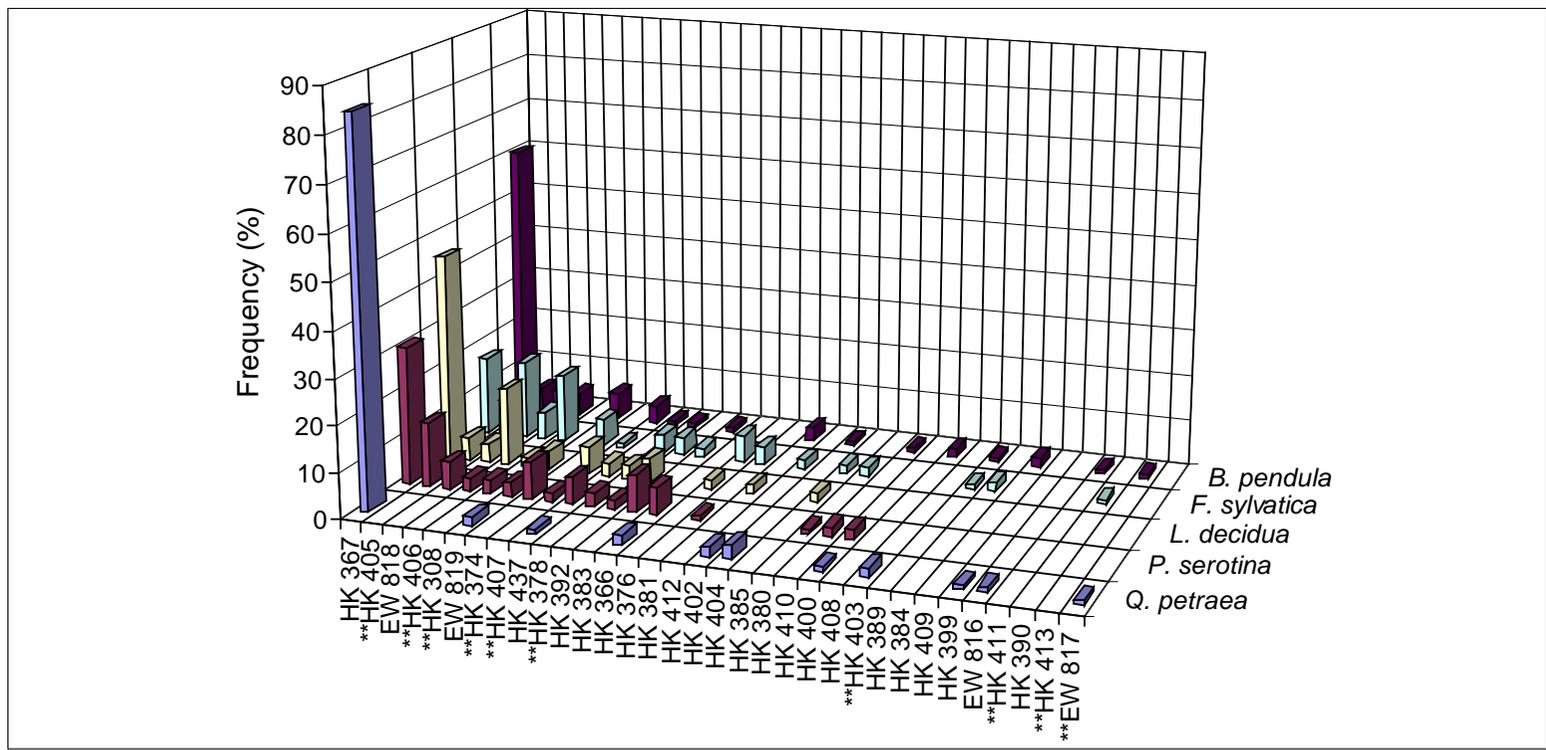
Fig. 2 – Frequency of microbiotal taxa in roots of *B. pendula*, *F. sylvatica*, *L. decidua*, *P. serotina* and *Q. petraea* estimated by the molecular method.